

EFFECTS OF CHRONIC ETHANOL CONSUMPTION ON SPLENIC DENDRITIC CELL FUNCTIONS

By

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Abstract

About 7.1% of Americans older than 18 have been reported to abuse alcohol. Chronic alcoholics are more susceptible to infections. In this regard, higher incidences of lung infection caused by several bacteria and viruses have been reported in humans. Hepatitis C virus (HCV), a primary cause of acute and chronic liver diseases, is also a major problem in alcoholics. Additionally, murine models of chronic ethanol consumption have been shown to be more susceptible to bacterial and viral diseases. We have recently revealed in a chronic ethanol murine model that generation of HCV viral NS5 protein specific CD4+ and CD8+ T-cell responses were impaired and CD8+ T-cell activity could be restored by adoptive transfer of dendritic cells (DCs) derived from isocaloric pair-fed control but not from ethanol-fed animals. These findings suggested that impaired cellular immunity may be due, in part, to alcohol induced intrinsic defects in DCs. In chapter 1, attempts were made to explore the cellular mechanisms of these defects that impair exogenous antigen presentation by splenic DCs. We observed a reduction in the ability of DCs to present exogenous OVA protein to primary and DO11 T-cells. Processing of endocytosed antigens were unaltered, yet peptide-MHCII complex formation and presentation on the cell surface of DCs was reduced after chronic ethanol exposure *in vivo*. The reduced T-cell activation was not due, entirely, to reduced peptide-MHCII presentation. Furthermore, addition of several cytokines produced by ethanol-exposed DCs back to DC:T cell co-cultures or neutralization of IL-10 were unable to restore the impaired T-cell activation suggesting that the process was not reversible by this stage. In chapter 3, ethanol induced changes in gene expression profile of mature and immature DCs were explored. Finally, in chapter 4, two chronic ethanol rat models were employed to investigate the influence of alcoholic liver disease (ALD) on DC function. In the ALD-sensitive Long Evans rats, DCs displayed a less-mature phenotype in terms of costimulatory molecule expression, cytokine secretion and allogeneic antigen presentation compared to ALD-resistant Fisher strain. These results suggest that liver disease produced by chronic ethanol consumption has a major effect on DC function.

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CHAPTER 1:
INTRODUCTION

1. MURINE MODELS OF CHRONIC ALCOHOL RESEARCH

Effects of acute and chronic ethanol consumption have been studied both *in vivo* and *in vitro*. *In vitro* and *in vivo* models of acute alcohol have recently been reviewed¹ and will not be discussed here in detail as this current work investigates the effects of chronic alcohol abuse on the immune responses in the context of dendritic cells. Briefly, *in vitro* models of acute alcohol studies are performed by supplementing the cell culture medium with varying concentrations of ethanol (1mM to 500mM). The optimal way for this is to place the culture plates in a chamber with wells that contain extra ethanol, which minimizes the evaporation of ethanol from the plate while the cells are in culture. *In vivo* models of acute ethanol include several invertebrate and vertebrate models by using various means of ethanol administration. These include voluntary alcohol consumption or involuntary force-feeding² (using gavage) or parenteral administration.^{3, 4} Usually, 2-24 hours of exposure to ethanol is considered acute exposure by investigators.

Chronic ethanol abuse is mostly studied with rat and mouse models; however, there are also reports in which long term ethanol exposure of macrophages and monocytes were investigated *in vitro*. In this context, exposure times varied from 48 hours to 5-7 days.^{5, 6} The rat and mouse models mostly utilize three distinct methods of ethanol administration. These methods vary in creating liver pathology (alcoholic liver disease (ALD)) and have different caloric contribution to animals' diet. Lieber-DeCarli is a high-fat liquid diet developed in 1967 and used during the course of this study as a means of ethanol feeding.^{6, 7} In this method, ethanol is given mixed in a liquid diet and that improves the consumption by minimizing the aversion compared to ethanol feeding in drinking water. For rats, ethanol constitutes 37% of the calories in this diet. This concentration is well tolerated and does not cause weight loss. In the control diet, ethanol derived calories are replaced with maltose-dextrin. For mice however, 37% ethanol has been found to be toxic; therefore the ethanol dose has been decreased to 25% of the calories taken⁸. Different than rats, mice on 25% ethanol diet are significantly lighter and gain less weight over time than their pair-fed control counterparts (Figure 1.1). When blood ethanol levels are measured in the early

morning, approximately 50mM concentrations were reached in Balb/c mice (Figure 1.2). Lieber-DeCarli ethanol diet results in severe steatosis and inflammation in rats (with the exception of Fischer rat strain). Mouse model, however, develops a milder liver disease compared with rats.⁹ The second method of ethanol administration is providing ethanol in the drinking water. This method has been utilized by several investigators¹⁰⁻¹² and proposed as a model to study effects of ethanol without liver disease as this method does not result in liver pathology.⁶ The third technique, developed by Tsukamoto-French¹³ allows the investigators to control precisely the amount of ethanol ingested by the mouse with the help of a catheter placed in the stomach. This model, called intragastric ethanol infusion model, has been employed to study alcoholic liver disease (ALD).

The first two chronic ethanol models were used by several investigators to dissect the ethanol induced abnormalities in immune cells and will be cited in the appropriate sections below.

2. ETHANOL METABOLISM

Ethanol metabolism is carried out by both oxidative and nonoxidative pathways (Figure 1.3). Alcohol dehydrogenase enzymes (ADH), cytochrome P450 (CYP2E1, 1A2, 3A4) and catalase are the enzymes that operate during oxidative ethanol metabolism. The majority of ethanol is metabolized by the liver via ADH; however, the stomach is also reported to contribute to ethanol metabolism via ADH in stomach cells. Non-liver tissues, such as brain, can also metabolize ethanol via enzymes cytochrome P450 (CYP2E1) and catalase.¹⁴ Other non-hepatic cells and tissues can also process ethanol through non-oxidative pathways.^{15, 16}

2. A. Oxidative pathways

The primary means of ethanol oxidation is through **ADH**, which yields acetaldehyde that is highly reactive and toxic to the cells. This reaction requires NAD⁺ reduction, and generates NADH; thus creates a highly reduced and therefore, vulnerable cytoplasmic environment to byproducts of ethanol metabolism such as acetaldehyde and reactive oxygen species (ROS).^{15, 16}

The second pathway of oxidative ethanol metabolism takes place in the microsomes (vesicles of endoplasmic reticulum) and is catalyzed by *cytochrome P450* isozymes CYP2E1, 1A2, 3A4. This pathway is induced in response to high levels of ethanol during acute and chronic alcohol consumption (CYP2E1 K_m =8-10mM ADH K_m =0.2-2mM) and generates acetaldehyde, ROS such as hydroxyethyl, superoxide anion and hydroxyl radicals¹⁵.

The third oxidative ethanol metabolism occurs in peroxisomes via the enzyme *catalase* in the presence of hydrogen peroxide, however; catalase metabolism is considered to be a minor pathway compared with other oxidative pathways. The byproduct of the pathway is also acetaldehyde.¹⁵

2. B. Nonoxidative pathways

There are two known nonoxidative pathways that eliminate ethanol from body, although the contribution of both pathways to ethanol metabolism is minimal compared to the oxidative pathways. The first pathway is catalyzed by fatty acid ethyl ester synthase and generates fatty acid ethyl esters (FAEE) due to the reaction of ethanol with fatty acids¹⁵. The second nonoxidative pathway is catalyzed by phospholipase D (PLD) and generates phosphatidyl ethanol.¹⁷ The effects of byproducts from both non-oxidative pathways on tissues and cells remain to be examined. FAEE is found in the circulation after ethanol intake and remains in the blood for long periods. The primary function of PLD is the production of phosphatidic acid from phospholipids and is very critical for many signaling processes. Phosphatidyl ethanol, however, is generated by PLD at the expense of phosphatidic acid. Therefore, PLD involvement in ethanol metabolism is likely to adversely affect many signaling pathways within cells.¹⁵

2. C. Byproducts of ethanol metabolism

Oxidation of NADH, generated by ADH and ALDH catalyzed reactions, back to NAD to balance the cellular NAD/NADH ratios requires more oxygen consumption by hepatocytes than usual, which results in an imbalanced oxygen distribution across the liver; thus, hypoxia will occur in certain regions of the liver tissue.^{15, 18}

The common byproduct of all oxidative pathways is acetaldehyde, which is rapidly metabolized to acetate, and can interact with lysine, cysteine and some aromatic amino acids to form adducts. Such protein modifications may occur in erythrocyte membrane proteins, lipoproteins, tubulin, hemoglobin, albumin, collagen and cytochrome enzymes. These adducts have been reported to invoke immune responses, and cause pathological conditions such as antibody dependent cell-mediated cytotoxicity, or macrocytosis, and even adversely affect nervous system.^{15, 19} Acetaldehyde is converted to acetate by oxidization in the mitochondria and may escape into the circulation. It is degraded to CO₂ in heart, muscles and brain and may affect metabolic pathways as well as the nervous system functions.²⁰

ROS are generated during ethanol metabolism by the action of CYP2E1 (during NADH oxidation). They can cause lipid peroxidation which results in malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) generation. Both of these compounds, alone, or in combination with acetaldehyde may form adducts with proteins²¹.

In summary, both oxidative and non oxidative ethanol metabolism yield several byproducts most of which were shown to exert toxic effects by interacting with proteins. Although the major ADH dependent oxidative pathway takes place in hepatocytes, the other oxidative and non-oxidative pathways may operate; thus generate abovementioned byproducts in other cell types. In addition, ethanol may pass through cell membranes and interact with many molecules including membranes lipids and proteins influencing several signaling pathways. The effects of ethanol described below may be due to a direct interaction of ethanol or through byproducts of ethanol metabolism.

3. ANTIGEN PRESENTING CELLS and MONOCYTES

There are three types of professional antigen presenting cells (APC): macrophages, B cells and dendritic cells. In this section, we will describe briefly the characteristic features of these cell types including monocytes, and the special role of DCs since ethanol has a direct effect on them.

Macrophages are endowed with advanced endocytic ability to internalize antigens in many forms: soluble or cell associated; through receptor mediated or nonspecific endocytosis. They can internalize up to 50% of their surface area during a phagocytosis event. On the other hand, such cells express low levels of MHCII and costimulatory molecules compared to B cells and DCs which makes them less efficient in priming naïve T cells. The major function attributed to macrophages is clearance of pathogens due to their exquisite phagocytosis and lysosomal machinery.²² Such cells can be found in lymphoid or nonlymphoid tissues. They express many Pathogen Associated Molecular Patterns (PAMPs) Recognition Receptors (PRRs) and are source of inflammatory cytokines.²³

B cells have modest endocytosis capacity. The Immunoglobulin g receptor on B cells allows recognition and internalization of corresponding specific soluble antigens after which they are presented on MHCII molecules. Current data suggest that B cells are unable to perform cross presentation. On the other hand, they express costimulatory molecules in abundance and also HLA-DM and HLA-DO which carry out the peptide loading onto MHCII molecules.²²

Monocytes are present mainly in circulation. They can phagocytose cells and toxic molecules followed by the release of cytokines and chemokines. Monocytes are not regarded as professional antigen presenting cells; however, they can quickly migrate to inflammatory areas and give rise to antigen presenting cells: inflammatory DCs and macrophages.²³

Both **DCs** and macrophages are distributed throughout the body and tissues, yet DCs have distinctive features that make them unique among APCs. In lymphoid organs DCs localize in areas where other APCs are absent. They especially accumulate within T cell areas in lymph nodes.²² DCs express very high amounts of costimulatory molecules. Unlike single antigen specific B cells they can take up any exogenous antigen and present them on MHCII molecules to CD4+ T cells, or on MHCI by a process called crosspresentation that is essential for CD8+ T cell priming against extracellular pathogens, tumors or viruses that do not infect DCs. DCs are several fold better at crosspresentation and regular antigen presentation than macrophages.²² The

efficiency of DCs at presenting antigens and priming naïve T cells is further supported by *in vivo* deletion of DCs or neutralization of macrophages: DC deletion resulted in major suppression of CD8+ T cell priming in contrast to macrophage neutralization.²⁴

Although MHCII presentation of antigens/peptides are mainly achieved by professional APCs, it has been recently reported that basophils,²⁵⁻²⁷ and Ito cells perform MHCII presentation of exogenous antigens and activate CD4+ helper T cells.²² Recently, DCs were proved to be dispensable for anti-helminth immunity whereas basophil depletion impaired the generation of immunity to helminthes.²⁶ Similarly, for papain (parasitic cysteine protease) induced Ova presentation to CD4+ T cells and generation of Th2 type responses, basophils has been shown to be necessary and sufficient to fulfill these functions.²⁴ In addition, endothelial and some epithelial and tumor cells were reported to express MHCII together with costimulatory molecules and HLA-DM, HLA-DO which function in the MHCII presentation pathway.²² Such examples of unconventional MHCII presenting cells, however, are exceptional. The major cell populations which activate the cellular adaptive immunity are the professional APCs, and DCs are the most effective member of this group.

3. A. DC maturation and Pathogen associated molecular pattern (PAMP) recognition receptors (PRRs)

The maturation is a reprogramming process in which DCs undergo anatomical and transcriptional/molecular changes in order for DCs to become fully competent to prime CD4+ and CD8+ T cells (i.e. activation, differentiation/polarization and proliferation of T cells).

In the immature/resting state, DCs are highly phagocytic; they constantly sample and process the exogenous antigens or lipids, and present them on MHCII, MHCI (via crosspresentation) or CD1 molecules, respectively. Endogenous antigens (lipid or protein) are continuously presented by MHCI and CD1 molecules. In the immature state, peptide-MHC molecules on the DC surface are rapidly turned over, and relatively low levels of costimulatory molecules and MHCII-MHCI are expressed on the DC surface.²² Stimulation with maturation ligands results in a transient

increase followed by a dramatic reduction in the phagocytic activity.^{28, 29} The cytoskeleton rearranges and enhances the dendritic structures. The migratory DCs move from tissues/mucosal surfaces to the draining lymph nodes. CCR7 expression increases (important for localizing DCs into T cell areas in the lymph nodes). In addition, antigen processing pathways start functioning more efficiently, acidification of the endocytic pathway is enhanced³⁰ and the peptide-MHC complexes on the DC surface dramatically increase. This is achieved by decreasing the recycling and increasing the stability of pMHCII molecules on the cell surface since MHCII synthesis is nearly shut down after a transient increase shortly after induction of maturation, at least in splenic DCs and Langerhans Cells (LC).³¹⁻³³ Maturation also augments the transcription and translation of costimulatory molecules, and secretion of several cytokines that ultimately and collectively contribute to the activation of T and polarization of CD4+ T cells into distinct subsets.

The maturation of DCs is induced by several stimuli including microbial products and inflammatory cytokines. The microbial products (proteins, lipids, DNA, RNA, bacterial/fungal cell wall) are sensed by pattern recognition receptors (PRRs) which are expressed on the surface of DCs and many innate immune cells.^{34, 35} Currently, PRRs consist of toll-like receptors (TLRs), C-type lectin receptors (CLRs), intracellular nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid inducible gene I (RIG) like receptors (RLRs). Members of RLRs and NLRs are cytosolic sensors that recognize intracellular ligands such as viral RNA³⁶ and bacterial peptidoglycan (or endogenous molecules),³⁷ respectively. CLRs are all transmembrane proteins usually used for cell-cell adhesion whose function in DCs have been shown to be involved in antigen uptake.³⁸ Mannose receptor (MR), Langerin, Dectin-1, DC-SIGN are notorious CLRs which have affinity (recognize as ligands) for the surface glycans of pathogens. TLRs are the best-characterized PRRs with 10 and 12 members in humans and mice, respectively. TLR1 through TLR9 are conserved in both species. TLRs are classified into two major groups based on their localization: TLR1, 2, 4, 5, 6 and 11 are expressed on the cell surface and recognize mainly microbial membrane components whereas TLR3, 7, 8, and 9 are localized

in intracellular vesicles such as endosomes, endoplasmic reticulum (ER), lysosomes or endolysosomes and sense microbial nucleic acids.³⁹ The most prominent ligand for TLR4 is gram negative bacteria membrane component lipopolysaccharide (LPS). TLR2 can interact with various ligands of fungal, bacterial, viral and parasitic origin which include lipopeptides from bacteria, zymosan from fungus, and hemagglutinin from measles virus. TLR2 heterodimerizes with TLR1 and TLR6 and acquires specificity for distinct lipopeptides. TLR5 recognizes bacterial flagellin. Nucleic acid sensor TLRs are also specific to different nucleic acids. TLR3 recognizes double strand viral RNA (dsRNA). Polyinosinic-polycytidylic acid (poly(I:C)) is experimentally used to stimulate TLR3 (as a mimic of viral infection) and induce type I interferon and proinflammatory cytokine secretion. TLR7, which is identified as a receptor for imidazoquinoline derivatives (imiquimod and resiquimod (R-848)), senses ssRNA from RNA viruses such as stomatitis, influenza A virus or HIV. TLR8 has a similar function to TLR7. Lastly, TLR9 recognizes unmethylated 2-deoxyribo (cytidine-phosphateguanosine) (CpG) motifs. In eukaryotes, CpG motifs in DNA are mostly methylated which allows self versus non-self distinction.

The common feature of these innate receptors is the activation of NFkB pathway which can lead to transcription of proinflammatory cytokines (such as TNF-alpha, IL-1beta and IL-6) and type I interferons through various TLR members.³⁹ On the other hand, each receptor can induce activation of unique adapter molecules culminating in different physiological outcomes. The functional heterogeneity of TLRs is exploited by DC populations in accord with their specific functions.⁴⁰⁻⁴³ Human and mouse pDCs, which are specialized for anti-viral immunity, are equipped with TLR7 and 9, whereas human cDCs express TLR1-6, 8 and 10. Interestingly, mouse cDCs express all TLRs except TLR7 in CD8+ DCs.⁴⁴ Some of the abovementioned ligands were used by several investigators to examine how ethanol affects DC maturation. The results of these studies are reviewed in the following sections.

3. B. Subsets of DCs

Since the discovery of DCs by Ralph Steinman⁴⁵ in 1973, Langerhans cells (LC) which was discovered by Paul Langerhans and mistakenly categorized as neuronal cells in the 1800s, has long been considered to be the prototype of DCs.⁴⁶ This simplistic view is only part of the current DC paradigm which consists of heterogeneous population of cells with different functions and bodily distribution for different subsets. DCs, scattered across the entire body, are classified based on the tissue location (splenic, thymic, lymph node, mucosal, interstitial), function-morphology (inflammatory, plasmacytoid), the surface markers expressed, and the lineage origin (myeloid, lymphoid) etc. [reviewed in ref. ^{46, 47}]. The investigations presented in this thesis were performed with splenic derived, conventional CD11c+ DCs without further separation into subsets. A brief summary of the most recent DC subset classification is as follows.

The entire DC content of mice fall into either of the following two categories: steady state and inflammatory DCs (Figure 1.4). *Steady state* DCs constitute the DC makeup of the body in non-inflammatory conditions and can be lymphoid resident, migratory, or peripheral. *Inflammatory DCs (iDCs)*, however, have recently been described and develop in response to pathogenic stimuli ^{47, 48} from monocytes and have been speculated to carry out crucial roles in peripheral tissues against infections.⁴⁶ *In vitro* generation of DCs from peripheral blood monocytes in the presence of GM-CSF and IL-4 notably gives rise to iDCs.^{47, 49} Steady state DCs include both *conventional* and *plasmacytoid* DCs (cDCs and pDCs). pDCs are believed to be important during viral infections as they produce substantial amounts of type I interferons.⁵⁰ pDCs are present in the bone marrow and the peripheral tissues, and are relatively long lived compared to cDCs. Also, they act as antigen presenting cells like cDCs.²³ cDC nomenclature includes both migratory (peripheral tissue resident) and lymphoid resident DCs. Lymph nodes home to both the resident (CD4+, CD8+ and Double Negative (DN)) and the migratory (CD11b+, CD103+ and LC) cDCs and pDC populations. Spleen, distinct from lymph nodes,

harbors both pDCs and cDCs but not the migratory DC component.⁵¹ All three splenic resident cDCs (CD4⁺, CD8⁺ and Double Negative (DN)) express the common surface marker CD11c.

Of interest, rat DCs have also been studied. However, our understanding of the subsets of DCs in this model organism is limited. Three subtypes of DCs have been described in rat spleen: OX62⁺CD11b⁺CD4⁺SIRPalpha⁺ (referred to as CD4⁺), OX62⁺CD11b⁺CD4⁻SIRPalpha⁻ (CD4⁻ DCs)⁵² and OX62⁻CD11b⁻CD4⁺SIRPalpha⁺ DCs that are the counterpart of human pDCs.^{53, 54} It was demonstrated that CD4⁻ DCs are the main producer of IL-12 and rat pDCs are responsible for type I IFN production.

This extensive heterogeneity in subsets is established with the help of local cues provided by the microenvironment in which the precursors differentiate into terminal DCs and likely to endow the each lineage with features that possibly cannot be replaced by another subtype which awaits confirmation by creation of subset specific DC deletion models. Despite this heterogeneity, all DCs all are able to take up process and present antigens to naïve T cells.⁴⁴

The lifespan of DCs in distinct tissues have also been studied. Splenic and mesenteric LN DCs are relatively short lived with approximate lifespan of 3 days. Thymic DCs, however, have both short and long-lived (~10 days) DCs. Langerhans cells either live longer or take longer for them to reach to cutaneous LNs.⁵⁵

3. C. Origin of DCs

Dendritic cells, like all blood cells, derive ultimately from hematopoietic stem cells (HSCs) of bone marrow. However, although pDCs are present, bone marrow contains very few developed cDCs⁴⁵ indicating a tissue specific developmental stage from precursors in DC life cycle. Indeed several reports show that peripheral lymphoid tissues (spleen, lymph nodes) and thymus^{56, 57} (though blood is also contributing) can generate its cDC content *in situ* from precursors without much contribution from blood or bone marrow.^{58, 59} Complete review of the precursors-DC subset relation is beyond the scope of this thesis and rapidly changing. Suffice it to say that HSCs differentiate into downstream common myeloid (CMP) and common lymphoid (CLP) precursors,

both of which were shown⁶⁰⁻⁶² to give rise to any of the steady-state DC subset currently known. This led to abandonment of the old lineage paradigms that DCs are of solely myeloid origin (before CLP was shown to give rise to DCs) and that CD8⁺ DCs are only of CLP origin.⁴⁴ In addition, the CLP and CMP are further divided into two populations based on the expression of FLT3 receptor. FLT3⁺ CLP and CMP were shown to be the precursors of both pDCs and cDCs.⁶³ Lastly, the ectopic expression of FLT3 receptor in FLT3 negative erythrocyte-megakaryocyte-restricted population led to development of DCs⁶⁴ signifying the important role for FLT3 and FLT3 ligand (FLT3L) during DC development. FLT3L is exploited by scientists to generate DCs both *in vitro* and *in vivo* and utilized in our studies to expand the number of DCs *in vivo* since the number of splenic DCs in mouse is few ($1-2 \times 10^6$ cells/spleen).

3. D. Antigen Presentation pathways

DCs are endowed with two distinct cell surface proteins, MHCI and MHCII, for presentation of peptides. In addition, the CD1 family of MHC-I like glycoproteins are utilized for presentation of lipids. This section will focus on MHCII presentation of exogenous antigens within the endocytic pathway as it is directly related to the studies presented in this thesis. MHCI and CD1 presentation of peptides and lipids are reviewed extensively elsewhere.^{65, 66} Nevertheless, a brief summary of them is given below.

3. D. 1. MHCI presentation

MHCI proteins can present peptides from a variety of endogenous and exogenous sources. These include defective ribosomal proteins (Drips),⁶⁷ cryptic proteins that are translated from alternative open reading frames and exon-intron boundaries, alternatively spliced transcripts translated into protein, cytosolic proteins, viral or intracellular pathogen proteins, exogenous proteins that follow the crosspresentation pathway.²² In addition, autophagy was recently shown to yield presentation by MHCI of viral peptides.^{68, 69} Endogenous proteins, which are usually tagged by ubiquitin, are processed by proteasome whose function can tightly be regulated by inflammatory stimuli. The peptide repertoire generated by the proteasome can be transported into

the endoplasmic reticulum (ER) by a specialized transporter molecule (TAP) for further trimming by ER resident peptidases (ERAAP), and subsequently loaded on MHCI molecules by the peptide loading complex (PLC) occurs.⁶⁵ Exogenous proteins, which are taken up and destined for MHCII presentation to CD4⁺ T cells along the endocytic pathway, can be diverted to the MHCI pathway and presented by MHCI to CD8⁺ T cells, a process called crosspresentation and cross priming. This process is crucial for priming of Cytotoxic T cells (CTLs) against tumors and viruses which do not directly infect DCs.⁷⁰ How antigens in the endosomes find their way to MHCI is controversial. There are studies which indicate that loading of exogenous peptides on MHCI can occur in the endosomes which contain an ER resident MHCI loading complex. In addition, proteins can also be transported from endosomes into the cytoplasm, followed by transport to ER for MHCI loading.⁷¹ Autophagy guided MHCI presentation is a recently discovered phenomenon and may follow similar routes to reach MHCI for crosspresentation.

3. D. 2. CD1 presentation

CD1 family is comprised of four members divided into three groups. Group 1 includes CD1a-c and presents mainly microbial antigens to clonally diverse T cells. Group 2 includes CD1d and presents lipids to Natural Killer T cells. Group 3 includes CD1e. CD1d and CD1b have been shown to bind endogenous lipids as well as the microbial lipids.⁶⁶ Humans express all isoforms whereas murine animals express only CD1d. The mechanisms of lipid loading of the CD1 are not delineated in detail for every members of this family, however, the surveillance of the endocytic pathway by CD1 members is considered to be the major pathway. CD1b was shown to move directly from Golgi to the plasma membrane, whereas CD1d was shown to be able to bind MHCII invariant chain (Ii) and therefore access the endocytic route from Golgi without being transported to the plasma membrane, although the importance of this pathway requires further study.⁷² Also, a major pathway for lipid loading is believed to occur within distinct endocytic compartments which receive CD1 family members delivered from the plasma membrane back into the cell.⁶⁶ In this regard, CD1a was shown to localize with recycling

endosomes whereas CD1b and mouse CD1d was shown to traffic through late endosomes and lysosomes, and CD1c revealed to be promiscuous to all three locations.⁷³

3. D. 3. MHCII presentation

Peptides utilized for MHCII presentation may be derived from a variety of sources: exogenous antigens taken up by various means of endocytosis, endosomal self proteins, cytosolic or nuclear proteins directed to lysosomes or proteins processed via autophagy. Since the investigations presented here deal with exogenous antigen presentation, we will give an overview of uptake pathways and processing of the exogenous antigens through the endocytic route.^{22, 74}

3. D. 3. A. Mechanisms of Antigen uptake by DCs

Dendritic cells can internalize exogenous antigens through a number of ways. Uptake of large particles, bacteria, dead or necrotic cells are achieved through formation of phagosomes around them in an actin dependent fashion by a process called *phagocytosis*.⁶⁵ Small particles or soluble proteins that do not interact with specific cell surface receptors are internalized by DCs via *macropinocytosis*, a process by which the antigen/particle is engulfed with the extracellular fluid in which it dissolves in also actin dependent manner.^{65, 49, 75} In addition, DCs can utilize *receptor mediated endocytosis* for antigen uptake which may be a pinocytotic (e.g clathrin mediated uptake) or even a phagocytotic process. Mannose receptor, DEC205, DC-SIGN, langerin (C type lectin family members) and Fcγ are prominent receptors that are exploited by DCs for antigen specific endocytosis. Receptor mediated endocytosis endows DCs with the ability to efficiently internalize antigens in limited concentrations. By differentially expressing the receptors in distinct subsets of DCs or utilizing a specific receptor DCs can divert the endocytosed antigens to specific presentation pathways.^{65, 76, 77}

3. D. 3. B. Antigen processing within the endosomes

Exogenous antigens, after endocytosis, undergo processing within the endosomes. This process involves several proteolytic enzymes that cleave the antigen of interest and the proactive MHCII through which both molecules are prepared for MHCII loading of the peptide (reviewed

in ref. ⁷⁴). The endocytic pathway is composed of early endosomes (EE), late endosomes (LE) and lysosomes with increasing acidity in each phase of endosome maturation creating the optimal conditions for the protease activity. The EE-LE-Lysosome axis is a very dynamic hub that receives newly synthesized MHCII molecules and proteases from Golgi as well as the plasma membrane which accumulates newly synthesized MHCII-li as a result of leaky protein sorting. Also, vesicles bearing peptide-MHCII complexes bud off the EE-LE-Lysosome axis (in almost every step) to replenish the plasma membrane resident MHCII-peptide molecules.

MHCII molecules are synthesized in a pro form which is bound to a chaperone protein called “li” that occupies its peptide binding cleft, and retains MHCII within the endosomes with its endosomal retention signal until after it is replaced with the peptide. li is cleaved by numerous proteases in a stepwise manner generating li22, lip10 and finally CLIP intermediates. Although legumain (AEP) enzyme initiates the li cleavage, AEP knock out mouse has been reported to show no defect in MHCII presentation indicating the presence of compensatory mechanisms⁷⁸ as have been the case for many proteases in this process.⁶⁵ The cleavage of lip10 in DCs, was shown to be mediated by Cathepsin S whose absence was associated with increased number of endosomes, which could be explained by the fact that lip10 cleavage by Cathepsin S results in removal of the endosomal retention signal. Upon generation of the final intermediate CLIP, which no longer carries the endosomal target signal, the appropriate peptide is immediately exchanged with CLIP by a reaction that involves H2DM. This process was confirmed with H2DM deficient APCs, whose surface was shown to be occupied by mostly CLIP-MHCII complexes.⁷⁹

3. D. 3. C. Different antigen uptake pathways are employed by DCs for targeting antigens to MHCI molecule for crosspresentation or for direct MHCII presentation.

DCs and other APCs have been studied in terms of allocation of different antigen uptake mechanisms to distinct presentation pathways by Burgdorf et al. Mannose Receptor (MR) mediated endocytosis is utilized by DCs for crosspresentation of exogenous antigens to CD8+ T

cells; pinocytosis of antigens, however, was shown to be employed for presentation to CD4⁺ T cells. Macrophages, on the other hand, use MR-endocytosed antigen for CD8⁺ T cell activation; pinocytosis and Scavenger Receptor endocytosed antigens were employed for CD4⁺ T cell activation. These observations⁷⁷ were made with *in vitro* bone marrow derived DCs; so how this translates to subsets of DCs *in vivo*, whether a subset specifically expresses a receptor more abundantly than the other or a different antigen routing is employed depending on the receptor utilized within the same cell, or if both mechanisms are in operation require further investigation. Indeed, Dudziak et al. substantiated the first hypothesis by differentially targeting OVA antigen via chimeric antibodies to two distinct subsets of splenic DCs. This study⁷⁶ demonstrated that CD8⁺205⁺ DCs diverted OVA antigen endocytosed through Dec205 into MHCI pathway, whereas the CD8⁺-DCIR2⁺ population, which endocytosed OVA through DCIR2, sent the OVA antigen mostly to the MHCII pathway. By creating human Dec205⁺ transgenic DCs and targeting the antigen to the same receptor on both subsets of DCs, they showed that preferential use of distinct presentation pathways in CD8⁺ and CD8⁻ splenic DC subsets was due to differential expression of molecular components of distinct antigen presenting pathways in both DCs rather than differential receptor expression within these cells.

3. E. DC-T cell interface: T cell activation and polarization

The priming and subsequent differentiation and proliferation of T cells by mature, peptide-presenting DCs involve myriads of expressed cell surface molecules as well as cytokines secreted by DCs, and the corresponding receptor molecules expressed on the surface of T cells. Activated DCs search for the appropriate T cell clones compatible with their peptide –MHC complexes by scanning the T cell pool in the vicinity within the lymphoid organ through transient contacts.⁸⁰ The right T cells, then, are engaged by DCs through formation of stable contacts between the two cells surfaces referred to as the immunological synapse (IS). IS formation allows the rearrangement and clustering of numerous cell adhesion and activatory or inhibitory molecules at the DC: T cell interface for a time period that lasts up to 20 hours.^{80, 81} Although the MHC-T cell

Receptor signaling (TCR) precedes the synapse formation, IS (and the components of the IS) has been proposed to have significant roles in enhancing the T cell activation and directing the effector T cell fate.⁸²⁻⁸⁴ The DC side of the synapse, compared to T cell side, is less well understood and the specific costimulatory molecules and soluble factors secreted by DCs will be briefly discussed below.⁸⁴

There are three principal signals necessary for the activation, differentiation and proliferation of a T cell into appropriate effector cell: The signal 1 is conveyed by peptide-MHC complex to TCR; the signal 2 is transmitted by costimulatory molecules like CD80 (B7.1) and CD86 (B7.2) to CD28/CTLA4; and the third signal (though not as pronounced as the former two signals) can be either a membrane bound molecule or a secreted cytokine and has been shown to polarize T cells into different effector populations as evident in Th1-Th2-Th17 and regulatory T cell differentiation from the same naïve CD4⁺ T cell given the correct combination of cytokines.⁸⁵ The requirement for the signal 2 for activating a T cell came from the work by Lafferty et al., which gave rise to the two signal model.⁸⁶ Subsequently, the absence of signal 2 was shown to lead to unresponsiveness of T cell (anergy) instead of activation.^{87, 88} B7.1 and B7.2 are the earliest costimulatory molecules defined.⁸⁹⁻⁹¹ They can both bind to CD28 and CTLA4 co-receptors on the T cell surface with a higher affinity to the latter. Although costimulatory molecules were initially thought to be necessary for activation only, the observations with CTLA4^{-/-} mice (showed fatal inflammation and excessive T cell activation) revised this hypothesis.⁹² There are two major divisions of costimulatory/coinhibitory molecules. Immunoglobulin (Ig) or sometimes called B7/CD28 family and tumor necrosis factor receptor family (TNFR) [reviewed⁹³]. B7 family members (most of which are inhibitory) are B7.1, B7.2, inducible T cell costimulatory ligand (ICOSL), programmed cell death ligand (PDL-1), PDL-2 (B7-DC), B7-H3, B7-H4, B7S3 and BTNL2. The studies which reveal that costimulation (or coinhibition) can control not only anergy-activation fate but also Treg development from naïve T cells⁹⁴ cloud the boundary between signal 3 and signal 2 but, illustrate the degree of regulation that can be

achieved by membrane bound factors in determining T cell fate. TNFR family member costimulatory molecules recruit TNFR associated adapter proteins to their cytoplasmic domains. This family of costimulators includes CD40, CD154, 41BB-L, OX40-L, CD70, and CD30L.

In addition to the membrane bound molecules, soluble factors (which are viewed as Signal 3) also contribute to effector CD4⁺ T helper cell differentiation.^{95, 96} There are a number of helper T cell subsets defined to date which rely on distinct cytokines for their differentiation and maintenance (Figure 1.5): Th1, Th2, Th17, follicular Th, inducible regulatory T cells (iTreg). Th1/Th2 division was proposed by Coffman and Mossman, 20 years ago and formed the basis for generation of two different kinds of adaptive immunity, namely cellular and humoral responses to intracellular bacteria/viruses and helminthes/parasites, respectively.⁹⁷ According to Th1/Th2 paradigm, most viruses and intracellular bacteria induce Th1 differentiation which arms the CD8⁺ T cells and macrophages for the clearance of the pathogens, whereas helminthes/parasites activates the Th2 pathway from naïve CD4⁺ T cells which in turn, activates B cells and results in humoral immunity and memory B cell generation. Recently, studies that have been conducted in autoimmune mouse models revealed a new CD4⁺ T cell subset, Th17, which is responsible for inflammatory responses during autoimmune reactions. Subsequently, Th17 cells have emerged as the third subset along with Th1/2 with significant biological functions to extracellular pathogens (i.e. fungus) in the development of host defense⁹⁸. Antigen presenting cells, exclusively DCs, were shown to assume distinct phenotypes after an encounter with or phagocytosis of distinct pathogens. Differentially activated such DCs can release numerous cytokines which ultimately drive differentiation of naïve CD4⁺ T cell to the corresponding fates.⁹⁹ The T cell intrinsic transcription factors that dictate the differentiation of naïve T helper cells (Th0) into individual subsets can be found elsewhere.⁸⁵ We will briefly describe the cytokines (of DC origin) that instruct¹⁰⁰ Th0 to differentiate into subsets as discussed above. Differentiation of Th0 cells to Th1 is achieved by IL-12. CD8⁺ DCs isolated from IL-12 deficient mice has been shown to fail to induce Th1 responses *in vivo* after transfer to wild type

animals whereas the IL-12 competent CD8⁺ DCs succeed.^{101, 102} These studies also revealed that recombinant IL-12 given with antigen pulsed DCs can induce Th1 responses. IFN- γ is a Th1 signature cytokine produced largely by Th1 cells and act on Th1 differentiation in an autocrine manner.¹⁰³ The differentiation of Th0 to Th2 is performed by IL-4.⁹⁹ IL-12 down-regulation is necessary for the Th2 induction and maintenance since IL-12 expression can restore the Th1 phenotype in established Th2 clones.¹⁰⁴ IL-4, IL-5 and IL-13 are signature cytokines of Th2 cells and IL4 acts in an autocrine fashion on Th2 cell differentiation as well. Th17 cell differentiation from Th0 is best achieved in the presence of TGF- β and IL-6.¹⁰⁵⁻¹⁰⁷ IL-21 is also thought to carry out the functions of IL-6.¹⁰⁸ IL-23, which was initially believed to guide Th0 to Th17 differentiation, is now thought to work either for maintenance of or during priming by Th17. The cytokines specifically secreted by Th17 include IL-17, IL-17F, IL-21 and IL-22. Finally, the last CD4⁺ T-cell subset to be discussed is inducible Tregs, which has regulatory/inhibitory function that is crucial in preventing autoimmunity in the peripheral tissues. They can be differentiated from naïve Th0 in the presence of TGF- β and IL-2.⁸⁵ iTregs produce large quantities of TGF- β , and are notorious for foxp3 transcription factor expression to maintain Treg phenotypic properties.

4. EFFECTS OF ETHANOL ON IMMUNITY

Chronic alcohol consumption is associated with increased susceptibility to infections by numerous pathogens including extracellular and intracellular bacteria and viruses.⁶ In this regard, lung infections caused by several bacterial and viral strains such as *S. pneumonia*, *L. pneumonia*, *K. pneumonia* and *H. influenza* have been reported to have an increased incidence in humans. A chronic ethanol fed mouse model (Lieber DeCarli diet fed) revealed more severe pulmonary tuberculosis with increased organism growth and reduced CD4⁺ and CD8⁺ T cell responses.¹⁰⁹ Jerrels et al.¹¹⁰ and Meyerholz et al.¹¹¹ demonstrated in a different chronic alcohol fed murine model (ethanol added into the drinking water) that clearance of respiratory syncytial virus (RSV)

was inhibited and susceptibility to *Hemophilus influenza* was increased. In addition to lung infections, anti microbial immunity to other infections are also adversely affected by ethanol.⁶ In humans more severe *Lysteria monocytogenes* infection was observed in alcoholics.¹¹² In a chronic ethanol-fed mouse model, susceptibility to the same pathogen was also increased accompanied by reduced immune function.^{113, 114} Hepatitis C virus infection is also a major problem in alcoholic populations and is a primary cause of accelerated liver diseases. Almost half of the alcoholics with clinical symptoms of liver disease test positive for HCV which is four times the rate of infection observed in alcoholics without liver disease.^{115, 116} This observation is based on HCV seroprevalence analyses. Indeed, HCV infectivity in alcoholics with liver disease increases up to ~64-94% when tested by real-time PCR analyses.¹¹⁷⁻¹¹⁹ The current treatment of chronic HCV is based on IFN- α and ribavirin co-administration to patients and is 50 to 90% effective in clearing the virus depending on the viral genotype.¹¹⁶ In addition, chronic alcohol consumption has been shown to blunt the response to anti-viral therapy. A comparative study between drinkers and non-drinkers showed that interferon-ribavirin therapy is effective in ~53% of non-drinkers, ~43% of those drinking less than 70 grams/day, and ineffective in those patients drinking more than 70g/day.¹²⁰ Thus, heavy drinkers are not only likely to have higher persistence of HCV, and hence to develop liver complications, but also are unresponsive to the current anti-viral therapy against chronic HCV infection.

Multiple components of the both innate and adaptive immune system, T cells, B cells, Natural killer cells (NK), monocytes, macrophages, neutrophils, and recently dendritic cells were shown to be adversely effected by ethanol. Diverse interactions of ethanol with this broad spectrum of immune cells have been extensively reviewed.^{6, 16, 121, 122} Herein, we present a brief summary of the deleterious effects of ethanol on antigen presenting cells: macrophages, B cells, DCs, and monocytes.

4. A. Effects of Ethanol on Macrophages, Monocytes and B cells

Influence of ethanol on macrophage-monocyte maturation induced by different TLR ligands, costimulatory molecule expression and allogeneic reactivity to T cells were studied by different investigators. Pruett et al. showed that acute ethanol exposure (administered by oral gavage) suppresses polyI:C induced TLR3 signaling by inhibiting p38K MAPK activation, p-cJun and p65 nuclear translocation which manifests as a reduction in IL-6, IL-12 and CXCL9 mRNA and protein production in mouse peritoneal *macrophages*.² This study has been extended to other TLR members in a subsequent report. In a mouse model, in which ethanol was intraperitoneally delivered, (up to 65mM blood ethanol levels were reached), Goral et al. showed that zymosan, LPS and CpG induced activation of TLR2, TLR4 and TLR9 mediated signaling, respectively, were impaired by acute ethanol exposure in *macrophages*. Ethanol suppressed p38 and ERK1/2 MAPK phosphorylation and IL-6 and TNF-alpha production through these receptors.^{3,4}

The deleterious effects of ethanol on the peripheral blood monocytes (PBMC) in humans and animal models have been extensively studied. *In vitro* exposure (25mM) of *human monocytes* to ethanol was shown to down-regulate TNF-alpha, IL-1Beta and up-regulate IL-10 and TGF-beta mRNA and protein levels upon stimulation with endotoxin (LPS) or staphylococcal enterotoxin B (SEB).¹²³ Acute alcohol intake studied 4 h after ingestion or *in vitro* exposure to ethanol (25 to 100 mM) also reduced allogeneic T cell activation by *human PBMC*. Mixed leukocyte reaction cultures with monocyte stimulator cells derived from acute exposure group showed decreased IFN-gamma and elevated levels of IL-10, IL-13 and this phenomenon was not due to a change in CD80, CD86 and CD40 expression.¹²⁴ In addition to macrophages, the TLR4 pathway was investigated in monocytes as well. Mandrekar et al. showed that acute *in vitro* (25 mM) alcohol exposure of *human monocytes* and CHO cell line expressing TLR4 and CD14, respectively, inhibited LPS induced NFkB signaling pathway. Ethanol achieves this by interfering with the nuclear translocation, DNA binding and transcriptional activity of NFkB which is mediated by

decreased phosphorylation of the p65 subunit of NFkB through inhibition of its kinase, IKKbeta.¹²⁵

The mechanisms of anti-inflammatory IL-10 induction in monocytes in response to ethanol have recently been studied. Drechesler et al. revealed that acute *in vitro* ethanol exposure of human PBMC augmented activity of heme oxygenase 1 (HO-1) and upregulated IL-10 in an heme oxygenase 1 (HO-1) dependent manner since the inhibition of HO-1 prevented LPS induced IL-10 elevation.¹²⁶ HO-1 is thought act through p38 MAPK as ethanol exposure resulted in an increase in LPS induced p38 phosphorylation (unlike macrophages in binge alcohol mouse model (Kovacs)³). Subsequently, Norkina et al. showed that IL-10 expression could be modulated by acute ethanol exposure via parallel pathways such as Src/STAT3 and Src/MAPK/STAT3, AP-1, and Sp-1.¹²⁷ The same investigators further studied the stimulation of the STAT pathway by ethanol. Suppressors of cytokine signaling (SOCS) are negative regulators of STAT proteins, and were shown to modulate cytokine induced activation of the STAT pathway. Norkina et al. demonstrated that pretreatment of human monocytes with ethanol results in desensitization of the STAT pathway to activation with cytokines IL-6 and type I interferon alpha due to increased SOCS1/3 activation.¹²⁸ Based on these findings we can hypothesize that elevated IL-10 levels during chronic ethanol exposure in mice and rats are probably mediated through signaling pathway components other than STATs since SOCS are also activated during chronic ethanol feeding and will be inhibiting STAT pathway.

Several studies described ethanol induced abnormalities in B cell function, and distribution in response to ethanol exposure.¹²⁹ Human studies show that long term alcohol consumption causes reduced B cell distribution in peripheral blood.¹³⁰⁻¹³² Increased IgM and IgA secretion by purified B cells before and after stimulation with immunoglobulin ligands (Staphylococcus aureus Cowan I) were found in patients with alcoholic liver cirrhosis.¹³³ Drew et al. also observed that IgA levels were elevated in the plasma of alcoholics with or without liver disease.¹³⁴ On the other hand, induction of protective humoral immunity/antibody secretion against Hepatitis B

virus (HBV) which requires the aid of T helper cells was reported to be suppressed in alcoholics after HBV vaccination.¹³⁵⁻¹³⁷ Moreover, antibody (IgG) generation against HCV NS5 protein after genetic immunization in a chronic ethanol murine model was also shown to be impaired.¹³⁸

4. B. Effects of Ethanol on DCs

Effects of acute or chronic ethanol exposure on human and murine monocyte or bone marrow derived, circulating, splenic and hepatic DCs have been studied by different groups of investigators. Their studies focused on allogeneic or specific peptide presentation, costimulatory molecule expression, cytokine secretion by DCs. In addition, investigations were performed regarding DC maturation with different ligands, differentiation-turnover, and migratory capacities.

Alloreactivity of immature DCs either generated *in vitro* from monocytes (MDC) in the presence of 25 mM ethanol, or those derived from monocytes isolated from human subjects who consumed ethanol 24 h before the isolation was shown to be suppressed.¹³⁹⁻¹⁴¹ This suppression was augmented when monocytes were from HCV positive subjects.¹⁴⁰ Additionally, tetanus toxoid antigen presentation by the MDC to T cells (recall responses) was shown to be diminished as well. Interestingly, the same study did not find any abnormalities in alloreactivity assays when MDCs are matured with LPS. Mixed leukocyte reactions with ethanol exposed DCs were also associated with decreased IL-2, IL-12, and increased IL-10 secretion.

Contrary to the *in vivo* or *in vitro* **acute** alcohol studies, peripheral blood DCs (not *in vitro* monocyte derived) obtained from **chronic** alcoholic subjects without liver disease showed increased secretion of IL-1beta, IL-6, IL-12 and TNF-alpha, whereas from those with liver disease had very low levels of IL-1beta and TNF-alpha.^{142, 143} Earlier work also reported higher levels of monocyte derived inflammatory cytokines¹⁴⁴ and higher expression of CD80 and CD86 on the macrophage cell surface.¹⁴⁵ Unlike these reports obtained with chronic alcoholic human blood circulating DCs and macrophages, Aloman et al., have found reduced secretion of inflammatory cytokines in mature and reduced expression of costimulatory molecules by mouse

splenic conventional CD11c⁺ DCs in the resting state in chronic alcohol fed murine model consistent with the acute alcohol effects observed *in vitro* and *in vivo*.⁸ In addition, Long Evans rat derived DCs, after 8 weeks of chronic ethanol feeding, showed the same pattern of reduced pro-inflammatory, and increased anti-inflammatory cytokine secretions, thus confirming the previous observations in a different system. Interestingly, the Fisher rat strain showed higher IFN-gamma and IL-12p40 but similar TNF-alpha and IL-10 levels in ethanol fed group compared to control diet fed animals which resembles findings observed in the human study described above.⁹

In vitro ethanol exposure (~8 days) was shown to inhibit FLT3L driven generation of myeloid and plasmacytoid DCs from bone marrow.¹⁴⁶ Likewise chronic ethanol feeding was associated with reduced number of LCs in the skin of mice.¹⁴⁷ In accord with these findings, Aloman et al. and Edsen-Moore et al. have reported reduced number of DCs in spleens of chronic ethanol fed murine models.^{8, 10} Edsen-Moore et al. also demonstrated that number of DCs in thymus increased. Interestingly, when healthy bone marrow was transferred into irradiated mice that are on the ethanol diet, such mice were able to restore DC numbers in the spleen comparable to levels of mice consuming the control diet. Neither did the investigators find a difference in DC turnover rate in spleen or bone marrow nor DC precursor numbers in the bone marrow between ethanol and control fed animals. This finding seems at odds with ethanol inhibition of DC differentiation *in vitro* and the reduction in splenic DC number *in vivo*. This discrepancy might be explained by the fact that the majority of the cDCs in the spleen and partly in thymus were replenished by the lymphoid organ resident precursors with little contribution from blood/bone marrow⁵⁸ that may need to be taken into account. It is also probable that the irradiation conditions might provide different signals compared to normal steady state conditions so that the effect of ethanol on differentiation of DCs from bone marrow was undetectable.

Effects of ethanol on bone marrow derived *plasmacytoid* and *myeloid* (after *in vitro* ethanol exposure) as well as *splenic* and *hepatic* cDCs after 8 weeks of ethanol feeding have been investigated. Alloreactivity of DCs were assessed in terms of stimulating T cell proliferation before and after CpG stimulation.¹⁴⁶ Ethanol suppressed alloreactivity with the exception of CpG matured hepatic DCs. In addition, significant reduction in CD40, CD80, and CD86 surface expression by bone marrow derived pDCs and cDCs were reported in both immature and CpG matured cells. Hepatic pDCs and myeloid DCs (mDCs) were resistant to maturation with CpG and splenic pDCs and mDCs showed reduced MHCII, CD86, CD80 in both immature and CpG matured cells. Our murine chronic alcohol model study suggest that, after maturation with LPS or poly(I:C), splenic cDCs can compensate for the reduced costimulatory molecule expression in the resting state (with the exception of CD86).⁸ These distinct responses of DCs to ethanol might be related to the length of stimulation time with ligands or indicate that ethanol exerts distinct phenotypic changes in response to maturation with different TLR ligands.

Lastly, the influence of ethanol on the trafficking of DCs from periphery to the secondary lymphoid organs was also recently investigated. Lau et al. found that chronic ethanol exposed (ethanol was given in the drinking water) hepatic but not splenic DCs migrated in greater numbers into the popliteal lymph nodes from the periphery after transfer to the footpad of mice independent of CCR7 and CD11a expression.¹⁴⁸

4. C. Effects of Ethanol on Immune Responses After Genetic Immunizations And the role of Defective DCs

To study the effect of ethanol on cellular and humoral immune responses to HCV proteins, our laboratory has established a mouse model which can generate an immune response by intramuscular (IM) immunization of the animals with plasmids encoding selected HCV viral proteins. Thus, following genetic immunization, DCs or fibroblasts at the site of immunization take up, express, process and present the peptides of those proteins transiently expressed from the plasmids which ultimately generate a detectable cellular as well as humoral immune response.

Both HCV core and nonstructural NS5 encoding plasmids generate detectable cellular and humoral immune responses by this method.¹⁴⁹ In a subsequent study, chronically ethanol fed animals were shown to generate significantly reduced CD4+ T cell proliferation and CD8+ T cell activity after genetic immunization with HCV core encoding plasmid.^{146, 138} This reduction in cellular immunity was successfully reversed by co-immunization with a plasmid encoding IL-2 or GM-CSF.^{150, 151} Restoration of ethanol-induced impairment in cellular immune response by GM-CSF expression implied that ethanol might alter antigen presentation by professional antigen presenting cells (APC). This idea was also supported by a mixed leukocyte reaction experiment in which ethanol treated stimulator cells but not responder cells resulted in decreased T cell proliferation.¹²⁴ Similar to findings observed with the genetic immunization model, Heinz and Waltenbaugh showed that in OVA sensitized mice, delayed hypersensitivity responses to OVA re-challenge was significantly suppressed which was indicative of inhibition of Th1 type responses.¹⁵²

To explore the possible suppressive effects of ethanol on DCs, Aloman et al. have recently compared the properties of CD11c+ DCs isolated from control and chronic ethanol fed mice, and found significant impairment in DC function.⁸ Activation of allogeneic T cells by splenic cDCs isolated from ethanol fed mice was significantly lower than the isocaloric pair-fed control group. Moreover, cytotoxic T lymphocyte (CTL) activity generated after immunization with NS5 expressing plasmid against NS5 protein expressing target cells was shown to be significantly reduced in chronic ethanol fed animals. In an elegant experiment, they revealed that the inhibition of CTL activity in ethanol fed animals can be fully corrected by syngeneic transfer of control DCs to mice at the time of immunization with the NS5 plasmid, indicating that the defect observed in CD8+ T cell mediated cytotoxicity is brought about by DC malfunction. In addition, both ethanol and control DCs had similar phagocytic and pinocytotic capability as well as MHC I and MHC II molecule expression levels. On the other hand, similar to studies in macrophages and monocytes (acute ethanol exposure models), the expression levels of cytokines produced by DCs were found

to be significantly altered in the ethanol-fed group. In this regard, increased IL-10, IL-1B and decreased IL-12, IFN- γ , TNF- α and IL-6 levels were observed in LPS stimulated and matured DCs isolated from ethanol fed mice. Apparently, the cytokine profiles of ethanol fed DCs were distorted towards a Th2 polarizing state.

5. RESEARCH AIMS:

The data from past research presented above on reduced DC alloreactivity, impaired tetanus toxoid antigen presentation by ethanol exposed DCs and restoration of impaired CTL response after transfer of healthy DCs into ethanol fed mice suggested that T cell priming/activation functions of DCs are substantially altered by long term ethanol exposure. Impaired T cell priming/activation by DCs may have been caused by several factors defective in DCs, alone or in combination; in either additive or synergistic manner. These may include reduced expression of (a) activating costimulatory signals, or overexpression of inhibitory molecules on the cell surface of DCs; (b) reduced secretion of cytokines by DCs, or (c) defects associated with antigen uptake, processing and presentation pathways in response to ethanol. Although changes in maturation induced cell surface markers expression and cytokine secretion profiles of DCs were studied, the effects on the processing and presentation of exogenous and endogenous antigens remained elusive. Osna et al recently reported in hepatoma cells that ethanol suppressed proteasomal degradation of Hepatitis C virus core peptides by 20S and 26S. This inhibition was dependent on ethanol metabolizing enzyme CYP2E1.^{153, 154} Finally, Heinz and Waltenbaugh reported that presentation of OVA to DO11.10 CD4⁺ T cells isolated from transgenic mice were suppressed when T cell- DC co cultures assessed in terms of IFN-gamma production.¹⁵²

Thus, our investigations focused on exploring the effects of ethanol on particularly presentation of exogenous antigens by DCs, and were structured as follows:

1. In Chapter 2 we aimed to explore how ethanol inhibits presentation of exogenous antigens, specifically ovalbumin protein, to CD4⁺ T cells
2. In Chapter 3, with a genome wide microarray experiment, we attempted to identify new candidate genes and gene products that might contribute to immune suppression produced by chronic ethanol exposure in immature and LPS matured DCs.
3. In Chapter 4; we have utilized two rat models of chronic ethanol consumption; one sensitive to alcoholic liver disease (ALD), and the other resistant, and compared the phenotype and function of DCs in both strains in response to chronic ethanol feeding with the aim of exploring the role of liver disease on DC dysfunction.

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CHAPTER 1 FIGURES AND LEGENDS

Figure 1. 1

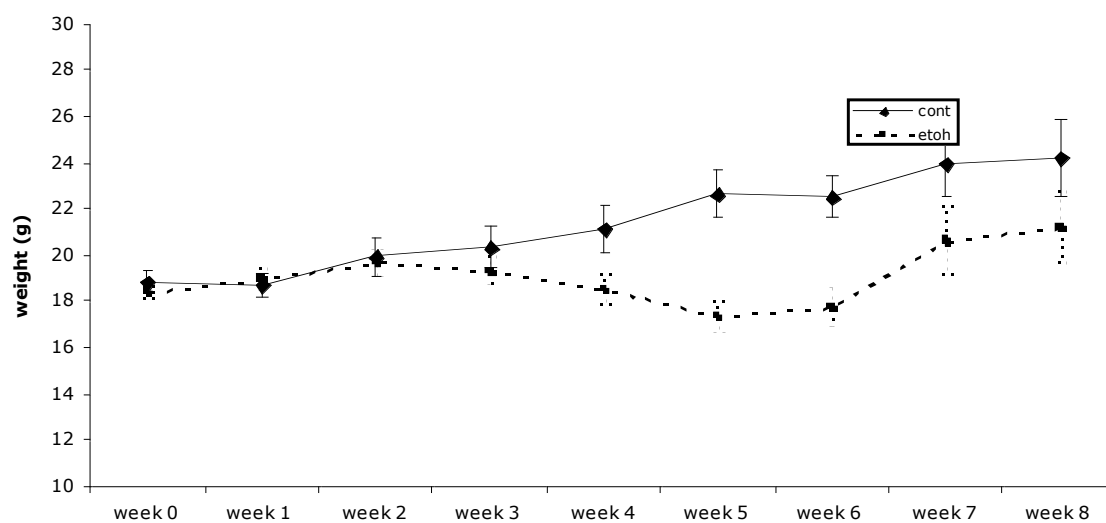


Figure 1.1 Weight chart for Balb/c mice during ethanol feeding. Control diet-fed mice are heavier during the adjustment period (week 4-7) to 24% ethanol (caloric). However, the weight difference between the control and ethanol groups decreases towards week 8th. n = 10 mice per group.

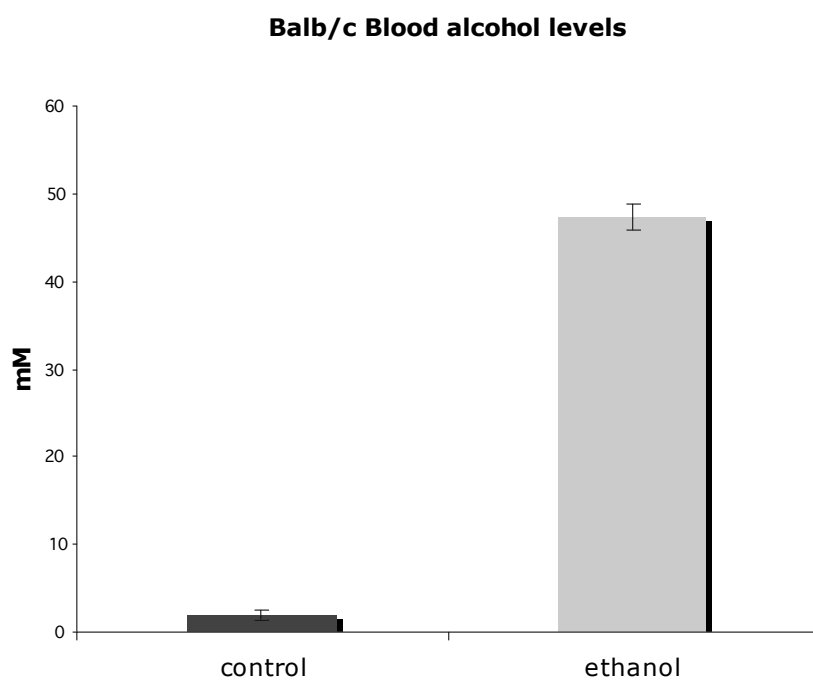
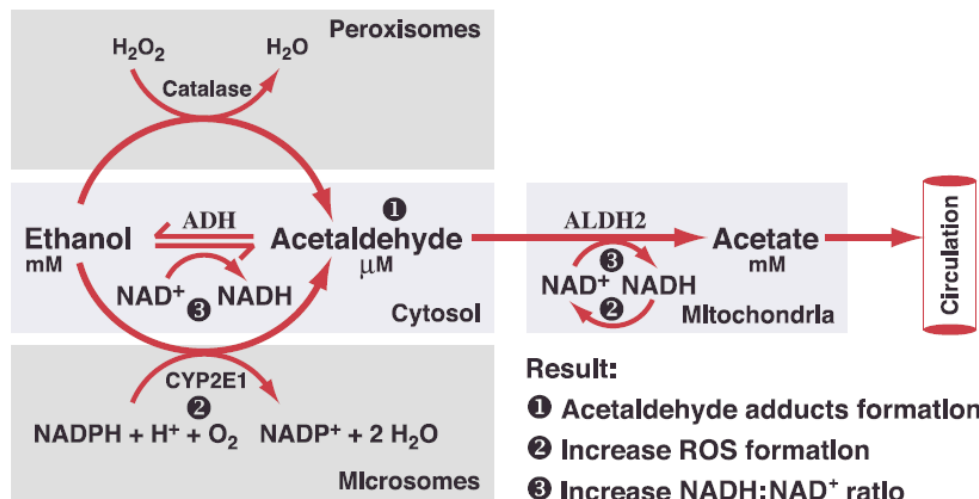
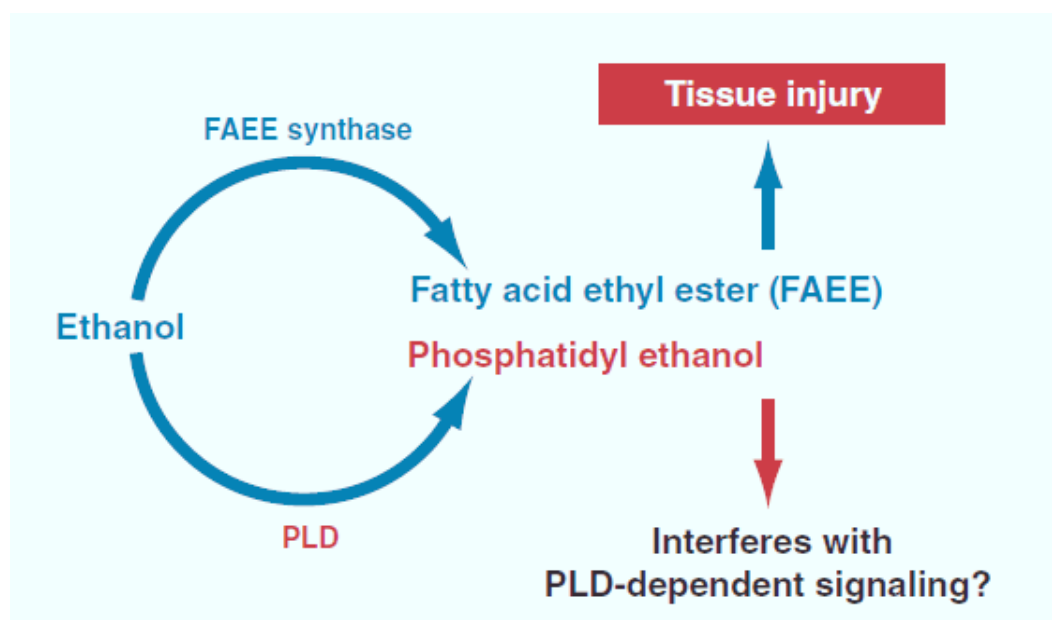
Figure 1. 2

Figure 1.2 Blood alcohol levels (BAL) of Balb/c mice during ethanol feeding. Ethanol concentration in the serum of mice on ethanol or control diet was measured via Analox GM7 analyzer. BAL reached up to ~50 mM in ethanol-fed mice.

Figure 1.3. A**Figure 1.3 A. Oxidative ethanol metabolism pathways (adapted from Zakhari S, 2006).**

There are 3 major oxidative pathways. Ethanol metabolism is primarily achieved by alcohol dehydrogenase (ADH). ADH functions in the cytoplasm and metabolizes most of the ethanol in hepatocytes. The second pathway (alternate pathway) is carried out in the presence of excess ethanol by cytochrome P450 enzymes, such as CYP2E1 and other cytochrome P450 isozymes, which function in microsomes/ER vesicles. Catalase mediated pathway is the third oxidative pathway and takes place in peroxisomes. All three pathways yield acetaldehyde as byproduct, which is readily oxidized to acetate. Acetaldehyde can form adducts with several proteins and thus affect their activities. In addition, ROS are produced during $NADH$ oxidation, particularly, during the alternate pathway. Balancing the increased $NADH/NAD$ ratio, as a result of ADH and ALDH action, results in more energy consumption by liver than usual and may cause hypoxia.

Figure 1.3. B**Figure 1.3 B. Non-oxidative ethanol metabolism pathways (adapted from Zakhari S, 2006).**

Ethanol is non-oxidatively metabolized by two pathways. The first pathway involves fatty acid ethyl ester (FAEE) synthase. FAEE is produced and released into the serum after ethanol intake. Its effect on tissues needs to be determined. The second pathway involves phospholipase D (PLD) and results in generation of phosphatidyl ethanol. The primary function of PLD is phosphatidic acid generation from phospholipids which is important for cell signaling. Phosphatidyl ethanol is generated at the expense of phosphatidic acid. Thus, many signaling processes may adversely be affected by ethanol metabolism.

Figure 1. 4

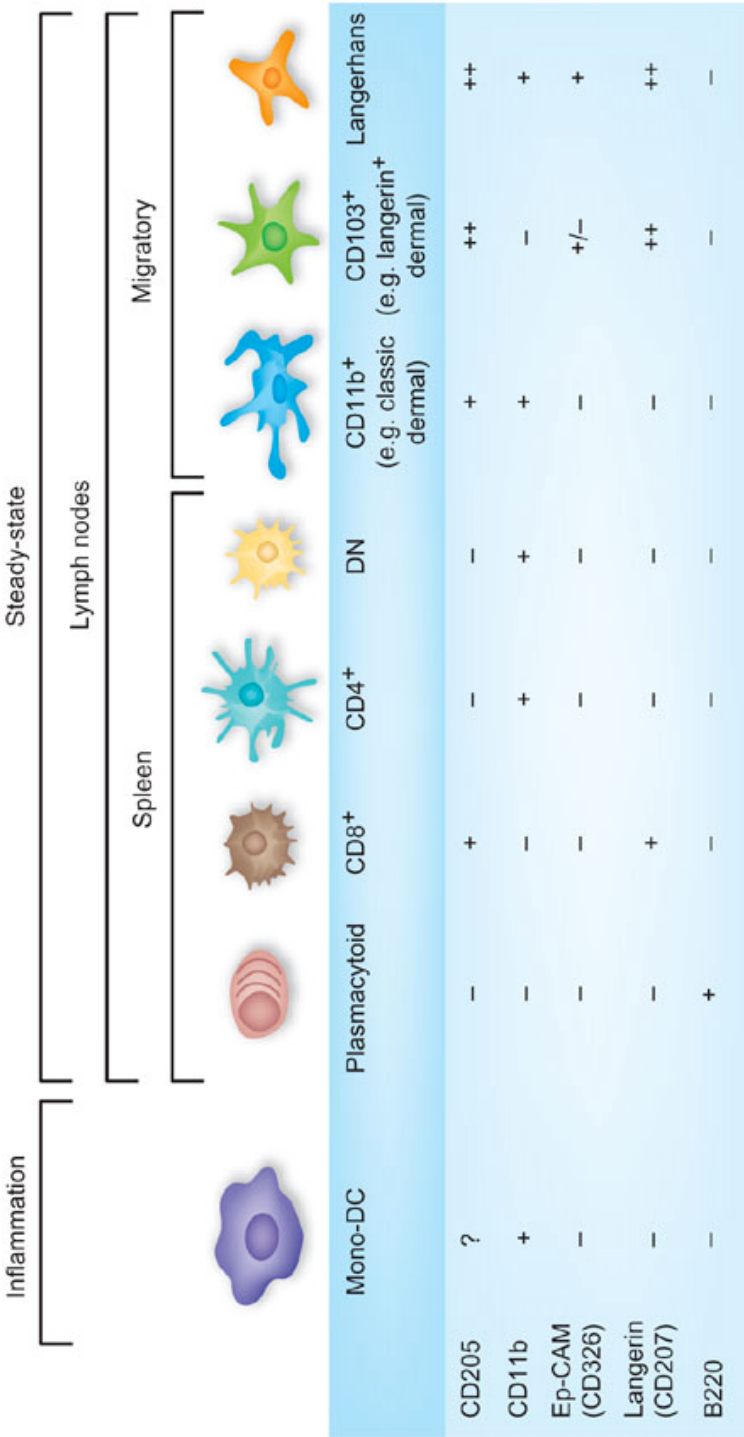


Figure 1.4 Dendritic cell subsets (Adapted from Heath & Carbone 2009). DCs at the lymphoid organs or other body surfaces can be categorized based on their cell surface markers and functions. (See text for details)

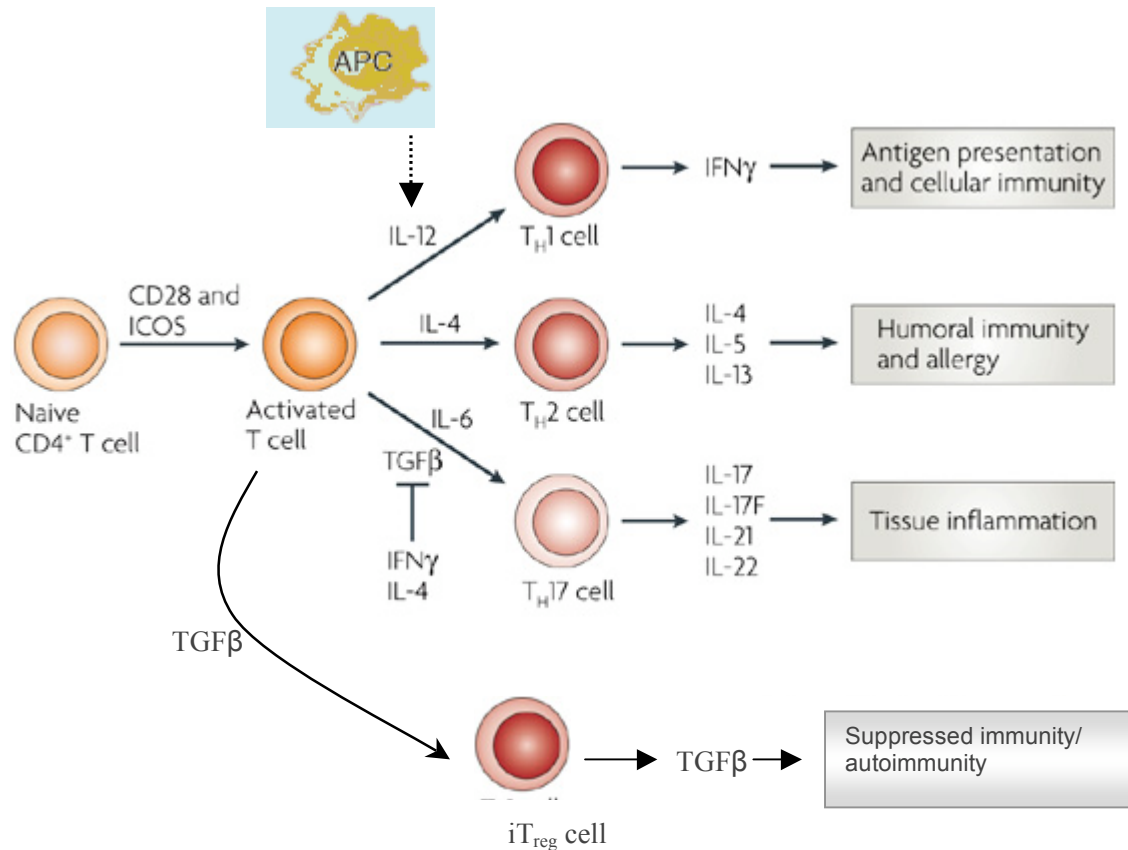
Figure 1. 5

Figure 1.5 T helper cell subset differentiation, polarizing and signature cytokines (Adapted from Dong C, 2008). After activation by DCs through the T-cell receptor and co-stimulatory molecules such as CD28 and inducible T-cell co-stimulator (ICOS), naive CD4⁺ T cells can differentiate into one of three lineages of effector T helper (TH) cells: TH1, TH2 or TH17 cells. These Th cells produce different cytokines and have distinct immunoregulatory functions. Interferon- γ (IFN γ) produced by TH1 cells is important in the regulation of antigen presentation and cellular immunity. The TH2-cell cytokines interleukin-4 (IL-4), IL-5 and IL-13 regulate B-cell responses and anti-parasite immunity and are crucial mediators of allergic diseases. TH17 cells have been shown to express IL-17, IL-17F, IL-21 and IL-22 (and IL-26 in humans) and to regulate inflammatory responses. CD4⁺ T cells can also be induced to give rise to iTregs which

suppress immune responses. TGF β , transforming growth factor- β , mediates this differentiation and is generated by iTregs as well.

CHAPTER 2:

ETHANOL SUPPRESSES EXOGENOUS ANTIGEN PRESENTATION BY SPLENIC

CD11c⁺ DENDRITIC CELLS

All of the experiments in this chapter were conducted by Ahmet Eken.

**ETHANOL SUPPRESSES EXOGENOUS ANTIGEN PRESENTATION BY SPLENIC
CD11C+ DENDRITIC CELLS**

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Abstract:

Background: Ethanol consumption suppresses effector T-cell function generated by genetic immunizations against hepatitis C virus nonstructural protein 5 (NS5). However, complete restoration of this defect could be achieved by adoptive transfer of DCs from control but not ethanol-fed mice. These observations suggest that the depressed effector T-cell responses may be due, in part, to intrinsic defects associated with antigen presenting and/or T-cell priming functions by DCs. Here, we explored the effects of ethanol on exogenous antigen presentation by DCs via MHCII molecules. **Methods:** 6-8 weeks old Balb/c or CBA/caj mice were fed ethanol or isocaloric control diet for 8 weeks. Twelve and seven days before sacrifice, mice were injected with Flt3L expression plasmid to expand the splenic DC population. DCs were purified with CD11c+ microbeads and utilized for antigen presentation, processing and peptide-MHC Class II (pMHCII) complex detection assays. IL-2 was measured via ELISA in T-cell-DC co-cultures as an indicator of antigen presentation. Antigen processing and pMCHII complexes were detected by flow cytometry.

Results: We observed that ethanol not only suppressed allogeneic peptide presentation to T-cells by both Balb/c and Cba/caj DCs, but also altered presentation of exogenous ovalbumin (OVA) protein and peptide 323-339 to an OVA specific DO11 T-cell line and OVA sensitized primary T-cells. Chronic ethanol consumption in mice did not appear to inhibit processing of exogenous antigens within the endocytic pathway. In contrast, lower amounts of peptide-MHC class II complexes were found on the DC cell surface which suggests a peptide loading or transport defect. When DCs were stripped off the contributing signals to the immunological synapse (i.e. costimulator-cytokines) by fixation, there was no difference in DC function between ethanol and control-fed mice with respect to T-cell activation. Likewise, neither supplementing the DC-T-cell co-culture with DC derived cytokines such as TNF- α , IL-6, IL-12p70 and IFN- γ (suppressed by ethanol), nor neutralizing IL-10 (up-regulated by ethanol) with antibodies was able to restore impaired antigen presentation. **Conclusions:** Ethanol inhibits exogenous and allogeneic antigen

presentation by DCs. This inhibition is mediated by synergistic effects of reduced formation peptide-MHC II complexes and intrinsic defects in DC cytokine secretion. Therefore, DCs are a critical target cell for the suppressive effect of ethanol on the host immune response to exogenous antigens.

Introduction:

Long-term excessive ethanol consumption is associated with increased susceptibility to bacterial and viral infections.¹⁻³ Ethanol has been reported to inhibit functions of multiple components of the immune system. Both innate immune cells such as neutrophils, monocytes, macrophages and recently DCs as well as B and effector T-cell components of adaptive immunity were adversely affected by ethanol *in vitro* and/or *in vivo*. Several signaling pathways including cytokine receptors, Toll-like receptors (TLR) 2, 3, 4, 9 and their downstream targets such as NF- κ B, as well as signal transducers and activators of transcription (STAT) were reported to be negatively affected by acute and chronic exposure within these cells.⁴⁻⁹ As a result, secretion of pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 were shown to be suppressed in response to both acute and chronic ethanol exposure.

In this regard, we provided evidence in a murine chronic ethanol model that CD4⁺ T-cell proliferation and cytotoxic T lymphocyte (CTL) responses generated by genetic immunizations against hepatitis C virus (HCV) core and nonstructural protein 5 (NS5) were substantially reduced compared to isocaloric pair-fed control mice.¹⁰⁻¹² Further investigations revealed that CTL activity could partly be restored by co-immunization with an IL-2 expressing plasmid, and fully be restored by co-immunization with GM-CSF expressing plasmid in the ethanol-fed mice, suggesting that antigen-presenting cells may be an additional target of ethanol action to promote impaired CD8⁺ T-cell priming. Indeed, subsequent studies demonstrated that adoptive transfer of splenic DCs from control but not ethanol-fed mice restored viral specific CTL activity in the chronic ethanol-fed animals.¹³ This finding generated the hypothesis that depressed effector T-cell functions in response to ethanol feeding may be due, in part, to intrinsic defects in DCs exposed to ethanol through alteration of antigen presenting functions. This idea was supported by the findings that alloreactivity of antigen presenting cells isolated from ethanol-fed mice was impaired compared with those obtained from control-fed mice, whereas ethanol feeding had no effect on alloreactivity (assessed by T-cell proliferation) when healthy APCs were co-cultured

with T-cells isolated from ethanol-fed mice.¹⁴ Such DC intrinsic defects that subsequently result in abnormal T-cell activation were revealed as impaired costimulatory molecule expression, cytokine secretion. However, the role of antigen processing-presentation pathways in this process remains elusive.

DCs are a heterogeneous population of professional antigen presenting cells (APCs) of crucial importance during the activation,^{15, 16} as well as recall phase¹⁷ of the adaptive immune responses against viral and bacterial pathogens and tumor cells. There is a growing list of distinct DC subsets;¹⁸ however, they are all specialized to take up, process, and present exogenous and endogenous antigens to CD4+ T-cells by MHCII and to CD8+ T-cells by MHCI molecule presentation, respectively.¹⁹ Recently, ethanol was shown to inhibit MHCI presentation of peptides by interfering with the proteasomal degradation of antigens in hepatoma cells in a CYP2E1 dependent fashion.^{20, 21} However in DCs, the influence of ethanol on antigen processing and presentation following the uptake of antigens from the extracellular milieu, as well as the relative contribution of cytokines released by DCs versus peptide-MHCII complex to ethanol induced impaired T-cell activation have not yet been experimentally addressed.

In this investigation, we explored the effects of ethanol on antigen processing and presentation following endocytosis by DCs. It was observed that ethanol not only impairs allogeneic peptide presentation, but also influences presentation of exogenous antigens by DCs. Ethanol does not inhibit the processing of exogenous antigens within the endocytic pathway. Higher peptide-MHCII complex formation was found on the control DC cell surface compared to ethanol exposed DCs *in vivo*, which may suggest a peptide loading or transport abnormality induced by ethanol. Despite the higher abundance of peptide-MHCII complex on the surface of control DCs, this quantitative difference alone was not sufficient to explain reduced T-cell activation exhibited by DCs following ethanol exposure. Likewise, this T-cell inhibition was independent of increased IL-10 and reduced TNF-alpha, IL-6, IL-12p70 and IFN- γ cytokine secretion since the neutralization of the former and addition of the latter cytokines into the culture

did not restore the reduced T-cell activation generated by ethanol effect on DCs. Thus, this study gives new insight into how ethanol may suppress cellular immunity against infections by interfering with processing and presentation of exogenous antigens in conventional CD11c⁺ splenic DCs.

Materials and Methods:

Antibodies and Reagents

FITC-CD11c, PE-CD11c, PE-TcRbeta, PerCp-CD8a, anti-mouse-FITC, anti-rabbit-FITC, anti-PE-41BB-L, anti- PE-B7DC, anti- PE-B7-H3, anti- PE-B7-H4, anti- PE-OX40L, anti- PE-ICOSL, and anti-FITC-CD86 were all product of Ebiosciences. Ovalbumin, hen egg lysozyme, LPS and rabbit anti-Ova (C 6534) were purchased from Sigma. Culture media for DCs and DO11 cells were Clone food (CF) that included 25mM HEPES-buffered RPMI supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 5 mg/mL gentamicin, 100 U/mL penicillin G, 2 mmol/L glutamine, 5×10^{-5} 2-mercaptoethanol, 1 mmol/L sodium pyruvate, and 100 mg/mL streptomycin (all from Cambrex Bio Science Walkersville, Inc, Walkersville, MD).

Animals

Six weeks old female Babl/c (H2^d), CBA/caj (H2^k), CBA/j (H2^k) and C57BL/6 mice from Jackson or Harlan Laboratories were used for ethanol feeding and the described experiments. All the procedures were approved by the Lifespan Animal Care and Use Committee.

Ethanol Feeding Regimen

Six weeks old female Balb/c or CBA mice were placed on chronic ethanol diet (Bioserv Results Liquid Diet; Bioserv, Frenchtown, NJ) that was prepared according to manufacturer's instructions as previously described.¹³ Mice were fed either the liquid ethanol or the isocaloric control diet for 8 weeks. Ethanol in the diet was gradually increased from 1.25 % (wt/vol) to 2.5% by the second, and 3.75% by the third week. Twelve days before the mice were sacrificed; they were injected with Flt3L plasmid to expand the DCs *in vivo*.

Generation of DCs

To boost the number of DCs obtained from the spleen, mice were given hydrodynamic injections with 10 µg of FLT3L encoding plasmid in 2 ml saline via the tail vein twice as previously described²². The injections were performed 12 and 7 days to the end of 8 weeks-feeding period. The animals were sacrificed at the end of 8th week. DCs were isolated from spleens using CD11c Microbeads (Miltenyi Biotech) and a magnetic separator. The purity of the CD11+ population was >95% after each isolation process.

Alloreactivity assays

Allostimulation assays were performed with negatively selected T-cells isolated from C57BL/6 spleens with pan T-cell purification microbeads (Miltenyi Biotech). Stimulator DCs were isolated from Balb/c or CBA/caj strains after 8 weeks of ethanol or isocaloric pair feeding followed by Flt3L treatment. After isolation, 10^7 DCs were incubated with or without 200 ng/ml LPS overnight. DCs were washed three times with serum free medium and co-cultured with varying number of T-cells for 3 days (for ELISA assays) or 6 days (for proliferation assays) at 37°C. For ELISA and thymidine incorporation assays, 10^5 T-cells were plated in 96-well round bottom plates. For CFSE assays 10^6 T-cells were plated in 24-well plates.

Antigen Degradation assays

Both OVA and GFP degradation were measured by two different methods. OVA degradation was assayed as previously described.²³ Briefly, OVA protein was covalently conjugated to 3µm latex beads (Polysciences) according to the manufacturer's instructions. Conjugation was confirmed by flow cytometry. 1×10^7 CD11c+ DCs derived from ethanol or control groups were re-suspended in 200 µl of CF medium. Protease inhibitors (Roche mini tablet) were added to the medium as control. Another control was also included and pulsed with unconjugated beads. All tubes were incubated at 37°C for 5 min prior to pulse with beads. 18 µl of OVA-bead or bead-only suspension was added and thoroughly mixed with the cells. After 15 min of incubation at 37°C, 1 ml cold medium was added to stop phagocytosis and the free beads were washed away

by overlaying the cell suspension on a 2 ml FBS floatation cushion. After 5min of centrifugation at 150 g 4°C, the pellet was resuspended in 1 ml medium and the overlaying step was repeated twice. Next, the cells were resuspended in 2 ml CF medium and incubated at 37°C. At various times, 300 µl was taken from tubes; spun, and lysed overnight, followed by staining with rabbit anti-OVA and FITC-anti rabbit antibodies. The samples were then analyzed by FACSCalibur.

The GFP degradation assay was performed with conjugating GFP to 3 µm immunomagnetic beads (Calbiochem) using the manufacturer's instructions. Flt3L expanded DCs derived from ethanol and control spleens were incubated with the conjugated or unconjugated beads for 5 h. The phagocytic cells were selected using magnets, and the free beads were separated by lympholyte density gradient centrifugation. Cells containing beads were incubated at 37°C, stained at different times with PE-CD11c, and examined on FACSCalibur for GFP loss. As controls, GFP-beads pulsed and fixed phagocytic cells or unconjugated bead pulsed cells were included in the analysis.

HEL48-61 presentation

CD11⁺ splenic DCs were cultured in CF containing 1mg/ml HEL protein for 25 hours. Cells (1×10^6) were removed at different times, washed with HBSS and stained with PE-CD11c and mouse Aw.3.18 antibody,²⁴ followed by staining with FITC-anti mouse antibody. CD11c positive cells were gated and analyzed for FITC signals via FACSCalibur analyses. Mean Fluorescent Intensity at each time point was subtracted from time point "0" and divided by, time point "0".
Relative MFI = $(tx - t_0) / t_0$.

Antigen Presentation Assays

Stimulator DCs, isolated from ethanol or control diet-fed mice, were pulsed with soluble OVA (1 mg/ml) or at indicated concentrations of OVA peptide at 10^7 cells/ml in CF for 20 hours or 2 hours (short pulse). Then, cells were extensively washed and used for co- culture with responder T-cells. Responder T-cells were either OVA specific D011 CD4⁺ T-cell hybridoma or

sensitized primary T-cells. When D011.10 cells were employed for the ELISA assays, IL-2 levels were measured at 18 h of co-culture.

To sensitize T-cells, balb/c mice were immunized s.q. on the back with 100 µg of OVA-CFA (Complete Freund's Adjuvant) and (incomplete) emulsion on day 0 and 7, respectively. The animals were sacrificed on day 12 to 15 and T-cells were isolated from the spleen using the pan T or CD4⁺ T-cell isolation microbead kits (Miltenyi Biotec). For ELISA measurements, 10⁵ T-cells were co-cultured with 10⁵ to 10² DCs in 96-well plates for 3 days. For the proliferation assays, 10⁶ T-cells were cultured with 5x10⁵ to 10⁴ DCs for 6 days.

Cell proliferation assay

CFSE (Molecular Probes) labeling of T-cells was performed with 5 µM CFDA. Cells were labeled in a 50 ml Falcon tube with 10⁷/ml cells in HBSS supplemented with 5% FBS. Cells were washed three times with 10 volumes of HBSS with 5%FBS and subsequently used for co-culture experiments. On day 6, the cells were harvested and stained with PE-TcRb and PerCp-CD8a. TcRbeta⁺ cell were gated and CD8⁺ and negative cells were analyzed for CFSE dilution.

Thymidine was added to 96-well plates at 1 µCi/well concentration 18 h before measurement. After labeling, cells were transferred to 96-well V-bottom plates, extensively washed (3x) and then radioactivity was determined by a TopCount scintillation counter.

ELISA assays

IL-2 (ebioscience) measurements were performed according to manufacturer's instructions.

Cytokine add-back experiments

The indicated amounts of cytokines were added to antigen presentation assay plates prior to DC and T-cells addition. Neutralizing anti IL-10 antibody was tested at 1, 10, 50 and 100 µg/ml concentrations for effectiveness. 10 µg/ml was found optimal and added into co-cultures prior to DC: T-cell addition.

Costimulatory molecule expression

Freshly isolated or mature DCs cultured overnight with 200 ng/ml LPS or polyI:C for 24 hours stained for costimulatory molecule expression, as well as for CD11c. The CD11c⁺ cells were gated and analyzed for the expression of the indicated costimulatory molecules.

Results:

Ethanol suppresses allogeneic antigen presentation

First, we confirmed the inhibitory effect of ethanol on allogeneic peptide presentation by CD11c⁺ DCs to T-cells as previously described.^{13, 25, 26} In this context, CD11c⁺ DCs isolated from spleens of both Balb/c and CBA/caj mouse strains were co-cultured with enriched T-cells derived from C57BL/6 mice spleens. Proliferation of CD4⁺ and CD8⁺ T-cells and IL-2 secretion in co-cultures were detected by CFSE labeling and ELISA, respectively. Ethanol suppressed both proliferation of and IL-2 secretion by T-cells in DCs-T-cell co-cultures (Figure 2.1.A-D).

Ethanol suppresses presentation of exogenous antigens to DO11 and primary T-cells

Next, we determined whether ethanol altered the presentation of exogenous proteins by DCs to syngeneic T-cells. To this end, presentation of soluble full length OVA by CD11c⁺ splenic DCs isolated from ethanol or control diet-fed Balb/c mice to either DO11 ova specific T-cell hybridomas or primary T-cells isolated from ova immunized Balb/c mice were compared. For the hybridoma T-cell line, IL-2 secretion, and for the primary T-cells both IL-2 secretion and CD8⁺ and CD4⁺ T-cell proliferation were measured as an indicator of T-cell activation by DCs in co-cultures. We observed that OVA loaded DCs derived from isocaloric pair-fed control mice presented antigen more efficiently to DO11 than those obtained from ethanol-fed group (Figure 2. 2A). However, when full length OVA presentation to primary CD4⁺ or pan T-cells was tested with respect to IL-2 secretion and T-cell proliferation, there were no difference between DCs derived from control and ethanol-fed mice (Figure 2. 2B and data not shown). However, the very same primary T-cells demonstrated significantly impaired IL-2 secretion during presentation of OVA323-339 peptide by DCs following incubation with the peptide confirming the DO11 T-cell

response (Figure 2. 3E a-b). This discrepancy may be explained by the fact that pulsing DCs with a single type of peptide will occupy the majority of the MHCII molecules, hence, increase the sensitivity of the assay to a unique T cell clone among primary T-cells whereas pulsing DCs with a full length protein will generate numerous epitopes and decrease the sensitivity.

Ethanol does not alter the kinetics of exogenous antigen degradation following endocytosis

Previously studies revealed that ethanol had no effect on either phagocytosis nor pinocytosis of exogenous antigens by DCs.¹³ Osna et al. reported that ethanol inhibited proteasomal degradation of peptides, thus presentation by MHCI molecules in hepatocytes.²¹ To explore the possible inhibitory influence of ethanol on the processing of exogenous antigens taken up through endocytic process (as a potential mechanism responsible for depressed T-cell activation) we compared the degradation kinetics of endocytosed OVA protein in DCs isolated from control or ethanol-fed balb/c mice. To this end, latex beads were conjugated with OVA, and CD11c+ splenic DCs were briefly pulsed with the beads. Cells were washed to remove free beads and incubated at 37°C.²³ At various times, DCs were harvested, lysed and stained with ova specific and FITC-coupled secondary antibodies, and analyzed by flow cytometry (Figure 2. 3A). As control for antigen degradation by DCs protease inhibitors were added. Moreover, uncoated beads were included as an additional control for signal specificity. There was comparable and equal degradation in DCs derived from both ethanol and control animal groups (Figure 2. 3B). This finding was recapitulated by the use of another protein (GFP) which confirms the results observed with the OVA antigen (Figure 2. 3C).

Providing DCs with OVA323-339 peptide does not overcome the ethanol inhibitory effects

To ensure that impaired antigen processing, in ethanol exposed DCs, is not responsible for diminished DO11 T-cell activation, we pulsed DCs with a short ready-to-present OVA peptide (OVA323-339) rather than the full length OVA. If the processing of proteins by DCs was defective, then, it is expected that providing cells with a short peptide fragment may overcome this inhibition. These experiments revealed that, when DCs were pulsed with low doses of OVA

323-339 peptide, presentation of the peptide by DCs derived from ethanol-fed animals was impaired to both DO11 T-cells and OVA sensitized primary T-cell (CD4+ and Pan T-cell). This result suggests that a different target for ethanol, other than antigen processing may be responsible for reduced T-cell activation by DCs (Figure 2. 3D-E).

Ethanol influences the abundance of peptide-MHCII complexes on DC surface

In order to assess the possible effects of ethanol on the presentation of specific peptide-MHCII complexes along the surface of DCs, we employed the monoclonal Aw3.18 antibody that detects HEL48-61 peptide-MHCII complex in the I-A k genetic background (Figure 2. 4A).²⁴ The kinetics of pHEL48-61-MHCII accumulation on the surface of CD11c+ DCs isolated from ethanol or control diet-fed CBA/caj mice spleens were compared at various times after pulsing cells with HEL protein for approximately 24 h. We detected higher amounts of pMHCII complexes in DCs isolated from control-fed mice (Figure 2. 4B.a). We also examined the kinetics of peptide-MHCII complex formation by pulsing DCs briefly (2 h) and subsequently monitored peptide-MHCII formation for 26 h. Higher levels of peptide-MHCII formation were observed on DCs derived from control compared to the ethanol-fed group. DCs derived from both groups of animals comparably maintained peptide-MHCII complexes on the cell surface for as long as 26 h indicating a similar turnover rate for cells surface peptide-MHCII complexes in both groups of animals (Figure 2. 4B.b).

High peptide-MHCII is not sufficient to differentially activate T-cells

To test whether lower levels of peptide-MHCII complexes found on DCs isolated from ethanol-fed mice was responsible for reduced DO11 T-cell activation, we fixed DCs after a 20 h pulse with OVA. Our hypothesis was that, if the reduced peptide presentation on the cell surface of DCs derived from ethanol-fed mice was sufficient to explain inefficient T-cell activation, by eliminating cytokine secretion by DCs via fixation would still render control DCs capable of activating DO11 T-cells more potently than DCs derived from ethanol-fed animals (Figure 2. 5A). Such an assay in which DCs are fixed prior to co-culture with T-cells emphasized the

additional need for appropriate DC cytokine secretions. However, we did not observe a significant difference in OVA presentation between fixed DC derived from chronic ethanol-fed and control animals, although control DCs were slightly better stimulators which is consistent with higher peptide-MHCII complexes (Figure 2. 5B). This finding indicates to us that impaired presentation of exogenous antigens by ethanol DCs must be due to multiple actions of ethanol on the cell such as alterations in the cytokine secretion pattern in addition to the abundance of peptide-MHCII complexes formed on DCs.

Adding back cytokines into DC:T-cell co-culture did not restore ethanol inhibition

Having demonstrated that the difference in peptide-MHCII complex abundance between ethanol and control DCs was not sufficient to explain all of the reduction in antigen presenting capacity of DCs, we explored the role of cytokines secreted by DCs. It is of interest that IL-12p70, TNF- α , IL-6 and IFN- γ secretion have been reported to be down-regulated in DCs derived from ethanol-fed mice after LPS maturation.¹³ Thus, we added back these cytokines at different concentrations into DC-T-cell co-cultures in which DO11 T-cell activation was tested by exposure of such cells to full length OVA loaded DCs. We were unable to restore this ethanol mediated T-cell suppression when the cytokines were added alone or in combination (Figure 2. 6A). In addition, IL-10 was neutralized in the co-cultures either alone or in combination with the addition of the abovementioned cytokines to test whether the T-cell activity could be restored to T-cell activation levels by DCs derived from control-fed mice. However, such experiments failed to improve the inhibition produced by ethanol which suggest that other, yet to be defined, action of ethanol may be operational.

Analysis of Costimulatory molecules

The expression of costimulatory molecules on the DC surface was assessed in response to chronic ethanol exposure. Previously, CD80, CD86, CD40 levels have been examined by us and others.^{13, 25} We extended these experiments to include other costimulatory molecules which may have activating and/ or inhibitory functions as an explanation for reduced T-cell priming. To do

this, immature or LPS and PolyI:C matured DCs were stained with anti 41BB-L, anti-B7DC, anti-B7-H3, anti-B7-H4, anti-OX40L, anti-ICOSL antibodies and as control with anti-CD86 (Figure 2. 7). Immature DCs expressed only CD86 and ICOSL with a reduced expression of the former in DCs derived from ethanol-fed animals as previously described. After maturation with LPS or polyI:C, DCs did not express 41BBL, B7-H3 and B7-H4. However, CD86, ICOSL, OX40L and B7-DC (PD-L2) were highly expressed. In addition, CD86 and B7-DC surface expression was reduced in mature DCs exposed to chronic ethanol in mice. Interestingly, higher expression levels of ICOSL was observed in DCs derived from ethanol-fed mice after maturation with both ligands. OX40L levels were comparable between ethanol and control. Thus chronic ethanol consumption has a major effect on costimulatory molecule expression on DCs which may contribute to depressed T-cell activation against exogenous antigens.

Discussion:

Suppressive effects of ethanol exposure on the ability of DCs to present endogenous peptides to allogeneic T-cells have been demonstrated by several *in vitro* and *in vivo* investigations.²⁵⁻²⁹ Inhibitory effects of ethanol on DC presentation of exogenous antigens have recently been explored as well.^{26, 30} In this regard, Mandrekar et al. showed that *in vitro* exposure of monocyte derived human myeloid DCs to ethanol inhibited presentation of tetanus toxoid antigen to memory T-cells.²⁶ Heinz and Waltenbaugh revealed that Ovalbumin presentation by CD8+ splenic DCs to DO11.10 CD4+ T-cells, purified from transgenic mice, were impaired. Furthermore, these co-cultures were characterized by reduced secretion of IL-2, IL-12p70, IFN- γ , IL-17 and IL-6.³⁰ Generation of NS5 specific CTL activity through genetic immunizations was also observed to be impaired in a chronic ethanol-fed murine model. Collectively, these studies suggest that priming of both CD4+ and CD8+ T-cells by DCs were diminished as consequence of chronic ethanol exposure.¹³ Despite accumulating evidence that reveal impaired allogeneic and autologous antigen presentation by DCs derived from chronic ethanol-fed mice compared to DCs obtained from animals on an isocaloric pair-fed control diet-fed, underlying cellular mechanisms

responsible for reduced antigen presentation have remained elusive. Previously, we demonstrated that chronic ethanol exposure did not impact antigen uptake pathways of DCs.¹³ Therefore, in this investigation, the fate of exogenous OVA protein following the uptake by DCs was explored with the aim of determining the possible effects of ethanol on the processes taking place from antigen uptake to the presentation on the cell surface to T-cells.

We utilized two mouse strains, the Cba/caj strain and previously used Babl/c strain to study the chronic ethanol exposure. Cba/caj strain is employed because it carries the H2IA^k haplotype of MHCII molecule which was required for peptide-MHCII complex detection experiments. LPS-matured DCs derived from both strains, after 8 weeks of ethanol feeding, are significantly impaired in presenting endogenous peptides to allogeneic T-cells compared with DCs derived from isocaloric pair-fed control when co-cultures were assessed with respect to IL-2 secretion and T-cell proliferation (Figure 2.1A-D). These results support previous findings on alloreactivity of bone marrow derived, splenic and hepatic murine DCs, or monocyte derived inflammatory DCs.²⁵⁻²⁸ To further study exogenous antigens presentation, DCs were pulsed with full length OVA, followed by co-culture with the DO11 T-cell line. The presentation of OVA was significantly suppressed by chronic ethanol exposure in vivo (Figure 2.2A) as indicated by impaired IL-2 secretion by T-cells. This result with the DO11 cell line also confirmed observations made with the DO11.10 transgenic mouse³⁰. In order to extend these finding to primary T-cells, DCs pulsed with full length OVA were co-cultured with T-cells derived from Balb/c mice previously immunized with full length OVA protein in Freund's adjuvant. Full length OVA loaded DCs derived from both ethanol and control diet-fed mice appeared to be equal stimulators of CD4+ and CD8+ T-cell proliferation and IL-2 secretion (Figure 2.2B and data not shown). However, when the DCs were pulsed with low concentrations of OVA 323-339 peptide instead of full length OVA (the epitope to which DO11 T-cells are reactive), both CD4+ T-cells and Pan T-cells activation by DCs prepared and isolated from ethanol-fed mice were impaired (Figure 2.3E). The observation that chronic ethanol exposure impaired antigen presentation to primary CD4+ and

CD8⁺ T cell by DCs pulsed with OVA 323-339 whereas the effect disappeared when DCs were pulsed with full length OVA could simply be explained by the loss of sensitivity in the latter case. The reason for that perhaps is that while the majority of the MHCII molecules in OVA323-339 peptide pulsed DCs will be occupied by this single epitope, full length OVA pulsed DCs will generate several 15-24aa fragments that may bind MHCII molecules. This explanation may have merit since the activation DO11 T-cell line (which has specificity to a single epitope) was significantly suppressed by DCs derived from ethanol-fed mice and pulsed with full length OVA. These observations collectively reveal that ethanol inhibits not only alloreactivity but also presentation of exogenous antigens by DCs.

To examine the influence of ethanol on the processing of exogenous proteins the degradation kinetics of endocytosed full-length OVA protein or GFP after uptake by DCs were compared (Figure 2.3). Both methods revealed that antigens appear to be processed at comparable rates by DCs derived from ethanol and control groups with an apparent short delay of degradation observed by control DCs (Figure 2. 3B-C). In order to ensure that the impaired T-cell activation by ethanol exposed DCs was not due to a protein processing abnormality, we compared the presentation of short OVA323-339 peptide by DCs isolated from ethanol and control-fed animals. We hypothesized that if the reduced presentation of OVA peptides by DCs pulsed with full length OVA protein was due to a processing dysregulation, then supplementing the ethanol DCs with short epitope would overcome the inhibition. However, the ethanol DCs were unable to present the short peptide properly to both DO11 T-cell line and primary T-cells (CD4⁺ T and Pan T-cells) as well, indicating that ethanol may have altered another component of the cellular antigen presentation pathway other than antigen processing. We pursued investigation, to ensure that the peptides generated during processing were properly loaded on and presented by MHC class II molecules. Thus, the formation of peptide-MHCII complex on the surface of DCs in response to chronic ethanol feeding was examined. Hen egg lysozyme (HEL) protein pulsed DCs (continuous pulsing) obtained from ethanol and control diet-fed animals were subsequently compared. A

relatively high level of peptide 48-61-MHCII complex was detected on the cell surface of control DCs compared to ethanol exposed DCs. In addition, some DCs were briefly pulsed (2 h) and examined for 25 h to assess whether the rapid turnover of the complexes on the DC cell surface was responsible for reduced peptide-MHCII presentation in ethanol DCs. We observed that the peptide-MHCII complexes were sustained on the cell surface over time in both DCs derived from ethanol and control-fed animals. However, the quantitative difference between two groups remained the same (Figure 2.4.B.a). These observations suggest an abnormality in peptide loading, peptide-MHCII transport to cell surface in ethanol exposed DCs which will require further investigation. Aloman et al. reported comparable levels of MHCII expression in polyI:C and LPS matured ethanol and control group splenic DCs, whereas Lau et al. observed reduced MHCII expression after CpG maturation.^{13, 25} Therefore, it is also possible that ethanol may alter the amount of MHCII molecule expression on DCs.

Antigen presentation to T-cells involves immunological synapse formation by which the DCs deliver three principal signals to initiate CD4⁺ T-cell activation and polarization.^{31, 32} The first signal is conveyed by T-cell receptor (TCR) – MHCII interaction; the second is delivered by costimulatory molecule-co-receptor engagement; and the third signal is transmitted by soluble factors (cytokines) that polarize the T-cell. We have reasoned that by eliminating signal 3, and to a certain extent signal 2, one can assess whether peptide-MHCII abundance difference that exists between ethanol and control DCs functionally translate into activation of T-cells. To this end, OVA pulsed DCs were fixed and used as antigen presenters (Figure 2.5B). They were co-cultured with T-cells, but were less potent activators compared to unfixed DCs since there would be no cytokine secretion by DCs under these experimental conditions. Second, fixation also altered the dynamic structure of the cell membrane; therefore, a true synapse formation would not occur since the organization of costimulatory molecules around TCR-MHCII complex requires a dynamic cell membrane. Thus, T-cell activation in these co-cultures could be primarily attributed to TCR-MHCII interaction. In this context, we observed specific DO11 T-cell activation by OVA

loaded DCs compared to an antigen specificity control such as HEL loaded DCs. Although a higher trend in T-cell activation by control DCs was observed compared to DCs derived from ethanol-fed mice, the level did not achieve statistical significance, which indicated that either costimulatory molecules or soluble factors secreted by DCs are required to create the observed differential T-cell activation exhibited between control and ethanol DC. Ethanol may substantially inhibit these two synaptic components and promote an additive effect in affecting the differential T-cell activation, in the setting of a known peptide-MHCII complex formation on the cell surface of ethanol exposed DCs. In all likelihood, chronic ethanol exposure probably alters all three components of DC:T cell synaptic formation and subsequent antigen specific T-cell activation.

Aloman et al. reported that chronic ethanol feeding of mice resulted in decreased IL-12, IL-6, TNF- α , IFN- γ and elevated IL-10 secretion by mature splenic CD11c⁺ DCs. We have hypothesized that if the soluble factors are of prime importance in mediating the inhibition of antigen presentation, adding back those cytokines (which were suppressed), or neutralizing IL-10 (which was up-regulated) could then restore the suppressive effect of ethanol on DC function. Neither “add-back” experiments of IL-12, IL-6, TNF- α , IFN- γ , nor neutralizing IL-10 alone or in combination, restored the impaired DO11 T-cell activation by DCs derived from ethanol-fed animals (Figure 2. 6). The functionality of anti-IL-10/and neutralization was separately tested by measurement of Th1 and 2 cytokines in Concanavalin A stimulated splenocytes (data not shown). Thus, we may suggest that the abovementioned cytokines (signal 3), whose secretions were altered, are not solely responsible for the reduced DO11 T-cell activation by ethanol exposed DCs. These results, taken in the context of OVA presentation, are different from the findings by Szabo et al. on allogeneic presentation. Unlike these allogeneic assays, where IL-12 addition restored the impaired T-cell proliferation driven by ethanol exposed allogeneic DCs, we observed that defective OVA antigen specific presentation by DCs derived from ethanol-fed animals could not be restored by addition of recombinant IL-12 back to DC:T cell co-cultures when T –cell

responses assessed in terms of IL-2 secretion.³³ However, the difference in experimental approaches including the antigen of interest may explain these discrepancies.

We also explored the role of costimulatory molecules (signal 2) expressed by DCs. The reduced expression of CD80, CD86, CD40 by mature (CpG) and immature splenic DCs derived from chronic ethanol-fed murine models has been established. To gain further insight into levels of activating and inhibitory costimulatory molecules, additional experiments were performed detailing OX40-L, ICOS-L, B7DC, B7-H3, B7-H4, 41BB-L and CD86 expression by DCs (Figure 2.7). We observed that B7-H3, B7-H4 and 41BB-L were not expressed by neither polyI:C nor LPS matured or immature DCs. Again, CD86 was down regulated in both resting and mature DCs as previously described. OX40-L exhibited comparable expression in ethanol and control DCs following maturation. However ICOS-L was consistently found to be highly expressed in ethanol exposed LPS and polyI:C matured DCs. Recently, high ICOS-L expression was reported to induce naïve CD4⁺ T-cells to produce IL-10, and these cells subsequently were found to serve as Tregs.³⁴ Finally, B7DC levels were consistently lower in mature DCs derived from ethanol-fed animals; yet its immunologic function and significance will require further study.

In summary, our investigations demonstrate that both endogenous antigen presentation by DCs to allogeneic T-cells and exogenous OVA presentation to DO11 and primary T-cells were impaired during chronic ethanol consumption in mice. Our results suggest unaltered processing of exogenous antigens in the endocytic pathway. However, there was reduced peptide-MHCII complex presentation by DCs derived from chronic ethanol-fed mice. Higher peptide-MHCII complexes were found on the surface of control DCs; yet this phenomenon was not sufficient to differentially activate T-cells without the contribution of DC derived secreted cytokines or costimulatory molecules or both. In addition, reduced T-cell activation by ethanol exposed DCs cannot be restored by addition of cytokines such as IL-12, IL-6, TNF- α , IFN- γ back to DC:T-cell co-cultures or by neutralizing IL-10. These results suggest that the reduced T-cell activation by ethanol exposed DCs is complex and may involve synergistic effects of abnormalities in

costimulatory molecule levels, cytokines secretions, as well as reduced peptide-MHCII abundance on DC cell surface.

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CHAPTER 2 FIGURES AND LEGENDS

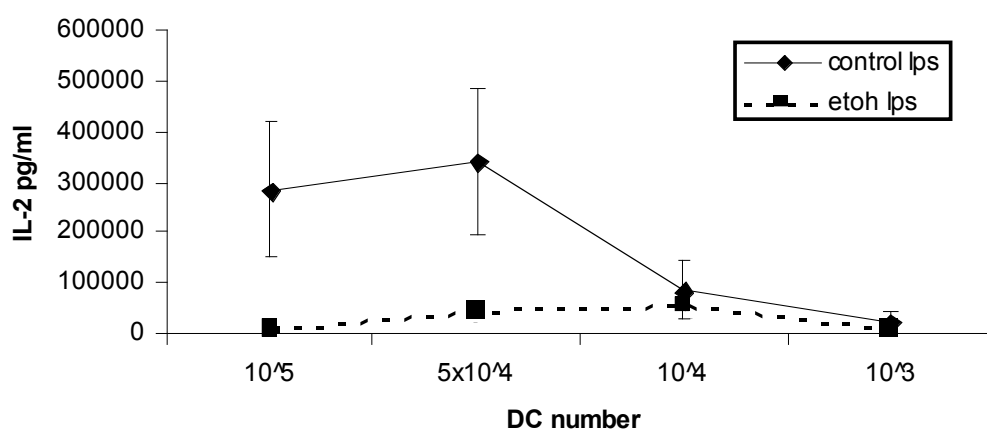


Figure 2.1.A. Chronic ethanol exposure in mice suppresses endogenous peptide presentation by CBA/caj DCs to allogeneic C57BL/6 mouse T cells (Assessed in terms of IL-2 secretion).

LPS matured splenic CD11c⁺ DCs were purified from ethanol or isocaloric pair-fed control diet CBA/caj mice and cultured for 3 days with negatively selected T-cells obtained from C57BL/6 mice. IL-2 levels in co-cultures were measured via ELISA as indicator of T-cell activation.

Control DCs were significantly more potent in activating T cells ($p < 0.05$).

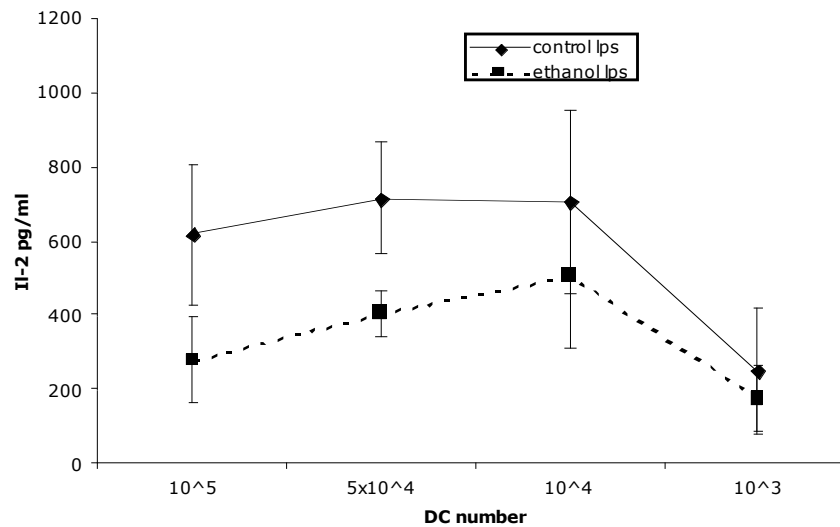


Figure 2.1.B. Chronic ethanol exposure in mice suppresses endogenous peptide presentation by Balb/c DCs to allogeneic C57BL/6 mouse T cells (Assessed in terms of IL-2 secretion).

DCs were purified from ethanol or control diet-fed Balb/c mice, and cultured with C57BL/6 T cells. Balb/c DCs derived from control diet-fed mice were also significantly more potent activators of T-cells ($p < 0.05$).

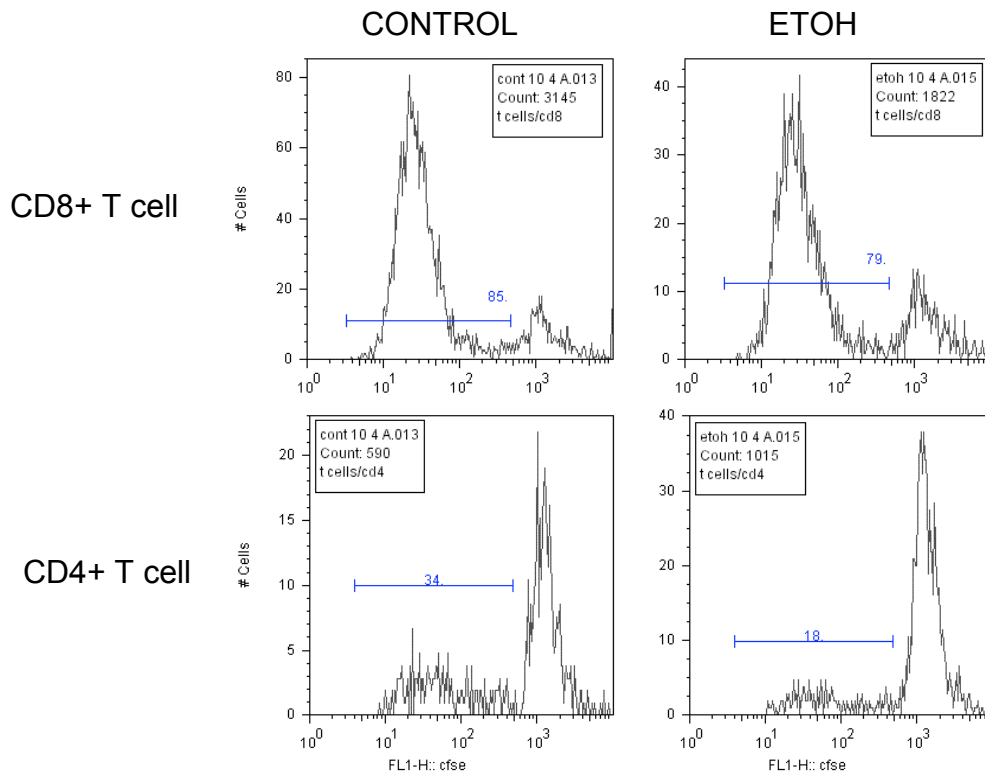


Figure 2.1.C. Chronic ethanol exposure in mice suppresses endogenous peptide presentation by CBA/caj DCs to allogeneic C57BL/6 mouse T cells (Assessed in terms of T cell proliferation). T cell proliferation responses were assessed by CFSE dilution via Flow cytometry. Ethanol or control diet-fed CBA/caj mice derived DCs were co-cultured with C57BL/6 derived T cells. Labeled T-cells were cultured for 5 days with DCs, T-cell receptor beta (TCRb)⁺CD8⁺ or TCR⁺CD8⁻ cells were gated for analyses. Proliferation was reduced in ethanol DC co-cultures (Data presented is representative of several experiments).

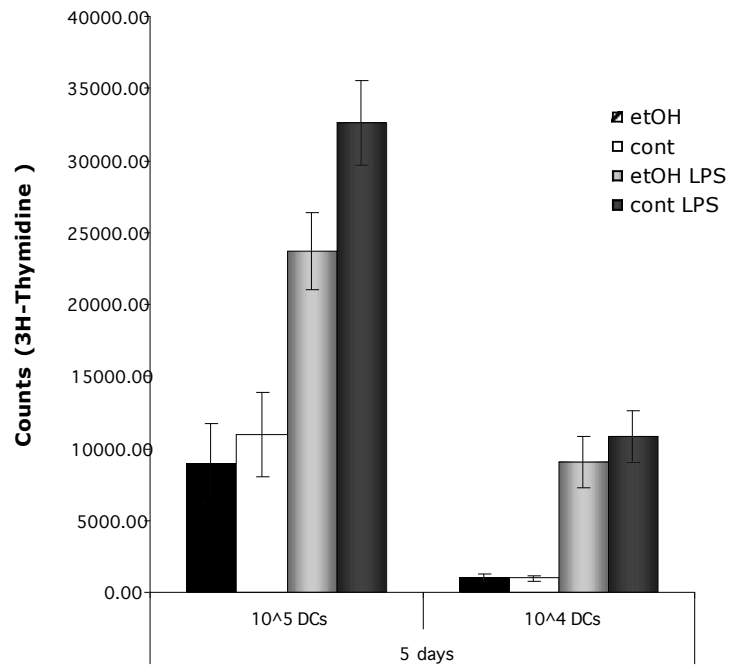


Figure 2.1.D. Chronic ethanol exposure in mice suppresses endogenous peptide presentation by Balb/c DCs to allogeneic C57BL/6 mouse T cells (Assessed in terms of T cell proliferation) Ethanol or control diet-fed Balb/c DC were co-cultured with C57BL/6 derived T-cells. ³H-thymidine incorporation was used to monitor cell proliferation. T-cell proliferation induced by mature Balb/c DCs derived from chronic ethanol-fed mice was significantly reduced ($p < 0.05$).

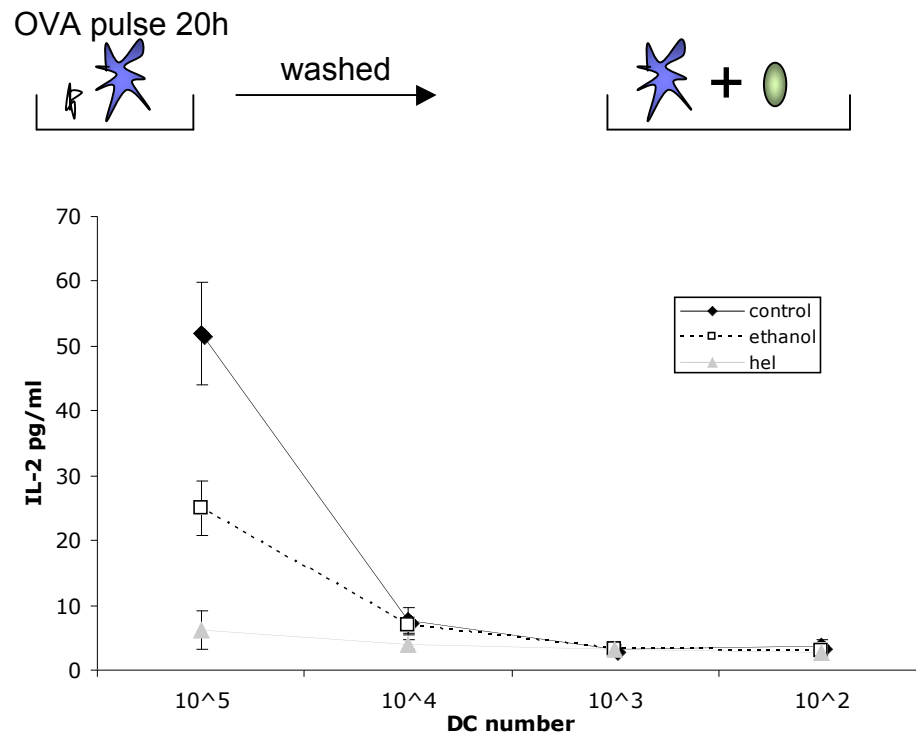


Figure 2.2.A. Chronic ethanol consumption impairs presentation of exogenous full length OVA protein by DCs to DO11 T cells. DCs obtained from ethanol or control diet-fed Balb/c mice were pulsed with full length OVA for 20 h and co-cultured with DO11 T-cell line overnight. Subsequently, IL-2 levels were measured in the supernatant via ELISA as an indicator of antigen presentation capability of DCs. DCs derived from control-fed mice presented OVA more efficiently than those derived from the ethanol-fed animals ($p < 0.05$).

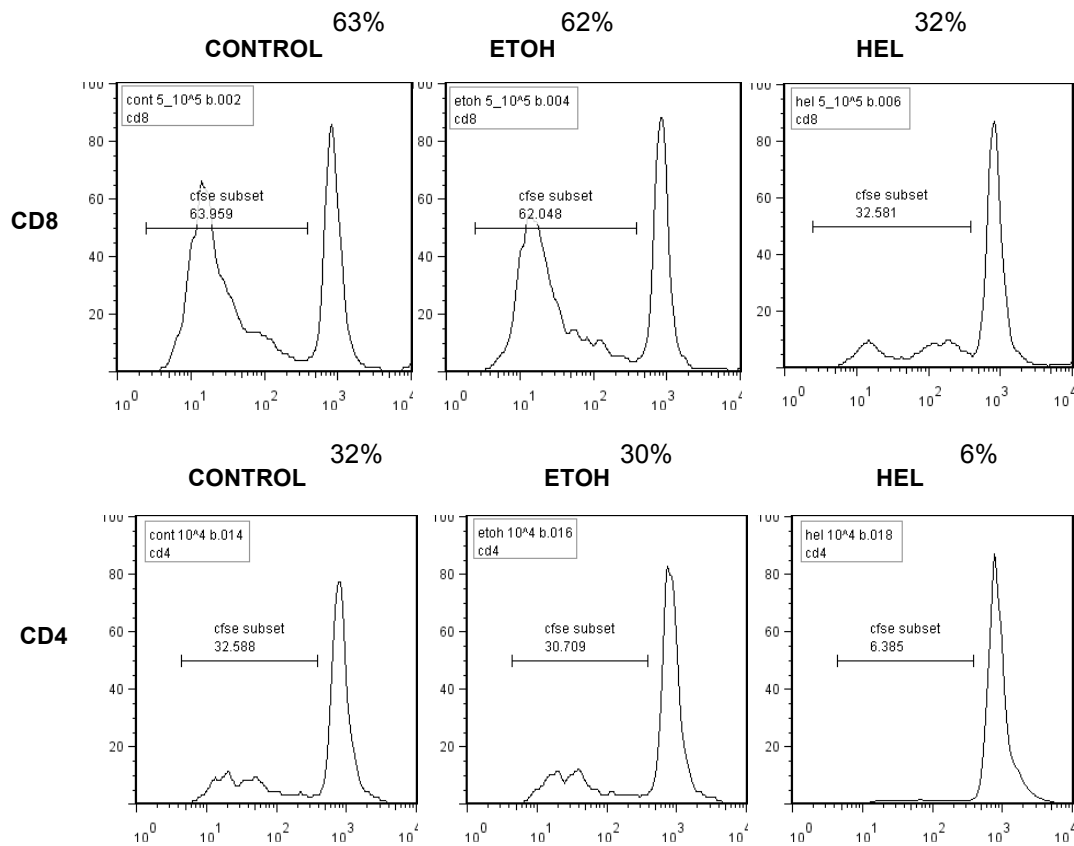


Figure 2.2.B. Chronic ethanol consumption does not effect presentation of exogenous full length OVA antigen to primary T cells. Proliferation of primary CD4⁺ and CD8⁺ T cells purified from OVA immunized mice were monitored by dilution of CFSE 5 days after culture with OVA or Hen egg lysozyme (HEL) pulsed DCs. Both ethanol and control DCs stimulated comparable T-cell proliferation.

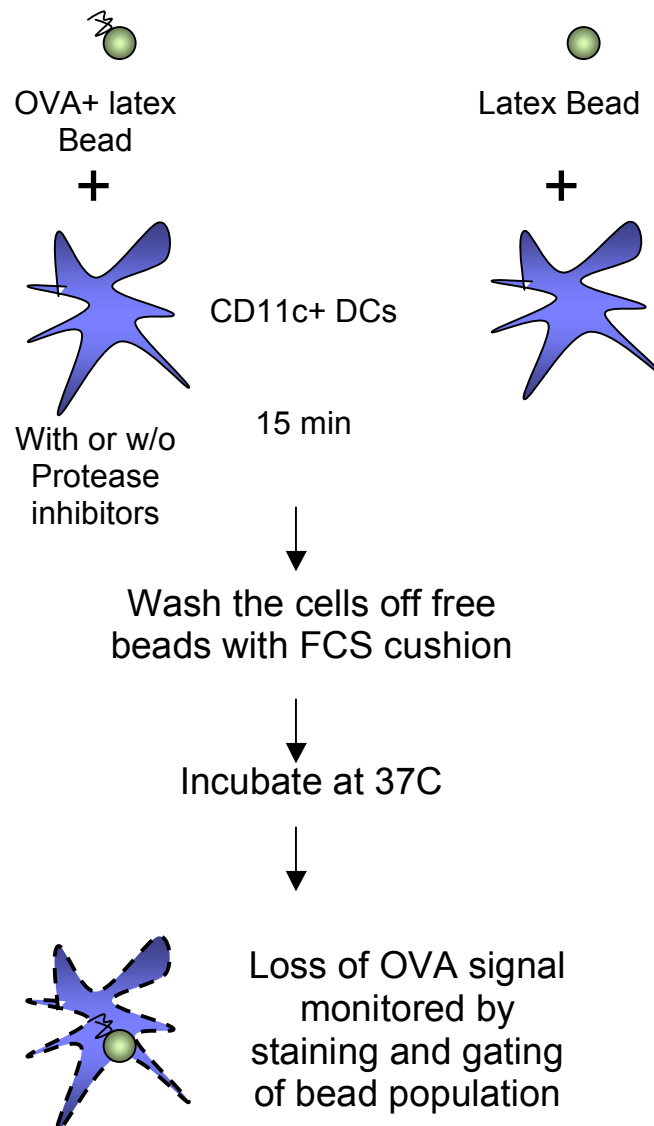


Figure 2.3.A. Chronic ethanol consumption in mice does not alter the ability of DCs to process exogenous endocytosed antigens. Schematic description of the antigen degradation assay using 3 μ m latex OVA coated bead pulsed CD11c⁺ DCs presented in (B).

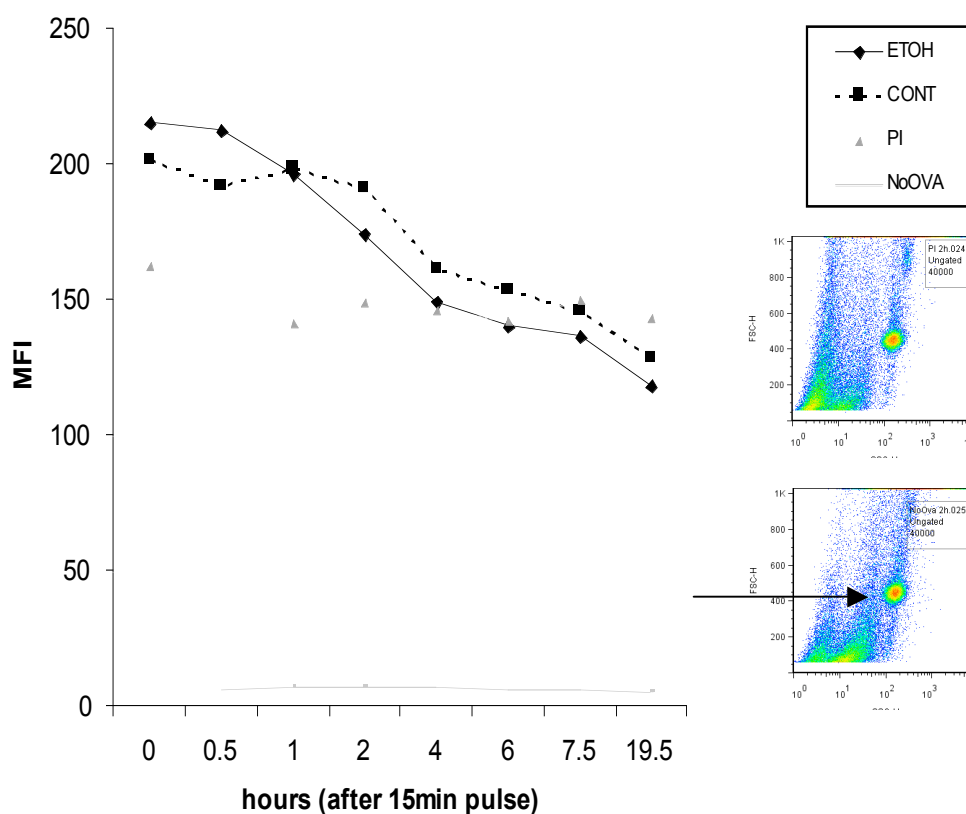


Figure 2.3.B. Chronic ethanol consumption in mice does not alter the ability of DCs to process exogenous endocytosed OVA protein. DCs derived from control and ethanol-fed mice degraded OVA with similar kinetics. As a control, DCs were pulsed with uncoated beads (NoOVA) or OVA coated beads in the presence of protease inhibitor cocktail (PI). The former (negative control) showed no signal; the latter (positive control) showed uniform signal across different time points.

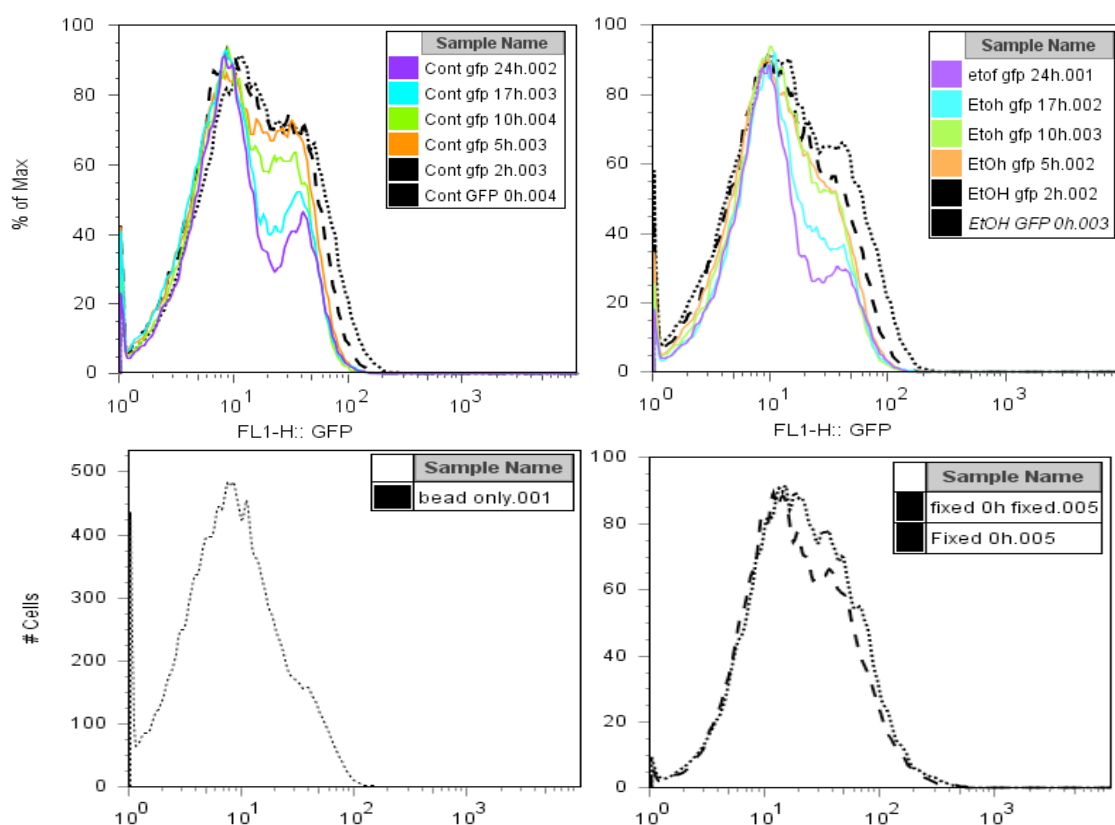


Figure 2.3.C. Chronic ethanol consumption in mice does not alter the ability of phagocytic cells to process exogenous endocytosed GFP. Antigen degradation assay was performed with GFP protein coated beads. Unlike experiment presented in Figure 2.3B, FLT3L expanded splenocytes were pulsed with GFP coated magnetic beads or uncoated beads (bead only). Phagocytic cells were selected through a magnetic field, cultured at 37°C and monitored over time for degradation of GFP. As further controls, DCs were pulsed with GFP-bead and fixed to ensure that the signal loss is specific for proteolytic degradation.

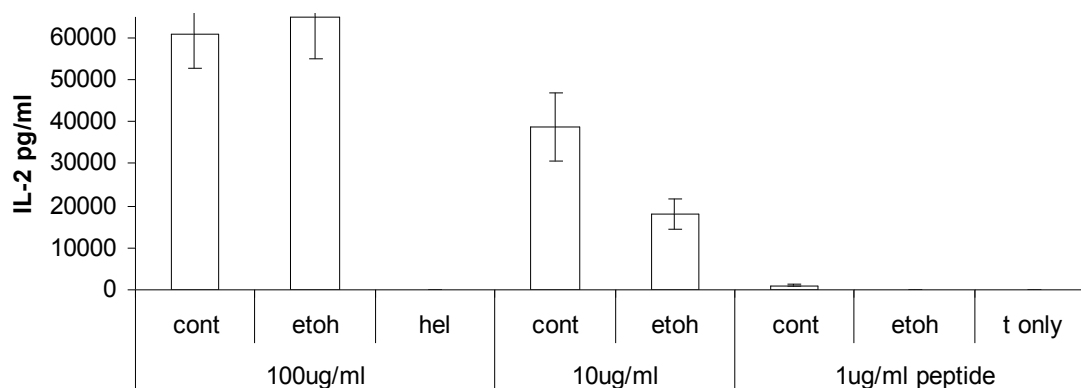
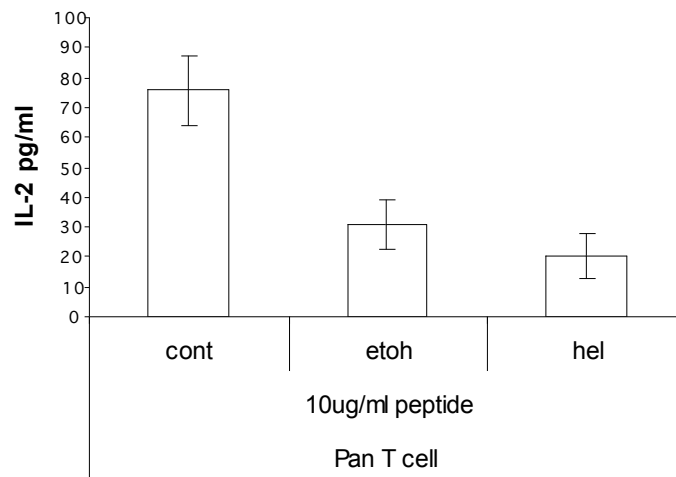


Figure 2.3.D. Providing DCs with ready-to-present OVA323-339 peptide at low doses did not restore impaired antigen presentation to DO11 T cells. DCs were pulsed with OVA323-339 peptide at different concentrations and co-cultured with DO11 T-cells for 18 h. Next, IL-2 levels were measured via ELISA. DCs derived from chronic ethanol-fed mice were unable to restore impaired OVA antigen presentation. The (hel) contains DCs pulsed with HEL protein and used as specificity control. The (t only) is used as a negative control and lacks DCs.

a)



b)

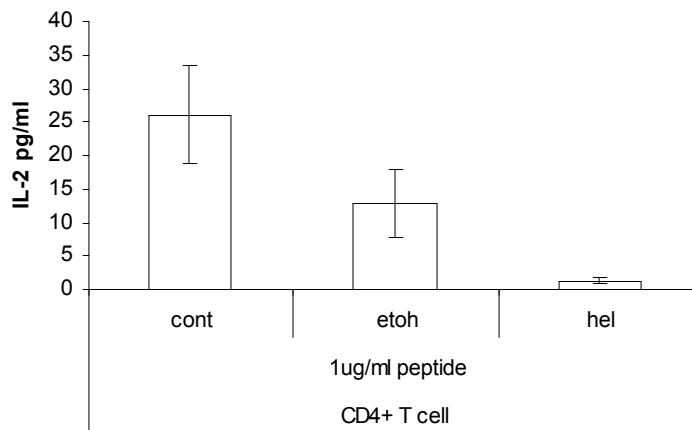


Figure 2.3.E. Providing DCs with ready-to-present OVA323-339 peptide did not restore impaired antigen presentation to Primary T cells. DCs were pulsed with OVA323-339 peptide and co-cultured with pan T-cells **(a)** or CD4⁺ T-cells **(b)** isolated from mice previously immunized with OVA protein. Next, IL-2 levels were measured via ELISA. DCs derived from chronic ethanol-fed mice were unable to restore impaired OVA antigen presentation. The (hel) contains DCs pulsed with HEL protein and used as specificity control.

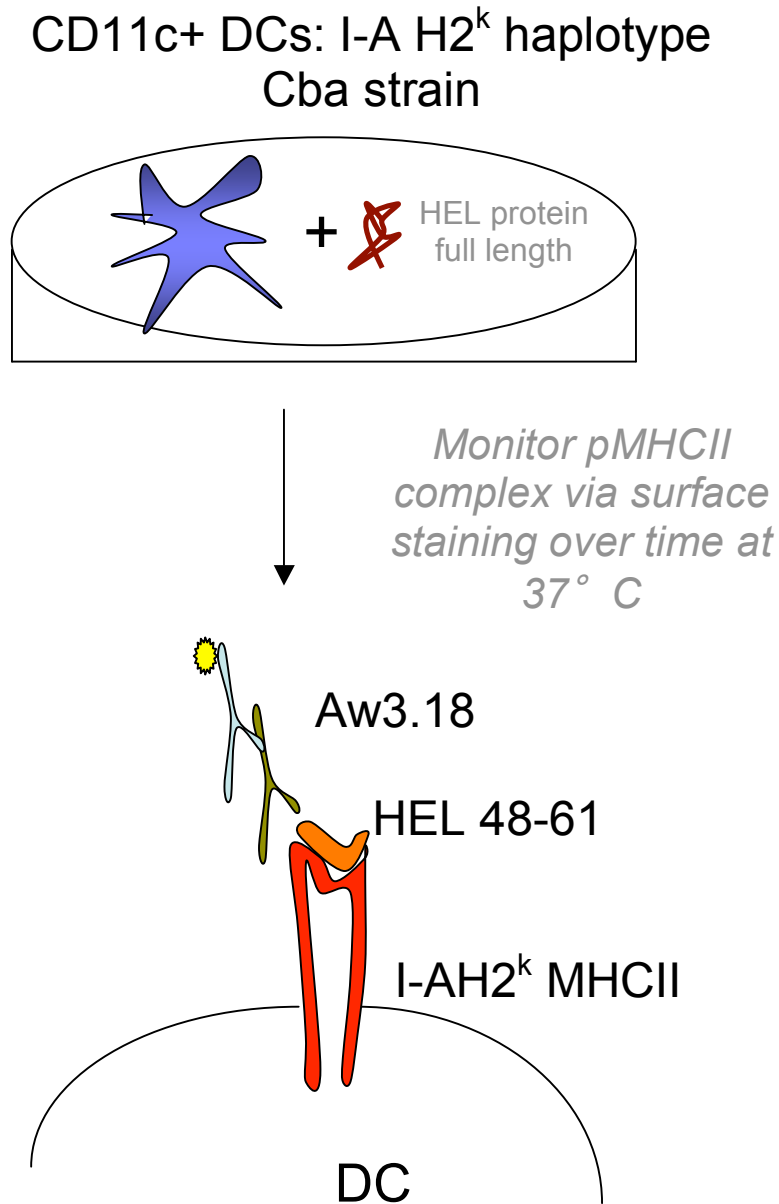


Figure 2.4.A. Chronic ethanol consumption in mice results in reduced peptide-MHCII complex presentation on the cell surface of DC. Schematic description of the strategy for analyzing HEL48-61-MHCII complex formation on the cell surface

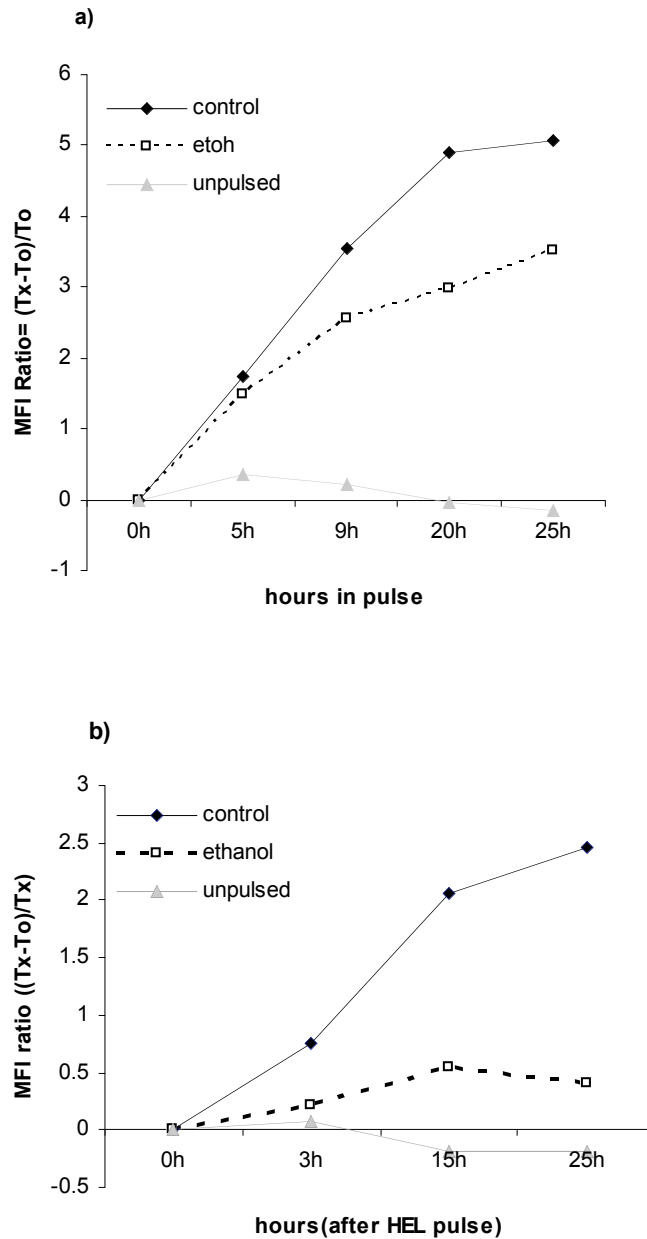


Figure 2.4.B. Chronic ethanol consumption in mice results in reduced peptide-MHCII complex presentation on the cell surface of DC. a) CD11c⁺ splenic DCs isolated from control or ethanol diet-fed mice were pulsed with HEL protein. At different time points, they were stained with AW3.18 antibody followed by FITC-anti mouse-IgG and visualized via FACSCalibur. The signal for each time point is calculated by (tx-t0)/t0. Unpulsed DCs were used

as negative control. DCs derived from control diet-fed mice displayed higher pMHCII levels compared with those isolated from ethanol-fed animals. The graph is representative of three independent experiments. **b)** DCs are pulsed with HEL for only 2 h, washed, and incubated at 37°C for analyses of peptide-MHCII complex in order to determine whether the turnover of pMHCII is different in response to ethanol exposure. pMHCII complexes were sustained on the cell surface comparably by DCs derived from ethanol and control diet-fed mice.

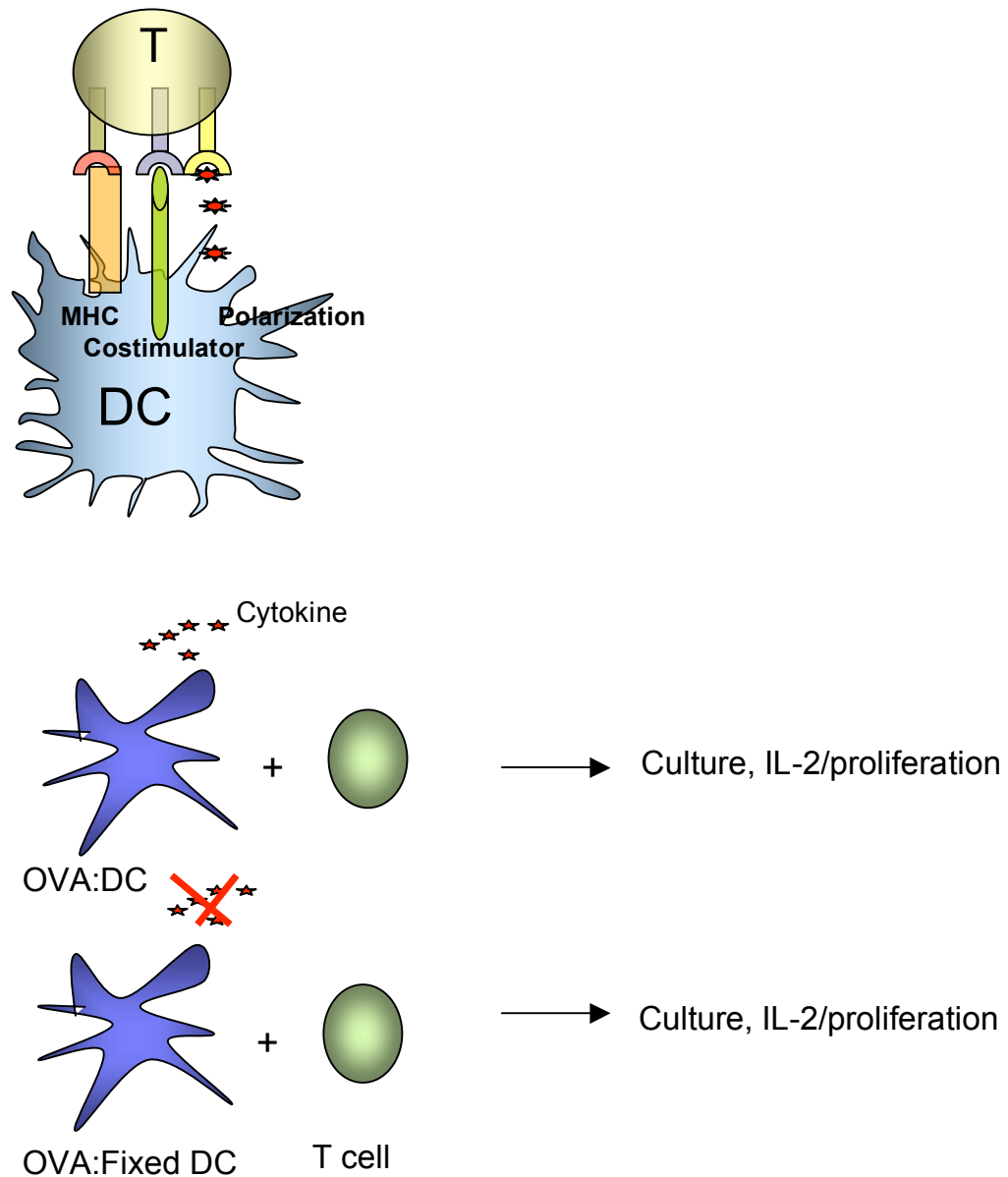


Figure 2.5.A. High levels of p-MHCII formation on DCs alone were not sufficient to differentially activate T cells. The top figure shows three principal signals provided by DCs to activate T-cells. Bottom figure is a schematic description the design of the assay in Figure 2.5B. DCs were fixed with formaldehyde to eliminate signal 2 (costimulator) and signal 3 (cytokines).

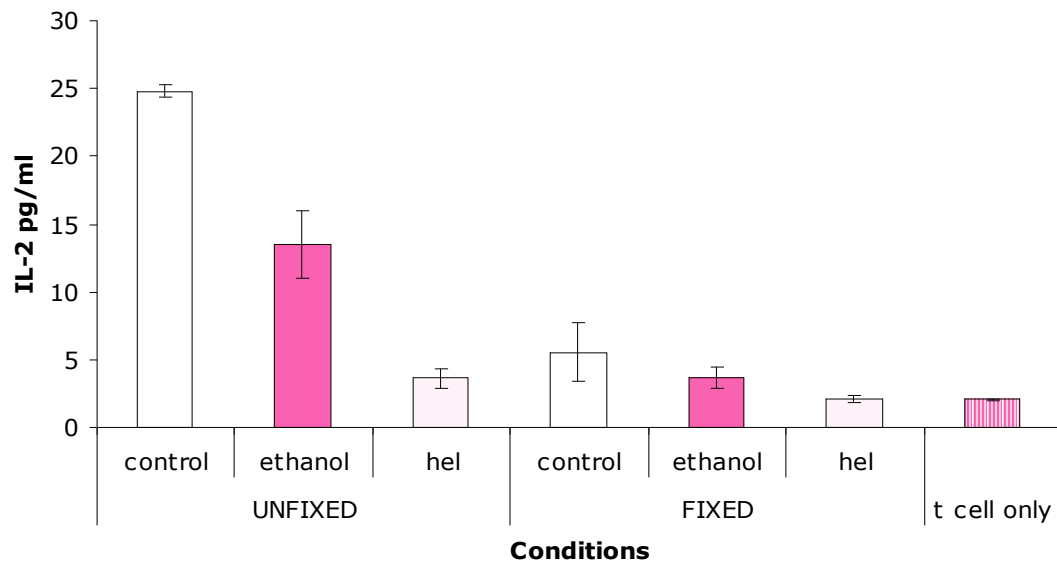


Figure 2.5.B. High levels of p-MHCII formation on DCs alone were not sufficient to differentially activate T cells. OVA or HEL-pulsed CD11c⁺ splenic DCs were fixed before co-culture with DO11-T cells. Subsequently, IL-12 levels were measured after 18 h culture by ELISA. Fixed DCs derived from both ethanol and control diet-fed mice presented OVA comparably in the absence of DC cytokine secretion and/or dynamic membrane environment indicating that pMHCII abundance alone on the cell surface of DCs isolated from control-fed mice was not sufficient to differentially activate T cells.

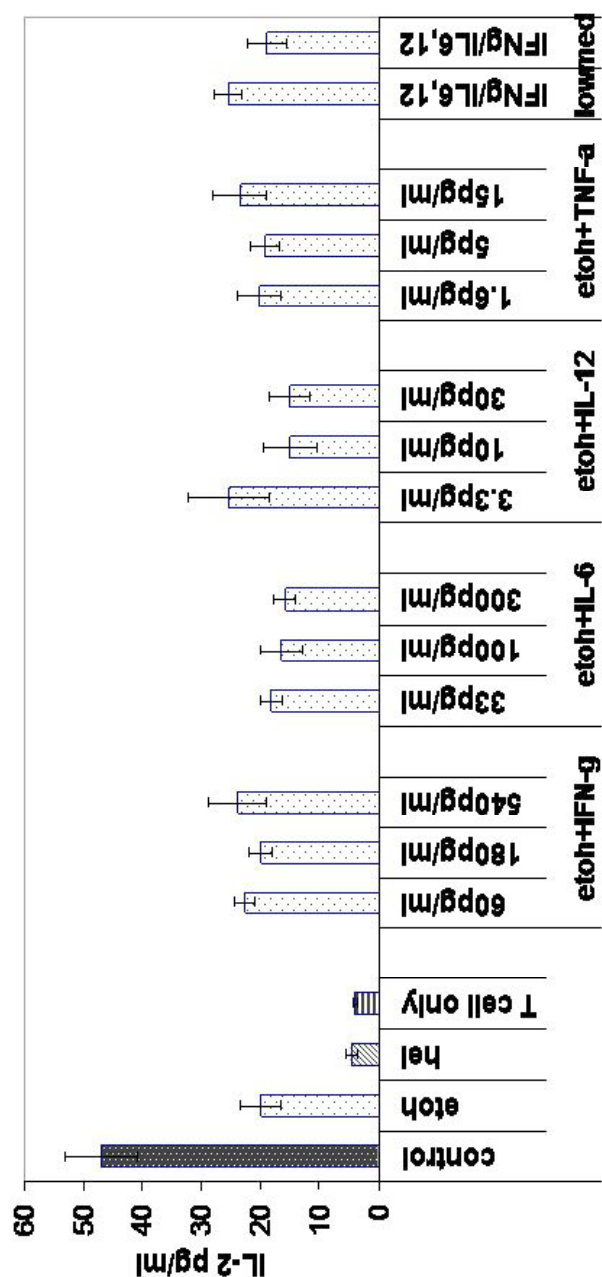


Figure 2.6.A. Adding back cytokines to DC: T cell co-cultures does not restore the inhibitory effect of ethanol T-cell activation. TNF-alpha, IL-12, IL-6 and IFN-gamma were added at indicated concentrations into DC: DO11 T-cell co-cultures in an attempt to restore the reduced T-cell activation produced by DCs derived from ethanol-fed mice. IL-2 levels were measured after 18 h culture as an indicator of T-cell activation. No improvement was observed in OVA presentation of DO11 T cells by DCs derived from ethanol-fed mice

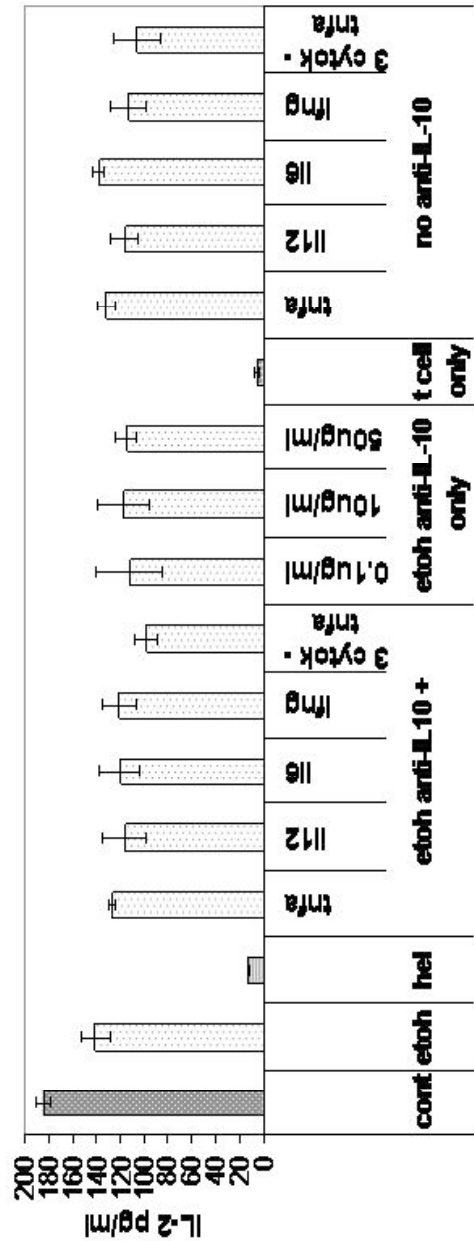


Figure 2.6.B. Neutralization of IL-10 in DC: T cell co-cultures does not restore the inhibitory effect of ethanol T-cell activation. IL-10 was neutralized with anti-IL-10 polyclonal antibody in OVA loaded DC:T-cell co-cultures. IL-2 levels were measured with ELISA after 18 h of culture. Neutralization of this anti-inflammatory cytokine did not improve OVA presentation by DCs derived from ethanol-fed animals to DO11 T cells. (3 cytokine-Tnf-α) received all three recombinant cytokines except TNF-α.

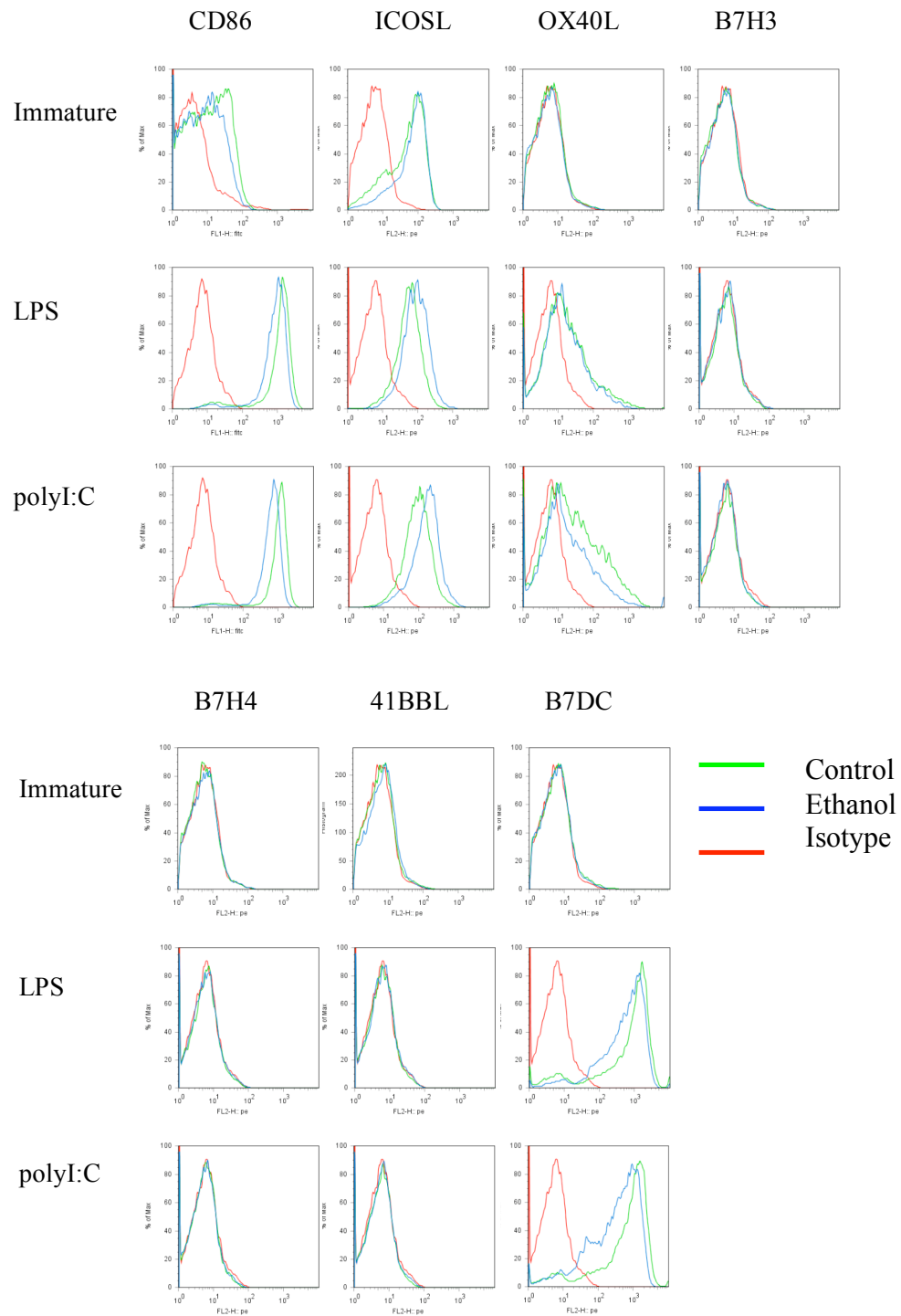


Figure 2.7. Inhibitory and activating costimulatory molecules are differentially expressed in DCs after chronic ethanol exposure. DCs freshly isolated from ethanol or control diet-fed Balb/c mice or after maturation for 24 h with LPS or polyI:C were stained with antibodies for the indicated costimulatory molecules as well as CD11c. The CD11c⁺ high cells were gated and

analyzed for the expression of indicated costimulatory molecules. ICOS-L was up-regulated whereas B7-DC and CD86 were down regulated in DCs derived from ethanol fed mice. OX40-L was unchanged; B7-H3, B7-H4 and 41BB-L were not expressed by DCs at all. The results are representative of three independent experiments.

CHAPTER 3:
ETHANOL ALTERS GENE EXPRESSION PROFILE OF IMMATURE AND LPS
MATURED DCs

All of the experiments in this chapter were conducted by Ahmet Eken.

Introduction:

As detailed in Chapter 1 and 2, several studies revealed that chronic ethanol exposure in mice alters antigen presentation by DCs, as well as expression and secretion of costimulatory molecules and cytokines, respectively. During chronic ethanol consumption, DC expression of a number of cytokines namely IL-12p70, IL-6, TNF- α , IFN- γ was reduced, whereas IL-10 was increased. However, we do not know whether the suppressive effects of ethanol is limited to genes that encode for these cytokines, or if there are other direct or indirect targets of ethanol that involve signaling pathways that regulate the expression of the above mentioned cytokine genes. It is also likely that ethanol affects genes that are related to antigen processing presentation pathways in DCs. Therefore, to have a better understanding of how chronic ethanol exposure mediates suppression of DC function, we compared gene expression profiles of immature and LPS matured DCs derived from ethanol and control diet fed mice.

Materials and Methods:***Isolation and Stimulation of DC***

DCs were isolated from ethanol or isocaloric pair-fed control Balb/c mice after 8 weeks of feeding. Splenic DCs were purified via CD11c microbeads (from Miltenyi Biotec) and stimulated with 200 ng/ml LPS in Clone food medium (described in Chapter 2 materials & methods) for 24 hours. The cells were seeded at a density of 10^7 DCs/5 ml in 5 cm dishes. The cells were collected 24 h later and used for RNA extraction. Freshly isolated DCs were used as immature DCs for RNA extraction as well.

Microarray Analysis

LPS stimulated mature and immature DCs were lysed with Trizol reagent and total RNA was extracted via Qiagen RNeasy Mini Kit. The samples were sent to Yale Keck Microarray Center for the analysis. The quality of the RNA was confirmed by Bioanalyzer and was excellent.

Mouse 430-2.0 Affymetrix chip (~39 000 transcripts) was used for the hybridization experiment.

Data Analyses: Data analyses for mature DCs were performed by Keck microarray facility and the genes significantly altered ($p < 0.05$) were categorized based on the proposed function of the genes. Analyses of immature DCs was performed using environment R in collaboration with Dr. Jean Wu at Brown University Statistics Department. *Gcrma* corrected & *quantile* normalized data was tested with both t-test and SAM. For the t-test, the cut off was $p \leq 0.01$ & $M\text{-value} \geq 1$. For SAM, $FDR = 0.19$ was regarded as an acceptable false discovery rate.

Results:

Differentially regulated genes in immature DCs in response to chronic ethanol exposure

Twenty transcripts were significantly altered by ethanol in immature freshly isolated splenic CD11c⁺ DCs and they were of interest and potential biological significance. These include lectins, growth factor receptors, and chemokines. To our surprise we have not detected any alterations in transcripts associated with cytokines, DC maturation process, antigen presentation pathway, or costimulatory molecules/or presumed regulators using this approach. A complete list of differentially regulated genes is given in Table 3.1 (page 104). The next step will be validation of transcript expression with real time PCR and these studies are in progress.

Differentially regulated genes in mature DCs in response to chronic ethanol exposure

In contrast to the immature DC microarray results, after maturation of DCs by LPS, 44 genes were found to be differentially regulated following long term ethanol feeding of mice. Of interest were the genes that carry out immune regulatory functions or metabolic processes which are important for antigen presentation. A list of these genes is depicted in table 3.2 (page 105), and the biological role of these transcripts are briefly described below to emphasize the relevance of their products for DC immunobiology.

Table 3.1

A list of differentially regulated transcripts in *immature* DCs after chronic ethanol exposure. (-) sign indicates down-regulation; the genes in this group are highlighted in red. (+) sign indicates up-regulation; the genes in this group are in black font color.

GENE NAMES	SYMBOL	FOLD CHANGE
RIKEN cDNA 4833422F24 gene	4833422F24Rik	2.59
MAM domain containing 2	Mamdc2	2.31
killer cell lectin-like receptor subfami	Klrg1	-2.60
chemokine (C-X-C motif) ligand 7	Cxcl7	-6.71
leukemia inhibitory factor receptor	Lifr	2.12
epithelial membrane protein 1	Emp1	3.04
F-box protein 30	Fbxo30	-2.34
ryanodine receptor 1, skeletal muscle	Ryr1	2.37
peroxisomal biogenesis factor 11a	Pex11a	2.26
translocation protein 1	Tloc1	-2.01
chemokine (C-X-C motif) ligand 4	Cxcl4	-2.69
keratin 80	Krt80	2.22
hydroxyacylglutathione hydrolase-like	Hagh1	2.32
endothelial differentiation, lysophospha	Edg2	2.47
fibroblast growth factor receptor 1	Fgfr1	3.11
4-nitrophenylphosphatase domain and non-	Nipsnap1	2.08
hydroxyacylglutathione hydrolase-like	Hagh1	2.07
TGFB inducible early growth response 3	Tieg3	2.31
leucine zipper transcription regulator 2	Lztr2	2.97
killer cell lectin-like receptor family	Klre1	-2.58

Table 3.2

Differentially regulated transcripts in *mature* DCs after chronic ethanol exposure. (-) sign indicates down-regulation, (+) sign indicates up-regulation.

Activation of immune responses	Fold change
CD1d1 antigen	(-2.14)
Myeloid differentiation primary response gene 88	(+2.46)
Histocompatibility 2, class II antigen A, alpha	(-1.62)
IL12b	(-1.52)
TNF receptor superfamily, member 5 (CD40)	(-1.52)
CD81 antigen	(-1.52)
Antigen processing-presentation	
Transporter 2, ATP-binding cassette, subfamily B (MDR/TAP)	(-2.83)
Legumain	(+7)

MyD88

Myeloid differentiation primary response gene 88 (Myd88) is a universal adapter molecule for all TLRs except TLR3.¹ The cytosolic portion of TLRs contains a Toll/interleukin-1 (IL-1) receptor (TIR) domain which is also found in the adaptor molecules that interact with TLRs. Myd88 is one such adapter with a TIR domain. MyD88 deficient mice were shown to be unresponsive to ligands for TLR2, TLR 4, TLR 5, TLR 7, and TLR 9.² The disease models also revealed that MyD88 deficient mice are resistant to the toxic effect of LPS, and are immunocompromised in terms of their ability to handle pathogens such as *Staphylococcus aureus*, *Plasmodium berghei*, *Toxoplasma gondii*, *Listeria monocytogenes*, and *Leishmania major*.³⁻⁸ The role of MyD88 in ethanol induced liver injury has been studied recently, and MyD88 deficient mice were shown to be susceptible to ethanol induced liver injury, whereas TLR4 deficient mice were protected, which suggests a role for MyD88 independent pathway (perhaps TRIF dependent) for TLR4 in generation of ethanol and endotoxin induced liver disease.⁹ On the other hand, to the best of our knowledge, there is no report with regard to the role

of MyD88 in mediating the effects of ethanol in DC maturation and/or cytokine secretion despite the conserved role of MyD88 in abovementioned members of TLR family.

CD81

CD81 is a transmembrane protein, a member of tetraspanin family, and expressed by endothelial epithelial and hematopoietic (with the exception of erythrocytes, platelets and neutrophils) cells. Several studies suggest that CD81 plays a role in HCV infection of hepatocytes as a receptor/co-receptor. CD81 binds HCV envelope protein E2.¹⁰ CD81 is also expressed on B cells, DCs and T cells. CD81^{-/-} B cells showed enhanced activation after stimulation of both B cell receptor and TLR4, suggestive of an inhibitory role for CD81 in B cells.¹¹ CD81^{-/-} mice also failed to generate proper Th2 responses, and *in vitro*, IL-4 production by CD81^{-/-} T cells after B cell stimulation was impaired.¹² CD81 is also expressed in DCs. In both APCs and T cells, CD81 was shown to cluster in the immunologic synapse central region.¹³

Legumain

Asparaginyl endopeptidase (AEP)/legumain is an asparagine-specific cysteine proteinase in animals. Legumain is an ortholog of plant vacuolar processing enzyme (VPE), which processes the exposed asparagine residues of various vacuolar proteins. AEP localizes in lamp 2 positive late endosomes, and Lgmn^{-/-} mice are characterized with enlarged lamp2⁺ late endosomes which later fuse with lysosomes. The processing of the lysosomal proteases, cathepsins B, H, and L, from the single-chain forms into the two-chain forms was completely defective in the Lgmn^{-/-} mice.¹⁴ Despite the apparent role of Lgmn in the endosomes and particularly Cathepsin L maturation, Lgmn^{-/-} DCs were able to present OVA and myelin oligodendrocyte glycoprotein antigens properly to primary T cells suggestive of compensatory proteases or no role for Lgmn in MHCII presentation.¹⁵

Transporter 2 (Tap2)

Tap2 is a membrane protein which is a member of ATP binding cassette transporter (ABC) family. Tap2 makes a heterodimer with Tap1 and composes the TAP complex which transports

proteasomal degradation products (peptides) from cytosol across endoplasmic reticulum for loading onto MHCI.¹⁶ The importance of TAP complex was demonstrated by cell lines that lack Tap1 and Tap2 which were deficient in presentation of antigens to CD8+ T cells.^{17, 18} MHCI is a heterodimer of polymorphic alpha and non-polymorphic Beta2 chain which is stable only when bound by the peptides.¹⁹ Thus, TAP not only loads peptides onto MHCI but also is crucial in generation of stable MHCI molecules and transport to the cell surface.

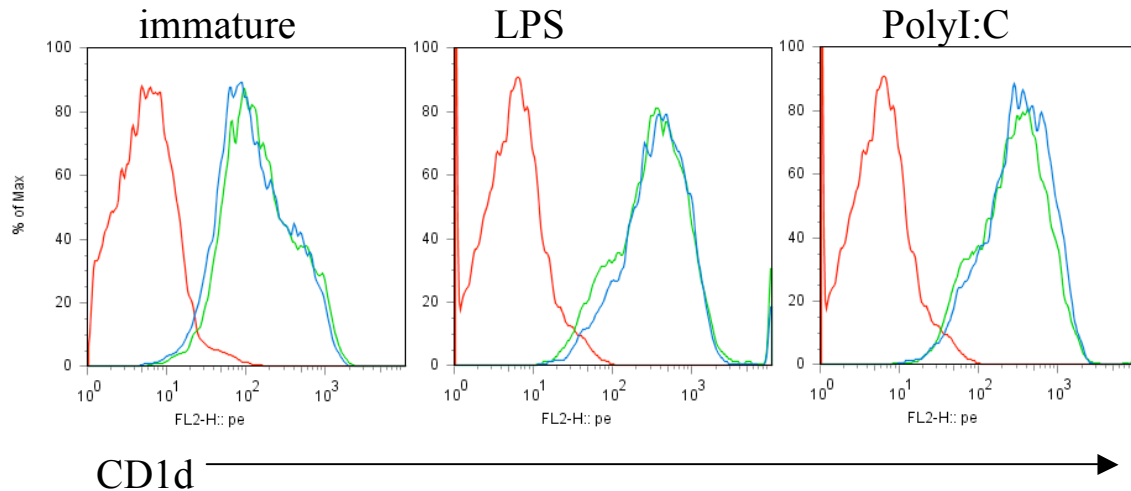
CD1d1

Murine CD1d1 or Cd1d is the only member of CD1 family that is expressed by muroids.²⁰ CD1d is a MHCI like glycoprotein which presents self and exogenous microbial lipid antigens to NKT cells. The importance of CD1d in host defense against pathogens have been demonstrated by several studies using different infection models. Increased intestinal colonization of *P. aeruginosa*, *E. coli*, *S. aureus*, and *L. gasseri* was reported in Cd1d-/- mice after intragastric administration compared with WT mice.²¹ Cd1d-/- mice was also reported to be more susceptible to Theiler's murine encephalomyelitis (TMEV) infection with signs of more severe neurological defects²² and *Toxoplasma gondii* infection.²³

To confirm the microarray data, cell surface expression of CD1d molecule by both mature (LPS and polyI:C matured) and immature DCs isolated from ethanol and isocaloric pair fed animals were evaluated. Comparable level of expression was observed by control and ethanol DCs (Figure 3.1). This may simply indicate that transcript level data is not a match for protein data, or that CD1d is a false positive hit that came up during the analysis of the microarray. Further real-time PCR analysis will be required to answer these questions.

Figure 3.1

Cell surface expression of CD1d molecules by immature and mature DCs after chronic ethanol feeding of mice. There is comparable expression of CD1d by DCs isolated from both ethanol and isocaloric pair fed control mice.



MHCII alpha

MHCII is a heterodimer comprised of two homologous chains (alpha and beta), both of which are composed of 2 domains (a1-a2 and b1-b2 respectively). Alpha and beta polypeptides are assembled in the ER. This assembly and formation of stable MHCII requires a type two integral membrane protein called the invariant chain (Ii). This functional heterotrimer is then trafficked to endosomes and is loaded with 15-24 aa long peptide fragments generated in the endocytic pathway for presentation on the cell surface as described in chapter 1.

IL-12b

IL-12b or IL-12p40 is the 40kD subunit of IL-12 (p70) cytokine. The other subunit of IL-12p70 is p35. As described in chapter 1, IL-12p70 heterodimer drives Th1 differentiation from naïve Th0 cells and IFN-gamma and IL-2 synthesis. IL-12b subunit also heterodimerizes with p19 and form a different cytokine IL-23 which operates in maintaining Th17 cell phenotype of CD4+ T cells.

CD40

CD40 is a costimulatory molecule that belongs to TNFR family. CD40 is expressed by DCs and other APCs as well as T cells. CD40L (CD154) is the ligand of CD40 and was initially found on activated T cell. However, CD40L is also expressed by DCs. CD40 expression is upregulated

upon maturation of DCs.²⁴ The ligation of CD40 via anti-CD40 mAbs or CD154 induces upregulation of costimulatory molecules, adhesion molecules, and the Th1-polarizing cytokine IL-12 in both mouse and human DCs.^{25, 26} CD40^{-/-} mice were impaired in generating T-dependent antibody responses. The study of tumors in CD40^{-/-} mice revealed that, transfer of CD40^{+/+} DCs into CD40^{-/-} mice rescued these animals from death caused by tumor challenge.²⁷ CD40 is also suspected to be involved in differentiation of Th0 to distinct subsets.²⁴

Thus, these experiments were quite revealing regarding the putative genetic targets of chronic ethanol consumption which could potentially alter DC function and may explain, in part, some of the findings observed with DCs regarding antigen presentation as described above.

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CHAPTER 4:
DENDRITIC CELL DYSFUNCTION IN THE ALCOHOLIC LIVER DISEASE
SENSITIVE RAT

The experiments in this Chapter were conducted in collaboration with Dr. Dechun Feng.

Dendritic Cell Dysfunction in the Alcoholic Liver Disease Sensitive Rat

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Abstract

We have compared dendritic cell (DC) function derived from the alcoholic liver disease (ALD) sensitive Long-Evans (LE) and resistant Fisher rat strains to evaluate whether the influence of ethanol on DCs was dependent, in part, on the presence of ALD. **Methods:** The LE and Fisher rats were fed an ethanol-containing or isocaloric control liquid diet for 8 weeks. DCs were isolated from the spleen by magnetic beads coated with anti-OX62 antibodies after expansion with hFlt3L plasmid. Maturation markers on DCs such as CD86, CD80, CD40 and MHC-II were analyzed by flow cytometry with or without LPS and Poly I:C additions. Cytokine levels including TNF- α , IFN- γ , IL-12p40 and IL-10 were measured in DC culture supernatant. The antigen presentation ability of DCs was assessed by measuring allostimulatory activity. **Results:** Only LE rats developed ALD characterized by liver injury, elevated ALT levels, and steatosis. Chronic ethanol feeding decreased CD86 and CD40 expression in LE but not Fisher rats. Reduced TNF- α , IFN- γ , IL-12, proinflammatory and enhanced IL-10 cytokine production was found in DCs isolated from ethanol-fed LE but not Fisher rats. Allostimulatory activity was also reduced in chronic ethanol-fed LE compared to the Fisher strain. **Conclusions:** Our results demonstrate that ALD sensitive LE rats exhibited characteristics of a suppressed DC phenotype after chronic ethanol feeding, which suggests an important role for liver disease in altering the host cellular and humoral immune responses. Therefore, both ALD and ethanol act to impair DC function.

Introduction

Alcohol-induced disorders have become a serious health problem in the United States. About 7.1% of Americans older than 18 years meet the criteria for alcohol abuse (1). All cell types within the liver including hepatocytes, immune cells and hepatic stellate cells may be adversely influenced by ethanol since it affects metabolic, immunologic, and inflammatory cellular processes.

Ethanol consumption is closely related to the progression of HCV infection. Excessive ethanol intake increases HCV RNA levels in serum which is accompanied by impairment of cellular immunity (2, 3). Heavy ethanol abuse greatly exacerbates the risk of cirrhosis among patients with persistent HCV infection (3, 4). The increased risk of HCV progression in alcoholic patients may be attributed to impaired cellular immunity to viral infection induced by chronic ethanol abuse (2). Long-term ethanol ingestion may broadly influence immunity (5, 6). Previous experimental studies suggest that CD4⁺ T cell proliferation and cytotoxic T-lymphocyte (CTL) activity responses to NS5 or HCV core proteins were suppressed by chronic ethanol consumption (3, 7, 8). Further investigations reveal that viral antigen specific impairment of CD4⁺ T cell proliferation and CTL activity may be restored by IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) additions (7, 8). These findings imply the potential involvement of antigen presenting cells (APC) or DC dysfunction in altering the host cellular immune response induced by ethanol.

The influence of chronic ethanol exposure on DC function was further explored using a murine model (9). DCs derived from mice fed ethanol for 8-weeks exhibited decreased maturation marker expression such as CD40 and CD80 necessary for antigen presentation although endocytic uptake of proteins exhibited by immature DCs was not affected. Further studies confirmed that allostimulatory activity of DCs derived from ethanol fed mice was impaired compared to cells isolated from isocaloric pair-fed control animals. Ethanol feeding was shown to suppress pro-inflammatory cytokine (TNF- α , IFN- γ , IL-12 and IL-6) production and

secretion by DCs when stimulated with LPS or Poly I:C; in contrast, IL-10 levels were increased. More importantly, impaired CTL responses to hepatitis C virus (HCV) NS5 protein observed in ethanol-fed mice could be restored by syngeneic transfer of DCs derived from isocaloric pair-fed controls but not ethanol-fed animals (9).

The results from this murine model establish the inhibitory effects of ethanol on DC function and illustrates how it impairs anti-viral immune response (9-13). Similar inhibitory actions of ethanol on human DCs have been observed by others (14-16). However, it is difficult to establish whether the alteration of DC function is due entirely to ethanol alone (without liver disease) or ethanol plus ALD using this murine model since there was lack of progressive hepatocyte injury, oxidative stress, mitochondrial dysfunction, severe steatosis, and fibrosis upon continued alcohol abuse. Recently, it was observed, unexpectedly, that an outbred Long-Evans (LE) rat strain exhibited many of the pathological, biochemical, and molecular features of ethanol-induced hepatotoxicity while on the Lieber-DeCarli liquid diet, which have not been previously observed in any other rodent model (17-20). In this context, we compared the features of ethanol sensitive LE rats with two other inbred strains i.e., Fisher and Sprague-Dawley (18-21); other than mild steatosis, Fisher rats did not develop liver disease whereas Sprague-Dawley had an intermediate phenotype. We hypothesized that DC function may be differentially altered in the ALD sensitive and resistant rat strains. To test this idea, we fed both the LE (sensitive) and Fisher (resistant) rats with the Lieber-DeCarli liquid diet for 8 weeks and compared results to the isocaloric pair-fed controls with respect to the characteristics and downstream effects of ethanol and ALD on DC function.

Materials and Methods

Media and Reagents

DCs were cultured in HEPES-buffered RPMI-1640 medium (Sigma-Aldrich St Louis, MO) supplemented with 2 mM L-Glutamine, 1% non-essential amino acid 5×10^{-5} M β -2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin and heat inactivated 10% fetal bovine serum. Other chemicals (LPS, poly I:C) were purchased from Sigma-Aldrich.

Rats and ethanol feeding regimen

The 150~230 gram, male Fisher and LE rats (Harlan Laboratories, Indianapolis, IN) were fed ad libitum with ethanol containing or isocaloric control liquid diet (Bioserv Frenchtown, NJ) for 8 weeks. After 8 weeks, the animals were injected rapidly I.V. via the tail vein with a plasmid for DCs generation and then sacrificed. Blood was collected by cardiac puncture. Liver tissue was excised and processed for histological analysis and biochemical assays. All animal protocols have been reviewed and approved by the Lifespan Animal Care and Use Committee.

Histology

Histologic changes in the liver were assessed by routine hematoxylin-eosin (H&E), and Oil Red O staining. Slides were scanned with Aperio Scanscope CS (Aperio Technologies, Inc., Vista CA).

Biochemical assays

Serum alanine aminotransferase (ALT) levels were measured by using a commercially available kit (Thermo Fisher Scientific Inc., Waltham, MA). Serum ethanol levels were measured by Analox GM7 analyzer (Analox Instruments, Lunenburg, MA). Liver lysates were used to measure triglyceride concentrations according to manufacturer's instructions (BioVision, Inc., Mountain View, CA).

Generation of DCs

To increase the rat DC population *in vivo*, 300 µg of the plasmid pUMVC3-hFLex, expressing the secreted portion of the human Fms-like tyrosine kinase receptor 3 ligand (hFlt3L) (Vector Core Laboratory, University of Michigan), was dissolved in 25 ml Ringer's solution and injected directly into the tail vein of rats within 15 seconds using the hydrodynamic gene delivery protocol (22). The plasmid was injected twice (days 0 and 6), and spleens were harvested on day 12. Single spleen cell suspensions were prepared in serum-free medium after red blood cells were lysed. The OX62⁺ DCs were isolated with magnetic beads coated with monoclonal anti-DC (OX62) antibodies after 14.5% Nycodenz gradient centrifugation enrichment according to the to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The DCs obtained by this method from both the ethanol and control diet-fed Fisher and LE rats were used for subsequent assays.

Cytokine production

To evaluate cytokine production, DCs derived from ethanol and control diet fed rats were cultured at 24-well flat-bottom plates at 1×10^6 cells in 1 ml of medium. Cells were stimulated with 0.2 µg /mL, or 1 µg/mL of LPS or 1 µg/mL, or 10 µg/mL of poly I:C. After 24 hours, the supernatants were collected and the levels of IFN- γ , TNF- α , IL-12p40 and IL-10 were quantified using enzyme-linked immunosorbent assay kits purchased from eBioscience (San Diego, CA), Invitrogen (Carlsbad, CA) or BD Biosciences (San Jose, CA) and performed according to the manufacturer's instructions.

Allostimulatory Activity

T cells were isolated from the spleens Fisher and LE rats by a commercial available pan T-cell isolation kit that uses immunomagnetic beads according to the manufacturer's protocol (Miltenyi Biotec). The DCs isolated from ethanol and control pair-fed Fisher and LE rats were added in triplicate at various ratios to 1×10^5 T lymphocytes in 96-well flat-bottom plates (T cells

from Fisher rats added to DCs from LE rats, as well as T cells from LE rats added to DCs from Fisher rats). Cultures were maintained in IMDM (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1mM HEPES, 5×10^{-5} M β -2-mercaptoethanol, and 5% normal rat serum for 3 days. The secretion of IL-2 was measured by enzyme-linked immunosorbent assay kits purchased from R&D systems (Minneapolis, MN). This set of experiments evaluated antigen presentation ability before and after maturation of DCs by 0.2 µg/ml of LPS for 24 hours.

Flow Cytometry Analysis

Cells (5×10^5) were incubated with purified anti-rat CD16/32 to block Fc receptors for 5 minutes and then incubated with PE or FITC labeled antibodies. Antibodies were diluted in Hank's balanced salt solution containing 2% fetal bovine serum, which also served as the wash medium. Samples were analyzed with a FACScalibur (BD Biosciences). The following antibodies were used for cell surface staining: PE-anti-rat CD103 (OX62, eBioscience), FITC-anti-rat CD80 (AbD Serotec, Kidlington, UK), FITC-anti-rat CD4 (BD Biosciences), FITC-anti-rat CD86 (BD Biosciences), FITC-anti-rat CD40 (BD Biosciences), FITC-anti-rat MHC-II (BD Biosciences) and along with the recommended isotype controls.

Statistical Analysis

Results were analyzed using the SigmaStat statistics program (Jandel Scientific, San Rafael, CA). Individual means were compared using a non-paired Student *t* test. Data derived from > 2 groups were compared by one-way analysis of variance followed by a Tukey Dunn test to identify the groups that differed. Differences at $p < 0.05$ were considered significant.

Results

LE rats develop liver injury and steatosis whereas Fisher rats do not

We fed Fisher and LE rats with the ethanol containing diet for 8 weeks. The body weight was similar in animals consuming the control liquid diet, compared to those given the normal diet (chow fed). Rats consuming the ethanol liquid diet showed a slightly reduced ($p = \text{NS}$) bodyweight compared with matched animals fed the isocaloric control diet in both rat strains (Fig.4.1 A). Blood ethanol concentration was measured; no difference in blood levels was found in ethanol-fed Fisher compared to LE rats (Fig.4.1 B), suggesting that both strains had similar ethanol intake. Liver triglyceride content and ALT levels were comparable in ethanol and isocaloric pair-fed control Fisher rats. However, liver triglycerides and ALT levels were significantly increased in the chronic ethanol-fed LE rats compared with animals that received the isocaloric pair-fed control diet (Fig.4.1 C and D). These observations, confirmed by histology, suggest that liver injury and steatosis were present in LE compared to Fisher rats during chronic ethanol feeding; indeed H&E staining demonstrated striking micro-and macrovesicular steatosis, and hepatocyte ballooning degeneration in ethanol fed LE but not in Fisher rats (Fig.4.1 E). Likewise, oil red staining revealed substantial hepatic steatosis in LE compared to Fisher rats (Fig.4.1 F). Taken together, LE but not Fisher rats developed histologic features of liver injury and steatosis after 8-week of ethanol feeding which further substantiated previous findings (17-21).

DC expansion by hydrodynamic delivery of the hFlt3L plasmid

To obtain sufficient DCs for subsequent experiments, we employed hydrodynamic delivery of the hFlt3L encoding plasmid (pUMVC3-hFlex) to expand this population in the spleen. Using this procedure, the total number of splenocytes was increased from $3\sim4\times10^8$ to $8\sim10\times10^8$ in Fisher rats and from $5\sim7\times10^8$ to $8.5\sim10.5\times10^8$ in LE rats on the chronic ethanol diet ($p=\text{NS}$). About 30% splenocytes in Fisher rats and 10% splenocytes in LE rats were OX62 positive after hFlt3L plasmid injection compared to about 4% OX62 positive cells in both Fisher and LE rat spleens prior to injection of this plasmid. There were no significant differences in the efficacy of

expansion in animals on the isocaloric pair-fed diet compared to the ethanol-fed group (Fig.4.2 A and C). Two different subset of rat DCs were characterized by the expression of the surface CD4 marker as described previously (23, 24). DCs were isolated from control and ethanol fed Fisher and LE rats by magnetic activated cell sorting (MACS), then stained them for OX62 and CD4+ expression. As shown in Fig.4.2 B and D, the percent of OX62+ CD4- DCs was strikingly increased while the percent of OX62+ CD4+ cells decreased; suggesting that hFlt3L expression principally expanded the OX62+ CD4- subpopulation.

Ethanol decreased expression of CD86 and CD40 in DCs from LE rats.

We examined cell surface maturation marker expression. Freshly isolated DCs expressed relatively low levels of CD86, CD40, CD80, which is consistent with previous results demonstrating that Flt3L treatment induced equal expansion of immature DCs (25, 26). There were no differences in the expression of these maturation markers in control and ethanol fed Fisher and LE rats except for a slightly lower CD40 in DCs isolated from Fisher control diet-fed animals compared to the ethanol group (Fig.4.3 A and B). DCs were cultured overnight with or without LPS/Poly I:C additions. In Fisher rats, we observed higher CD86 expression in ethanol-fed rats stimulated with LPS low (0.2 µg/ml), Poly I:C low (1 µg/ml) and high (10 µg/ml) groups; all other maturation markers were unchanged with ethanol feeding (Fig.4.3 A). In contrast, DCs derived from chronic ethanol fed LE rats revealed significantly lower expression of CD86 in overnight culture with LPS/Poly I:C (Fig.4.3 B). We observed substantially lower CD40 expression in overnight cultures with LPS in ethanol fed LE rats as well (Fig.4.3 B). These results demonstrated that chronic ethanol feeding was associated with decreased expression of key cell surface molecules such as CD86 and CD40 required for activating T cells in the ALD sensitive LE but not the resistant Fisher rat strain.

Ethanol reduced pro-inflammatory and increased anti-inflammatory cytokine production in DCs derived from LE rats.

Several important cytokines (TNF- α , IFN- γ , IL-12 and IL-10) secreted by DCs were measured; these molecules are considered critical for determining the Th1/Th2 balance and subsequent anti-viral and anti-bacterial immune responses. Cytokine production by DCs after LPS or Poly I:C stimulation were determined by ELISA. All pro-inflammatory cytokines were significantly suppressed in DCs derived from chronic ethanol fed LE rats compared with isocaloric pair-fed controls after LPS or Poly I:C stimulation (Fig.4.4). In Fisher rats, the TNF- α levels were similar in DCs isolated from ethanol and control diet-fed groups after LPS and poly I:C stimulation whereas IFN- γ and IL-12 were found to be surprisingly higher in the chronic ethanol-fed group (Fig.4.4). In contrast, IL-10 levels were similar in LPS stimulated DCs isolated from isocaloric pair-fed controls and ethanol-fed Fisher rats; IL-10 levels were slightly higher after poly I:C stimulation in DCs derived from these animals. However, there was a substantial increase in IL-10 production in both LPS and poly I:C stimulated DCs derived from ethanol-fed LE rats compared to isocaloric pair-fed controls (Fig.4.4). Therefore, major alterations were observed (suppression of pro-inflammatory and upregulation of anti-inflammatory cytokines) in the production and secretion patterns exhibited by DCs when comparing the ALD sensitive LE to resistant Fisher rat strains after consuming the chronic ethanol and isocaloric pair-fed control diets for 8 weeks.

Allostimulatory activity exhibited by DCs

The capability of antigen presentation by DCs was evaluated by measuring allostimulatory activity. Before maturation induced by LPS, DCs from all groups had a very low ability to initiate proliferation of naïve allogeneic T cells (data not shown). DCs matured by LPS showed a higher capacity to initiate proliferation. In LE rats, ethanol feeding significantly decreased DCs allostimulatory activity as indicated by low IL-2 production when compared with DCs isolated from controls and co-cultured with naïve allogeneic T cells. However, in the ALD resistant Fisher rats, no significant difference in IL-2 production was observed by chronic ethanol feeding as

compared to animals on the control diet when their DCs were co-cultured with naïve allogeneic T cells (Fig.4.5).

Discussion

In the current study, DCs were expanded for functional analysis by using the hydrodynamic delivery of hFlt3L plasmid to rats (27-30). Comparisons were made with respect to DC function in ALD sensitive and resistant rat strains to determine the possible role of ALD in the etiology of DC functional impairment in the context of chronic ethanol exposure (7-9).

DCs have been used for therapeutic vaccine development against chronic viral infection like HCV and tumor immunotherapy due to their unique properties of antigen processing, presentation and stimulation of CD4⁺ and CD8⁺ immune responses (9, 27-29). It is difficult to generate sufficient numbers of DCs for characterization and vaccine studies in rats (9, 29). We have previously developed protocols to expand large numbers of DCs in mice by using hydrodynamic gene delivery of the hFlt3L expression plasmid pUMVC3-hFLex (9, 27, 28), which increased DC populations about 5-10 fold. There have been no reports regarding DC expansion in rats. In this study, we developed a protocol for rats based on a modification of the murine procedure. In these investigations, the plasmid dose was increased from 10 µg in 2 ml saline (mouse) to 300 µg in 25 ml Ringer's solution for the rat based on established criteria (22, 31). The DC expansion efficiency was similar to mice in the Fisher rats where more than 25% of the splenocytes were OX62 positive DCs compared to 4% in non-treated animals. Although the expansion efficiency was lower in LE rats (about 10% splenocytes were OX62 positive DCs), the hydrodynamic plasmid delivery greatly increased the splenic DC numbers (Fig.4.2). The reason for this phenomenon may be due, in part, to the presence of liver disease in the LE rat since expression of the hFlt3L plasmid depends on transfection of functional hepatocytes following hydrodynamic injection (22). However, unlike mice, we did not find an adverse influence of ethanol feeding on DC expansion efficiency in both Fisher and LE rat strains (Fig.4.2). The mechanisms for lack of an ethanol effect in this regard are unknown but may relate, in part, to species variation or genetic differences.

The enriched DC population was characterized by staining for CD8⁺ and CD4⁺ cell surface markers. Consistent with previous observations, all OX62 positive cells were CD8 negative (32) (data not shown). Two DC subsets were identified based on whether they expressed CD4 or not as previously described (23, 24, 32). Although the ontogeny of DC subsets in rats has not been extensively studied, CD4⁻ DCs appeared to be a homogenous population of cells with myeloid-like morphological features and CD4⁺ DCs appeared much more heterogeneous regarding their morphology and to a lesser extent their phenotype (24). After the hydrodynamic delivery of the hFlt3L expression plasmid, CD4⁻ DCs were preferentially expanded in both Fisher and LE rats (Fig.4.2). These findings were consistent with previous studies in mice, where CD11c⁺CD11b⁺ myeloid DCs comprise more than 80% of total CD11c⁺ DCs population following Flt3L expansion (28). Such observations suggest that myeloid DCs are also the main target of Flt3L induced expansion in the rat.

There is a wide spectrum of alcohol-related liver pathology in humans. Alcohol is not only a direct hepatotoxin but is also considered to be a “permissive” agent that causes liver injury-(33, 34). Several rodent models of ALD have been established using the Lieber-DeCarli ethanol-containing liquid diet as well as the Tsukamoto-French intragastric feeding approach with or without lipid additions to the ethanol-containing diet (35). The use of such animal models has contributed to a better understanding of ALD development, and how the severity of liver injury may be influenced by factors other than ethanol, such as nutrition (fat intake), oxygen deprivation, gene regulation and immune dysfunction. However, small animal models that recapitulate the hepatic histologic, biochemical and molecular changes indicative of human ALD have been difficult to develop (35).

Reasons for lack of a small ALD rodent model are poorly understood. Most murine and rat strains develop very mild liver injury and steatosis. However, recent studies revealed that certain rat strains are either highly susceptible or totally resistant to ALD when fed the Lieber-DeCarli liquid diet (18-21). Indeed, LE rats remarkably reveal ALD features such as insulin resistance,

decreased cell survival signaling, hepatic steatosis, hepatocellular injury, hepatocyte apoptosis, increased oxidative stress and enhanced collagen gene and TNF- α expression when placed on an ethanol diet 8 weeks or longer (17-21). In contrast, Fisher rats do not develop liver disease or any of the above features found in the LE strain (Fig.4.1).

Thus, to determine how ALD affected DC function, such cells were isolated from control or ethanol fed Fisher and LE rats after hFlt3L expansion. Maturation marker expression including CD86, CD40, CD80, MHC-II were analyzed by flow cytometry in freshly isolated DCs and those cultured with/without different concentrations of maturation stimuli such as LPS/Poly I:C for 24 hours. The CD86, CD40, and CD80 expression levels were relatively low in freshly isolated DCs from both control and ethanol fed Fisher or LE rats; after overnight culture, expression levels were significantly increased. Furthermore, expression of these maturation markers were increased in the presence of LPS/Poly I:C in a dose dependent manner (Fig.4.3 A and B). MHC-II expression was found to be high in freshly isolated DCs and significantly increased following overnight culture with or without LPS/Poly I:C stimulation.

In Fisher rats, expression levels of these markers were found to be similar in freshly isolated or cultured DCs. The CD40 levels were marginally lower ($p=NS$) in freshly isolated DCs from ethanol fed rats, but this difference disappeared after overnight culture with/without LPS/Poly I:C additions. Surprisingly, CD86 levels were slightly higher ($p=NS$) in ethanol fed Fisher rats compared with animals that received the control diet.

In contrast, CD86 and CD40 expression were significantly lower ($p < 0.01$) in ethanol fed LE rats compared with controls after overnight culture with/without LPS/Poly I:C stimulation. Consistent with cell surface maturation marker expression, it was found that pro-inflammatory cytokines production and secretion including TNF- α , IFN- γ , IL-12p40 was substantially suppressed while IL-10 was greatly increased in ethanol-fed LE rats compared with controls following LPS/Poly I:C stimulation, whereas in Fisher rats, the TNF- α production was comparable in DCs isolated from both ethanol fed and controls (i.e., animals fed the isocaloric

control diet *sans* ethanol). Moreover, IFN- γ and IL-12p40 concentrations were higher in ethanol fed Fisher rats than controls (Fig.4.4A and B). Finally, no difference in TNF α levels was observed in ethanol and control diet fed DCs isolated from the ethanol and isocaloric pair-fed Fisher strain. Overall there is a propensity to generate Th2-type immune responses because the secretion of three critical cytokines (namely TNF- α , IFN- γ , and IL-12) promote the Th1 differentiation pathway of CD4 $^{+}$ and CD8 $^{+}$ into antigen-specific functional cells is strikingly impaired. In addition, IL-10 is increased in ethanol-fed animals which provides another boost toward a robust Th2-type response observed only in the ALD sensitive LE rat.

Previous studies revealed that ethanol feeding may also reduce allostimulatory activity of DCs in mice and humans (9, 12, 14-16). We explored the influence of ethanol feeding on allostimulatory activity in Fisher and LE rats. As expected, ethanol feeding greatly suppressed the allostimulatory activity of DCs derived from LE rats after LPS maturation. However, in the Fisher rat strain, no difference was observed in the allostimulatory activity of DCs (Fig.4.5). Interestingly, results from a human study were similar to our current observations in Fisher rats i.e., increased secretion of IL-1 β , IL-6, IL-12, and TNF- α was found in peripheral blood DCs derived from chronic alcoholic patients without liver disease. In contrast, DCs prepared from the blood of patients with chronic ethanol-induced liver cirrhosis and continued active ethanol intake had abnormally low production of IL-1 β and TNF- α by peripheral blood derived DCs which suggest an important role for ALD in the pathogenesis of DC dysfunction (36).

In summary, our results revealed that the ALD sensitive LE rat exhibited substantial suppression of DC function after chronic ethanol feeding compared to the ALD resistant Fisher strain. These findings suggest an important role for ALD in altering the host's cellular and humoral immune responses to viral and bacterial infections, in addition to ethanol effects which further impairs DC function.

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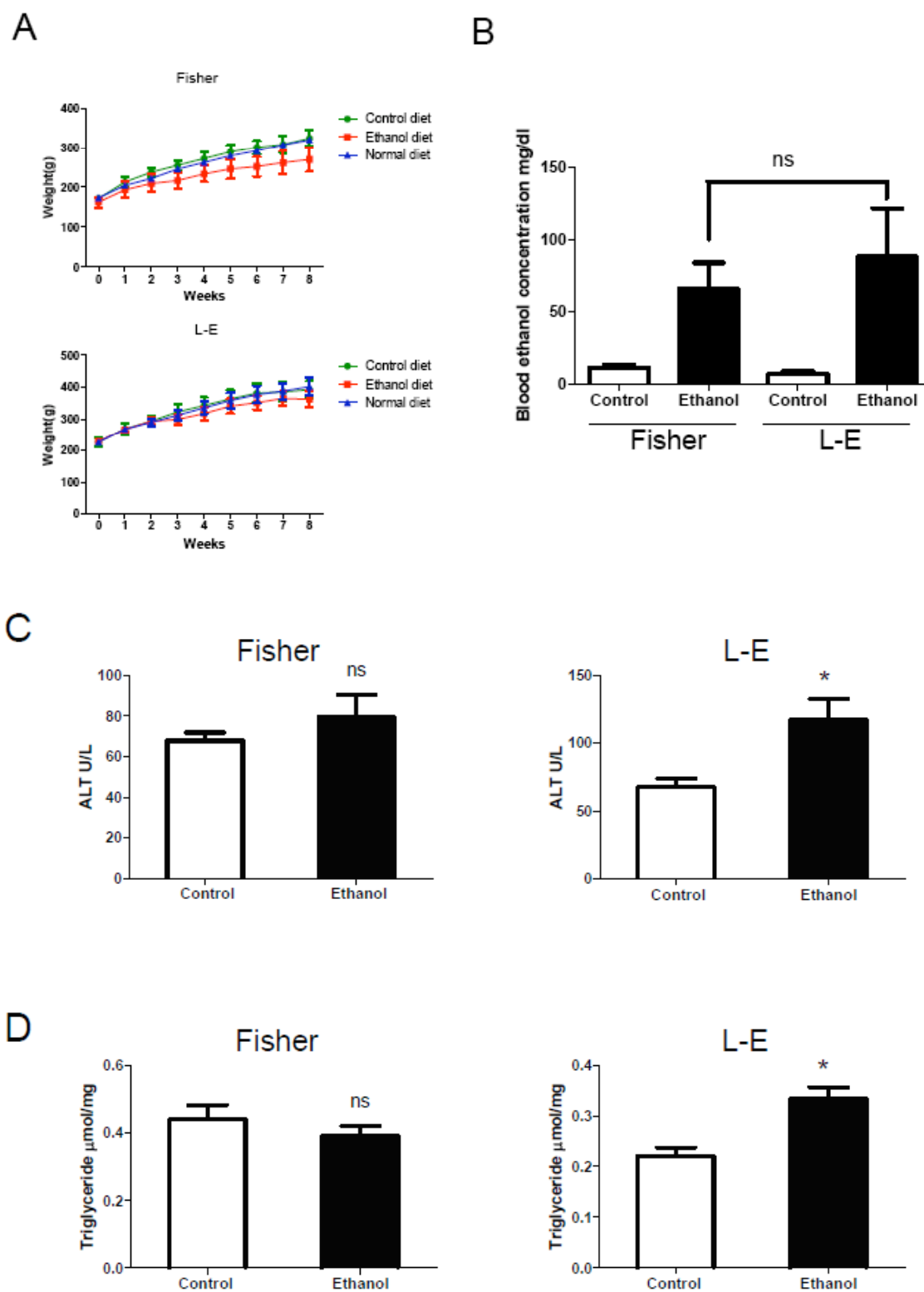
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CHAPTER 4 FIGURES AND LEGENDS

Figure 4.1



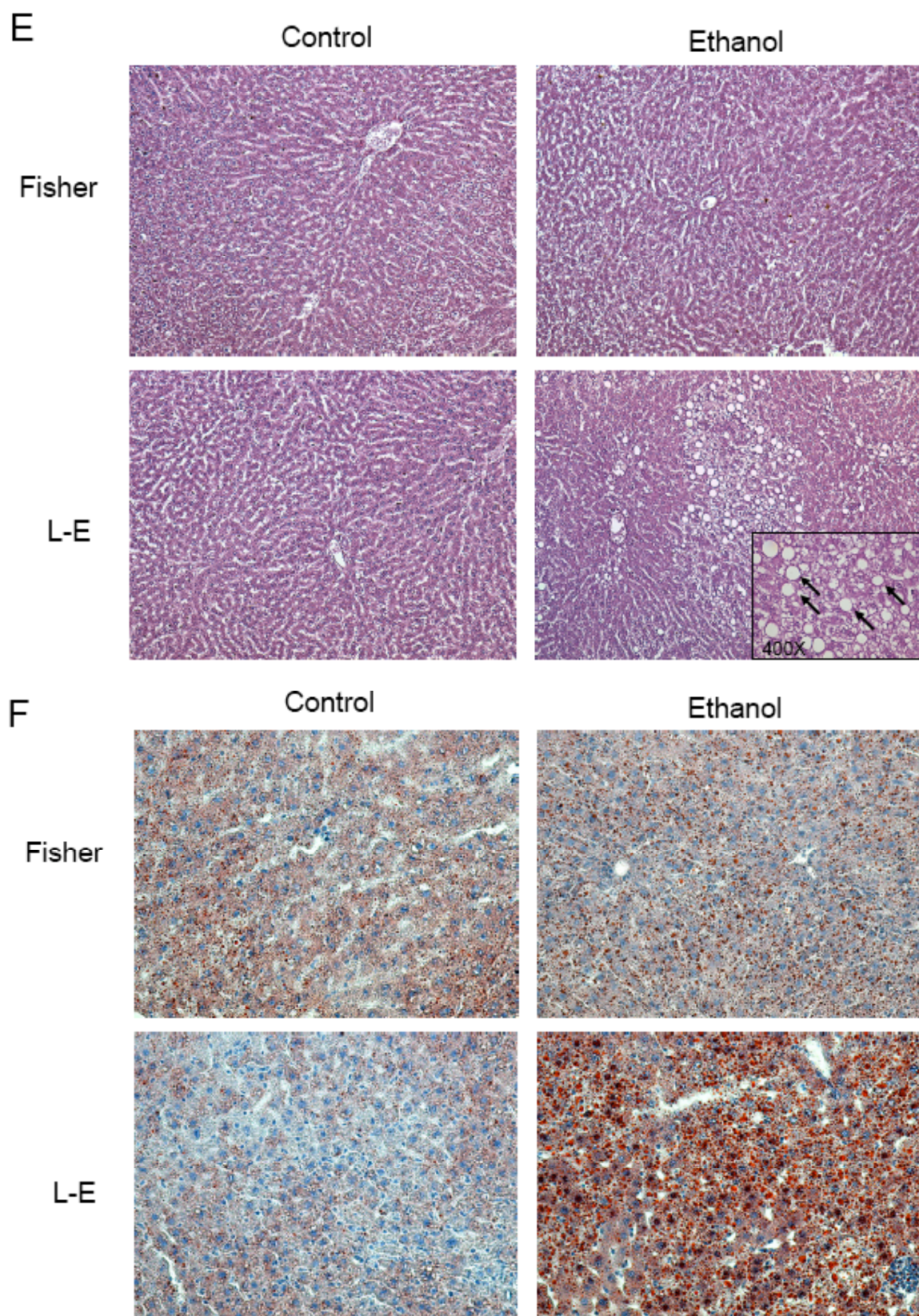


Fig. 4.1. LE but not Fisher rats developed ALD. (A) Changes in body weight of Fisher and LE rats on ethanol and isocaloric pair-fed control liquid or chow-fed normal diets for 8-weeks **(B)** Blood ethanol concentrations of Fisher and LE rats on isocaloric control or ethanol liquid diets. Measurement of liver triglyceride **(C)** or serum ALT levels **(D)** derived from Fisher and LE rats on control or ethanol liquid diets for 8 weeks, * $P < 0.05$. H&E **(E)** and Oil Red O staining **(F)** of Fisher and LE rat liver. Arrows indicate robust macro-and microsteatosis. There was hepatocyte ballooning degeneration of hepatocytes as well (magnification, 100× for H&E, 200× for Oil Red O staining, and 400X for the insert). There were six animals in the ethanol and pair-fed groups.

Figure 4.2

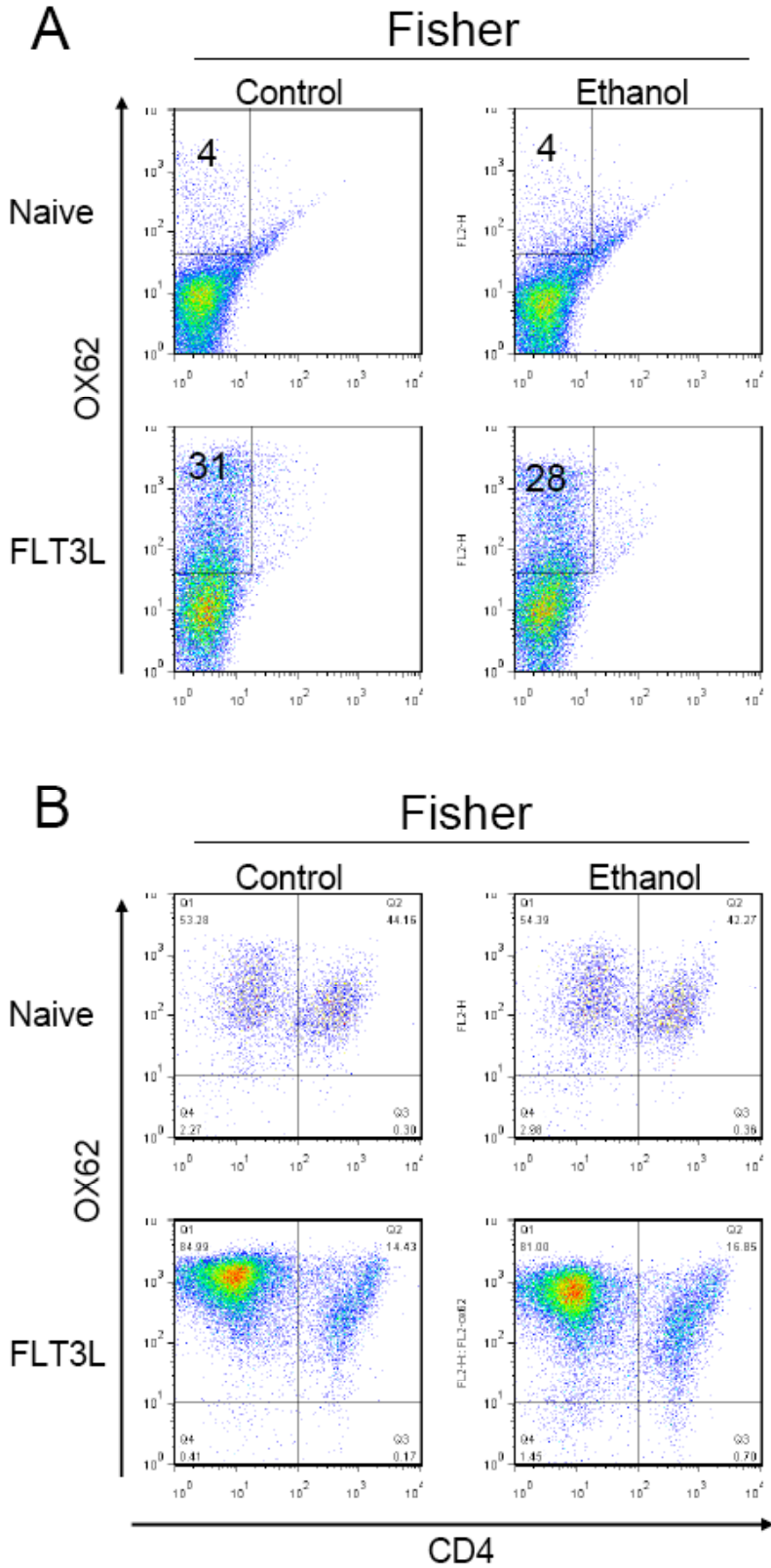


Figure 4.2

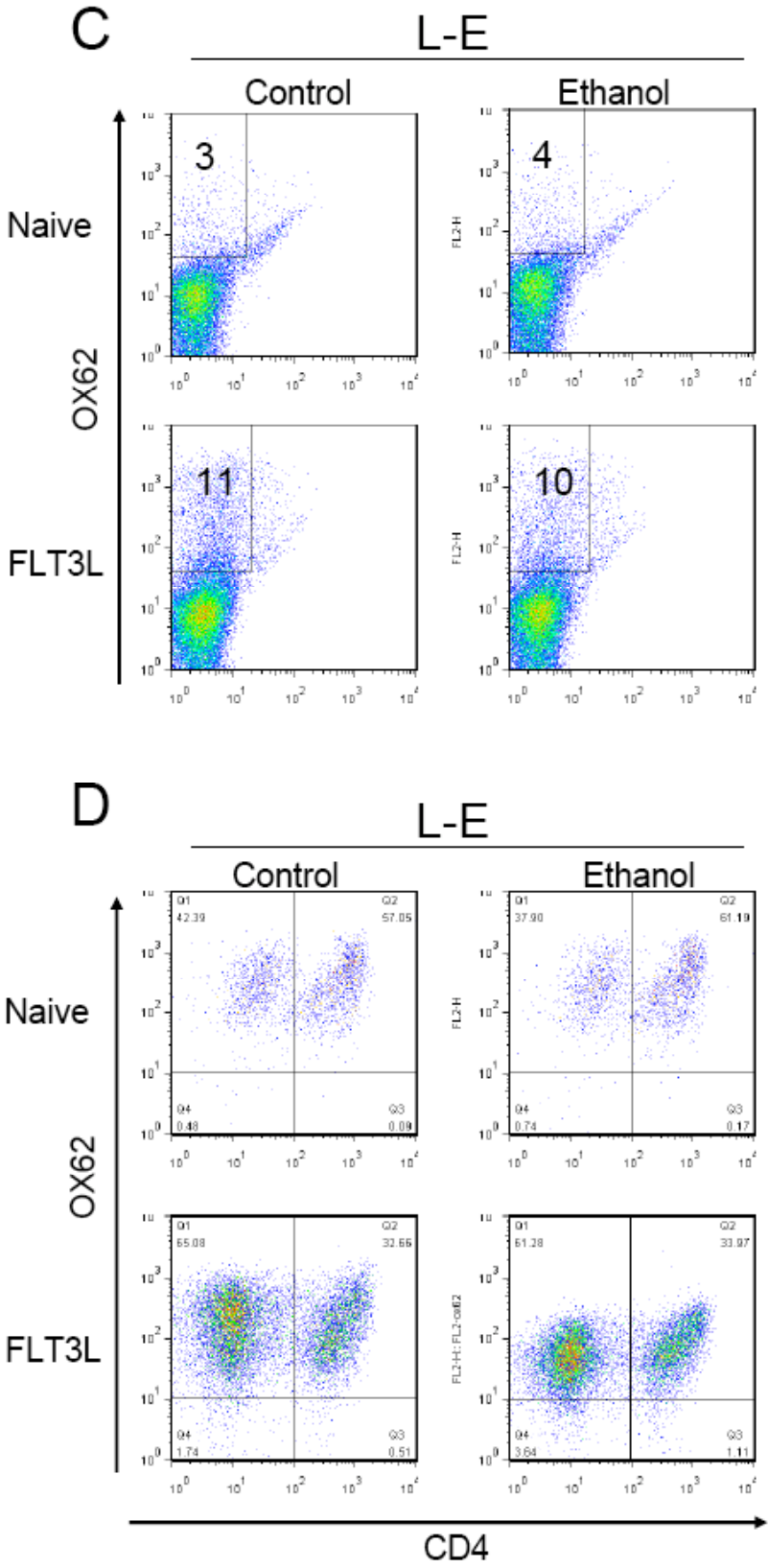
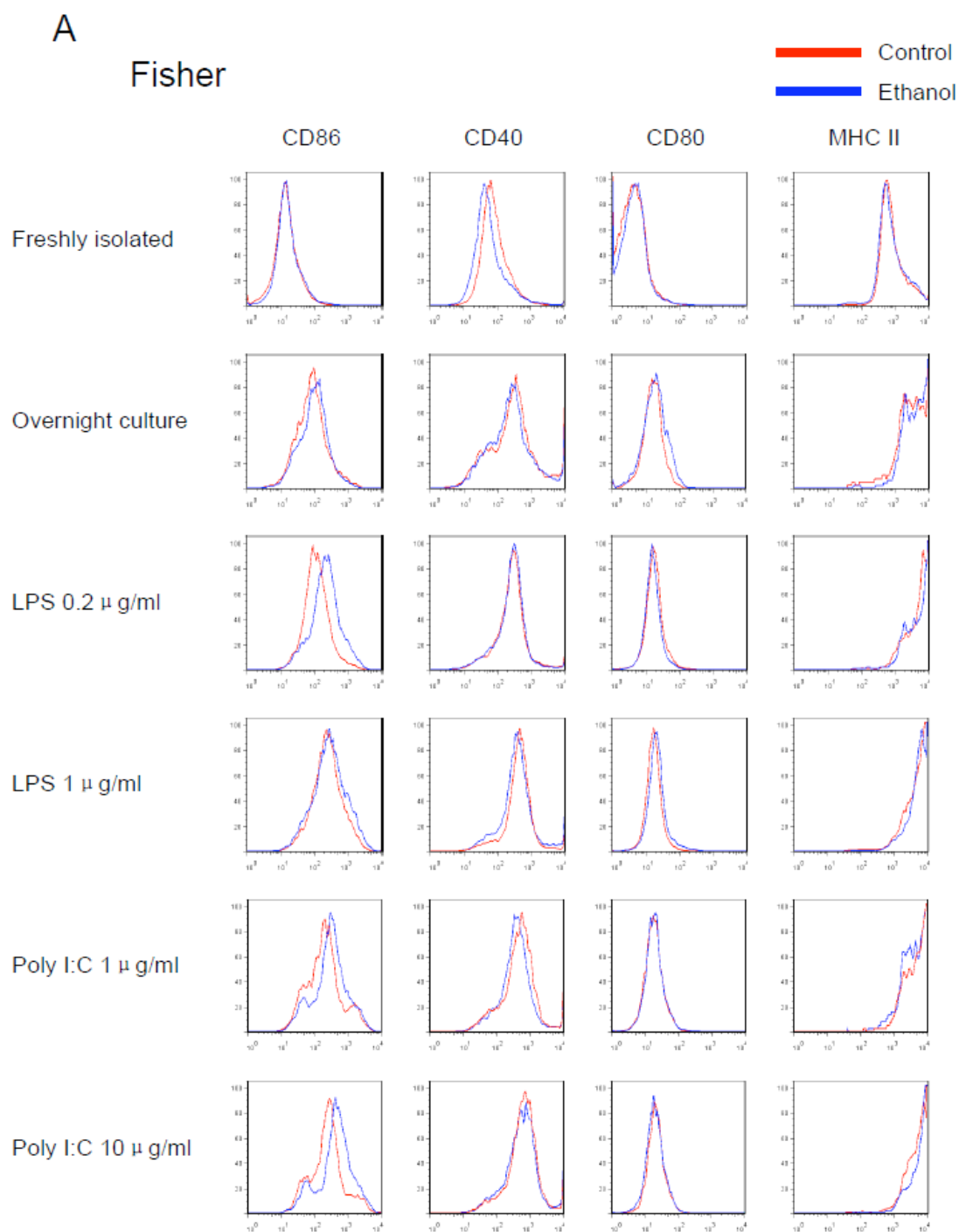


Fig. 4.2. Isolation and characterization of DCs from the spleen following expansion by hydrodynamic delivery of hFlt3L encoding plasmid. Flow cytometry analysis of splenocytes isolated from Fisher **(A)** or LE rats **(C)** before and after hFlt3L plasmid administration. Flow cytometry analysis of DCs purified from Fisher **(B)** and LE rats **(D)** by magnetic beads before or after hFlt3L plasmid administration. Thus, hydrodynamic delivery and expression of the hFlt3L plasmid greatly expands the DC population in rats. The experiment was repeated three times with similar results.

Figure 4.3



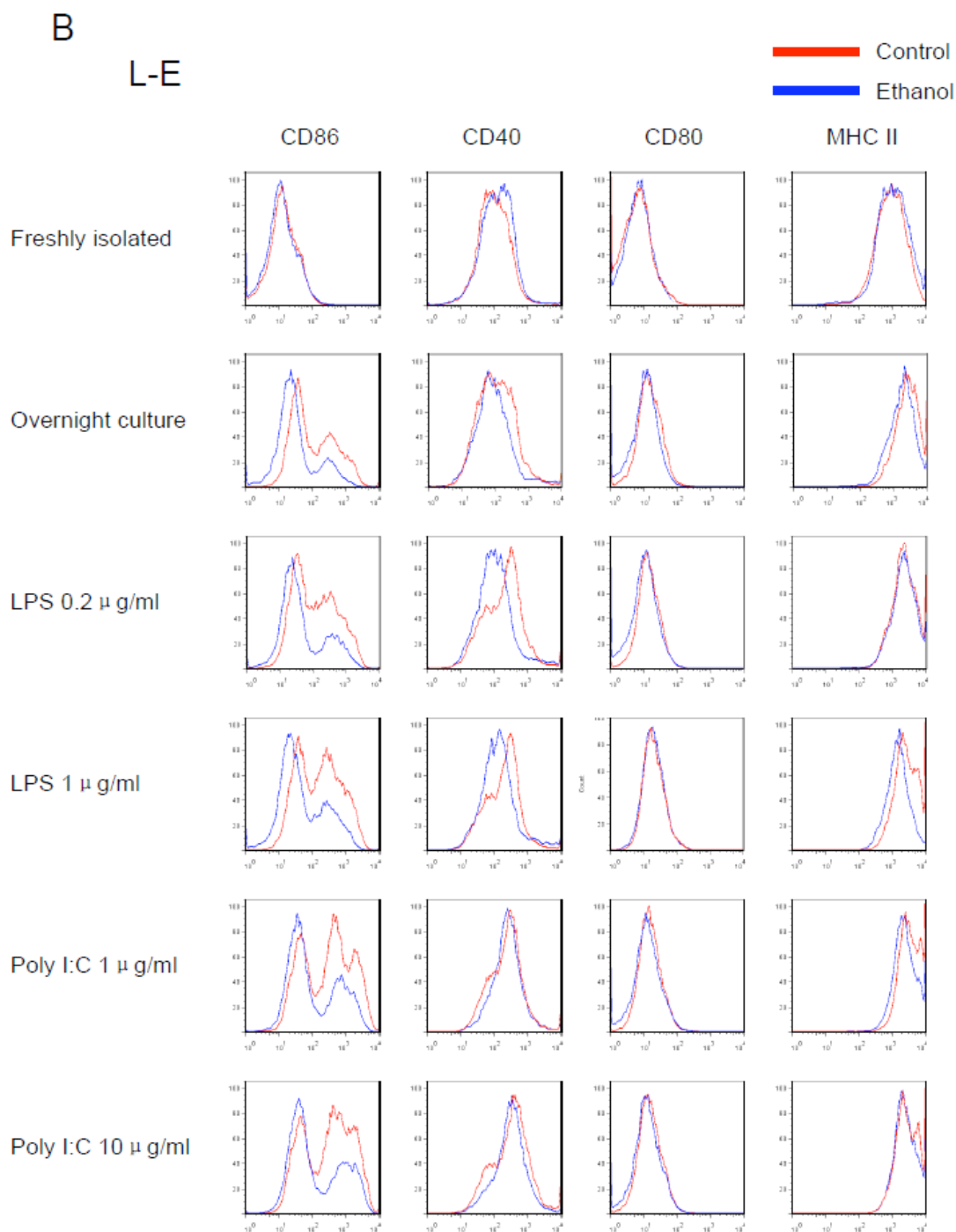


Fig. 4.3. Flow cytometry analysis of maturation marker expression. DCs were purified by magnetic beads from control or ethanol fed Fisher and LE rats after hFlt3L expansion. These isolated DCs were studied both fresh and after overnight culture with or without LPS, and Poly I:C additions. DCs were evaluated for CD86, CD40, CD80 and MHC-II expression. **(A)** Results

from DCs derived from Fisher rats on control and ethanol diet and **(B)** LE rats. Note that chronic ethanol feeding decreases CD86 and CD40 expression on DCs derived from LE but not in Fisher rats. The experiment was repeated three times with similar results.

Figure 4.4

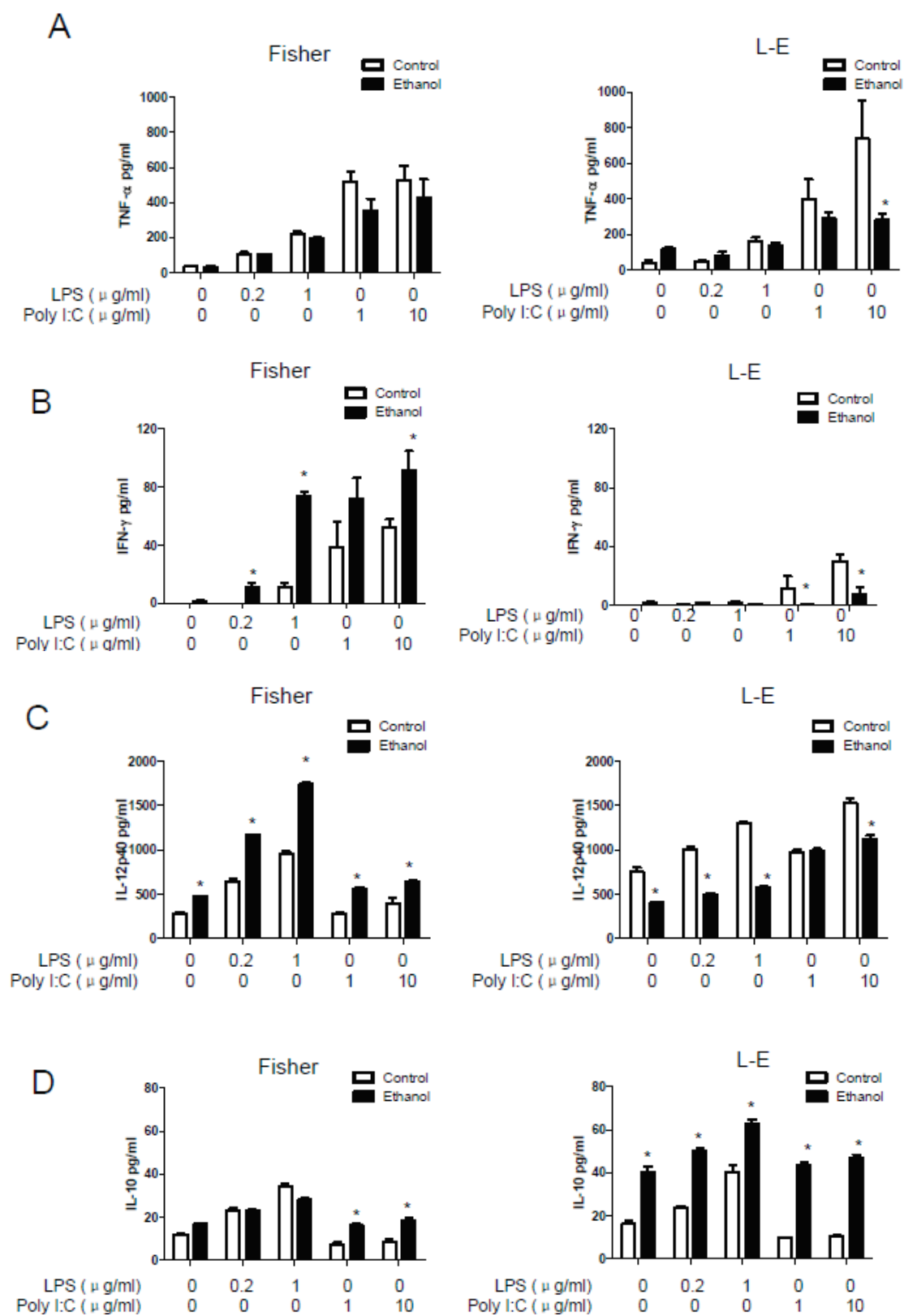


Fig. 4.4. Cytokine production by DCs after LPS or Poly I:C stimulation. DCs were purified by magnetic beads from the spleens of control or ethanol fed Fisher and LE rats after hFlt3L expansion. These DCs were cultured for 24 hours with or without LPS or Poly I:C. Cell culture supernatants were collected for cytokine measurement by ELISA. **(A)** TNF- α levels, *P<0.05, **(B)** IFN- γ levels, *P<0.01, **(C)** IL-12p40 levels, *P<0.01. **(D)** IL-10 levels, *P<0.01. It is apparent that chronic ethanol feeding inhibits pro-inflammatory and increases anti-inflammatory cytokine production in the ALD sensitive LE rat. The experiment was repeated three times with similar results.

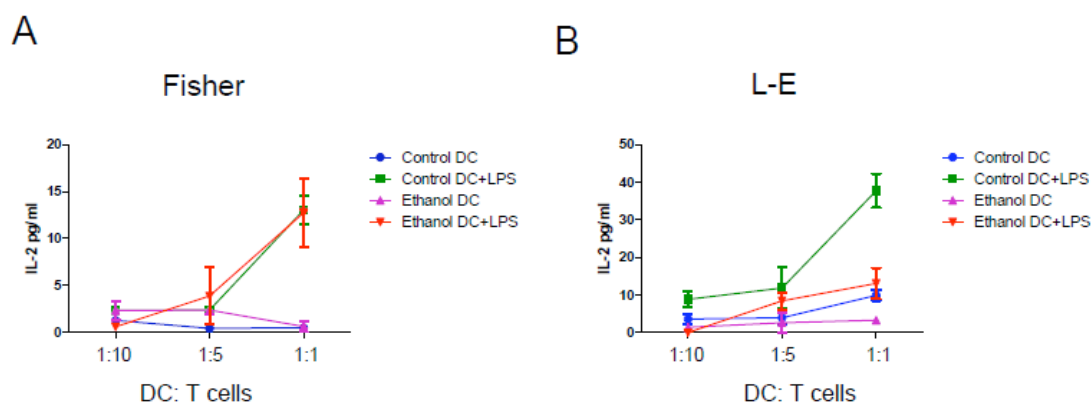
Figure 4.5

Fig. 4.5. Allostimulatory activity exhibited by DCs before or after LPS maturation. DCs were purified by magnetic beads from the spleen of control, ethanol fed Fisher and LE rats after hFlt3L expansion. DCs were cultured with/without 0.2 $\mu\text{g/ml}$ LPS as a maturation stimulus. After 24 hours of culture, DCs were collected and co-cultured with naïve allogeneic -T cell purified by magnetic beads from LE or Fisher rats as indicated ratio. Supernatants were collected for IL-2 levels as measured by ELISA after 3 days culture. **(A)** IL-2 levels of Fisher DCs co-cultured with LE T cells, and **(B)** IL-2 levels of LE DCs co-cultured with Fisher T cells. The experiment was repeated three times with similar results.

CHAPTER 5:
CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

In Chapter 1, an overview was presented on DC immunobiology emphasizing the findings of several studies on the effects of acute and chronic ethanol consumption on APCs, in particular, DCs. Chapters 2-4, which comprise the main body of this work, details how chronic ethanol consumption may alter DC function and downstream cellular processes in both mouse and rat models. Given the central role of DCs in generation of cell mediated immunity through priming of CD4⁺ and CD8⁺ T cells, the higher incidences of viral and bacterial infections in chronic alcoholics as well as the reports revealing exacerbated bacterial and viral infections in several chronic alcohol-fed murine models could be explained, in part, by the inhibitory effects of ethanol on DCs. Indeed, several investigations made it clear that both acute and chronic ethanol exposure has adverse effects on costimulatory molecule expression, alloreactivity, cytokine secretion and antigen presentation functions of DCs both in humans and mice (Chapter 7). These findings became impetus for further investigation and helped shape the hypothesis that: Suppressed effector T cell immune responses, and thus, the susceptibility to infections in chronic alcoholics, may partly be due to defects generated in DCs by chronic ethanol exposure.

Previous research on hepatocytes in culture demonstrated defective MHCI presentation of endogenous viral peptides, which was due to impaired proteasomal degradation in response to *in vitro* ethanol exposure.¹ In addition, MHCII presentation of exogenous antigens such as tetanus toxoid and ovalbumin by DCs to memory T cells as well as transgenic DO11.10 primary T cells isolated from mice were impaired, respectively, after long-term ethanol exposure.^{2,3} In chapter 2, we studied the characteristics of this suppression produced by ethanol on exogenous antigen presentation. Antigen presentation by DCs to CD4⁺ T cells is a process that requires engagement of 3 signals at DC- T cell interface: 1) MHCII-TCR interaction, 2) costimulatory molecule-receptor interaction and 3) soluble cytokines secreted by DCs to polarize CD4⁺ T cells differentiation and drive their expansion.^{4,5} In Chapter 2, in a sequential manner, the roles these

three signals conveyed by DCs in creating a functionally less active T cell at the end of DC: T-cell engagement were investigated in a setting of chronic ethanol consumption. The conclusions from these investigations can be summarized as follows:

1. Presentation of endogenous peptides by DCs to allogeneic T cells (both CD4⁺ and CD8⁺) is impaired after chronic ethanol feeding of CBA/caj strain as well the Balb/c strain. It is noteworthy that Cba/caj has not been used previously for ethanol studies. In addition to the altered alloreactivity, presentation of exogenous OVA protein and/or OVA 323-339 peptide to DO11 T cells and the primary CD4⁺ T and pan T cells was impaired as well. Although DO11.10 transgenic mice was used in a previous study, DO11 T cell line and the OVA specific primary T cells generated via immunizations have not been utilized in antigen presentation assays (in ethanol context). Therefore, these results, generated with different responder T cell types, are novel and support the findings by Heinz et al. and Mandrekar et al. that chronic ethanol exposure inhibits exogenous antigen presentation by DCs.

2. To investigate the effect of ethanol on signal 1(MHCII-peptide complex) conveyed by DCs to T cells, we first examined the impact of ethanol on degradation of antigens endocytosed by DCs, which ultimately leads to generation of peptide MHCII complexes. Our data indicate that DCs derived from both ethanol and control diet-fed animals can properly process the endocytosed antigens. This finding was further substantiated by an assay where DCs were pulsed with a short OVA323-339 peptide by which the DCs derived from ethanol-fed mice were expected to restore the defects in antigen presenting function if the abnormal processing of the antigens was responsible for the reduced antigen presentation. Supplementing the DCs derived from ethanol-fed mouse with ready-to-present peptide did not restore the antigen presentation defect, suggesting involvement of processes other than antigen processing in DC dysfunction produced by chronic ethanol feeding.

3. The influence of ethanol on the abundance of signal 1 (peptide-MHCII complex) generation by DCs was further explored by investigating the formation of Hen egg lysozyme (HEL) peptide₄₈₋₆₁-MHCII complex formation on the surface of DCs. These experiments revealed that although antigens are processed properly, ethanol exposure reduced the peptide-MHCII presentation on the surface of DCs. This reduction may be due to a problem in the loading of peptides onto MHCII, transport to cell surface, or as a result of low levels of MHCII expression in DCs derived from ethanol-fed mice. This difference was not due to a rapid turnover of peptide-MHCII as the DCs from both ethanol and control diet-fed mice maintained them comparably.

4. To distinguish whether the low peptide-MHCII complex level is the main actor that causes reduced antigen presentation and T-cell activation, or if the signal 2 (costimulatory molecules) and 3 (cytokines secreted) are contributing, we fixed OVA pulsed DCs and then utilized them as antigen presenting cells. We expected fixed DCs derived from control diet-fed mice to better activate T-cells based on the observation that DCs from control-fed animals had higher amounts of peptide-MHCII complexes on the cell surface. In contrast, we observed similar levels of T-cell activation by fixed DCs derived from ethanol and control-fed animals. This may indicate that, the difference in peptide-MHCII complex levels between ethanol and control group DCs is not sufficient per se to explain the antigen presentation defect induced by ethanol. Additionally, the cytokines secreted by DCs and or costimulatory molecules also play a role in this process. Chronic ethanol exposure has been shown to suppress both cytokine secretion and costimulatory molecule expression by DCs. So these defects may be additive or synergistic to promote reduced antigen-presentation, and hence, T-cell activation.

5. Attempts were made to restore the impaired antigen presentation by DCs isolated from ethanol-fed mice to DO11 T cells by adding back the cytokines TNF- α , IL-12, IL-6, IFN- γ or neutralizing IL-10 in the co-cultures. The results revealed that abnormal production profile of these cytokines per se was also insufficient to explain the reduced T cell activation by

DCs exposed to chronic ethanol. It is noteworthy that cytokines were added in combination or alone in an attempt to restore the defect and resulted in little or no improvement in T-cell responses. Unlike our findings with antigen specific T cell activation (with IL-2 read-out), impaired stimulation of allogeneic T-cell proliferation by DCs (exposed to ethanol) was repaired by the addition of recombinant IL-12 into cultures, indicating distinct mechanisms of action produced by ethanol for depressed alloreactivity and autologous antigen presentation⁶.

6. Finally, we examined the expression of a number of costimulatory molecules by DCs in response to chronic ethanol exposure. B7-H3 and B7-H4 have been reported to have T cell inhibitory effects; yet these molecules were not expressed by either mature or immature DCs. In addition, 4-1BBL was also reported to be expressed by DCs and acts to prolong effector T cell responses. However, 4-1BBL was not expressed by mature (LPS and polyI:C) or immature splenic DCs in our experiments as well. B7-DC (PD-L2), described as an inhibitory costimulatory molecule, was down regulated by DCs derived from ethanol-fed animals. CD86, a key DC costimulatory molecule, was down-regulated in response to chronic ethanol exposure as previously reported. The ICOS-L, which is an activating costimulatory molecule, was substantially higher in DCs derived from ethanol-fed mice. Although CD86 down-regulation after ethanol feeding is consistent with reduced antigen presentation by DCs, the ICOS-L and B7DC modulation will require further study before firm conclusions can be made.

In Chapter 3, new target genes were identified in DCs that could mediate suppressive effects of chronic ethanol exposure. In this regard, we compared the transcription profiles of immature CD11c⁺ splenic mouse DCs derived from control and ethanol diet-fed animals, as well as those matured in the presence of LPS *in vitro*. The analyses of immature DCs revealed only 21 differentially regulated genes involved in various cellular processes that include lectins, growth factors and chemokines. Analyses of gene expression data obtained from mature DCs, however, revealed alterations in the expression of 44 genes that carry out metabolic, immunologic, cofactor

and cell proliferation functions. The functions of the most relevant genes with respect to potential DC function were described in Chapter 3 and await confirmation studies by real time PCR followed by protein level analysis, both of which are currently underway. Among these 44 genes are TAP2, Legumain, MyD88, MHCI alpha, CD40, CD81, IL-12b and CD1d. Indeed, TAP2 has a very important function in MHCI loading, whereas legumain is a protease that functions in endosomes. MyD88 is an adapter molecule common to all TLRs, which is important for DC maturation. CD40 and CD81 are costimulatory molecules. Additionally, IL-12 secretion by mature CD11c⁺ splenic DCs has been shown to be down regulated confirming the observation we made in mRNA level. We also stained immature and mature DCs for CD1d to assess the cell surface protein expression as a preliminary validation of the microarray results. Although immature DCs derived from control diet-fed animals had slightly higher CD1d, after maturation with LPS and polyI:C, protein levels were comparable in DCs derived from ethanol and control-fed animals by immunostaining and flow cytometry analysis. Whether there is a discrepancy between protein and transcript levels or CD1d is a false positive hit will be determined after real time PCR analysis.

In Chapter 4, two rat strains, Long Evans (LE) and Fisher, were employed to study the suppressive effects of ethanol and liver disease on rat DC function. These two strains were chosen because: LE rats have been shown to be more susceptible to alcohol, and developed many features of the alcoholic liver disease (ALD) such as progressive hepatocyte injury, oxidative stress, mitochondrial dysfunction, severe steatosis, and fibrosis upon chronic ethanol feeding whereas Fisher strain was resistant to ALD. With this unique comparative animal system, we aimed to explore the impact of presence of ALD on DC functions in a chronic ethanol consumption setting. The findings of this investigation can be summarized as follows:

1. To expand DCs in rats we utilized for the first time the FLT3L plasmid injection protocol optimized for rats. This method proved successful in expanding the total DC number in the rat spleen with a particular bias towards OX62+ CD4- population.

2. We observed differences in costimulatory molecule expression pattern of LE and Fisher rat DCs in response to chronic ethanol feeding. LPS (low dose) and polyI:C (low and high dose) matured DCs from Fisher rats expressed higher CD86, whereas those derived from LE rats had substantially lower levels of both CD86 and CD40 molecules expression on the cell surface.

3. Fisher and LE rat DCs displayed unique cytokine secretion profiles in response to chronic ethanol exposure as well. Interestingly, LPS and polyI:C matured Fisher rat DCs derived from ethanol-fed animals produced higher amounts of IFN-gamma, IL-12p40 and IL-10 compared to DCs obtained from isocaloric pair-fed control animals. In contrast, mature LE rat DCs isolated from ethanol-fed animals showed reduced IFN-gamma, IL-12p40 and TNF-alpha secretion compared to DCs isolated from isocaloric pair-fed animals. In addition, IL-10 secretion by LE rat DCs derived from ethanol-fed animals was increased compared with Fisher strain DCs.

4. Finally, antigen presentation capabilities of DCs from both strains to allogeneic T cells also revealed significant differences. Chronic ethanol feeding impaired the alloreactivity of LE rat derived DCs, whereas DCs derived from Fisher strain were unaffected by ethanol.

These results obtained from Fisher strain are similar to those of human peripheral blood DCs purified from chronic alcoholics without liver disease, which were characterized by increased secretion of pro-inflammatory cytokines. Additionally, our findings reveal that antigen presenting function of DCs were severely altered when chronic ethanol consumption was accompanied by ALD. It is likely that the genetic factors that predispose LE rat liver tissue to the development of ALD are also operational in DCs and thus the DCs of this strain are more susceptible to ethanol like hepatocytes. On the other hand, ALD may also indirectly affect the functions of immune cells including hepatic DCs in the liver microenvironment; yet this hypothesis needs to be substantiated experimentally.

In chapter 6, we present a study conducted in collaboration with Dr. Wintermeyer in which we generated immunity to Hepatitis C Virus (HCV) non- structural (NS5) protein 5 through *in vivo* targeting of this protein to DCs. It was demonstrated that by *in vivo* expansion of DCs in mice through FLT3L plasmid injection, one can target viral antigens (NS5 in this case) coupled to microparticles to DCs; and subsequently generate cellular immunity *in vivo* as demonstrated by the reduced growth rate of implanted NS5 expressing tumors in immunized mice. This anti-viral immune response was composed of both CD8⁺ and CD4⁺ T cells arms as shown by the presence of a robust CTL activity to NS5 expressing target cells and secretion of IFN-gamma and IL-2 by splenocytes, respectively. Therefore DCs may be useful for immunotherapy of HCV in alcoholics with persistent viral infection as well.

Finally, Chapter 7 includes a review contributed by many investigators studying the adverse effects of ethanol on the course of HCV infection in both immune and liver cells. The “HCV, Alcohol and Dendritic Cell Dysfunction” part of this article is a minireview contributed by us, and describes the findings from our lab in the past decade, as also reviewed in Chapter 1.

Ongoing studies and Future directions

One primary focus is to follow up and validate the significance of the genes differentially regulated in response to chronic ethanol exposure obtained from mature DCs, and is currently being pursued with real time PCR analyses. We will later evaluate protein expression and compare it to the mRNA data.

In Chapter 2 we investigated presentation of exogenous antigen via MHCII molecules. However, DCs can present exogenous proteins by MHCI molecules through cross presentation as well. Indeed, CD8⁺ DCs have been shown to be specialized for crosspresentation. Therefore, we would like to determine if the crosspresentation by DCs is altered by chronic ethanol exposure.

Our observations revealed that epitope specific T cells proved to be more sensitive for antigen presentation experiments. To evaluate crosspresentation to CD8⁺ T-cells, we planned to employ C57BL/6 mice which carry an engineered TCR transgene specific to 8-mer SIINFEKL OVA peptide (257-264aa) in the context of H2K^b haplotype. Syngeneic C57BL/6 H2K^b mice will be fed a chronic ethanol diet and DCs will be isolated for crosspresentation experiments. These experiments will be performed with both full length OVA protein and SIINFEKL peptide to investigate the presence of any abnormality related to protein processing, which involves proteasomal and endocytic degradation pathways. Further experiments can be designed to dissect the exact contribution and role of proteasomal and endocytic antigen processing in DCs to crosspresentation dysfunction, if that occurs. Additionally, there is a specific antibody commercially available that can detect H2K^b MHCI-SIINFEKL peptide complex, which would hopefully reveal whether peptide-MHCI complex formation on the cell surface of DCs occurs properly after long term ethanol exposure *in vivo*.

We have briefly described distinct subsets of splenic DCs in Chapter 1: pDCs, and conventional DCs such as CD8⁺, CD4⁺ and Double negative (DN). Another potential approach would be to pursue comparisons of effects of ethanol on allogeneic and autologous presentation along with secretion of Th1-Th2 and Th17 cytokines and costimulatory molecule expression by these DC subsets.

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CHAPTER 6: APPENDIX 1

**GENERATION OF CELLULAR IMMUNE RESPONSES TO HCV NS5 PROTEIN
THROUGH IN VIVO ACTIVATION OF DENDRITIC CELLS**

The experiments in this Chapter were performed by or in collaboration with Dr. Philip
Winterrmeyer.

Generation of cellular immune responses to HCV NS5 protein through *in vivo* activation of dendritic cells

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SUMMARY. Chronic hepatitis C (HCV) infection is a substantial medical problem that leads to progressive liver disease, cirrhosis, and hepatocellular carcinoma (HCC). The aim of this study was to achieve sustained cellular immune responses *in vivo* to a HCV nonstructural protein using dendritic cell (DC)-based immunization approach. We targeted the HCV NS5 protein to DCs *in vivo* by injecting microparticles loaded with this antigen. The DC population was expanded in BALB/C mice (H-2^d) by hydrodynamic injection of a plasmid pUMVC3-hFLex expressing the secreted portion of the human Fms-like tyrosine kinase receptor-3 ligand (hFlt3). Mice were subsequently injected with microparticles coated with HCV NS5 protein via the tail vein. Cellular immune responses were determined with respect to secretion of INF γ and IL2 by CD4⁺ cells and cytotoxic T-lymphocyte (CTL) assays *in vitro*; inhibition of

tumour cell growth was employed for the assessment of CD8⁺ generated activity *in vivo*. We found that Flt3L treatment expanded the DC population in the spleen to 43%, and such cells displayed a striking upregulation of CD86 as well as CD80 and CD40 co-stimulating molecules. Viral antigen-specific T_H1 cytokine secretion by splenocytes was generated, and CTL activity against syngeneic NS5 expressing myeloma target cells was observed. In addition, these cells inhibited tumour growth indicating that NS5-specific robust CTL activity was operative *in vivo*. Thus, the capability of activating DCs *in vivo* using the methods described is valuable as a therapeutic vaccine strategy for chronic HCV infection.

Keywords: cellular immune response, cytotoxicity, dendritic cells, hepatitis C virus.

INTRODUCTION

Hepatitis C virus (HCV) infection is caused worldwide by a positive strand RNA virus [1]. Exposure to HCV results in acute infection in approximately 15% of persons; however, in the remaining 85%, there is the establishment of persistent viral infection. Most individuals with chronic HCV have mild to no clinical symptoms; however, there is often the development of progressive liver disease, cirrhosis, and hepatocellular carcinoma (HCC). Therefore, chronic HCV infection is a substantial medical problem on a global basis. The mechanism(s) responsible for persistent viral infection are unclear, but may involve alterations in the host cellular immune response to HCV.

In this regard, dendritic cells (DCs) are highly specialized antigen-presenting cells (APC). DCs are characterized by their ability to take up, process, and present peptides in the

context of MHC Class I and Class II molecules to effector T-cells. Thus, DCs act at the interface between innate and adaptive immune responses [2]. Upregulation of co-stimulatory molecules is essential and influenced by several factors such as activation of Toll-like receptors or binding of CD40 by the CD40 ligand. Interactions of DCs with T-cells allows for highly efficient antigen presentation and the generation of viral-specific cellular immunity [3].

There is increasing evidence that DC-based vaccination represents an attractive approach to elicit sustained antiviral responses to HCV structural and nonstructural proteins [4–6]. To develop and employ a successful immunological approach against HCV, it is important to understand the essential process(es) in the host that leads to clearance of viral infection. Current studies suggest that HCV eradication from infected persons requires the early appearance of strong CD8⁺ cytotoxic T-cell (CTL) activity in the context of a vigorous and sustained CD4⁺ T-cell proliferation culminating in the secretion of T_H1-type cytokines in response to stimulation by multiple structural and nonstructural viral proteins [7–9]. Because DCs interact with both CD8⁺ and CD4⁺

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T-cells, they play a pivotal role in the transition from innate to adaptive immunity. Interestingly, there is emerging data that suggests chronic HCV infection is associated with impaired DC activity [10,11]. Defective DC function has been observed in recent studies where chronic alcohol consumption has been found to alter the properties of DCs and resulted in impaired cellular immune responses against the HCV NS5 protein [12]. Taken together, these studies emphasize the importance of robust DC function for successful eradication of chronic HCV infection.

The purpose of this study was to achieve sustained cellular immune responses to a HCV nonstructural protein *in vivo* using a DC-based immunization approach. In this study, we attempted to target the HCV NS5 protein to DCs *in vivo* by injecting microparticles coated with this antigen. Previous studies revealed that antigen-loaded beads represent an excellent vehicle for targeting DCs *in vitro* because they are very efficiently internalized by such cells [13]. The results suggest that DCs are activated *in vivo* and induced a viral-specific CD4⁺ and CD8⁺ cellular immune response following prior DC expansion with Flt3L administration and subsequent intravenous (i.v.) injection of NS5 protein-coated microparticles.

MATERIALS AND METHODS

Mice

Six- to eight-week-old female BALB/c mice (H-2^d) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and kept under specific pathogen-free conditions in the animal facility of Rhode Island Hospital. Experiments were conducted according to animal protocols reviewed and approved by the Lifespan Animal Care and Use Committee.

Culture conditions

Splenocytes derived from immunized and control mice were cultured in serum-free HEPES-buffered RPMI 1640 medium (Cambrex Bio Sciences, Walkersville, MD, USA) supplemented with 2 mM L-glutamine, 1% essential and nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5 U/mL gentamycin (all from Sigma-Aldrich, St Louis, MO, USA). Cultivation medium for splenocytes obtained from immunized animals was additionally supplemented with heat-inactivated 10% foetal bovine serum (FBS).

Coating of magnetic beads with HCV NS5

Immunomagnetic beads (Calbiochem, EMD Bioscience, San Diego, CA, USA) with a diameter of 1.3 µm were suspended in CDI buffer (Sigma-Aldrich, St Louis, MO, USA) and incubated for 10 min at 50 °C, pelleted and washed three

times in borate buffer (pH 8-9) (Fluka/Sigma-Aldrich, St Louis, MO, USA). After the final wash, borate buffer was added with or without 100 µg of NS5 protein (RDI Fitzgerald, Concord, MA, USA), followed by an overnight incubation at room temperature under constant slow agitation. Then beads were pelleted through magnetic forces, and the supernatant was removed by pipetting; the beads were then washed and resuspended in 200 µL Hank's balanced salt solution (HBSS). To prevent bead aggregation, centrifugation was omitted in all steps.

In vivo enrichment of dendritic cells

Expansion of the DC population before immunization was achieved by using methods previously described [2,14]. Briefly, the plasmid pUMVC3-hFLex expressing the secreted portion of human Fms-like tyrosine kinase receptor-3 ligand (hFlt3; Vector Core Laboratory, University of Michigan) was injected twice (day 0 and 6) into the tail vein of mice (hydrodynamic gene delivery); the first course of immunization was then performed on day 12 after the first hFlt3L plasmid injection.

Immunization

Four groups of mice were immunized after *in vivo* expansion of DCs with hydrodynamic injection of an hFlt3L expression plasmid. Mice were injected with 100 µg of beads or beads coated with HCV NS5 protein and resuspended in 200 µL HBSS intravenously into the tail vein. The immunization schedule is described in Table 1.

Intracellular cytokine staining (ICS) and flow cytometric analysis

ICS and flow cytometry were performed in accordance with methods previously described [2,14]. In brief, $1-5 \times 10^5$ cells were incubated with excess anti-mouse CD16/32 (clone 93, rat isotype) to block Fc-receptor, then stained with 1 µg PE-, FITC-, or PerCP-labelled antibody specific for the following mouse cell surface markers: CD11c (clone N418), CD4 (clone L3T4), CD8α (clone Lyt-2), CD40 (clone 1C10); the recommended isotypes controls were included. ICS was

Table 1 Immunization schedule

Day 0	First hydrodynamic injection of Flt3L plasmid
Day 6	Second hydrodynamic injection of Flt3L plasmid
Day 12	First immunization (beads with or without NS5 protein)
Day 26	Second immunization
Day 40	Sacrifice animals for immunological studies Injection of tumour cells (4×10^6)
Day 47	Weigh tumours

performed with anti-mouse IFN- γ (clone XMG1.2) or anti-mouse IL-2 (clone JES6-5H4), and the Cytofix/Cytoperm Kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. All antibodies were purchased from eBiosciences (San Diego, CA, USA) if not otherwise indicated.

Proliferation assay

Cells (4×10^6) were labelled with 1.0 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) in HBSS by incubation for 15 min at 37 °C. Then, the cells were centrifuged, resuspended in prewarmed medium, and incubated for another 30 min to ensure complete modification of the probe. The labelled cells were incubated for 5 days in 6-well plates containing 5 mL culture medium supplemented with 10% FBS and 1 μ g/mL HCV NS5. The cells were then washed, stained for CD4 or CD8, and evaluated for proliferation by flow cytometry.

Measurement of cytokine secretion

To quantify cytokine production, splenocytes derived from immunized and nonimmunized animals were cultured at 5×10^5 cells/200 μ L/well in 96-well flat-bottom plates at 37 °C. Cells were stimulated with 0.1–1 μ g/mL recombinant HCV NS5 protein. After 24 h, the supernates were collected, and the levels of IFN- γ and IL-2 were quantified using commercial ELISA kits purchased from eBiosciences according to manufacturer's instructions.

In vitro cytotoxicity assay

The CTL activities expressed by splenocytes derived from immunized and control mice were assessed following *in vitro* stimulation in which 1×10^8 cells in 30 mL of culture medium were incubated with recombinant murine IL-2 (5 U/mL; eBiosciences, San Diego, CA, USA) and recombinant HCV NS5 protein (0.3 μ g/mL; RDI Fitzgerald, Concord, MA, USA). After three days culture, the cells were harvested, and a standard 4-h ^{51}Cr release assay was performed. The ^{51}Cr labelled parental syngeneic murine myeloma cells (SP2/0); (ATCC, Manassas, VA, USA) and stably expressing clones of HCV NS-5 (SP-2/21-NS-5) were used as the targets. All assays were conducted in quadruplicate. Antigen-specific lysis is calculated using the following formula: $[(E-M)/(T-M)] \times 100$; T = maximum release, M = spontaneous release, E = experimental release.

Tumour challenge model

CTL activity was assessed *in vivo* by tumour challenge in accordance with methods described previously [2,14]. Briefly, stably expressing HCV NS5 cells (SP-2/21-NS-5) were harvested and washed three times in serum-free medium. The

backs of mice were shaved, and each animal was inoculated s.c. with 4×10^6 SP2/21-NS5 cells in the left flank and with parental SP2/0 cells in the right flank. Cells were resuspended in 100 μ L serum-free medium. On day 15, mice were euthanized; tumours were dissected, measured, and weighed.

Statistical analysis

Results were analysed using the SigmaStat 3.0 statistics program (Jandel Scientific, San Rafael, CA, USA). Individual means were compared using a nonpaired Student's *t*-test. When comparing more than two groups, a one-way ANOVA was performed followed by a Tukey test to determine which groups differed significantly ($P < 0.05$).

RESULTS

Expansion and maturation of dendritic cell populations *in vivo*

To expand DCs in BALB/c mice prior to immunization, we employed the technique of hydrodynamic delivery of the hFlt3-L expressing plasmid (pUMVL3hFlex) as described [15]; the total number of splenocytes was increased tenfold to approximately 4×10^8 cells/spleen of which 25% were positive for CD11c [13]. This represents approximately 100-fold expansion of the murine DC splenic population [6,13]. In this context, animals were immunized with NS5 or poly IC-coated beads. It was not possible to determine the number of amplified DCs in individual animals following immunization. Nevertheless, we characterized the DC population following i.v. injection of NS5-coated magnetic microparticles in animals with or without hydrodynamic injection ($\times 2$) of the Flt3L expression plasmid. Splenocytes were isolated and bead-containing phagocytic cells were separated using MACS[®] columns. Isolated cells were then stained with antisera to CD11c and CD11b, and double-positive cells were designated as DCs. Without Flt3L treatment, DCs represented 6.9% of the total cell population. However, following Flt3L injection, 4.3% of the cells in the spleen were characterized as DCs as shown in Fig. 1. In addition, all such cells were found to contain NS5-coated microbeads by direct microscopic examination, and thus the HCV antigen-coated microparticles reached the spleen by IV injection (data not shown). Furthermore, injection of beads coated with poly I:C, a ligand to the Toll-like receptor 3 expressed on DCs, led to a striking upregulation of CD86 (Fig. 2a), as well as CD80 and CD40 (Fig. 2b,c). Again, it appears that the coated beads reached their intended target cells in the spleen and matured the DC population.

In vitro cytotoxicity assay

A standard ^{51}Cr -release assay was employed to measure CTL present in the animals immunized with NS5-coated microparticles when compared to beads alone. The target

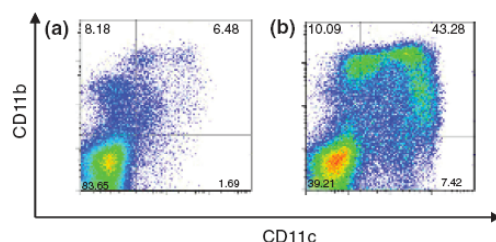


Fig. 1 Expansion of CD11b⁺ and CD11c⁺ cells following hydrodynamic delivery of an Flt3L expressing plasmid. Panel A represents before and Panel B represents after administration of Flt3L. Note that the percentage of double-positive cells increased from 6.5% to 43.3% of the splenic population.

SP2/21-NS5 cells were a derived cell line that constitutively expresses NS5-related peptides following stable transfection of an NS5 expressing plasmid [6]. The control cells were derived from the parental SP2/0 myeloma cell line. Highly significant CTL activity was observed in Flt3L-pretreated mice immunized with NS5-coated beads at various effectors to target cell ratio(s). In contrast, splenocytes derived from animals immunized with beads alone had little, if any, NS5-specific cytotoxicity (Fig. 3). There was no CTL activity directed against the control SP2/0 cells derived from mice immunized with NS5-coated, or noncoated beads (data not shown). These results suggest that i.v. immunization of NS5-coated microparticles in Flt3L-expanded DC populations are capable of activating HCV-specific CTL activity. It then became of interest to determine if such CTL activity was operative *in vivo*.

Tumour challenge model for assessment of *in vivo* CTL activity

Generation of CD8⁺ CTL viral-specific immune response is a key to the eradication of HCV infection in the liver. We performed a tumour challenge to determine whether antigen-specific CTL activity was present *in vivo*. In this experimental paradigm, animals were inoculated with tumour cells that expressed NS5 protein (SP2/21-NS5), or the nonexpressing parental SP2/0 cell line (control). DCs were expanded by hydrodynamic injection of the Flt3L expressing plasmids on day(s) 0 and 6, respectively. On day 12, animals were immunized with HCV NS5-coated beads i.v. via the tail vein; an immunization boost was performed 2 weeks later. Control animals received no immunization, immunization with beads alone, or immunization with soluble recombinant NS5 protein as an additional control. After the second immunization, mice were inoculated with 4×10^6 tumour cells. To provide intra-animal comparisons, mice received NS5 expressing cells in the left flank and the SP2/0 parental cells in the right flank simultaneously. Two weeks later, tumours were dissected, removed, and weighed.

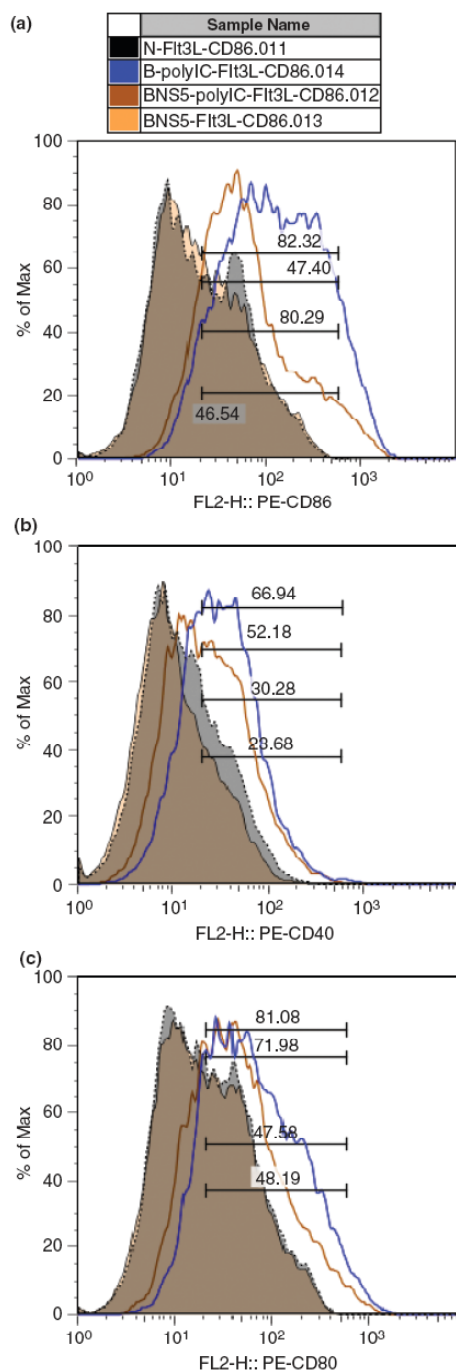


Fig. 2 Expression of co-stimulatory molecules CD86 (Panel A), CD40 (Panel B), and CD80 (Panel C) on DC surface after Flt3L injection or/and immunization with beads coated with/without NS5, poly I:C. Mice were injected with different coated microbeads and sacrificed 12 h after injection for harvesting of splenocytes. Splenocytes were stained with anti-CD11c as a marker for DCs, and anti-CD80, anti-CD86, and anti-CD40 as indicators of maturation. CD11c⁺ cells were gated and analysed for earlier mentioned markers. DCs obtained from mice that were injected solely with Flt3L expression plasmid (Black dotted line/grey filling) were already CD86 and CD80 positive: 47.40% and 47.58%, respectively. Injection of NS5-coated microbeads (light pink line and filling) did not increase neither CD86 nor CD80 expression: 46.54% and 48.19%, respectively. Intravenous injection of microparticles coated with the TLR3-ligand poly I:C, a potent DC maturation stimulus upregulated all three markers regardless of NS5 (82.32%, 66.94%, 81.08%, CD86, CD40, and CD80 levels, respectively, for those received beads-poly I:C and Flt3L represented by blue line; 80.29%, 52.18%, 71.98%, CD86, CD40, CD80 levels, respectively, for those received beads-NS5-poly I:C and Flt3L represented by orange line). This confirms that protein that is coated on microbeads reaches DCs where it is processed effectively. Percentile bars from top to bottom across all three panels are for the indicated line: blue (Bead-poly I:C-Flt3L), orange (Bead-NS5-poly I:C-Flt3L), black (Flt3L only), light pink (Bead-NS5-Flt3L).

As shown in Fig. 4, there was no difference in growth rate between the NS5 expressing tumour cells and the SP2/0 nonexpressing parental cells in immunized mice that

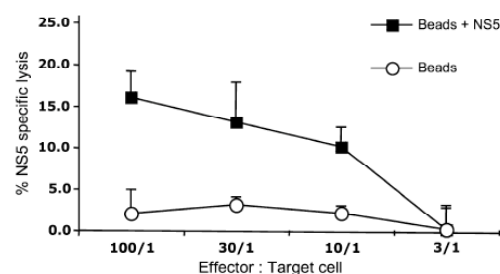


Fig. 3 *In vitro* CTL activity generated against NS5 expressing target cells. Splenocytes derived from immunized mice were restimulated with NS5 protein for 4 days. Restimulated cells were placed in co-culture with NS5 expressing or parental SP2/0 myeloma cell lines. Target cells were labelled with Cr⁵¹ chromium, and target cell lysis was measured by release of Cr⁵¹ chromium into the cell culture medium. Nonspecific SP2/0 cell lysis has been subtracted from the lysis of NS5 expressing cells (SP-2/21-NS5). Note that animals immunized with uncoated beads showed no CTL activity compared to beads coated with NS5 protein.

received no beads, uncoated beads, or NS5 soluble protein as shown in Fig. 4a,c,d. In contrast, animals immunized twice with NS5-coated beads showed a dramatic reduction in the growth rate of NS5 expressing tumour cells when compared to the control SP2/0 parental tumour cell line (Fig. 4b). These results suggest that the Flt3L-expanded DC population that ingested the NS5-coated microparticles generated robust CTL activity *in vivo* as measured by reduction in tumour growth.

HCV viral antigen-specific cytokine production

Natural clearance of HCV infection is strongly associated with a sustained T_H1 immune response. Therefore, we assessed the ability of splenocytes derived from immunized mice to secrete T_H1-type cytokines such as INF γ , and IL-2. We compared cytokine secretion in animals pretreated with or without Flt3L. As shown in Fig. 5a,b, both INF γ and IL-2 secretion were increased in an NS5 dose-dependent manner in animals that received both NS5-coated beads and Flt3L prestimulation when compared to mice who received NS5-coated beads without Flt3L expansion of DCs. The specificity of the CD4⁺ immune response to NS5 is indicated by cytokine production following stimulation with an increasing concentration of NS5 protein. There was no INF γ and IL-2 production by splenocytes following immunization with beads alone or immunization with NS5 protein (data not shown).

DISCUSSION

Similar to most phagocytic cells, DCs are equipped with receptors which include members of the C-type lectin family that mediate antigen uptake. This phagocytic process is initiated through the calcium-dependent binding of C-type lectins to carbohydrate-bearing pathogen-derived antigens through highly conserved carbohydrate recognition domains (CRD). Some of these receptors appear to be ubiquitously expressed on phagocytic cells such as macrophages, monocytes, B-cells, neutrophils, and DCs [16]. Two different types of transmembrane C-type lectins are expressed on DCs distinguished by their molecular characteristics. The prototype C-type-1 lectin is the macrophage mannose receptor (MMR; CD206). This molecule contains 8 CRD, a N-terminal cysteine-rich domain, and a region involved in antigen transport [16]. A homologue to the MMR is DEC-205 (CD205), which is a second member of the type I lectins. Unlike the MMR, it is expressed specifically and uniquely on DCs and thymic epithelial cells. Investigations reveal that antibodies binding to DEC-205 are presented with a 100-fold higher efficiency to effector T-cells than nonrelevant antibodies [17]. In addition, when antigen is conjugated to an anti-DEC-205 antibody and specifically taken up by DCs residing in lymphoid tissue, evidence suggest that peptide epitopes are presented with high efficiency to CD4⁺ and CD8⁺ T-cells [18].

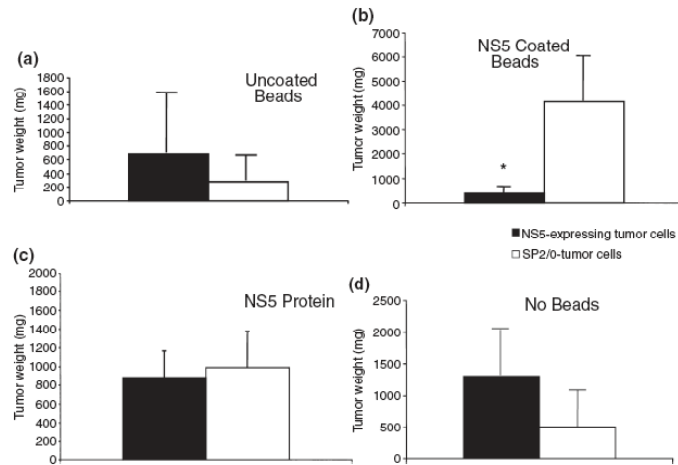


Fig. 4 Generation of CD8⁺ CTL activity *in vivo* using a tumour challenge model. Mice were administered Flt3L expression plasmid via hydrodynamic delivery and then immunized $\times 2$ with microparticles that were either uncoated (a), coated with NS5 protein (b), or following i.v. injection of NS5 protein alone (c), or no beads (d). Two weeks after the last immunization, mice were inoculated with hybridomas expressing NS5 protein (SP2/21-NS5) in the left flank and a hybridoma cell line negative for NS5 (SP2/0) in the right flank simultaneously. Animals were sacrificed two weeks later, and the tumours were excised and weight measured. Note the striking reduction in tumour growth rate in Panel B where animals are immunized with NS5-coated beads, and showed potent inhibition in growth rate of NS5 producing tumours. Animals immunized with uncoated beads, NS5 protein alone, or no beads showed no effect on tumour growth rate. The anti-tumour effect was antigen specific as shown in Panel B because there was no effect on the growth rate of the parental, nonexpressing NS5 cell line. * $P < 0.01$.

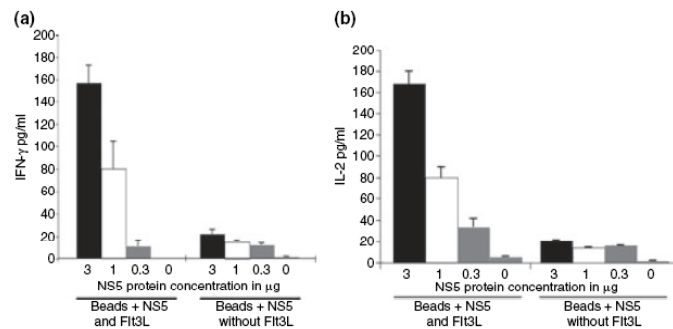


Fig. 5 Demonstration of CD4⁺ activity against NS5. Splenocytes from immunized mice were restimulated for 18 h with NS5 protein at various concentrations. INF- γ (a) and IL-2 (b) were determined in cell culture supernate by ELISA. Note that animals immunized with NS5-coated beads and pretreated with Flt3L expression plasmids showed a striking dose-dependent increase in both INF- γ and IL2 expression when compared to animals immunized with NS5-coated beads without prior Flt3L ligand treatment. Animals immunized with beads alone had no effect on INF- γ or IL2 expression (data not shown).

To explore the role of DCs in generating anti-viral immune responses to HCV, it was necessary to highly enrich the DC population *in vivo* via the injection of FMS-like tyrosine kinase 3 ligand (Flt3L). Recombinant human (rh) Flt3L exhibits a substantial, time dependent, and reversible increase in the number of functionally mature DCs derived

from spleen, bone marrow, liver, lymph nodes, and gut-associated lymphoid tissue [19]. Furthermore, an increase in the weight of the liver of Flt3L-treated mice has been observed, which correlates with a striking proliferation of DEC205⁺ and CD86⁺ DCs within the nonparenchymal cell (NPC) population [15,19,20]. In contrast, other procedures

used to amplify DCs from bone marrow-derived cells *in vitro* involve the addition of GM-CSF to cells in culture; however, the Flt3L stimulation approach leads to a uniform enrichment of all DC subtypes, which is in contrast to GM-CSF stimulation [21,22].

To amplify DC populations, hydrodynamic-based administration of a plasmid expressing Flt3L effectively transfects hepatocytes *in vivo* [6]; rapid injection of a large volume (2 mL in 5 s) that exceeds the cardiac output of the mouse. The resulting high hydrostatic pressure in the inferior vena cava causes an inversion of the blood flow containing the expression plasmid, and transfects organs such as spleen, liver, heart, and kidney with high efficiency [15]. Two hydrodynamic injections of the Flt3L expression plasmid, the splenocyte population was expanded up to tenfold (5×10^7 – 5×10^8). The proportion of functionally mature DCs in the splenic cell population increased 30% compared to 1% found in normal spleen [6].

There is increased interest in the use of DCs in therapeutic vaccination of viral diseases such as HCV [6]. They have high level of antigen-presenting capability and are a key component in the transition from the innate to an adaptive immune response. Thus, numerous studies reveal the importance of using DC-processed antigens in immunization strategies [4,6,13,18,23–29]. The most common procedure employs DCs as vaccine vectors through prior *ex vivo* loading of these cells with the antigen of interest and subsequent adoptive transfer into vaccinated animals [4–6,13]. Indeed, this approach has reached clinical application [24,30,33].

Another novel approach targets the antigen to DCs *in vivo* instead of loading such cells with the antigen *ex vivo* prior to immunization. Bonifaz *et al.* presented an attractive model, i.e. antigen, coupled to the DC-specific DEC 205 antibody, initiates uptake into the cell by a receptor-mediated process followed by DC antigen processing [18]. Immunization studies induced high-level stimulation of antigen-specific CD4⁺ and CD8⁺ T-cell responses. Thus, it is possible to target antigens to DCs *in vivo*, and subsequently stimulate a robust cellular immune response. Another successful approach employs expression plasmids targeted to DCs following encapsulation into microspheres; encapsulated antigens are processed in the context of MHC class molecules and presented to T-cells to stimulate a cellular immune response [31–33]. Therefore, additional studies demonstrate that DCs are a promising vector to produce antigen-specific immunological responses [34,35].

Recently, a more straightforward and direct strategy for antigen loading of DCs has been proposed. It has been shown that magnetic microbeads may serve as carriers for antigen and are taken up efficiently by DCs *in vitro*. Furthermore, these studies suggest that DCs generated in mice through hydrodynamic tail vein injection of Flt3L expression plasmid will take up antigen-loaded beads with varying efficiency [18]. More important, it was reported that to induce a strong T-cell response, it was necessary to combine the process of

antigen loading of DCs with a second maturation stimulus using antibodies to CD40 [4]. These observations support the concept that in the absence of a second stimulus, such as crosslinking of CD40 or ligand binding to TLR receptors as well as generating CD80 and CD86 interaction with T-cells may render DCs tolerogenic [28]. In the present study, we propose a novel preclinical vaccination strategy for HCV, which enabled us to combine currently established *in vitro* methods with an *in vivo* approach. Of central importance is the use of an Flt3L expressing plasmid for the expansion of the DC population *in vivo*. Indeed, the clinical use of this protein ligand has been reported [35]. By pretreating BALB/c mice prior to vaccination, the expanded lymphoid repertoire is slanted towards a high expansion of the DC population that is sufficiently immature to allow for antigen-coated microparticle uptake. Importantly, such DCs still possess the distinct property of T-cell priming. To our knowledge, this is the first study to reveal that enrichment of DCs through Flt3L pretreatment enables the establishment of a substantial viral antigen-specific immune response upon single injection of antigen-loaded microspheres. Although these antigen-coated beads are engulfed by other phagocytic cells, there is a preferential ingestion of the microparticles by the expanded DC population. Indeed, Flt3L exposure produces DCs with a maturation status that is still low enough to keep their phagocytic properties while being sufficiently mature to induce antigen-specific T-cell responses without requiring additional co-stimulation such as crosslinking of CD40 or toll-like receptors activation via poly I:C or LPS [4].

This approach functionally fulfils the requirements for an effective vaccine strategy against HCV in a preclinical animal model system, in that, there is induction of a strong CD8⁺ CTL response accompanied by sustained CD4⁺ T-cell activity [11]. In this context, it was possible to induce a significant CD4⁺ T-cell response resulting in an NS5-specific induction of IFN γ and IL-2 secretion by splenocytes following two immunizations. Cytokine production was NS5 specific because it was clearly dependent upon the protein concentration in the splenocyte restimulation assay. However, we were unable to detect intracellular cytokine staining by flow cytometric analysis. It was apparent from earlier studies that to reach detectable cytokine levels by intracellular cytokine staining requires strong additional immunogenic stimuli such as LPS when *ex vivo* loaded DCs were used for immunization [10]. However, the CD8⁺ CTL biological activity was robust as shown by the suppression of tumour growth of syngeneic NS5 expressing myeloma cells in immunized mice. This finding confirms that the NS5-specific CTL response measured *in vitro* is operative *in vivo* and highly active. Our studies further revealed that it was essential to use microbeads as carriers to achieve efficient uptake of the immunogen by DCs because i.v. injection of soluble NS5 protein did not induce detectable T-cell responses as demonstrated in the tumour challenge experiments and Th1-type cytokine secretion.

This preclinical model provides evidence that DCs can be activated directly *in vivo* to generate HCV-specific cellular immune responses. It will be of interest to employ this same approach using biodegradable microparticles because we have shown that such microbeads are easy to use as carriers for *in vivo* targeting of antigens to DCs. Because Flt3L ligand has been administered to humans in clinical vaccine studies [35], the combination of immunization with a biodegradable microparticle antigen carrier system appears to be a promising approach. Thus, the capability of activating DCs *in vivo* using the methods described herein have value as a therapeutic vaccine strategy for chronic HCV infection.

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CHAPTER 7: APPENDIX 2
ALCOHOL AND HEPATITIS C VIRUS-INTERACTIONS IN IMMUNE
DYSFUNCTIONS AND LIVER DAMAGE



Alcohol and Hepatitis C Virus–Interactions in Immune Dysfunctions and Liver Damage

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Hepatitis C virus infection affects 170 million people worldwide, and the majority of individuals exposed to HCV develop chronic hepatitis leading to progressive liver damage, cirrhosis, and hepatocellular cancer. The natural history of HCV infection is influenced by genetic and environmental factors of which chronic alcohol use is an independent risk factor for cirrhosis in HCV-infected individuals. Both the hepatitis C virus and alcohol damage the liver and result in immune alterations contributing to both decreased viral clearance and liver injury. This review will capture the major components of the interactions between alcohol and HCV infection to provide better understanding for the molecular basis of the dangerous combination of alcohol use and HCV infection. Common targets of HCV and alcohol involve innate immune recognition and dendritic cells, the critical cell type in antigen presentation and antiviral immunity. In addition, both alcohol and HCV affect intracellular processes critical for hepatocyte and immune cell functions including mitochondrial and proteasomal activation. Finally, both chronic alcohol use and hepatitis C virus infection increase the risk of hepatocellular cancer. The common molecular mechanisms underlying the pathological interactions between alcohol and HCV include the modulation of cytokine production, lipopolysaccharide (LPS)-TLR4 signaling, and reactive oxygen species (ROS) production. LPS-induced chronic inflammation is not only a major cause of progressive liver injury and fibrosis, but it can also contribute to modification of the tissue environment and stem cells to promote hepatocellular cancer development. Alteration of these processes by alcohol and HCV produces an environment of impaired antiviral immune response, greater hepatocellular injury, and activation of cell proliferation and dedifferentiation.

Key Words: Antigen-Presenting Cells, CD4⁺ T Cells, CD8⁺ T Cells, Cytotoxic T Lymphocytes (CTL), Cytokines, CYP2E1, Dendritic Cells (DCs), HCV, HCV Core Protein, NS5a Protein, Hepatocytes, Hepatocellular Carcinoma (HCC), Innate and Adaptive Immunity, Lieber-DeCarli diet, Lipopolysaccharide (LPS), Mitochondrial Ca²⁺, Mitochondrial Dysfunction, Mn-Superoxide Dismutase (SOD2), Proteasome, Reactive Oxygen Species (ROS), Toll-Like Receptor 4 (TLR4), Transgenic Mice.

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HEPATITIS C VIRUS (HCV) infects an estimated 4 million Americans and 170 million people worldwide. About 80% of all acute infections develop into chronic infections, which is a cause of progressive liver disease characterized by continuing hepatocellular inflammation and progression from mild to advanced fibrosis and to cirrhosis. Patients with cirrhosis also have a very high risk for developing hepatocellular carcinoma (HCC). Nearly 40% of all chronic liver disease cases in the United States and 27% (in the United States) and up to 90% (in Japan) of HCC cases have an underlying chronic HCV infection (Blonski and Reddy, 2008; Singal and Anand, 2007). While a pegylated interferon/ribavirin-based anti-HCV therapy is available, it results in a sustained virological response in only approximately 55% of the patients with chronic infection (Asselah et al., 2009). Study of the underlying mechanisms of HCV infection and pathogenesis has been a very active research area since HCV was first identified (Kuo et al., 1989) and still is in need for better disease prevention and intervention.

HCV is a positive-stranded RNA virus and a member of Flaviviridae. Its genome has approximately 9,600 nucleotides containing a large open reading frame coding for a

polyprotein. Once made, this polyprotein is processed into ten separate proteins. Among these, core (nucleocapsid protein), NS3 (helicase/protease), NS5a, and NS5b (RNA polymerase) have been implicated in HCV-related tissue damage and carcinogenesis. Based on phylogenetic studies, HCV variants are classified into 6 major genotypes (Simmonds et al., 2005). The most common variants found in the United States are genotype 1. Interestingly, this genotype, as well as genotype 4, is more resistant than genotypes 2 and 3 to the standard pegylated interferon/ribavirin therapy (Lemon et al., 2007).

The natural history of HCV infection and pathogenesis is influenced by both genetic and environmental factors. Among these, alcohol abuse is the most consistent environmental risk factor and independently associated with a much reduced HCV clearance and accelerated disease course (Siu et al., 2009). From a study of a U.S. veteran cohort, patients with alcohol use disorder (AUD, including current and past use) are less than half as likely to have spontaneous viral clearance compared to those without AUD (Pisesecki et al., 2004). It was recognized soon after HCV was identified that, in alcoholics, HCV infection positively correlates with clinical severity of liver disease (Mendenhall et al., 1991; reviewed in Hutchinson et al., 2005). Studies since have established that HCV and heavy alcohol use synergistically accelerate the progression of the most severe liver diseases, cirrhosis (Corrao and Aricò, 1998), and HCC (Hassan et al., 2002; Yuan et al., 2004). The mechanisms by which alcohol affects liver damage and impairs immune elimination of the HCV virus, however, are yet to be fully understood.

HCV and alcohol separately can cause both immune impairment and tissue damage (reviewed in Szabo and Mandrek, 2009; Tsukamoto et al., 2009; Rehmann, 2009; Lanford et al., 2009). This review focuses on 4 recently described aspects of alcohol/HCV interactions that significantly impact on viral clearance and pathogenesis. (i) Dendritic cell (DC) function. DCs are critical to the adaptive immune response because they are the central cell type, via antigen presentation, for activating antiviral effector $CD4^+/CD8^+$ lymphocytes. Previous observations on $CD4^+/CD8^+$ cell dysfunction in alcoholics have led to the closer examination of the effect of alcohol on DC cell function. (ii) Proteasome activity and reactive oxygen species (ROS). Intracellular proteasome activity is important for antigen processing and presentation on virus-infected cells. This is the mechanism through which these cells become recognizable by antiviral immune cells. Reduced proteasome activity is associated with alcohol-induced oxidative stress. The identification of ROS induction in hepatocytes by HCV core protein has led to the investigation of the combined effect of alcohol and HCV on proteasome activity. (iii) Mitochondrial function and ROS in tissue injury. ROS-induced mitochondrial dysfunction plays a major role in alcohol-induced tissue injury. Mitochondria are also one of the intracellular targets for HCV core protein. The role of mitochondria as targets and effectors of HCV/alcohol-induced liver injury is thus an active area of research. (iv) Inflammation and hepatocellular cancer (HCC). Both alcohol and chronic

HCV infection are associated with an increase in the level of circulating lipopolysaccharide (LPS), a well-known inflammation inducer. It has been found only recently that Toll-like receptor 4 (TLR4), a receptor for LPS, plays a key role in the generation of liver cancer cells with stem cell characteristics.

Together, the combined effects of HCV and alcohol on various host cell types, via modulating ROS production, LPS signaling, and cytokine production, produce an environment of impaired antiviral immune response, greater hepatocellular injury, and activation of cell proliferation and dedifferentiation responsible for the range of diseases seen in patients.

HCV, ALCOHOL, AND DENDRITIC CELL DYSFUNCTION

Chronic alcoholics have a high incidence of HCV infection (Oshita et al., 1994; Schiff and Ozden, 2003; Siu et al., 2009), which may be because, in part, of the action of alcohol on the cellular immune response to epitopes that reside on viral structural and nonstructural proteins. Studies have been performed in animal models where genetic immunization has been employed to generate viral-specific antibody and $CD4^+$ and $CD8^+$ responses to HCV core protein (Encke et al., 1998; Geissler et al., 1997a; Stylianou and Saklatvala, 1998; Tokushige et al., 1996). Low-level expression of HCV proteins encoded by such plasmids either in muscle or fibroblasts following DNA inoculation led to activation of dendritic cells (DCs) at the site of immunization or in distant draining lymph nodes to subsequently prime antiviral immune responses. Chronic alcohol feeding had a substantial suppressive effect on the generation of viral antigen-specific $CD4^+$ and $CD8^+$ cellular immune activity when HCV core was employed as the immunogen. Of interest, this inhibitory effect of alcohol could be partially, or completely, reversed by co-immunization with an interleukin-2 (IL-2)- or granulocyte macrophage colony-stimulating factor (GM-CSF)-expressing plasmid (Encke and Wands, 2000; Encke et al., 1998; Geissler et al., 1997a, 1999; Stylianou and Saklatvala, 1998; Tokushige et al., 1996). Such experimental results are consistent with the concept that DNA-based immunization was enhanced by cytokine production at the site of antigen presentation and that DCs could be an important cellular target during chronic alcohol consumption (Encke and Wands, 2000; Encke et al., 1998; Geissler et al., 1997a, 1999; Stylianou and Saklatvala, 1998; Tokushige et al., 1996). Similar results were obtained using a plasmid encoding for the HCV NS5 nonstructural protein as well (Encke et al., 1998; Rehmann and Nascimbeni, 2005). Taken together, these investigations reveal that DCs, differentiated in vivo in the context of chronic alcohol feeding, may have intrinsic functional defects that could partially explain the depressed cytotoxic T-cell lymphocyte (CTL) activity previously observed after DNA-based immunization using HCV core and NS5 as the immunogens (Aloman et al., 2007; Encke and Wands, 2000; Encke et al., 1998; Geissler et al., 1997b,c).

Dendritic cells are known to play a crucial role in generating immune responses to viral proteins. In this regard, DCs are highly specialized antigen-presenting cells. Such cells are known to take-up, process, and present small peptide fragments (8 to 10 amino acids) to effector T cells. They generally elicit an adaptive immune response or, paradoxically, a tolerogenic reaction that depends in part on how the antigen is presented and its concentration. To initiate a successful adaptive immune response, DCs need to express a variety of co-stimulatory molecules on their cell surface (Banchereau and Steinman, 1998). Upregulation of their activity is mediated by several factors including TLR–ligand interactions or binding of CD40 to the CD40 ligand on the cell surface. These interactions between DCs and T cells promote sustained stimulation and improve the efficiency of antigen presentation. DCs are ideal for immunomodulatory strategies because their activity is crucial for eliciting a robust immune response to viral peptides. It is important to note that DC-based immunization was recently shown to be a suitable approach to elicit sustained immune responses to HCV proteins of the type necessary for viral eradication from the liver (Encke et al., 2005; Kuzushita et al., 2006). DCs are equipped with receptor mediating antigen uptake, which include members of the C-type lectin family. It is known that the phagocytic process is initiated through the calcium-dependent binding of C-type lectins to carbohydrate-bearing pathogen-derived antigens using highly conserved carbohydrate-recognition domains. Some of these receptors appear uniquely on phagocytic cells such as macrophages, monocytes, B lymphocytes, neutrophils, and DCs (Figdor et al., 2002).

One of the major effects of long-term alcohol consumption on the biology of DCs *in vivo* appears to be at the level of cytokine production. DCs generated from alcohol-fed mice had a greater ability to produce IL-1 β , the initial cytokine that drives lymphocyte proliferation (Aloman et al., 2007). However, secretion of IL-6, another cytokine implicated in the overall proliferative response, is impaired. It is noteworthy that there is a propensity to generate TH2-type cytokine responses because the secretion of TNF α , IFN- γ , and IL-12, which promote the TH1 differentiation pathway of CD4 $^{+}$ and CD8 $^{+}$ into viral-specific functional cells, is strikingly impaired by chronic alcohol. Indeed, IL-10 is increased in alcohol-fed mice, which provides another boost toward the TH2-type immune response. These *in vivo* findings provide information on cytokine production by DCs from mice fed alcohol long-term followed by LPS and poly-I:C stimulation. These *in vivo* observations in mice support previous *in vitro* findings on human DCs expanded in the presence of alcohol (Dolganiuc et al., 2003; Mandrekar et al., 2004). However, chronic alcohol consumption may also affect antigen uptake, degradation, processing, and transport of peptides to the cell surface in the context of MHC class I and II molecules to interact with and stimulate viral-specific immune responses, and further studies will be required to examine these possibilities.

It was important to further characterize the role of DCs *in vivo* in animals with an intact immune system. Therefore, the model of DNA-based immunization and syngeneic transfer of nonselective splenic DCs has been employed. Studies were performed with the NS5 viral antigen because previous studies revealed that a DNA construct expressing this protein generates a strong cellular immune response (Encke and Wands, 2000). The strategy involved the concept that syngeneic DC transfer would allow one to determine whether the effects of alcohol on cellular and humoral immune responses could be reversed by simply co-immunizing with DCs isolated from normal animals, and whether these effects could be inhibited by DCs generated from alcohol-fed mice.

In addition, it was critical to evaluate CTL responses after splenic DC transfer to a HCV nonstructural protein such as NS5 (Aloman et al., 2007). Surprisingly, it was found that normal DCs can correct a functional defect in the cellular immune response as measured by CTL activity previously altered by long-term alcohol feeding. The correction of CTL activity after transfer of normal DCs to animals on the chronic alcohol diet provided strong evidence that it is the effect of alcohol on DCs rather than a direct effect of alcohol on CD8 $^{+}$ lymphocyte function. In addition, pair-fed control mice receiving DCs from alcohol-fed animals were unable to drive an effective CTL response and developed partial tolerance with respect to CTL activity against the HCV NS5 protein (Aloman et al., 2007).

In summary, *in vivo* generation of DCs combined with DC transfer and DNA-based immunization revealed that alcohol-induced dysfunction of DCs is a major factor responsible for the reduced cellular immune response to HCV-related peptides. Such observations may have relevance to persistent HCV infection in alcoholic patients and identify a critical cell type that is at-risk for the effects of alcohol. Therefore, one of the major cellular targets in the immune system for long-term activity of alcohol are DCs with subsequent impaired generation of robust CTL activity to viral structural and nonstructural proteins that may be critical for viral clearance (Dolganiuc et al., 2003; Mandrekar et al., 2004). More important, such observations open new strategies to enhance antiviral immune responses in alcoholics and, particularly, in the setting of long-term ethanol consumption by improving DC function. Thus, DCs represent one of the major cellular components of the immune system sensitive to chronic alcohol effects and subsequently impair the generation of CD8 $^{+}$ CTL and CD4 $^{+}$ proliferative activity against epitopes on HCV viral proteins that may be essential for viral resolution (Aloman et al., 2007; Dolganiuc et al., 2003; Edsen-Moore et al., 2008; Heinz and Waltenbaugh, 2007; Kuzushita et al., 2006; Mandrekar et al., 2004; Szabo et al., 2004). Further studies on the biology of DCs and how alcohol affects their function will lead to a better understanding of the acquisition and persistence of HCV infection and also will provide opportunities to devise vaccine strategies to eradicate persistent HCV infection in chronic alcoholics.

DIFFERENTIAL REGULATION OF PROTEASOME ACTIVITY BY HCV CORE PROTEIN AND ETHANOL IN LIVER CELLS

The generation of viral peptides for antigen presentation in virus-infected liver cells is another site at which alcohol may modify the immune response to HCV. The proteasome is a multi-catalytic enzyme that degrades about 80% of intracellular proteins, including signal transduction factors. In addition to protein degradation, the proteasome also generates peptides for MHC class I-restricted antigen presentation. The chymotrypsin-like and the trypsin-like activities of the proteasome are related to antigen presentation because of their ability to cleave peptide bonds after hydrophobic and basic amino acids, respectively (Goldberg et al., 2002; Qian et al., 2006).

Proteasome exists in equilibrium of 2 particles, 20S proteasome and 26S proteasome. For protein degradation by 26S proteasome, proteins are marked by ubiquitin, a small 8.5-kDa protein that is covalently attached to protein substrate. Proteins subjected to degradation have multiple ubiquitin molecules covalently attached to generate polyubiquitin chain binding to an internal lysine (lysine 48) residue. In contrast, 20S proteasome degrades nonubiquitylated proteins, most of which are oxidatively modified (reviewed by Donohue et al., 2007 and Osna and Donohue, 2007). Usually, oxidative modification (adduction) of proteins makes them more susceptible to degradation (Curry-McCoy et al., 2009; Grune et al., 1998).

Proteasome function is apparently susceptible to oxidative stress that forms adducts with protein carbonyls, 4-hydroxynonenal (4-HNE), and 3-nitrotyrosine derived from peroxynitrite (Bardag-Gorce et al., 2005; Kessova and Cederbaum, 2005; Osna et al., 2004). Other studies have revealed a reduction in proteasome activity as a result of ethanol metabolism in recombinant hepatoma (VL-17A) cells that express both CYP2E1 and alcohol dehydrogenase (ADH) (Donohue et al., 2006; Osna et al., 2003, 2007). This inhibition of proteasome function was CYP2E1 dependent (Dey and Cederbaum, 2006; Kessova and Cederbaum, 2005; Osna et al., 2003) and correlated with generation of intracellular oxidants. In the liver, the levels of oxidative stress induced by multiple agents, including ethanol and viral proteins, inhibit proteasome activity (Osna et al., 2004, 2008).

HCV proteins are able to induce oxidative stress in the liver. Specifically, outer mitochondrial membrane-associated HCV core protein elevates generation of ROS by mitochondrial electron transport complex I, resulting in a decrease in mitochondrial glutathione (GSH) and mitochondrial depolarization, which can be augmented by simultaneous endoplasmic reticulum (ER) oxidative stress (Korenaga et al., 2005a; Otani et al., 2005). This effect of core protein is further potentiated by exposure of cells to ethanol (Otani et al., 2005). Thus, the combined action of HCV core protein and ethanol may interfere with proteasome activity, thereby affecting the events that are downstream from proteasome-dependent

generation of peptides for MHC class I-restricted antigen presentation in liver cells, a crucial process for recognition of infected hepatocytes by cytotoxic T lymphocytes. Previously, we have demonstrated the critical role of oxidative stress-modified proteasome activity for the peptide hydrolysis as well as the presentation of peptide-MHC class I complex on liver cell surface (Osna et al., 2007, 2009).

While studying the effects of HCV core protein on proteasome function in CYP2E1-expressing hepatoma cells, we found that HCV core protein enhanced this enzyme activity in intact Huh7 cells, but suppressed proteasome in ethanol-treated cells. These results have been previously presented (Osna et al., 2008). Because glutathione ethyl ester (GSH-EE), *N*-acetyl cysteine (NAC), uric acid, diallyl sulfide (DAS, CYP2E1 inhibitor), and catalase reversed the effects of core protein on proteasome activity, the activation of proteasome by core protein was attributed to oxidative stress, which at low levels enhanced proteasome activity. However, when HCV core/CYP2E1-expressing cells were exposed to ethanol, a higher intensity of oxidative stress induced by both HCV core protein and ethanol suppressed proteasome activity, indicating a dual regulation of proteasome activity by differential levels of oxidative stress. In addition to oxidative stress-related proteasome regulation, HCV core protein-proteasome interactions, which involved the proteasome activator (PA28) and were potentiated by mitochondrial and microsomal cell fractions, activated 20S proteasome in cell-free system, in the absence of oxidative stress. Remarkably, when cell fractions were purified from ethanol-exposed cells, the activating effect of HCV core on proteasome was attenuated.

In vivo studies in hepatocytes of HCV core-expressing versus nonexpressing mice fed with ethanol showed similar pattern of proteasome activation by core protein and suppression by ethanol feeding as observed in vitro. These changes, at least in part, could be attributed to oxidative stress because induction of ROS by ethanol and thiobarbituric acid reactive substances (TBARS) levels was higher in ethanol-fed HCV core protein expressing mice than in ethanol-fed controls (Osna et al., 2009; manuscript in preparation). Enhanced ROS production by mitochondria of these transgenic mice has been previously demonstrated (Korenaga et al., 2005a; see subsequent section). These data suggest that both HCV core and ethanol induce oxidative stress, which accordingly corresponds to the magnitude of proteasome activity inhibition in hepatocytes induced by either ethanol or ethanol + HCV core protein. Overall, both HCV core protein and ethanol appear to regulate proteasome activity by overlapping mechanisms. HCV core protein activates proteasome activity both via a low level of oxidative stress as well as a direct effect. However, when ethanol is added, either in cells or in mouse models, there is a further increase in oxidative stress and a net decrease in proteasome activation (Osna et al., 2008).

In conclusion, proteasome activity in HCV core protein-expressing cells is regulated by differential levels of oxidative

stress: core protein-induced low oxidative stress enhances proteasome activity, while high oxidative stress induced by the combined action of core protein and ethanol suppresses proteasome activity. These core protein/ethanol-mediated changes in proteasome function are confirmed by *in vitro* and *in vivo* experiments and may suggest the reduced proteasome-dependent hydrolysis of antigenic peptides, which limits the supply of peptides for MHC class I-restricted antigen presentation, thereby suppressing the display of these peptides in the context of MHC class I on ethanol-metabolizing liver cells.

MITOCHONDRIA, OXIDATIVE STRESS, AND HCV/ALCOHOL-INDUCED LIVER INJURY

Hepatocytes are the major target cell type for HCV infection/replication (Lemon et al., 2007). Hepatic oxidative stress and mitochondrial abnormalities are nearly universally observed in patients with chronic hepatitis C and appear to be an intrinsic component of the disease. They are manifested by elevations of oxidized derivatives of lipids, proteins, and nucleic acids (Fujita et al., 2007; Konishi et al., 2006; Paradis et al., 1997), decreases in antioxidant content (Jain et al., 2002; Larrea et al., 1998; Mahmood et al., 2004; Sumida et al., 2000; Vendemiale et al., 2001; Yadav et al., 2002), and ultrastructural abnormalities of mitochondria (Barbaro et al., 1999). While some degree of oxidative stress is a characteristic of any inflammatory disease, it occurs with greater frequency and magnitude for hepatitis C than for other liver diseases (Valgimigli et al., 2002) and it correlates with greater severity of inflammation, the presence of insulin resistance (Mitsuyoshi et al., 2008; Vidali et al., 2008b), more rapid progression of fibrosis, and the development of HCC (Maki et al., 2007). In addition, successful viral clearance of hepatitis C results in a decrease in the biomarkers of oxidative stress concomitantly with an improvement in the clinical sequelae (Serejo et al., 2003).

Hepatitis C interacts synergistically with other factors to worsen liver disease. The most well-known example of this phenomenon is that heavy alcohol consumption in HCV-infected individuals predisposes to more rapid fibrosis progression, a greater incidence of HCC, and impaired response to therapy (Siu et al., 2009). In addition, HCV is a risk factor for toxicity of acetaminophen as well as anti-retroviral agents in HIV-co-infected patients (Nguyen et al., 2008). Mitochondrial effects are prominent in each of these disease processes. There has thus been considerable investigation of how HCV-induced mitochondrial changes occur and their contribution to combined HCV/alcoholic liver disease.

Mechanisms of HCV-Induced Mitochondrial ROS Production

An understanding of HCV-induced mitochondrial effects has emerged from studies in HCV model systems including hepatoma cells expressing viral proteins, cells replicating the

HCV RNA, cells infected with the JFH1 cell culture strain of HCV, and transgenic mice with constitutive or inducible expression of HCV proteins. Each of these systems has shown evidence of viral protein-mediated ROS production, lipid peroxidation, and activation of stress kinase pathways (Machida et al., 2006a; Piccoli et al., 2009; Wang and Weinman, 2006). The most important observation is that increased mitochondrial ROS production is a direct consequence of viral proteins and does not require the full process of viral infection. Several transgenic mouse models for hepatitis C reproduce the oxidative stress phenotype, even in the absence of inflammation (Chang et al., 2008; Moriya et al., 2001; Okuda et al., 2002). These findings demonstrate that in the case of oxidative stress, direct effects of viral proteins predominate over specific events in viral RNA replication.

Four different HCV proteins, core, NS3, NS4a, and NS5a, have been shown to cause oxidative stress. Core protein appears to be the primary cause of HCV oxidative stress as it is both sufficient and in some cases necessary for the phenomenon (Piccoli et al., 2009), and cells expressing HCV core protein have multiple mitochondrial abnormalities (Moriya et al., 2001; Okuda et al., 2002; Piccoli et al., 2007). The effect is entirely Ca^{2+} dependent and is secondary to both ER stress and direct effects on the mitochondrial Ca^{2+} -uptake mechanism. Core protein, like all the viral proteins, is initially produced in the ER and its expression causes ER stress (Benali-Furet et al., 2005). This results in the activation of the unfolded protein response and release of the Ca^{2+} with diminished retention within the ER lumen (Piccoli et al., 2007; Tardif et al., 2005; Qadri et al., 2004).

At the same time, core protein more directly interacts with the mitochondria as well. Core localizes to mitochondria (Korenaga et al., 2005b; Schwer et al., 2004; Suzuki et al., 2005), and a specific sequence in the C terminal portion of the molecule serves as a targeting sequence to the mitochondria-associated membrane fraction of the ER. This is a point of close contact between the ER and the mitochondrial outer membrane (Williamson and Colberg-Poley, 2009). Core protein then serves as a modulator of the mitochondrial Ca^{2+} uniporter causing more rapid Ca^{2+} entry and increased net mitochondrial Ca^{2+} accumulation for a given extramitochondrial Ca^{2+} concentration (Li et al., 2007). This effect occurs in cells expressing core as well as in isolated mitochondria incubated with recombinant core protein. It is thus a direct mitochondrial effect. The consequence is to make the process of Ca^{2+} transfer from ER to mitochondria more efficient with a net increase in baseline matrix Ca^{2+} and a dramatically increased rise in mitochondrial Ca^{2+} in response to agonists or ER stress. This Ca^{2+} influx increases mitochondrial superoxide production, which then initiates an amplification phenomenon whereby oxidation of GSH leads to inhibition of electron transport and increased superoxide production from complex I. Under some circumstances, such as in the presence of alcohol and exogenous peroxides, this can be sufficient to trigger mitochondrial inner membrane permeabilization and cell death (Abdalla et al., 2005; Otani et al., 2005).

Other viral proteins increase mitochondrial ROS production as well, although the mechanisms of these are less well understood. NS5a also causes ER stress, ER Ca^{2+} release, and mitochondrial Ca^{2+} uptake (Dionisio et al., 2009; Gong et al., 2001), although it has not been shown to directly modulate mitochondrial Ca^{2+} -uptake mechanisms. The NS3/NS4a complex forms a protease that binds to the mitochondrial outer membrane where it cleaves an important innate immune signaling molecule, MAVS (Horner and Gale, 2009). It has been shown to increase mitochondrial ROS production and regulate mitochondrial apoptosis pathways (Nomura-Takigawa et al., 2006; Selimovic and Hassan, 2008). An additional viral protein, the alternate reading frame protein F, has recently been shown to localize to mitochondria, but the functional significance of this is not yet known (Ratinier et al., 2009).

Two other mechanisms contribute to HCV oxidative stress as well. Plasma membrane-associated NADPH oxidase in macrophages and neutrophils is induced by the HCV NS3 (Bureau et al., 2001; Thoren et al., 2004) and this contributes to oxidative stress in the setting of inflammation. An additional contributor is secondary to HCV-induced iron overload. HCV infection or HCV polyprotein expression in mouse liver results in a suppression of hepatic hepcidin production. This results from an oxidative stress-dependent alteration in transcriptional activity (Miura et al., 2008; Nishina et al., 2008). This iron accumulation that results further increases lipid peroxidation, induces hepatic steatosis, and contributes to pathogenesis. Because alcohol has a similar effect, it may contribute to the synergistic liver injury (Harrison-Findik et al., 2006).

Consequences of HCV-Induced Oxidative Stress

The weight of evidence suggests that oxidative stress in hepatitis C serves largely as an incomplete host cell response that inhibits viral replication and enhances apoptotic and necrotic clearance of infected cells. In a series of elegant experiments, Choi et al. demonstrated that ROS-induced increases in Ca^{2+} inhibits viral replication in replicons or in vitro (Choi et al., 2004, 2006). This conclusion is further supported by data showing direct antiviral effects of lipid peroxidation (Huang et al., 2007), arsenic-related oxidative stress (Kuroki et al., 2009), and oxidant-induced ERK activation (Yano et al., 2009). In contrast, other studies have shown that the oxidative stress produced by alcohol metabolism specifically increases viral replication (McCartney et al., 2008), although it is possible that this may be mediated by other aspects of alcohol metabolism. Oxidative stress does clearly play a role in chronic disease pathogenesis. There is a consistent correlation between greater degrees of oxidative stress markers and more severe disease (Cardin et al., 2001; Jain et al., 2002; Mahmood et al., 2004; Valgimigli et al., 2002; Vendemiale et al., 2001; Yadav et al., 2002) and it is an independent risk factor for fibrosis (Vidali et al., 2008a).

Mitochondrial ROS and HCV/Alcohol Pathogenesis

It is well established that alcohol also causes hepatic oxidative stress and mitochondrial dysfunction (Mantena et al., 2008), although the characteristics of this differ somewhat from that in HCV. Alcohol appears to cause more global decreases in mitochondrial electron transport complex activities, whereas the defect in hepatitis C is more specific for complex I. Synergistic mitochondrial electron transport inhibition and ROS production has therefore been proposed as a mechanism for liver injury in HCV/alcohol-consuming patients. Evidence in support of this hypothesis has largely come from experimental model systems, and its strength is dependent on the validity of these models for human disease.

Cell culture models for alcohol-induced liver cell injury are limited because hepatoma cells and even primary hepatocytes fail to express the most important alcohol-metabolizing enzymes including ADH, aldehyde dehydrogenase (ALDH), and CYP2E1. Nonetheless, hepatoma cells stably transfected with CYP2E1 have been used as a model for alcoholic liver injury, and this approach has been tried with Huh7 hepatoma cells expressing HCV core protein (Otani et al., 2005) or replicating HCV RNA (McCartney et al., 2008). These systems show synergistic effects of alcohol and HCV on ROS production, oxidation of the glutathione pool, mitochondrial depolarization, and oxidant-induced cell death. As cell death because of HCV and alcohol could be prevented by antioxidants, it appears that synergistic ROS formation was essential for the cytotoxic effects (Otani et al., 2005).

Transgenic mice expressing HCV proteins are more useful models as they have the potential to better reproduce tissue injury in vivo. The limitation is primarily that alcohol consumption fails to reproduce the full range of alcoholic liver injury seen in humans. The Lieber-DeCarli voluntary feeding model tends to produce steatosis without significant inflammation, and higher-dose alcohol feeding through continuous intragastric feeding is technically difficult and still does not fully recapitulate human disease. Nonetheless, several groups have reported the results of alcohol feeding via the Lieber-DeCarli method to HCV core protein transgenic mice. These have found that the combination of HCV core and alcohol increased lipid peroxidation, cytokine production, and MAP-kinase activation to a greater degree than that seen with either alcohol or core protein alone (Perlemuter et al., 2003; Tsutsumi et al., 2003).

In mice expressing the HCV proteins core, E1, E2, and p7, HCV protein expression mildly sensitizes mice to alcohol-induced hepatic steatosis (Tumurbaatar et al., 2008; and manuscript in preparation). In this transgenic mouse model, it appears that high levels of mitochondrial Mn-superoxide dismutase (SOD2) protect the liver from developing a severe disease in response to alcohol. Decreasing SOD2 levels by heterozygous gene deletion exacerbates the synergy between HCV and alcohol, producing a histological steatohepatitis in some animals (Tumurbaatar et al., 2008). These findings do suggest that HCV and alcohol trigger a superoxide-dependent

injury cascade in sensitive individuals. In human populations, homozygosity for a frequent polymorphism in the mitochondrial targeting sequence of SOD2 results in up to a 50% decrease in liver mitochondrial SOD2 levels (Martin et al., 2009). The relevance of these mouse models to human disease is thus improving.

In aggregate, the evidence supports the concept that the combined effects of alcohol and HCV on mitochondrial superoxide production initiate an injury cascade in sensitive individuals. Future studies need to examine the downstream effectors of this injury, their role in human disease, and the potential contribution of targeted antioxidant therapy in these diseases.

LIVER CANCER DEVELOPMENT AND STEM CELLS UNDER THE INFLUENCE OF HCV AND ALCOHOL

Hepatocellular carcinoma is highly prevalent in the world, especially in Africa and Asia, does not respond well to conventional therapy, has a high mortality, and is one of the most catastrophic consequences of combined HCV and alcohol effects on the liver (Okuda, 2000).

Alcohol synergistically enhances the progression of liver disease and the risk for liver cancer caused by hepatitis C virus (HCV). Recent studies have identified effects on TLR4 as a primary contributor to HCV/alcohol-induced HCC. TLR4 is induced by hepatocyte-specific transgenic (Tg) expression of the HCV nonstructural protein NS5A, and this induction mediates synergistic liver damage and tumor development by alcohol-induced endotoxemia (Machida et al., 2009). The stem/progenitor cell marker, Nanog, is upregulated as a novel downstream gene by TLR4 activation and is responsible for the presence of cancer progenitor cells (CD133/Nanog-positive cells) in liver tumors of alcohol-fed NS5A Tg mice (Machida et al., 2009). Transplantation of p53-deficient hepatic progenitor cells transduced with TLR4 results in liver tumor development in mice following repetitive LPS injection, but concomitant transduction of Nanog short-hairpin RNA abrogates this outcome (Machida et al., 2009). Despite the common understanding that TLR4 is one of the pattern-recognition receptors expressed predominantly by innate immune cells such as macrophages and lymphocytes,

our study demonstrates that hepatocytes can be the primary cellular site of both TLR4 upregulation and its pathologic consequences in the context of HCV infection. Therefore, the TLR4-dependent mechanism synergizes liver disease by HCV and alcohol and is partly dependent on Nanog, a TLR4 downstream gene.

Although transduction of Nanog expression is required for tumorigenesis, Nanog alone is not as effective as TLR4 activation in production of liver tumors (Machida et al., 2009). It thus appears that TLR4 activation induces other tumor-driver genes that cooperatively work with Nanog to cause liver oncogenesis. Thus, Nanog is essential for TLR4-dependent oncogenesis, but it alone is poorly oncogenic. TLR4 promoter upregulation by NS5A is mediated by PU.1, Oct-1, and AP-1 elements (Machida et al., 2006b). The similar transcriptional mechanism may underlie TLR4 induction in primary hepatocytes.

The finding that Nanog is transcriptionally upregulated in HCV/alcohol-associated liver tumorigenesis suggests that appearance of a stem cell phenotype may have a role in HCC. Recent studies of HCC have centered on cancer stem cells (CSC), including detection of CSC in cancer, identification of CSC markers, and isolation of CSC from human HCC cell lines. The liver has a high regenerative potential, and hepatic small oval progenitor cells around the peripheral branches of the bile ducts, the canals of Hering, can differentiate into biliary epithelial cells and hepatocytes (Roskams et al., 2004). These oval liver progenitor cells share molecular markers with adult hepatocytes [albumin, cytokeratin 7 (CK7), CK19, oval cell markers (OV-6, A6, and OV-1), chromogranin-A, NCAM (neural cell-adhesion molecule)] and fetal hepatocytes (α -fetoprotein) (Table 1) (Roskams, 2006; Roskams et al., 2004). They are also positive for more common stem cell markers such as CD34+, Thy-1+, c-Kit+, and Flt-3+ (FMS-like tyrosine kinase 3) (Burke et al., 2007). Thus, it currently remains unclear whether these stem cells are derived from the bone marrow and just migrate to this periportal niche or whether they represent true resident liver stem/progenitor cells. Binding of stroma-derived factor-1 α (SDF-1 α) to its surface receptor CXCR4 activates oval hepatic cells (Hatch et al., 2002). CSC were identified as CD117+/CD133+ hepatic precursors in regenerating liver tissue (Craig

Table 1. Markers for Liver Cancer Stem Cells

Gene name	Other name	Function	Species	Organ	References
CD133	Prominin 1 (PROM1)	Glycoprotein, membrane protrusions	Human, Mouse	Liver, Brain	a
CD49f	Integrin α 6 (ITGA6)	Cell adhesion, cell signaling	Mouse	Liver	b
CD90	Thy-1	Glycophosphatidylinositol (GPI) anchor	Mouse	Liver	c
CD44	Hyaluronic acid receptor	Cell adhesion and migration, metastasis	Mouse	Liver, Breast	d
CD117	KIT	C-kit receptor Cytokine receptor	Mouse	Liver	e
CK19	Cytokeratin 19	Biliary lineage marker	Mouse	Liver	f
OV-6	Oval cell marker	Early progenitor cells	Human	Liver	g
CD34	Glycoprotein	Cell-cell adhesion factor	Mouse	Liver, Leukemia	h
AFP	α -fetoprotein	Fetal counterpart of serum albumin	Mouse	Liver	i

a: Ma et al., 2007, 2008; Ho et al., 2006; Singh et al., 2004; Shmelkov et al., 2005; Rountree et al., 2008. b: Ma et al., 2007; Rountree et al., 2008. c: Ma et al., 2007. d: Ma et al., 2007; Al-Hajj et al., 2003. e: Ma et al., 2007. f: Tirnitz-Parker et al., 2007; Libbrecht et al., 2001. g: Libbrecht et al., 2001. h: Lapidot et al., 1994. i: Chiba et al., 2006.

et al., 2004) and a CD45⁺/CD90⁺ subpopulation of tumor cells in HCC (Yang et al., 2008). The CD90⁺ cells are not present in the normal liver and, when injected into immunodeficient mice, create tumors repeatedly.

In human HCC and HCC cell lines, specifically CD133⁺ cells, not CD133⁻ cells, had the ability to self-renew, create differentiated progenies, and form tumors (Ma et al., 2007). This coincided with the expression of genes associated with stem/progenitor status, such as β -catenin, NOTCH, BMI, and OCT3/4. When compared to CD133⁻ cells, the CD133⁺ cells isolated from the HCC cell lines showed higher expression of CD44 and CD34, but both CD133 subpopulations displayed similar expression for CD29, CD49f (integrin α 6), CD90, and CD117 (Ma et al., 2007). The role of CSC in HCV/alcohol-associated liver carcinogenesis is thus an active area of investigation.

In summary, alcohol and HCV NS5A synergistically induce liver tumor development via induction and activation of TLR4 in mice. The importance of Nanog as a direct downstream gene of TLR4 in this oncogenesis has also been identified. Pharmacologic inhibition of TLR4 signaling may become a novel therapeutic strategy for alcohol/HCV-associated liver tumors.

SUMMARY AND FUTURE DIRECTIONS

Research studies discussed in this review demonstrate that alcohol and HCV independently and jointly alter immune and cellular pathways to result in liver damage (Fig. 1). Both alcohol and HCV activate pathways of ROS production, cytokine production, induction of TLR expression, and proteasome activity. Together, these effects modulate antigen presentation, dendritic cell function, and development of a liver stem cell phenotype. Experimental evidence support the hypothesis that alcohol and hepatitis C virus can have additive negative effects on functions of multiple cell types in the liver including hepatocytes, immune cells, and stem cells, thereby affecting antiviral immunity, hepatocyte survival, liver regeneration, and oncogenesis in favor of HCV survival and replication. While HCV has various ways to affect cell functions, the core protein of the HCV virus appears to be a key modulator of many of these cellular functions.

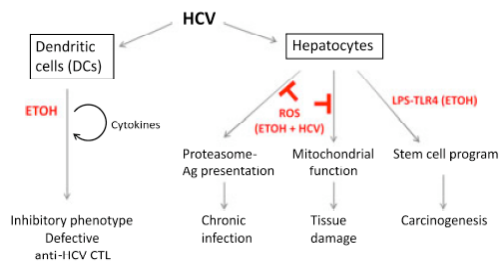


Fig. 1. A schematic of the interactions between alcohol and HCV and their impact on immune cells and liver cells. Ag, antigen.

It is important to note that none of the studies described here were conducted in the context of replicating HCV or the entire HCV genome. While this does not necessarily diminish the significance of the outlined studies, confirmation in an authentic replication/infection system of the modulating effects of HCV proteins and alcohol on cellular and molecular function awaits further studies. It is useful to note, however, that replication of the JFH1 strain of HCV in Huh7.5 cells has nearly identical effects as core protein on mitochondrial ROS production (Wang et al., 2009) and proteasome inactivation (Osna, unpublished observations).

Increasing evidence suggests that the combination of alcohol use and HCV amplifies abnormalities of the functions of DCs that otherwise are induced by the individual insults. In response to LPS stimulation, DCs isolated from alcohol-fed mice had decreased IFN γ and IL-12 but increased IL-10 production (Aloman et al., 2007). This observation is remarkably in parallel to the effect of HCV core protein on human myeloid DCs (Dolganiuc et al., 2004, 2006). The role of HCV core protein in induction of dendritic cell defects appears to be central as impaired CTL responses in mice immunized with a DNA construct expressing HCV core protein showed decreased activity that could be corrected by transfer of DCs from control but not from alcohol-treated mice. A similar inhibitory effect of HCV core protein has been shown in human monocyte-derived DCs along with an amplified inhibition of DC T-cell activating capacity with the combination of alcohol and HCV infection (Dolganiuc et al., 2004).

The pathogenic role of HCV core protein is also evident in hepatocytes. While mitochondrial oxidative stress is primarily induced by the HCV core protein, NS3, NS4a, and NS5a proteins as well as the replicating JFH1 culture strain of HCV can induce ROS and mitochondrial injury (Li et al., 2007). Studies by the Weinman group demonstrated that through disturbing mitochondrial Ca²⁺ transport from the ER, HCV core protein can amplify mitochondrial superoxide production. While alteration of the mitochondrial electron transport by chronic alcohol has been demonstrated in several previous studies, recent evidence suggests a combined effect of alcohol and HCV core protein on mitochondria. The increased cell death because of HCV and alcohol could be prevented by antioxidants, suggesting an important role for ROS (Korenaga et al., 2005a).

Recent interesting findings link proteasome function and oxidative stress in alcoholic liver disease and HCV infection. Data presented by Osna indicate that the combined action of HCV core protein and ethanol may interfere with proteasome activity (Osna et al., 2008). Such change in proteasome activity was suggested to contribute to alterations in proteasome-dependent generation of peptides for MHC class I-restricted antigen presentation in hepatocytes.

While clinical evidence for the combined effects of HCV infection and alcohol in hepatocellular cancer development is well established, only recent developments provide molecular basis in potential explanation for promotion of HCC by alcohol plus HCV infection. Alcohol administration promotes

the expression of Nanog, a stem cell/progenitor marker in the liver in an I.PS/TLR4-dependent manner (Machida et al., 2009). Furthermore, the highest expression of Nanog was found in alcohol-fed HCV NS5a-transgenic mice after repetitive LPS injections (Machida et al., 2009). These observations raise important aspects in the potential role of gut-derived LPS in alcoholic liver disease in the development of HCC. Interestingly, increased levels of circulating LPS were reported not only in models of alcoholic liver disease but also in patients with chronic HCV infection and this was associated with increased inflammatory cell activation likely as a result of a loss of TLR tolerance (Dolganiuc et al., 2007). Thus, ethanol-induced increase in portal and systemic endotoxin levels likely promote HCV-induced inflammation and HCC development.

Together, the combined effects of alcohol and HCV infection seem to emerge both at molecular and cellular levels, suggesting that amplification of the negative effects of alcohol by HCV infection and vice versa deserves further careful investigations.

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