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CHRONIC AND ACUTE ALCOHOL EXPOSURE PREVENTS MONOCYTE-DERIVED DENDRITIC CELLS FROM MATURING IN RESPONSE TO MICROBIAL STIMULI

Brigitta Buttari

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Tutor: Dott.ssa Rachele Riganò

Coordinatore: Prof.ssa Paola Sinibaldi-Vallebona

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ABSTRACT

Research demonstrating alcohol's adverse effects on functions of the immune system supports clinical evidence of an increased incidence of infectious diseases, such as pneumoniae, tuberculosis and hepatitis C, as well as a greater susceptibility to cancer in humans chronically abusing alcohol. While the association of alcohol abuse with increased deaths from infections was made over 75 years ago, only in the past 15 years has serious investigative efforts been made to understand the role of alcoholic exposure on immune dysfunction. Among the many alcoholics whose socioeconomic status is poor, overcrowded living conditions and limited access to health care may increase the likelihood of contracting and spreading disease. In addition, many alcoholics suffer from malnutrition and liver disease, conditions that may themselves compromise the immune system's capacity to resist infection. Evidence from human and animal studies in vivo as well as from experiments in vitro suggests that alcohol abuse may exert adverse immunomodulatory effects on innate and adaptive immune responses. Alcohol-induced immune dysfunctions depend on various factors including the dose and duration of alcohol exposure (chronic vs acute) and on the presence and characteristics of additional stimuli such as microbial molecules.

Defects of antigen presenting cells (APC) appear to be pivotal in the alcohol-induced alterations of cell-mediated immunity and in decreased antigen (Ag)-specific T cell proliferation. Dendritic cells (DCs) are the most potent APCs. The functional state of DC maturation and their activation degree control immunity and tolerance.

The complex nature of the linkage between alcohol consumption, altered host immune responses, and infection remains controversial and incompletely understood. Most importantly, there is a need to develop a multimodal approach, including components of immune modulation and immune restoration, to repair possible damages in the host defence system induced by alcohol ingestion.

In this study we addressed this research need in: 1) to understand how alcohol exposure induces immune alterations that contribute to the occurrence of particular kinds of infectious diseases showing a higher incidence in alcohol abuser population than in the general population and 2) to develop strategies to reduce the occurrence of alcohol-induced immune alterations.

To clarify the immunological effects exerted by alcohol abuse on human monocyte-derived DCs we investigated whether in vivo chronic alcohol exposure alters the ability of monocytes from alcoholics to differentiate and mature in vitro into functional DCs. To extend current information on direct alcohol-induced changes in DCs, we evaluated whether in vitro acute ethanol (EtOH) treatment of differentiated immature DCs (iDCs) generated from chronic alcoholics and healthy control subjects impairs DC maturation. Using immunochemical and cytofluorimetric analysis we determined the phenotype and functions (endocytosis, migration, cytokine production and allostimulatory ability) of monocyte-derived DCs and analyzed the ability of iDCs to respond to the microbial product lipopolysaccharide (LPS). We also wanted to find out whether EtOH-treated antigen-stimulated DCs correctly primed naïve T lymphocytes, thus inducing T helper 1 (Th1) cell polarization.

As a possible approach in the prevention of alcohol's adverse effects on the immune system we investigated whether cromoglycate-like' anti-allergic drugs -- by their well established property to stabilize membrane lipid polarity -- might exert in vitro an adjuvant effect on monocytes and DCs exposed to EtOH. For this purpose we evaluated in vitro activity of cromoglycate on DC phenotype and function exposed to EtOH in the well-established model of DC differentiation and maturation.

Our results showed that alcoholics' monocytes differentiated into immature DCs with an altered phenotype and functions (alc-iDCs). Alc-iDCs showed fewer $CD1a^+$ cells, weaker CD86 expression and higher HLA-DR expression associated with lower

endocytosis and allostimulatory functions than did iDCs from healthy subjects (control-iDCs). Despite these impairments, alc-iDCs produced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) in large amounts. LPS stimulation failed to induce full phenotypical and functional alc-iDC maturation. In vitro acute EtOH exposure also prevented alc-iDCs and control-iDCs from maturing in response to LPS. T-cell priming experiments showed that EtOH treatment prevented LPS-stimulated control-DCs from priming and polarizing naïve allogeneic T cells into T helper 1 (Th1) cells, thus favouring a predominant T helper 2 (Th2) environment. Furthermore, this thesis establishes a new adjuvant effect of cromoglycate on the level of DC differentiation and maturation and suggests that cromoglycate may represent an innovative strategy to reverse the impaired immune response in alcoholics thus improving the immune system's capacity to resist infection.

Key Words: Alcoholics, Ethanol, Dendritic cells, Costimulatory molecules, Cytokines.

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Presentations/Abstracts at Scientific Meetings

1) Mancinelli R., Riganò R., Buttari B., Margutti P., Ortona E., Profumo E., Colasanti T., Delunardo F., Mazzoli C., Ceccanti M. Abuso alcolico e sistema immunitario. XIV Congresso Nazionale della Società Italiana di Tossicologia, Istituto Superiore di Sanità, Roma, 6-9 febbraio 2006.

2) Buttari B., Profumo E., Mancinelli R., Ceccanti M., Riganò R. Inhibition of antigen-presenting cell functions by ethanol. 4th National Conference SIICA, Brescia, 8-11 giugno 2005. Minerva Medica 2005; 96 (suppl.1), n.3: 65.

3) Buttari B., Profumo E., Mancinelli R., Attilia ML., Ceccanti M., Riganò R. Ethanol and its metabolites impair the maturation and function of monocyte-derived dendritic cells obtained from alcoholics and healthy subjects. 10th Congress of the European Society for Biomedical Research on Alcoholism, Canterbury, 4-7 Settembre 2005. Alcohol and Alcoholism 2005; 40 (suppl.1) : i35.

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ABBREVIATIONS

Abbreviation Definition

Ag Antigen

AIDS Acquired immune deficiency syndrome

alc-iDCs alcoholics' monocytes differentiated into immature DCs

ALD Alcoholic liver disease

APC antigen presenting cells

BSA bovine serum albumin

CD Cluster of Differentiation

control-iDCs healthy subjects' monocytes differentiated into immature DCs

DCs Dendritic cells

DNA Acido desossiribonucleico

ELISA Enzyme Linked Immunosorbent Assay

EtOH ethanol

ERK1/2 Extracellular-regulated kinases 1 and 2

FACS Fluorescence Activated Cell Sorting

FBS Fetal bovine Serum

FITC Fluorescein Isothiocyanate

fMLP N-formyl-L-methionyl-L-leucyl-L-phenylalanine

FSC Forward Scatter

HEPES 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid

HIV immunodeficiency virus

IFN-γ Interferon-gamma

Ig Immunoglobulin

iDCs immature dendritic cells

IL-1β interleukin-1β

IL-2 interleukin-2

IL-6 interleukin-6

IL-12 interleukin-12

IL-13 interleukin-13

IL-17 interleukin-17

LPS Lipopolysaccharide

MAC Mycobacterium avium complex

MACS Magnetic Activated Cell Sorting

MAPK Mitogen-activated protein kinase

MLR Mixed lymphocyte reaction

MFI Mean Fluorescence Intensity

MHC Class I major histocompatibility complex

MIF Migration inhibition factor

NK Natural killer cells

NF-κB Nuclear factor kB

PBS Phosphate Buffered Saline

PBLs Peripheral blood monocytes

PBMCs Peripheral blood mononuclear cells

PMA Phorbol 12-Myristate 13-acetate

rhGM-CSF Recombinant human granulocyte-macrophage colony-stimulating factor

rhIL-4 Recombinant human interleukin 4

Th1 T helper 1

Th2 T helper 2

TNF Tumour Necrosis Factor

TLR Toll like receptor

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Chapter 1 Literature Review

1.1 Introduction

Research demonstrating alcohol's adverse effects on functions of the immune system supports clinical evidence of an increased incidence of infections and autoimmune diseases among alcoholics (F. Paronetto, 1993; I.G. McFarlane, 2002). In trauma and burn patients acute excessive alcohol intake can increase the incidence of sepsis (M. Lois et al., 1999; D.M. Boe et al., 2003; H. Friedman et al., 2003).

Alcohol-induced immune dysfunctions depend on various factors including the dose and duration of alcohol exposure (chronic vs acute) and on the presence and the characteristics of additional stimuli such as microbial molecules. While the association of alcohol abuse with increased deaths from infections was made over 75 years ago, only in the past 15 years has serious investigative efforts been made to understand the role of alcoholic exposure on immune dysfunction. The more recent studies from both humans and animals suggest that alcohol produces significant alterations in immune regulatory function. These abnormal immune regulatory effects produce either immunodeficiency or autoimmune features and can lead to clinical disease.

Among the many alcoholics whose socioeconomic status is poor, overcrowded living conditions and limited access to health care may increase the likelihood of contracting and spreading disease. In addition, many alcoholics suffer from malnutrition and liver disease, conditions that may themselves compromise the immune system's capacity to resist infection.

The complex nature of the linkage between alcohol consumption, altered host immune responses, and infection remains controversial and incompletely understood. The development of improved laboratory techniques to investigate functions of the immune system may help research to delineate better immunologic abnormalities in persons who abuse alcohol as well as in animal models of alcohol use.

1.2 Alcoholics and infections

1.2.1 Alcoholics and tubercolosis

Although the incidence of tuberculosis has declined dramatically in this century, pockets of the disease among the indigent alcoholic population have been a major factor in preventing its eradication in this country.

Historically, tuberculosis has been one of the great scourges of mankind. Almost one-third of all deaths in Europe in the 19th century were caused by this infection (Des Prez and Heim 1990). Contagion has been associated particularly with crowded and impoverished living conditions--and their associate malnourishment--settings associated with high rates of alcoholism. Much has been written about the relationship between alcoholism and tuberculosis (Tapper 1980; Des Prez and Heim 1990; Hudolin 1975). Alcoholics--homeless, indigent alcoholics in particular--are more susceptible to acquiring the infection, and present a greater challenge to caregivers in adhering to prescribed therapies.

With improvement in living conditions and the arrival of effective antituberculous drug therapy, the incidence of tuberculosis has declined dramatically in the 20th century (Des Prez and Heim 1990). As the incidence of tuberculosis in the general population has fallen, recalcitrant pockets of disease among the homeless, many of who are alcoholic have been a prominent factor in preventing eradication of the disease in this country (Rhodes and Hudson 1969). Recently, the rate of decline has begun to level off, and, in the last few years, the incidence of tuberculosis has actually begun to rise in areas of the country heavily affected by the human immunodeficiency virus (HIV) epidemic (Bloch et al. 1989).

Tuberculosis is an infection caused by the organism Mycobacterium tuberculosis. It is acquired by inhaling infectious "droplet nuclei." particles of respiratory secretions produced by coughing. Prolonged exposure to these droplet nuclei is usually required for a person to become infected. Until the human host develops adequate immunity, the slow-growing bacteria continue to multiply within these cells. The infected cells spread to regional lymph nodes in the chest, and subsequently disseminate via the bloodstream to other organs of the body (Des Prez and Heim 1990).

The medical literature is replete with articles describing an association between alcoholism and the acquisition of tuberculosis. (Jones et al. 1954; Olin and Grzybowski 1966). Numerous other studies have noted an association between alcoholism and pulmonary tuberculosis (Brown and Campbell 1961; Miline 1970; Coetzee et al. 1988; Hudolin 1975). A survey of tuberculous patients in Australia (Miline 1970) revealed that the level of alcohol consumption was correlated with the risk of acquiring tuberculosis.

The explanation offered for the greater susceptibility among alcoholics in general to pulmonary tuberculosis is largely hypothetical. In indigent alcoholics, it has been attributed to the crowded, dormitory-style living conditions in which they exist (Tapperr M.L., 1980; Olin and Grzybowski 1966; Pincock 1964; Brown and Campbell 1961). Prolonged exposure to infectious droplet nuclei is usually necessary for an individual to become infected, and such an environment would greatly increase the chances of an individual inhaling large numbers of these droplet nuclei, and after being infected, of then coughing them back out to infect others.

In addition, alcohol has a significant inhibitory effect on cell-mediated immunity, the primary host defence against tuberculosis (MacGregor 1986). Impaired cell-mediated immunity and reduced resistance to tuberculosis have been described in animals and humans with deficiencies of protein and vitamins A, B_{12}, C, and D.

These same factors that allow for primary infection with tuberculosis in the alcoholic may increase the risk of a more serious second infection from a subsequent exposure (Nardell et al. 1986). Reports generally suggest that, in addition to suffering a greater frequency of tuberculosis, indigent alcoholics present with more severe disease as well. Homeless alcoholics have very disorganized lifestyles, and, therefore, are more likely to come to medical attention later in the course of their illness. A review of 2,800 cases of pulmonary tuberculosis reported to the Health Department of Victoria, Australia, between 1961 and 1966 (Miline 1970), showed that these alcoholics tended to present with more advanced disease.

Consistent with the greater extent of disease when they first present with tuberculosis, alcoholics appear to have a higher risk of respiratory failure and death during the initial hospitalization period (Barnes et al. 1988). Nevertheless, unless the infection is far advanced, the alcoholic with tuberculosis who takes his or her medications should respond as well to therapy as the non-alcoholics (Kok-Jensen 1972).

When indigent alcoholics do relapse with tuberculosis, their erratic pattern of medication use is more likely to lead to infection with bacteria resistant to the standard anti-tuberculous drugs. This is because intermittent exposure to these drugs allows the bacteria to continue to survive and multiply; those organisms that have mutated during multiplication and developed resistance to one or more of the medications have a survival advantage over those that have not. These resistant strains of tubercle bacilli are then spread to others. In the previously noted outbreak of tuberculosis in a Boston shelter for the homeless, 31 of the 42 cases were infected with antibiotic-resistant strains (Nardell et al. 1986).

1.2.2 Alcoholics and bacterial pneumonia

Among the infections, bacterial pneumonia has the strongest and best-documented association with alcohol abuse. Benjamin Rush published one of the earliest reports to link alcohol consumption and pulmonary infections in 1785. In “An Inquiry Into the Effects of Ardent Spirits Upon the Human Body and Mind”, Rush listed tuberculosis and pneumonia as "infectious sequelae" of alcoholism. Sir William Osler, in his Principles and Practice of Medicine (1909), stated that alcoholism was "perhaps the most potent predisposing factor" to pneumonia. Indeed, the frequency and severity of pneumonia are so pronounced among alcohol abusers that physicians have historically been convinced that the alcohol-consuming patient is, in fact, an immuno-compromised host.

Bacterial pneumonia is the clinical manifestation of infection caused by multiple host and pathogen interactions involving characteristics of the invading microorganism, immunologic defences, nutrition, metabolism, and environmental exposure. As most patients with pneumonia do not have a clinically recognized genetic defect in their host defence system, their disease is typically the outcome of an acquired imbalance resulting from a failure of the immune system to destroy or rid itself of invading pathogens. Because the invading pathogens encounter a highly integrated system of defence mechanisms, which comprises both mechanical components and immunologic components, infectious challenges to the lower respiratory tract rarely cause pneumonia in healthy people under normal circumstances.

Inhalation of airborne pathogens may result in pneumonia, as may direct spread from a contiguous source, or spread from the bloodstream. Bacterial pneumonia, however, often results from the aspiration of bacteria residing in the oropharynx.

The major factor influencing the development and clinical course of pneumonia is the integrity of the host defence system. Alcohol is known to disrupt many aspects of normal lung phagocytic defenses. Alcohol has been shown to impair many critical functions of these defences, including adherence, phagocytosis, oxygen-radical generation,

bactericidal capacity, and cytokine production. Furthermore, alveolar macrophages obtained from the lungs of alcohol-consuming patients show defective uptake and killing of bacteria in comparison with alveolar macrophages obtained from normal subjects.

One of the most important components of the defensive system is the recruitment of neutrophils from the bloodstream into the lower respiratory tract. Alcohol, however, impairs the neutrophil's ability to enter infected tissue sites and their ability to phagocyte and kill intracellular pathogens; these are ones of the most consistent adverse effects of alcohol on the host defense system.

1.2.3 Alcoholics and HIV

Each year thousands of people are infected with the human immunodeficiency virus (HIV) that results in acquired immune deficiency syndrome (AIDS). The virus may lie dormant for years, but when it awakens it selectively infects cells of the immune system, thus destroying the cells specifically designed to protect the body from such foreign invaders. The majority of AIDS patients die from rare opportunistic infections or cancers those are able to attack with little resistance from the host's impaired immune system. AIDS research generally concentrates on three mechanisms: infection, spread of the virus within the host, and treatment of the diseases commonly found in AIDS victims, such as pneumocystic pneumonia and Kaposi's sarcoma. Alcohol abuse may play a role in each of these mechanisms by compounding the strain on the immune system.

Alcohol has been proposed as a cofactor in HIV infection at all stages, including initial infection, spread of the virus within the immune system, and onset of symptoms. This role as a cofactor may be attributed to alcohol's many effects on immune functions in humans (Gluckman et al. 1977; Smith et al. 1980; Young et al. 1978).

The effects that alcohol has on the immune system and HIV infection may depend on a person's drinking habits. Variables such as quantities consumed, duration and frequency of consumption, and the presence of liver disease may be important to consider in attempting to determine the effects of alcohol on HIV infection. Alcohol effects on HIV may be quite significant in the chronic alcoholic, or heavy drinker, and negligible in the occasional drinker. In addition, because many of the effects of alcohol on immune function are reversible, the timing of alcohol exposure relative to the different stages of HIV infection may influence the effect(s) that alcohol has on the infection.

In vivo and in vitro studies suggest that alcohol may increase the susceptibility of phagocytes to initial infection with HIV and may impair the ability of these cells to eliminate HIV in the early stages of infection when viral burden is low. Thus, alcohol may increase the potential for phagocytes to serve as reservoirs for HIV. Alcohol affects not only the function of immune system cells but also their numbers. Exposure to alcohol results in pronounced effects on the numbers of lymphocytes, such as T-cells, in the peripheral lymphoid organs (spleen, lymph nodes, and thymus) and in the circulating blood. Alcoholics have significantly reduced numbers of white blood cells, or leukocytes, in their blood (MacGregor 1986; Smith et al. 1980; Spagnuolo and MacGregor 1975; Young et al. 1978). This may be due, in part, to liver disease in these individuals associated with chronic alcoholism. However, similar effects of alcohol have been observed in animal models in the absence of liver disease. For example, consumption of alcohol by mice results in a loss of cells in the peripheral lymphoid organs and in the circulating blood (Jerrells et al. 1990b; Saad and Jerrells 1991).

Although alcohol reduces the absolute numbers of immune system cells that HIV can infect, the remaining cells may be more susceptible to initial infection once they come into contact with the virus. In fact, one group of investigators has reported an increased expression of HIV proteins after alcohol treatment of lymphocytes in vitro followed by

HIV infection (Saravolatz et al. 1990). Further support of this contention is provided by the findings of Bagasra and associates (1989). These investigators harvested blood cells (lymphocytes) from healthy individuals who had recently consumed alcohol and, subsequently, infected the cells with the HTLV-IIIB strain of HIV in vitro. They observed an increase in syncytia (disabled cell clusters) formation and HIV proteins in these cultures as compared with control cultures not exposed to alcohol.

An alcohol-induced loss of cells in the immune system may accelerate the onset of clinical manifestations in HIV-infected individuals. Research has shown that alcohol preferentially impairs T-cell--mediated immune functions (Bagasra et al. 1988; Jerrells et al. 1990b; Saad and Jerrells 1991). One possible mechanism for impairment after exposure to alcohol is a resulting inability of the cells to respond to the cytokines that stimulate T-cell helper functions (Jerrells et al. 1990a; Norman et al. 1991; Weinberg and Jerrells 1991). Many of the events influenced by CD4+ T-cells that activate an immune response, such as cell growth, activation, and cytokine production, also stimulate HIV replication.

It could be suggested that alcohol impedes the course of HIV infection through its effects on the immune system. Not only does alcohol reduce the absolute numbers of immune system cells that HIV can infect, but also it impairs T-cell activation that can result in progression of HIV infection.

Because macrophages and T-cells are important in the defence against infections, the effects of alcohol on these cells may greatly increase the risk of secondary infections in people with HIV. Chronic alcoholics, in general, have a higher than normal incidence of pathogenic and opportunistic infections, including bacterial pneumonia (MacGregor 1986); tumors of the head, the neck, and the gastrointestinal tract (Breeden 1984); and lymphocyte-mediated (in part) chronic liver disease related to autoimmunity (Mutchnick et al. 1980). This already increased susceptibility to infection may be an important role for alcohol as an indirect cofactor that determines the susceptibility of an individual to

progressive HIV infection. Secondary opportunistic infection resulting from severe immunosuppression is one of the primary causes of death in people with HIV infection. Thus an alcohol-induced decrease in the ability to eliminate these infections may decrease the life expectancy of these individuals.

1.2.4 Alcoholics and viral hepatitis

Viral hepatitis can cause as much damage to the liver as alcoholism. Research is now indicating that alcoholics are at high risk of contracting viral hepatitis, compounding their risk of liver damage and cirrhosis.

Both excessive alcohol consumption and viral hepatitis have been known to cause cirrhosis (Haberman and Weinbaum 1990). Hepatocellular carcinoma is also associated with patients afflicted with alcoholism or viral hepatitis, although it is relatively rare in the United States (Dusheiko 1990).

Several studies suggest that alcoholic patients have an increased risk of viral hepatitis. The reasons for the increased risk are unclear but may be related to social habits and conditions sometimes associated with alcoholism (e.g., intravenous drug use, poverty, "unsafe" sexual habits). The risk of severe liver injury in alcoholics, including cirrhosis and hepatocellular carcinoma, may be compounded by the dual effects of both alcohol and viral hepatitis (Nalpas et al. 1991). The therapy for cirrhosis is often related to its cause: when caused by excessive alcohol consumption, complete abstinence can be effective even in late stages of the disease (Powell and Klatskin 1968); when caused by viral hepatitis, cirrhosis can progress despite abstinence from alcohol. Therefore, if alcoholics are at high risk of contracting viral hepatitis, preventive measures should be instituted to avoid further liver damage.

Commercially available vaccines against hepatitis B have been developed during the last 10 years. The first vaccines was derived from plasma, and although there was initial fear, of HIV transmission from the vaccine, there has been no evidence of such transmission. The most recent vaccine is produced by recombinant DNA and has virtually replaced the use of the plasma-derived vaccine.

Many epidemiologic studies have reported a higher than average occurrence of hepatitis viral infections in alcoholics, specifically hepatitis B and C viral infections. These infections can occasionally lead to chronic infections and can contribute to the progression of liver disease in alcoholic patients.

Several studies in urban areas have reported that alcoholics have an increased prevalence of hepatitis B infection (Chopra et al. 1980; Mills et al. 1981; Goudeau et al. 1981; Basile et al. 1981; Boron et al. 1986; Jacobson et al. 1992). The mechanism for the increased risk of hepatitis B infection are unclear but have been attributed to several factors: increased intravenous drug use, history of blood transfusions, decreased immune functioning associated with alcoholism, increased risk of unsafe sexual contact, and socioeconomic conditions.

Not only may alcoholics have an increased risk of contracting hepatitis B, but also once infected, hepatitis B may occasionally exacerbate liver disease in these patients. Several studies have reported that alcoholic patients with advanced liver disease (e.g., cirrhosis) have a higher prevalence of hepatitis B markers than alcoholics with less severe forms of alcoholic liver disease (Mills et al. 1981; Orholm et al. 1981; Buffet et al. 1982). However, other studies have failed to detect an association between cirrhosis and hepatitis B markers in alcoholics (Gludd et al. 1982; Bassendine et al. 1983; Saunders et al. 1983; Mendenhall et al. 1991).

Vaccination of alcoholics has been considered because of their increased risk of hepatitis B infections. However, studies have suggested that alcoholics may have a

decreased immunologic response to hepatitis B vaccine. Mendenhall and colleagues (1988) reported that response rate to the vaccine was 89 percent in non-alcoholics controls, 70 percent in alcoholics without liver disease, and 18 percent in alcoholics with liver disease. Degos and co-workers (1986) also reported reduced response rates in alcoholics with cirrhosis.

Currently, use of hepatitis B vaccines may not offer sufficient protection in some alcoholic patients. Further research is needed to improve vaccination strategies.

It is not certain whether alcoholism alone increases the risk of contracting hepatitis C, thus, further studies are needed to determine whether alcoholism significantly increases the risk of hepatitis C infection in the absence of intravenous drug use.

Although rate of infection may not be increased in alcoholics, studies suggest that hepatitis C viral infections may aggravate alcoholic liver disease. Several studies have reported that a large number of patients with alcoholic liver disease were positive for anti-HCV.

It is not certain whether alcoholism alone increases the risk of contracting hepatitis C. The proportion of alcoholics who had antibodies to hepatitis C virus was variable in three urban studies

There has been little evidence linking alcoholism to the other types of hepatitis viruses. Studies conflict on whether or not hepatitis A infections are increased in alcoholics (Gluud et al. 1982; Stigendal et al. 1984). Hepatitis D infections are more frequent in intravenous drug users, though the prevalence of this infection in alcoholics who are not intravenous drug users has not been well studies. Hepatitis E viral infections are rare in developed countries.

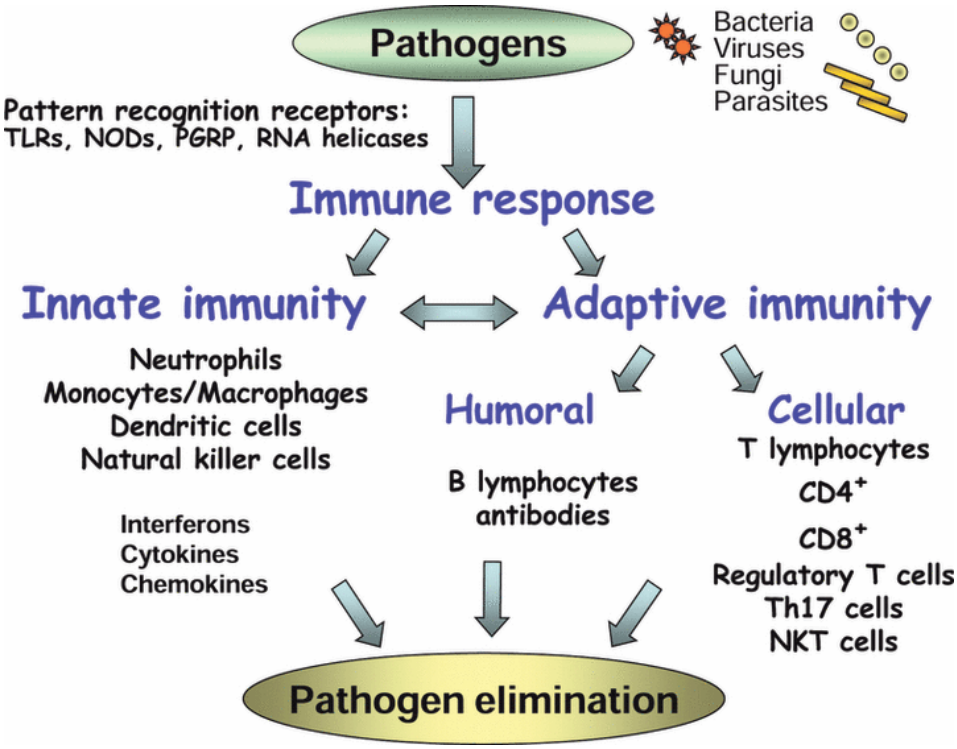

Figure 1.1 Immunocells in immunosurveillance and pathogen clearance.

1.3 Alcohol and immune system

1.3.1 Alcohol adverse effects on lymphoid organs and lymphocyte distribution

Early studies of alcohol's influence on immune responses examined whether alcohol alters the numbers or ratios of lymphocytes and lymphocyte subpopulations found in the blood. These studies consistently showed a decrease in the numbers of lymphocytes isolated from the blood of alcoholic humans or from laboratory animals that had consumed alcohol over a period of several weeks. These lymphocytes also responded abnormally to in vitro stimulation by mitogens or antigens, suggesting an adverse effect on their capacity to react appropriately to infection (Grossman et al. 1988; Roselle et al. 1988). Improved technology for characterizing and isolating different types of lymphocytes (B-cells, T-cells, and various T-cell subpopulations) have allowed investigators to determine alcohol's effects on the numbers and functions of lymphocyte populations. One group of researchers has found numbers of circulating helper T-cells and cytotoxic or suppressor T-cells to be reduced in alcoholics (Roselle et al. 1988). The same study observed that T-cell deficits were temporary and at least partially reversible following abstinence from alcohol (Roselle et al. 1988). Alterations in circulating lymphocyte numbers could contribute to increased susceptibility to infection. In contrast, other investigators have found no abnormalities in the numbers of lymphocytes within particular subpopulations or in relative proportions (ratios) of various T-cell subpopulations isolated from the blood of alcoholics (Jovanovic et al. 1986; Ishimaru and Matsuda 1990). Differences between these reports and those of Roselle and co-workers remain to be explained.

Researchers also have examined effects of alcohol on the histology of lymphoid organs (the thymus and spleen) in laboratory animals. Alcohol intake has been shown to

decrease thymic weight and reduce the number of cells found in the thymus. This effect was accompanied by a decrease in responsiveness of thymocytes to mitogen stimulation, indicating impairment in these cells' capacity to undergo the proliferation and differentiation integral to effective T-cell responses (Jerrells et al. 1986; Grossman et al. 1988; Jerrells et al. 1989; Chang et al. 1990; Jerrells et al. 1990b). Studies of the spleen have demonstrated similar reductions in cell numbers and decreased mitogen responses following alcohol administration, with similar implications for resistance to disease (Jerrells et al. 1988).

Saad and Jerrells (1991) have published a detailed investigation of alcohol's effects on lymphocyte populations and lymphoid organs in mice. The authors suggest that alcohol may influence the migration of thymocytes within the thymus, and as a result, may alter normal patterns of T-cell development and migration from the thymus to peripheral blood and lymphoid tissues. Further documentation of abnormalities in thymocyte characteristics (and in a variety of other immunologic parameters) are found in studies of fetal alcohol syndrome (Ewald 1989; Ewald and Walden, 1988).

1.3.2 Alcohol effects on antibody production

Even though alcoholics demonstrate normal numbers of circulating B-cells (Roselle et al. 1988), they frequently demonstrate elevated levels of serum antibodies, or immunoglobulins (Ig), particularly those of the IgG and IgA classes (Nouri-Aria et al. 1986). Elevated serum antibody levels may be related to liver disease and may indicate impaired regulation of B-cell function (Nouri-Aria et al. 1986). Experiments that have attempted to explain these abnormalities have found that alcohol can have both direct and indirect effects on antibody-producing B-cells. However, for the most part these experiments have demonstrated that alcohol inhibits B-cell production of antibody, results

that are difficult to reconcile with clinical findings. In vitro experiments have demonstrated that physiologic doses of alcohol can suppress antibody production through direct effects on B-cells (Aldo-Benson et al. 1986; Aldo-Benson 1989). The biochemical mechanisms involved in this inhibition are unclear, since, in these studies, alcohol had no apparent effect on those membrane and intracellular changes that normally occur when B-cells are stimulated to produce antibody. In vivo experiments have suggested that alcohol may suppress antibody production through indirect effects on B-cells. To produce antibodies, B-cells usually require "help," in the form of cytokines released from T-cells. Thus indirect effects of alcohol on antibody responses may result from effects on participating T-cells. Researchers can identify such an effect by comparing alcohol's actions in two experimental types of B-cell antibody responses: T-cell-dependent antibody responses and T-cell-independent antibody responses. B-cell responses that are T-cell dependent will not generate optimal amounts of antibody unless T-cell help is present. B-cell responses that are T-cell independent will produce antibodies without T-cell help. Thus a substance that interferes with T-cell help (but has little or no direct influence on B-cell functions) would alter T-cell--dependent antibody responses but have no effect on T-cell--independent antibody responses.

Researchers have shown that, compared with non-alcoholic control subjects, alcoholics generate normal antibody responses to the Streptococcus pneumoniae vaccine (Pirovino et al. 1984), but generate poor responses to the hepatitis B vaccine (Mendenhall et al. 1988). The vaccine used to immunize against Streptococcus pneumoniae induces a T-cell--independent response, while the vaccine used to immunize against hepatitis B virus induces an optimal antibody response only with help from T-cells. Thus, the above experiments suggest that alcohol suppresses antibody production by inhibiting T-cell production of cytokines needed for B-cells to respond normally.

1.3.3 Alcohol adverse effects *on dendritic cells*

DCs are the most potent APCs. Owing to their unique ability to stimulate naïve T cells. The functional state of DC maturation and their activation degree control immunity and tolerance (Ueno H et al., 2007).

In chronic alcoholics without liver disease, increased secretion of pro-inflammatory cytokines (i.e. IL-1β, IL-6, IL-12 and TNFα) by circulating DC has been reported (F.J Laso et al., 2007), whereas subjects with alcoholic liver cirrhosis and at least 1 yr after alcohol withdrawal, and patients with active alcohol intake, showed decreased numbers of circulating DC and reduced secretion of these cytokines. Chronic alcoholism is associated also with numerical, phenotypical, and functional changes in circulating peripheral blood DCs. Early work on DC and alcohol was confined to human peripheral blood monocyte-derived DC propagated in vitro (P. Mandrekar et al., 2004; A. Dolganiuc et al., 2003) When cultured for 7 days with a low concentration (25 mM) of alcohol, monocyte-derived DC showed reduced CD80 and CD86 expression in addition to altered cytokine production and displayed reduced allostimulatory capacity in MLR, compared to control DCs (P. Mandrekar et al., 2004, and A. Dolganiuc et al., 2003). Surprisingly, NF-κB activation, which is normally upregulated during DC maturation, was not affected by in vitro alcohol exposure (P. Mandrekar et al., 2004). These alcohol-exposed DC induce anergy in naïve T cells, thereby preventing their proliferation.

Prolonged alcohol exposure has been shown to inhibit the development and function of mouse myeloid and plasmacytoid DCs derived from bone marrow precursors in vitro, in a dose-dependent manner (A.H. Lau et al 2006). Furthermore, the function of murine alcohol-treated DC, when stimulated with herpes simplex virus or the toll like receptor-(TLR)-9 ligand CpG, was inhibited compared to control DCs, as determined by maturation marker expression in addition to a reduced capacity to stimulate naïve allogeneic T-cell proliferation. In vivo, alcohol-exposed DCs were poorer at priming naïve

T cells after adoptive transfer to recipients. Interestingly, alcohol-exposed DCs primed T cells that produced more IL-10 when re-stimulated with alloAg ex vivo. The reduced allostimulatory capacity of alcohol-exposed DCs can be ascribed to their immature phenotype and perhaps also to their higher inhibitory molecule expression (i.e. PD-L1, programmed death ligand-1) relative to co-stimulatory B7 molecule expression (A.H. Lau et al 2006).

The influence of chronic alcohol consumption on DC, freshly isolated from mice, has also been assessed. Reduced numbers of DCs in the spleen, but increased numbers in the thymus, are seen in mice given 20% ethanol in drinking water for up to 28 weeks (M.R. Edsen-Moore et al., 2008). These changes could not be ascribed to altered DC precursor numbers, differentiation or turnover rate. In a similar manner, liver and spleen DCs were affected differentially by alcohol exposure, with alcohol exerting a less marked inhibitory effect, as determined by phenotypic and functional characteristics (A.H. Lau et al 2006) on liver DC (the latter are inherently more resistant to maturation than splenic DC (T.L. Sumpter et al., 2007). Although classic DC phenotypic maturation markers, such as CD40, CD80 and CD86, were all expressed at low levels on both control and alcohol-exposed, freshly isolated DC, alcohol-exposed liver and spleen DC were both poorer stimulators of naïve allogeneic T cells in MLR compared to control, freshly isolated liver and spleen DC. These data indicate that alcohol uses another mechanism, independent of classic costimulatory pathways, to affect the T-cell stimulatory capacity of immature liver and spleen DC.

Interestingly, when immature hepatic and splenic DC were tested for their capacity to prime naïve T cells in vivo, hepatic DC from chronic alcohol-exposed mice displayed increased ability to prime T cells compared to control hepatic DC. By contrast, splenic alcohol-exposed DCs had reduced capacity to prime naïve T cells compared to control splenic DCs that corresponded with in vitro phenotypic maturation and functional data

(A.H. Lau et al., 2007). However, the enhanced priming ability of alcohol-exposed hepatic DCs did not correspond with and, in fact, conflicted with phenotypic and in vitro functional data. Examination of DCs migration to draining lymph nodes in vivo revealed enhanced migration of alcohol-exposed hepatic DCs (A.H. Lau et al., 2007). The altered migration of hepatic alcohol-exposed DCs was found to be independent of both CC chemokine receptor 7 and CD11a expression.

Prolonged in vivo alcohol exposure does not affect the endocytic capacity of mouse splenic DCs (C. Aloman et al., 2007). Unlike TLR9 activation, stimulation of splenic DC with TLR3 and TLR4 ligands led to upregulation of the maturation markers CD40 and CD86, with no difference between alcohol and control groups. However, similar to previous findings with TLR9-activated DC, the allostimulatory capacity of alcohol-exposed DC was impaired. Alcohol-exposed splenic DC produced enhanced levels of IL-1β and IL-10, but decreased TNFα, IL-12, IFN-γ and IL-6. Similarly, another study found decreased production of the same cytokines, in addition to IL-17A, but observed increased IL-13 from mouse splenic DCs exposed to alcohol in vivo (R. Heinz and C. Waltenbaugh; 2007).

Like chronic alcohol abuse, acute alcohol abuse also has immune regulatory potential. Studies in human volunteers indicate that acute alcohol abuse compromises accessory cell function (P. Mandrekar et al.,2004; B. K. Verma et al., 1993: T.R. Jerrells et al., 2002;) thus impairing antigen-(Ag-) specific T cell proliferation (F.J. Laso et al., 1999).

When humans consume alcohol acutely, blood monocyte-derived DCs exhibit reduced ability to present tetanus toxoid and impaired T-cell allostimulatory capacity (P. Mandrekar et al., 2004; G. Szabo et al., 2004a and b). In a study on in vivo alcohol consumption and in vitro alcohol treatment on myeloid DCs obtained from healthy individuals, Mandrekar et al (P Mandrekar et al., 2004] have shown that acute alcohol

exposure primarily impairs DC differentiation, leaving maturation almost unchanged, thus causing T cell anergy through mechanisms that involve decreased IL-12 induction.

1.3.4 Alcohol adverse effects on natural killer cells

Natural killer (NK) cells are thought to play an important role in the destruction of virus-infected and cancerous cells. Meadows and co-workers (1989) have found that alcohol induces specific abnormalities in NK cell function. These researchers observed that NK cells from mice that had ingested alcohol over a 1-2 week period showed a decreased (in comparison with control animals) capacity to kill tumor cells in vitro. This deficit was observed even in the presence of interleukin-2, a cytokine that normally enhances NK killing. In contrast, other investigators have shown that alcohol may enhance NK cell activity when comparatively lower doses of alcohol are consumed (Saxena et al. 1981).

No information is currently available to explain this low dose enhancement versus higher dose impairment of NK cell function. However, the phenomenon may not be limited to this particular immune response. Similar data have been generated in alcohol-fed laboratory rats, using a delayed-type hypersensitivity skin test as an in vivo measure of immunity (Dehne et al. 1989). In these animals, low doses of alcohol (0.5-2 grams per kilogram of body weight per day) enhanced mitogen-induced hypersensitivity reactions, while higher doses (6 grams per kilogram of body weight per day) depressed these responses. The clinical significance of alcohol's apparent bimodal effects is unclear and warrants further investigation.

1.3.5 Alcohol effects on neutrophils and macrophages

A primary role of neutrophils, macrophages, and other phagocytic cells is to locate, ingest, and kill microorganisms that invade body tissues. This process is extraordinarily

complex, involving migration of these cells to the site of infection, attachment (adherence) to the vascular endothelium at the site of infection, engulfment of the microorganism, and intracellular destruction of the microorganism by means of proteolytic enzymes and toxic oxygen-containing compounds. As indicated in the studies described below, alcohol probably affects multiple events in this pathway. Adherence is a normal property of neutrophils that is controlled by the expression of a surface glycoprotein called Mac-1. Non-stimulated neutrophils express Mac-1, and neutrophils that are exposed to infectious or chemical agents become even "stickier" as a result of increased Mac-1 expression. Alcohol has been shown to inhibit the in vitro adherence of human neutrophils following stimulation with a neutrophil-activating chemical (N-formyl-L-methionyl-L-leucyl-L-phenylalanine, or fMLP) (MacGregor et al. 1988; Nilsson et al. 1991). The same study found that alcohol had no adverse effect on adherence of non-stimulated neutrophils or their normal, non-stimulated expression of Mac-1; however, alcohol did inhibit the increased expression of Mac-1 following stimulation with fMLP. MacGregor (1988) has examined the in vitro migration of neutrophils isolated from the blood of alcholic patients with liver disease. Normal, nonstimulated movement of neutrophils from alcholic patients with cirrhosis was not significantly different from that of healthy control subjects. But when nuetrophils from these patients were stimulated with a bacterial substance, lipopolysaccharide (LPS), chemotaxis (measured in terms of neutrophil movement toward a neutrophil-attracting factor) was reduced. These investigators also characterized a substance in patient serum, not found in the serum of healthy control subjects that appeared to cause the observed inhibition. Such alterations in the directed movement of neutrophils suggest an impairment in their ability to locate and effectively eliminate infectious microorganisms. However, in the same study, an in vivo (and therefore perhaps more clinically relevant) test of neutrophils migration revealed no differences between alcoholic

patients with cirrhosis and healthy controls. The discrepancy between in vitro and in vivo experimental results is not currently understood.

Alcohol also inhibits macrophage functions. The most notable effect of alcohol seems to be on the suppression of phagocytosis, as determined in experiments that test the efficiency with which macrophages engulf various kinds of particles. Some researchers have shown that macrophages taken from alcohol-consuming animals demonstrate an impaired ability to phagocytes red blood cells or yeast cells (Watson et al. 1988; Bagasra et al. 1988). Alcohol also may inhibit the macrophage's ability to kill phagocytosed microorganisms: one study has shown that alcohol suppresses the generation of toxic oxygen products within macrophages, compounds that assist in this microbial killing (Dorio 1990).

Other researchers have examined the effect of alcohol on the clearance of bacteria from the lungs, a process in which pulmonary macrophages play an important role. Green and Kass (1964) observed, in experiments using rats, that alcohol consumption inhibited the efficiency with which these animals eliminated bacteria from their lungs. In similar experiments, Nelson and co-workers (1990) have found that acute alcohol administration to mice inhibited the intrapulmonary killing of Staphylococcus aureus and Klebsilla pneumoniae, bacteria that commonly cause pneumonia and other infections in alcoholics. Reduced killing probably resulted, in part, from detrimental effects of alcohol on bactericidal activities of pulmonary macrophages.

Bermudez and colleagues (1992) have observed that, following infection with bacteria belonging to the Mycobacterium avium complex (MAC), mice that have ingested alcohol show a significant increase in the number of viable MAC organisms found in liver, spleen, and appendix compared with control animals. These results suggest that alcohol impairs the ability of macrophages to eliminate MAC from these tissues. A related study revealed that cytokine-stimulated human macrophages and mouse Kuppfer cells (a type of

macrophage found in the liver) killed significantly fewer MAC organisms, when treated control macrophages (Bermudez and Young 1991). Because MAC bacteria can cause serious disseminated disease in persons who have AIDS (Young et al. 1986), alcohol-induced defects in the killing of these organisms may be of clinical significance for alcoholics who have AIDS. As yet, however, there is no clear evidence to show that alcohol use or abuse increases AIDS patients' susceptibility to MAC or other infectious diseases (TR Jerrels, 1988).

Interestingly, a study by Wickramasinghe (1986) has shown that human macrophages can metabolize alcohol to acetate. This alcohol metabolite may impair protective functions of macrophages or other cells of the immune system.

1.3.6 Alcohol effects on cell cytokine production

Many cells of the immune system secrete cytokines, substances that enhance intercellular communication and function. Researchers have characterized numerous cytokines that influence a wide variety of functions in the immune system. Short-term (as few as 8 days) alcohol consumption by laboratory animals has been shown to inhibit the in vitro production of migration inhibition factor (MIF) (Roselle et al. 1989; Dehne et al. 1989). MIF, a cytokine produced by T-lymphocytes, may contribute to the retention and accumulation of macrophages and other phagocytes at a site of infection. Clinically, decreases in MIF activity may result in fewer phagocytic cells at that site--and thus a decreased ability to localize and eradicate infection.

A study by Kaplan (1986) revealed that alcohol could inhibit mitogen-stimulated T-cell proliferation, a response that depends on T-cell production of a cytokine known as IL-2. Kaplan also found that alcohol has no effect on the ability of stimulated T-cells to produce IL-2 or to express IL-2 receptors--meaning that inhibition of T-cell proliferation

could not be explained by a lack of IL-2 or the absence of the receptor necessary to respond to this cytokine. These findings have been confirmed and extended by other investigators, including Jerrells and co-workers (1989, 1990a) who have hypothesized that alcohol somehow interferes with the T-cell's ability to utilize IL-2.

Alcohol consumption also has been shown to alter serum levels of TNF, a cytokine that helps to generate non-specific inflammatory responses. Elevated serum levels of TNF observed in alcoholics with severe liver disease suggest that TNF may contribute to inflammation and damage to the liver (Bird et al. 1990). However, in experiments using animals, acute administration of alcohol has been shown to blunt the increase in serum TNF induced by injection with a substance derived from bacterial cell walls (D'Souza et al. 1989). It was not clear whether these alcohol-induced alternations resulted from changes in production release, or degradation of TNF. In vitro experiments have established that alcohol suppresses TNF and other cytokine production by LPS-stimulated monocytes and macrophages. One study found that production of TNF by monocytes isolated from the blood of cirrhotic patients is depressed in comparison with TNF production by monocytes from age-and gender-matched healthy control subjects (Mozes et al. 1989). Other investigators have noted that LPS-stimulated white blood cells from alcoholic patients shown an increased production of TNF as well as the cytokines IL-1 and IL-6 (McClain and Cohen 1989; Deviere et al. 1990). Impairments in production of TNF, IL-1, and IL-6 could profoundly impair host inflammatory responses that are critical for resistance to bacterial infection.

1.3.7 Alcohol effects on expression of histocompatibility proteins

Singer and colleagues (Parent et al. 1987; Singer et al. 1989) observed that alcohol increases the surface expression of Class I major histocompatibility complex (MHC) proteins on several different types of cells. This increase, which was correlated with

increased intracellular protein synthesis, may have critical implications for physiologic immune responses. Expression of Class I MHC proteins is required for the recognition of virus-infected cells by T-cells and also may be necessary for T-cell recognition of tumor cells and foreign cells introduced by organ transplantation. Therefore, alterations in the expression of this complex by alcohol may cause alterations in immune responses that serve to eliminate virus infections and cancer.

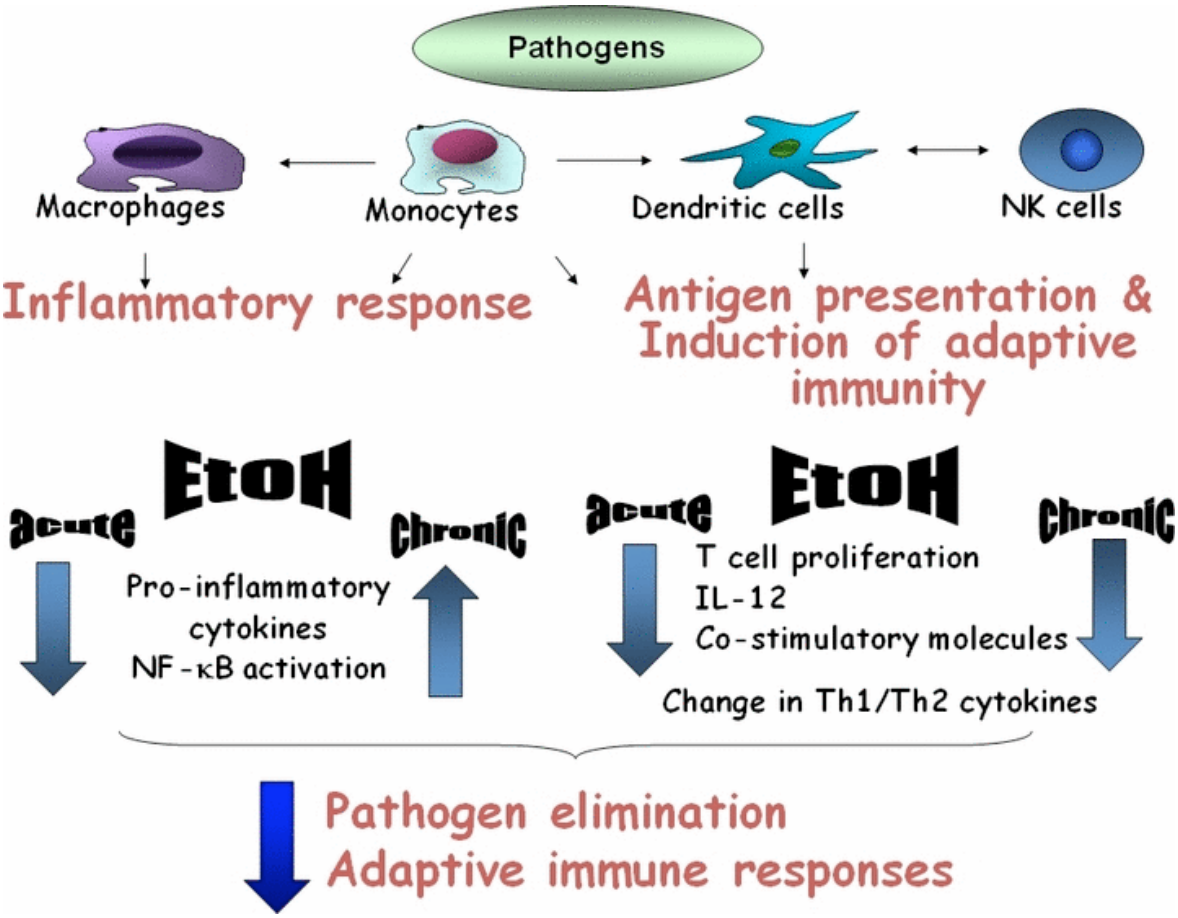

Figure 1.2 Alcohol effects on immune cells during pathogens infections

1.4 Alcohol, pattern-recognition receptors and lipid rafts

Considering that most of the detrimental effects of alcohol on immunity are related to infections, recent studies turned attention to the role of pattern recognition receptors that play a key role in recognition of invading pathogens (Takeuchi and Akira, 2007a,b) and are expressed on innate immune cells as well as on some parenchymal cells.

The family of TLRs that includes 11 mammalian receptors has been partially studied in relation to alcohol. Most studies investigated the LPS-induced inflammatory responses in monocytes, macrophages, or dendritic cells. LPS, a component of Gram-negative bacteria, is recognized by TLR4 with participation of the co-receptors, CD14 and MD2 (Takeuchi and Akira, 2007a,b). Upon encounter with pathogens or after LPS stimulation, these pattern-recognition receptors activate intracellular signaling, most notably via the transcription factor NF-kB, which results in the induction of a variety of pro-inflammatory genes.

Each TLR has a unique extracellular domain that allows specific ligand recognition. The intracellular Toll/IL-1 receptor (TIR) domains share considerable homology, but there are sufficient differences to cause different adapter molecules to be used by some TLRs (Beutler B, 2006; Takeda K, 2005; Miggin SM, 2006; Miyake K, 2006). Uniquely, TLR4 can associate with both of these adaptor molecules (B Beutler, 2006; K Takeda, 2005; SM Miggin, 2006; K Miyake, 2006). CD14, a membrane-associated receptor that has a GPI-linked transmembrane but not intracellular domain, transduces signals by interacting with other signaling molecules, including TLRs (Miyake K, 2006). CD14, similar to other GPI-linked proteins, resides in lipid rafts (M Triantafilou, 2004a). Lipid rafts, also called detergent-resistant domains, are plasma membrane microdomains characterized by insolubility in non-ionic detergent and enrichment in cholesterol and sphingomyelin (LJ Pike, 2003; JB Helms, 2004).

Upon stimulation with specific ligand the recruitment of membrane-associated TLRs, such as TLR2 and TLR4, and other components of TLR complex occurs into lipid rafts (Triantafilou M, 2004a,b,c and 2002; Dolganiuc AG, 2006a). Recently studies described results indicating that acute ethanol (EtOH) exposure alters LPS-induced redistribution of TLR4 receptor components to lipid rafts, that LPS-induced TLR4 signaling is dependent on normal raft structure, that EtOH also alters the reorganization of the actin cytoskeleton, that EtOH decreases TLR4 and CD14 clustering and co-localization, and that these changes are associated with decreased production of TNF-alpha a key pro-inflammatory cytokine (Dai Q, 2006; Dai Q, 2005). Acute EtOH treatment interfered with TLR4 recruitment to lipid rafts at doses that occur in humans after consumption of three to four standard drinks. Attenuation of TLR4 and CD14 recruitment to lipid rafts by EtOH was associated with reduction in LPS-induced TLR4 downstream signaling events and inflammatory cell function (AG Dolganiuc 2006a,b; PD Mandrekar, 2006, SP Oak, 2006). The effects of EtOH on lipid rafts and TLR4 demonstrate that EtOH has significant modulating potential on receptor recruitment to lipid rafts, resulting in functional changes in inflammatory cell functions that may contribute to alcohol induced modulation of the immune cell functions.

While studies have general consensus on the inhibitory effects of acute alcohol on LPS/TLR4-mediated inflammatory cascade activation, the effects of acute alcohol on other TLR-induced monocyte/macrophage responses are less clear. In human monocytes TLR2 ligand-induced TNFα production and NF-κB activation was not inhibited by acute alcohol (Oak et al., 2006). In contrast, murine macrophages had attenuated responses to TLR2 or TLR9 ligand stimulation (Goral et al., 2004). In murine, macrophages acute alcohol inhibited LPS-induced IL-6 production and this was associated with transient down-regulation of the extracellular-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) (Goral et al., 2004). Acute ethanol treatment in RAW

264.7 murine macrophages increased IL-10 production but inhibited IL-6 and IL-12 levels (Pruett et al., 2005). Besides TLR4, acute alcohol altered TLR3-induced gene expression to affect the IFN-related amplification loop, which could be responsible for suppression of several effector molecules of inflammation (Pruett et al., 2004a,b). It has been shown that alcohol inhibits the recruitment of the LPS receptors, TLR4 and CD14 into the lipid rafts on the cell surface (Dai and Pruett, 2006;Dolganiuc et al., 2006a,b; Blanco et al., 2008). Interestingly, TLR2 recruitment to the lipid rafts was not affected by acute alcohol; this correlates with the lack of inhibition of TLR2-induced TNFα production (Dolganiuc et al., 2006; Oak et al., 2006).

In contrast to the inhibitory effects of acute alcohol, prolonged alcohol treatment results in an augmentation of macrophage TNFα production and inflammatory cascade activation. In rat and mouse liver macrophages, Kupffer cells, chronic alcohol exposure in vivo resulted in an increased production of TNFα after ex vivo LPS stimulation (Kishore et al., 2004). This was associated with increased ERK and Egr1 activation and early growth response protein (Egr) 1 mice were protected from alcohol-induced liver disease (Pritchard and Nagy, 2005). Consistent with the peripheral blood monocytes from patients with alcoholic liver disease (ALD) show increased production of TNFα (McClain et al., 1999). Chronic ethanol consumption increased expression of co-stimulatory molecules CD80 and CD86 on TLR9-activated CD11b+ splenocytes contributing to systemic immunodysregulation including T-cell activation (Cook et al., 2004). As discussed above, lung alveolar macrophages are not only suppressed after acute alcohol exposure but even after chronic alcohol exposure, the functions of alveolar macrophages remained impaired (Guidot et al., 2000).

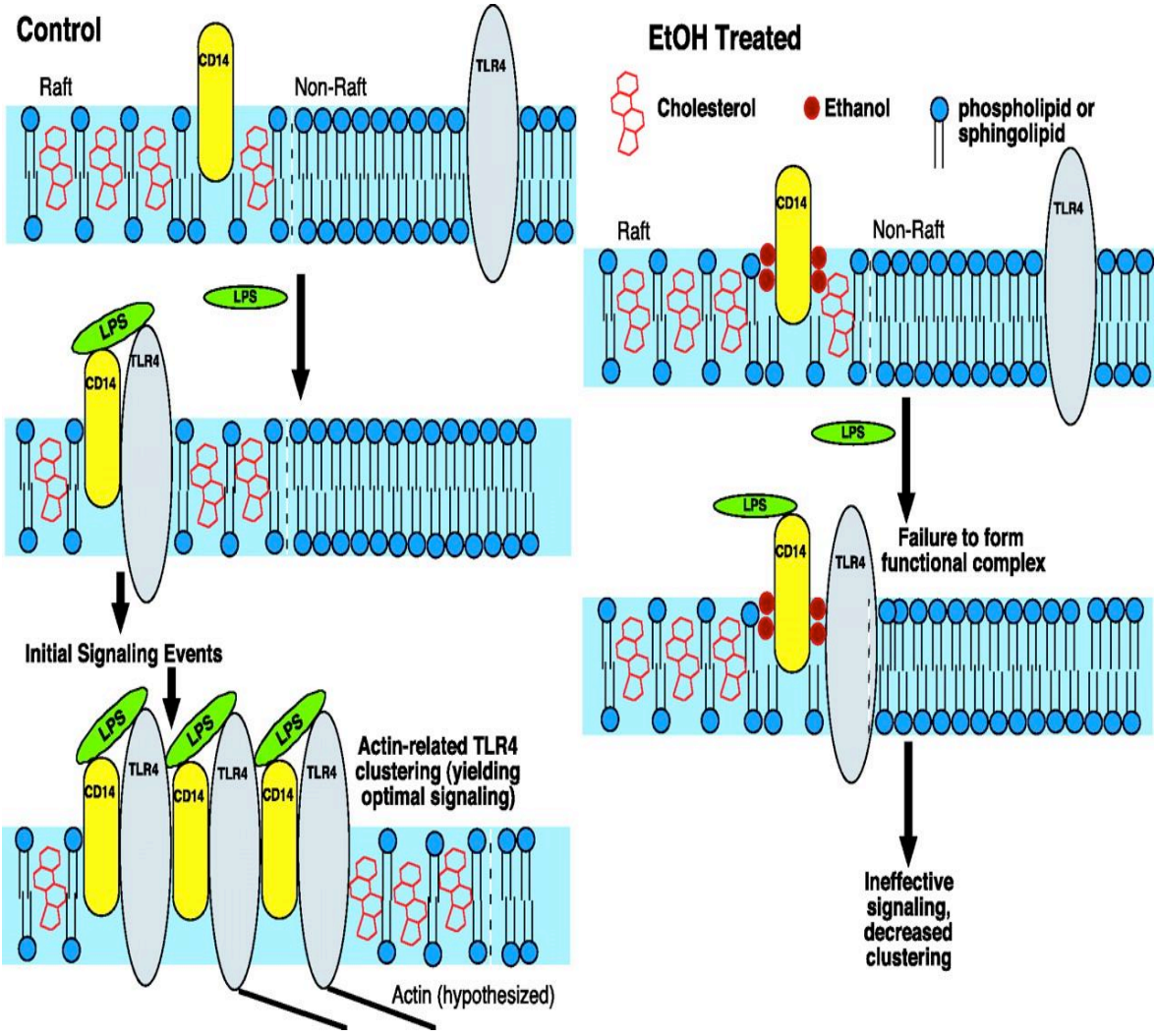

Figure 1.3 TLR4, ethanol, and lipid raft (by Szabo G et al, J Immunol. 2007)

1.5 Cromoglycate drugs

The 'cromoglycate-like' anti-allergic drugs (cromolyns) are a group of compounds of which sodium cromoglycate and sodium nedocromil are the exemplars. The family also embraces lodoxamide, traxanol and amlexanox as well as some H1 antagonists such as ketotifen, azelastine, pemirolast and olopatidine, many of which appear to share a similar pharmacology with cromoglycate (E.B. Cook, 2002). Contemporary reviewers are unanimous in attributing the anti-asthmatic activity of the cromoglycate-like drugs to their anti-inflammatory properties (P.J. Barnes, 1993; R.M. Viscardi, 1997; E.R. Bleecker, 1996), although the exact mechanism of action of this group of drugs has proved elusive. Early experiments (J.S. Cox, 1967; J.S. Cox and R.E., 1970a,b,c; T.S. Orr, 1989) led to the concept that these drugs acted mainly on mast cells to suppress mediator release and to stabilize membrane lipid polarity, but the balance of evidence now suggests that this is unlikely to be their only clinically significant action and that mast cells are not their sole target.

It is more likely that they work by inhibiting the response of sensory C fibres to the irritant capsaicin, inhibiting local axon reflexes involved in asthma, and may inhibit the release of preformed T cell cytokines and mediators involved in asthma (Garland, 1991). They may also inhibit chloride channels (Heinke S, 1995) and thus may inhibit the: 1) exaggerated neuronal reflexes triggered by stimulation of irritant receptors on sensory nerve endings (e.g. exercise-induced asthma); 2) release of preformed cytokines from several type of inflammatory cells (T cells, eosinophils) in allergen-induced asthma. Finally they may act by inhibiting calcium influx. Of particular relevance on cromoglycate

mechanism of action is the observation that it can also suppress eicosanoid generation (S. Mattoli, 1990; T. Radeau, 1993). There have been several previous attempts to link cromoglycate action to activation of signaling pathways and modification of potential down-stream molecular targets. Treatment of mast cells with cromoglycate results in the phosphorylation of (at least) four intracellular protein substrates including the erythrocyte band 4.1 group protein moesin (C. Theoharides, L., 2000; T.C. Theoharides, 1980) and there have been scattered reports of an interaction between cromoglycate and PKC (S.K. Bansal, 1997; A.M. Lucas and S. Shuster, 1987; R. Sagi-Eisenberg, 1985). However, most researchers seem to have considered that these drugs inhibit, rather than stimulate, this enzyme, although differing experimental protocols and crucially, timing, obscure clear interpretation of this issue.

There have also been previous reports (Y. Oyama, 1997; T. Shishibori, 1999), of a link between the cromoglycate and the annexin system in that these drugs have an affinity for some S100 proteins that are intracellular binding partners for members of the annexin family of proteins.

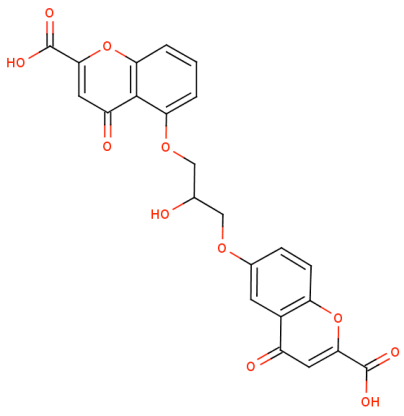

Figure 1.3 Salt sodium of cromoglycate.

1.6 Aims

Precisely how chronic and acute alcohol abuse alters human innate immunity thus causing immune dysfunction remains unclear. Having more information on this topic is important in designing new therapeutic strategies for improving immune responses to pathogens, tumours and vaccinations in alcohol abusers.

The first aim of this thesis was to clarify the immunological effects exerted by alcohol abuse on human monocyte-derived DCs. For this purpose we investigated whether in vivo chronic alcohol exposure alters the ability of monocytes from alcoholics to differentiate and mature in vitro into functional DCs. To extend current information on direct alcohol-induced changes in DCs, we evaluated whether in vitro acute EtOH treatment of differentiated immature DCs (iDCs) generated from chronic alcoholics and healthy control subjects impairs DC maturation. Using immunochemical and cytofluorimetric analysis we determined the phenotype and functions of DCs and analyzed the ability of iDCs to respond to the microbial product LPS. We also wanted to find out whether EtOH treated LPS-stimulated DCs correctly primed naïve T lymphocytes, thus inducing Th1 cell polarization.

The second aim of this thesis was to design a new therapeutic strategy as a possible approach in the prevention of alcohol's adverse effects on the immune system and in the improving the alcoholics' resistance to infections. For this purpose we investigated whether cromoglycate-like' anti-allergic drugs -- by their well established property to stabilize membrane lipid polarity -- might exert in vitro an adjuvant effect on monocytes and DCs in the well-established model of DC differentiation and maturation occurring in the presence of EtOH.

Chapter 2 Materials and Methods

2. Materials and Methods

2.1 Antigen and drug

Phenol-purified lipopolyssaccharyde (LPS, strain 0111:B4 Escherichia coli) and cromoglycate drug was purchased from Sigma-Aldrich (Milan, Italy).

2.2 Blood donors

The procedures for enrolment and study protocols were fully approved by the institutional review board of the Sapienza University of Rome. Seven alcohol-dependent patients (five men and two women; aged 18-60 years) consecutively admitted for treatment to the Alcohologic Reference Center for the Latium Region, Sapienza University of Rome, were eligible for participation. To be included patients had to be drinking more than 90 g of EtOH/day until the day of study entry, and to be constantly negative for hepatitis B surface antigen and antibodies to hepatitis C virus and to human immunodeficiency virus. Exclusion criteria were polydrug abuser patients with a diagnosis of chronic liver disease confirmed by clinical and ultrasonographic studies, and patients with a diagnosis of malnutrition confirmed by anthropometric measurements according to published criteria (Bishop et al, 1981). Blood samples from seven sex- and age-matched healthy blood donors from the Transfusion Center at the Sapienza University of Rome, were used as controls. Control subjects had no previous liver disease, ongoing infection or autoimmune processes and consumed less than 15 g of ethanol/day. Written informed consent was obtained from patients and controls, and the study was conducted in accordance with the Helsinki Declaration of 1975 and 1983.

2.3 Generation of DCs and T lymphocytes

Dendritic cells were generated from peripheral blood monocytes, as described previously (Buttari et al, 2005). In brief, PBMCs were isolated by density gradient separation (Lympholyte, Cedarlane Oxford, UK). $CD14^+$ monocytes were purified by incubation with anti-CD14-coated microbeads (Miltenyi Biotec Belgish, Gladbach, Germany), followed by sorting with a magnetic device (MiniMacs Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were incubated for 5 days in RPMI 1640 supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin (GIBCO, Life Technologies, Paisley, UK), 5×10^{-5} M 2-mercaptoethanol (Merck Milano, Italy) and 10% Fetal bovine Serum (Hyclone Laboratories Logan, UT) containing 100 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF; R&D System MN, USA) and 1000 U/ml of recombinant human interleukin 4 (rhIL-4; R&D System) to obtain iDCs. iDCs untreated and treated with 0.5, 10, 25, 50, or 100 mM EtOH (Merck), were cultured for 18 hours with or without 100 ng/ml of phenol-purified LPS. Phenotypic DC maturation was assessed by flow cytometric analysis. $CD4^+$ T cells were purified from PBMCs by negative selection using the untouched $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Negatively selected $CD4^+$ T cells were depleted of $CD45RO^+$ cells using anti-CD45RO-coupled magnetic beads and LD negative selection columns (Miltenyi Biotec) to obtain negatively-selected $CD45RA^+$ T cells. The purity of negatively selected $CD4^+CD45RA^+$ cells was analyzed by direct staining for membrane expression of CD45RA and of CD4 using phycoerythrin (PE)-conjugated monoclonal antibody (mAb) to CD45RA and fluorescein isothiocyanate (FITC)-conjugated mAb to CD4 (BD-Biosciences, San Josè, CA, USA). A portion of the negatively selected $CD4^+CD45RA^+$ T cells was cryopreserved for later use in T cell priming experiments.

2.4 DC phenotype

For flow-cytometric analysis of surface molecule expression, DCs were harvested, and 2 x 10^5 cells per sample were resuspended in phosphate-buffered saline (PBS). Cells were then incubated with saturating concentrations of the various fluorochrome-conjugated mAbs for 30 min at 4°C. The stained cells were washed twice in PBS and analyzed by flow cytometry on a FACSCanto using CellDIVA or CellQuest software (BD-Biosciences). The following mouse anti-human mAbs were used: PE-conjugated mAbs to CD1a, CD80, CD86, CCR5 and HLA-DR, and FITC-conjugated mAbs to CD14, CD83, CCR7 and CD40 (BD-Biosciences). Typically, iDC cultures contained > 90% $CD1a^+$ $CD14^-$ cells. Flow cytometric results were expressed as median ± SD of the positive cell percentages (CD1a, CD14, CD83, CCR5 and CCR7) and of the mean fluorescence intensity (MFI) (CD80, CD86, CD40 and HLA-DR). Apoptosis was determined by ApoAlert Annexin V-FITC Apoptosis Kit (annexin V-FITC with propidium iodide) according to the manufacturer's recommendations (Clontech Laboratories Palo Alto, CA, USA).

2.5 DC endocytosis

Because DCs lose their high endocytic activity during maturation, we assessed the effect of in vivo and in vitro alcohol exposure on mannose receptor-mediated endocytosis measured as the cellular uptake of FITC-dextran and quantified by flow cytometry. Cells ($2x10^5$ per sample) were incubated in RPMI 1640 medium with the addition of FITC-dextran (1 mg/ml; molecular mass 40.000, Sigma) for 30 min at 37°C or at +4° C (for control binding). After incubation, DCs were washed twice with PBS and fixed with 1% formaldehyde. At least $5x10^3$ cells per sample were analyzed.

2.6 DC migration

We investigated the ability of DCs to migrate in response to the chemokines MIP-1α/CCL3 (a CCR5 ligand) and MIP-3β/CCL19 (a CCR7 ligand) (R&D System). The chemokines were diluted in migration media RPMI 1640 + 0.5 % wt/vol BSA and 600 µl placed in each well for measurement of chemotaxis. Transwells (Costar, Cambridge, MA) with 8 µm polycarbonate membrane pore size were loaded with 100 µl DCs ($1x10^5$ cells/well), placed in the 24-well plates, and incubated in a humidified atmosphere of 5% CO_2 in air for 3 hours at 37°C. The numbers of migrating DCs harvested from the lower chambers were counted by FACS (60 second counts).

2.7 DC cytokine production

Because phenotypic DC maturation increases cytokine production, we determined by ELISA the cytokine content in stimulated and unstimulated DC culture supernatants collected at 18 h. Levels of IL-12p70, TNF-α, IL-10 and IL-6 were determined by ELISA (OptEIA kits; BD-Biosciences) following the manufacturer's instructions. The limits of detection were as follows: IL-10, TNF-α: 16 pg/ml; IL-12p70: 7.8 pg/ml; IL-6: 2.2 pg/ml.

2.8 DC allostimulatory ability

Because features of DC function in vivo are critical for antigen presentation and T cell activation, we evaluated the allostimulatory ability of stimulated and unstimulated DCs in a standard mixed lymphocyte reaction (MLR). Although this assay is not specific for a given antigen, it provides adequate information on the overall antigen-presenting function of DCs. The ability of DCs to stimulate allogeneic T cells was assessed and irradiated DCs (30 Gy) were used as stimulator cells. Allogeneic T cells ($1x10^5$ cells/well) were incubated with the irradiated DCs for 3 days at a different responder/stimulator ratios (1:4 to 1:32 DC:T) in a 96-well round bottom plate. On day 2, 0.5 µCi/well ^{3}H-methyl-thymidine (Amersham, Life Science, Milan, Italy) was added to each well. After a further 18 h at

37°C, cells were harvested on glass fiber filter paper (Wallac, EG&G Company, Turku, Finland), using an automatic cell harvester (Harvester 96, MACH III M, TOMTEC Orange, CT, USA). ^{3}H-methyl-thymidine uptake into cell DNA was measured by reading samples in a β counter (1450 Microbeta Plus, Wallac). Net counts per minute (cpm) of triplicate cultures were measured. Supernatants from the MLR samples were tested for IFN- γ production by ELISA. The limit of detection for IFN- γ was 3 pg/ml.

2.9 Nuclear factor-κB (NF-κB) translocation

The NF-κB p65 transcription factor assay kit (Active Motive Carlsbad, CA, USA) was used to monitor NF-κB activation. Unstimulated DCs and DCs stimulated for 45 min at 37°C, in 5% CO_2 with EtOH (0.5, 10, 25 and 50 mM) and LPS (100 ng/ml) were lysed, protein was quantified, and equal amounts of lysates were used to test activated levels of p65 subunits with the antibodies directed against the subunits bound to the oligonucleotide containing the NF-κB consensus binding site. As a positive control, we used a HeLa cell extract, and to monitor the specificity of the assay, we used NF-κB wild-type and mutated consensus oligonucleotides, according to manufacturer's instructions.

2.10 T-cell priming assay

To find out whether EtOH-treated DCs primed naïve T lymphocytes, negatively selected naïve allogeneic T cells were cultured with EtOH-treated DCs at a ratio of 20:1. LPS-matured DCs were used as positive control to prime IL-4- or IFN-γ-expressing T cells. Activated T cells were expanded for 10 days with recombinant IL-2 (30 U/ml; Roche Molecular Biochemicals Indianapolis Indiana, USA), added on day 5, in a 24-well plate in complete medium to obtain polyclonal T cell lines to be analysed for IL-4 and IFN-γ expression, by flow cytometry. In brief, 10^{6} cells were stimulated with 10^{-7} M phorbol 12-myristate 13-acetate plus 1 μg/ml ionomycin for 4 h in the presence of 10 μg/ml brefeldin

A (all reagents Sigma Aldrich). Cells were labelled with anti-CD3 PerCP (BD-Biosciences) (5 μl/10^4 cells, 30 minutes on ice), and then cells were fixed with FACS lysing solution, treated with FACS permeabilizing solution (BD-Biosciences), stained with a predetermined optimal concentration of anti-cytokine mAb or appropriate isotype mAb control and analyzed on a FACSCanto (BD-Biosciences). Variables evaluated were the pattern of cytokine expression on the $CD3^+$ population. Cells were gated according to light scatter properties to exclude cell debris. A minimum of 10,000 viable cells was analyzed for each sample. Results were processed using Diva software (BD-Biosciences).

2.11 Statistical Analysis

Median and range, as well as the 25^{th} and 75^{th} percentiles, were calculated for each variable under study. Unless otherwise stated all values are expressed as mean ± SD. All the statistical procedures were performed by STATA 8.1 statistical package. The Mann-Whitney U and Kruskal-Wallis non-parametric tests were used to evaluate the statistical significance of intergroup differences in all the tested variables. P values < .05 were considered statistically significant.

Chapter 3 Results

3. Results

3.1 In vivo chronic alcohol exposure impairs in vitro monocyte differentiation to DCs

Flow cytometry analysis showed that whereas healthy control monocytes cultured with GM-CSF and IL-4 differentiated to the typical immature DC phenotype ($CD1a^+$, $CD86^+$, $CD80^+$, $CD40^+$, HLA-DR^+, $CD83^-$) (control-iDCs), monocytes obtained from alcoholics differentiated to an altered immature DC phenotype (alc-iDCs) (Fig. 1A, 1B). Alc-iDCs contained fewer CD1a-immunoreactive cells than control-iDCs (P = .0012 by Mann-Whitney non parametric tests, n = 7 experiments) (Fig. 1A). They also expressed CD86 more weakly than control iDCs (P = .0040) and showed significantly higher HLA-DR expression (P = .0060) (Fig. 1C).

Flow cytometric analysis of CCR5 and CCR7 chemokine receptor expression detected similar CCR5 levels in control- and alc-iDCs (MFI: 25±15 and 20±15). Neither control iDCs nor alc-iDCs expressed CCR7 (data not shown). As chemokine receptor expression analysis suggested, alc-iDCs and control-iDCs both migrated in response to MIP-1α/CCL3 and did so in similar numbers. Conversely, neither alc-iDCs nor control-iDCs migrated in response to MIP-3β/ CCL19 (Fig. 1D).

In flow cytometry investigating antigen endocytosis, alc-iDCs took up significantly smaller amounts of FITC-dextran antigen than control-iDCs (MFI: 2235±502 vs 2875± 547, P = .042, n = 7 experiments) (data not shown).

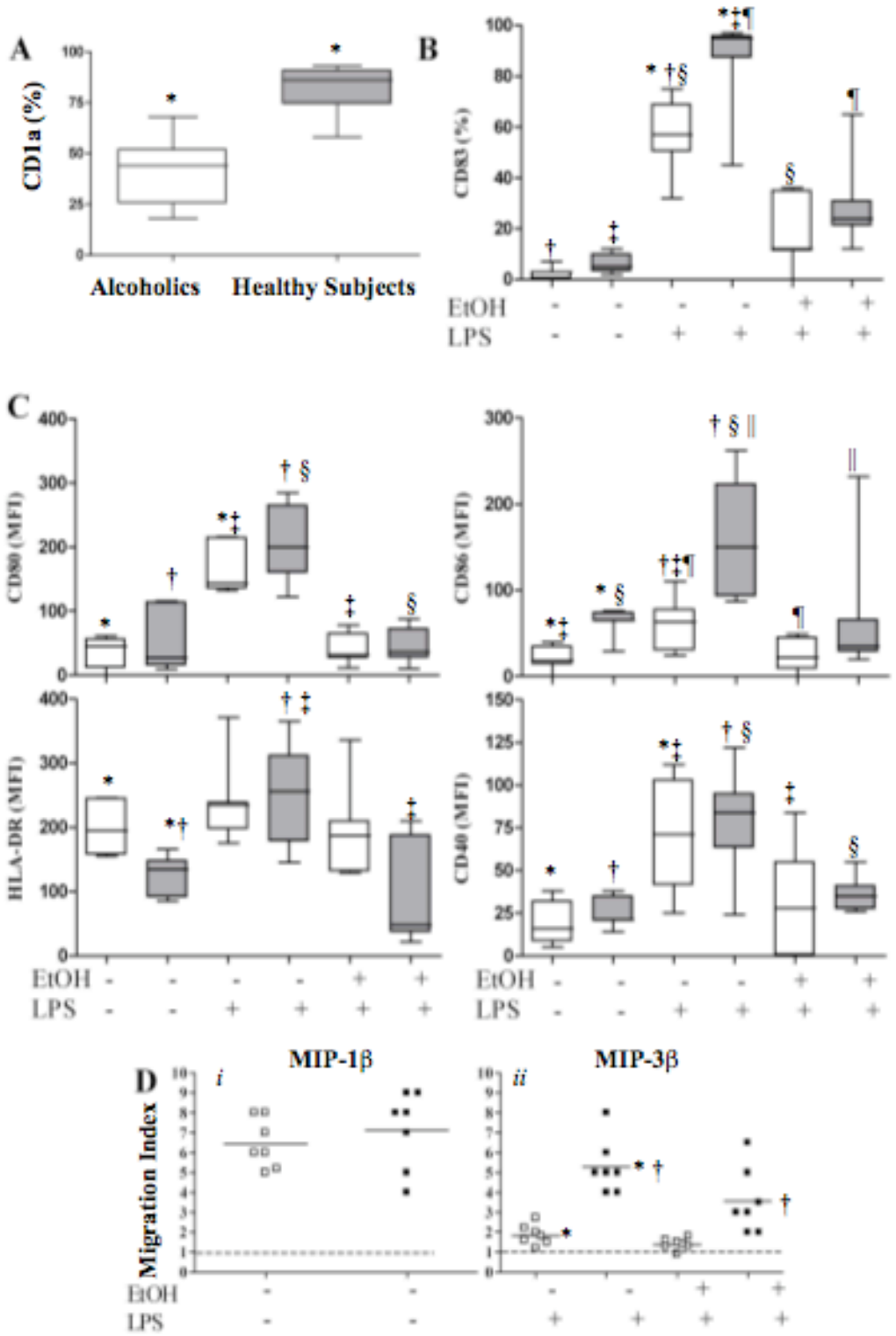

Fig. 3.1. In vivo and in vitro alcohol exposure impairs the ability of monocytes to differentiate and mature into functional dendritic cells (DCs). Human immature DCs (iDCs) were generated from alcoholics (□) and from healthy blood donors (■). Five-day

iDCs were cultured with or without LPS (100 ng/ml) in the presence or not of EtOH (25 nM). DC surface molecule expression and migratory capacity were analyzed by flow cytometry, as described in the "Materials & Methods" section. (A) The appearance of DC differentiation marker CD1a (* P = .0012) was analyzed in five-day iDCs generated from alcoholics and healthy blood donors. (B) The appearance of DC maturation marker CD83 (*†‡P = .0180, § P = .0023, ¶ P = .0012), (C) the upregulation of costimulatory and activation molecules CD80 (*† P = .0180, ‡ § P = .0006), CD86 (* P = .0040, † P = .0060, ‡ § P = .0180, ¶ P = .0111, || P = .0175), HLA-DR (* P = .0060, † P = .0180, ‡ P = .0111) and CD40 (*† P = .0180, ‡ P = .0379, § P = .0262) were analyzed after 18 hours of culture. Results are expressed as positive cell percentages (A, B) and mean fluorescence intensity (MFI) (C) of seven independent experiments. Notched boxes represent 25th and 75th percentile values; the horizontal lines and the vertical lines correspond to the median value and the 10th and 90th percentiles. (D) Migration of DC derived from alcoholics (□) and from healthy blood donors (■) towards the chemotactic stimuli MIP-1β and MIP-3β. (*† P = .0006). The horizontal lines correspond to the arithmetic means.

Samples were analyzed on a FACSCanto cytofluorimeter using CellDIVA software (BD Biosciences). P values by the Mann-Whitney test.

3.2 In vivo chronic alcohol exposure impairs in vitro full DC maturation in response to LPS

Experiments investigating whether in vitro LPS-induced DC maturation overcame the iDC defects related to chronic alcohol exposure detected a significantly lower percentage of CD83-immunoreactive cells and significantly weaker CD86 expression in LPS-stimulated alc-iDCs (alc-DCs) than in LPS-stimulated control-iDCs (control-DCs) (CD83: P = .0178; CD86: P = .0060 by Mann-Whitney non parametric tests, n = 7 experiments) (Fig. 1B, 1C). CD83-immunoreactive cells were nevertheless more numerous (Fig. 1B) and CD80, CD86 and CD40 expression was stronger in alc-DCs than in alc-iDCs (CD83 P = .0180; CD80 P = .0180; CD86: P = .0178; CD40 P = .0180) suggesting their partial maturation (Fig. 1C).

Chemokine receptor analysis showed that CCR7 was expressed in smaller amounts in alc-DCs than in control-DCs (MFI: 46±20 vs 23±18 P = .043) (data not shown). In accordance with this finding, fewer alc-DCs than control-DCs migrated in response to MIP-3β/CCL19 (number of migrated cells: 20±13 vs 48±13 P = .009, n = 7 experiments) (Fig. 1D).

Flow cytometric analysis of antigen endocytosis showed that alc-DCs and control DCs took up similar amounts of FITC-dextran antigen (data not shown).

3.3 In vitro ethanol treatment impairs full DC maturation

In alc-iDCs and control-iDCs, in vitro alcohol exposure further impaired final LPS-stimulated DC maturation (Fig. 1). In dose-response experiments the lowest EtOH dose able to impair control-iDC maturation was 25 mM, equivalent to a blood alcohol level of 0.1 g/dl (data not shown). None of the EtOH doses tested induced cytotoxicity as determined by staining apoptotic/necrotic cells with annexin V and propidium iodide (data not shown).

When LPS-stimulated iDCs were treated with EtOH during maturation, the number of CD83-immunoreactive cells resulted lower in alc- and control-DCs than in EtOH-untreated LPS-stimulated DCs (alc-DCs: P = .0023; control-DCs: P = .0012) (Fig. 1 B) and so did CD80, CD86, HLA-DR and CD40 expression (alc-DCs: CD80 P = .0006; CD86 P = .0175; CD40 P = .0379; control-DCs: HLA-DR P = .0111, CD40 P = .0262) (Fig. 1C).

When LPS-stimulated control- and alc-iDCs were treated with EtOH, CCR7 expression_was not significantly up-regulated (data not shown). Accordingly, in vitro EtOH treatment reduced the ability of control-DC to migrate in response to MIP-3β/CCL19. The reduced number of migrating control-DCs matched numbers in alc-DCs (P ≤ .0385) (Fig. 1D).

In flow cytometry investigating antigen endocytosis, alc-DCs and control-DCs treated with EtOH during LPS maturation retained the ability of iDCs to internalize FITC-dextran (alc-DCs: P ≤ .0389; control-DCs: P ≤ .0389) (data not shown).

3.4 Chronic and acute alcohol exposure impairs cytokine production by DCs

In ELISA to detect cytokine production in culture supernatants, alc-iDCs produced significantly larger amounts of TNF-α and IL-6 than control-iDCs (TNF-α: P = .031 and IL-6: P = .0181) (Fig. 2A). When alc- and control-iDCs were stimulated with LPS, the production of all cytokines increased but alc-DCs produced significantly lower amounts of IL-12p70 and TNF-α than control-DCs (P = .0350 and P = .0253). Alc-iDCs stimulated with LPS and treated with EtOH produced significantly lower amounts of IL-12p70 and IL-10 and higher levels of IL-6 than EtOH-untreated alc-DCs (P = .0111, P = .0041 and P = .0041). TNF-α production remained unchanged. Control iDCs stimulated with LPS and treated with EtOH produced lower amounts of IL-12 and TNF-α and higher amounts of IL-6 than EtOH-untreated control DCs (P = .0012 P = .0023 and P = .0006).

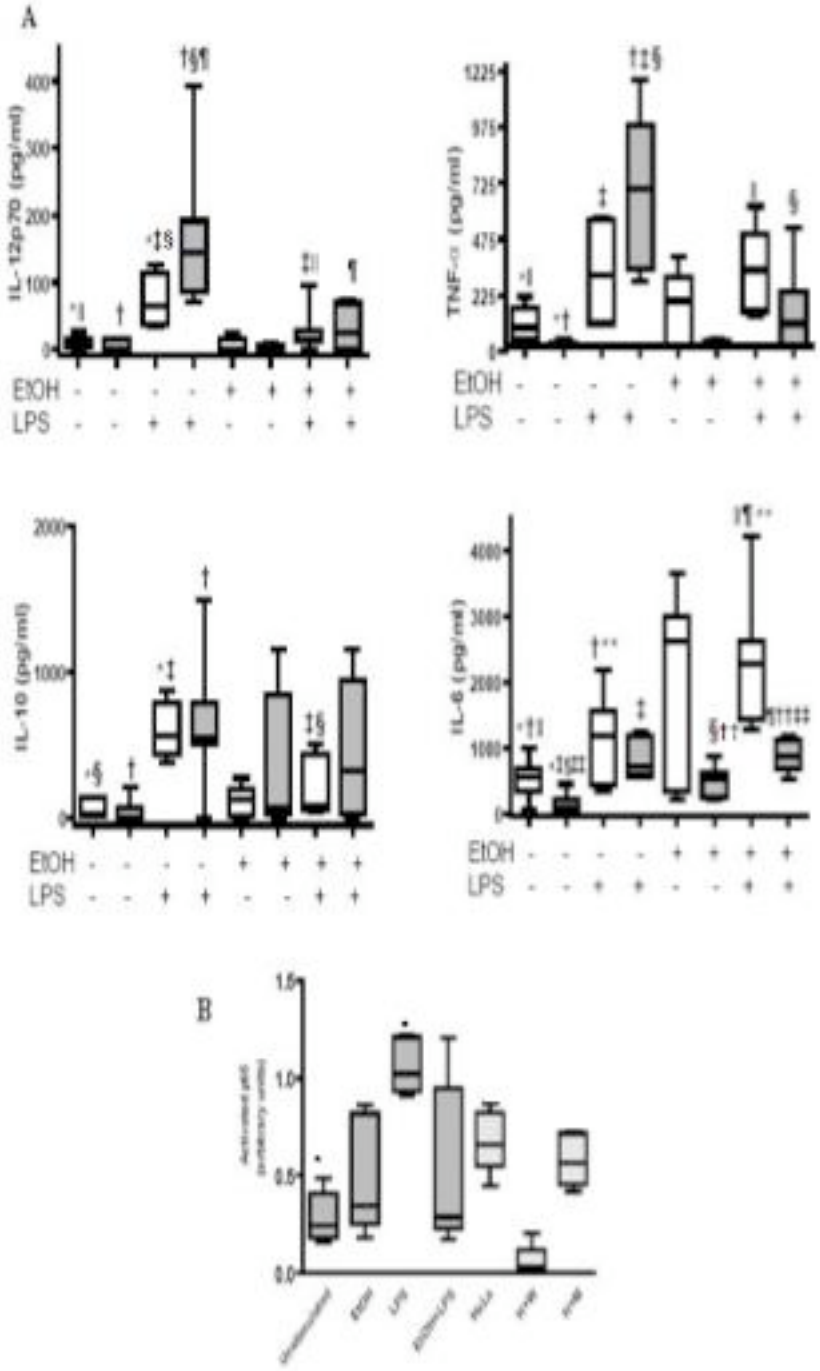

Fig. 3.2. Alcohol exposure impairs DC cytokine production and NF-kB nuclear translocation. (A) Alc-iDCs (□) and control-iDCs (■) were cultured with or without LPS (100 ng/ml) and EtOH (25 nM) or left unstimulated. Supernatants were collected after 18 hours to measure IL-12p70, TNF-α, IL-10 and IL-6 by specific enzyme linked immunosorbent assay (ELISA) experiments. Notched boxes represent 25th and 75th percentile values; the line in the middle and the vertical lines correspond to the median

value and both the 10th and 90th percentiles. (IL-12p70: * † P = .0180; ‡ P = .0111; § P = .0350; || P = .0280; ¶ P = .0012; TNF-α: * P = .0310; † P = .0180; ‡ P = .0253; § P = .0023; || P = .0425; IL-10: * P = .0180; † P = .0280; ‡ P = .0041; § P = .0343; IL-6: * ‡§|| P = .0181; † P = .0280; ¶ P = .0017; **P = .0041; ††‡‡ P = .0006. (B) Control-iDCs (■) were stimulated with or without LPS (100 ng/ml) and EtOH (25 nM) or left unstimulated for 45 minutes. Cellular extracts were obtained after cell lysing, protein quantified, and equal amounts of lysates were used to test activated levels of the p65 subunit with antibody directed against the subunits bound to the oligonucleotide containing the NFkB consensus binding site. A HeLa cell extract (□) was used as a positive control alone or in the presence of wild-type or mutated consensus oligonucleotide. Notched boxes represent 25th and 75th percentile values; the line in the middle and the vertical lines correspond to the median value and both the 10th and 90th percentiles. (* P = .0043; †‡ P = .0087; n = 5 experiments). P values by the Mann-Whitney test.

3.5 Chronic and acute alcohol exposure impairs NF-κB nuclear translocation in LPS-stimulated iDCs

LPS-stimulated control-iDCs showed significant massive NF-κB p65 nuclear translocation (P = .0043 by Mann-Whitney non parametric tests, n = 5 experiments). EtOH added to LPS-stimulated control iDCs inhibited NF-κB p65 nuclear translocation (Fig. 2B). The assay was specific, because incubating a HeLa extract with an unbound wild-type consensus oligonucleotide abolished binding of both subunits, whereas incubating the HeLa extract with a mutated consensus oligonucleotide left NFκB binding unchanged (P = .0087).

3.6 Chronic and acute alcohol exposure impairs DC allostimulatory function

When we investigated the antigen-presenting function of iDCs by testing these cells in an MLR, alc-iDCs induced significantly lower allogeneic T-lymphocyte proliferation than control iDCs (at 1:4, 1:8 and 1:16 DC/T cells ratios P ≤ .0027) (Fig. 3A). Experiments investigating whether in vitro LPS-induced DC maturation overcame the iDC defective allostimulatory functions related to chronic alcohol exposure showed that alc-DCs induced significantly lower allogeneic T-lymphocyte proliferation than control DCs (at all calculated DC/T cell ratios P ≤ .0040) (Fig. 3A). When EtOH was added in vitro during DC maturation it further reduced the degree of alloantigen-induced T cell proliferation in LPS-stimulated alc-iDCs (at 1:8, 1:16 and 1:32 DC/T cells ratios P ≤ .0280) and impaired the allostimulatory ability of LPS-control-DCs (at 1:8, 1:16 and 1:32 DC/T cells ratios P ≤ .0280) (Fig. 3A).

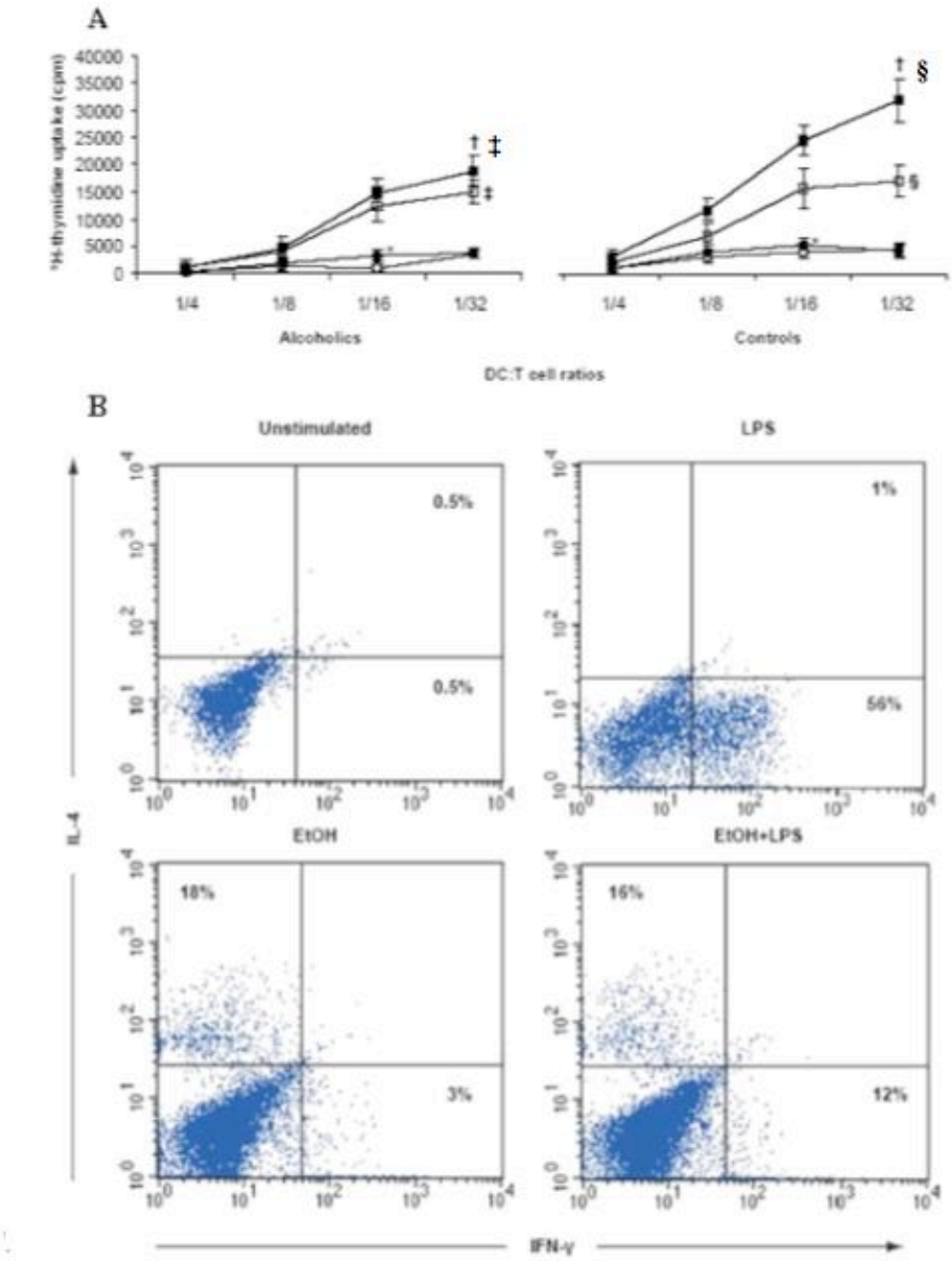

Fig. 3.3 Alcohol exposure impairs allostimulatory function of DCs and prevents them from correctly priming and polarizing naïve allogeneic T cells. (A) Five-day human DCs were stimulated with LPS (100 ng/ml) (■), EtOH (25 nM) (●), LPS plus EtOH (□) or left unstimulated (❍). After 18 hours, DCs were extensively washed and cultured with allogeneic T lymphocytes (1 x 105 cells/well) for 3 days at various stimulator-responder ratios (1:4 to 1:32 DC/T). Proliferation of allogeneic T cells was measured by 3H-methyl-

thymidine incorporation. Data are presented as mean cpm ± SD of seven independent experiments. (at 1:4, 1:8 and 1:16 DC/T cell ratio, alc-iDCs vs control-iDCs: * P = .0027; at all DC/T cell ratio, alc-DCs vs control-DCs: † P = .0040; at 1:8, 1:16 and 1:32 DC/T cell ratio, EtOH-treated alc-DCs vs alcohol-DCs and EtOH-treated control-DCs versus control-DCs: ‡ § P = .0280). P values by the Mann-Whitney test. (B) DCs derived from healthy subjects were stimulated with LPS (100 ng/ml) and/ or EtOH (25 nM) or left unstimulated for 18 hours. A total of 5 x 104 DCs were used to stimulate 1 x 106 allogeneic naïve negatively selected CD4+CD45RA+ T cells. Activated T cells were expanded with rhIL-2 (30 U/ml). On day 10, T-cell lines were stimulated with PMA and ionomycin for 4 hours in the presence of brefeldin A. Cells were stained with anti-hu-CD3PerCP and processed for intracellular labelling with anti-hu-IFN-γ-FITC and anti-hu-IL-4-PE. The numbers show the percentage of activated CD3+ cells producing the cytokine. Samples were analyzed on a FACSCanto cytofluorimeter using CELLDiva software (BD Biosciences). The figure shows a representative experiment from five with similar results.

3.7 In vitro ethanol treatment prevents DCs from correctly priming and polarizing naïve allogeneic T cells

In cell culture experiments designed to find out whether EtOH added in vitro during DC maturation impaired the ability of DCs to prime and polarize allogeneic naïve CD4+ CD45RA+ into typical T helper type 1 (Th1) cells, cytofluorimetric analysis showed a smaller number of IFN-γ-producing T cells and a larger number of IL-4-producing cells in control-iDCs stimulated with LPS and treated with EtOH than in samples stimulated with LPS and EtOH-untreated (12% vs 56% and 16% vs 1%) (Fig. 3B). Most naïve T cells (18%) co-cultured with EtOH-treated control-iDCs turned into typical IL-4 producing T cells (Th2). Only a small percentage (3%) differentiated into IFN-γ-producing T cells.

3.8 Characterization of DCs differentiated from monocytes in the presence of cromoglycate and EtOH

Monocyte cultures induced to differentiate in immature DCs in the presence of cromoglycate and EtOH showed a clear-cut induction of marked expression of the CD1a antigen (evaluated as an increase in the positive cells and as an increase in the mean fluorescence intensity, control iDCs vs SCG plus EtOH: $p < .006$), which is considered a crucial receptor in the pathogen-derived glycolipid presentation by DCs to initiate antimicrobial responses (Fig. 4 A). The same cells showed also an up-regulation of costimulatory molecule CD86 compared to control iDCs (Fig. 4 B). These data indicate that cromoglycate treatment in the presence of EtOH, increases the number of differentiated iDCs and the CD86 expression. The irreversible commitment of cromoglycate-treated DCs to undergo an advanced differentiation process was suggested by the finding that, upon cromoglycate removal, cells retained a DC phenotype without adhering to the plastic surface, whereas EtOH–treated cells acquired a macrophage features and readily re-adhered to culture plates unless preventively stimulated to terminally

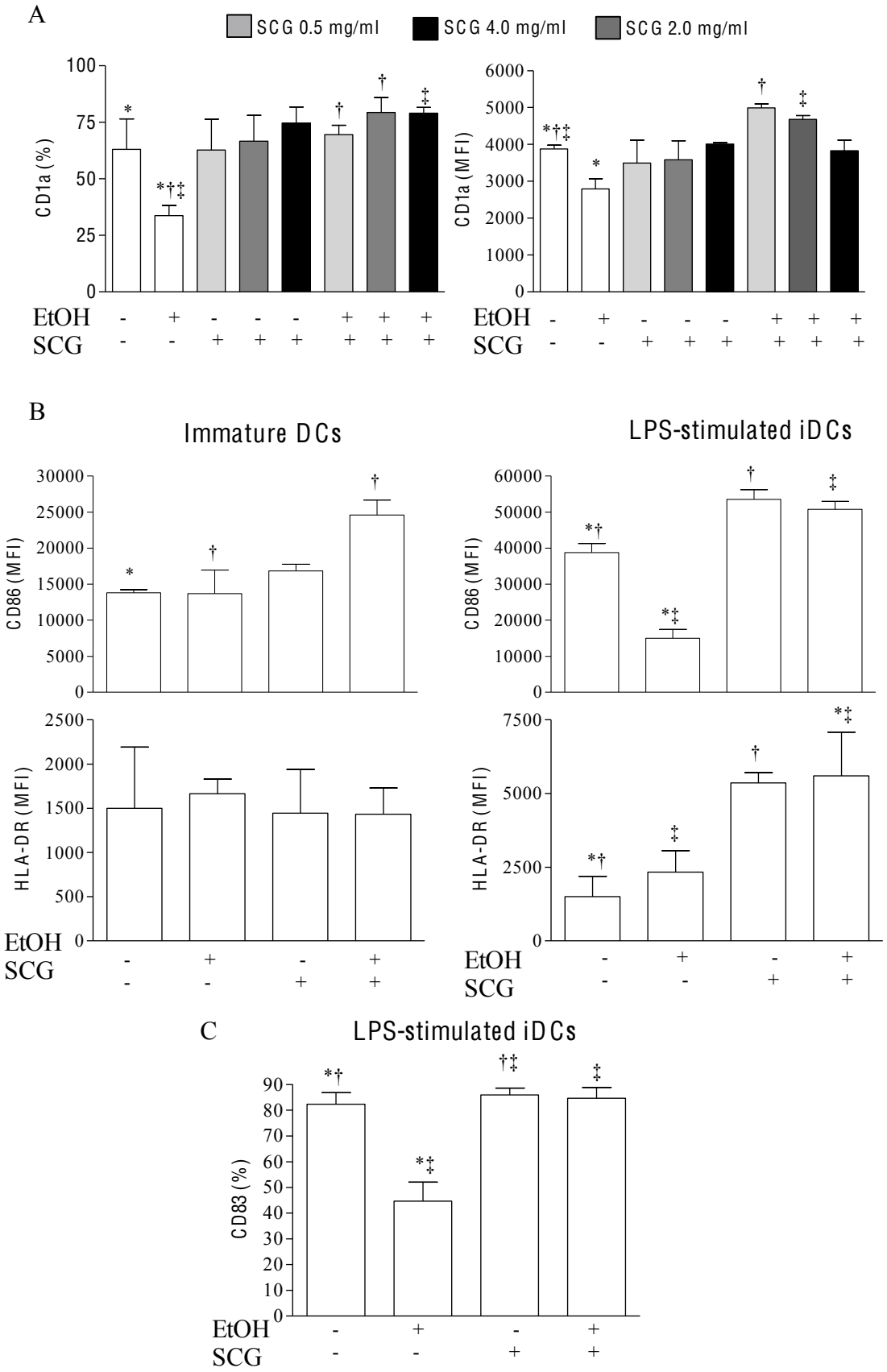

Fig. 4. In vitro cromoglycate effects on the expression of CD86 costimulatory molecule and HLA-DR on dendritic cells (DCs) induced to differentiate and mature in the presence of EtOH.

In vitro cromoglycate effects on the differentiation of human monocytes into DCs. Human monocytes were induced to differentiate in the presence of GM-CSF plus IL-4 in immature DCs with or without EtOH (25 mM), and cromoglycate (0.5; 2; 4 mg/ml). Afterwards the same cell samples were stimulated with LPS (100 ng/ml) to induce maturation for 18 hours. Expression of cell surface molecules was assessed by flow cytometry as described in the "Materials and methods" section. (A) In vitro cromoglycate induced a clear-cut induction of CD1a expression in a dose-dependent manner. Results are expressed as mean ± SD of the positive cell percentages from three independent experiments. Significant differences are indicated (*†P < .006; ‡ P < .03 by Student's paired t test). (B) The upregulation of costimulatory and activation molecules CD86 and HLA-DR by cromoglycate (2 mg/ml), with or without EtOH (25 mM), in iDCs and LPS-stimulated DC and (C) the appearance of DC maturation marker CD83. Results are expressed as mean fluorescence intensity (MFI) (B) and positive cell percentages (C) of three independent experiments. (*† P < .004; ‡ P < .03 by Student's paired t test). Samples were analyzed on a FACSCanto cytofluorimeter using CellDIVA software (BD Biosciences).

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differentiate by LPS (data not shown). Cromoglycate-treated DCs proved to be fully susceptible to undergo activation/terminal differentiation after LPS treatment, as revealed by the enhanced expression of costimulatory (CD86) and accessory (HLA-DR) molecule as well as by a massive CD83 induction (Fig. 4 B and C). Furthermore, in vitro cromoglycate exposure during DC differentiation restored the full iDC ability to respond properly to LPS stimulation, as showed by the up-regulation of CD86 and HLA-DR and the higher number of CD83+ DCs in comparison to cells pre-treated with EtOH alone before LPS stimulation (Fig. 4 C).

3.9 Functional activities of DCs in vitro generated from cromoglycate drug*-treated monocytes.*

We performed functional experiments aimed to evaluate the ability of cromoglycate to restore the proper functional activities of LPS-stimulated DCs when added during iDCs differentiation in the presence of EtOH. Allogeneic mixed leucocyte reaction (MLR) and IFN-production by allogeneic PBLs were chosen because, although they are not specific for a given antigen, it provides adequate information on the overall antigen-presenting function of DCs.

In an allogeneic MLR-experiments, we evaluated the proliferative response of allogeneic monocyte-depleted PBLs when co-cultured with different numbers of DCs generated in the presence of cromoglycate drug, EtOH or both and induced to mature by LPS. As shown in Fig. 5, at 1:20 DC/T cell ratio, iDCs obtained in the presence of cromoglycate drug and cromoglycate drug plus EtOH showed a better capability to simulate the proliferation of allogeneic lymphocytes than EtOH-treated iDCs or unstimulated iDC (Fig. 5Ai) ($P < .03$). A stronger proliferative response was observed in MLR experiments after LPS stimulation ($P < .006$; Fig. 5Ai). Of note, DCs generated from the same donor in the presence of EtOH and SCG elicited a higher proliferative response than DC generated in the presence of

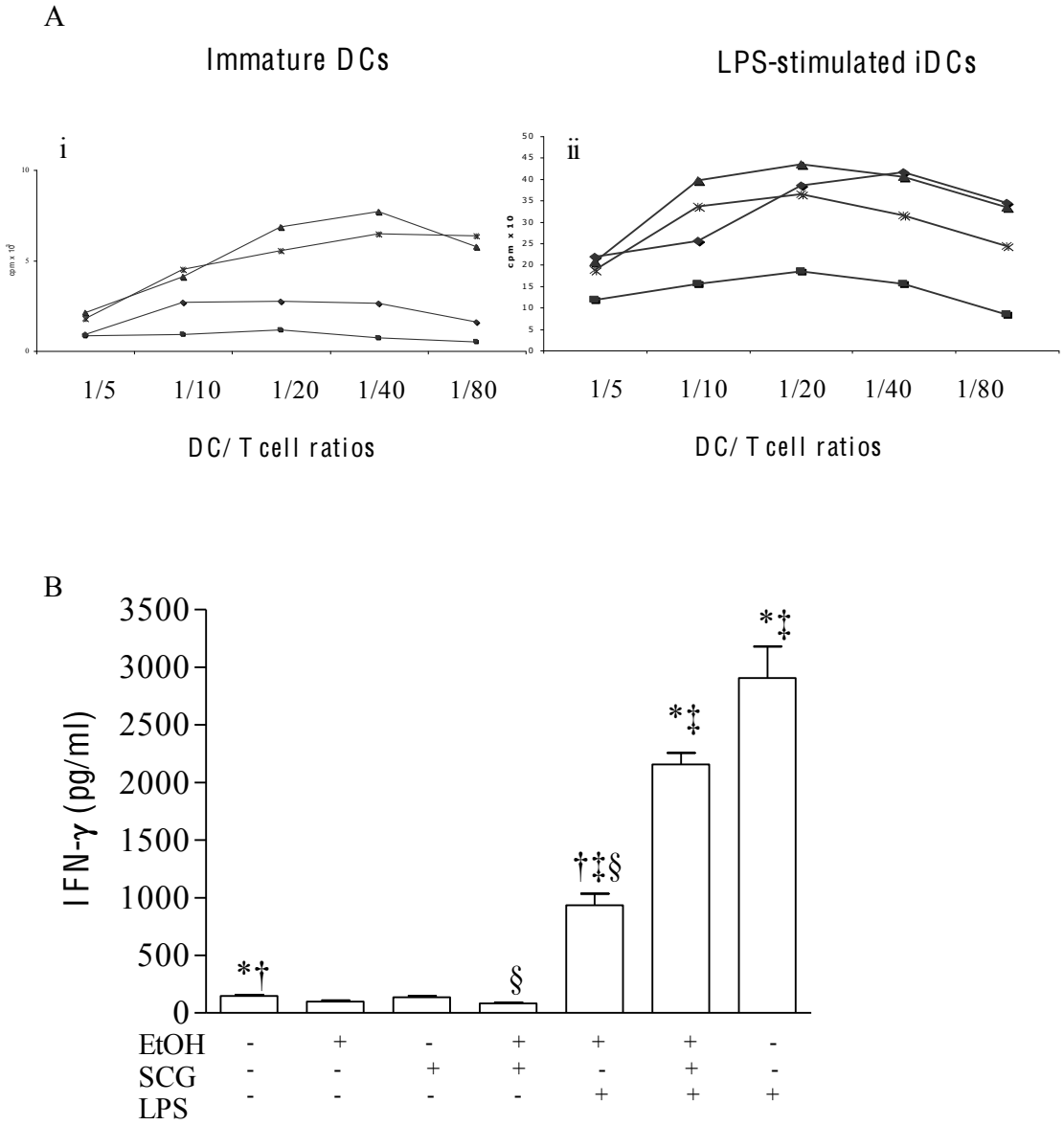

Fig. 5. In vitro cromoglycate effects on the ability of dendritic cells (DCs) to induce allogenic T cell proliferation and Th1 differentiation. (A, i) Five-day human immature DCs differentiated from monocytes in the presence of EtOH (25 nM) (■), cromoglycate (2 mg/ml) (▲) cromoglycate plus EtOH (✳) or left unstimulated (◆), (A, ii) afterwards the same cell samples were stimulated with LPS (100 ng/ml) for 18 hours to the induction of terminal maturation phenotype (LPS-stimulated DCs). Immature and LPS-stimulated DCs were extensively washed and cultured with allogeneic T lymphocytes (1 x 105 cells/well) for 3 days at various stimulator-responder ratios (1:5 to 1:80 DC/T). Proliferation of allogeneic T cells was measured by 3H-methyl-thymidine incorporation. Data are

presented as mean cpm ± SD of triplicate cultures. A representative experiment of three with comparable results is shown. (B) IFN-γ production in the supernatants from the MLRs was tested at the time of performing the lymphocyte proliferation assay. Allogeneic PBLs were stimulated with DC samples for three days (at 1:20 DC7/T cell ratio). Results are expressed as mean ± SD of three independent experiments. (*† $P < .004$; ‡ §$P < .03$ by Student's paired t test).

EtOH alone. This was expected, as DCs generated with EtOH and SCG exhibited a higher level of costimulatory molecules, as showed above (Fig. 1C). A specific feature of MLRs generated with mature DCs developed in the presence of cromoglycate drug plus EtOH was the considerable IFN-gamma production, which was definitively higher than that found in the corresponding co-cultures using LPS-stimulated iDCs generated in the presence of EtOH (Fig. 5 B), suggesting a prominent capability of cromoglycate drug DCs to promote and restore a Th1 response.

Chapter 4 Discussion

4. Discussion

In this study we provide new information showing that in vivo chronic alcohol exposure prevents monocytes from differentiating and maturing into functional DCs. We also provide in vitro evidence that acute ethanol treatment acts directly on differentiated iDCs generated from alcohol-dependent patients and healthy controls preventing them from maturing correctly in response to the microbial stimulus LPS. An unexpected finding was that in vitro EtOH–treated control iDCs turned them into typical Th2 cells. Our in vitro findings suggest that cromoglycate drug exerts an adjuvant effect on monocytes and DCs render lower susceptible to EtOH-induced alteration in phenotype and functions.

Immature DCs from alcoholics and healthy controls differed in both phenotype and function. Alc-iDCs typically exhibited lower percentages of CD1a-immunoreactive cells, weaker CD86 and stronger HLA-DR expression than control iDCs, impaired allostimulatory function (reduced MLR response) and reduced endocytic ability. Although the impaired allostimulatory function of alc-iDCs may in part reflect their weak CD86 and CD1a surface expression it contrasts with their strong surface HLA-DR expression.

Functional evidence of the impairment caused by chronic alcohol abuse on DC differentiation came in our study from the reduced endocytic ability of alc-iDCs. In line with our findings in DCs, a previous study has described defective monocyte phagocytic function in patients with alcoholic cirrhosis (Silvain et al. 1995). Alcohol may change cell membrane properties by disrupting protein-lipid interactions (Chiou et al, 2004). Whether in vivo chronic alcohol exposure affects the expression of the membrane mannose receptor or of other endocytic receptors is a question for further research.

Despite the phenotypical and functional impairments, alc-iDCs produced the proinflammatory cytokines TNF-α and IL-6 in large amounts. Increased alcohol-induced cytokine production in DCs agrees with the high circulating levels of proinflammatory cytokines found in alcoholics (Dominguez-Santalla et al. 2001; Germann et al. 1997).

Alcohol may contribute to proinflammatory activation of monocytes (Germann et al. 1997; RT Cook 1998; Hanna et al. 2000; González-Quintela et al. 2003) by regulating cytokine secretion at a post-transcriptional level and by increasing intestinal permeability to bacterial LPS. Alcohol may also impair monocyte-derived DCs through epigenetic alterations, such as acetylation, methylation of histones, and hypo- and hypermethylation of DNA (Shukla et al. 2006). The epigenetic effects arise mainly from EtOH-induced metabolic stress, generated by oxidative and non-oxidative EtOH metabolism, and dysregulated methionine metabolism.

Further information on DC impairment induced by in vivo chronic alcohol exposure came from our experiment investigating phenotypical and functional changes in DCs stimulated to mature by LPS. Microbial LPS stimulation of alc-iDCs only partially restored the alc-iDC impairment thus inducing a semi-mature phenotype (Thyssen et al. 2007) characterized by reduced CD83 and CD86 expression associated with low IL-12 and TNF-α production and with impaired allostimulatory function. This evidence suggests that chronic alcohol exposure prevents DC from differentiating and maturing thereby making them unable to sense properly and respond to LPS and possibly to other microbial stimuli. A similar conclusion was reached by Mandrekar et al, (Mandrekar et al. 2004) in iDCs obtained from normal volunteers after in vivo acute alcohol intake.

The impaired allostimulatory function of LPS-stimulated alc-iDCs may in part reflect their semi-mature phenotype characterized by reduced CD83 and CD86 expression associated with low IL-12 and TNF-α production. Whereas the functional relevance of the costimulatory molecule CD86 is well documented (AJ Coyle AJ and Gutierrez-Ramos JC. 2001), the precise functions of CD83 remain unknown (Scholler et al. 2001; Kruse et al. 2000). CD83 is a cell-surface membrane glycoprotein whose expression is largely restricted to DCs (Cao et al. 2005) and may have important roles during intercellular interactions (Kruse et al. 2000).

The decreased percentage of CD83-immunoreactive cells in LPS-stimulated alc-iDCs, associated with an impaired cytokine profile, may contribute to T-cell generation with altered activation and polarization (Waltenbaugh et al. 1998).

In accordance with their semi-mature phenotype, alc-DCs were less able than control-DCs to migrate in response to the chemokine MIP-3β/CCL19, a typical feature of mature DCs (Rao et al. 2004).

Several findings in our study provide evidence that in vitro acute alcohol exposure directly prevents DCs from maturing. For example, EtOH-treated-LPS-stimulated DCs typically exhibited the semi-mature phenotype characterized by a low percentage of CD83-immunoreactive cells and weak costimulatory molecule expression. These findings partially agree with the reduced expression of the costimulatory molecules CD80 and CD86 associated with a sufficient expression of the maturation marker CD83 on alcohol-treated DCs from controls compared with non alcohol-treated DCs observed by Mandrekar et al (Mandrekar et al. 2004). The discrepancy between their results and ours could depend on differences in culture time (72 hours in the study of Mandrekar et al vs 18 hours in our study). A possible explanation is that, because immature DCs contain preformed intracellular CD83 and its rapid surface expression upon activation is post-translationally regulated in a process involving glycosylation, EtOH may delay but not prevent surface CD83 induction.

The phenotypical alterations we observed were also associated with marked functional impairment (retained ability to internalize FITC-dextran, reduced ability to migrate, reduced allostimulatory ability and altered cytokine production). Many studies show that DCs secrete more IL-12 during the earlier stages of their maturation. This increased secretion influences their migration to lymphoid organs and terminal maturation (Kalinski et al. 1999; Ebner et al. 2001).

Alcohol-induced delayed DC maturation, if proved, could have as a functional consequence a transient immunosuppressive effect on the ability of DCs to respond promptly and to eliminate pathogens. The major functional impairment caused by acute in vitro EtOH exposure arose from the low IL-12p70 and IL-10 cytokine production by LPS-stimulated DCs. In our experiments, maturation was more severely impaired in DCs from chronic alcoholics than in DCs from healthy subjects. The high inflammatory response (IL-6 production) shown by DCs from alcoholics probably reflects in vivo chronic alcohol exposure of monocytes. The degree of endotoxemia in alcoholics would have also a profound effect upon their immune status.

Starting an efficient immune response depends crucially on the degree of DC maturation. Mature DCs are the unique DC phenotype able to prime and polarize naïve T lymphocytes. T-cell priming experiments showed that_DCs, treated with EtOH during maturation, polarized naïve T lymphocytes into IL-4-producing Th2 cells thus inducing a low number of IFN-γ-producing Th1 cells. Unexpectedly, EtOH treated control-iDCs were also able to prime naïve T lymphocytes into typical Th2 cells. Evidence that EtOH polarizes the immune response toward Th2-driven humoral immunity (as IgE production) is reported in ethanol-consuming mice (Starkenburg et al. 2001; Krolewiecki et al. 2001).

A possible mechanism to explain why Th2 cells increased is that EtOH inhibited IL-12 production (Peterson et al. 1998) thus inducing an IL-12/IL-10 imbalance that may promote a tolerogenic state in alcoholics. This hypothetical mechanism notwithstanding we cannot exclude the possibility that EtOH induces mediators, such as OX-40L, that are directly involved in Th2 cell polarization (Kuriyama H et al. 2006).

Our finding that EtOH exposure during DC maturation inhibits nuclear translocation of NF-κB -- a common element in the promoter region of proinflammatory Th1 cytokine genes -- might explain why EtOH-treated DCs from healthy subjects produce IL-12p70 and TNF-α in low amounts. Controversial results exist with regard to NF-κB

activation during maturation of DCs exposed to EtOH (Mandrekar et al. 2004; Mandrekar et al. 2006; Oak et al. 2006). In contrast, in a study on in vitro EtOH-treated DCs from healthy subjects, Mandrekar et al. (Mandrekar et al. 2004 and 2006) found no changes in NF-κB activation between DCs generated with or without EtOH treatment, suggesting that alcohol leaves NF-κB activation and hence DC maturation unchanged. The discrepancy between our findings and the data from Mandrekar et al probably depends on the purity of LPS used to stimulate DCs. In another study, the same investigators found that acute alcohol intake may attenuate or augment inflammatory responses through NF-κB activation depending on the TLR triggered (Oak et al. 2006). They found that alcohol attenuated TLR-4- but not TLR-2-induced NF-κB activation. Our data on DCs are in accordance with this finding on monocytes, because DC stimulation with the phenol-purified LPS, used in our study, exclusively involves TLR-4 triggering, confirming that alcohol reduces NF-κB activation and hence DC maturation in response to microbial stimuli involving TLR-4.

The cromoglycate drug exerts an anti-asthmatic activity most probably by its anti-inflammatory properties (P.J. Barnes, 1993; R.M. Viscardi, 1997; E.R. Bleecker, 1996), although some evidence now suggests that this is unlikely to be the only clinically significant action and that mast cells are not the sole target. A recent study established that cromoglycate acts on human mononuclear cell membrane in a stabilizing fashion on lipid polarity (Zimmer G, 2006). Ethanol can perturb lipid rafts, resulting in disruption of the membrane receptor function, signaling and impaired immune cell functions. (Fernandez-Lizarbe, 2008; AG Dolganiuc 2006; PD Mandrekar, 2006, SP Oak, 2006). The aforementioned findings prompted us to investigate whether the treatment of monocytes and DCs with the ‘cromoglycate-like’ anti-allergic drug is also able to prevent alcohol membrane perturbation in lipid raft structure as a possible approach in the prevention of alcohol's adverse effects on the immune system.

Our in vitro findings suggest for the first time that cromoglycate exerts an adjuvant effect on monocytes and DCs. The addition of this drug during the differentiation and maturation process of DCs exposed to EtOH overcome the cell impairment in phenotype as well as in function, restoring the full iDC ability to respond properly to LPS. The data presented herein indicate that this drug in vitro was able to increase the number of differentiated iDCs and the immune-stimulatory properties of mature DCs. The maturation effects induced by LPS in the presence of cromoglycate proved to be superior to those achieved by the LPS alone. Moreover, MLR experiments generated with mature DCs developed in the presence of cromoglycate drug plus EtOH showed a considerable IFN-γ production by T cells, suggesting a prominent capability of cromoglycate-treated DCs to promote and restore a Th1 response.

A limitation of our study is that we assessed the effects of acute alcohol exposure only in in vitro experiments. Even though our study provides no information on whether the abnormalities we detected are present also in DCs derived from human healthy volunteers with no previous alcohol abuse history and exposed to alcohol before blood donation, it substantially confirms and extends previous findings by Mandrekar et al (Mandrekar et al. 2004) on the effect of in vivo acute alcohol exposure. Another limitation of this study is that our experiments on NF-κB activation and the priming ability of in vitro EtOH-treated DCs were conducted only on DCs from buffy coats, owing to the paucity of monocyte-derived DCs obtained from alcoholics. Further studies are also necessary to investigate whether the impairment observed in DCs derived from alcoholics is also present in DCs from alcoholics who had stopped alcohol intake at least one year previously.

Overall the results of our study elucidate an underlying molecular mechanism of immunomodulation by cromoglycate drug, suggesting their benefit in the treatment of alcohol-driven immunological disorders. However, cromoglycate drug-induced

amplification of co-stimulatory and accessory molecules on stimulated DCs as well as cromoglycate drug direct effects on T cell need to be further investigated.

In conclusion we provide evidence that chronic alcohol exposure affects the ability of monocyte-derived DCs to respond properly to a pathogen-related molecule such as LPS confirming that alcoholics are less able than other individuals to control infections and cancer (Messingham et al. 2002; S Nelson an JK Kolls, 2002; RR MacGregor, 1986; RT Cook, 1998) and respond to vaccinations (Degos et al.1986; Hanna et al. 2000). Our evidence that EtOH polarizes the immune response toward Th2-driven immunity, deregulating the Th1 response may explain the immunological impairments observed in alcohol abusers (Pavia et al. 2004; S Nelson and JK Kolls, 2002; González-Quintela et al. 2003; Thyssen et al. 2007). Our findings showing immunological changes induced by acute in vitro alcohol treatment of DCs derived from healthy non alcoholic individuals underline the risk of a transient impairment of the innate immune system after acute alcohol consumption. This transient immune suppression may increase the individual risk of infectious diseases developing, especially if alcohol abuse and pathogen exposure coincide. Our evidence that EtOH polarizes the immune response toward Th2-driven immunity, deregulating the Th1 response may explain the immunological impairments observed in alcohol abusers, for example diminished immunity to infection, exacerbated proinflammatory responses and enhanced pro-allergic Th2 responses (Pavia et al. 2004; S Nelson and JK Kolls. 2002; González-Quintela et al. 2003; Thyssen et al. 2007).

Overall our results in this study strengthen previous findings that alcohol exerts immunological effects on innate immune response thus leading to immune deregulation. The findings of the specific mechanisms of immune deregulation induced by chronic and acute alcohol exposure obtained in the first part of the present thesis, suggested us to explore the use of cromoglycate as innovative strategy to reverse the impaired immune response in alcoholics. Our preliminary findings indicate that cromoglycate drug may

benefit alcoholics in the treatment of alcohol-driven immunological disorders. Further work is required to understand the signaling pathways responsible for the adjuvanticity of cromoglycate drug on the level of DC differentiation and maturation during EtOH exposure.

Chapter 5 References

5. References

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