Effect of low ethanol concentrations on the production and stability of Interferon γ

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List of abbreviations

%	percent
°C	degree centigrade
‰	per mille
ADCC	antibody dependent cellular cytotoxicity
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variances
AP	activated protein
APC	antigen presenting cell
APS	ammonium persulfate
ATF	activating transcription factor
BCG	Mycobacterium bovis
bidest.	double distilled
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic AMP
CD	cluster of differentiation
cDNA	complementary DNA
Con A	Concanavalin A
CREB	cAMP response element binding protein
CREB-ATF	CREB-activation transcription factor
C_T	threshold cycle
d	day
dd	double distilled
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DMEM	Dulbecco modified eagle medium
dNTP	desoxyribonukleosidtriphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked-immunosorbent assay
EMSA	electrophoretic mobility shift assay
EPO	erythropoetin
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	figure
FI	fluorescence intensity
g	gram
GADD	growth arrest and DNA-damage inducible
GAF	γ-activating factor
GAPDH	glycerinaldehyde 3 phosphate dehydrogenase
GAS	IFN-γ-activated sequence
GH	growth hormone

GRB2	growth factor receptor-bound protein 2
h	hour
H_2SO_4	sulfuric acid
HCl	hydrochloride acid
HEPES	(N-[2-hydroxylethyl]piperazine-n'-[2-ethanesulfonic acid])
HRP	horse radish peroxidase
HS	horse serum
ICAM	intercellular adhesion molecule
ICSPB	IFN consensus binding protein
IFN	interferon
IFNAR1	IFN-α-receptor
IFNGR2	IFN-γ-receptor
IFN-γ	interferon gamma
Ig	immunoglobulin
IgG	immunoglobulin G
IL	interleukin
IL12Rβ1	IL-12-receptor subunit β1
IL12Rβ2	IL-12-receptor subunit β2
iNOS	inducible nitric oxide synthase
IRAK	insulin receptor activated kinase
IRF-1	IFN-regulator factor 1
IRS	insulin receptor substrate
IκB	inhibitor of κB
Jak	janus kinase
JH	Jak homologous
JNK	c-Jun-NH2-terminal kinase
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
l	liter
LPS	lipopolysaccharide
M	mol
MAPK	mitogen-activated protein kinase
MEF2C	myocyte enhancer factor 2 c
MEK	MAPK-kinase
MEKK	MAPKK-kinase
MEOS	microsomal ethanol oxidizing system
MHC	major histocompatibility complex
MKK	Mitogen-activated protein kinase kinase
mRNA	messenger RNA
MyD88	myeloid differentiation primary response gene 88
n.c.	negative control
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor kappa B
NK cells	natural killer cells
NKSF	natural killer cell stimulatory factor
OD	optical density
p.c.	positive control
p38	serin-threonin-kinase

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PGE	prostaglandin E
PHA	phytohemagglutinin
PI3K	phosphoinositid-3-kinase
PIAS	protein inhibitors of activated stats
PMSF	phenylmethanolsulfonyl fluoride
PTB	phosphotyrosine binding
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
Ras	rat sarcoma
rcf	relative centrifugal force
RNA	ribonucleinacid
RT	reverse transcription
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodiumdodecylsulfate
SEB	Staphylococcal enterotoxin b
SEM	standard Error of the Mean
SH2	Src homologous 2
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription
Stim.	stimulation
T-bet	T-box expressed in T cells
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF6	TBF receptor-associated factor 6
TYK	tyrosine kinase
vs.	versus
WHO	world health organization
YY	ying-yang protein

1 Introduction

Chronic alcohol abuse is a major social and medical problem in western countries. For the year 2000, WHO reported that 6 % of the male and 3.8 % of the female adult population of Germany was alcohol dependent (WHO Global Status Report on Alcohol, 2004). In 2005, 14.940 German people died in consequence of chronic alcohol abuse (Statistisches Bundesamt, 2005).

Deleterious effects of alcohol use on health have long been described, including symptoms, which are associated with a damaged liver and increased illness and death seen in alcohol abusers. One of the least appreciated medical complications of alcohol abuse is an altered immune regulation leading to immunodeficiency and autoimmunity. Impairment of the immune system, which serves as defense against infections and uncontrolled tumor growth, increases a person's risk for developing various illnesses and certain types of cancer (Szabo, 1997). The consequences of the immunodeficiency include high susceptibility to bacterial and viral infections (Cook, 1998).

Alcoholics have long been recognized to be at a greater risk of community-acquired pneumonias (Friedman *et al.*, 2003) and are twice as likely to die of pneumonia. The incidence of tuberculosis is 15- to 200fold higher than in a control population. The increase in both, the frequency and the severity of infections in ethanol-abusing individuals is attributed to immune dysfunction (Messingham, 2002).

In addition, the most destructive complication of alcoholism, liver disease and liver failure result from a dysregulated immune response. In this case, chronic alcohol consumption leads to an increase in the expression of a number of inflammatory mediators, including cytokines, reactive oxygen and nitrogen species, and chemokines (Tilg and Diehl, 2000). One of the primary mechanisms causing liver damage in response to ethanol exposure is due to activation of Kupffer cells that is causing an increase in TNF-α production and reactive oxygen species, leading to the progression of fatty liver, inflammation, and fibrosis (Thurmann, 1998).

Kupffer cells are critical to the onset of ethanol-induced liver injury because of their function as the first site of exposure to gut-derived lipopolysaccharide/endotoxin (LPS). Since alcohol consumption is associated with impaired barrier function (Parlesak *et al.*, 2000) and increased serum levels of LPS, the increase in TNF-α after alcohol consumption is at least in part due to an increase in LPS exposure (Bode *et al.*, 1997). Additionally mechanisms of Kupffer cell activation during chronic alcohol exposure may involve the activation of autoimmunity in response to oxidative damage to hepatic proteins and phospholipids (Albano, 2002, Vidali *et al.*, 2003).

Studies during the last three decades have demonstrated that alcohol has multiple effects on the host immune responsiveness. These effects are characterized by depletion of circulating lymphocyte populations and altered lymphoid organ architecture and immune functions (Friedman *et al.*, 2003).
Many of the functions of immunocompetent cells are regulated by a network of cytokines that are able of effecting local and systemic immune and inflammatory responses. Cytokines produced by lymphocytes such as interleukins and interferons regulate the functions of immune cells as well as non-immune cells (e.g. nerve cells, hormone-producing cells). The effect of either chronic or acute alcohol use on cytokine production might be the key element to understand immune dysregulation seen in alcohol abusers.
Altered cytokine balance caused by alcohol is leading to a new insight on the regulation of the immune system although much remains to be learned of the basic immune disorders of the alcoholic (Cook, 1998).
Since IFN-γ together with IL-12 is crucial for the induction of cell-mediated immune response, ethanol-induced changes in the production of those cytokines might be a key factor to compromised immunity. Consequently, in the absence of appropriate IFN-γ stimulation a preferential induction of the humoral immune response may occur. The accompanying lack of cell-mediated response would make the individuals more susceptible to infections that require a T cell response. Furthermore, decreased IFN-γ levels are likely to contribute to additional cytokine abnormalities and thereby to further impairment of cell-mediated immune response (Szabo, 1997).
A reason we conducted the current study was to determine the effect of ethanol on Interferon γ production and to examine possible mechanisms by which ethanol is modulating intracellular signaling leading to a suppression of IFN-γ expression in NK-92 cells.
Studies that focused on ethanol-mediated changes of IFN-γ expression have already been carried out and showed a significant suppression of serum levels of IFN-γ in alcoholics. The inhibitory action of ethanol on IFN-γ production is supported by *in vivo* and *in vitro* findings (Wagner *et al.*, 1992, Chen *et al.*, 1993, Laso *et al.*, 1997, Waltenbaugh *et al.*, 1998, Starkenburg *et al.*, 2001, Szabo *et al.*, 2001, Dokur *et al.*, 2003). The question remains to be answered on which level ethanol is able to interfere with intracellular signaling pathways.

1.1 Alcohol – damage to the immune system

An overwhelming amount of evidence reveals that both acute and chronic alcohol exposure suppresses all branches of the immune system, including early responses to infection and the tumor surveillance system (Cook, 1998, Diaz *et al.*, 2002, Nelson and Kolls, 2002, Messingham *et al.*, 2002). For example, there is a decrease in the ability to recruit and activate germ-killing white blood cells (Deaciuc, 1997, Szabo *et al.*, 1999). In addition, epidemiological data have identified chronic alcohol consumption as a significant risk factor for upper alimentary tract cancer, including cancer of the oropharynx, larynx and the esophagus and of the liver. The increased risk attributable to alcohol consumption of cancer in the large intestine and in the breast is much smaller (Pöschl, 2004).

The association between alcohol exposure and the risk of developing an alcohol-related disease is multifactorial and there is a considerable individual variation, with a particular female susceptibility. This highly variable individual susceptibility is influenced apart from sex by different factors such as age, race, hormones and body mass as well as genetic and environmental factors (Diaz *et al.*, 2002).

Although more severe alcohol-induced morbidity is generally preceded by high levels of alcohol consumption and long duration of drinking, both development and progression of lesions are highly variable. Liver cirrhosis and pancreatitis are usually associated with excessive alcohol consumption. Conditions that occur include digestive disturbance, fatty infiltration of the liver, gastrointestinal bleeding, neuropsychological impairment, nutritional deficiency, peripheral neuropathy and skeletal myopathy (Thakker, 1998).

The two toxicity types of ethanol most readily discussed are hepatoxicity and neurotoxicity, but there is a persistent and widespread ambivalence to recognizing 'intrinsic toxicity of alcohol'. Today it is generally accepted that drinking alcohol regularly for years is toxic to almost every tissue in the body (Diaz, 2002).

In the last few years, researchers have become more knowledgeable about the influence of alcohol consumption, both in moderate and high amounts, on the immune system.

A survey of the consequences of ethanol abuse on health status and immune system is the objective of the following chapter.

1.1.1 Human diseases related to alcohol abuse

Evidence supports the idea that both acute and chronic exposure to alcohol have negative effects on several biologic systems, including the immune system (Díaz *et al.*, 2002 and Nelson and Kolls, 2002). Chronic exposure to ethanol leads to alcoholic liver damage and increased morbidity and mortality due to infectious diseases (Cook, 1998 and Watson, 1994). For example, individuals who consume alcohol, compared with those who do not, are twice as likely to die of pneumonia (Cortese *et al.*, 1992 and Esposito, 1984). Moreover, the incidence of tuberculosis is significantly greater in people who abuse alcohol than in those who consume moderate amounts of alcohol (Cook, 1998 and Friedman *et al.*, 1987). Thus, infections in ethanol-consuming individuals who consume large amounts of alcohol are both more frequent and more severe (Cook, 1998), in part because of ethanol-induced dysregulation of immune responses. In addition to the increased incidence of infectious disease in alcoholics, acute (or binge), ethanol exposure has detrimental effects on inflammatory and immune responses (Messingham *et al.*, 2002).

Investigation of the potential relationship between alcohol use and HIV-1 infection is evolving. In case of alcohol abuse and HIV infections, two essential questions remain: Does prior or concurrent alcohol ingestion increase the susceptibility to infection at the time of exposure, and on the other hand, does alcohol abuse by infected individuals increase the rate of progression from HIV infection to AIDS and profound immune deficiency. However, further research is needed to understand cellular and intracellular mechanisms by which ethanol consumption may modulate the biology and clinical course of HIV-1 infection (Szabo, 1999).

The other viral disease where alcohol consumption has been shown to adversely affect the natural course of the disease is Hepatitis C. Recent reports show evidence that alcohol consumption promotes clinical progression and liver damage in patients with chronic hepatitis C infection (Wiley *et al.*, 1998). Prevalence of HCV is 3-fold to 30-fold higher in alcoholics compared with the general population. Patients with HCV infection and alcohol abuse develop more severe fibrosis with higher rate of cirrhosis and hepatocellular cancer compared with non-drinkers. Increased oxidative stress seems to be the dominant mechanism for this synergism between alcohol and the HCV. Additionally, it was shown that response rates to interferon in alcoholics were significantly lower. As for HIV infections, the answer whether alcoholism in some way predisposed the individual to HCV infections is still to be elucidated (Singal and Anand, 2007).

Several less common infections are more apparent in the chronic alcoholic: lung abscess, emphysema, spontaneous bacterial peritonitis, diphtheria and meningitis (MacGregor *et al.*, 1997).

A disastrous medical complication of chronic alcohol abuse is alcoholic liver disease with eventual liver failure. In 2005, 10.007 German people died as a consequence of alcohol liver disease (Statistisches Bundesamt, 2005). The liver is predominantly damaged by chronic alcohol intake, since the liver is the central organ of ethanol metabolism (Messingham *et al.*, 2002).

It has become commonly accepted that immune mechanisms are partially responsible for the onset and/or progression of alcoholic liver disease (ALD). This observation is often overlooked because of the many other complications of alcohol abuse such as malnutrition, ingestion of high saturated fats, vitamin deficiency, drug abuse, and smoking (Thiele, 2004).

In alcoholic liver disease, Kupffer cells in the liver are activated by lipopolysaccaride (LPS) caused from a breakdown in the intestinal wall permeability. This phenomenon has been called 'leaky gut' and occurs when alcohol increases gut permeability, causing bacteria from the intestinal tract to escape into the blood stream (Keshavarzian et al., 1999). When LPS is present, it activates Kupffer cells to release TNF-alpha and superoxides that result in an inflammatory response. Already very small amounts of LPS can stimulate Kupffer cells to release these proinflammatory cytokines (Duryee, 2004). Once these cytokines are initiated, inflammation and necrosis occurs to hepatocytes and other cell types of the liver.

Alcoholic liver disease has often been associated with circulating antibodies and lymphocytes specific to hepatic antigens (Klassen, 1995). Circulating antibodies specific to acetaldehyde adducts and hydroxyethyl-free radicals have also been found to correlate with ALD (Clot, 1995). This provides a possible mechanism of autoantibody production wherein metabolites of ethanol modify hepatic self-antigens and induce an autoimmune response against the liver.

When metabolites of alcohol are present in the liver, the immune response is tremendously increased. Toxicity due to the chemical breakdown of these metabolites and the inflammation of immune cells increase the number of hepatocyte damage. Self-proteins from hepatocytes could become modified with acetaldehyde, malondialdehdye, or both. These self-proteins could bind to and be taken up by macrophage, endothelial cells, or dendritic cells and presented to T cells. If this occurs, reactive cytotoxic T cells or the production of antibody could aid in damaging the liver (Thiele, 2003).

1.1.2 Alterations of the immune system

Healthy individuals protect themselves against microbes by many different mechanisms including innate (non-specific) and acquired (specific) immunity. Elements of innate immunity exist prior to exposure to microbes and include phagocytes such as neutrophils and macrophages, natural killer (NK) cells, circulating molecules, i.e. complement and macrophage-derived soluble mediators.

Acquired immunity is triggered by exposure to foreign substances (antigens) and involves an integrated system of host defense in which numerous cells and molecules function cooperatively. Acquired immunity features humoral and cell-mediated immune responses as a result of a complex cross-talk between T and B lymphocytes, antigen presenting cells (monocytes, macrophages, dendritic cells, B lymphocytes), and also utilizes specific antibodies and lymphocyte-derived cytokines. However, exogenous agents that affect any of these components of the immune system can impair this well-orchestrated defense mechanism against pathogens.

Alcohol has been shown as one of the modulators of host defense. Impaired immunity in patients with chronic alcohol use has long been described in the medical literature (Jerrels 1993, Cook 1995, MacGregor and Louria, 1997). Chronic and even acute, moderate alcohol use can increase host susceptibility to infections caused by bacterial and viral pathogens. Impaired host defense after alcohol exposure appears to be linked to a combination of decreased inflammatory response, altered cytokine production and abnormal reactive oxygen intermediate generation. Furthermore, cellular immunity, particularly antigen-specific immune response, is impaired by both acute and chronic alcohol use (Szabo, 1999).

While malnutrition, vitamin deficiency and advanced liver cirrhosis can contribute to some of the immune abnormalities in chronic alcoholics, alcohol itself is a potent modulator of the immune system. Increasing evidence from human and animal studies *in vivo* as well as from experiments *in vitro* suggests that alcohol use can indeed modulate the immune system at various levels (Szabo, 1999).

The unfavorable effects of chronic alcohol abuse include a general decrease in the main cellular components of the immune system (Laso *et al.*, 1997) and are reported to be caused by impaired cell proliferation (Brodie and Watson), increased apoptosis (Slukvin and Jerrells, 1995), or reduced production of particular antibody (Helm *et al.*, 1996). Brodie *et al.*, (1992) suggested a Ca^{2+} membrane channel inhibition and subsequent suppression of c-fos induction as the major effects of ethanol on B cell proliferation. For immunoglobulin A (IgA) or immunoglobulin G (IgG), the results of clinical studies show an immunoglobulin increase in patients with ethanol-induced liver cirrhosis (Mühlbauer *et al.*, 2001). The latter report indicates a spontaneously higher B lymphocyte secretion rate in alcoholics for IgA and IgG (but not IgM), which is described being

due to increased T lymphocyte-derived B cell differentiation factors. Gluud and Tage-Jensen (1983) monitored increased IgM, IgA, and IgG concentrations in patients with liver cirrhosis. However, ethanol seems to negatively affect the $CD19^+/CD5^+$subset of B cells in alcoholic patients with alcoholic hepatitis, decreasing their concentration (Laso *et al.*, 1997).

Immunosuppression associated with chronic alcohol use is characterized by reduced antigen-specific T cell response and impaired delayed-type hypersensitivity. T lymphocyte activation and proliferation are also dependent on the cell surface signals received during cell-cell interactions. Although T lymphocyte functions can be directly affected by ethanol, decreased antigen presenting cell function appears to be a key element in the ethanol-induced decrease in cell-mediated immunity. Chronic alcohol consumption is associated with reduced antigen-specific T cell proliferation due to insufficient accessory cell function. (Szabo *et al.*, 2001).

Additionally, the T cell proliferation-inhibiting effects of ethanol-induced monocyte-derived cytokines and mediators (TGF-β, IL-10, PGE2) might be important (Szabo *et al.*, 1993). In addition, a preferential induction of Th2 vs. Th1 immune response has been suggested, based on the increased immunoglobulin levels seen in chronic alcoholics (Szabo, 1999).

There have been reports of reduced natural killer (NK) activity in human alcoholics. Cell surface markers and fresh NK activity in controls and alcoholics showed abnormalities in both phenotype and function (Cook, 1997).

In contrast to cell types that show a decrease in number and depressed function, other cell types are enhanced in their activity. Polymorphonuclear neutrophils (PMNs) are involved primarily in the elimination of microbial antigens at sites of infection. However, they are also implicated in the initiation of tissue injury in alcoholic liver disease. Migration of PMNs is controlled by a series of events triggered by infection, injury, or both. The most prominent factors involved in these processes are proinflammatory mediators, such as, reactive oxygen species (ROS), cytokines, chemotactic lipids and peptides, and complement products. Additionally circulating IL-8 levels correlate with neutrophilic infiltration in patients with severe alcoholic hepatitis (Maltby *et al.*, 1996).

1.1.3 Mechanism of alcohol-induced modulation of immune response

1.1.3.1 Cellular interactions of the immune system

In the past several years, research in immunology has demonstrated a dramatic degree of interaction among the different types of immunocompetent cells. The insights from that work have suggested new fields for investigation the immune alterations in alcoholism.

Among the lymphocytes, T cells play a central role. They can inhibit or stimulate B cells to produce antibodies and they are the source of many regulatory cytokines, but also interact with monocytes and interact and regulate other T cell subclasses. It is therefore of importance to evaluate their interaction and possible alteration after both acute and chronic alcohol exposure (Szabo, 2002).

Encountered microbes or antigens are internalized and microbial peptides are produced by proteolytic cleavage and are bound to MHCII molecules. Both peptide and MHCII are displayed on the surface of dendritic cells (DCs) or macrophages. Antigen-presenting cells then interact with antigen-specific receptors on $CD4^+$ T helper cells. This interaction is critical in determining the subsequent type 1 or type 2 nature of the effector immune response. The different types show defined patterns of cytokine secretion (Romagnani, 1994). Type 1 responses lead to cell-mediated immune responses such as delayed-type hypersensitivity (Macatonia *et al.*, 1995). Both IL-4 and IL-10 are associated with type 2 responses (Mosmann and Sad, 1994) and up-regulate antibody production.

Ethanol consumption diminishes type 1 IL-12/IFN-γ (Mandrekar *et al.*, 2004, Starkenburg, 2001) production, but up-regulates type 2-dependent IgE responses in both human and mice (Gonzalez-Quintela *et al.*, 2004 and Latif *et al.*, 2002). This is attributed to the effect of ethanol on diminished type 1 cytokine secretion and to the influence of APC on $CD4^+$ T cell maturation.

1.1.3.2 Alcohol and cytokine network

One of the most important developments in immunology in recent years has been the discovery of a vast network of regulator molecules called cytokines. Changes in their balance have profound effects on the function of immune cells.

Induction of inflammatory cytokines by pathogens is the initial step in the host's immune defense. Whereas acute alcohol exposure seems to dampen inflammatory cytokine production, which is pivotal to innate immune activation after pathogen contact, chronic alcohol abuse is associated with inflammatory processes (Crews *et al.*, 2006, Szabo, 1999). Furthermore, the presence of already developed diseases, such as alcohol liver diseases affects the cytokine balance as well (Messingham *et al.*, 2002).

Ethanol-mediated changes in monocytes cytokine production

Alcohol can adversely affect the innate immune responses: Acute alcohol can inhibit proinflammatory cell activation pivotal to innate immune activation. This down-regulation in the production of proinflammatory cytokines involves the inhibition of NF-κB-mediated intracellular pathways (Mandrekar *et al.*, 2004). Physiological innate immune responses to pathogens involve the induction of proinflammatory cytokines followed by induction of antiinflammatory cytokines by the same pathogen that provide a mechanism for resolution of inflammation. IL-10 has antiinflammatory properties as it inhibits TNF-α production and is also a negative regulator of antigen-specific T cell activation through inhibition of IL-12 production by antigen presenting cells such as monocytes and dendritic cells (Xia and Kao, 2003). Moderate alcohol consumption augments LPS-induced IL-10 production in monocytes. Taking together, these observations suggest that acute, moderate alcohol consumption has dual antiinflammatory effects by inhibiting proinflammatory and augmenting antiinflammatory cytokines in monocytes (Szabo *et al.*, 2001).

In contrast, chronic alcohol us in humans is associated with increased proinflammatory cytokine activation (Mandrekar *et al.*, 2004). Importantly, in ALD there is activation of monocytes and macrophages resulting in massive increases in proinflammatory cytokines including TNF-α, IL-1 and IL-6 and the chemokine IL-8 (McClain *et al.*, 1999).

In vitro treatment of human monocytes with 25 mM alcohol for 7 days revealed, that chronic alcohol treatment resulted in augmentation of LPS-induced TNF-α production. Consistent with this induction of proinflammatory cytokine production, there was an up-regulation of NF-κB activation by LPS after chronic alcohol treatment. The activation of monocytes responses can be modulated by ethanol at different stages. In this case, the recognition of pathogens by innate immune cells with the help Toll-like receptors is important. Toll-like receptor 4 sense bacterial LPS, whereas TLR 2 is able to recognize diverse pathogens including Gram-positive bacteria and certain viral proteins (Lien *et al.*, 1999).

Acute alcohol treatment inhibited TLR4 (LPS)-induced TNF-α production at the protein and mRNA levels, whereas TLR2-induced TNF-α production was not inhibited by alcohol. Additionally acute alcohol augmented TNF-α protein and mRNA levels in the presence of TLR2 and TLR4 costimulation in human monocytes. Opposite changes in IL-10 production in TLR4 and TLR4 plus TLR2-stimulated cells were obtained, when cells were treated with ethanol. In this case, acute alcohol augmented IL-10 in TLR4 stimulated, but inhibited IL-10 in TLR4 plus TLR2-costimulated monocytes (Szabo, 2001). In summary, the regulation of pro- and antiinflammatory cytokines and the modulation by ethanol is depending on both, the length of alcohol treatment and on the complexity of costimulatory signals (Crew, 2006).

The other antiinflammatory cytokine that can control inflammation and inhibit antigen-specific T cell proliferation is TGF-β. Alcohol at physiologically relevant concentrations can induce TGF-β production in monocytes and augment TGF-β production in response to a bacterial challenge *in vitro* (Szabo *et al.*, 1992). Ethanol-induced elevation in TGF-β may have multiple implications for the immune system, including inhibition of inflammatory cytokine production by monocytes and other cells, inhibition of T cell proliferation and augmentation of Th2-type immune response.

Alcohol intake changes pro- and antiinflammatory mediators in the lung

Changes in cytokine production seem to play an important role in the development of ADRS since the acute lung injury is induced by an intense inflammatory response to the initial result, such as sepsis and trauma, with an exuberant release of proinflammatory cytokines including TNF-α, IL-1β, IL-6 and IL-8 into the alveolar space. This is followed by an influx of activated neutrophils and other immune cells (Ware and Matthay, 2000).

On the other hand alveolar macrophages from alcoholics' secret less TNF-α when stimulated with LPS *in vitro* (Omidvari *et al.*, 1998). In animal models chronic ethanol treatment is associated not only with suppression of TNF-α secretion, but also with suppression of reactive oxygen species and reactive nitrogen species (Standiford and Danforth, 1997).

TGF-β could be one important candidate that might be able to explain the underlying mechanism of alcohol-mediated susceptibility to acute lung injury. TGF-β is a pluripotent cytokine with multiple effects on tissue injury and repair during lung injury (Pittet *et al.*, 2001). There was no evidence of release or activation of TGF-β into the alveolar airspace during ethanol ingestion alone, but consistent with the clinical observations ethanol-fed rats released approximately 5 times more activated TGF-β into the airspace during endotoxemia (Bechara *et al.*, 2004).

Further, bronchoalveolar fluid from endotoxemic ethanol-fed rats was able to induce a significant permeability defect in intact alveolar epithelial monolayers, derived from control-fed rats, and this permeability defect was completely inhibited by neutralizing antibodies to TGF-β (Bechara *et al.*, 2004).

Increased TGF-β expression could also contribute to the significant immune suppression that renders alcoholics at high risk for pneumonia. TGF-β is secreted by regulatory T cells and is able to down-regulate both Th1 and Th2 responses, which is beneficial in modulating the inflammatory response of acute inflammation. Excessive TGF-β activation as consequence of ethanol consumption would be deleterious to a normal immune response to invading pathogens (D'Souza El-Guindy *et al.*, 2007).

Finally, impaired responsiveness to granulocyte/macrophage colony-stimulating factor (GM-CSF) was found in alveolar macrophages from ethanol-fed rats. Macrophages showed a decrease in GM-CSF receptor expression on their surface membrane and a decreased expression and nuclear binding of the associated transcription factor PU.1 (Joshi *et al.*, 2005).

This leads to a global defect in innate immune functions including suppression of TNF-α and other proinflammatory cytokines needed to respond to pathogens. In parallel, ethanol induced expression and activation of TGF-β can acutely damage the already vulnerable alveolar epithelium.

Taken together, changes in cytokine production renders alcohol abusers to both 'inflammatory' diseases such as acute lung injury and to infectious diseases such as pneumonia in which the effects of alcohol are immune suppressing (Guidot, 2000).

Alcoholism and neurodegeneration

Cytokines are potent regulators of neuroinflammation, implicated in a variety of brain diseases. Immune mediators are found in a wide diversity of neurological disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis and amyotrophic lateral sclerosis (Nguyen *et al.*, 2002). Results of many studies implicate an association between peripheral endotoxin (LPS), increase in systemic cytokine synthesis, neuronal activation and altered behavior in alcohol abusers (Rivest *et al.*, 2000). In this case, receptor-signaling molecules including CD14 and TLR4 localized within the brain are activated through systemic LPS and therefore, endotoxin in addition to already activated systemic cytokines has direct effects on the brain, resulting in changes in behavior including activity, sleep pattern and social interaction (Kelley *et al.*, 2003).

Additionally in chronic alcoholism, Kupffer cells in the liver are activated by gut-derived LPS to synthesize large amounts TNF-α and other cytokines (Chen *et al.*, 1998).

Systemic TNF-α has shown to activate macrophage-like cells in the brain to increase synthesis of additional cytokines and create a long-lasting positive feed forward process of increased inflammation (Qin *et al.*, 2004). Elevated levels of TNF-α are associated with demyelization and neurodegeneration (Li *et al.*, 2005). In long-term alcohol abuse neurodegeneration and loss of both cortical gray and white matter and enlargement of the ventricles has been observed by imaging and postmortem studies (Crews *et al.*, 2004). Elevated TNF-α level therefore might be one mechanism of neurotoxicity associated with alcohol abuse, since TNF-α appears to potentate glutamate excitotoxicity by inhibiting glutamate transporters. Prolonged alcohol consumption associated with long-lasting increase in TNF-α may create a hyperglutaminergic state and promote degeneration of both gray and white matter (Zou and Crews, 2005).

Inflammatory mediators and the pathogenesis of ALD

The progression of alcoholic liver injury is characterized by the initial appearance of fatty liver, followed by inflammation, necrosis and apoptosis, followed by fibrosis and then by cirrhosis (Tilg and Diehl, 2000). Every stage of ALD is associated with a pronounced inflammatory and cytokine response (Wheeler, 2006).

One of the primary mechanisms for increased production of inflammatory mediators in response to ethanol is due to activation of Kupffer cells in the liver. Kupffer cells are the resident macrophages in the liver and are critical to the onset of ethanol-induced liver injury. Ablation of Kupffer cells prevents the development of fatty liver and inflammation in rats chronically exposed to ethanol (Thurman, 1998).

Kupffer cells are the first site of exposure to gut-derived LPS and since alcohol consumption is associated with impaired barrier function of the intestinal mucosa (Parlesak *et al.*, 2000), increased exposure of Kupffer cells to LPS may induce TNF-α production and ethanol-induced liver injury (Chen, 1998). The important role for TNF-α in early ethanol-induced liver disease is the fact, that the livers of mice lacking TNF-α receptor 1 were completely resistant to the effects of chronic alcohol (i.e., steatosis, inflammation and mild necrosis) (Yin *et al.*, 1999).

Evidence that gut-derived bacterial toxin plays an important role in the initiation and progression of ALD in humans was already published two decades ago (Bode *et al.*, 1982) and is supported by numerous experimental and clinical studies showing that increased gut permeability is associated with alcohol abuse (Parlesak *et al.*, 2005). Direct evidence of increased LPS translocation across the gut mucosa caused by ethanol was obtained in experiments using rats (Mathurin *et al.*, 2000). Additionally, endotoxemia was found more frequently in patients with alcohol cirrhosis than in patients with non-alcoholic cirrhosis and even more important transient endotoxemia was found in non-alcoholic subjects following acute heavy drinking (Fukui *et al.*, 1991, Bode *et al.*, 1993).

Additionally to the increased exposure, chronic ethanol consumption increases the expression of CD14, part of the LPS receptor complex on the surface of Kupffer cells as well as the key CD14 signaling molecules such as members of the mitogens-activated protein kinase family (MAPK), ERK1/2 and p38 (Kishore *et al.*, 2001 and 2002).

Kupffer cells are activated during early alcohol-induced liver injury and that this process involves gut-derived endotoxin. LPS is known to bind CD14, a cell surface protein found on mononuclear cells, including Kupffer cells. CD14, without a transmembrane-spanning domain, requires an association with toll-like receptors (TLR) to initiate an intracellular signal., Recent experimental evidence has demonstrated that mice deficient in CD14 endotoxin receptor or TLRs are resistant to ethanol-induced liver injury (Wheeler, 2001).

In addition to cytokines, such as TNF-α, Kupffer cells are also capable of producing large amounts of potentially damaging free radicals including superoxide. Indeed, products of oxidative stress can be measured in rodent livers treated chronically with ethanol. One potential source of superoxide within macrophages including Kupffer cells is the phagocytic NADPH oxidase system. This multisubunit enzyme complex is assembled on activation by a variety of stimuli and serves an important function in immune-mediated pathogen destruction (Wheeler, 2001).

This initial activation of the hepatic macrophages then sets into motion a cascade of inflammatory events where next to TNF-α other cytokines are produced, depending on the stage of liver disease. Some of the initial cytokines that are also induced in early stage of ALD are IL-12, IL-4 and IL-10. In a second step, cytokine profile is directed either to a strong inflammatory response (IFN-γ and IL-12) or to a profibrogenic cytokine response (IL-4, IL-5 and IL-13).

In contrast to acute inflammatory reactions, characterized by vascular changes, oedema and neutrophilic inflammation, fibrosis typically results from chronic inflammation, defined as an immune response that persists for several months and in which inflammation, tissue remodeling and repair processes occur simultaneously.

The Th2 cytokines IL-4, IL-5, IL-13 and IL-21 each have distinct roles in the regulation of tissue remodeling and fibrosis. Although the extent to which IL-4 participates in fibrosis varies in different diseases, it has long been considered a potent profibrotic mediator. In fact, studies have suggested that IL-4 is nearly twice as effective as TGF-β (Buttner *et al.*, 1997, Fertin *et al.*, 1991) another potent profibrotic cytokine that has been extensively studied. IL-13 shares many functional activities with IL-4 because both cytokines exploit the same IL-4R/STAT6 signaling pathways (Zurawski *et al.*, 1993). IL-13 was identified as the dominant effector cytokine of fibrosis (Aliprantis *et al.*, 2007).

IL-5 and eosinophils have also been shown to regulate tissue fibrogenesis. The differentiation, activation and recruitment of eosinophils are highly dependent on IL-5, and eosinophils are an important source of fibrogenic cytokines, including TGF-β and IL-13. In mice deficient in IL-5 and/or CCL11 (eotaxin), tissue eosinophilia was abolished and the ability of $CD4^+$ Th2 cells to produce the profibrotic cytokine IL-13 was significantly impaired (Mattes *et al.*, 2002). Thus, one of the key roles of IL-5 and eosinophils may be to facilitate production of important profibrotic cytokines like IL-13 and/or TGF-β, which function as the key mediators of fibrosis.

Finally, similar to IL-5, IL-21/IL-21R signaling was recently shown to promote fibrosis by facilitating the development of the $CD4^+$ Th2 response. IL-21R signaling was also critical for Th2 cell survival and for the migration of Th2 cells to the peripheral tissues (Frohlich, 2007). In addition to supporting the development of Th2 responses, IL-21 also increased IL-4 and IL-13 receptor expression on macrophages (Pesce *et al.*, 2006).

Interleukin 13-secreting $CD4^+$ cells regulate fibrogenesis indirectly by promoting TGF-β1 production, which has been linked with the development of fibrosis since TGF-β1 is a potent activator of stellate cells and collagen production. In macrophages, the primary level of control is not in the regulation of TGF-β 1 mRNA expression, but in the regulation of both the secretion and activation of latent TGF-β 1. Once activated, TGF-β signals through transmembrane receptors that trigger signaling intermediates known as Smad proteins, which modulate transcription of important target genes, including procollagen I and III (Roberts *et al.*, 2003).

Together these data highlight the importance of Th2 cytokine response in liver disease and their role in liver fibrogenesis. The transition to fibrotic liver disease follows a transition from a Th1 response to a profibrogenic Th2 cytokine production (Wheeler, 2004).

1.1.4 Effects of ethanol on IFN-γ production

Chronic alcohol consumption has been largely associated with alterations of the immunes system, increased susceptibility to opportunistic microbes (Watson *et al.*, 1994) and higher incidences of certain forms of cancer (Arjona *et al.*, 2004). Together with IL12, IFN-γ is important for the cell-mediated immunity and for the host defense against infection. Since chronic alcohol abuse is correlated with reduced serum levels of IFN-γ, alcohol-related changes of IFN-γ production may be the key factor in explaining compromised immunity in alcohol abusers (Szabo 1998).

Interferon γ is an important immunoregulatory protein responsible for several immunological effects, including induction of the Fc receptor, major histocompatibility complex class I and II expression, regulation of cytokine gene expression (IL-1, IL-6 and tumor necrosis factor) and activation of immune effector cells, including B lymphocytes and monocytes.

The effect of chronic or acute alcohol treatment on Interferon γ production has been center of a number of *in vivo* and *in vitro* studies: *In vitro* studies focused either on isolated immunocompetent cells from experimental animals or healthy humans. Upon isolation, cells were incubated with and without ethanol for certain time periods, followed by measurement of cytokine production, intracellular cytokine levels or cytokine mRNA. On the other hand, animals or humans (non-alcoholics and alcoholics) were exposed to certain alcohol doses *in vivo* and subsequent measurement of serum levels of cytokines was performed (Deacius, 1997).

The following chapter summarizes the results of these different *in vivo* and *in vitro* studies.

Vicente-Gutiérrez and colleagues 1991 conducted the first study that focused on immune alterations seen in alcoholic cirrhosis and the serum levels of Interferon γ and other cytokines.

Forty patients diagnosed having alcoholic cirrhosis with histopathological confirmation were divided in three groups according to the Child-Pugh classification of severity of liver disease. The control group consisted of 23 healthy volunteers. Patients with AC had significantly reduced plasma levels of IFN-γ in comparison to controls. No significant difference was noted between the cirrhotic patients classified according to the severity of liver disease.

A direct immunosuppressive effect of ethanol leading to an inhibition of Interferon γ secretion was confirmed in 1992 when Peripheral Blood Mononuclear Cells (PBMC) were isolated from healthy donors (to exclude any indirect effect of malnutrition or liver disease seen in chronic alcoholics with AC). Isolated cells were mitogen-stimulated either with Con A or PHA and treated with ethanol for 72 hours at concentrations ranging from 0 to 100 mM. Cell viability was not altered after incubation in ethanol-containing medium. The IFN-γ concentrations in cell culture supernatant were significantly decreased. The inhibition was dose-dependent and already detectable at very low ethanol concentrations (6.25 mM) (Wagner *et al.*, 1992).

In this study, it was demonstrated for the first time that ethanol at low and moderate concentrations inhibits mitogen-induced Interferon γ secretion by human PBMC of healthy donors *in vitro*.

Sixty-one alcoholics and 49 controls were included to a study by Windle *et al.*, 1993 to investigate Interferon γ serum levels and NK cell activity. Patients were screened using the Michigan Alcoholism Screening Test (MAST); alcohol consumption was determined by asking standard quantity-frequency questions for each alcoholic beverage to calculate the average number of drinks per day. For Interferon γ measurement, 20 ml of venous blood were obtained, lymphocytes were isolated and activated with Staphylococcal Enterotoxin B (SEB). The average number of drinks per day was 6.04 in alcoholics compared to 0.81 in controls. The Interferon γ release by isolated cells from alcoholics was 133 units compared to 568 units in control group. This significant decrease in Interferon γ production by cells isolated from alcoholics compared to control group confirms former results, indicating the ethanol-induced IFN-γ suppression in alcohol abusers and the alterations in the activity of isolated lymphocytes.

In humans, it is difficult to assess the contribution of ethanol to the modulation of cytokine release that may be affected by additional factors such as malnutrition. Therefore, Chen *et al.*, (1993) used the murine model to study the effect of *in vivo* chronic and *in vitro* acute ethanol exposure on cytokine production without liver cirrhosis found in humans.

Animals were fed the Lieber-DeCarli liquid diet for five months. Twenty-seven % of the total calories were emerged from alcohol. Control groups were fed an isocaloric liquid diet ad libitum. Calories that were contributed by ethanol were substituted with maltose dextran. Spleen cell suspensions were prepared and treated with various ethanol concentrations ranging from 0.1–1.0 % for 72 hours. The IFN-γ release by spleen cells was reduced after ethanol incubation *in vitro*. However, there were no significant differences in IFN-γ production by spleen cells from mice fed ethanol or control diets. In addition, no significant liver damage occurred in ethanol-fed mice; food consumption and growth was not affected too. The fact that IFN-γ production was reduced, regardless, whether splenocytes were obtained from chronically ethanol-fed mice of from control-fed mice suggests a direct downregulation of IFN-γ expression by ethanol in this *ex vivo* animal model (Chen *et al.,* 1993).

This is in agreement with studies where ethanol-feeding had no effect on IFN-γ production in ethanol-fed mice by the group of Galluci and Meadows (1996). In this case, mice were given water or 20 % (w/v) ethanol ad libitum as the sole fluid source for 2 weeks. Enriched NK cells from ethanol-fed mice and controls were tested for proliferation upon IL-2 stimulation and for DNA synthesis by [3H]Thymidine uptake. The NK cells of ethanol fed mice showed significantly reduced proliferation and a significant lower Thymidine uptake than those of water-fed controls. The percentage of apoptotic nuclei was also determined but remained constant in both groups. The expression of IFN-γ was assessed using intracellular cytokine staining. Ethanol consumption did not affect the IL-2-induced expression of this cytokine at either time point (Galluci and Meadows, 1996).

In contrast, a significantly lower IFN-γ expression was observed in mice splenocytes isolated from chronically alcohol-fed mice compared to controls (Wang *et al*., 1995). Splenocytes were obtained from mice fed a liquid diet with 5 % (v/v) ethanol for 11 weeks. Cells were isolated and cultured in the presence of Con A for 72 hours to induce IFN-γ expression. The cytokines were measured by sandwich ELISA. Con A-stimulated IFN-γ expression was significantly suppressed in splenocytes by chronic dietary ethanol compared to the controls.

The goal of the study by Laso *et al.* was to evaluate the role of NK cells in the production of cytokines in patients with chronic alcoholism, analyzing the possible relationship between cytokine production and alcohol induced liver disease. A total of 30 chronic alcoholic patients – 11 without liver disease (AWLD) and 19 diagnosed to have liver cirrhosis – were included. Production of IFN-γ was measured in NK-enriched Peripheral Blood Mononuclear Cells after stimulation with IFN-α and IL-2. Only cirrhotic patients with a prolonged ethanol withdrawal period displayed abnormal production. Cytoplasmatic IFN-γ levels of NK-enriched Peripheral Blood Mononuclear

Cells of AWLD patients showed an increase in intracellular IFN-γ levels. This is was measured in ALC patients who were actively drinking. ALC patients who had withdrawn from alcohol showed no changes in IFN-γ levels compared to controls but instead had a higher level of Th2-derived cytokines such as IL-4 (Laso *et al.*, 1997).

Two years later the same group conducted a study that focused on the effect of ethanol on the imbalanced production of Th1/Th2 cytokines in chronic alcoholism. Again, actively drinking and abstinent patients with or without alcoholic liver cirrhosis were examined. This suggest the existence of a Th1 pattern of cytokine production in alcoholics of both groups, which was normalized in patients with more than 1 year of withdrawal., In contrast, cytoplasmatic expression of IL-4 was detected in a higher proportion of cells, and these patients therefore showed a predominant Th2 pattern of cytokine production (Laso *et al.*, 1999).

Alterations in the Th1/Th2 balance were also observed in ethanol-consuming mice in which chronic alcohol consumption polarizes the immune response away from Th1-mediated immunity.

After 7 to 10 days of ethanol consumption by experimental animals, cell-mediated immunity is impaired and humoral immunity is enhanced (Jerrells *et al.*, 1995). This is evident by the production of the Th1-related cytokine IFN-γ.

Most immune responses involve multiple cell interactions leading to the question, whether the impairment of T cell function results from the direct inhibition of helper T cells or from the inhibition of the cells which process and present antigen to Th cells.

Since antigen presenting cells (APC) influence the development of Th1 and Th2 adaptive immune response through IL-12, the ethanol-mediated decrease of interleukin-12 production by APC is important for Th2 polarization. To investigate the first onset of alteration in immune parameters during ethanol consumption in terms of alterations in Th1 and Th2 function, Starkenburg *et al* (2001) used C57BL/6 and BALB/c mice. Animals were fed a liquid ethanol diet containing 30 % ethanol-derived calories or were pair-fed a liquid control diet for 3 to 10 days. To functionally assess the kinetic role of alcohol on immune function, spleen cells from BALB/c mice were used as APCs in coculture with purified T cells from naïve, solid diet-fed DO11.10 mice. Already after a ethanol consumption of 3 days inhibited IFN-γ production by the transgenic T cells in response to ovalbumin stimulation by ~40 %. Ethanol impairment of the IFN-γ response continued in subsequent days. Single-cell cytokine (ELISPOT) data indicated that ethanol consumption affected IFN-γ but not IL-2 secretion. This was explained by a reduced splenic cellularity and MHC class II expression in ethanol-fed mice, resulting in functional alterations of Th1 associated IFN-γ production (Starkenburg *et al.*, 2001).

As mentioned above, a reduced T cell response and suppression of IFN-γ expression have been linked to an antigen presenting cell defect, a reduced IL-12 production and a Th2 type cytokine induction in animal models of chronic alcohol feeding. The question remains if the same mechanisms are involved in reduced T cell proliferation and IFN-γ production after acute alcohol intake in healthy volunteers. In healthy individuals, the effect of acute, moderate alcohol intake (2 ml of vodka/kg body weight) on IFN-γ production of LPS-stimulated whole blood samples was investigated at different post-alcohol time points. Compared to the pre-alcohol or 16-hour post-alcohol state, IFN-γ levels increased at the 4-hour post-alcohol time point. In addition, the PHA stimulation of isolated mononuclear cells 16 hours after alcohol consumption led to significantly lower IFN-γ concentrations in supernatants of cells obtained from alcohol consumers in comparison to those isolated from control donors (Szabo *et al.*, 1998).

Furthermore, the reduced IFN-γ production was associated with a reduced T cells activation by accessory cells (monocytes). This observations lead to the conclusion that even one occasion moderate alcohol intake can reduce allostimulatory T cell activation via decreasing accessory cell function. The increased IL-10 and IL-13 concentrations also observed together with reduced IFN-γ production are likely to contribute to both the reduced T cell proliferation and the suppressed monocyte accessory cell function (Szabo 2001).

There is an association between chronic alcohol consumption and an increased risk of cancer. Suppression of the immune system can be considered as one mechanism by which ethanol could increase the incidence or progression of cancer. Natural killer cells are belonging to the first line of defense, not only against infections, but also play a significant role in the cellular resistance to malignancy and tumor metastasis. Alcohol-induced changes in the gene and protein expression of perforin, granzyme B and IFN-γ could be a mechanism involved in the suppression of NK cell cytolytic activity by chronic ethanol consumption.

This mechanism was investigated in male Fischer 344 rats either pair-fed an isocaloric liquid diet or fed an ethanol-containing liquid diet for two weeks. Ethanol substitution provided approximately 37 % ethanol-derived calories. NK cell cytolytic activity of splenic lymphocytes was determined against YAC-1 lymphoma cells by a standard 4-hr chromium-51 release cytolytic assay. Gene expression was evaluated by real-time PCR. Alcohol diet in rats at 2 weeks decreased the mRNA expression of IFN-γ as compared to pair-fed animals. Additionally, IFN-γ release by splenocytes of alcohol-fed animals was suppressed significantly. Hence, ethanol seems to act both at transcriptional and translational levels without changing splenocyte or total NK cell number (Dokur *et al.*, 2003).

The latest study on ethanol-related changes on IFN-γ production was done in 2004 and focused on circadian rhythms of granzyme B, perforin and IFN-γ and the cytolytic activity of NK cells. It was already shown, that chronic alcohol consumption reduces granzyme B, perforin and IFN-γ and therefore NK cell activity but since alterations in the body's biological rhythms can lead to serious diseases including cancer, the question was whether NK cells follow a circadian rhythm and if so, to analyze the effect of chronic alcohol consumption.

Rats were fed an ethanol-containing diet for 14 days. At different times over a day, concentration of both IFN-γ protein and mRNA levels of the spleen tissue was measured.

Under an ethanol-free diet, the highest levels of IFN-γ mRNA were obtained during the daytime, the lowest at nighttime. Chronic ethanol feeding blunted the circadian rhythm although mRNA levels were not significantly different between the alcohol and the control group.

However, at protein level, the circadian rhythm of alcohol-fed rats was at highest level during the dark phase and at the lowest during the light phase. In addition, the amplitudes, which normally characterize a circadian rhythm, were markedly reduced. Suggesting, that the circadian rhythm of IFN-γ in splenocytes was disrupted by chronic ethanol consumption (Arjona *et al.*, 2004).

In conclusion, it is well established that chronic alcoholism is associated with important immunological abnormalities involving the production of IFN-γ as well as other cytokines, and changes can also be seen after acute one occasion alcohol consumption. The type of change observed depends not only on the existence of liver disease, but also on the status of ethanol intake and the constancy of withdrawal. Reports on the production of IFN-γ analyzed in chronic alcoholism are difficult to compare and the information provided is controversial. Accordingly, low, unchanged or increased serum levels have been reported in chronic alcoholism. The same is true for its production by isolated PBMC of ethanol-fed mice after *in vitro* stimulation. Such a variability of results could be related in part to the relatively short time of these cytokines and to circadian variations in their production (Laso *et al.*, 1999).

Results that are more consistent were obtained, when isolated cells were used, in this case acute ethanol incubation led to a reduction of IFN-γ production in most of the *in vitro* studies conducted.

Table 1-1. Effects of ethanol on *in vitro* stimulated IFN-γ production by various cell types (according to V. Gloy)

references	**species, subjects**	***in vivo* alcohol exposure/ consumption**	**cell type**	***in vitro* stimuli**	**alcohol effects on IFN-γ**
Wagner *et al.*, 1992	human, healthy	-	Peripheral Blood Mononuclear Cells	1.ethanol 0-100mM, (72h of incubation)	↓ production at 24h of culture, also reduced spontaneously without PHA or Con A
				2. exchange for ethanol-free medium with Con A, 0.25μg/ml or PHA, 5μg/ml	
Laso *et al.*, 1997	human, alcoholics without liver disease (AWLD), with alcoholic liver cirrhosis (ALC)	90g EtOH /day > 5 years, AWLD: active EtOH intake, ALCET: ALC + active EtOH intake ALCAW: ALC + EtOH withdrawal > 1 year	Peripheral Blood Mononuclear Cells, enriched for NK cells stimulated *ex vivo*	IL-2, 500 units/ml	↓ production only in ALCAW, unchanged in ALCET, unchanged in ALCAW, at 72h of incubation
Szabo 1998	human, healthy	acute, moderate alcohol consumption of 2ml of vodka/ kg body weight	whole blood samples stimulated *ex vivo*	LPS, 0.1μg/ml	↑ production at the 4h post-alcohol time point; ↓ production at 16h
			isolated mononuclear cells stimulated *ex vivo*	PHA, 0.2 μg/ml	↓ production at the 16h post-alcohol time point
Szabo *et al.*, 2001	human, healthy	acute, moderate alcohol consumption of 2 ml of vodka per kg body weight, peripheral blood samples taken after 18hr	isolated mononuclear cells stimulated *ex vivo*	PHA, 0.2 μg/ml	↓ production at 72h of incubation
Chen *et al.*, 1993	mice	chronic feeding, diet with 27% ethanol-derived calories for 5 month	spleen cells stimulated *ex vivo*	ethanol, 0.1-1.0% v/v; LPS, 10μg/ml or Con A, 2μg/ml	↓ production at 24h and 72h of culture, also in control fed mice
Wang *et al.*, 1995	mice	liquid diet with 5% (v/v) ethanol for 11 weeks	splenocytes stimulated *ex vivo*	Con A, 5μg/ml	↓ production at 72h

Legend: Con A: Concanavalin A; LPS: Lipopolysaccharide; NK cells: Natural killer cells; PHA: phytohemagglutinin

Table 1-1 continued.

references	**species, subjects**	**alcohol exposure/ consumption**	**cell type**	**stimuli**	**alcohol effects on IFN-γ**
Galluci & Meadows 1996	mice	chronic feeding, drinking water with 20% w/v ethanol for 2 weeks ad libitum	splenic cells enriched for NK cells *ex vivo* stimulated	IL-2, 20pg/ml	unchanged intracellular levels, at 24h and 48h
Waltenbaugh *et al.*, 1998	mice	chronic feeding, diet with 30% ethanol-derived calories for up to 11 days; effects seen after 5 days	spleen cells, *ex vivo* in coculture with transgenic T cells (receptor specific for OVA)	ovalbumin (OVA), 18μM	↓ production, at 48h of incubation
Starkenburg *et al.*, 2001	mice	chronic feeding diet with 30% ethanol-derived calories for 12 days	spleen cells (APCs source) *ex vivo* co-cultured with transgenic T cells	ovalbumin, 18μM	↓ production at 72h of culture
Dokur *et al.*, 2003	rats	chronic feeding, diet with 37% ethanol-derived calories for 14 days	whole-spleen tissue	-	↓ mRNA expression
			isolated splenocytes *ex vivo* stimulated	Concanavalin A, 5μg/ml	↓ production at 24h of culture
Dokur *et al.*, 2005	rats	feeding ad libitum without ethanol	splenocytes enriched for NK cells	after 24h of cell culture, additional incubation with 100mM ethanol	↓ intracellular levels at 18h of incubation

Table 1-2. In vivo effects of ethanol on the levels of IFN-γ in serum/ cytoplasma of experimental animals and clinical humans (according to V. Gloy)

references	species/ subjects	alcohol exposure /consumption	cell type	alcohol effects on IFN-γ
Vicente-Gutierrez *et al.*, 1991	human	patients with alcoholic cirrhosis	-	↓ serum levels
Windle *et al.*, 1993	human	alcohol abusers	-	↓ serum levels
Laso *et al.*, 1999	human, alcoholics without liver disease (AWLD), with alcoholic liver cirrhosis (ALC)	90g EtOH /day > 5 years, AWLD: active EtOH intake, ALCET: ALC + active EtOH intake ALCAW: ALC + EtOH withdrawal > 1 year	isolated peripheral blood T cells (PBL): CD4+ and CD8+	AWLD: ↑ levels in cytoplasm ALCET: ↑ levels in cytoplasm but lower than in AWLD, ALCAW: unchanged
Lin *et al.*, 1998	rats	chronic feeding 36% ethanol-derived calories for 6 weeks	-	hepatic steatosis, ↑ detectable protein levels in serum, ↑ mRNA levels in the liver
Arjona *et al.*, 2004	rats	chronic feeding, liquid diet : 8.7% ethanol v/v for 14 days	Splenocytes	disruption of circadian rhythm of protein and mRNA levels in cell lysates

1.2 Interferon γ – signals, mechanisms and functions

Interferon γ (IFN-γ) coordinates a diverse array of immunological functions through transcriptional regulation of relevant genes. This following chapter reviews the current understanding of IFN-γ signal transduction and its cellular effects as well as its role in host defense. The influence on other immunocompetent cells and its significance during infection is described, including up-regulation of pathogen recognition, antigen processing and presentation, the antiviral and microbiocidal properties, immunomodulation and leukocyte trafficking.

1.2.1 Characteristics of Interferon γ

The cytokine IFN-γ belongs to the family of interferons, which are closely related by their ability to protect cells from viral infections. Based on several criteria, the IFN molecules have been divided into two distinct classes. The first class is named type I IFN and includes IFN-α and IFN-β molecules, which are the classical interferons induced in response to viral infections (Teixeira *et al.*, 2005).

The second class is solely composed by IFN-γ (type II), which is not related to the type I IFN at both the genetic and the protein levels. Although IFN-γ displays most of the biologic activities that have been described to the other IFNs, it has a lower specific antiviral activity, but presents more immunomodulatory properties than the type I interferons (Farrar and Schreiber, 1993).

The overall organization of the IFN-γ gene is very similar in different species, comprising (Boehm *et al.*, 1997) four exons and three introns. The genomic DNA structure is strongly conserved and has been maintained among all species analyzed, and DNA sequence has greater identity in the promoter DNA sequence than in the protein-coding regions (Young *et al.*, 1995).

The human IFN-γ protein consists of 166 amino acids of which 23 amino acids represent a hydrophobic signal sequence that is removed before secretion. There are two N-linked glycosylation sites and the protein appears to exist functionally as a non-covalent homodimer (Young *et al.*, 1995). Two polypeptide chains self-associate in an antiparallel fashion, producing a molecule that exhibits a twofold axis of symmetry with an apparent molecular weight of 34 kDa (Farrar and Schreiber, 1993, Bach *et al.* 1997). Only the dimer displays biologic activity, possibly because it is the only conformation of the molecule that can induce IFN-γ receptor (IFN-γR) dimerization (Farrar and Schreiber 1993).

For a long time, the production of IFN-γ has been considered to be restricted to activated natural killer (NK) cells, $CD4^+$ T helper-1 (Th1) cells, and $CD8^+$ T cytotoxic cells (Farrar and Schreiber 1993, Boehm *et al.* 1997).

However, we now know that these cells are the most potent, but not the only sources of IFN-γ. Several studies have identified additional IFN-γ-secreting cell types, including γδ T cells, NKT cells, macrophages, dendritic cells, naive $CD4^+$ T cells, and even B cells (Frucht *et al.* 2001, Szabo *et al.* 2003). During the adaptive phase of immune response, IFN-γ is mainly produced by activated T cells, whereas NK and NKT cells seem to be the main source during the innate phase (Young *et al.*, 1995).

IFN-γ production is controlled by cytokines secreted by APCs, most notably Interleukin (IL)-12 and IL-18. These cytokines serve as a bridge to link infection with IFN-γ production in the innate immune response (Gollob *et al.*, 2000). Macrophage recognition of many pathogens induces secretion of IL-12 and chemokines. These chemokines attract NK cells to the site of inflammation, and IL-12 promotes IFN-γ synthesis in these cells. In macrophages, NK and T cells, the combination of IL-12 and IL-18 stimulation further increase IFN-γ production (Fukao *et al.*, 2001). Negative regulators of IFN-γ production include IL-4, IL-10, transforming growth factor β and glucocorticoids (Schroder *et al.*, 2004).

1.2.2 Interferon γ receptor

The biological actions of IFN-γ can be attributed to its ability to activate or inhibit the expression of specific target genes. All biological actions of IFN-γ require binding to specific cell surface receptors, expressed on virtually all types of cells (Bach *et al.*, 1996).

Functional IFN-γ receptor (IFNGR) is comprised of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains and associated signaling machinery. IFNGR1 and IFNGR2 chains belong to the class II cytokine receptor family, a class of receptors that binds ligands in the small angle of a V formed by the two Ig-like folds that constitute the extracellular domain (Tau and Rothman, 1999).

Although binding of IFN-γ to the receptor is the function of the IFNGR1 chain, IFN-γ binds with a higher affinity in the presence of the IFNGR2 chain. Signaling is initiated by the formation of a multisubunit complex that contains both IFN-γ receptor chains and IFN-γ (Igarashi *et al.*, 1994).

Both IFNGR chains lack intrinsic kinase/phosphatase activity and so must associate with signaling machinery for signal transduction. The IFNGR1 intracellular domain contains binding motifs for the Janus tyrosine kinase (Jak) 1 and the cytosolic factor, signal transducer and activator of transcription (STAT) 1. The intracellular region of IFNGR2 contains a non-contiguous binding motif for recruitment of Jak2 kinase for participation in signal transduction. The IFNGR2 chain is not tyrosine phosphorylated during signal transduction (Heim *et al.* 1995).

1.2.3 IFN-γ-associated signal transduction

Interferon γ primarily signals through the Jak-STAT pathway, a pathway used by over 50 cytokines, growth factors and hormones to affect gene regulation. Jak-STAT signaling involves sequential receptor recruitment and activation of members of the Janus family of kinases (Jaks 1–3 and Tyk2) and the STATs (STATs 1–6, including STAT5a and STAT5b) to control transcription of target genes via specific response elements.

Signal transduction starts with an interaction of the IFN-γ homodimer with two IFN-γR1, thereby inducing IFN-γR1 dimerization and the subsequent recruitment of two IFN-γR2 to the complex. Each chain is constitutively associated with a specific Janus kinase (JAK) (the IFN-γR1 with JAK1 and the IFN-γR2 with JAK2) (Igarashi *et al.*, 1994). The aggregation of the receptor components brings inactive JAKs into close proximity with one another. Once clustered, JAKs are reciprocally activated through sequential auto and transphosphorylation events. After activation, JAKs then phosphorylate a specific tyrosine residue near the C-terminus of the IFN-γR1, which serve as a docking site to the binding of STAT1 (Heim *et al.* 1995).

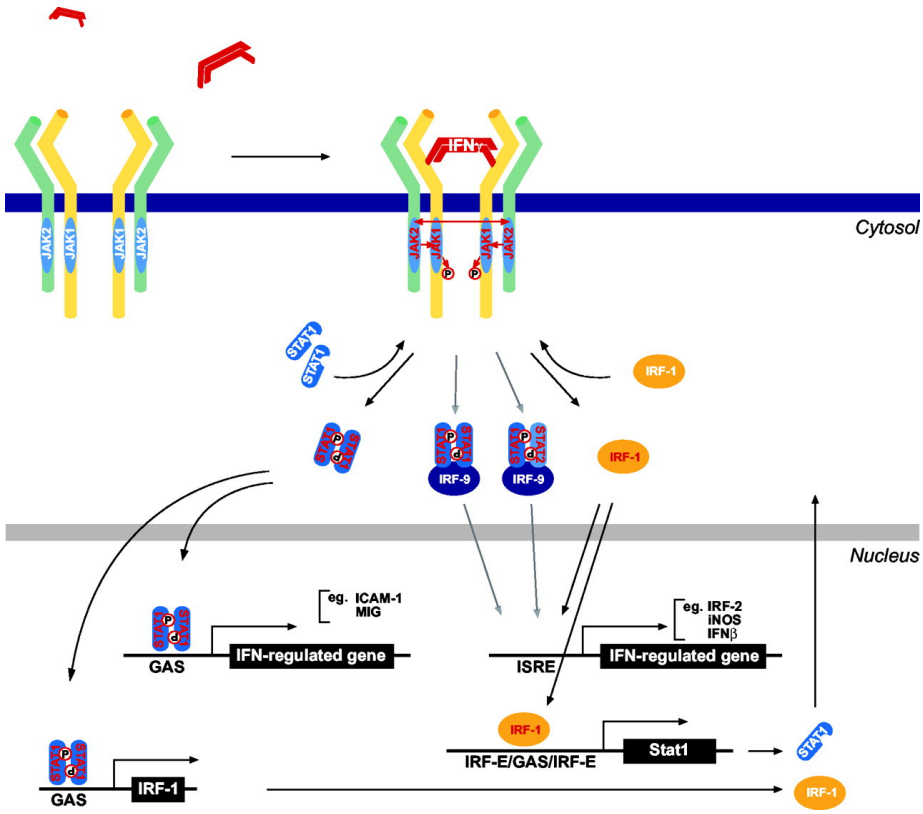


Figure 1-1. The current paradigm for IFN-γ signal transduction JAK: Janus kinase, STAT: signal transducer and activator of transcription, IRF: Interferon regulating factor, GAS: Interferon-γ activated sequence, ICAM: Intercellular adhesion molecules, MIG: mitogen-inducible gene, INOS: inducible nitric oxide synthase, IRF-E: IRE: IRF-binding element, ISRE: IFN-stimulated response element (Schroder *et al.*, 2004)

The recruitment of STAT1 is followed by its phosphorylation on tyrosine residue 701 by the receptor-associated JAKs. This phosphorylation leads to a rapid dissociation of the receptor and to the formation of STAT1 homodimers (Greenlund *et al.*, 1995). At some point during the early phase of activation, STAT1 is also phosphorylated on serine 727 by a process involving phosphatidylinositol 3-kinase (PI3-K) and Akt that is required for maximal transcriptional activity (Nguyen *et al.*, 2001). The dissociated STAT1 homodimer enters the nucleus and binds to promoter elements to initiate or suppress transcription of IFN-γ-regulated genes (Darnell *et al.*, 1998). STAT1 homodimers bind DNA at GAS elements of consensus sequence (Decker *et al.*, 2002).

In summary, by activating the cytosolic transcription factor STAT1, IFN-γ initiates the transcription of a number of genes containing STAT1-binding sites in their promoter regions. Many of these induced genes are transcription factors that are able to further drive the regulation of the next wave of transcription. The total number of IFN-γ-regulated genes is estimated to be ~500 (Boehm *et al.*, 1997). It has been demonstrated that IFN-γ upregulates the transcription of genes related to antigen presentation, Th1 phenotype development, chemokine-based recruitment of monocytes, T cells, eosinophils and basophils, cellular adhesion, immunoglobulin heavy chain class switch, cytokine network, apoptosis, lymphocyte activation, and others (Teixeira *et al.*, 2005).

The key function of STAT1 in mediating IFN-γ signal transduction is indicated by the phenotype of STAT1−/− mice, which showed largely similarity to IFNGR1−/− mice during IFN-γ stimulation. In addition, activated STAT1 protein can form a heterocomplex with p48 and this heterodimer can bind to other DNA sequences (interferon-stimulated response elements, ISRE) present in some IFN-γ-inducible genes, and thus activate their transcription (Majumder *et al.*, 1998).

1.2.3.1 Signal Transducer and Activator of Transcription 1 (STAT1)

Phosphorylation of STAT1 at S727 is essential for maximal ability to activate transcription of target genes. A number of different stimuli induce STAT1 serine phosphorylation, including type II IFN, lipopolysaccharide (LPS) and IL-2, IL-12, tumor necrosis factor α (TNF-α), and platelet-derived growth factor (Gollob *et al.*, 2000). This may be a mechanism, whereby S727 serves as road for modulation of IFN-γ signaling by independent extracellular stimuli. For example, LPS signaling increases STAT1 S727 phosphorylation independently of Y701 phosphorylation in macrophages, thereby augmenting cellular responses to IFN-γ (Schroder *et al.*, 2004).

The ability of STAT1 to activate or repress gene transcription depends on the presence of other transcription factors binding to the promoter element and STAT1 interaction with other factors. STAT1 activation is necessary but not sufficient for transcription of a number of genes. Likewise, factors such as oncostatin M, which participate in Jak-STAT signaling to produce activated STAT1, do not induce transcription of IFN-γ-inducible genes. STAT1 interaction with transcription factors such as IRF-9, upstream stimulatory factor-1, specificity protein-1, and heat shock factor-1 have been reported and are likely to influence specificity of DNA binding and transactivator ability. In some promoters, transcription is maximal when more than one GAS-binding transcriptional activator binds to tandem GAS sites in the target gene promoter (Zhang *et al.*, 1998).

The mechanism of STAT1 entry into the nucleus is still controversial, but the involvement of importin-α-1 (NPI-1) is implied. Nuclear entry of STAT1 is apparent at 15 min and almost complete after 30 min exposure to IFN-γ (Lillemeier *et al.*, 2001).

Functional STAT1 is crucial to host response to infection. KO of STAT1 renders mice extremely sensitive to infection by viral and microbial pathogens (e.g. vesicular stomatitis viruses, mouse herpes virus and L. monocytogenes).

1.2.3.2 Interferon Regulatory Factor 1 (IRF-1)

Transcription factors belonging to the interferon regulatory factor (IRF) family are necessary for Th1 development. The IRF family includes at least 10 members and their expression is either constitutive and/or induced upon treatment with IFN-γ and other cytokines or in response to viral infections (Coccia *et al.*, 1999).

Members of the IRF family share similar structure, including an amino-terminal DNA-binding domain containing multiple tryptophan residues and a carboxy-terminal transcriptional activation or repression domain (Taniguchi *et al.*, 2001).

IRF-1 drives inducible expression of many target genes through interaction with the IRF-E site. This specificity overlaps with the ISRE consensus site, recognized by ISGF3, which is induced by

type I IFN and to a lesser extent, type II IFN. In this way, IRF-1 is able to induce a subset of the full spectrum of IFN-inducible genes (Schroder *et al.*, 2004).

One characteristics of IRF-1−/− mice is a defect in the development of thymic $CD8^+$ cells, although maturation of $CD4^+$ cells was normal (Matsuyama *et al.*, 1993). The defect in $CD8^+$ TCR-α/β^+ cells and decreased levels of MHC class I are a consequence of reduced expression of transporter associated with antigen processing-1 (TAP-1) and the low molecular weight protein-2 (LMP-2) (Penninger, 1998). Mice lacking TAP-1, LMP-2 or IRF-1 all have a similar phenotype, characterized by a developmental block in MHC class-I-restricted thymocytes (Hombach, 1995).

This MHC class I defect in IRF-1−/− mice only partially explains the impaired positive and negative selection of T cells in the thymus of these mice (Penninger *et al.*, 1998). IRF-1 also seems to regulate genes in developing T cells that are crucial in signal transduction in thymocytes and lineage-specific differentiation of $CD8^+$ cells. Although $CD4^+$ T cell maturation occurred normally in IRF-1−/− mice, profound phenotypic changes were detected: IRF-1−/− mice possessed a greater number of memory/effector $CD4^+$ T cells at the expense of the naive cell subset; and all $CD4^+$ T cells (memory and naive) displayed an altered profile of inducible cytokine production. After stimulation, a decreased production of IL-2 and IFN-γ (Th1 cytokines) and an increased production of IL-3, -4, -5 and -6 (Th2 cytokines) were observed. This shift to Th2 cytokine production has important implications for the clearance of pathogens, since the balance between the Th1- and Th2-related cytokines will determine whether the immune response is protective, non-protective, or pathogenic (McElliot *et al.*, 1997). These results illustrate a role for IRF-1 in the homeostasis of T cell subset frequencies and cell functions.

IRF-1−/− mice also exhibited a severe natural killer (NK) cell deficiency (Ogasawara *et al.*, 1998). NK cell development was restored when IRF-1−/− bone marrow cells were cultured in the presence of IL-15, a cytokine that induces proliferation of mitogen-activated $CD4^+$ and $CD8^+$ T cells and is also crucial to NK cell activation, cytotoxicity, cytokine production and proliferation (Nguyen *et al.* 1999).

1.2.3.3 Negative regulation of IFN-γ signaling

Elevated levels of IFN-γ can be dangerous, and it is clear that both IFN-γ production and the response to this cytokine must be tightly regulated in order to achieve a balance between beneficial and harmful effects. Other cytokines including IL-4, IL-10, and IL-13 contribute to some extent to this regulation by antagonizing IFN-γ functions. In addition, negative regulators act to limit signal transduction in response to IFN-γ (Brysha *et al.*, 2001).

STAT1 activation is inhibited within 1 h of IFN-γ treatment, despite the continued presence of extracellular IFN-γ, and so mechanisms must exist to control the extent of ligand stimulation of IFN-γ signaling. These mechanisms (Figure 1-2) involve every level of the pathway (Darnell *et al.*, 1998) and include downregulation of receptor/ligand complex, degradation of signaling intermediates and inactivation of positive regulators by dephosphorylation, and activation of specific suppressors (Starr *et al.*, 1999).

In case of IFN-γ, following signal transduction, the IFN-γ/IFNGR1 complex internalizes and enters the endosomal pathway, where the complex dissociates. In many cell types, the IFNGR1 chain recycles to the cell surface in its uncoupled, dephosphorylated form, and the ligand is degraded (Starr *et al.*, 1999). Dephosphorylation of the activated IFNGR1 subunit occurs rapidly following stimulation with IFN-γ (Stark *et al.*, 1998).

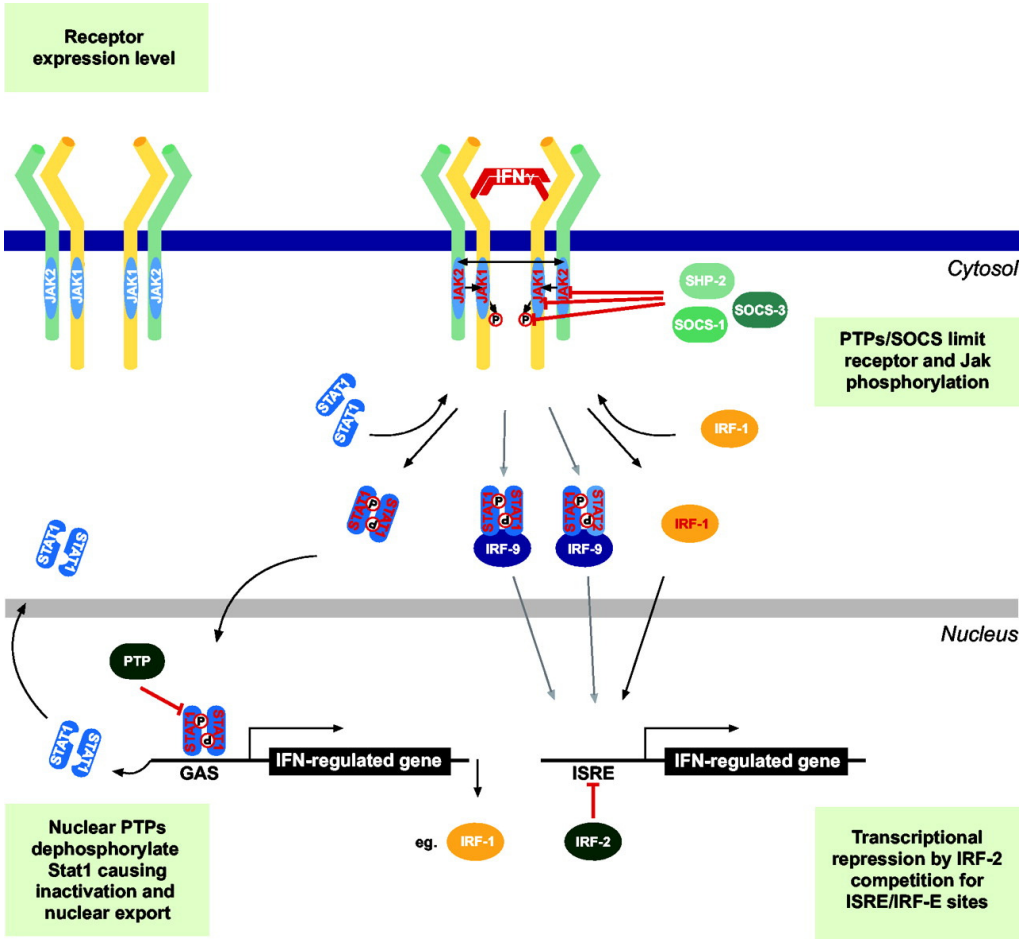


Figure 1-2. Negative regulation of IFN-γ signaling. JAK: Janus kinase, STAT: signal transducer and activator of transcription, IRF: Interferon regulating factor, GAS: Interferon-γ activated sequence, IRF-E: IRF-binding element, ISRE: IFN-stimulated response element, SOCS: suppressors of cytokine signaling PTP: tyrosine phosphatase (Schroder *et al.*, 2004).

One of the preferentially inducible targets of IFN-γ is a specific feedback inhibitor, SOCS-1, which associates with Jak1/2, interfering with tyrosine kinase activity and inhibiting downstream IFN-γ signaling. SOCS-1 is an intracellular SH2 domain-containing protein and is part of a family of eight proteins each of which comprise a C-terminal SOCS box (Krebs *et al.*, 2001). SOCS-1 expression is induced by IFN-γ and overexpression of SOCS-1 inhibits IFN-γ signaling (Brysha *et al.*, 2001). On the other hand, mice lacking SOCS-1 die from a complex disease characterized by liver degeneration and massive inflammation (Kile *et al.*, 2001). Another SOCS protein, SOCS-3, is also induced by IFN-γ and negatively regulates IFN-γ signaling, although perhaps less effectively than SOCS-1 (Schroder *et al.*, 2004).

1.2.4 Molecular effects of Interferon γ

1.2.4.1 Class I antigen presentation pathway

Types I and II IFN upregulate multiple functions within the class I antigen presentation pathway to increase the quantity and diversity of peptides presented on the cell surface in the context of class I MHC. Upregulation of cell-surface class I MHC by IFN-γ is important for host response to intracellular pathogens, as it increases the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity (Hisamatsu *et al.*, 1996).
IFN-γ stimulation induces a replacement of the constitutive proteasome subunits with 'immunoproteasome' subunits. Inducible proteasome replacement is thought to be a mechanism by which IFN-γ can increase the quantity, quality and repertoire of peptides for class I MHC loading. The quantity is increased, as overall expression levels of proteasome are increased. The cleavage specificity of the immunoproteasome may allow production of peptides better able to bind class I MHC and thereby increase efficiency in the system. As a whole, this serves to increase levels and diversity of epitopes presented for $CD8^+$ T cell recognition in the context of class I MHC and thus increases immune surveillance (Groettrup *et al.* 2001).

1.2.4.2 Class II antigen presentation pathway

Of the IFNs, only IFN-γ can efficiently upregulate the class II antigen-presenting pathway and thus promote peptide-specific activation of $CD4^+$ T cells (Mach *et al.*, 1994). IFN-γ treatment further upregulates class II MHC molecules in cells constitutively expressing class II MHC, such as B cells, DCs, and cells of the monocyte/macrophage lineage. IFN-γ is also able to induce class II MHC expression in cells that do not constitutively express this gene, such as skin fibroblasts, vascular endothelial cells, thyroid epithelial cells and astrocytes (Chang *et al.*, 1995).

As a result of class II MHC molecule induction, these cells acquire an ability to function as antigen-presenting cells, and can thus participate in the process of specific immune recognition (Mach *et al.*, 1994).

IFN-γ is known as the most potent inducer of class II MHC expression but is also important for the expression of other MHC-encoded genes involved in antigen-presentation such as TAP-1 and TAP-2, and proteasome subunits. IFN-γ promotes the translocation of antigenic peptides from the cytosol to the endoplasmatic reticulum, where the peptide binds to the MHC molecules. IFN-γ alters the proteolytic specifity of proteasomes, facilitating the generation of peptides for antigen presentation (Tanaka *et al.* 1994).

1.2.4.3 IFN-γ and the development of Th1 response

Beginning with the activation of the T cell receptor (TCR) by the appropriate peptide-MHC complex, naive $CD4^+$ T cells rapidly undergo a differentiation process that leads to the development of two functionally distinct cell subsets. These subsets are characterized by the production of cytokine pattern. Th1 cells that secrete IFN-γ are efficient in eliminating intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which affect humoral immunity to helmintic parasites and are responsible for immune responses to persistent allergens (Abbas *et al.*, 1996). IL-12 and IL-4 are known to be the major Th1- and Th2-inducing cytokines, respectively (Abbas *et al.*, 1996). The Th1/Th2 balance is extremely important and may determine whether the immune response is appropriate or leads to severe immunopathologies. Overproduction of Th1 cytokines has been implicated in delayed-type hypersensitivity reactions and autoimmune diseases. On the other hand, the basis for allergic disorders remains on the dysregulation of the Th2 phenotype (Abbas *et al.*, 1996).

IFN-γ is the principal Th1 effector cytokine, and it has a crucial role in Th1 differentiation. IFN-γ has the ability to act in a great number of cell types that are involved in Th differentiation. It induces IL-12 production by antigen presenting cells (APC), such as dendritic cells and macrophages (Snijders *et al.*, 1998, Szabo *et al.*, 2003). These APCs provide the first contact of naive $CD4^+$ T cells with the antigen; therefore, this IL-12 production is of great importance on the differentiation pathway towards a Th1 phenotype. In addition to its role on APC, IFN-γ exerts effects on the $CD4^+$ T cells themselves. This cytokine is capable of enhancing the development of Th1 effector cells from BALB/c mice by increasing naive $CD4^+$ T cells responses to IL-12 (Wenner *et al.*, 1996).

IFN-γ also exerts direct inhibitory effects on Th2 cytokines, reducing the levels of IL-4 and IL-5 production. The IFN-γ signaling pathway activates T-bet protein, the Th1-specific and Th2-suppresing transcription factor (Lighvani *et al.*, 2001, Afkarian *et al.*, 2002). In fact, ectopically expression of T-bet was able to repress IL-4 and IL-5 in Th2 cells (Szabo *et al.* 2000).
On the other hand, the Th2-induced transcription factor GATA-3 specifically controls the expression of Th2 cytokines (Zhang *et al.*, 1999).
Loss of IL-4 receptor responsiveness may be another mechanism that suppresses Th2 development in polarizing Th1 cells (Huang and Paul, 1998). Other studies have shown that IFN-γ directly suppresses IL-4 gene expression through IRF-1 and 2, which bind to three distinct IL-4 promoter sites and act as transcriptional repressors (Elser *et al.*, 2002).
Besides the counteracting roles of IFN-γ in the Th2 differentiation process, IFN-γ has a role in inhibiting the proliferation of Th2 cells. Th1 cells decreased their expression of the b chain of the IFN-γR while Th2 cells did not, suggesting a mechanism by which IFN-γ could inhibit selectively the proliferation of Th2 clones (Pernis *et al.*, 1995).

1.2.4.4 Effect on B cells

B cells contribute to the humoral immunity by production of antibodies. IFN-γ also has regulatory roles in the control of immunoglobulin isotypes produced by activated B cells (Vilcek *et al.*, 1998).
The best-characterized action of IFN-γ directed toward B cells is the ability to influence Ig heavy-chain switching. Ig class switching is significant because the different Ig isotypes promote distinct effector functions in the host. By favoring the production of certain Ig isotypes while inhibiting the production of others, IFN-γ can facilitate interactions between the humoral and cellular effector pathways of the immune response and increase the host defense against certain bacteria and viruses. *In vitro*, IFN-γ is able to direct immunoglobulin class switching from IgM to the IgG2a-subtype in LPS-stimulated murine B cells and to IgG2a and IgG3 in murine B cells that have been stimulated with activated T cells. Moreover, IFN-γ blocks IL-4-induced Ig class switching in murine B cells from IgM to IgG1 or IgE (Stark *et al.*, 1998).
Unlike the upregulating effect of IFN-γ on surface MHC expression in macrophages, the induction of MHC class II expression by IL-4 in B cells is downregulated. Thus, IFN-γ reduces the capability of antigen recognition and presentation by B cells (O'Neill *et al.*, 1999).
IFN-γ was shown to be involved in the regulation of proliferation and apoptosis of lymphocytes, too. It is able to inhibit lipopolysaccharide (LPS-)-induced B cell proliferation, antigen recognition and presentation by B cells (O'Neill *et al.*, 1999).

1.2.5 Interferon γ – role in host defense

1.2.5.1 Host defense against viral infection

During viral infections, some of the most prominent cytokine produced are the interferons, named for their ability to interfere with viral replication. The IFN system regulates innate and adaptive immunity to viral infection. Viral invasion directly triggers induction of type I IFNs, through a mechanism involving IRF-3 and IRF-7 (Malmgaard, 2004).

IFN-γ-induced antiviral mechanisms include induction of key antiviral enzymes, most notably PKR, which is a serine/threonine kinase greatly induced by type I and II IFN stimulation. PKR is inactive in its constitutive form and requires an activating signal for autophosphorylation. dsRNA, a necessary intermediate in replication of RNA viruses, is the best characterized activator of PKR, although other agents such as heparin are able to activate PKR. Association of PKR with dsRNA is likely to cause a conformational change that unmasks the catalytic domain responsible for PKR autophosphorylation. PKR is then activated for dsRNA-independent phosphorylation of specific cellular substrates (Goodbourn *et al.*, 2000).

One of these substrates is the eIF-2α subunit, a rate-limiting factor in the normal cellular translational machinery. Phosphorylation by PKR prevents recycling of eIF-2–GTP from its guanosine 5'-diphosphate-bound form, thereby inhibiting viral and cellular protein synthesis (Schroder *et al.*, 2004) resulting in rapid inhibition of translation (Stark *et al.*, 1998).

1.2.5.2 Antimicrobial functions

One of the most important effects of IFN-γ on macrophages is the activation of microbiocidal effector functions. Macrophages activated by IFN-γ display increased pinocytosis and receptor-mediated phagocytosis as well as enhanced microbial killing ability. IFN-γ-activated microbiocidal ability includes induction of the NADPH-dependent phagocyte oxidase (NADPH oxidase) system, priming for NO production, tryptophan depletion and upregulation of lysosomal enzymes promoting microbe destruction (Decker *et al.*, 2002).

Macrophages kill bacteria, viruses, protozoa, helminthes, fungi and tumor cells primarily by production of ROS and reactive nitrogen intermediates (RNI) via induction of the NADPH oxidase system and iNOS, respectively (Stark *et al.*, 2005).

IFN-γ also promotes microbe destruction by augmenting surface expression of the high-affinity FcγRI on mononuclear phagocytes, promoting antibody-dependent, cell-mediated cytotoxicity. Complement-mediated phagocytosis is also upregulated by IFN-γ through increased complement secretion and complement receptor expression on mononuclear phagocytes (Decker *et al.*, 2002).

1.2.5.3 Immunomodulation and leukocyte trafficking

The ability of IFN-γ to coordinate the transition from innate immunity to adaptive immunity distinguishes it from the other IFNs. Mechanisms by which IFN-γ coordinates this transition include aiding in the development of a Th1-type response, directly promoting B cell isotype switching to IgG2a, and regulation of local leukocyte-endothelial interactions (Collins *et al.*, 1993)

Another important action is the ability of IFN-γ to augment the expression of the adhesion receptor ICAM-1 on vascular endothelial cells, leading to an increased adhesion of LFA-1 expressing cells. In this activity too, IFN-γ acts cooperatively with TNF-α or IL-1, leading to the recruitment of lymphocytes to a local inflammatory site (Boehm *et al.*, 1997).

Unstimulated leukocytes cycle continuously between the blood and the lymph. IFN-γ and NO produced at the site of inflammation cause local dilation of the blood vessels, thereby decreasing the local blood flow rate and causing gathering of blood in leaky vessels. Specific leukocyte subsets are instructed by the cytokine/chemokine milieu to extravasate into the tissue via interactions between adhesion molecules presented on leukocyte and endothelial surfaces. IFN-γ regulates this process by upregulating expression of chemokines (e.g. IP-10, MCP-1, MIG, MIP-1α/β, RANTES) and adhesion molecules (e.g. ICAM-1, VCAM-1) (Boehm *et al.*, 1997).

Many IFN-γ-inducible genes are also TNF-α-inducible, and these genes are often superinduced by the combination of these factors TNF-α is a macrophage-derived cytokine secreted in response to LPS, which can act in an autocrine manner to mediate many LPS-induced effects via NF-κB. IFN-γ priming of TNF-α responses is responsible for the priming of a subset of LPS-induced genes. In many cases, synergistic induction by the combination of these factors may be a result of the combined presence of STAT1 and NF-κB-binding sites in the promoter elements of responsive genes (Pine, 1997). Synergy may also be a result of cross talk between the IFN-γ and TNF-α signaling pathway.

Synergy between LPS- and IFN-γ-induced transcription factors in expression of target genes also contributes to the priming phenotype. Genes such as IRF-1, IP-10, ICAM-1, and iNOS contain STAT1 and NF-κB-binding sites in their promoter, and maximal transcription requires both signals. This is a demonstrated in IFN-γ-dependent gene hyperinduction in response to LPS for a number of genes and is likely to be a global mechanism for the synergistic, coordinate regulation of large coexpression groups (Gao *et al.*, 1997).

1.2.6 Interferon γ in disease and therapeutic application

A better understanding of the roles of IFN-γ in the intact organism is emerging from studies with animals in which either the gene encoding IFN-γ or the gene encoding the IFNGR1 chain of the IFN-γ receptor has been inactivated by gene targeting (Huang *et al.* 1993).

IFN-γ −/− and IFNGR1−/− mice showed no overt developmental defects, and their immune system appeared to develop normally. However, these mice show deficiencies in natural resistance to bacterial, parasitic and viral infections. The lack of functional IFN-γ is a decreased ability to resist infections with Listeria, Mycobacteria, with parasites such as Leishmania and viruses (Kamijo *et al.*, 1993).

Other changes seen in those animals include an increased resistance to the toxic action of bacterial LPS and a decreased production of some other cytokines such as TNF-α, IL-1, IL-6, and IL-12 (Vilcek *et al.*, 1998)

Patients with inactivating mutations of the human IFNGR1 or IFNGR2 chains show clinical indications similar to the mouse models. Human loss-of-function mutations in the IFNGR1 or IFNGR2 chain are closely associated with severe susceptibility to poorly virulent mycobacteria (Doffinger *et al.*, 2000).

In addition to recurrent infection, infants with deficient production of IFN-γ exhibited decreased neutrophil mobility and NK cell activity, highlighting the importance of IFN-γ in the inflammatory response and immunoregulation. It is interesting that natural IFN-γ polymorphisms have been correlated with increased longevity. It has been proposed that a slightly dampened inflammatory status caused by an IFN-γ polymorphism, which is not able to significantly affect the individual's ability to clear infections, may prevent inflammation-related diseases such as cardiovascular disease, neurodegeneration and diabetes (Campell *et al.*, 1991, Lio *et al.*, 2002).

Allergic diseases have been closely related to the Th2 immune responses, which are characterized by high levels of interleukin IL-4, IL-5, IL-9 and IL-13. These cytokines are key players in the development of chronic allergic inflammatory disorders, usually characterized by hyperresponsiveness, reversible obstruction and inflammation of the airways. Accumulating evidence have shown that altering cytokine-producing profile of Th2 cells by inducing Th1 responses may be protective against Th2-related diseases such as asthma and allergy. Interferon γ, the principal Th1 effector cytokine, has shown to be crucial for the resolution of allergy-related immunopathologies. In fact, reduced production of this cytokine has been correlated with severe asthma (Teixeira *et al.*, 2005).

The suppressive effects of IFN-γ on allergic diseases have been shown to be mediated by various mechanisms such as the regulation of allergen presentation to T lymphocytes, differentiation of naïve T cells toward Th1 phenotype and inhibition of Th2 cell differentiation, inhibition of effector cell recruitment to the site of inflammation, induction of apoptosis in T cells and eosinophils, blockage of IgE isotype switch in B cells and induction of nitric oxide (NO) production.

In fact, the potent inhibitory property of IFN-γ on Th2 responses and allergic inflammation has suggested that it might be a possible treatment approach in such diseases. However, initial studies have shown an unexpected toxicity and several side effects related to IFN-γ administration to allergic patients (Teixeira *et al.*, 2005).

In view of its other immunoregulatory actions, recombinant IFN-γ has been tested in humans. Many of the trials were in patients with malignancies. The value of IFN-γ in neoplastic diseases has not been confirmed in controlled clinical studies. The use of IFN-γ for the treatment of some chronic infectious affecting macrophages (leishmaniasis, toxoplasmosis, tuberculosis) is under consideration. In Germany, low doses of IFN-γ are licensed for the treatment of patients with rheumatoid arthritis (Vilcek *et al.*, 1998).

1.3 Mechanisms in regulation of Interferon γ expression

1.3.1 Cellular source of Interferon γ

Interferon γ is a pleiotropic cytokine that plays an essential role in both the innate and the adaptive phase of the immune response. Natural killer (NK) cells, $CD8^+$ and $CD4^+$ Th1 cells are the most potent, but not the only, source of IFN γ. A number of studies have identified additional IFN-γ-secreting cell types, including macrophages, dendritic cells, naïve CD4 T cells and even B cells (Szabo *et al.*, 2004).

1.3.1.1 Natural Killer (NK) cells

NK cells constitute a population of bone marrow-derived, low-density, large granular lymphocytes that make up 10–15 % of circulating PBMC, but in some organs, e.g., the liver, they represent up to 45 % of tissue-infiltrating lymphocytes (Whiteside *et al.*, 1994). Phenotypically, they are defined phenotypically by their expression of CD56 and lack of CD3 (Gong *et al.*, 1994).

Natural killer (NK) cells are lymphocytes of the innate immune system that are involved in the early defense against microbial infection or tumor transformation. Thus, playing an important role in host defense against pathogens and malignancy (Cooper *et al.*, 2001). They are capable of spontaneously killing tumor and virus-infected cells, which have downregulated one or more major histocompatibility complex (MHC) molecules and/or expressed certain stress antigens on their surface. They kill target cells without MHC restriction and prior activation. By killing virus-infected cells and causing the release of proinflammatory substances, e.g., TNF-α, NK cells provide the necessary signal to the immune system for inducing virus-specific immunity (Ahmad *et al.*, 2003).

Upon stimulation, NK cells secrete large amounts of cytokines including Interferon γ (IFN-γ), tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and chemokines such as CC chemokine ligand 3 (CCL3), macrophage inflammatory protein 1-α (MIP1-α), CCL4 (MIP1-β) and CCL5 (RANTES)(Cooper *et al.*, 2001).

1.3.1.2 Natural killer cells – functions in host defense

Natural killer (NK) cells constitute the first line of host defense against invading pathogens. They are activated in an early phase of a viral infection. NK cells are also cytotoxic, namely by inducing apoptosis of cells recognized as targets. NK cells identify their targets through a set of activating or inhibitory receptors that recognize pathogen-encoded molecules, self-proteins whose expression is upregulated in transformed or infected cells, or self-proteins that are expressed by normal cells but downregulated by infected or transformed cells (Ahmad *et al.*, 2003).

The production cytokines and chemokines are important in initiating an inflammatory response and in determining the nature and strength of the pathogen-specific immunity. NK cells represent a major source of IFN-γ in addition to activated T cells. An immediate production of this cytokine from NK cells is a crucial factor in inducing effective antiviral, cellular immunity in the host. In addition to directly killing virus-infected cells by releasing cytotoxic molecules such as perforin and granzymes, NK cells also kill target cells by Fas/FasL, TNF-α, and TRAIL/DR-4 and DR-5 interactions. The NK cell-secreted, soluble factors such as IFN-γ and TNF-α also play a role in inhibiting virus replication by inducing an antiviral state in host cells. Furthermore, NK cells play important immunoregulatory roles by interacting with T and B cells and APC (Cooper *et al.*, 2001).

In vivo studies in animal models have demonstrated that depletion of NK cells may result in the inability of the host to control viral infections (Walzer *et al.*, 2005).

The infected host usually responds to a viral infection by enhanced NK cell activity. Viruses may directly activate NK cells by encoding a viral protein that is recognized by an activating receptor on their surface; Viruses can also activate NK cells indirectly by inducing expression of stress-inducible proteins or cytokines in the host. The virus-induced cytokines that activate NK cells include type 1 IFN (IFN-α and -β), IL-15, IL-18, IL-12, and IL-21. These cytokines positively affect different aspects of NK cell activation, proliferation and survival (Walzer *et al.*, 2005).

In summary, NK cells were originally identified by their ability to kill malignant and virally infected cells without prior sensitization. However, the complexity of balancing signals required to control NK cell function and Interferon γ production highlights the importance in host defense and their immunomodulatory properties.

The next chapter deals with one of these signals, Interleukin 12, the most important activator of NK cells and Interferon γ production.

1.3.2 Regulation of Interferon γ production by Interleukin 12

Interleukin 12 is one of the most efficient inducers of IFN-γ gene expression, either acting alone or in synergy with other cytokines (Young *et al.*, 1997).

1.3.2.1 Interleukin 12 – characteristics and biological effects

Interleukin 12 is a heterodimer composed of two disulfide-linked subunits, p35 and p40 encoded on different chromosomes. The p40 subunit is homologous to cytokine receptors, whereas the p35 subunits are similar to other soluble cytokines comprising four α-helices such as IL-6 and granulocyte colony-stimulating factor (CG-CSF). When coordinately expressed within a cell, biologically active p70 is secreted. This cytokine is produced by a variety cells including monocytes, neutrophils and B cells, but the major producer of IL-12 are macrophages and dendritic cells (Trinchieri *et al.*, 1994).

It is induced by pathogenic organisms, including Gram-positive and Gram-negative bacteria, parasites, viruses and fungi. Microbial products induce T cell-independent production of IL-12 by cells of the innate immune system via Toll-like receptor signaling (Watford *et al.*, 2004).

IL-12 is also produced in a T cell-dependent manner through the engagement of antigen-presenting cells with CD40 ligand on T cells (Jacobson *et al.*, 1995).

IL-12 regulates both the innate and adaptive immunity. A major action is the induction of IFN-γ production in NK, T cells, B cells and even antigen-presenting cells. IFN-γ promotes cell-mediated immunity, which in turn is essential for the response to intracellular pathogens. IL-12 also induces T cell proliferation and enhances the cytolytic activity of NK and T cells (Trinchieri *et al.*, 1994).

The development of naïve CD4 T cells into either Th1 or Th2 cells that produce IFN-γ or IL-4 is a process that is essential for an effective adaptive immune response. IL-12 is the main cytokine that regulates the Th1 differentiation (Watford *et al.*, 2004).

1.3.2.2 The Interleukin 12 signal transduction pathway

Interleukin 12 signals through the IL-12 receptor complex composed of the IL-12Rβ1 and the IL-12Rβ2 chains (Szabo *et al.*, 2003), which are structurally related to the type I cytokine receptor superfamily. The affinity of IL-12 for either subunit alone is low, but coexpression of both subunits generates human IL-12 high-affinity binding sites. IL-12p40 interacts predominantly with the β1 subunit, whereas p35 interacts with the β2 subunit. IL-12 receptor subunits are expressed on T cells, natural killer cells and DC. Receptor expression correlates with cellular responsiveness to IL-12 (Watford *et al.*, 2004).

Like other cytokine receptors, IL-12 subunits lack intrinsic enzymatic activity. Instead, IL-12Rβ1 binds the Janus kinase (Jak) family member Tyk 2, whereas IL-12Rβ2 associates with Jak2 (Presky *et al.*, 1996). Signal transduction by IL-12 is initiated by ligand-induced autophosphorylation and transphosphorylation of receptor-associated Jaks, and the Jaks in turn phosphorylate tyrosines located in the intracellular domain of the receptor subunits. These phosphorylated tyrosines serve as docking sites for signal transducers and activators of transcription (STAT) and potentially other signaling molecules (Watford *et al.*, 2004).

There are seven members of the STAT family of transcription factors that share a number of features critical for transcription factor functions: an N-terminal STAT dimerization domain, a DNA-binding domain, a Src homology (SH2) domain, a conserved tyrosine residue and a C-terminal transactivation domain. One of the important STATs activated by IL-12 is STAT4 (Watford *et al.*, 2004).

Upon cytokine stimulation, STAT4 becomes phosphorylated on a conserved tyrosine residue and forms dimers through the intermolecular association of the SH2 domain of one STAT molecule with a conserved phosphotyrosine of another (Watford *et al.*, 2004).

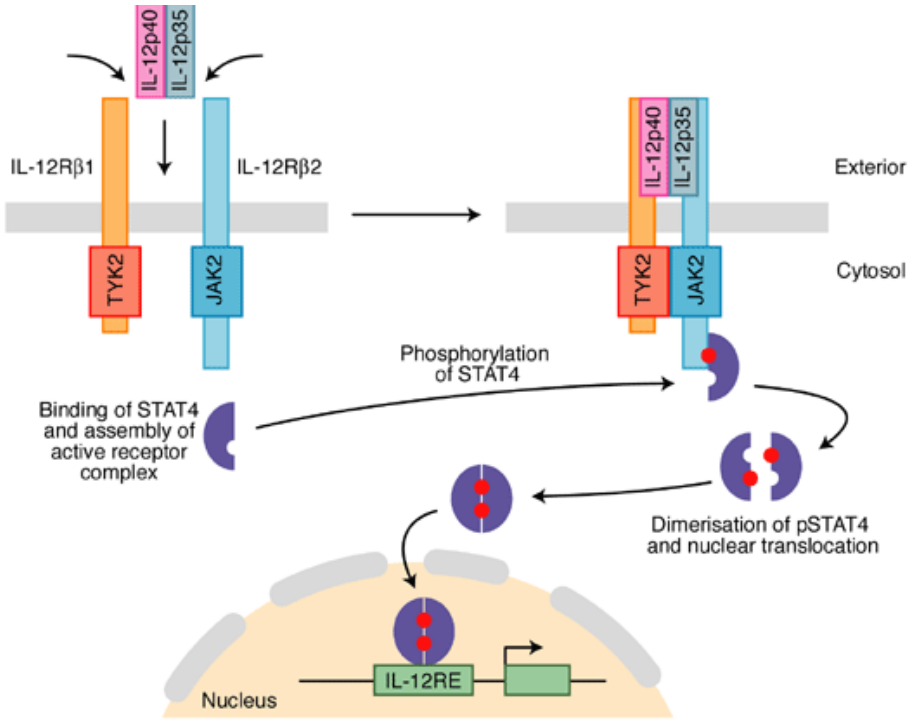


Figure 1-3. Signaling pathway of Interleukin 12 (Lammas *et al.*, 2003)

Only as dimers, STATs can translocate to the nucleus and bind to DNA. Apart from direct binding of STAT4 to response elements, transcriptional activation can also require other transcription factors. STATs can form complexes with these transcription factors and initiate or suppress transcription (Shuai *et al.*, 2003).

1.3.2.3 Inhibitors of IL-12-induced Interferon γ production

During many infections, there is an initial peak of NK cell activity followed by a dramatic decrease a few days later This is also seen in clinical trials in which the treatment with a single dose of IL-12 augmented both the cytolytic activity and IFN-γ production in cancer patients. Following IL-12 treatment, however, led to a dramatical decrease in cytokine production indicating a loss of responsiveness, which contributes to an ineffectiveness of IL-12 treatment in clinical trials (Wang *et al.*, 2000).

At least two cytokines (IL-10 and TGF-β) have been shown to play a role in the inhibition of NK cell responses during infection. TGF-β, a product of many cell types including macrophages and NK cells, has been shown to inhibit NK cell IFN-γ production (Liebermann *et al.*, 2002).

Therefore, TGF-β is described as an immunosuppressive antiinflammatory cytokine. The inhibition of IFN-γ gene expression is mediated via binding of SMAD (Small Mothers Against Decapentaplegic) proteins to the IFN-γ promotor region. In addition, TGF-β inhibits T-bet activity leading to an indirect inhibitory effect on IFN-γ production (Young 2006).

The situation with IL-10 is more complex since both inhibitory and stimulatory effects have been attributed to this cytokine. In contrast to the inhibitory effects of IL-10 on accessory cell functions, IL-10 has immunostimulatory effects on NK cell proliferation and cytotoxicity. In combination with IL-18, it even augments the ability of NK cells to produce IFN-γ (Liebermann *et al.*, 2002).

In addition, type I IFNs are potent inhibitors of IL-12 production. The effects of IL-10 and of type I IFNs (which belong to the same cytokine superfamily) seem to depend on the applied individual model and system (Liebermann *et al.*, 2002).

The suppressor of cytokine signaling family (SOCS) is known to inhibit intracellular pathways of a number of cytokines. In case of IL-12/IFN-γ, SOCS-1 has been shown to inhibit both signaling pathways (Eyles *et al.*, 2002). In addition, SOCS-3 has been reported to be a negative regulator of IL-12 signaling by preventing STAT 4 binding to the IL-12Rβ2 subunit (Yamamoto *et al.*, 2003).

Another family of transcriptional inhibitors, the protein inhibitors of activated STATs (PIAS) also negatively regulates cytokine signaling. Binding of PIAS to STAT4 is preventing STAT4-dependent gene transcription (Arora *et al.*, 2003, Shuai *et al.*, 2003).

1.3.3 Regulatory pathways

1.3.3.1 Cofactors of IL-12-induced Interferon γ production

Although IL-12 alone can stimulate NK cells to produce IFN-γ, when in case of combination with other cytokines it has more potent effects, leading to the production of high levels of IFN-γ.

TNF-α alone fails to stimulate NK cell production of IFN-γ, but when combined with IL-12, a potent synergy occurs (Lieberman *et al.*, 2002).

Other cytokines related to acute inflammation are also stimuli that synergize in NK cell activity. The closely related proinflammatory cytokines IL-18 and IL-1 can both enhance IL12-induced production of IFN-γ *in vitro* with IL-18 being much more potent than IL-1. In some infections, NK cell production of IFN-γ has a 95 % reduction in IL-18−/− mice (Fehninger *et al.*, 1999, Lieberman *et al.*, 2002).

Other cell surface ligands have shown to enhance NK cell responses and IFN-γ production. One involved in the regulation of innate responses is CD44 that is able to enhance the production of IL-12 and IFN-γ. Following infections NK cells express the activated form of CD44, which is able to bind low molecular weight hyaluronic acid, normally found at sites of inflammation, suggesting a possible mechanism that regulates innate resistance at local site of infection (Lieberman *et al.*, 2002).

A common feature of many stimuli that enhance the effects of IL-12 is the activation of NF-κB family of transcription factors. Thus IL-1, IL-18, TNF-α, as well as signaling through CD44 is dependent on NF-κB. Since there is an NF-κB binding site in the promoter of IFN-γ, it can be concluded that both STAT4 and NF-κB are required in the production of high levels of IFN-γ (Lieberman *et al.*, 2002).

1.3.3.2 Induction of Interferon γ independently of IL- 12/STAT4

IL-12 and STAT4 play a critical role in the activation of NK cell responses and resistance to infections. However, IL-12/STAT4 independent pathways lead to NK cell production of IFN-γ.

Other transcription factor, such as NF-κB (as described above) and the T-box transcription factor, T-bet have been implicated in the NK cell IFN-γ gene expression (Lieberman *et al.*, 2002).

Two other cytokines are potent IFN-γ inducers: IL-2 and IL-15. Like IL-12, IL-2 also utilizes the Jak/STAT pathway, but primarily activates STAT3 and STAT1. However, in NK cells IL-2 is able to phosphorylate STAT4, which correlates with the ability of IL-2 to enhance IFN-γ production (Lieberman *et al.*, 2002).

Another cytokine that mediates similar biological functions as IL-12 is IL-23, secreted by activated dendritic cells. IL-23 is a heterodimer of the p40 subunit of IL-12 and a subunit p19 (Oppmann *et al.*, 2001). The IL-23 receptor complex consists of an IL-12Rβ1 chain and an IL-23R chain related to IL-12Rβ2 and gp130. The Jak/STAT pathway is utilized in IL-23 signaling with activation of Jak2 and Tyk2 and subsequent STAT1 and STAT3, STAT4 and STAT5 activation. IL-23 is not able to initiate the Th1 differentiation but may be involved in sustaining IFN-γ production (Szabo *et al.*, 2004).

1.3.4 Regulation of Interferon γ gene expression

The IFN-γ gene is remarkably similar in genomic structure among all species where DNA was cloned and sequenced. The intron and exon sizes are very similar between species too, and interestingly, the promoters are even more conserved than the exons (Young 1996).

1.3.4.1 Methylation of the IFN-γ promoter region

Promoter DNA methylation is a major epigenetic mechanism for silencing genes and establishing commitment in cells differentiating from their precursors (Tong *et al.*, 2005). DNA methylation changes chromatin structure and possibly inhibits the recruitment of transcription factors to the target genes. In fact, certain transcription factors (i.e., CREB) bind with a low affinity to the methylated recognition elements, and this reduction in affinity has been correlated to low promoter activity (Yano *et al.*, 2003). In the conserved promoter region of IFN-γ, there are a number of potential methylation sites. In the human IFN-γ promoter, there is a conserved CpG target for methylation at position -54 critical for promoter functions. The importance of this site is reflected in the fact that it is present in mouse, rat and canine promoters, indicating that it has been conserved through evolution (Young 1996). In analysis of the murine $CD4^+$ T helper cells, a correlation was observed between the degree of methylation and IFN-γ gene expression. In addition to the data obtained with the murine T cells, a similar correlation between gene expression and IFN-γ promoter methylation was observed in primary human T cells (Visconti *et al.*, 2000).
The consequences of promoter methylation were also observed in the recruitment of transcription factors to the responsive regions. Among several transcription factors that have been shown to recognize the methylation-sensitive promoter region of the IFN-γ gene, the recruitment of phospho-CREB and CREB to the promoter were significantly lower in Th2 cells as compared with Th1 cells. In summary, the methylation of the promoter plays an important role in the regulation of the IFN-γ gene expression (Yano *et al.*, 2002).

1.3.4.2 Enhancer and silencer elements in the IFN-γ DNA

The levels and cell specificity of gene expression are generally controlled by distal regulatory modules (enhancers and silencers) that may be located in introns or intergenic regions and are able to potentiate or repress gene transcription. Typically, multiple regulatory modules are associated with a single gene, and different combinations of modules are used to control gene expression in different cell types at different developmental stages or under different conditions of stimulation (Lee *et al.*, 2001).

Hardy *et al.* identified a DNAse-hypersensitive site in the promoter and first intron, indicating that specific regions of the genomic DNA are accessible to DNASe I. The presence of these regions suggests that they are involved in the transcriptional control of the IFN-γ gene (Hardy *et al.*, 1989).

Sequence analysis revealed the presence of clustered binding sites for three relevant transcription factors, the Th1-restricted transcription factor T-bet and the inducible transcription factors NFAT and AP-1. Lee *et al.* (2001) confirmed by chromatin immunoprecipitation (ChIP) assays that the highly conserved non-coding sequences were occupied by these transcription factors and that the region possessed functional enhancer activity.

1.3.4.3 Transcription factors in IFN-γ gene expression

Transcriptional control of IFN-γ is mediated by multiple positive and negative regulatory elements located proximal and distal to the 5'-end of the coding sequence and within the first intron of the IFN-γ gene (Young *et al.*, 2004). These elements bind a number of transcription factors, including NF-κB, NFAT, STATs, T-bet, AP-1, CREB-ATF, GATA-3 and Ying-Yang 1, all of which cooperate to regulate induction of IFN-γ gene expression (Kiani *et al.*, 2001, Malmgaard *et al.*, 2004).

The number of regulatory sites and transcription factors discovered lately to be involved in IFN-γ gene expression is still growing and makes it quite difficult to fully understand IFN-γ gene regulation. In addition, the requirement of transcription factors is depending on both cell type and inducing signal (Sica *et al.*, 1997).

1.3.4.3.1 Nuclear Factor kappa B (NF-κB)

At least seven NF-κB binding elements have been identified in the human IFN-γ genomic DNA. These elements are present in the promoter and first, second and third intron. The importance of the NF-κB proteins in regulating IFN-γ expression is dependent on the extracellular signals that the T cell or natural killer cell receives (Young, 1996).

NF-κB is often activated by cell stimuli that synergize with IL-12. The combination of STAT4 activation by IL-12 and NF-κB by IL-18 is likely to be required for optimal IFN-γ production. Other cytokines that are able to influence IFN-γ production such as IL-1 and TNF-α also act through NF-κB activation as well (Lieberman *et al.*, 2002).

NF-κB can indirectly promote IFN-γ production through expansion of cells producing the cytokine or through induction of IFN-γ inducing cytokines such as IL-2 (Malmgaard, 2004).

1.3.4.3.2 T-box expressed in T Cells (T-bet)

T-bet is the only known member of the T-box family of transcription factors specifically expressed in the lymphoid system, with its expression restricted to the spleen, thymus, lymph node and lung. All T-box transcription factors contain a 200-amino acid DNA-binding domain called the T-box. Genes within the T-box family are not only conserved across diverse species, but have also been maintained throughout evolution (Szabo *et al.*, 2003).

T-bet is known to activate the IFN-γ promoter both in NK and Th1 cells. Several T-bet-responsive elements in the promoter region of IFN-γ have been identified (Maalmgaard, 2004).

In CD4 T cells, T-bet is rapidly and specifically induced in developing Th1 but not Th2 cells. T-bet expression appears to be controlled by two signaling pathways: the TCR and the IFNγR/STAT1 signal transduction pathways, but not by the IL-12/STAT4 pathway. Thus, IFN-γ from diverse sources such as NK cells, macrophages and dendritic cells induces expression of T-bet, which can cause chromatin remodeling of the IFN-γ gene. This increase in local IFN-γ causes a positive feedback loop, promoting Th1 differentiation (Szabo *et al.*, 2004).

T-bet also induces IL-12Rβ2 chain expression, followed by optimal IFN-γ production through IL-12/STAT4 signaling (Mullen *et al.*, 2001).

1.3.4.3.3 Nuclear Factor of Activated T cells (NFAT)

Transcription factors of the nuclear factors of activated T cell (NFAT) family were originally identified in lymphocytes as a nuclear complex binding to the interleukin-2 (IL-2) promoter in activated T cells (Shaw *et al.*, 1988). Until today five members of the NFAT family have been isolated and characterized (NFAT1−5). Their expression is not limited to T cells, since one member is present in almost every cell type including other cells of the immune system as well as non-immune cells (Hogan *et al.*, 2003). Four of these proteins are regulated by calcium signaling. The only non-calcium-regulated NFAT-protein is NFAT5 (Lopez-Rodriguez *et al.*, 1999).

NFAT1, NFAT 2 and NFAT4 are expressed in by T cells and their role in the regulation of T cell function and development has been extensively characterized.

The activation of NFAT proteins is induced by receptors coupled to the calcium-signaling pathway, such as the antigen receptors that are expressed by T- and B cells, the Fcγ receptors that are expressed by monocytes and natural killer cells, or the Fcε receptors that are expressed by mast cells (Macian, 2005).

In Th cell differentiation NFAT proteins act together with STAT proteins to determine the Th1/Th2 lineage. During early stages of naïve T cell activation NFAT proteins bind to the promoters of both IFN-γ and IL-4, after T cell activation has been initiated in either direction, the inappropriate locus is progressively silenced. This cell-type specific binding is accompanied by changes in chromatin accessibility and histone acetylation (Avni *et al.*, 2002).

Whether individual NFAT proteins have selective roles in Th cell activation is quite controversial. Since the loss of NFAT1 promotes Th2 differentiation and NFAT1 deficiency impairs IFN-γ production leading an increase in IL-4 production and increased IgG1 and IgE titers. By contrast, NFAT2-deficient T cells show impaired production of IL-4 and other Th2 cytokines, indicating that NFAT2 is required for Th2 promotion, whereas NFAT1 and NFAT4 promote Th1 activation. However constitutively active forms of NFAT1 and NFAT2 are able induce the transcription of Th1 and Th2 cytokines (Kiani *et al.*, 2001, Monticelli *et al.*, 2002).

1.3.4.3.4 GATA-binding protein 3 (GATA-3)

A binding site for GATA-3 has been found in the promoter region of IFN-γ, and binding to this regulator element is correlated with the inhibition of IFN-γ gene expression in developing Th1 cells. In addition, this gene regulation is independent of GATA-3-induced IL-4 expression (Malmgaard 2004).

1.3.4.3.5 Small Mothers Against Decapentaplegic (SMAD)

In case of infections with Gram-negative organisms and obligate intracellular pathogens, both pro- and antiinflammatory cytokines are released to modulate IFN-γ expression by NK cells. TGF-β is an immunosuppressive cytokine implicated in the negative regulation of IFN-γ. SMAD proteins are regulators of transcription, which transduce signals from TGF-β receptors. In most cases, TGF-β signaling is accomplished by SMAD2, SMAD3 and SMAD4 (Yu *et al.*, 2006).

The interaction of pro- and antiinflammatory pathways is important for integrated responses to the cell's total output: proinflammatory cytokines such as IL-12, IL-15 and IL-18 (important in IFN-γ expression) antagonize antiinflammatory TGF-β signaling by downregulating TGF-βRII, SMAD2 and SMAD3 (Carson 1995). On the other hand, TGF-β was found to repress monokine-induced IFN-γ gene expression and T-bet expression via SMAD-depending signaling (Yu *et al.*, 2006).

1.3.4.3.6 Activator Protein 1 (AP-1)

The AP-1 family of transcription factors consists of several Fos- and Jun-related proteins. Dimers of proteins from the Fos and Jun family bind to AP-1-responsive DNA sequences via leucin zipper structure. Heterodimers have higher DNA affinity and transcriptional activity than homodimers. AP-1 family members can also form complexes with other transcription factors, such as NF-κB and NFAT. AP-1-modulated gene expression can either result in activation or repression depending on the on the partner of dimerisation. The activation of AP-1 is regulated through kinases. In case of c-Jun phosphorylation by protein kinase C and MAPK c-Jun N terminal kinase (JNK) enhance DNA binding activity of c-Jun. AP-1 is known as an important transcription factor in immunocompetent cells, but its role in IFN-γ induction is quite controversial (Foletta *et al.*, 1998).

Barbulescu *et al.*, (1997) investigated the involvement of AP-1 in IFN-γ regulation. A complex of c-Jun/ATF-2 (activating transcription factor 2) seems to be involved in the modulation of IFN-γ gene expression. Upon stimulation with IL-18, phosphorylated c-Jun accumulated in the nucleus of mouse T cells leading to an enhanced c-Jun binding to the AP-1 element. In contrast, IL-12 showed no enhancement of c-Jun activation. Surprisingly, stimulation with both cytokines led to a far higher binding activity compared to the stimulation with IL-18 alone. The authors therefore conclude that IL-12 resulted in STAT4 phosphorylation and IL-18 induced c-Jun activation. Furthermore, the dimeric complex of STAT 4 and c-Jun was able to activated gene expression through binding to the AP-1 response element (Nakahira *et al.*, 2002).

Findings in the human NK3.3 cell line support a similar mechanism, where IL-2 and IL-12 in combination stimulate IFN-γ, but only IL-2 is able to induce the AP-1 transcription factor (Ye *et al.*, 1995). Subsequently, increased transcription activity and binding of AP-1 was only present in IL-2-stimulated cells (Azzoni *et al.*, 1996).

Regarding the fact that STAT4 was not found to bind to the IFN-γ promoter region, the authors favor a model were STAT4 is able to induce IFN-γ gene expression through the enhancement of AP-1-binding to the promoter site (Barbulescu *et al.*, 1998).

1.3.4.3.7 Yin-Yang 1 (YY-1)

Yin-Yang is a ubiquitous DNA binding protein with both enhancer and silencer activities, being able to bind to a highly conserved region in the IFN-γ promoter region. This region was characterized as a silencer element, able to bind a multiprotein-complex. The complex contained an AP-2 like protein and the DNA binding protein Yin-Yang (Young *et al.*, 2002).

Further studies have identified at least one more functional YY-1 binding site in the IFN-γ promoter. Additionally, this binding site overlaps with an AP-1 binding site.

The fact that overexpression of c-Jun compensated the repressive effect of YY-1, suggests a model, where binding of YY-1 suppresses IFN-γ expression in the absence of activating stimuli. Upon cell stimulation, the activation of AP-1 leads to the competition of AP-1 and YY-1 for the overlapping binding region. AP-1 displays YY-1 from the DNA and initiates gene expression (Ye *et al.*, 1996).

2 Objectives

Many studies conducted by different groups already described the immunomodulatory properties of alcohol. Acute ethanol treatment is associated with immunosuppression caused by changes in cytokine patterns. Interferon γ is considered to play a key role in alcohol-mediated cytokine expression.

The aim of the present study was to investigate the mechanisms that lead to alcohol-induced Interferon γ suppression. The question was whether ethanol is able to interfere with intracellular signaling molecules or transcription factors, if mRNA expression was influenced or if posttranscriptional mechanisms caused changes in Interferon γ production.

Consequently, the goal of the current study was to:

- find a suitable model to study the ethanol-related changes in cytokine expression
- induce cytokine production through specific stimulances
- study ethanol-related changes in cytokine production
- investigate intracellular signaling pathways and the effect of ethanol
- examine changes in mRNA-expression of IFN-γ
- determine intracellular IFN-γ accumulation
- elucidate posttranscriptional effects of ethanol
- identify the unknown protein that is able to either bind or degrade IFN-γ as a consequence of ethanol-treatment

3 Material and Methods

3.1 Material

3.1.1 Cell line and PBMC

Cells	Source
Buffy Coats (heparinized, cell-enriched)	Blutzentrale Katharinenhospital, Stuttgart, Germany
NK-92 cells, human natural killer lymphoma (ACC 488)	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany

3.1.2 Chemicals and reagents

3.1.2.1 Cells and cell culture

Product	Manufacturer
2-Propanol, ROTIPURAN® ≥ 99.8 %, p.a., ACS, ISO	Carl Roth GmbH & Co, Karlsruhe, Germany
Alpha Modification of Eagle's Medium, without Ribosides & Deoxyribosides, with Earle's salts, with L-Glutamine, endotoxin tested, sterile filtered	PAA Laboratories GmbH, Pasching, Austria
Biocoll (1.077 g/ml)	Biochrom Berlin, Germany
Carbon dioxide	Sauerstoffwerke Friedrichshafen, Germany
Fetal Calf Serum (FCS)	PAA Laboratories GmbH, Pasching, Austria
Horse Serum (HS)	PAA Laboratories GmbH, Pasching, Austria
Penicillin-Streptomycin (10 000 IU/ml – 10 000 µg/ml)	PAA Laboratories GmbH, Pasching, Austria
Phosphate Buffered Saline (PBS)	PAA Laboratories GmbH, Pasching, Austria
Recombinant Human Interleukin-2 (IL-2)	Pierce Biotechnology Inc., Rockford, USA
RPMI 1640, ohne Phenolrot, mit Glutamin	PAA Laboratories GmbH, Pasching, Austria
RPMI 1640, ohne Phenolrot, ohne Glutamin	PAA Laboratories GmbH, Pasching, Austria

3.1.2.2 Cell incubation experiments

Product	Manufacturer
Albumin, bovine serum, Fraction V, approx. 99 %, Protease-free	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Alpha Modification of Eagle's Medium, without Ribosides & Deoxyribosides, with Earle's salts, with L-Glutamine, endotoxin tested, sterile filtered	PAA Laboratories GmbH, Pasching, Austria
Dulbecco's PBS (phosphate buffered saline), without Ca & Mg	PAA Laboratories GmbH, Pasching, Austria
Ethanol, ROTIPURAN®, > 99.8 %, p.a.	Carl Roth GmbH & Co., Karlsruhe, Germany
LPS	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
monoclonal anti-human IFN-γ R1 Ab	R&D Systems Europe GmbH, Wiesbaden-Nordenstadt, Germany
Penicillin-Streptomycin (10000 IU/ml – 10000 µg/ml)	Life Technologies, Paisley, USA
PHA (Phytohemagglutinin)	PAA Laboratories GmbH, Pasching, Austria
Protease Inhibitor Cocktail for use in cell culture	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Protease Inhibitors - Aprotinin from bovine lung, cell-culture-tested - Bestatin hydrochloride - Leupeptin - trans-Epoxysuccinyl-L-Leucylamido-(4-Guanodino)Butane, E64 - Pepstatin A	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Recombinant human Interleukin 12	R&D Systems Europe GmbH, Wiesbaden-Nordenstadt, Germany
Recombinant human Interferon γ	R&D Systems Europe GmbH, Wiesbaden-Nordenstadt, Germany
Salmon Sperm DNA solution	Gibco Life Technologies, Eggenstein, Germany
SYTOX Green	Molecular Probes, Göttingen, Germany
Triethylamin	Riedel-de Haen, Seelze, Germany
Triton X-100	Boehringer Mannheim GmbH, Mannheim, Germany
Trypan blue solution, 0.4 %, liquid, sterile-filtered, cell-culture-tested	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

3.1.2.3 ELISA (Enzyme Linked Immunosorbent Assay)

Product	Manufacturer
Fetal Calf Serum (FCS)	PAA Laboratories GmbH, Pasching, Austria
H_2SO_4	Carl Roth GmbH & Co., Karlsruhe, Germany
Human Phospho-IFN-gamma R1 DuoSet IC	R&D Systems Europe GmbH, Wiesbaden-Nordenstadt, Germany
KCl	Merck KGaA, Darmstadt, Germany
KH_2PO_4	Carl Roth GmbH & Co., Karlsruhe, Germany
MBL™Human IL-18 ELISA kit	MEDICAL & BIOLOGICAL LABORATORIES CO., LTD, Nagoya, Japan
Na_2CO_3	Merck KGaA, Darmstadt, Germany
$Na_2HPO_4 \cdot 2\ H_2O$	Merck KGaA, Darmstadt, Germany
NaCl	Carl Roth GmbH & Co., Karlsruhe, Germany
$NaHCO_3$	Fluka Chemie AG, Buchs, Schweiz
OptEIA™ ELISA Sets - Human TNF-α - Human TGF-β - Human IL-2 - Human IL-10 - Human IL-12 - Human IFN-γ - Human IP-10	BD Bioscience, Heidelberg, Germany
OptEIA™ Substrate Reagent Set - Substrate Reagent A (Hydrogenperoxid) - Substrate Reagent B (Tetramethylbenzidin)	BD Bioscience, Heidelberg, Germany
Tween® 20	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

3.1.2.4 Fluorescence Activated Cell Sorting (FACS)

Product	Manufacturer
Dulbecco's PBS (phosphate buffered saline), without Ca & Mg	PAA Laboratories GmbH, Pasching, Austria
FCS (Fetal Calf Serum)	PAA Laboratories GmbH, Pasching, Austria
IOTest®-Ab - CD3-FITC/CD4-PE - CD3-FITC/CD16 + CD56-PE - CD45-FITC/CD14-PE - CD3-FITC/CD19-PE - CD3-FITC/CD8-PE - IG1-FITC/IgG1-PE	Immunotech, Marseille, France

PE-conjugated IgG1 Ab	Beckman Coulter
Phycoerythrin (R-Pe)-conjugated monoclonal anti human IL-12Rβ1 Ab Clone 69310	R&D Systems Europe GmbH, Wiesbaden-Nordenstadt, Germany
Phycoerythrin (R-Pe)-conjugated monoclonal anti-human IL-12Rβ2 Ab Clone 2B6	BD, Biosciences Pharmingen, San Diego, USA
Sodium azide	Merck AG, Darmstadt, Germany

3.1.2.5 Sample preparation (cell lysates, nuclear extracts)

Product	Manufacturer
β-Glycerol phosphate	Roth, Karlsruhe, Germany
Benzamidine	Roth, Karlsruhe, Germany
Dithiothreitol	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Dulbecco's PBS (Phosphate buffered Saline), without Ca & Mg	PAA Laboratories GmbH, Pasching, Austria
EDTA	Roth, Karlsruhe, Germany
Glycerol	Merck, Darmstadt, Germany
HEPES	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
KCl	Merck, Darmstadt, Germany
$MgCl_2$	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
NaF	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
NP-40 Alternative	Calbiochem, Bad Soden, Germany
Phenylmethanesulfonyl	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Protease-Inhibitors - Pefablock - Pepstatin - Chymostatin - Aprotinin - Leupeptin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium orthovanadate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sodium-Pyrophosphate	Merck, Darmstadt, Germany
Tris-HCL	Roth, Karlsruhe, Germany
Triton X-100	Merck, Darmstadt, Germany

3.1.2.6 Protein assay

Product	Manufacturer
BSA (bovine serum albumin)	Merck, Darmstadt, D
Bio Rad Protein Assay Dye Reagent concentrate	Bio-Rad Laboratories, München, D

3.1.2.7 Western blotting

Product	Manufacturer
6-Aminocapronacid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
APS	Carl Roth GmbH & Co, Karlsruhe, Germany
Blotting grade blocker non fat dry milk	Carl Roth GmbH & Co, Karlsruhe, Germany
BSA (Bovine Serum Albumin)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
GAPDH/anti mouse	kind gift of Ute Albrecht, Düsseldorf
Glycerol, approx. 95 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Glycine for electrophoresis, minimum 99 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Mercaptoethanol 38 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Methanol 100 %	Carl Roth GmbH & Co, Karlsruhe, Germany
NaCl	Carl Roth GmbH & Co, Karlsruhe, Germany
Phospho-c-jun (Ser63)(54B3) Rabbit mAb	Cell Signaling Technology®, Inc., USA
Phospho-STAT4 Rabbit polyclonal Ab	Zymed, San Francisco, USA
Pierce ECL Western Blotting Substrate	PIERCE, Rockford IL, USA
Polyacrylamide Mix 30 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Polyclonal Goat Anti-Rabbit Immunoglobulines/HRP	Dako Cytomation, Glostrup, Denmark
Rainbow Marker	Amersham, Buckinghamshire, UK
SDS, ultrapure, ≥ 99 %	Carl Roth GmbH & Co, Karlsruhe, Germany
STAT4 Rabbit polyclonal Ab	Santa Cruz Biotechnology, Santa Cruz, USA
TEMED (N,N, N',N'-Tetramethylenediamine)	Fluka, Buchs, Germany
Tris PUFFERAN®, ≥ 99 %, p.a.	Carl Roth GmbH & Co, Karlsruhe, Germany
Tween® 20	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

3.1.2.8 RNA-Isolation

Product	Manufacturer
2-Propanol, ROTIPURAN®, > 99,8 %, p.a., ACS, ISO	Carl Roth GmbH & Co., Karlsruhe, Germany
DEPC (Diethylpyrocarbonat), > 97 %	Carl Roth GmbH & Co., Karlsruhe, Germany
Ethanol, ROTIPURAN®, > 99,8 %, p.a.	Carl Roth GmbH & Co., Karlsruhe, Germany
Trichlormethan/Chloroform, > 99 %, DAB	Carl Roth GmbH & Co., Karlsruhe, Germany
TRIzol® Reagent	Invitrogen GmbH, Karlsruhe, Germany

3.1.2.9 cDNA-Synthesis

Product	Manufacturer
Dichloridphenyltrichlorethan (DTT)	Invitrogen GmbH, Karlsruhe, Germany
dNTP Mix (Deoxynucleotide Mix),	Eppendorf AG, Hamburg, Germany
First Strand Buffer (5 ×)	Invitrogen GmbH, Karlsruhe, Germany
Random Primer (3 µg/µl)	Invitrogen GmbH, Karlsruhe, Germany
Reverse Transcriptase (200 U/µl)	Invitrogen GmbH, Karlsruhe, Germany

3.1.2.10 PCR

Product	Manufacturer
10 × Hot-Master™-Taq-Buffer, pH 8.5; 25 mM Mg^{2+}	Eppendorf AG, Hamburg, Germany
18S Competimers (Classic II)	Ambion Inc., Austin, TX, USA
18S Primer Pair (Classic II)	Ambion Inc., Austin, TX, USA
2 × iQ™ SYBR® Green Supermix	Bio-Rad Laboratories GmbH, München, Germany
dNTP Mix (Deoxynucleotide Mix), 10 nM each dNTP	Eppendorf AG, Hamburg, Germany
Hot-Master™ Taq DNA Polymerase (5 U/µl)	Eppendorf AG, Hamburg, Germany
NucleoSpin® Extract II Kit	Macherey-Nagel GmbH & Co. KG, Düren, Germany

3.1.2.11 Electrophoresis

Product	Manufacturer
Ethidiumbromid	Carl Roth GmbH & Co., Karlsruhe, Germany
Boric acid	Carl Roth GmbH & Co., Karlsruhe, Germany
Track-It™ 100 bp DNA Ladder	Invitrogen GmbH, Karlsruhe, Germany

SeaKem® LE Agarose	Cambrex Inc., Rockland, ME, USA
Tris-(hydroxymethyl)-aminomethan	ICN Biomedicals GmbH, Eschwege, Germany
Ethylenediaminetetraacetic acid disodium salt dihydrate, for molecular biology, > 99 %	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

3.1.3 Equipment and expendable items

3.1.3.1 Generally used items

Product	Manufacturer
Beakers, volumetric flask, graduated cylinder, Erlenmeyer flask	Carl Roth GmbH & Co., Karlsruhe, Germany
Aluminum foil	
Still	Heraeus Holding GmbH, Hanau, Germany
SANOclav, autoclave	Wolf GmbH, Bad Überkingen-Hausen, Germany
Heat sterilization oven	Memmert GmbH + Co. KG, Schwabach, Germany
Digital balance 510-37 (± 10 mg)	Gottl. Kern & Sohn, Albstadt, Germany
Analytical balance (± 0.01 mg)	Sartorius, Wolfinger GmbH, Germany
Vortex-Genie 2	Scientific industries Inc., Bohemia N.Y., USA
Centrifuge 3200, table centrifuge	Eppendorf AG, Hamburg, Germany
Reaction tubes 1.5 ml, 2 ml	Eppendorf AG, Hamburg, Germany
Pipettes	Eppendorf AG, Hamburg, Germany
Multipette® plus	Eppendorf AG, Hamburg, Germany
Glass pipettes 10, 20 ml	Multimed Wicker GmbH, Kirchheim u. Teck, Germany
Disposable pipettes 10 ml, 20 ml	Sarstedt AG & Co., Nümbrecht, Germany
Disposable pipette tips	Sarstedt AG & Co., Nümbrecht, Germany
Pasteur pipettes	Hirschmann Laborgeräte. GmbH & Co, Eberstadt, Germany
Falcon Tubes, Cellstar® PP tubes with screw cap, 15 ml, 50 ml	Greiner, Frickenhausen, Germany
Parafilm M, LABORATORY FILM	Pechiney Plastic Packaging, Chicago, USA
Gentle Skin®, gloves	Rösner- Mautby Meditrade® GmbH, Kiefersfelden, Germany

3.1.3.2 Cell culture

Product	Manufacturer
Culture bottles 25 cm^2, 75 cm^2	Sarstedt AG & Co., Nümbrecht, Germany
Culture bottles 175 cm^2	Greiner Bio-One GmbH, Frickenhausen, Germany
MCO-20AIC Automatic CO_2 Incubator	Sanyo Electric Biomedical Co. Ltd., Bad Nenndorf, Germany
Fireboy plus, Bunsen burner	INTEGRA Biosciences AG, Chur, Switzerland
Pipettus®	Hirschmann Laborgeräte GmbH & Co, Eberstadt, Germany
Laminar flow Biosafe 2	Dipl.-Ing W. Ehret GmbH, Emmendingen, Germany

3.1.3.3 Cell incubation experiments

Product	Manufacturer
GS-6R centrifuge	Beckman Coulter GmbH Krefeld, Germany
Light optical microscope	Olympus, Hamburg, Germany
Neubauer counting chamber	Assistent, Kirchheim u. Teck, Germany
Disposable injection, sterile, 20 ml	B. Braun Melsungen AG, Germany
Filter unit Filtropur S0.2	Sarstedt AG & Co., Nümbrecht, Germany
TC-plate 24 well, sterile, with lid	Greiner bio-one Cellstar® GmbH, Frickenhausen, Germany
TC-plate 96 well, sterile, U-shape	Greiner bio-one Cellstar® GmbH, Frickenhausen, Germany
Lid For microplate, sterile, standard	Greiner bio-one Cellstar® GmbH, Frickenhausen, Germany
Combitips plus 1 ml, 5 ml	Eppendorf AG, Hamburg, Germany
Centrifuge 5417 R	Eppendorf AG, Hamburg, Germany
Plastic boxes with lids	Emsa Werke Wulf GmbH & Co, Emsdetten, Germany
BioFolie	Vivascience, Göttingen, Germany
Fluorstar	BMG, Freiburg, Germany

3.1.3.4 ELISA

Product	Manufacturer
Nunc-Immuno™microplatte (Maxi Sorp™Surface)	Nunc, Wiesbaden, Germany
Multi-channel pipette 300	Eppendorf, Hamburg, Germany
little plastic vessels	
ϕ 340 pH/Temperature Meter	Beckman Inc., Fullerton, CA, USA

3.1.3.5 FACS

Product	Manufacturer
BD FACSCalibur	BD Bioscience, Heidelberg, Germany

3.1.3.6 Sample preparation (cell lysates, nuclear extracts)

Product	Manufacturer
Cooling centrifuge 5417 R	Eppendorf AG, Hamburg, Germany

3.1.3.7 Protein assay

Product	Manufacturer
Cary 1 Bio UV-Visible Spectrophotometer	Varian, Inc., Darmstadt, Germany
Disposable kuvette (semimicro-) PMMA MB layer depth 10 mm	MBT BRAND, Gießen, Germany

3.1.3.8 Western Blotting

Product	Manufacturer
Mini Vertical Gel System	Neolab, Heidelberg, Germany
Electrophoresis Power Supply EPS 300 and 600	Pharmacia, Sweden
Electroblotting System, Semi-Dry Hep-1 Panther	Owl Separation Systems, Portsmouth, USA
Thermostat 3401	Eppendorf AG, Hamburg, Germany
PVDF-Membrane Hybond-P	Amersham, Buckinghamshire, UK
Hyperfilm™ ECL	Amersham, Buckinghamshire, UK
Whatman® chromatography paper	Whatman International Ltd., England

3.1.3.9 RNA-Isolation, cDNA-Synthesis and PCR

Product	Manufacturer
Cary 1 Bio UV-Visible Spectrophotometer	Varian Inc., Darmstadt, Germany
Mastercycler Gradient	Eppendorf AG, Hamburg, Germany
RoboCycler™ 40 Accessories	Stratagene, La Jolla, CA, USA
iCycler™ Thermal Cycler	Bio-Rad Laboratories GmbH, München, Germany
Electrophoresis Power Supply EPS 300	Pharmacia Biotech
Multi®-Tubes RNAse-/DNAse-free (2.0 ml; 1.7 ml, 0.65 ml)	Carl Roth GmbH & Co., Karlsruhe, Germany
Multi ™-Dolphin Tubes, certified RNAse/DNAse free (2.0 ml)	SorensonTM BioScience, Inc. Carl Roth GmbH + Co., Karlsruhe, Germany
Thin-wall 8-Tube Strip	Biozym Scientific GmbH, Oldendorf, Germany
Biosphere filtered Tips, sterile, non-pyrogenic, DNA-free, RNAse-free, ATP-free (10 µl; 100 µl; 1000 µl)	Sarstedt AG & Co., Nümbrecht, Germany
Digital camera E.A.S.Y 440K	Herolab GmbH, Wiesloch, Germany

3.2 Software

Product	Manufacturer
MS-Office	Microsoft GmbH
Corel Photo-Paint 8	Corel Inc., Canada
TINA 2.09g	Raytest Isotopenmessgeräte, Straubenhardt, Germany
iCycler-Software	Bio-Rad Laboratories GmbH, München, Germany
Statistica Version 6.0	StatSoft, Inc., Tulsa, USA
Slide Write Plus, Version 6	Advanced Graphics Software Inc., Carlsbad, USA
BMG-FluoStar 1.0	BMG, Offenburg, Germany
Photometer Software (easykin)	SLT, Crailsheim, Germany

3.3 Methods

3.3.1 Cells and cell cultivation

With respect to cell handling, cell culture procedure and cell incubation experiments, sterile working conditions were applied. Materials and equipment were primarily disinfected, sterilized or autoclaved before their application at the laminar flow. UV-light was turned on 30 minutes before and after using the laminar flow.

3.3.1.1 Preparation and activation of human Peripheral Blood Mononuclear Cells

For studying the effect of low ethanol concentrations on cytokine expression, we used PHA-activated or LPS-stimulated human Peripheral Blood Mononuclear Cells (PBMC). The change of cell composition within 4 day of PHA-activation was measured using flow cytometry as well as surface expression of to different IL-12 receptor subunits.

Cell culture medium		
RPMI 1640	500 ml	82.5 %
FCS (heat inactivated)	100 ml	16.5 %
Penicillin-Streptomycin (10 000 IU/ml – 10 000 µg/ml)	6 ml	

PBMC were isolated from buffy coats of healthy donors through gradient density centrifugation using Ficoll. In a first step, donor blood was diluted 1:2 using RPMI-Medium. The suspension was then layered carefully on 14 ml of Ficoll. After centrifugation at 400 g for 30 min (without brake), the interphase was carefully collected and diluted 1:1 with RPMI. PBMCs were washed twice and suspended in RPMI with glutamine. Cell number was determined and PBMC were cultured at a starting concentration of 1×10^6 cells/ml.

For T cell activation studies, PBMC were cultured in RPMI 1640 containing 16.5 % FCS, 2 % L-glutamine, 0.01 % penicillin-streptomycin and were stimulated with 2 µg/ml PHA at indicated times.

PHA solution (24 µg/ml)	
PHA	1.2 mg
RPMI-Medium	50 ml

3.3.1.2 NK-92 cell line

NK-92 cell line	
Cell type	human natural killer lymphoma
DSMZ no.	ACC 488
Origin	established from the peripheral blood of a 50-year-old man with non-Hodgkin lymphoma (large granular lymphocyte) in 1992; cells were described as having azurophilic granula and strong cytotoxic activity
Morphology	cells growing in clumps in suspension
Doubling time	ca. 40–50 hours

Human NK cell lymphoma NK-92, an interleukin-2 dependent cell line, is one of few cell lines with phenotype and functions similar to normal peripheral blood NK cells. NK-92 may serve as a model for NK cell cytokine studies. IL-2, IL-12 and IL-18 regulate NK cell cytokine production.
Since natural killer cells are the main source of Interferon γ, we established the NK-92 cell line in our laboratory. NK-92 cells were used to investigate the effect of different ethanol concentrations on Interferon γ protein synthesis, mRNA expression, transcription factor activation (AP-1, STAT4), intracellular accumulation and proteolytic degradation upon IL-12 stimulation.

Cell culture medium		
α-Modification of Eagle's medium, α-MEM	500 ml	75.1 %
FCS (heat inactivated)	83 ml	12.5 %
horse serum (heat inactivated)	83 ml	12.5 %
IL-2 solution	333 µl	
IL-2		10 ng/ml

Cultivation was carried out in a humidified atmosphere at 37 °C and 5 % CO_2. Cells were subcultured every two to three days and split with a density 1:5 or 1:10.

3.3.2 Cell incubation experiments

Before starting experiments, cell number and seeding density were determined. To obtain a sufficient amount of cells, cell number was increased by successively raising the size and number of cell culture bottle and volume of applied cell suspension and cell culture medium

The Neubauer counting chamber was used for determining the cell number. Therefore, the cells were spinned down for 10 minutes at 1500 rpm and afterwards the cell pellet was suspended in 10 ml starvation medium. For the counting procedure, cell suspension was diluted with Trypan blue solution in a proportion of 1:50. Cells of four squares were counted, each with a defined volume of 0.1 µl. Calculation of cell number per ml was as follows:

Cell number/ml = MV × DF × 10^4	
MV	counted cell number divided by number of counted squares
DF	dilution factor

For the incubation experiment, cell density was 2.5 × 10^6 cells/ml. Cell suspension was then diluted with starvation medium to obtain the required total volume for the corresponding experiment.

Starvation medium		
α-Modification of Eagle's medium, α-MEM	500 ml	
FCS (heat inactivated)	12.5 ml	2.5 %

After determination of cell density, cells were seeded in cell culture plates and underwent a starvation phase of 4–16 hours (depending on experiment and examined factor) in the incubator with 37 °C, 5 % CO_2 and 94 % humidity, where NK-92 cells were maintained in starvation medium in order to avoid the influence of IL-2, FCS and HS on IFN-γ production.

IL-12 stock standard		
IL-12	5 µg	0.02 %
BSA PBS solution 0.1 %	25 µl	

IL-12 incubation solution		
IL-12 stock standard diluted in α-MEM	1 : 111	1.8 µg/ml
BSA-PBS-solution 0.1 %	25 µl	

Ethanol incubation solution 36 ‰	
Ethanol, ≥ 99.8 %	227.7 µl
Starvation medium	47.7 ml

Ethanol incubation solution 24 ‰	
Ethanol incubation solution 36 ‰	2.7 ml
Starvation medium	1.4 ml

Ethanol incubation solution 12‰	
Ethanol incubation solution 36 ‰	1 ml
Starvation medium	2 ml

At the beginning of each experiment, cells were incubated at five different experimental conditions: negative control, i.e. neither treated with IL-12 nor ethanol, a positive control, i.e. cells treated exclusively with IL-12, and three conditions, where cells were treated with IL-12 and incubated either with 1 ‰, 2 ‰, or 3 ‰ ethanol. The final concentration of the IL-12 solution in each well was 75 ng/ml. Every experiment was performed in duplicates. Additionally, ethanol-treated cells were kept in alcohol vapor chambers.

Ethanol solution/alcohol vapor chamber		
deionized water	ethanol ≥ 99,8 %	
200ml	252 µl	1 ‰
200ml	506 µl	2 ‰
200ml	758 µl	3 ‰

These alcohol vapor chambers were plastic boxes with lids containing open beakers with an aqueous solution of the corresponding ethanol concentration. Additionally, the lids were equipped with a gas-permeable plastic foil allowing gas diffusion. Alcohol vapor chambers were kept in the incubator. The negative and positive controls were kept in the incubator. Samples were taken at indicated times. Regarding sample preparation and determination of IFN-γ levels, see section sample preparation.

IFN-γ solution		
IFN-γ standard	90 ng/vial	24 ng/ml
Starvation medium	3750 µl	

Experiments were protein stability and degradation was examined, IFN-γ was added to each sample at a final concentration of 2000 pg/ml.

3.3.3 Cell viability measurement

To confirm that applied ethanol concentrations were not toxic to isolated PBMC or NK-92 cells, cell viability was assessed using SYTOX Green Nucleic Acid Stain.

SYTOX Green solution		
Sytox Green (5 mM)	4.2 µl	10 µM
RPMI	ad 2100 µl	

Triton X-100 solution		
Triton X-100	2 ml	4 %
RPMI	48 ml	

DNA stock standard		
Ready mixed Salmon Sperm DNA	10 mg	1 %
Deionized water	1 ml	
DNA stock standard was aliquoted and stored at –20°C		

Principle. This method is based on the fact that polar nucleic acid stains such as SYTOX Green are cell-impermeant, i.e. excluded from live cells, but permeates cell walls of cells with damaged plasma membranes or dead cells by penetrating into the nucleus and staining DNA. In this case, SYTOX Green is used in combination with Triton X-100. After brief incubation of cells with the SYTOX Green nucleic acid stain, dead cells fluorescence bright green (λ emission = 530 nm) when excited with a 470–490 nm (λ absorption) source (Haugland edit. 2002). After an additional incubation with Triton X-100, a non-ionic detergent, which lyses cells (Ahn *et al.*, 1997), fluorescence of total DNA, is measured. The calculated difference between the two measurements of fluorescence corresponds to the number of SYTOX Green-negative (i. e. viable) cells.

Assay procedure. Cells with a density of 6.25×10^5/ml were seeded in 96-well plates and incubated with ethanol medium at a final concentration 0, 1, 2, or 3 ‰ for 24 hours in an incubator of 37 °C and 5 % CO_2. Because of the intended fluorescence measurement, colorless RPMI medium was used. The measurement was repeated 10-times per experimental condition. In addition, ethanol-treated cells were kept in ethanol vapor chambers as already described. After incubation, cells were spinned down at 800 rpm for 10 minutes and cell supernatants (200 µl) were removed. Regarding DNA measurement, serially diluted DNA standards (8 concentrations, starting with 3000 ng DNA/well or 75 µg/ml, dilution ratio 1:3) were prepared of ready mixed Salmon Sperm DNA solution and run with each measurement as well. After addition of 10 µl SYTOX Green to each cavity and an incubation time of 5 minutes fluorescence was measured at 485 nm for the first time. After an additional incubation for 45 minutes with 50 µl Triton X-100 per well, fluorescence was measured again.

Calculation of results. The amount of living cells was calculated via the difference between total DNA (SYTOX Green positive cells after Triton X-100 treatment) and DNA of initially dead cells (SYTOX Green positive cells before Triton X-100 treatment). The amount of DNA of dead cells was calculated by the help of a calibration curve with a sigmoid function supported by Slide Write plus software:

Calculation of cell number per ml was as follows:

$$\text{Fluorescence intensity} = a0 + a1/(1+\exp(-(c[\log(\text{DNA per well})] - a2)/a3))$$

Coefficients of this equation were determined by non-linear iterative regression (Levenberg-Marquardt Algorithms). Due to a finally relative evaluation in the end, the relative amount of DNA of living cells can be referred to as the relative amount of living cells.

3.3.4 ELISA – Enzyme-Linked Immunosorbent Assay

At the end of each cell culture experiment, supernatants were collected and stored at –80 °C. Interferon γ secretion in response to IL-12 stimulation and/or ethanol incubation in cell culture supernatant from human PBMC or NK-92 cells were measured by Enzyme-linked Immunosorbant Assay. Commercially available kits (Human IFN-γ BD OptEIA™ ELISA Set) were used according to the supplier's protocol. If necessary supernatants were diluted 1:10 or 1:50.

Principle. ELISA is a solid-phase enzyme immunoblotting assay using immobilized antibody for assessing the antigen content of a sample. The 'sandwich' or two-site ELISA requires two antibodies that bind to epitopes on the antigen. One antibody binds as 'capture' antibody to the bottom of the microwell plate. The added antigen from sample is able to complex with the antibody. A labeled second antibody ('detection' antibody) is necessary to detect this complex. The enzyme bound to the second antibody, in this case horseradish peroxidase, is able to react with added colorimetric substrate. The more antigen the sample contains, the more detection antibody is bound, and a greater color change can be measured as increasing optical density (Wilson and Walker 2005, Lottspeich and Zorbas 1998).

Coating buffer		
$NaHCO_3$	4.2 g	0.84 %
$NaCO_2$	1.78 g	0.356 %
Deionized water	ad 500 ml	
pH-value adjusted at pH 9.5; aliquoted at 10.5 ml; stored at –20°C		

Coating reagent	
Coating buffer	10.5 ml
Capture antibody	42 µl
(per microwell plate, freshly prepared within 15 min. before usage)	

Assay procedure. Microwell plates were coated with 100 µl coating reagent per well, sealed with aluminum foil and incubated overnight at 4 °C.

25× Wash buffer		
NaCl	200 g	20 %
$Na_2HPO_4 \cdot 2H_2O$	29 g	2.9 %
KH_2PO_4	5 g	0.5 %
KCl	5 g	0.5 %
Deionized water	ad 1000 ml	
pH-value adjusted with NaOH at pH 7.0, aliquoted (40 ml); stored at –20°C		

1× Wash solution		
Wash buffer (1×)	900 ml	
Tween® 20	450 µl	0.05 %

Microwells were washed 3-times with 300 µl wash solution per well by an automated washer. After the last wash, the plates were tapped on an absorbent paper to remove residual wash solution.

Assay diluent		
FCS	16.5 ml	16.5 %
Wash buffer (1×)	ad 100 ml	
for two microwell plates, freshly prepared		

IFN-γ stock standard		
Recombinant human IFN-γ	90 ng/vial	90 ng/ml
Deionized water	ad 1.0 ml	
After reconstitution, stock standard was aliquoted and stored at –80°C		

Thereafter, microwells were blocked with 200 µl blocking buffer per well and incubated for 1 hr at room temperature under permanent agitation. Meanwhile, a serially diluted IFN-γ standard (dilution ratio 1:2) with the highest concentration of 1200 pg/ml and appropriate dilutions of samples were prepared. After another wash, 100 µl of each standard and sample were pipetted in duplicates into the microwell plate, followed by sealing the plate again and incubating it for another 2 hours at room temperature under permanent agitation.

Working detector	
Assay diluent	10.5 ml
Detection antibody	42 µl
Enzyme reagent	42 µl

per microwell plate, freshly prepared within 15 min before usage

After five total washes, the plate was incubated with 100 µl working detector per well for 1 hr.

Substrate reagent	
Substrate reagent A	5.5 ml
Substrate reagent B	5.5 ml

per microwell plate, freshly prepared within 15 min before usage

Stop solution		
H_2SO_4 (25 %)	25 ml	12.5 %
Deionized water	50 ml	

After a final wash (7-times), 100 µl of substrate reagent were added to each well and the plate was incubated unsealed under permanent agitation in the dark until intense blue stain appeared in wells with the highest standard IFN-γ concentration. The reaction was interrupted by adding 50 µl of stop solution to each well (color change into yellow). Optical density was measured at a wavelength of 450 nm with an ELISA plate reader within 30 minutes.

Calculation of results. For quantification of IFN-γ, IFN-γ concentration was calculated by the help of a standard curve supported by the 'Slide Write' program. Standards were run with each assay in duplicates. The function of the standard curve was as follows:

$$y = a0 + (a1 \times x) / (a2 + x)$$

Coefficients of this equation were calculated with the Levenberg-Marquardt algorithm. The standard curve was constructed by blotting the mean absorbance of each standard on the vertical axis (y-axis) versus the corresponding IFN-γ concentrations on the horizontal axis (x-axis).

3.3.5 Flow cytometry (FACS)

Flow cytometry is a technique for counting, examining and sorting cells suspended in a stream of fluid. It allows simultaneous multiparametric analysis of the physical and biological characteristics of single cells.

This method was used in a first step to characterize the cell population of isolated PBMC. This was important to describe the changes of lymphocyte subsets in PBMC treated with PHA for 4 days. Additionally two PE-conjugated antibodies were used to examine the influence of low ethanol concentrations on the expression of IL-12 receptor subunits on PHA-activated T cells.

Principle: A laser light of a single wavelength is directed onto a hydro-dynamically focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam; one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors. Each suspended cell passing through the beam scatters the light in some way and fluorescent chemicals found in the cell or on the surface are excited into emitting light at a lower frequency than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak) it is then possible to extrapolate various types of information. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e. shape of the nucleus), the amount and type of cytoplasmic granules or the membrane roughness.

In the field of molecular biology, it is especially useful when used with fluorescence tagged antibodies. These specific antibodies bind to antigens on the target cells and help to give information on specific characteristics of the cells being studied in the cytometer.

Staining buffer	
FCS	10 ml
Sodium azide	450 mg
PBS	490 ml

Assay procedure. PBMCs were isolated as described earlier and kept at a starting concentration of 1×10^6 cells/ml. After incubation, cell suspensions were washed twice with PBS and spinned down for 5 min. at 1500 rpm and 4 °C. The pellet was resuspended in 500 µl staining buffer. To six samples of each experiment (50 µl) 10 µl of each antibody combination (IOTest®-Antikörper: CD3-FITC/CD4-PE, CD3-FITC/CD16+ CD56-PE, CD45-FITC/CD14-PE, CD3-FITC/CD19-PE, CD3-FITC/CD8-PE) or isotype matched control (IgG1-FITC/IgG1-PE) were added.

Incubation lasted 30 min and was finished by adding 100–200 µl of staining buffer. Cells were washed twice with PBS, resuspended in 1.5 ml of staining buffer and analyzed by flow cytometry.

To examine IL-12R expression by cytofluorometric analysis cells were cultured for 6 days in 6 well plates either unstimulated or PHA activated and were stained with PE-conjugated Ab. The IL-12 receptor β1 and IL-12 receptor β2 R-Phycoerythrin-conjugated rat anti human monoclonal Ab react with the 12Rβ1 e.g. IL-12Rβ2 subunit expressed on T cells and NK cells. Cytofluorometric analysis for both receptor subunits was performed using standard methods.

1×10^5 cells were incubated with 10 µl of 2B6 or 2B10 for 45 min. Control samples were stained using isotype controls FITC-conjugated IgG1 Abs. The mixture of IgG1-FITC/IgG1-PE isotypic control permits the non-specific part of the staining obtained on leucocytes to be determined with combinations of IgG1-isotype antibodies conjugated to FITC or PE. During specific staining, the boundary between negative and positive events must be adapted for each experiment depending on the signal obtained with the isotypic control.

Table 3-1. Antibody combinations used to determine leukocyte subpopulations

Subpopulation	FITC-/PE-labeled Ab
B cells	CD3-FITC/CD19-PE
Th cells	CD3-FITC/CD4-PE
cytotoxic T cells	CD3-FITC/CD8-PE
NK cells	CD3-FITC/CD56-PE/CD16-PE
monocytes	CD45-FITC/CD14-PE
isotype control	IgG1-FITC/IgG1-PE

Table 3-2. Cell surface (CD-) marker on leukocyte subpopulations

Cell type					
Marker	**monocytes**	**Lymphocytes**			
		Th1/Th2 cells	**cytotoxic T cells**	**B cells**	**NK cells**
CD3	–	+++	+++	–	–
CD4	+	+++	–	–	–
CD8	–	–	+++	–	–
CD14	+++	–	–	–	–
CD16/56	–	–	–	–	+++
CD19	–	–	–	+++	–
CD45	++	+++	+++	+++	+++

Calculation of results: Isotype control monoclonal antibodies were used to estimate the non-specific binding of target primary antibodies to cell surface antigens. Non-specific binding is due to Fc receptor binding or other protein-protein interactions. Isotype controls were used at identical concentrations and staining conditions as the target primary antibodies. Before samples were analyzed, the FSC Amp Gain and SSC voltage was adjusted to appropriately display the scatter properties of the isolated PMBC. The isotype controls were necessary to place the negative population in the lower-left quadrant of the plot.
Stored data files were analyzed after acquisition in complete using WinMDI Ver. 2.8.

3.3.6 Sample preparation

In the following, sample preparation for western blotting, real-time reverse transcription PCR (RT-PCR), the determination of intracellular IFN-γ concentration and IFN-γ measurement in cell supernatants by ELISA are described. At each point in time of experimental specimen, collection of samples for each experimental condition was taken in duplicates. Protein concentration of cell lysate was determined in order to get a standardization factor. Regarding calculation of intracellular IFN-γ concentration, the amount of IFN-γ was related to the amount of protein from the same assay.

3.3.6.1 Supernatant samples

At each point in time of sample drawing, the whole content of each well was transferred into a reaction tube. In case of having a cell suspension, samples were spinned down for 10 minutes at 1400 rpm and 4 °C. The supernatant was removed and stored at –20 °C until measurement of IFN-γ concentration by ELISA.

3.3.6.2 RT-PCR samples

At the end of the stimulation experiment, cells were washed twice with phosphate buffered saline (PBS), lysed with 500 µl TRIzol reagent, transferred to a tube and frozen at –80 °C.

3.3.6.3 Cell lysate samples

Cell lysis buffer		
NaCl (5 M)	6.8 ml	136 mM
Tris-HCL (1 M, pH 7.4)	5 ml	20 mM
Glycerol	25 ml	10 %
EDTA (0.5 M, pH 8)	1 ml	2 mM
β-Glycerol-phosphate	2.76 g	50 mM
Na-Pyrophosphate	2.23 g	20 mM
Benzamidine (1 M)	1.1 ml	4 mM
Sodium orthovanadate (100 mM)	2.5 ml	1 mM
Deionized water, ad 250 ml, pH-value was adjusted at 7.4		
Triton X-100		1 %
Cell lysis buffer was aliquoted at 15 ml and stored at –20 °C		
Pefablock (200 mM)	15 µl	0.2 mM
Aprotinin (5 mg/ml)	15 µl	5 µg/ml
Leupeptin (5 mg/ml)	15 µl	5 µg/ml
Added only before usage of lysis buffer and after warming to 4 °C		

After removing the supernatant, the cell pellet was washed twice with PBS for 5 minutes at 1400 rpm and 4 °C. Then the cell pellet was lysed, by resuspending with 50 µl lysis buffer and subsequent incubation for 10 minutes on ice. A following centrifugation step for 20 minutes at 1400 rpm and 4 °C separated cell lysate from cell waste. Gained supernatant or cell lysate was stored at –80 °C.

3.3.6.4 Nuclear extract samples

Nuclear extract were prepared to examine STAT4 activation using western blot.

After removing the supernatant, the cell pellet was washed twice with PBS for 5 minutes at 1400 rpm and 4 °C. Then the cell pellet was lysed by resuspending with 200–800 µl buffer A and subsequent incubation for 10 minutes on ice. A following centrifugation step for 2 minutes at 1400 rpm and 4 °C separated cell lysate from nucleus fraction.

Supernatant was removed; pellet was again dissolved in 100–200 µl of buffer C and incubated for 30 min on ice. A second centrifugation step followed. Supernatant was then stored at –20 °C.

Buffer A		
HEPES (0.5 M, pH 7.9)	20 µl	10 mM
$MgCl_2$ (1 M)	0.75 µl	1.5 mM
KCl (1 M)	5 µl	10 mM
Deionized water, ad 100 ml. Buffer A was aliquoted at 1 ml and stored at –20 °C.		
DTT (1 M)	0.5 µl	0.5 mM
PMSF (250 mM)	1 µl	0.2 mM
Natriumorthovanadat	10 µl	1 mM
Aprotinin (5 mg/ml)	0.6 µl	3 µg/ml
Leupeptin (5 mg/ml)	5 µl	5 µg/ml
Pepstatin (5 mg/ml)	5 µl	5 µg/ml
Chymostatin (25 mg/ml)	5 µl	5 µg/ml
NaF (2.5 M)	2 µl	5 mM
NP-40 Alternative	1 µl	0.1 %
Added only before usage of buffer A and after warming to 4 °C		

Buffer C		
HEPES (0.5M, pH 7.9)	4 µl	20 mM
NaCl (5 M)	8.4 ml	400 mM
$MgCl_2$ (1 M)	150 µl	1.5 mM
EDTA (0.5 M, pH 8)	40 µl	0.2 mM
Glycerol	25 ml	10 %
Deionized water, ad 100 ml, buffer A was aliquoted at 1 ml and stored –20 °C.		
DTT (1 M)	0.5 µl	0.5 mM
PMSF (250 mM)	1 µl	0.2 mM
Natriumorthovanadat	10 µl	1 mM
Aprotinin (5 mg/ml)	0.6 µl	3 µg/ml
Leupeptin (5 mg/ml)	5 µl	5 µg/ml
Pepstatin (5 mg/ml)	5 µl	5 µg/ml
Chymostatin (25 mg/ml)	5 µl	5 µg/ml
NaF (2.5 M)	2 µl	5 mM
NP-40 Alternative	1 µl	0.1 %
Added only before usage of buffer A and after warming to 4 °C		

3.3.6.5 Protein samples

A small fraction of each diluted cell lysate or nuclear extract sample (20 µl) was stored at –20 °C for determining protein concentration via Bio Rad Protein Assay.

3.3.7 Protein assay

Protein concentration of cell lysate samples and nuclear extract samples was determined. For this purpose, the 'Bradford method' was used.

BSA stock standard		
BSA	100 mg	0.1 %
Deionized water	ad 10 0ml	

BSA standard solutions		
BSA stock standard (µl)	Deionized water (ml)	BSA concentration [µg/ml] (after adding Dye Reagent)
0	8	0
10	7.99	1
20	7.98	2
40	7.96	4
80	7.92	8
100	7.90	10
150	7.85	15
200	7.80	20
270	7.73	27
350	7.65	35
400	7.60	40

Standard solutions were aliquoted at 800 µl/vial and stored at –20 °C

Principle. The dye 'Coomasssie Brilliant Blue' binds to proteins. At low pH values, the free dye has absorption maxima of 470 and 650 nm, but when bound to protein, has an absorption maximum of 595 nm (Wilson and Walker 2005).

Assay procedure. Samples for protein analysis were diluted 1:200 with deionized water. Afterwards, the 'Dye Reagent' was added to samples and standards at a proportion of 1:5. After vortexing, absorption was measured at 595 nm.

Calculation of results. Protein concentrations were calculated based on a linear standard curve. Standards were run with each assay. The standard curve was obtained by plotting the mean absorbance for each standard on the ordinate (y-axis) versus the corresponding protein concentration on the abscissa axis (x-axis).

3.3.8 Western blotting

Western blotting was proceeded in order to examine if STAT4 or AP-1 are involved in IL-12-induced IFN-γ production in NK-92 cells. Separation of proteins was performed by SDS-PAGE (Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis). The electrotransfer of proteins was preceded in semi-dry conditions. Visualization was done by ECL method.

Principle. Western blotting is a technique for detecting electrophoretically separated proteins by electroblotting and subsequent immunodetection. The separated proteins in a PAA-gel are transferred onto a membrane (here: PVDF) via electroblotting. A sandwich of gel and membrane is located between two parallel electrodes and a current is passed, that allows the proteins to electrophorese out of the gel and onto the membrane, referred as blot. The use of enzyme-linked second antibodies enables to detect specific proteins. First, the primary antibody binds to the protein of interest. Secondly, the enzyme-linked secondary antibody (here: HRP, Horseradish peroxidase-linked IgG) detects the primary antibody. Concerning visualization, enhanced chemiluminescence method (ECL) was used. The linked enzyme HRP oxidizes the substrate luminol, followed by light emission. The emitted light is detected by exposing the blot to a photographic film (Wilson and Walker 2005).

SDS		
SDS	50 g	10 %
Deionized water	ad 500 ml	

APS		
APS	50 g	10 %
Deionized water	ad 500 ml	

Tris (pH 8.8)		
Tris-Base	45.5 g	1.5 M
Deionized water	ad 250 ml	

Tris (pH 6.8)		
Tris-Base	30.3 g	1 M
Deionized water	ad 250 ml	

Assay Procedure. At the beginning, the SDS-PAGE was prepared. A single cassette was formed with two glass plates and two Teflon spacers. The cassette was stacked upright in the casting stand. The SDS-PAA-gel was composed of two gel layers. Firstly, the separating gel was applied, overlaid with isopropanol and abandoned until it was set. Before stacking gel preparation, the isopropanol was poured off and the gel was washed with deionized water. Thereafter, the stacking gel was mixed and poured into the cassette to the top of the plate. A comb was inserted to form slots for the later applied samples. When the gel was polymerized, the comb was removed and the cassette was filled with electrode buffer.

Separating gel (7.5 %)		
deionized water	1.06 ml	
PAA-Mix (30%)	3.06 ml	
Tris Base 1.5 M (pH 8.8)	3.125 ml	
SDS 10 %	125 µl	0.26 %
APS 10 %	125 µl	0.26 %
TEMED	5 µl	

Stacking gel		
Deionized water	1.7 ml	
PAA-Mix (10 %)	425 µl	
Tris Base 1.0 M (pH 6.8)	313 µl	
SDS 10 %	25 µl	0.01 %
APS 10 %	25 µl	0.01 %
TEMED	2.5 µl	

APS and TEMED were added just before gel application, starting polymerization

5× Electrode buffer		
SDS	5 g	0.5 %
Tris Base	15.1 g	1.51 %
Glycin	94 g	9.4 %
Deionized water	ad 1 L	

Electrode buffer	
Electrode buffer, 5×	200 ml
Deionized water	ad 1 L

Loading buffer		
Glycerol	10 g	10 %
SDS	3 g	3 %
Tris-Base	757 mg	62.5 mM
Bromphenolblau	10 mg	0.01 %
2-Mercaptoethanol	8 ml	8% vol
Deionized water	ad 100 ml	

Meanwhile, samples were diluted to a predetermined concentration and volume in order to have the same concentration of protein (20 µg/µl) in each sample. For denaturation and reduction, samples were mixed with loading buffer (2-times concentrated) 1:1 and the mixture were heated to 95 °C for 5–7 minutes. For visualization of the migration front of the proteins in the gel, the dye molecule bromphenol blue was added to the loading buffer. Then each sample was loaded onto the gel with a 'Hamilton' syringe. To determine the protein size a colored molecular weight marker (Rainbow-marker) was used. For running the gel, the electrophoresis chamber was filled with electrode buffer and the cassette was placed in. The anode was connected to the bottom, the cathode to the top of the chamber and the first run of the gel was at 80 V for approximately 45 minutes until the bromphenol front reached the borderline between stacking gel and separating gel. The second run was at 120 V for approximately 2 hours and was stopped when the dye molecule reached the bottom of the gel. Then power was turned off, plates were separated, the separating gel was removed and the gel was decreased into a dish containing cathode buffer.

Anode buffer 1		
Tris-base	36.34 g	3.6 %
Methanol 100 %	200 ml	20 %vol
Deionized water	ad 1 L	

Anode buffer 2		
Tris-base	3.02 g	0.3 %
Methanol 100 %	200 ml	20 %vol
Deionized water	ad 1 L	

Cathode buffer		
6-Aminocapronacid	5.2 g	0.5 %
Methanol 100 %	200 ml	20 %vol
Deionized water	ad 1 L	

Thereafter, an electrotransfer of the proteins onto a PVDF membrane was performed. PVDF membrane (tailored to the same size as the gel) was activated by incubating it in methanol for several seconds, 5 minutes in deionized water and for 15 minutes in anode buffer 2. The transfer stack assembly consisted of 5 layers: 6 'Whatman' filter papers moistened with anode buffer 1 and placed on the anode plate of blotter, 3 papers moistened with anode buffer 2 and placed on top of the latter papers, followed by the activated PVDF membrane and the gel and completed by 6 papers moistened with cathode buffer. Finally, the cathode plate of blotter was placed on top of the transfer stack. The semi-dry transfer apparatus was connected to power and the limiting amperage was set to 0.8 mA/cm² of the gel surface. The transfer was performed in about 1 h and 15 min.

10× TBS, Tris-buffered saline		
Tris	24 g	2.4 %
NaCl	87 g	8.7 %
Deionized water	ad 1 L	
pH-value adjusted at 7.6 with pure HCl		

TBS-T		
TBS 10×	500 ml	
Tween® 20	5 ml	
Deionized water	ad 5000 ml	
Stored at 4 °C, used within one week		

Blocking solution		
BSA	2.5 g	5 %
TBS-T	ad 50 ml	
Deionized water	ad 1 L	

After blotting, PVDF membrane was washed 3 times for 20 minutes with TBS-T solution under permanent agitation. Afterwards, the surface of the membrane was blocked with 5 % BSA solution over night under permanent agitation and afterwards washed again as described before. The membrane was incubated with primary antibody diluted 1:500 in TBS-T for 2 hours at room temperature under permanent agitation. After a next wash, the membrane was incubated with secondary antibody diluted 1: 1000 with TBS-T for 1 hr. After a final wash, detection was carried out by using 'ECL Western Blotting Substrate'. Equal amounts of the development solutions A and B were poured onto the membrane. The membrane was then wrapped into foil and exposed to a photographic film for a few seconds. To demonstrate equal loading, the same membrane was reprobed by repeating the working procedure after blocking, using the antibody against GAPDH or Histone 1.

Analysis of results. Because of immunodetection and visualization via ECL, results were expressed as bands on the photo film. The bands of the positive and negative control were compared for documenting effects of IL-12 stimulation and ethanol treatment.

3.3.9 RT-Polymerase Chain Reaction (RT-PCR)

To examine changes in IFN-γ mRNA expression upon IL-12 stimulation or ethanol treatment RT-Polymerase Chain Reaction (RT-PCR) was used. After incubation experiments total RNA was isolated, quality and quantity was determined by photometry. Subsequently 1 µg of total RNA was reversed transcribed into cDNA. Real-time reverse transcription PCR analyses were performed.

3.3.9.1 Isolation of total RNA

Principle. TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi. During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. After removal of the aqueous phase, the DNA and proteins in the sample can be recovered by sequential precipitation.

Procedure. After incubation experiment 100 µl of TRIzol were added to each sample. Isolation of total RNA was started by incubation with 100 µl chloroform for 3 min at room temperature, followed by centrifugation for 15 min (11500 rcf/4 °C). The aqueous phase (RNA-phase) was completely removed. Total RNA can be precipitated using 250 µl ice-cold isopropanol. The following centrifugation step (10 min/11500 rcf/4 °C) leads to a RNA-pellet. This pellet is washed twice with 500 ml 75 % ethanol. Isolated RNA was dissolved in DEPC-H_2O samples were stored at −80°C.

3.3.9.2 UV spectroscopy of RNA yield

Quantity of isolated RNA was measured by UV spectroscopy. This is a commonly used and easy method for quantification of RNA. The absorbance of a diluted RNA sample was measured at wavelengths of 260 nm and 280 nm. The nucleic acid concentration was calculated using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

$$\text{cRNA [µg/ml]} = \text{OD260} \cdot 40\ \text{µg/ml} \cdot \text{dilution factor}$$

OD Optical density at 260 nm

Besides, of the quantity of RNA the purity was determined by 260/280-ratio. The absorbance at 260 nm is used to estimate the RNA concentration.

The other wavelength (280 nm) is used in the numerator of the ratio analysis to detect sample impurities. For pure RNA the 260/280 ratio is 1.8-2.0. Lower ratio levels are a sign of protein contamination.

3.3.9.3 cDNA Synthesis

Reverse Transcription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using total cellular RNA, a reverse transcriptase enzyme, a primer, dNTPs, and an RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction. The reverse transcriptase enzyme *'SuperScript™ Reverse Transcriptase'*(Invitrogen) was used according to the manufacturer's protocol.

nuclease-free water (DEPC-H_2O)	
DEPC (Diethylpyrocarbonate)	10 µl
dd H_2O	10 ml

RNA samples were diluted with sterile nuclease-free water to a concentration of 1 µg/11 µl. 1 µl of random-primer (Invitrogen) was added (150 ng/µl). A negative control tube only contained nuclease-free water instead of RNA. RNA was incubated in a thermal cycler with heated lid. Tubes were quickly chilled on ice.

RT core mix	
First strand buffer	4 µl
DTT (0.1 M)	2 µl
dNTP-Mix	1 µl
SupersScript II Reverse Transcriptase (200 U/µl)	1 µl

Meanwhile a RT core mix was prepared on ice, 8 µl was added to each sample, and tubes were spinned briefly. The RT was performed at 42 °C for 50 min, followed by a heating step of 70 °C for 15 min. Samples were then kept at 4 °C or stored at –20 °C.

3.3.9.4 Primer

PCR parameters such as suitable primers, annealing temperature and cycle number were optimized using PHA-induced PBMC as positive control. Three different human IFN-γ primer sequences were tested in advance. 18S rRNA was used as internal control to assure constant expression level across the sample set being studied.

IFN-γ oligonucleotides

source	sequence	melting point	length bp
Overbergh *et al.*, 2003	forward: 5'-TCAGCTCTGCATCGTTTTGG-3' reverse: 5'-AAGTCTACATCGCCTATTACCTTG-3'	60 °C 64 °C	110 bp
Rentzsch *et al.*, 2003	forward: 5'-AGCTCTGCATCGTTTTGGGTT-3' reverse: 5'-AACCCTACAACAGACCCACA-3'	62 °C 60 °C	108 bp
Rad *et al.*, 2002	forward: 5'-CTTGGCTTTTCAGCTCTGCATC-3' reverse: 5'-CCTTGAGAAAAGAATCCGTAAAACTTC-3'	66 °C 62 °C	151 bp

18S rRNA

source	sequence	melting point
Parlesak *et al.*, 2004	*forward*: 5'-AAGTCTTTGGGTTCCGGG-3' *reverse*: 5'-GGACATCTAAGGGCATCACA-3'	54 °C 60 °C

Oligonucleotide were ordered from Invitrogen GmbH in HPSF-purified form, solubilized with the respective amount of TE buffer and stored at –20 °C.

3.3.9.5 Real-time RT-PCR

Real-time reverse transcription analyses were performed in an iCycler™ Thermal Cycler according to the manufacturer's instructions. This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. As a template, cDNA at a concentration of 10 ng cDNA/ml for IFN-γ and 0.1 ng cDNA/ml for 18S rRNA was used. To 20 µl of reaction mixture 5 µl of cDNA sample was added.

Reaction mixture

Nuclease-free water	5.9 µl
2× iQ™ SYBR® Green Supermix	12.5 µl
12.5 µM Primer forward	0.8 µl
12.5 µM Primer forward	0.8 µl

Assay Procedure: Samples were loaded in 8er stripes and placed in the fluorescence thermocycler. Initial denaturation (95 °C for 180 s) of the hot start enzyme (iTaq™-DNA polymerase, Biorad) was followed by 45 cycles, each consisting of 95 °C for 30 s, the primer-specific annealing temperature for 30 s and the elongation phase for 120 s at 72 °C. At the end of each run melting curve profiles were obtained by cooling the sample to 65 °C for 15 s and heating at 0.20 °C/s up to 95 °C with continuous measurement of fluorescence to confirm amplification of specific transcript. Cycle-to-cycle fluorescence emission reading was monitored and analyzed using iCycler software.

3.3.9.6 Calculation of mRNA Copy Number

For the quantitative evaluation of the IFN-γ gene expression by Real-time PCR the calibration with DNA solutions of known copy number was necessary. Therefore, 75 µl of PCR product were isolated using NucleoSpin Extract II kit (Macherey-Nagel GmBH). The concentration of DNA was measured at 260 nm and the number of copies was calculated as followed:

$$\text{Number of copies/µl} = \text{cDNA [g/µl]} \cdot 6.022 \cdot 1023\ [\text{mol-1}]/\text{basepairs} \cdot \text{average MW of DNA}$$

MW	Molecular Weight of DNA	660 [g/mol]

DNA-Standard was then adjusted to 2×10^6 copies/µl and aliquoted to 50 µl to avoid multiple thawing and freezing cycles and stored at –20 °C. In each run, two samples of the standard curve were used to prepare serial dilutions and included as control.

3.3.10 Statistical evaluation

Data were initially evaluated for normal distribution. To compare means between two groups, Student's t-test was used. To calculate the significance of differences among means for more than two groups analysis of variance (ANOVA) was used. If the level of significance (p) was less than 0.05, levels of significance among negative control, IL-12 stimulated and ethanol incubations were evaluated with Dunnett's or Tukey's post hoc test. The statistical evaluation was performed with STATISTICA, Version 6.0.

4 Results

4.1 Interferon γ production by human Peripheral Blood Mononuclear Cells

4.1.1 Effect of PHA activation, LPS stimulation and ethanol treatment on IFN-γ production by human Peripheral Blood Mononuclear Cells (PBMC)

4.1.1.1 Effect of PHA activation and LPS stimulation

Interferon γ can either be stimulated by non-viral agents called superantigens such as PHA, or by LPS know as a potent inducer of human T-lymphocyte proliferation and cytokine production (Ulmer et al., 2000). In the present study, we tested the ability of both substances to stimulate Interferon γ production in Peripheral Blood Mononuclear Cells (PBMC).

1 × 106 cells were stimulated with PHA at a concentration of 2 µg/ml or LPS at a concentration of 1 µg/ml. The degree of stimulation varied from donor to donor, but was comparable to the level of response to PHA activation or LPS stimulation for all experiments.

In Table 4-1 and Figure 4-1, the concentration of IFN-γ in cell supernatant of unstimulated, PHA-activated or LPS-stimulated PBMC is compared. LPS at a concentration of 1 µg/ml caused a highly significant stimulation of IFN-γ production in isolated human PBMC. The addition of PHA at a concentration of 2 µg/ml led to a highly significant increase of IFN-γ production not only compared to untreated cells, but also compared to cells stimulated with 1 µg/ml LPS.

Table 4-1. Effect of PHA and LPS on the absolute IFN-γ concentration in supernatants of isolated human PBMC. Cells (10^6/2.4 ml) were cultured with medium alone or PHA (2 µg/ml) or LPS (1µg/ml) for 72 hours. Results are given as absolute mean values with SD; [s]F-test (ANOVA, monofactorial, significance of stimulation), post hoc test: Tukey's Honest-Significant-Difference-Test, n.c.: negative control, SD: standard deviation.

absolute IFN-γ concentration [pg/ml]						
					significance of stimulation	
stimulation	**N**	**mean**	**SD**	**[s]F-test**	**p (Tukey´s) vs PHA**	**p (Tukey´s) vs LPS**
n.c.	14	36	33	<0.0001	0.0001	0.0001
PHA	14	70842	58939			0.0001
LPS	14	2388	3146			

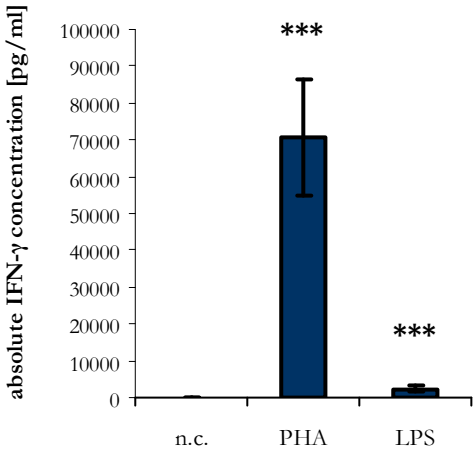


Figure 4-1. Comparison of absolute IFN-γ concentration in cell culture supernatants of untreated, PHA-activated and LPS-stimulated PBMC. IFN-γ levels were determined via ELISA. Results are given as absolute mean values ± SEM; [s]F-test (ANOVA), post hoc test: [*]Tukey's test (versus n.c.) [* * *] = p-value < 0.001.

4.1.1.2 Effect of ethanol treatment

To investigate the effect of different ethanol concentrations on IFN-γ concentration in cell supernatant of PHA-activated or LPS-stimulated PBMC, isolated cells were seeded in cell culture plates and were activated with PHA at a concentration of 2 µg/ml or LPS at a concentration of 1 µg/ml. Ethanol addition led to concentrations of 1 ‰ (22 mM) and 3 ‰ (66 mM). Additionally, ethanol-treated cells were kept in alcohol vapor chambers during incubation time to guarantee constant alcohol concentrations. Samples were taken 72 hours upon incubation start.

The results on the effect of low ethanol concentration on IFN-γ production by human PBMC are given in Table 4-2a and depicted in Figure 4-2.

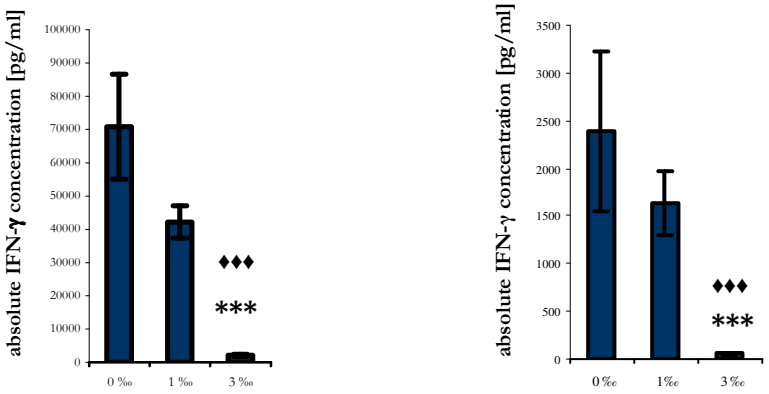
For further evaluation, absolute mean values were transformed into relative mean values. This was necessary since the degree of stimulation varied from donor to donor. The absolute value of IFN-γ concentration of PHA-activated PBMC without ethanol treatment was set to 100 % for PHA-activated cells with ethanol treatment. The absolute value of IFN-γ concentration of LPS-stimulated PBMC without ethanol was set to 100 % and used as a comparison for all LPS-stimulated preparations. Relative mean values are shown in Table 4-2b and Figure 4-3.

Table 4-2. Effect of two different ethanol concentrations on the IFN-γ production by PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA or LPS and treated with two different ethanol concentrations for 72 hours. IFN-γ was determined using ELISA. Table a) absolute mean values; b) relative mean values (PHA, 72 hours = 100 %, LPS, 72 hours = 100 %); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration); post hoc test: Tukey's Honest-Significant-Difference-Test).

a	absolute IFN-γ concentration [pg/ml]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	14	36	33	0.7398		
	1	14	35	22			
	3	14	28	33			
PHA	**0**	14	70842	58939	<0.0001	0.2554	**0.0001**
	1	14	42232	18173			**0.0001**
	3	14	2090	1369			
LPS	**0**	14	2388	3146	<0.0001	0.9503	**0.0001**
	1	14	1641	1248			**0.0001**
	3	14	56	47			

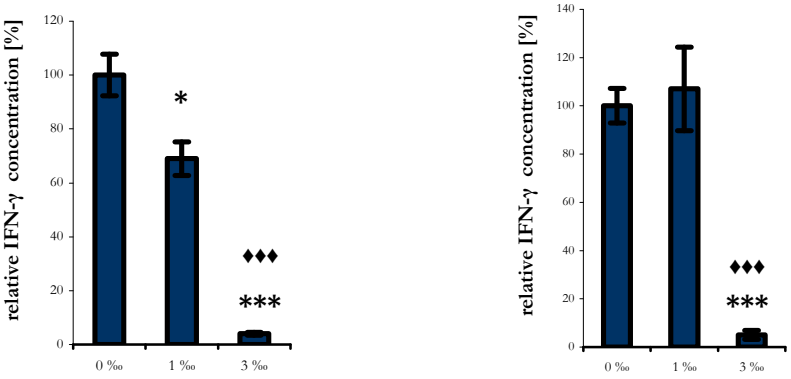
b	relative IFN-γ concentration [%]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	14	100	38	0.6596		
	1	14	166	154			
	3	14	167	347			
PHA	**0**	14	100	29	<0.0001	**0.016**	**0.0001**
	1	14	69	23			**0.0001**
	3	14	4	2			
LPS	**0**	14	100	27	<0.0001	0.8890	**0.0001**
	1	14	107	65			**0.0001**
	3	14	5	7			

Ethanol treatment resulted in a significant decrease of IFN-γ production. The absolute mean value in cell supernatants of PHA-activated PBMC treated with 66 mM (3 ‰) ethanol was suppressed in a highly significant manner compared to untreated cells. The same effect is seen in LPS-stimulated cells. The transformation of absolute values to relative values (PHA, 0 ‰ = 100 %) led to a highly significant reduction of IFN-γ production not only compared to untreated cells, but also compared to those incubated with 22 mM (1 ‰) ethanol.



a) b)

Figure 4-2. Absolute IFN-γ concentrations in cell culture supernatants of a) PHA-activated and b) LPS-stimulated PBMC. Data of 14 experiments performed in duplicates. Results are given as absolute mean values ± SEM; [e]F-test (ANOVA, significance of ethanol concentration); post hoc test: * Tukey's test (versus 0 ‰): **** = p-value < 0.001; ♦Tukey's test (versus 1 ‰): ♦♦♦ = p-value < 0.01



a) b)

Figure 4-3. Relative IFN-γ concentrations in cell culture supernatants of a) PHA-activated and b) LPS-stimulated PBMC. Data represent the relative mean values and SEM of 14 experiments performed in duplicates. [e]F-test (ANOVA, significance of ethanol concentrations) post hoc test: * Tukey's test (vs 0 ‰): * = p-value < 0.05, **** = p-value < 0.001; ♦Tukey's test (versus 1 ‰): ♦♦♦ = p-value < 0.001

4.2 Other cytokines produced by human Peripheral Blood Mononuclear Cells

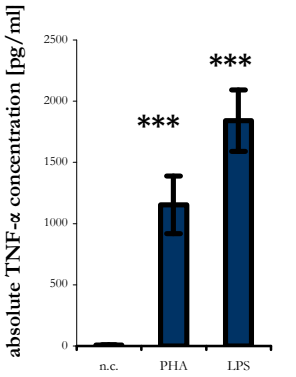
4.2.1 Effect of PHA and LPS stimulation on the production of seven cytokines by human Peripheral Blood Mononuclear Cells (PBMC)

To test the capability of PHA and LPS to stimulated T-lymphocyte cytokine production, seven IFN-γ-related cytokines were determined in cell supernatants of ethanol-treated PBMC using ELISA. Samples were drawn from LPS- or PHA-stimulated cells at 12 hours to test for TNF-α, at 32 hours upon incubation start to measure the concentrations of TGF-β, Interleukin 10, Interleukin 12 and Interleukin 18 as well as IP-10 and at 72 hours to determine Interleukin 2 concentrations.

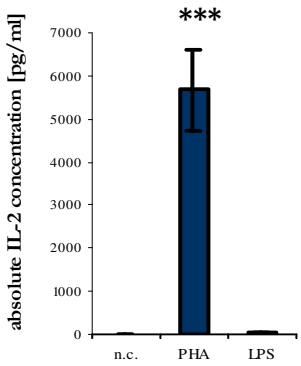
In Table 4-3 the absolute cytokine concentration of seven different cytokines in cell supernatants of PHA- or LPS-treated cells is given.

Table 4-3. Effect of PHA and LPS stimulation on the concentration of TNF-α (12 h), TGF-β (32 h), IL-2 (72 h), IL-10 (32 h), IL-12 (32 h), IL-18 (32 h) and IP-10 (32 h) in supernatants of isolated human PBMC. Cells (10^6/2.4 ml) were cultured with medium alone, with PHA (2 µg/ml) or LPS (1 µg/ml) for indicated hours. Results are given as absolute mean values with SD; [s]F-test (ANOVA), post hoc test: Tukey's test, n.c.: negative control

absolute cytokine concentration [pg/ml]							
					significance of stimulation		
Cytokine	**stimulation**	**N**	**mean**	**SD**	**[e]F-test**	**p (Tukey´s) vs PHA**	**p (Tukey´s) vs LPS**
TNF-α	**n.c.**	12	7	14	**<0.0001**	**0.0009**	**0.0001**
	PHA	12	1153	818			0.0516
	LPS	12	1840	870			
TGF-β	**n.c.**	12	234	152	0.9367		
	PHA	12	242	106			
	LPS	12	252	107			
IL-2	**n.c.**	14	13	13	**<0.0001**	**0.0001**	0.9998
	PHA	14	5675	3492			**0.0001**
	LPS	14	29	38			
IL-10	**n.c.**	8	5	3	**<0.0001**	**0.0242**	**0.0001**
	PHA	7	200	150			**0.0001**
	LPS	8	680	171			
IL-12	**n.c.**	12	2	3	**<0.0001**	**0.0001**	**0.0058**
	PHA	12	43	26			**0.0495**
	LPS	12	26	16			
IL-18	**n.c.**	4	8	3	0.4888		
	PHA	3	13	9			
	LPS	4	10	2			
IP-10	**n.c.**	12	785	1218	**<0.0001**	**0.0001**	0.9861
	PHA	12	18189	5336			**0.0001**
	LPS	12	577	588			



a)



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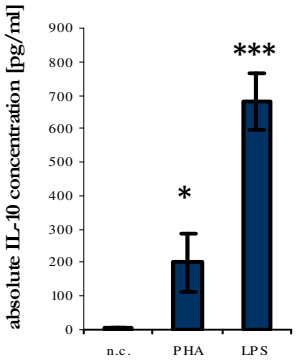

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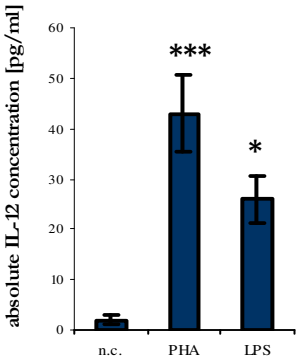

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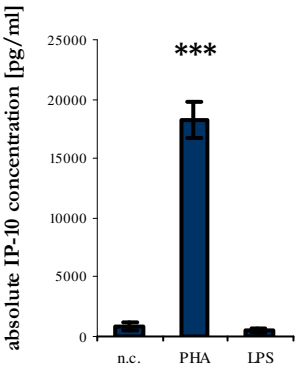


e)

Figure 4-4. Comparison of absolute cytokine concentrations in cell culture supernatants of untreated, PHA activated and LPS stimulated PBMC. a) TNF-α, b) IL-2, c) IL-10, d) IL-12 and e) IP-10. Cytokine levels were determined via ELISA. Results are given as absolute mean values ± SEM; ^{s}F-test (ANOVA, significance of stimulation) post hoc test: *Tukey's test (versus n.c.): * = p-value <0.05, *** = p-value < 0.001;

In our model system of isolated human PBMC, the stimulation with PHA or LPS had no significant effect on the production of TGF-β or Interleukin 18. For TNF-α, IL-2, IL-12 and IP-10 a highly significant increase in cytokine release can be observed upon PHA stimulation, additionally IL-10 production is induced.

LPS on the other hand caused a highly significant increase in TNF-α and IL-10 concentration and was also able to lead to higher IL-10 levels in cell culture supernatants. No effect of LPS is seen for IL-2 and IP-10 (see Figure 4-4).

4.2.2 Effect of ethanol on seven cytokines produced by Peripheral Blood Mononuclear Cells (PBMC)

To answer the question, if other cytokines produced by PHA- or LPS-stimulated cells were affected by low ethanol concentrations in the same way as IFN-γ, seven IFN-γ-related cytokines were determined in cell supernatants of ethanol-treated PBMC using ELISA.

To investigate the effect of different ethanol concentrations on cytokine concentration in cell supernatant of PHA-activated or LPS-stimulated PBMC, isolated cells were seeded in cell culture plates and were activated with PHA at a concentration of 2 μg/ml or LPS at a concentration of 1 μg/ml.

Ethanol addition led to concentrations of 1 ‰ (22 mM) and 3 ‰ (66 mM). Additionally, ethanol-treated cells were kept in alcohol vapor chambers during incubation time to guarantee constant alcohol concentrations. Samples were taken 12, 32 and 72 hours upon incubation start.

The following chapters 4.2.2.1 – 4.2.2.7 deal with the effect of ethanol on TNF-α, TGF-β, IL-2, IL-10, IL-12, IL-18 and IP-10 concentration in cell supernatants of isolated human PBMC.

4.2.2.1 Effect of ethanol treatment on TNF-α production by PBMC

As seen in chapter 4.2.1, TNF-α production can be induced in PBMC by both PHA and LPS stimulation. The addition of ethanol at a concentration of 66 mM to the cell culture medium led to a significant decrease only in LPS-stimulated cells (Table 4-4a and Figure 4-5), when absolute mean values are compared.

The transformation of absolute mean values into relative mean values, where absolute cytokine concentrations of PHA- or LPS-stimulated PBMC without ethanol was set to 100 %, led to relative mean values shown in Table 4-4b and Figure 4-6.

In this case, the addition of ethanol (66 mM) caused a highly significant decrease in relative values of TNF-α level in both LPS- and PHA-treated cells, but this decrease was not dose-dependent, since the addition of 22 mM of ethanol had no effect on TNF-α concentration.

Table 4-4. Effect of two different ethanol concentrations on the TNF-α production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 12 hours. TNF-α was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration; post hoc test: T-test: Tukey's test).

a	absolute TNF-α concentration [pg/ml]						
					significance of ethanol concentration		
Stimulation	ethanol concentration [‰]	N	mean	SD	[e]F-test	♦T-test vs 1 ‰	T-test vs 3 ‰
n.c.	0	12	7	14	0.0842		
	1	12	389	731			
	3	12	79	140			
PHA	0	12	1153	818	0.0893		
	1	12	1394	1282			
	3	12	529	665			
LPS	0	12	1840	870	0.0013	0.5512	0.0201
	1	12	2175	935			0.0013
	3	12	931	448			

b	relative TNF-α concentration [%]						
					significance of ethanol concentration		
Stimulation	ethanol concentration [‰]	N	mean	SD	[e]F-test	♦T-test vs 1 ‰	T-test vs 3 ‰
n.c.	0	12	100	54	0.0842		
	1	12	8800	15036			
	3	12	5515	12234			
PHA	0	12	100	4	<0.0001	0.3184	0.0001
	1	12	111	27			0.0001
	3	12	38	17			
LPS	0	12	100	14	<0.0001	0.0721	0.0001
	1	12	120	20			0.0001
	3	12	55	28			

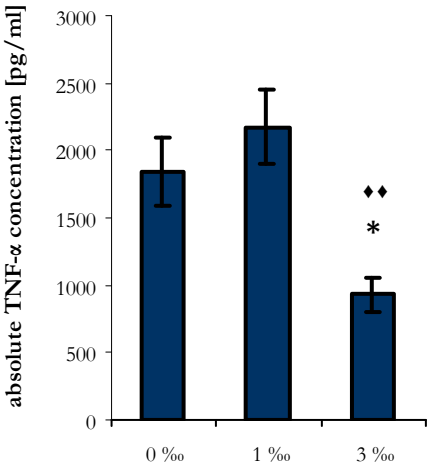


Figure 4-5. Absolute TNF-α concentration in cell culture supernatants of LPS-activated PBMC. Data of 12 experiments performed in duplicates. Results are given as absolute mean values ± SEM; ^{c}F-test (ANOVA, significance of ethanol concentration) post hoc test: *Tukey's test (versus 0 ‰): * = p-value < 0.05; $^{\blacklozenge}$Tukey's test (versus 1 ‰): $^{\blacklozenge\blacklozenge}$ = p-value < 0.01

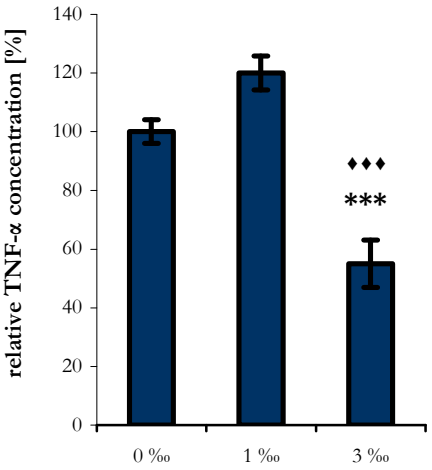


Figure 4-6. Relative TNF-α concentration in cell culture supernatants of LPS-activated PBMC. Data represent the relative mean of values and SEM of 12 experiments done in duplicates. ^{c}F-test (ANOVA, significance of ethanol concentration), post hoc test: * Tukey's test (versus 0 ‰): *** = p-value < 0.001. $^{\blacklozenge}$ Tukey's test (versus 1 ‰): $^{\blacklozenge\blacklozenge\blacklozenge}$ = p-value < 0.001.

4.2.2.2 Effect of ethanol treatment on TGF-β production by PBMC

Human Peripheral Blood Mononuclear Cells isolated from healthy donors showed no change in TGF-β production upon LPS- or PHA-activation. The results in the following Table 4-5 demonstrate, that the addition of ethanol had no effect on the TGF-β concentration as well, leading to the conclusion that in our cell culture model the production of TGF-β cannot be induced by LPS or PHA and is not affected by ethanol.

Table 4-5. Effect of two different ethanol concentrations on the TGF-β production by untreated, PHA-activated or LPS stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 32 hours. TGF-β was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration)

a	absolute TGF-β concentration [pg/ml]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	12	234	152	0.3852
	1	12	283	149	
	3	12	311	101	
PHA	**0**	12	242	106	0.6581
	1	12	261	88	
	3	12	284	135	
LPS	**0**	12	252	107	0.6373
	1	12	247	82	
	3	12	284	116	

b	relative TGF-β concentration [%]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	12	100	22	0.1146
	1	12	138	83	
	3	12	150	58	
PHA	**0**	12	100	11	0.3872
	1	12	119	53	
	3	12	129	70	
LPS	**0**	12	100	9	0.2709
	1	12	103	28	
	3	12	119	43	

4.2.2.3 Effect of ethanol treatment on Interleukin 2 production by PBMC

The following Table 4-6 shows the absolute (a) and relative (b) IL-2 concentration in cell supernatants of LPS- and PHA-activated human PBMC additionally treated with two different ethanol concentrations (22 mM and 66 mM). As seen in chapter 4.2.1, Table 4.3, only PHA was able to induce a significant IL-2 production.

Table 4-6. Effect of two different ethanol concentrations on the IL-2 production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 72 hours. IL-2 was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration)

a	absolute IL-2 concentration [pg/ml]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**
n.c.	**0**	14	13	13	**0.0463**
	1	14	13	7	
	3	14	29	28	
PHA	**0**	14	5675	3492	0.5701
	1	14	4531	2427	
	3	14	5640	3619	
LPS	**0**	14	29	38	0.2544
	1	14	21	5	
	3	14	37	21	

b	relative IL-2 concentration [%]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**[♦]T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	14	100	53	0.0398	0.9260	**0.0478**
	1	14	152	150			0.1066
	3	14	439	612			
PHA	**0**	14	100	29	0.1173		
	1	14	78	19			
	3	14	96	36			
LPS	**0**	14	100	47	**0.0201**	0.9706	**0.0301**
	1	14	109	68			0.0516
	3	14	203	158			

Comparing relative mean values of untreated and LPS-stimulated cells the addition of 66 mM of ethanol led to an increase in IL-2 production (Figure 4-7). Concerning the low amount of IL-2 produced in non-PHA-stimulated preparation, the question remains, whether the results for negative control and LPS-stimulation are of physiological relevance.

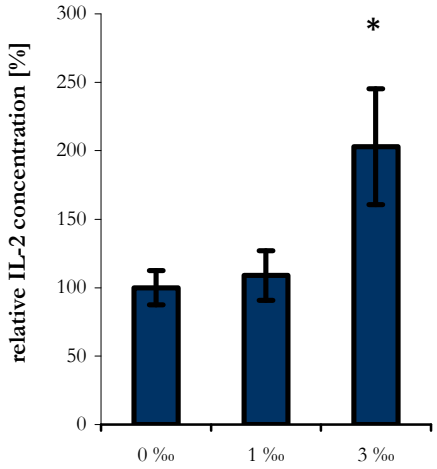


Figure 4-7. Relative IL-2 concentrations in cell culture supernatants of LPS-activated PBMC. Data represent the relative mean of values and SEM of 14 experiments done in duplicates. [e]F-test (ANOVA, significance of ethanol concentration), post hoc test: [*]Tukey's test (versus 0 ‰): [*] = p-value < 0.05

4.2.2.4 Effect of ethanol treatment on Interleukin 10 production by PBMC

After an incubation time of 32 hours, cell supernatants of ethanol-treated PBMC were removed and IL-10 concentration was determined using ELISA. The absolute concentrations are shown in Table 4-7a, the relative mean values in Table 4-7b.

Untreated cells were barely able to produce IL-10, whereas LPS led to the highest IL-10 concentrations in cell culture supernatants.

LPS-challenged cells additionally treated with 66 mM ethanol had a significant lower IL-10 concentration (Figure 4-8). This effect is even more prevalent in relative data, where IL-10 is decreased in incubations with 66 mM compared to untreated cells and additionally compared to incubations with 22 mM (Figure 4-9).

Table 4-7. Effect of two different ethanol concentrations on the IL-10 production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 32 hours. IL-10 was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration; post hoc test: T-test: Tukey's test).

a	absolute IL-10 concentration [pg/ml]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	8	5	3	0.1543		
	1	10	40	72			
	3	10	6	3			
PHA	**0**	7	200	150	0.2883		
	1	6	162	66			
	3	10	110	104			
LPS	**0**	8	680	171	**0.0239**	0.9648	**0.0409**
	1	8	655	160			0.0522
	3	8	424	270			

b	relative IL-10 concentration [%]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	8	100	37	**0.0003**	**0.0005**	0.7027
	1	8	256	83			**0.0024**
	3	8	127	71			
PHA	**0**	7	100	17	0.7682		
	1	6	90	26			
	3	8	79	84			
LPS	**0**	8	100	7	**0.0035**	0.9396	**0.0058**
	1	8	96	23			**0.0124**
	3	8	57	36			

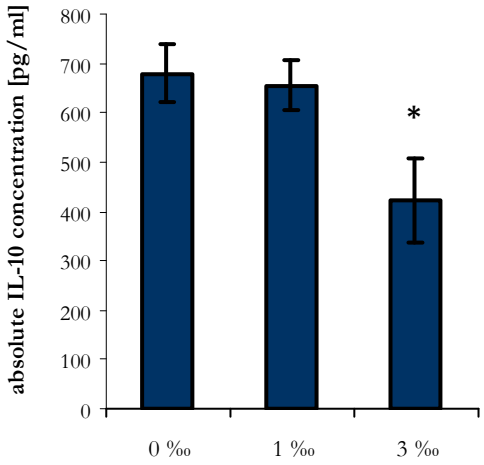


Figure 4-8. Absolute IL-10 concentrations in cell culture supernatants of LPS-stimulated PBMC. Data of 8 experiments performed in duplicates. Results are given as absolute mean values ± SEM; [e]F-test (ANOVA, significance of ethanol concentration) post hoc test: [*]Tukey's test (versus 0 ‰): [*] = p-value < 0.05

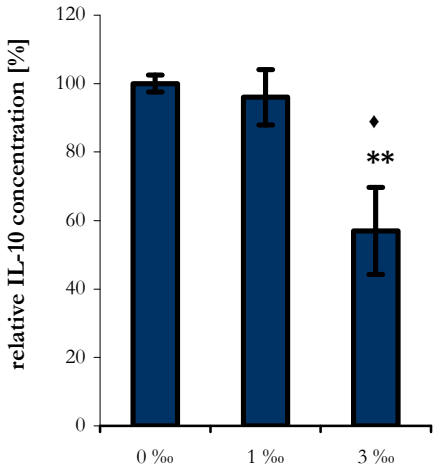


Figure 4-9. Relative IL-10 concentrations in cell culture supernatants of LPS-stimulated PBMC. Data represent the relative mean of values and SEM of 8 experiments done in duplicates. [e]F-test (ANOVA, significance of ethanol), post hoc test: [*] Tukey's test (vs 0 ‰): [**] = p-value < 0.01, [♦] Tukey's test (vs 1 ‰): [♦] = p-value < 0.05.

4.2.2.5 Effect of ethanol treatment on Interleukin 12 production by PBMC

Interleukin 12 production was measured 32 hours upon incubation start in cell supernatants of PHA- or LPS-treated isolated human PBMC with the addition of two ethanol concentrations. Only PHA-activated cells produced verifiable amounts of IL-12 within this period (Table 4-8). In supernatants of cells treated with 66 mM of ethanol the increase in IL-12 production was observed compared to both untreated and cells treated with 22 mM (Figure 4-10). These findings are pointed out by the transformation into relative data, where the addition of ethanol (66 mM) led to a highly significant increase in IL-12 production by PHA-activated cells (see Table 4-8b and Figure 4-11).

Table 4-8. Effect of two different ethanol concentrations on the IL-12 production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 32 hours. IL-12 was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration; post hoc test: T-test: Tukey's test).

a	absolute IL-12 concentration [pg/ml]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	12	2	3	0.4115		
	1	12	3	6			
	3	12	7	17			
PHA	**0**	12	43	26	**0.0007**	0.7765	**0.0010**
	1	12	55	34			**0.0061**
	3	12	114	62			
LPS	**0**	12	10	2	0.3567		
	1	12	12	4			
	3	12	12	4			

b	relative IL-12 concentration [%]						
					significance of ethanol concentration		
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	12	100.0	47	0.2159		
	1	12	226	183			
	3	12	846	1882			
PHA	**0**	12	100	13	**<0.0001**	0.6138	**0.0002**
	1	12	140	74			**0.0008**
	3	12	312	161			
LPS	**0**	12	100	33	0.2411		
	1	12	106	49			
	3	12	141	89			

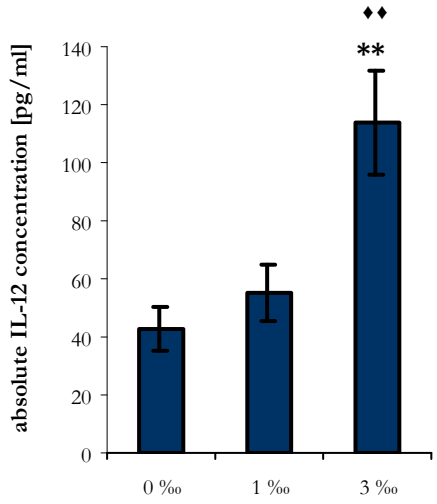


Figure 4-10. Absolute IL-12 concentrations in cell culture supernatants of PHA-activated PBMC. Data of 12 experiments performed in duplicates. Results are given as absolute mean values ± SEM; [e]F-test (ANOVA, significance of ethanol concentration) post hoc test: [*]Tukey's test (versus 0 ‰): [**] = p-value < 0.01; [♦]Tukey's test (versus 1 ‰): [♦♦] = p-value < 0.01

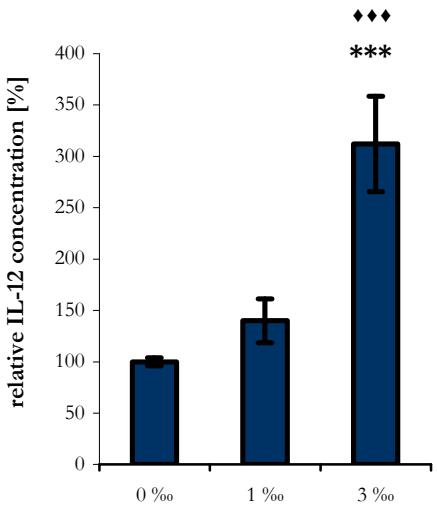


Figure 4-11. Relative IL-12 concentrations in cell culture supernatants of PHA-activated PBMC. Data represent the relative mean of values and SEM of 12 experiments done in duplicates. [e]F-test (ANOVA, significance of ethanol concentration), post hoc test: [*] Tukey's test (versus 0 ‰): [***] = p-value < 0.001, [♦] Tukey's test (versus 1 ‰): [♦♦♦] = p-value < 0.001.

4.2.2.6 Effect of ethanol treatment on Interleukin 18 production by PBMC

In our cell culture model, IL-18 was not produced in namable amounts, neither on LPS nor on PHA stimulation. The effect of ethanol on IL-18 production is therefore negligible (see Table 4-9).

Table 4-9. Effect of two different ethanol concentrations on the IL-18 production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 32 hours. IL-18 was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration).

a	absolute IL-18 concentration [pg/ml]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	4	8	2	0.0877
	1	8	13	8	
	3	8	6	3	
PHA	**0**	3	13	9	0.0854
	1	2	25	3	
	3	7	12	6	
LPS	**0**	4	10	2	0.7148
	1	6	12	4	
	3	7	12	4	

b	relative IL-18 concentration [%]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	4	100	29	0.2400
	1	4	174	152	
	3	4	58	26	
PHA	**0**	3	100	53	0.2436
	1	2	245	130	
	3	4	116	93	
LPS	**0**	4	100	15	0.6011
	1	4	123	35	
	3	4	119	43	

4.2.2.7 Effect of ethanol treatment on IP-10 production by PBMC

To compare the amount of IP-10 produced by PBMC treated with ethanol, PBMC were activated with PHA or LPS. The addition of ethanol led to final concentrations of 22 mM or 66 mM.

PHA-stimulation induced a 20-fold higher IP-10 production compared to untreated or LPS-challenged PBMC (Table 4-10). Significant effects of ethanol were only observed in non-stimulated cells, when absolute mean values were transformed to relative values. The amount of IP-10 traceable in cell supernatants of non-stimulated cells dropped to 69 % ± 22 % when cells were incubated with cell culture medium containing 66 mM ethanol (Figure 4-12).

Table 4-10. Effect of two different ethanol concentrations on the IP-10 production by untreated, PHA-activated or LPS-stimulated PBMC. PBMC (10^6/2.4 ml) were maintained in RPMI medium, activated by PHA (2 µg/ml) or LPS (1 µg/ml) and treated with two different ethanol concentrations for 32 hours. IP-10 was determined using ELISA. Table a): absolute mean values; b): relative mean values (related to concentrations in ethanol-free samples with equal stimulation); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration; post hoc test: T-test: Tukey's test).

a	absolute IP-10 concentration [pg/ml]				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	12	785	1218	0.6242
	1	12	394	383	
	3	12	689	1223	
PHA	**0**	12	18189	5336	0.1885
	1	11	20445	8785	
	3	12	23346	5831	
LPS	**0**	12	577	588	0.5074
	1	12	802	919	
	3	12	1036	1245	

b	relative IP-10 concentration [%]						
						significance of ethanol concentration	
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**	**♦T-test vs 1 ‰**	**T-test vs 3 ‰**
n.c.	**0**	12	100	14	**0.0320**	0.6741	**0.0282**
	1	12	90	42			0.1682
	3	12	69	22			
PHA	**0**	12	100	12	**0.0497**		
	1	12	102	51			
	3	12	132	30			
LPS	**0**	12	100	25	0.1416		
	1	12	143	101			
	3	12	168	97			

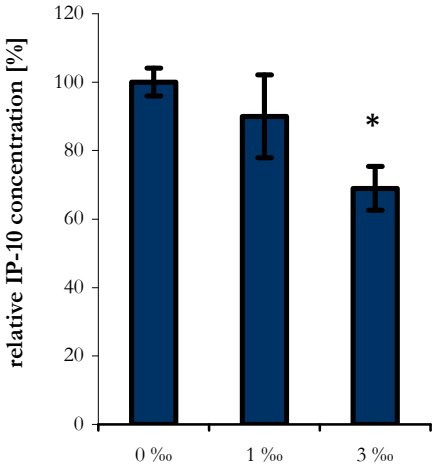


Figure 4-12. Relative IP-10 concentrations in cell culture supernatants of unstimulated PBMC. Data represent the relative mean of values and SEM of 12 experiments done in duplicates. [e]F-test (ANOVA, significance of ethanol), post hoc test: [*]Tukey's test (versus 0 ‰): * = p-value < 0.05

4.3 Proliferation of human Peripheral Blood Mononuclear Cells (PBMC)

4.3.1 Effects of PHA activation and ethanol treatment on proliferation of human Peripheral Blood Mononuclear Cells (PBMC)

4.3.1.1 Effect of PHA activation

Phytohemagglutinin is a commonly used activator of lymphocytes. PHA is a polypeptide belonging to the group a lectines. It is able to bind to certain surface-glycoproteins and -glycolipids on lymphocytes. Its structure is responsible for connecting T cells and antigen-presenting cells leading to polyclonal T cell activation. In this study, PHA confirmed its efficiency as a strong inductor of cytokine release and cell proliferation (Table. 4-11, Figure 4-13).

Results shown in Table 4-11 and Figure 4-13 accent the proliferative response of isolated PBMC treated with PHA.

The amount of DNA per cavity is almost stable in cells untreated within 9 days of *in-vitro* cultivation, whereas the addition of PHA leads to a three-times higher DNA content within 7 days of incubation and 9 days upon stimulation the amount of DNA is increased 3.6-fold.

Table 4-11. Effect of PHA stimulation on the absolute amount of DNA of human PBMC (10^4/120 µl) per cavity at incubation start and day 7 and 9 in cells treated 2 µg/ml PHA. The amount of total DNA was determined using SYTOX green. Absolute mean values, N: number of cases; SD: standard deviation, Inc: incubation, stim: Stimulation n.c.: negative control, ^{s}F-test: (ANOVA: significance of stimulation), post hoc test: *t-test: Student's t-test.

absolute amount of DNA/cavity [ng/cav]						
					significance of stimulation	
Inc [d]	stim	N	mean	SD	^{s}F-test	*t-test
0	n.c.	14	41	4		
	PHA	14	41	4		
7	n.c.	14	36	8	< 0.0001	< 0.0001
	PHA	14	110	24		
9	n.c.	14	37	12		< 0.0001
	PHA	14	132	44		

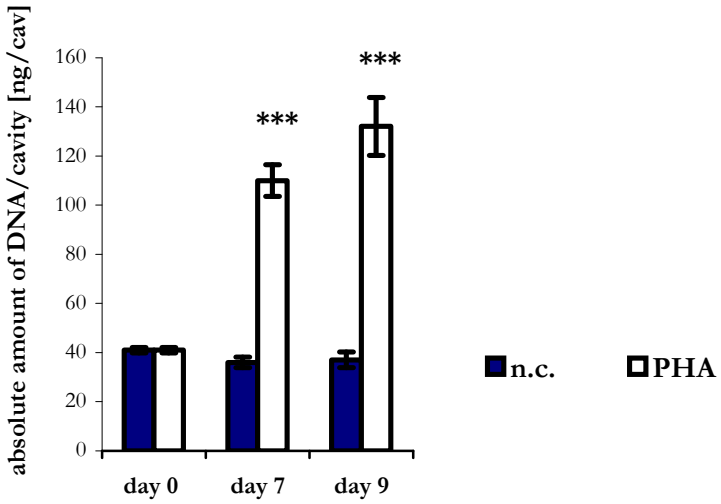


Figure 4-13. Absolute amount of DNA of PHA-activated PBMC per cavity. Data represent the absolute mean of values and SEM of 14 experiments done in duplicates. ^{s}F-test (ANOVA, significance of stimulation), post hoc test: *t- test: Student's t-test (versus n.c., at each time point):, *** = p-value < 0.001.

4.3.1.2 Effect of ethanol treatment

Many of the immunosuppressive effects of acute alcohol treatment have been linked to decreased production of inflammatory cytokines. On the other hand, a decline in cell number and viability can also be observed. To find out whether low ethanol concentrations used in this study are responsible for a lower cytokine release or if this is due to lower cell numbers, we used SYTOX Green to determine cell proliferation and viability.

The results on the effect of low ethanol concentration on the absolute amount of DNA of PHA-activated human PBMC are given in Table 4-12a.

For further evaluation, absolute mean values were transformed in relative mean values. The absolute amount of DNA per cavity of PHA-activated PBMC without ethanol treatment was set to 100 %. Relative mean values are shown in Table 4-12b.

Table 4-12. Effect of two different ethanol concentrations on the absolute and relative amount of DNA per cavity at incubation day 7 and 9 in cells treated with 22 mM and 66 nm of ethanol. The amount of total DNA was determined using SYTOX Green. Table a) absolute mean values; b) relative mean value (PHA, 72 hours = 100 %); N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration).

a	absolute amount of DNA/cavity [ng/cav]				
					significance of ethanol concentration
Inc	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**
day 7	**0**	14	110	24	0.8640
	1	14	106	26	
	3	14	105	26	
day 9	**0**	14	132	44	0.6591
	1	14	121	27	
	3	14	122	33	

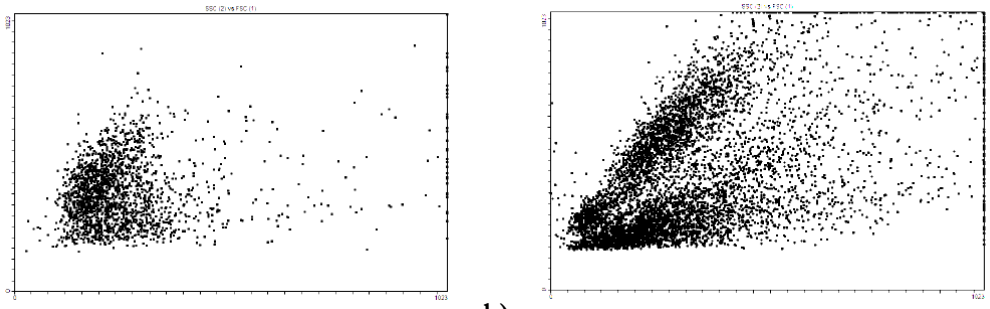
b	relative amount of DNA/cavity [%]				
					significance of ethanol concentration
Inc	**ethanol concentration [‰]**	**N**	**mean**	**SD**	**[e]F-test**
day 7	**0**	14	100	2	0.8880
	1	14	96	8	
	3	14	95	9	
day 9	**0**	14	100	5	0.6718
	1	14	96	18	
	3	14	96	18	

The results in Table 4-12 document no significant effect of low ethanol concentrations on proliferation compared to untreated cells. Neither the absolute amount nor the relative amount of DNA per cavity of PHA-activated human Peripheral Blood Mononuclear Cells (PBMC) is affected by alcohol concentrations of 22 or 44 mM (1 ‰ or 3 ‰).

4.4 Fluorocytometric analysis of human Peripheral Blood Mononuclear Cells (PBMC)

4.4.1 Effect of PHA-activation on morphological parameters of Peripheral Blood Mononuclear Cells (PBMC)

The morphological parameters of cells can be assessed as a response of a particle to an incident beam, resulting from interaction between reflection and refraction phenomena. Light scatter collected at a narrow angle from the incident beam (forward scatter, or FSC) can be considered proportional to the cell size; whereas light scatter collected at 90° (side scatter, or SSC) can be considered proportional to the cell granularity.



a) b)

Figure 4-14. Forward/Side-Scatter dot plot of a) freshly isolated human Peripheral Blood Mononuclear Cells and of b) cells stimulated with PHA for 4 days.

4.4.2 Expression of Clusters of Differentiation (CD) in PBMC

Immunofluorescent staining was used to identify and quantitate the number of cells that express specific surface antigens (CD marker). The following Figure 4-15 shows two color density plots of FACS scans done with freshly isolated Peripheral Blood Mononuclear Cells using different CD-antibody combinations. Isotype control monoclonal antibodies were used to estimate the non-specific binding of target primary antibodies to cell surface antigens. Isotype controls were used at identical concentrations and staining conditions as the target primary antibodies. Before samples were analyzed, the FSC Amp Gain and SSC voltage was adjusted to appropriately display the scatter properties of the isolated PMBC. The isotype controls were used to allocate the 'negative' population (99 % of the cells) in the lower-left quadrant of the plot. The percentage of cells that were considered PE-positive were then found in the upper left quadrant of the density plot, those FITC-positive in the lower right panel. The upper right panel (quadrant 2) contains all cells that express both CD-antigens, on their cell surface.

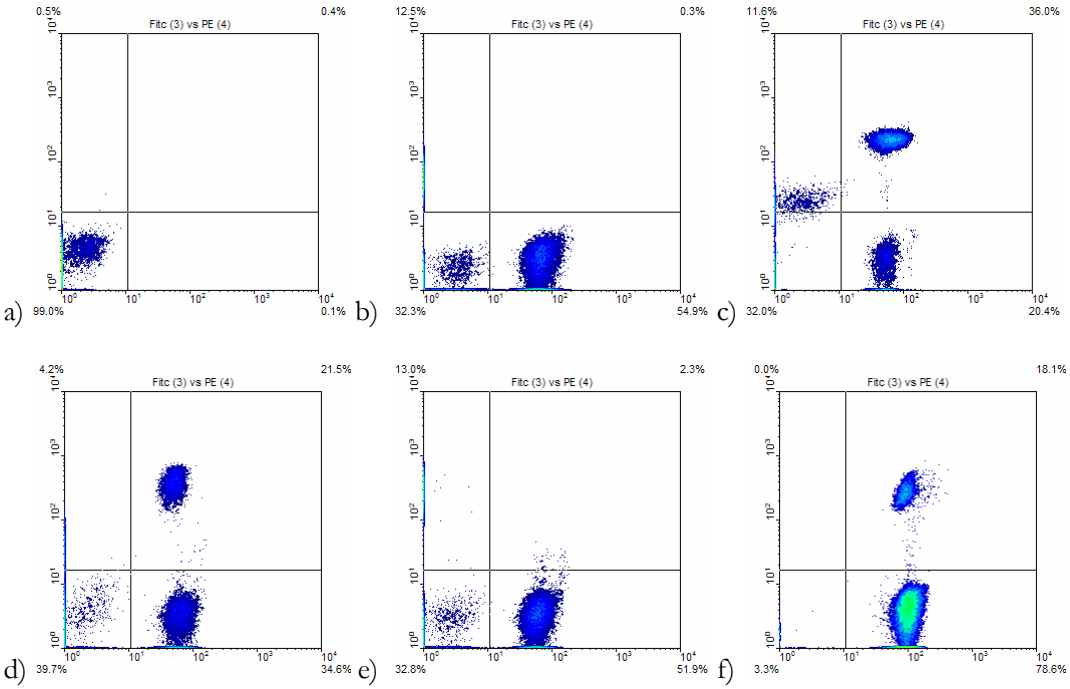


Figure 4-15. Flowcytometry results in representative cases. Two color analysis of freshly isolated PBMC. All samples were subsequently stained as described in material and methods with two-color antibody combinations containing b) CD3-FITC/CD19-PE, c) CD3-FITC/CD4-PE, d) CD3-FITC/CD8-PE, e) CD3-FITC/CD56-PE/CD16-PE, f) CD45-FITC/CD14-PE Using a) IgG1-FITC/IgG1-PE as isotype control conjugates to exclude background fluorescence.

4.4.3 The effect of PHA stimulation on cell composition of Peripheral Blood Mononuclear Cells

In vitro lymphocyte activation is a standard approach for evaluating cell-mediated response to a variety of stimuli, including polyclonal mitogens (i. e. PHA), antigens or cytokines. In this study, we wanted to investigate T cell activation using an assay that measures cell surface expression of certain CD antigens using multiparameter flow cytometry. The change in phenotype of lymphocyte subsets is shown in Table 4-5. The cells were analyzed directly upon isolation and 4 days untreated or PHA-activated.

Lymphocyte subsets were characterized as follows: B cells: $CD3^-/CD19^+$(Figure 4-15b, upper left panel), Th cells: $CD3^+/CD4^+$ (Figure 4-15c, upper right panel), cytotoxic T cells: $CD3^+/CD8^+$, Figure 4-15d, upper right panel), NK cells: $CD3^-/CD56^+/CD16^+$ (Figure 4-15e, upper left panel) Monocytes $CD45^+/CD14^+$ (Figure 4-15f, upper right panel).

The data prove the ability of PHA to stimulate T cell activation leading to 94 ± 2 % CD 3$^+$/CD56$^-$ cells during 4 days of incubation. At the same time, the percentage of monocytes dropped from 17 ± 1 % to 1 ± 2 % percent during PHA-activation, as well as the fraction of NK cells from 13 ± 1 % percent to 2 ± 1 % percent. On day 4 of incubation, no B cells were detected. The results of two independent experiments are shown in Table 4-13 and the percentage of each cell subset is diagrammed in Figures 4-16 to 4-18.

Table 4-13. Effect of 4-day-incubation and PHA stimulation on the phenotype of PBMC subtypes. Analyzed using FACSscan. The percentage of each cell subtype compared to total cell number is given as mean value. N: number of cases; SD: standard deviation, n.c.: negative control, Inc: incubation, stim: stimulation

	Relative cell number [%]				
Cell subtype	**Inc [d]**	**stim**	**N**	**mean**	SD
B-lymphocytes	0	n.c.	2	9	3
	4			3	0
		PHA		0	0
Th-lymphocytes	0	n.c.	2	35	1
	4			57	7
		PHA		61	2
Cytotoxic T cells	0	n.c.	2	21	1
	4			27	1
		PHA		34	9
NK-cells	0	n.c.	2	13	1
	4			11	7
		PHA		2	1
Monocytes	0	n.c.	2	17	1
	4			14	5
		PHA		1	2
CD3$^+$	0	n.c.	2	56	1
	4			84	11
		PHA		94	2

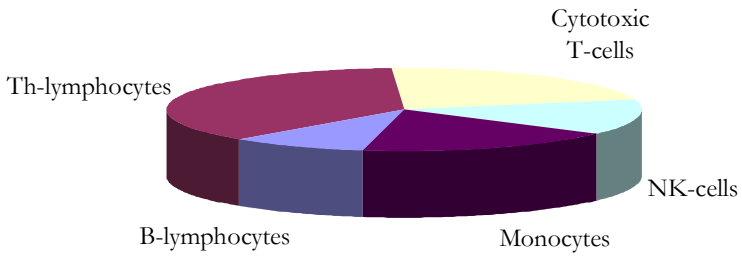


Figure 4-16. Percentage of each lymphocyte subset in freshly isolated PBMC compared to total cell number is given as mean value.

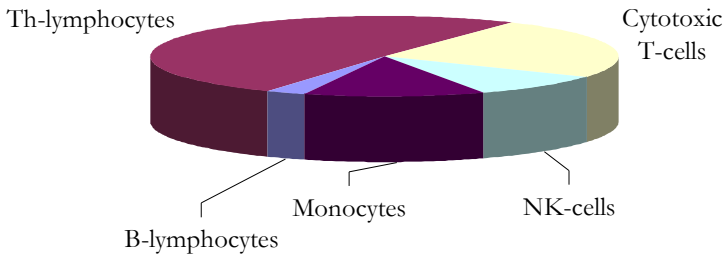


Figure 4-17. Percentage of each cell subtype in isolated PBMC left untreated for 4 days compared to total cell number is given as mean value.

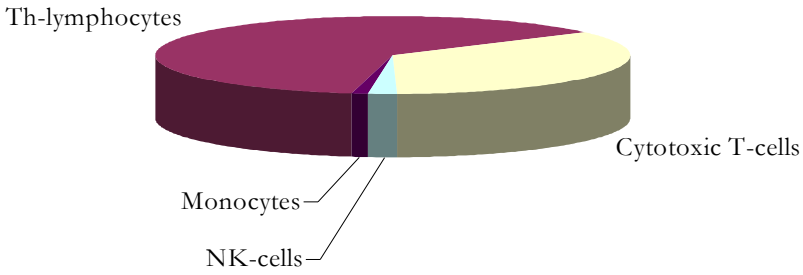


Figure 4-18. Percentage of each cell fraction in isolated PBMC stimulated with 2 µg/ml PHA for 4 days compared to total cell number is given as mean value.

4.4.4 Effect of PHA and ethanol on IL-12 receptor density on PBMC

To answer the question whether low levels of IFN γ production seen in ethanol-treated cells is due to a lower density of IL-12 receptor on cell surface, the expression of IL-12 receptor subunits was evaluated by flow cytometry

In PBMC isolated and stimulated with PHA for 6 days, 82 % of the cells were IL-12Rβ1$^+$ compared with only 20 % in untreated cells. Similar results were seen for the IL-12Rβ2 subunit, where 63 % of the PHA-treated were IL-12Rβ2$^+$ compared to 18 % in unactivated cells. Considering alcohol treatment of unactivated and PHA-stimulated PBMC (22 mM, 44 mM and 66 mM) for 6 days *in vitro*, we found no significant change in the expression of IL-12-Rβ1 or IL-12Rβ2 (Figure 4-19a, b and 4-20).

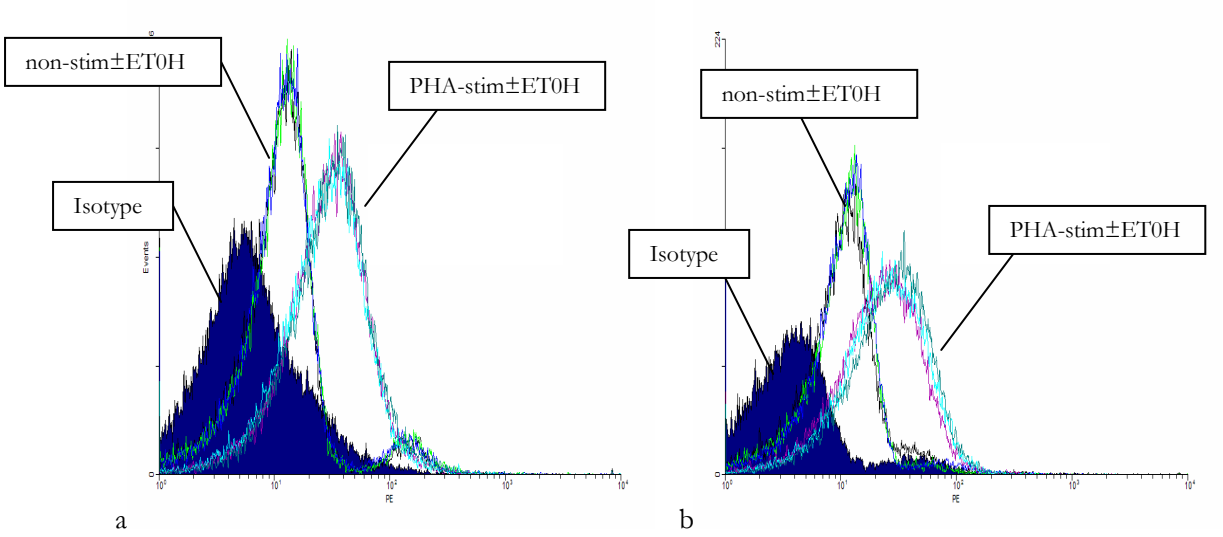


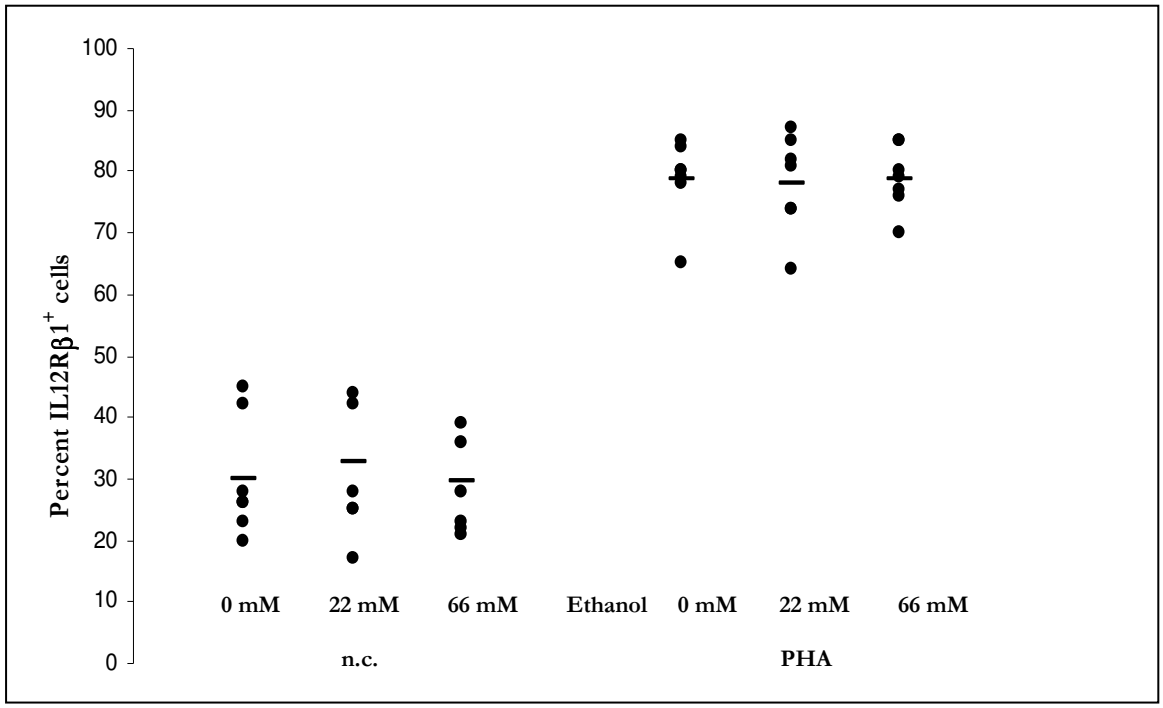
Figure 4-19. Flowcytometry results in representative cases. No change in IL-12 receptor density in PHA-stimulated T cells treated with three different alcohol concentrations. PBMC isolated from leukocyte-enriched buffy coats were stimulated with PHA (2 µg/ml) and treated with 22 mM, 44 mM and 66 mM ethanol for 6 days. The surface expression of a) IL-12-receptor subunit β1 and b) subunit β2 were assessed by FACS analysis. Appropriate isotype controls (■) were included for determination of non-specific binding. Neither unstimulated controls (— 0 ‰, — 1 ‰,— 3 ‰), nor PHA-activated cells (— 0 ‰, — 1 ‰,— 3 ‰) showed any difference in receptor density when treated with different ethanol concentrations.

Table 4-14. Expression of IL-12Rβ1 (a) and IL-12Rβ2 (b) by unstimulated and PHA-stimulated T cells isolated from cell-enriched whole blood from 6 healthy donors treated with two different ethanol concentrations. The percentage of cells expressing IL-12Rβ1 and IL-12Rβ2 cells were measured by flow cytometry. N: number of cases; SD: standard deviation; [e]F-test: (ANOVA: significance of ethanol concentration).

a	percentage of cells expressing IL-12Rβ1				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	6	31	10	0.9427
	1	6	31	12	
	3	6	29	7.8	
PHA	**0**	6	81	2.8	0.9456
	1	6	80	6.1	
	3	6	80	3.9	

b	percentage of cells expressing IL-12Rβ2				
					significance of ethanol concentration
Stimulation	**ethanol concentration [‰]**	**N**	**mean**	**SD**	[e]F-test
n.c.	**0**	6	16	3.1	0.7370
	1	6	17	3.7	
	3	6	18	4.1	
PHA	**0**	6	57	9.6	0.4504
	1	5	66	2.6	
	3	6	61	17	

a)



b)

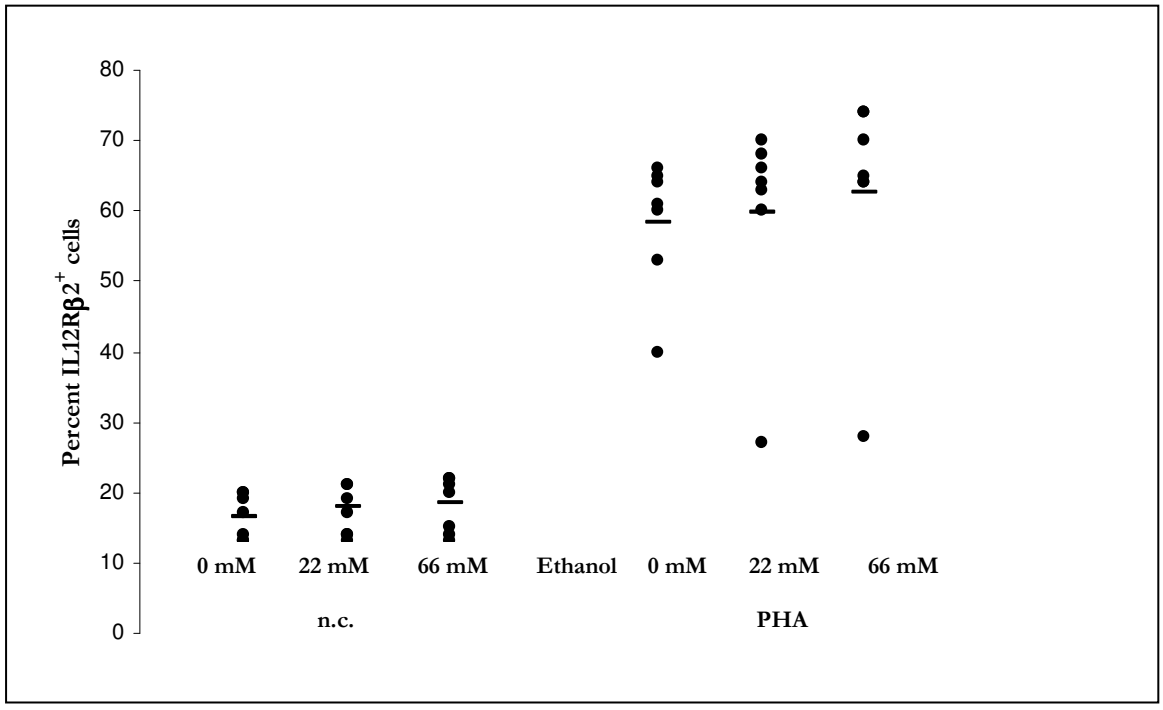


Figure 4-20. Expression of IL-12Rβ1 (a) and IL-12Rβ2 (b) by unstimulated and PHA-stimulated T cells isolated from cell-enriched whole blood from 6 healthy donors treated with two different ethanol concentrations. The percentage of cells expressing IL-12Rβ1 and IL-12Rβ2 cells were measured by flow cytometry. The horizontal line shows the mean values for each group. Cells show an increase in IL-12 receptor density when treated with PHA compared to unstimulated cells. The addition of ethanol to the medium did not alter the percentage of IL-12Rβ1$^+$ or IL-12Rβ2$^+$ cells.

4.5 Interferon γ production by NK-92 cells

4.5.1 Effect of IL-12 on the Interferon γ production by NK-92 cells

The ability of IL-12 to induce the production of Interferon γ in NK-92 cells and the kinetics of cytokine release was the focus of this experiment.

The absolute mean concentrations of IFN-γ after 6, 8, 10, 12, 14, and 24 hours did not rise significantly above the initial value in cell supernatants of non-stimulated NK-92 cells. IL-12 stimulation led to a significant increase of absolute mean values of IFN-γ concentration. IFN-γ production of NK-92 cells stimulated with IL-12 was significantly higher at 10, 12 and 24 hours compared to unstimulated cells (see Table 4-15 and Figure 4-21).

Although cells were kept in starvation medium for 16 hours without IL-12, IL-2, FCS and HS, 1093 pg/ml IFN-γ were detected as initial concentration.

For further evaluation, absolute mean values were transformed into relative mean values by defining the absolute mean value of IFN-γ concentration at 14 hours in the supernatant of IL-12 stimulated NK-92 cells as 100 %. In contrast to absolute mean values, incubation time had a significant effect on the relative mean values of IFN-γ in the supernatant of NK-92 cells stimulated with IL-12. The relative mean concentrations of IFN-γ were significantly higher at 10, 12 and 24 hours compared to the initial value. As seen for absolute mean values, IL-12 stimulation also increased relative mean values of IFN-γ concentration in the supernatant of NK-92 cells. Post hoc test (Tukey's HSD) indicated that relative mean IFN-γ concentration in the supernatant of NK-92 cell stimulated with IL-12 was significantly higher at 10, 12, 14 and 24 hours, compared to those not stimulated with IL-12 (Table 4-15 and Figure 4-21).

Table 4-15. Effect of IL-12 on IFN-γ production by NK-92 cells. NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS, or HS for 16 hours. Cells were replated and the medium was supplemented with or without 75 ng/ml IL-12. Samples of the supernatants were collected within 24 hours and IFN-γ was determined via ELISA. Table a) absolute mean values; b) relative mean values (IL-12, 14 hours = 100 %); N: number of cases; SD: standard deviation, [S]F-test (ANOVA, monofactorial: significance of IL-12 stimulation), post hoc test: Tukey's Honest-Significant-Difference test; [t]F-test (ANOVA, monofactorial: effect of time), post hoc test: *D-test = Dunnett's-test (6, 8, 10, 12, 14, 24 hours versus 0 h); n.c.: negative control; IL-12: cells stimulated with IL-12, Inc.: incubation.

a		absolute IFN-γ concentration [pg/ml]						
Inc [h]	N	n.c.		IL-12			significance of IL-12 stimulation	
		mean	SD	Mean	SD	[t]F-test	[S]F-test	[#]p(Tukey)
0	10	1093	426	1093	426	0.119	<0.001	-
6	10	1074	478	2861	2482			0.125
8	10	990	654	2763	1935			0.133
10	10	1042	446	3151	1788			**0.023**
12	10	1095	399	3243	1884			**0.018**
14	10	1068	444	2855	1099			0.124
24	10	1043	534	3253	2119			**0.013**

b		relative IFN-γ concentration [%]							
Inc [h]	N	n.c.		IL-12				significance of IL-12 stimulation	
		mean	SD	Mean	SD	[t]F-test	[*]p(Dunnett vs 0 h)	[S]F-test	[#]p(Tukey)
0	10	37	10	37	10	0.044	-	<0.001	-
6	10	37	12	97	78		0.092		0.056
8	10	32	20	91	62		0.155		0.070
10	10	36	11	109	54		**0.026**		**0.004**
12	10	38	9	111	56		**0.022**		**0.004**
14	10	37	12	100	28		0.068		**0.032**
24	10	37	16	117	69		**0.012**		**<0.001**

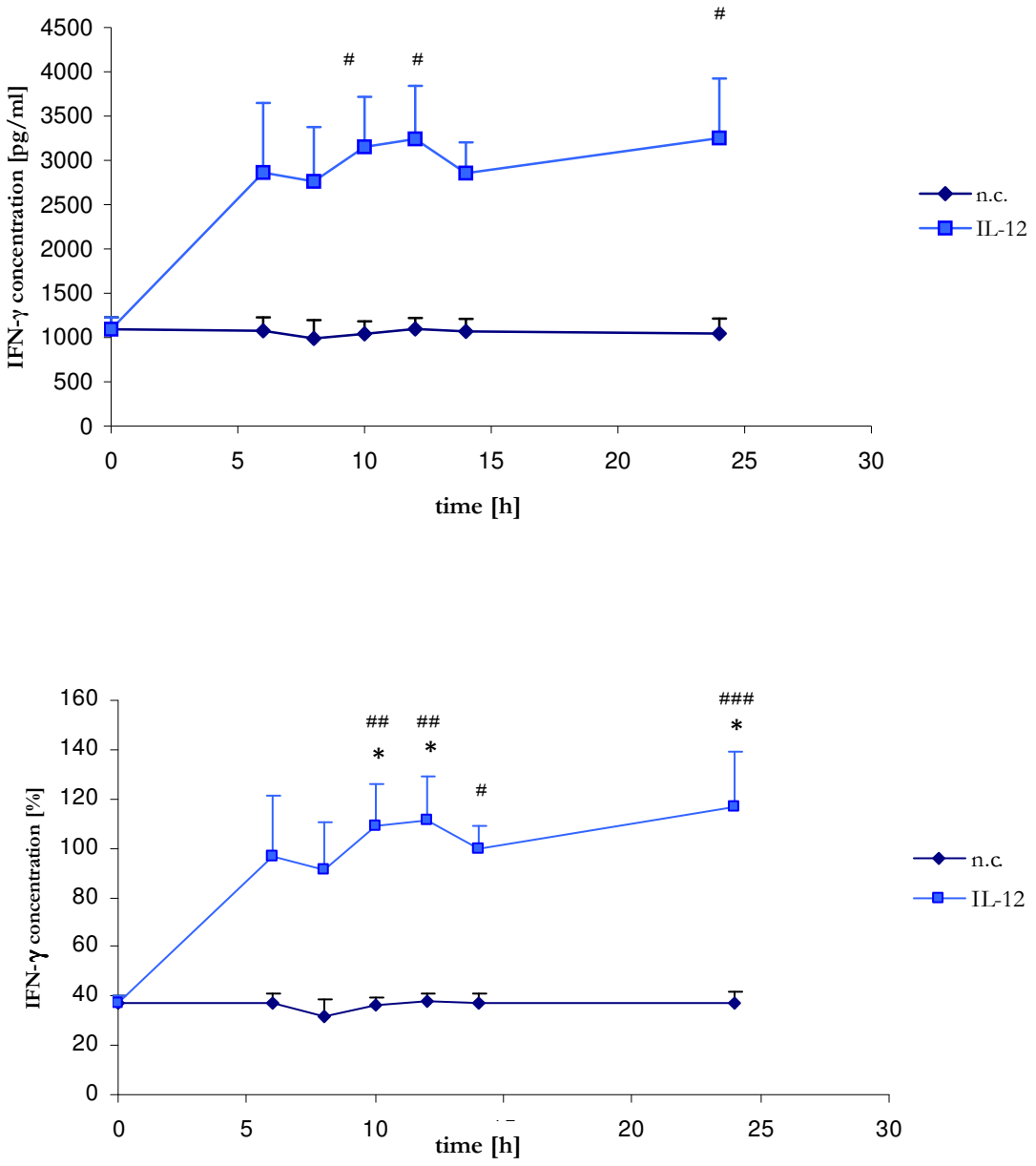


Figure 4-21. Effect of IL-12 on IFN-γ production by NK-92 cells. Cells were maintained in starvation medium without IL-12, IL-2, FCS, or HS for 16 hours. Cells were replated and incubated in the presence or absence of 75 ng/ml IL-12. Samples of the supernatants were collected within 24 hours and IFN-γ was determined via ELISA. Top: absolute mean values; Bottom: relative mean values ± SEM (IL-12, 14 hours = 100 %); ^{S}F-test (ANOVA, monofactorial: significance of IL-12-stimulation), post hoc test: $^{\#}$Tukey's Honest-Significant-Difference test; tF-test (ANOVA, monofactorial: effect of time), post hoc test: *D-test = Dunnett's-test (6, 8, 10, 12, 14, 24 hours versus 0 h); n.c.: negative control; IL-12: cells stimulated with IL-12, $^{*/\#}$ = p-value < 0.05; / $^{\#\#}$ = p-value < 0.01; $^{***/\#\#\#}$ = p-value < 0.001; n.c.: negative control; IL-12: cells stimulated with IL-12.

4.5.2 Effect of ethanol on IL-12-induced IFN-γ production by NK-92 cells

In a next step, we sought to investigate whether ethanol treatment of NK cells is leading to results comparable to those seen in PHA-activated PBMC.

The absolute mean value of IFN-γ level in the supernatants of IL-12 stimulated and with ethanol incubated cells decreased significantly within 24 hours with respect to each applied ethanol concentration, i.e. 1 ‰, 2 ‰ or 3 ‰ ethanol (Table 4-16, Figure 4-22).

Regarding treatment with 1 ‰ ethanol, IFN-γ concentration in cell supernatants of IL-12-stimulated NK-92 cells were significantly lower at 6 and 14 hours of incubation compared to initial values. For 2 ‰ and 3 ‰ treatment absolute mean values of IFN-γ level were significantly suppressed at each time point of sample drawing compared to concentrations at incubation start.

Comparing absolute mean values of IFN-γ concentration in the supernatants of IL-12 stimulated NK-92 cells with those of IL-12 stimulated and ethanol-treated cells, the addition of ethanol (1 ‰, 2 ‰ or 3 ‰) had a significant effect on IFN-γ levels. IL-12-induced IFN-γ production of exclusively IL-12 stimulated NK-92 cells was significantly higher compared to the absolute mean value of IFN-γ level in the supernatant of NK-92 cells incubated with IL-12 and either with 1 ‰, 2 ‰, or 3 ‰ ethanol after 6, 8, 10, 12, 14, and 24 hours.

For further evaluation, absolute mean values of IFN-γ levels were transformed into relative mean values. The absolute mean value at 14 hours of IL-12 stimulated cells was set to 100 %. The IFN-γ production decreased in a highly significant manner, when incubating NK-92 cells with ethanol. The relative mean values of IFN-γ level in the supernatant were significantly lower at each time point of sample drawing and for all applied ethanol concentrations (Table 4-16 and Figure 4-22).

Additionally IFN-γ concentrations in ethanol-containing preparations were below the concentration in cell supernatants of non-stimulated cells.

Table 4-16. Effect of ethanol on IL-12-induced IFN-γ production by NK-92 cells: NK-92 cells were maintained in starvation medium for 16 hours. Cells were replated and the medium was supplemented with 75 ng/ml IL-12 and in part with 1, 2, or 3 ‰ ethanol. Samples of the supernatants were collected and IFN-γ was measured by ELISA. Table a) absolute mean values ± SD, Table b) relative mean values ± SD (IL-12, 14 hours, without ethanol = 100 %); ᵗF-test (ANOVA, monofactorial): effect of incubation time (24 hours), post hoc test: *D-test= Dunnett's-test (6, 8, 10, 12, 14, 24 hours versus 0 h); N: number of experiments; SD: standard deviation, Inc: incubation. All concentrations of IFN-γ in the presence of any ethanol concentration were significantly lower (p<0.001).

a		absolute IFN-γ concentration [pg/ml]														
Inc [h]	N	IL-12			IL-12 1 ‰				IL-12 2 ‰				IL-12 3 ‰			
		IFN-γ		effect of time	IFN-γ		effect of time		IFN-γ		effect of time		IFN-γ		effect of time	
		mean	SD	ᵗF-test	mean	SD	ᵗF-test	#D-test	mean	SD	ᵗF-test	#D-test	mean	SD	ᵗF-test	#D-test
0	10	1093	426		1093	426		-	1093	426		-	1093	426		-
6	10	2861	2482		344	358		0.008	87	48		<0.001	59	35		<0.001
8	10	2763	1935		560	378		0.096	261[1]	334		<0.001	91	36		<0.001
10	10	3151	1788	0.119	514	568	0.048	0.060	99	39	<0.001	<0.001	73	25	<0.001	<0.001
12	10	3243	1884		695	675		0.313	109	52		<0.001	152	136		<0.001
14	10	2855	1099		454	450		0.031	147	84		<0.001	112	62		<0.001
24	10	3253	2119		633	598		0.190	348	455		<0.001	323	436		<0.001

b		relative IFN-γ concentration [%]														
Inc [h]	N	IL-12				IL-12 1 ‰				IL-12 2 ‰				IL-12 3 ‰			
		IFN-γ		effect of time		IFN-γ		effect of time		IFN-γ		effect of time		IFN-γ		effect of time	
		mean	SD	ᵗF-test	#D-test	mean	SD	ᵗF-test	#D-test	mean	SD	ᵗF-test	#D-test	mean	SD	ᵗF-test	#D-test
0	10	37	10		-	37	10		-	37	10		-	37	10		-
6	10	97	78		0.092	11	11		<0.001	3	1		<0.001	2	1		<0.001
8	10	91	62		0.155	20	11		0.022	12[2]	18		<0.001	3	1		<0.001
10	10	109	54	0.044	0.026	15	15	0.002	0.003	3	1	<0.001	<0.001	3	1	<0.001	<0.001
12	10	111	56		0.022	21	17		0.035	4	1		<0.001	6	6		<0.001
14	10	100	28		0.068	14	13		0.002	5	2		<0.001	4	1		<0.001
24	10	117	69		0.012	20	15		0.026	11	14		<0.001	11	14		<0.001

[1] N= 8

[2] N= 8

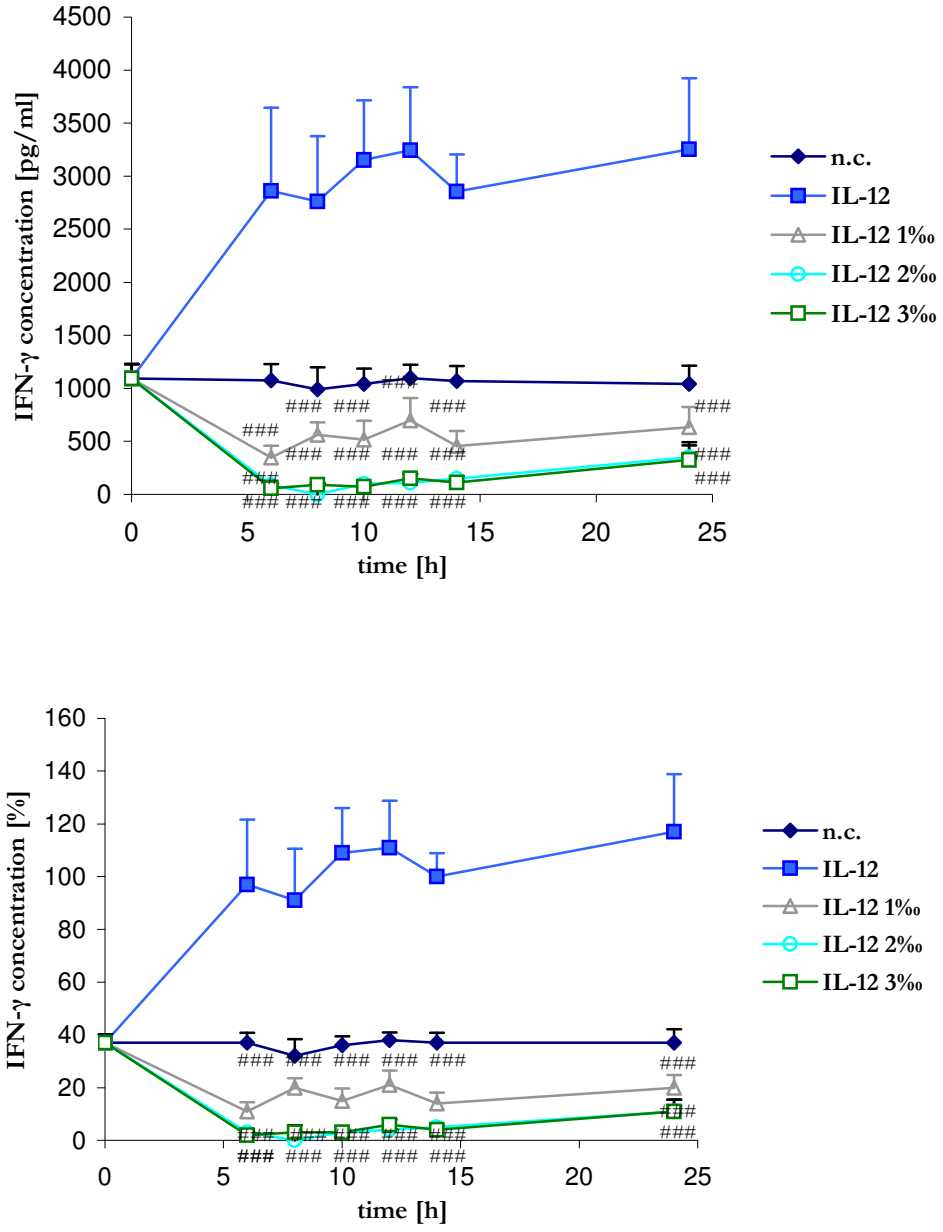


Figure 4-22. Effect of ethanol on IL-12-induced IFN-γ production by NK-92 cells. NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS or HS for 16 hours. Cells were replated and the medium was partly supplemented with 75 ng/ml IL-12 and with 1, 2, or 3 ‰ ethanol. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Top: absolute mean values; Bottom: relative mean values (IL-12, 14h, without ethanol = 100 %) ± SEM; post hoc tests: #Dunnett's-test (IL-12 versus IL-12 + 1, 2 or 3 ‰); # = p-value < 0.05; ## = p-value < 0.01; ### = p-value < 0.001; n.c.: negative control; IL-12: cells stimulated with IL-12; IL-12 + 1, 2 or 3 ‰: cells incubated with IL-12 and either 1, 2, or 3 ‰ ethanol.

4.6 Effect of ethanol incubation on cell viability

To determine the reduced ability of IL-12 to stimulate IFN-γ production under ethanol was due to changes in cytokine production or post-translational modulation and to exclude that ethanol in the concentrations applied had an effect on NK-92 cell survival we assessed a cell viability test using SYTOX Green in combination with Triton X-100.

On average, 59 % of the cells were still viable after 24 hours of incubation with starvation medium (= RPMI medium without additives) without ethanol. Regarding incubation with 1 ‰ ethanol, 65 % of the cells were still viable after 24 hours, regarding incubation with 2 ‰ ethanol 60 % of the cells were still viable and regarding incubation with 3 ‰ ethanol 62 % of the cells were still viable. Statistical evaluation did not indicate significant differences with respect to cell viability among incubations without ethanol and incubations with 1, 2, or 3 ‰ ethanol.

The results on cell viability upon ethanol treatment are given in Table 4-17.

Table 4-17. Effect on ethanol incubation on cell viability. Cells were plated and incubated in RPMI medium (without FCS, HS, IL-2) and without or either with 1, 2, or 3 ‰ ethanol for 24 hours. Results are given as mean values with SD of percent cells viable; [e]F-test (ANOVA) monofactorial, significance of ethanol

mean viable cells [%]				
ethanol	N	mean	SD	significance of ethanol
				[e]F-test
0	20	59	17	0.456
1	20	65	11	
2	20	60	14	
3	20	62	14	

These results lead us to the conclusion that low IFN-γ levels seen in cell supernatants of NK-92 cells treated with ethanol concentrations between 1, 2 and 3 ‰ are not due to a non-specific cytotoxic effects causing an increase in cell death.

4.7 IL-12-induced activation of transcription factors in NK-92 cells

4.7.1 Kinetics of IL-12-induced STAT4 phosphorylation

In previous experiments, it was shown that alcohol treatment had an inhibitory effect on IFN-γ production. As IL-12 is a potent inducer of IFN-γ production in NK-92 cells and STAT4 is the prominent signal transducer in IL-12 activation we studied the kinetics of STAT4 phosphorylation upon IL-12 stimulation using western blot analysis to establish an experimental setting for maximum STAT4 activation.

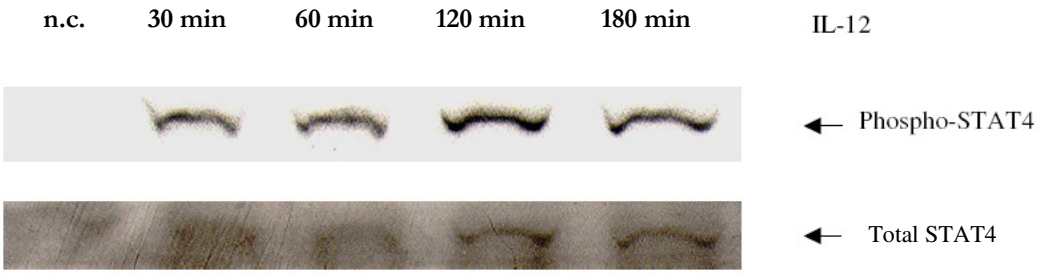


Figure 4-23. Activation kinetic of STAT4. NK-92 cells were incubated in the absence or presence of IL-12 (70 ng/ml) for the times indicated. Whole-cell extracts were prepared at the indicated time points. Extracts were resolved by SDS–PAGE, and then blotted with anti-phospho-STAT4 (upper panel). The filter was then stripped and reprobed with monoclonal anti-STAT4 to demonstrate equal loading (lower panel). Results are representative of three experiments.

Figure 4-23 shows the result of western blot analysis of STAT4 phosphorylation. Activated STAT4 is not detectable in untreated cells. IL-12 stimulation in contrast leads to STAT4 phosphorylation already traceable within 30 min of cytokine treatment. The highest level of activation is seen within 120 min of incubation. This time point was used for further experiments investigating the effect of ethanol incubation.

4.7.2 Effect of ethanol on IL-12-induced STAT4 phosphorylation

We showed that ethanol treatment at physiological concentrations leads to a dramatic decrease of IFN-γ expression in NK-92 cells. Aim of our research was to find out whether this is caused by suppression of STAT4 activation (the main transcription factor for IFN-γ induction).
Using whole cell lysates of ethanol-treated cells, we investigated the modulation of IL-12-induced STAT4 activation by acute alcohol treatment.

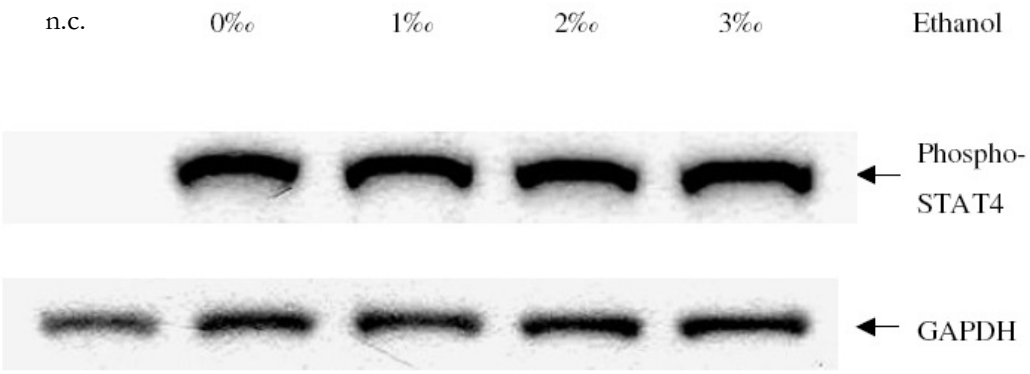


Figure 4-24. Western blot analysis of IL-12-induced STAT4 phosphorylation in NK-92 cells. NK-92 cells were plated and incubated with starvation medium in the absence (n.c.) or presence of 75 ng/ml IL-12 and 3 different ethanol concentrations. Samples of the cell lysate were used for western blotting. The antibody used was against phospho-STAT4. Blots were reprobed with the antibody for GAPDH to demonstrate equal loading. The membrane shown is representative for five independent experiments done.

As shown in previous experiments (4.7.1) IL-12-induced a strong activation of STAT4 2 hours upon stimulation. Alcohol used at physiologically relevant doses did not inhibit STAT4 phosphorylation as seen in Figure 4-24.
Cells did not response to ethanol incubation with a downregulation of IL-12-induced STAT4 activation as seen on protein level. Hence, low Interferon γ expression in alcohol-treated cells is not due to a significant reduction in STAT4 phosphorylation.

4.7.3 Effect of ethanol on STAT4 translocation

Considering the significant modulator potential of ethanol on the expression of IFN-γ gene that contains a STAT4 responsive element, we hypothesized that, though STAT4 activation through IL-12 stimulation is unchanged in ethanol-treated cells, the inhibition of translocation of transcription factors to the nucleus could explain changes in cytokine expression. Therefore, the effect of acute ethanol treatment was tested on the nuclear translocation of STAT4 dimers after IL-12 stimulation in NK-92 cells. Cells were stimulated with 75 ng/ml IL-12 for 2 hours in combination with three different ethanol concentrations. Nuclear extracts were prepared and tested for phospho-STAT4 levels by Western blot technique as described in Methods. NK-92 cell stimulated with IL-12 served as positive control for STAT4 translocation.

Figure 4-25 illustrates that 2 hours upon stimulation a substantial amount of phospho-STAT4 is translocated to the nucleus but the addition of ethanol does not lead to a change in translocation activity.

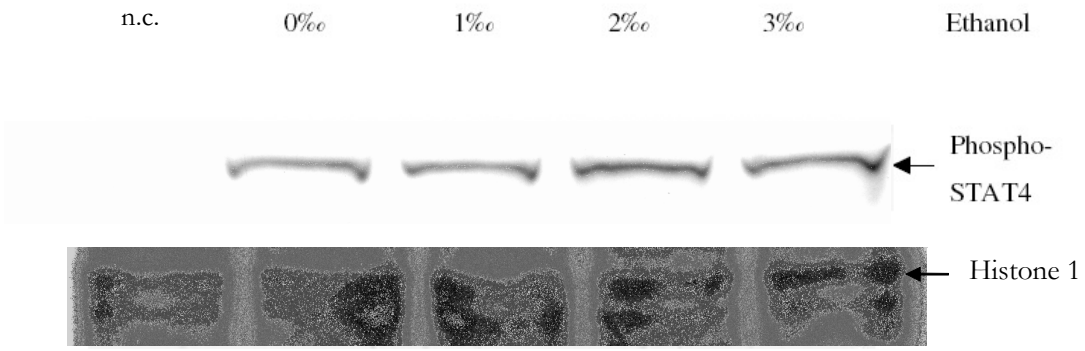


Figure 4-25. Phospho-STAT4 analysis of IL-12-induced nuclear translocation in NK-92 cells. NK-92 cells were plated and incubated with starvation medium in the absence (n.c.) or presence of 75 ng/ml IL-12 and 3 different ethanol concentrations. Samples of the nuclear extracts were used for western blotting. The antibody used was against phospho-STAT4. Blots were reprobed with the antibody for Histon 1 to demonstrate equal loading. The result shown is representative for five independent experiments performed.

4.7.4 IL-12-induced IFN-γ production by NK-92 cells is not mediated by AP-1

The may interact with other transcription factors previous to binding to the IFN-γ promoter to enhance its activation. This hypothesis is based on the recent observation that STAT4 interacts with c-Jun, a component of AP-1, to activate transcription (Malmgaard *et al.*, 2004). Therefore, we examined whether c-Jun is detectable in NK-92 cells and whether IL-12 stimulation is leading to an increase in c-Jun Phosphorylation.

Samples of the cell lysate and nuclear extract were tested. Samples of IL-12 stimulated and unstimulated cells were loaded onto the gel. As illustrated in Figure 4-26 AP-1 was not activated upon IL-12 stimulation. There was no band (indicating the position of phospho-c-jun) upon stimulation neither with nor without IL-12. This held true for the nuclear extracts as well as for the cell lysates. In NK-92 cells, IL-12-induced IFN-γ production is not mediated by AP-1.

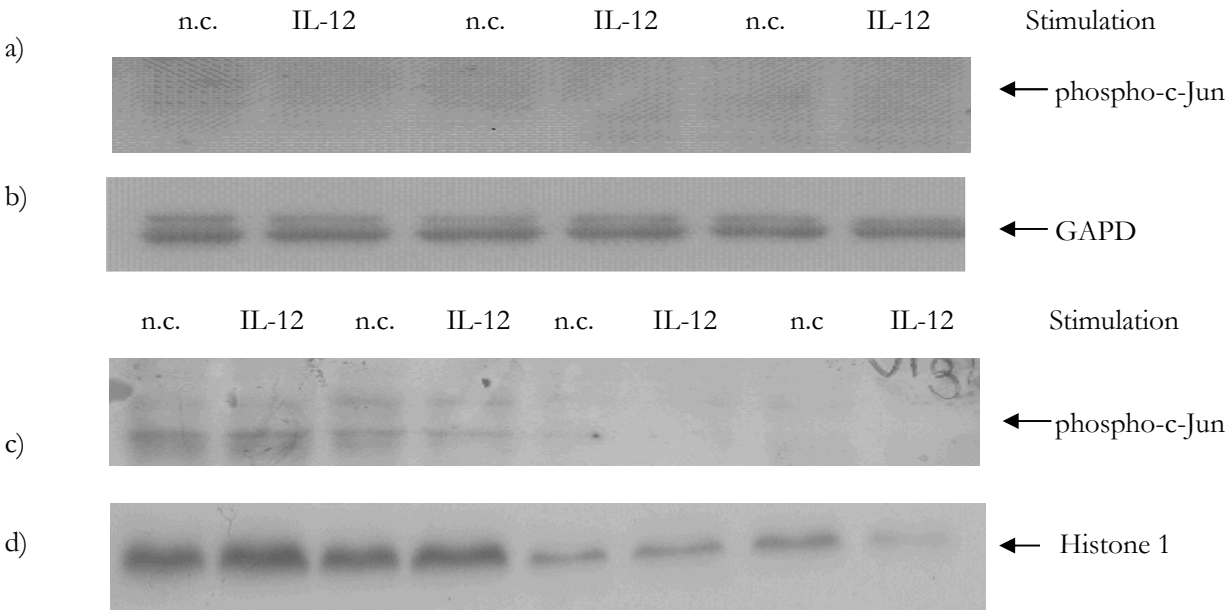


Figure 4-26. Phospho-c-Jun analysis of IL-12-induced activation of AP-1 in NK-92 cells. NK-92 cells were plated and incubated with starvation medium in the absence (n.c.) or presence of 75 ng/ml IL-12. Samples of the cell lysate and nuclear extract were used for western blotting. The antibody used was against phospho-c-Jun. Blots were reprobed with the antibody for GAPDH to demonstrate equal loading. Panel a) cell lysate samples of three independent experiments, Panel b) reblot of panel a, Panel c) nuclear extract of four independent experiments, Panel d) reblot of panel c for the presence of Histone 1. The arrow indicates the position of phospho-c-Jun.

4.8 IL-12-induced IFN-γ mRNA expression in NK-92 cells

4.8.1 Kinetics of IL-12-induced IFN-γ mRNA expression in NK-92 cells

Since changes in STAT4 phosphorylation and translocation are not responsible for ethanol-induced modulation of IFN-γ production the mechanisms behind alcohol-mediated downregulation of IFN-γ remains unrevealed. In the following experiments, we assessed the modulation of IFN-γ production at the mRNA and protein level.

To study the kinetics of IL-12-induced mRNA-expression, NK-92 cells were starved for 4 hours, replated and stimulated with 75 ng/ml IL-12 for up to 72 hours. In Table 4-18, the result of mRNA quantification using real-time PCR as described in Material and Methods are shown. The number of IFN-γ mRNA copies is related to those of 18S rRNA resulting in a relative copy number for each experiment done.

Table 4-18. Effect of IL-12 on relative IFN-γ mRNA expression in NK-92 cells. NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS or HS for 4 hours. Cells were replated and the medium was partly supplemented with 75 ng/ml IL-12. Samples of the supernatants were collected within 72 hours and IFN-γ mRNA expression was determined using real-time PCR. Relative number of copies [IFN-γ/18S rRNA); N: number of cases; SD: standard deviation, ^{s}F-test (ANOVA, monofactorial: significance of IL-12-stimulation), post hoc test: Tukey's-test; ^{t}F-test (ANOVA, monofactorial): effect of time (72 hours), post hoc test: *D-test = Dunnett's-test (0, 2, 4, 6, 8, 18, 24, 32, 48 and 72 hours vs. 0 h); n.c.: negative control; IL-12: cells stimulated with IL-12.

		relative number of IFN-γ mRNA copies [IFN-γ/18S rRNA]						
Inc [h]	N	n.c.		IL-12			significance of IL-12 stimulation	
		mean	SD	Mean	SD	*Dunnett	^{s}F-test	$^{\blacklozenge}$Tukey's-test
0	8	0.037	0.022	0.037	0.022		<0.001	
2	4	0.063	0.044	0.129	0.028	0.004		0.047
4	8	0.021	0.012	0.085	0.036	0.134		<0.001
6	4	0.058	0.035	0.175	0.057	<0.001		0.013
8	7	0.017	0.016	0.088	0.048	0.094		0.002
18	7	0.027	0.030	0.058	0.048	0.930		0.172
24	4	0.016	0.015	0.056	0.056	0.994		0.227
32	3	0.007	0.007	0.051	0.027	0.999		0.041
48	4	0.018	0.025	0.025	0.023	1.000		0.718
72	3	0.003	0.004	0.016	0.024	0.983		0.405

Between 2 and 8 hours, IL-12 stimulation led to a significant increase in relative IFN-γ mRNA. mRNA level at 2 and 6 hours did rise significantly above initial value. Cells not treated with IL-12 expressed a far smaller amount of mRNA.

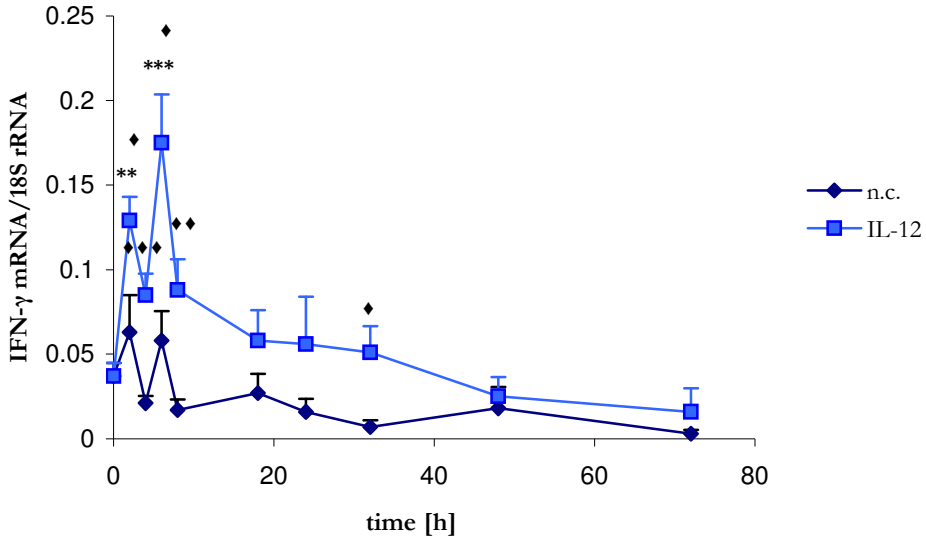


Figure 4-27. Effect of IL-12 stimulation on relative IFN-γ mRNA expression in NK-92 cells. Cells were maintained in starvation medium without IL-2, FCS or HS for 4 hours. Cells were replated and incubated in the presence or absence of 75 ng/ml IL-12. Samples were collected within 72 hours and IFN-γ mRNA expression was determined using real-time PCR. The results are given as relative copy number [IFN-γ/18S rRNA] ± SEM; post hoc test: *Dunnett's-test (monofactorial, significance of incubation time: 0, 4, 8, 18, 24, 32 48 and 72 h vs. 0 h); ♦Tukey's test (multifactorial, significance of IL-12 stimulation, IL-12 versus n.c.); $^{*/♦}$ = p-value < 0.05; $^{**/♦♦}$ = p-value < 0.01; $^{***/♦♦♦}$ = p-value < 0.001; n.c.: negative control; IL-12: cells stimulated with IL-12.

If we compare the relative copy number (IFN-γ/18S rRNA) at each incubation time point IL-12 stimulation led to a significant increase of mRNA expression at 2, 6 and 32 hours. At 4 hours of incubation, the IFN-γ mRNA expression is highly significant elevated. At six hours of stimulation, the mRNA concentration reaches a peak, with levels 3-fold higher compared to unstimulated cells. This time point was picked for investigation of ethanol-mediated changes in mRNA expression of NK-92 cells.

The mentioned results on the effect of IL-12 stimulation on IFN-γ mRNA expression of NK-92 cells are given in Figure 4-27.

4.8.2 Effect of ethanol on IL-12-induced IFN-γ mRNA expression in NK-92 cells

To investigate the intracellular mechanism of ethanol-driven downregulation of IFN-γ production by NK-92 cells, we examined whether ethanol (22 mM, 44 mM and 66 mM) could suppress expression of IFN-γ mRNA expression after IL-12 stimulation in 4 hour-starved NK cells. First, RT-PCR was performed with total RNA extracts from cells stimulated and ethanol-treated for 6 and 18 hours. IFN-γ mRNA transcripts were quantified in relative units compared to 18S rRNA transcripts for each culture condition. As expected, IL-12 enhanced IFN-γ mRNA expression 6 hours after incubation start, which is consistent with previous experiments. Surprisingly, however, ethanol did not lower expression of IFN-γ mRNA.

Thus, these results imply that suppression of IFN-γ production by NK-92 cells exposed to ethanol is not attributable to decreased IFN-γ gene transcription. The results are shown in Table 4-19 and Figure 4-28.

To examine long-term effects of ethanol on the IFN-γ -mRNA-expression RT-PCR was performed with total RNA extracts from cells stimulated and ethanol-treated for 18 hours. IFN-γ mRNA transcripts were quantified in relative units compared to 18S rRNA transcripts for each culture condition. In NK-92 incubated for 18 hours with IL-12 and ethanol no significant effect of IL-12 stimulation occurred between negative control and positive control ($p = 0.535$). Additionally ethanol incubation caused no change in IFN-γ expression (see Table 4-20).

Table 4-19. Effect of ethanol on IL-12-induced IFN-γ mRNA expression by NK-92 cells (6 hours). NK-92 cells were maintained in starvation medium without IL-2, FCS or HS for 4 hours. Cells were replated and the medium was supplemented with or without 75 ng/ml IL-12 and without or with 1, 2, or 3 ‰ ethanol. Samples were collected 6 hours after incubation start and relative IFN-γ mRNA copy number was determined via real-time PCR. Results are given as relative number of IFN-γ mRNA copies [IFN-γ/18S rRNA] ± SD; [c]F-test ANOVA, (monofactorial, significance of ethanol incubation); post hoc test: *Tukey's test (IL-12 versus IL-12 + 1, 2 or 3 ‰, and n.c.)

relative number of IFN-γ mRNA copies [IFN-γ/18S rRNA]								
				significance of ethanol concentration				
ethanol concentration [‰]	N	mean	SD	[c]F-test	p (Tukey's) vs. 0 ‰	p (Tukey's) vs. 1 ‰	p (Tukey's) vs. 2 ‰	p (Tukey's) vs. 3 ‰
n.c.	4	0.058	0.035		0.038	0.168	0.306	0.133
0	4	0.175	0.057	0.719		0.863	0.673	0.914
1	4	0.145	0.058				0.983	0.999
2	4	0.131	0.027					0.959
3	4	0.150	0.068					

Table 4-20. Effect of ethanol on IL-12-induced IFN-γ mRNA expression by NK-92 cells (18 hours). NK-92 cells were maintained in starvation medium without IL-2, FCS or HS for 4 hours. Cells were replated and the medium was supplemented partly with 75 ng/ml IL-12 and with 1, 2, or 3 ‰ ethanol. Samples were collected after 18 h and relative IFN-γ mRNA copy numbers were determined via real-time PCR. Results are given as relative numbers of IFN-γ mRNA copies [IFN-γ/18S rRNA] ± SD; [e]F-test ANOVA, (monofactorial, significance of ethanol incubation); post hoc test: [*]Tukey's test (IL-12 versus IL-12 + 1, 2 or 3 ‰, and n.c.)

relative number of IFN-γ mRNA copies [IFN-γ/18S rRNA]				
				significance of ethanol concentration
ethanol concentration [‰]	N	mean	SD	[e]F-test
n.c.	7	0.027	0.030	0.535
0	7	0.058	0.048	
1	3	0.039	0.040	
2	4	0.050	0.043	
3	4	0.076	0.075	

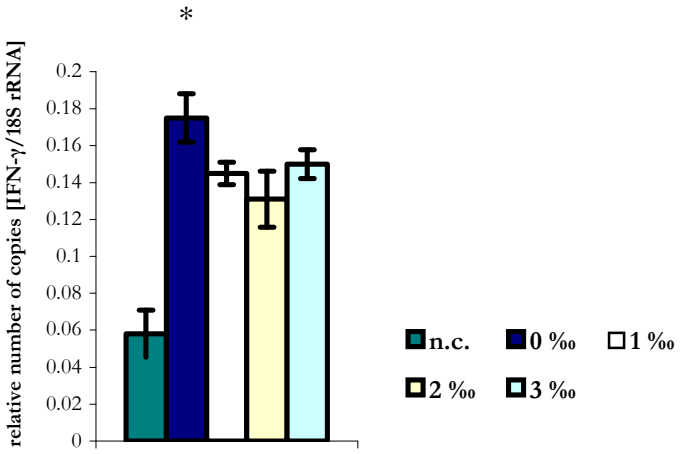


Figure 4-28. Effect of ethanol on IL-12-induced IFN-γ mRNA expression by NK-92 cells (6 hours). NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS or HS for 4 hours. Cells were replated and the medium was partly supplemented with 75 ng/ml IL-12 and with 1, 2 or 3 ‰ ethanol. Samples were collected after 6 h and relative IFN-γ mRNA copy numbers were determined via real-time PCR. Results are given as relative numbers of IFN-γ mRNA copies [IFN-γ/18S rRNA] ± SEM [e]F-test ANOVA, (monofactorial, significance of ethanol incubation); post hoc test: [*]Tukey's test (IL-12 versus IL-12 + 1, 2 or 3 ‰, and n.c.)

4.9 Intracellular IFN-γ concentration of NK-92 cells

4.9.1 Effect of IL-12 on intracellular IFN-γ concentration of NK-92 cells

Since there were no changes on mRNA-level of ethanol-treated NK-92 cells, we sought to determine intracellular IFN-γ concentrations in order to investigate whether the low protein concentrations in cell supernatant of alcohol-incubated cells were due to intracellular accumulation causing an ethanol-induced decrease of IFN-γ secretion into incubation medium.

In a first step, the intracellular changes of IFN-γ concentration upon IL-12 activation were determined.

Table 4-21. Effect of IL-12 on intracellular IFN-γ concentration of NK-92 cells. NK-92 cells were maintained in starvation medium for 16 hours. Cells were replated and incubated in the absence or presence of 75 ng/ml IL-12 for 24 hours. Cell lysates were used for measurement of IFN-γ levels via ELISA and related to the total protein content. a) absolute mean values; b) relative mean values (IL-12, 14 hours = 100 %); N: number of cases; SD: standard deviation, Inc.: incubation; ^{S}F-test (ANOVA, bifactorial): significance of IL-12 stimulation, ^{t}F-test (ANOVA, monofactorial): effect of time (24 hours), n.c.: negative control; IL-12: cells stimulated with IL-12.

a		IFN-γ mean [pg/mg protein]					
Inc [h]	N	n.c.		IL-12			significance of IL-12 stimulation
		mean	SD	mean	SD	^{t}F-test	^{s}F-test
0	10	195	223	195	223		
6	10	165	184	277	354		
8	10	183	120	356	443		
10	10	151	76	319	273	0.573	0.864
12	10	180	73	410	322		
14	10	194	95	395	274		
24	10	301	206	495	457		

b		IFN-γ mean [%]					
Inc	N	n.c.		IL-12			significance of IL-12 stimulation
		mean	SD	mean	SD	^{t}F-test	^{s}F-test
0	10	52	36	52	36		
6	10	42	31	65	55		
8	10	57	28	98	79		
10	10	47	28	90	54	0.147	0.796
12	10	66	46	99	22		
14	10	63	29	100	24		
24	10	87	47	113	75		

Incubation time had no significant effect on mean values of intracellular IFN-γ level, neither with respect to the absolute, nor to the relative data (absolute mean value of intracellular IFN-γ level at 14 hours upon IL-12 stimulation set to 100 %). Intracellular IFN-γ levels remained nearly constant within the incubation time. Bifactorial ANOVA (incubation time/IL-12 stimulation) indicated a significant effect of IL-12 stimulation neither on the absolute nor on the relative mean values of intracellular IFN-γ concentration. Table 4-21 and Figure 4-29 comprises the relative and absolute mean values of intracellular IFN-γ level of IL-12 stimulated and unstimulated NK-92 cells, as well as of statistical analysis as mentioned above.

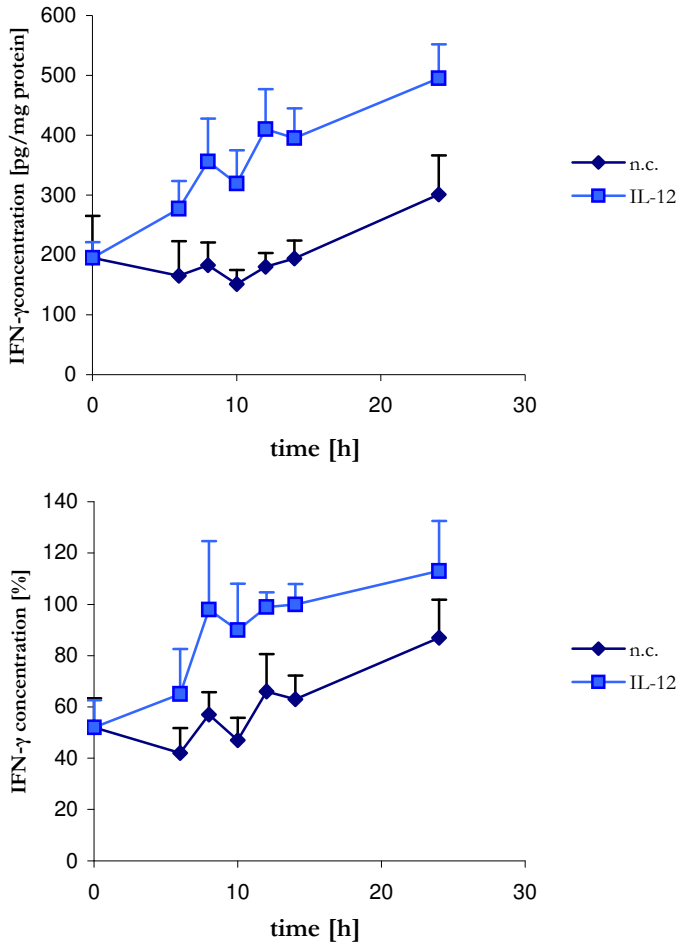


Figure 4-29. Effect of IL-12 on intracellular IFN-γ concentration of NK-92 cells. NK-92 cells were maintained in starvation medium for 16 hours. Cells were replated and incubated in the absence or presence of 75 ng/ml IL-12 for 24 hours. Cell lysates were used for measurement of IFN-γ levels via ELISA and related to the protein content. Top: Results are given as absolute mean values Bottom: Relative mean values ± SEM; N: number of cases; ^{S}F-test (ANOVA, multifactorial: significance of IL-12 stimulation, ^{t}F-test (ANOVA, monofactorial: effect of time), n.c.: negative control; IL-12: cells stimulated with IL-12.

4.9.2 Effect of ethanol on intracellular IFN-γ concentration of IL-12 stimulated NK-92 cells

Next, we investigated whether the ethanol-driven suppression of IFN-γ production from NK-92 cells was due to inhibition of IFN-γ secretion.

Incubation time had no significant effect on the absolute mean values of intracellular IFN-γ level of NK-92 cells incubated in the presence of IL-12 and either with 1, 2 or 3‰ ethanol (4.9.1).

No differences were indicated comparing absolute mean values of intracellular IFN-γ level of NK-92 cells upon IL-12 stimulation with those upon combined incubation with IL-12 and 1, 2, or 3 ‰ ethanol (Table 4-22, Figure 4-30). We therefore conclude that ethanol did not affect the intracellular IFN-γ concentration.

When evaluating data relatively (absolute mean value of intracellular IFN-γ concentration at 14 hours of IL-12 stimulated NK-92 cells set to 100 %), incubation time had no significant effect on the relative mean values of intracellular IFN-γ concentration of NK-92 cells incubated in the presence of IL-12 and 1 ‰ ethanol. Regarding the combined incubation of NK-92 cells with IL-12 and 2 or 3 ‰ ethanol, incubation time had a significant effect on the relative mean values of intracellular IFN-γ level. With respect to incubation with 2‰ ethanol, the relative mean values of intracellular IFN-γ level were significantly higher at 12 and 24 hours compared to the initial value. With respect to 3‰ ethanol, the relative mean values of intracellular IFN-γ level at 14 and 24 hours were significantly higher than the initial value.

Nevertheless, no significant differences were found comparing the relative mean values of intracellular IFN-γ level of exclusively IL-12 stimulated NK-92 cells, with those of the ethanol-treated cells throughout total incubation time of 24 hours.

Results on the effect of ethanol on intracellular IFN-γ concentration of IL-12 stimulated NK-92 cells are given in Table 4-22 and are diagrammed in Figure 4-30.

Based on these measurements, it appeared that the ethanol-induced suppression of IFN-γ production was not mediated through interference with IFN-γ secretion. Cells stimulated with IL-12 exclusively or with additional ethanol treatment did not react with significant changes of intracellular IFN-γ protein content. We therefore conclude that NK-92 cells keep a constant pool of cytokine; higher mRNA expression leading to higher protein biosynthesis is correlated with higher IFN-γ secretion.

Table 4-22. Effect of ethanol on intracellular IFN-γ concentration of IL-12 stimulated NK-92 cells. NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS or HS for 16 hours. Cells were replated and incubated with medium supplemented with or without 75ng/ml IL-12 and with or without 1, 2, or 3 ‰ ethanol within 24 hours. Samples of the cell lysate were collected and IFN-γ was determined via ELISA and related to the protein content. Table a) absolute mean values; Table b) relative mean values (IL-12, 14 hours = 100 %); N: number of cases; SD: standard deviation; [t]F-test (ANOVA, monofactorial) effect of time, post hoc test: *D-test= Dunnett's-test (6, 8, 10, 12, 14, 24 hours versus 0 h); [e]F-test (ANOVA, monofactorial) significance of ethanol treatment (IL-12 versus IL-12 + 1, 2, or 3 ‰); IL-12: cells stimulated with IL-12; IL-12 + 1, 2 or 3 ‰: cells incubated with IL-12 and either 1, 2, or 3 ‰ ethanol.

a absolute IFN-γ concentration [pg/ml]

Inc [h]	N	IL-12 1 ‰			IL-12 2 ‰			IL-12 3 ‰			significance of ethanol
		IFN-γ		effect of time	IFN-γ		effect of time	IFN-γ		effect of time	
		mean	SD	[t]F-test	mean	SD	[t]F-test	mean	SD	[t]F-test	[e]F-test
0	10	195	223		195	223		195	223		1.000
6	10	344	460		266	337		340	490		0.961
8	10	376	462		166	107		387	500		0.674
10	10	421	321	0.559	418	428	0.059	504	490	0.434	0.768
12	10	487	447		560	425		580	513		0.812
14	10	493	421		503	531		599	544		0.801
24	10	543	469		703	628		551	468		0.817

b relative IFN-γ concentration [pg/ml]

Inc [h]	N	IL-12 1 ‰			IL-12 2 ‰				IL-12 3 ‰				significance of ethanol
		IFN-γ		effect of time	IFN-γ		effect of time		IFN-γ		effect of time		
		mean	SD	[t]F-test	mean	SD	[t]F-test	*D-test	mean	SD	[t]F-test	*D-test	[e]F-test
0	10	52	36		52	36		-	52	36			1.000
6	10	88	79		59	51		1.000	70	80		0.988	0.793
8	10	93	75		56	21		1.000	91	81		0.731	0.603
10	10	109	34	0.078	98	44	**<0.001**	0.458	112	47	**0.006**	0.329	0.692
12	10	116	47		142	49		**0.022**	138	55		0.070	0.140
14	10	114	36		127	75		0.078	145	70		**0.043**	0.337
24	10	146	109		176	131		**0.001**	181	133		**0.002**	0.529

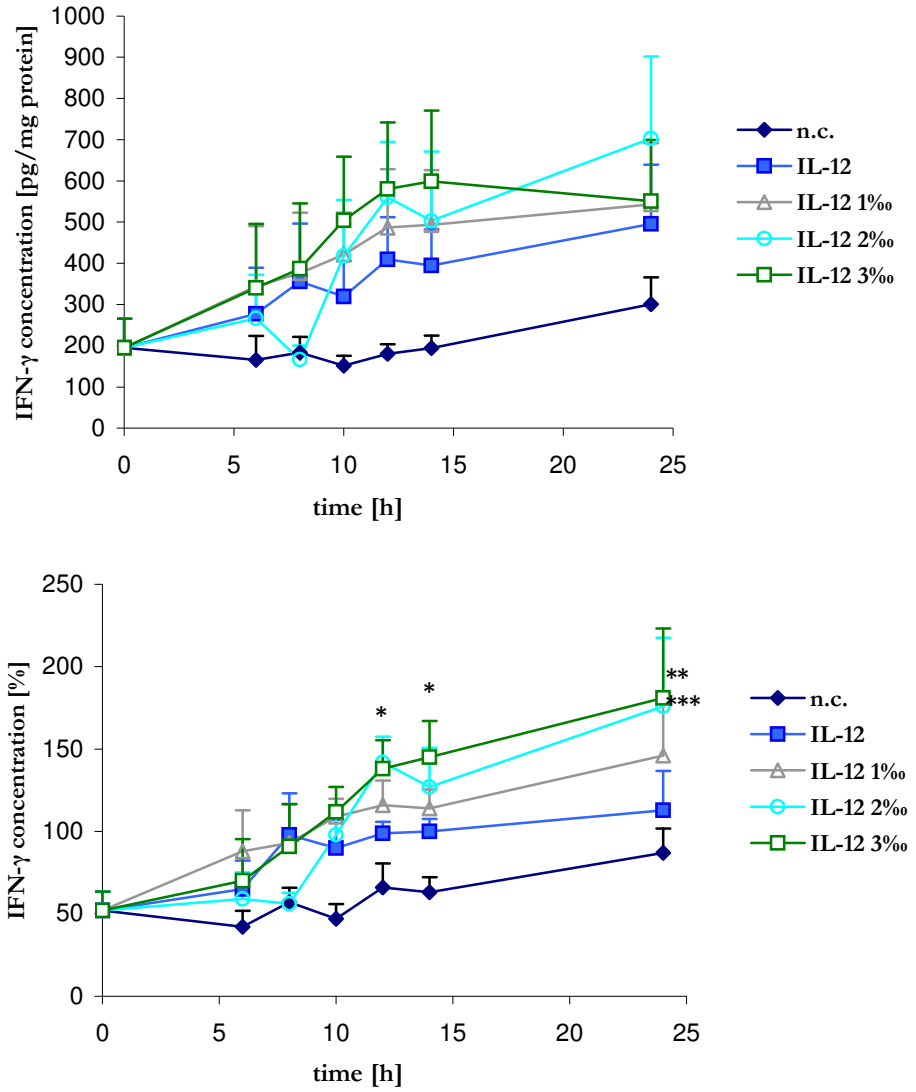


Figure 4-30. Effect of ethanol on intracellular IFN-γ concentration of IL-12 stimulated NK-92 cells. NK-92 cells were maintained in starvation medium without IL-12, IL-2, FCS or HS for 16 hours. Cells were replated and the medium was supplemented with or without 75 ng/ml IL-12 and with or without 1, 2, or 3 ‰ ethanol for 24 hours. Samples of the cell lysate were used for measurement of IFN-γ via ELISA. IFN-γ levels were related to the protein content. Results are given as relative mean values ± SEM (IL-12, 14 hours = 100 %), ANOVA (monofactorial, significance of incubation time), post hoc test: *Dunnett's-test (6, 8, 10, 12, 14, 24 hours versus 0 h) * = p-value < 0.05; **= p-value < 0.01; *** = p-value < 0.001; n.c.: negative control; IL-12: cells stimulated with IL-12; IL-12 + 1, 2 or 3 ‰: cells incubated with IL-12 and either 1, 2, or 3‰ ethanol.

4.10 Detectability of IFN-γ in the presence ethanol-treated NK-92 cells or their cell-free supernatant

Since no significant difference occurred in mRNA expression and intracellular accumulation of IFN-γ, we asked whether ethanol treatment is leading to a more rapid degradation of IFN-γ once released from NK-92 cells instead of a lower production by the cells themselves. To address this question, cells were kept in starvation medium containing 2.5 % FCS for four hours at a density of 2.5×10^6 cell/ml. At the end of starvation phase, cells were spinned down and resuspended in new medium containing 2.5 % FCS. Cells and cell culture supernatant were replated in 24 well plates. To examine the detectability of IFN-γ, recombinant human IFN-γ was supplemented to the cell-free supernatant at a concentration of 2 ng/ml and samples were taken at 0, 10 and 70 min upon IFN-γ addition.

Table 4-23. Effect of ethanol on the detectability of IFN-γ in presence of the cell-free supernatant of NK 92 cells. Absolute data. NK-92 cells were maintained in starvation medium for 4 hours. Cells were pretreated with different ethanol concentrations (1, 2, or 3 ‰), spinned down and cell culture supernatant was replated in 24 well plates and supplemented with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Table a) absolute mean values ± SD; Table b) [e]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]Tukey's-test (0 versus 1, 2, or 3 ‰).

a	absolute IFN-γ concentration [pg/ml]								
Inc [min]	N	0 ‰		1 ‰		2 ‰		3 ‰	
		mean	SD	mean	SD	mean	SD	mean	SD
10	8	1512	458	175	89	49	16	47	16
70	8	1537	452	327	216	66	25	56	15

b				
Inc [min]	significance of ethanol concentration			
	[e]F-test	[#]Tukey's test: p.c. vs. 1 ‰	[#]Tukey's test: p.c. vs. 2 ‰	[#]Tukey's test: p.c. vs. 3 ‰
10	<0.001	<0.001	<0.001	<0.001
70	<0.001	<0.001	<0.001	<0.001

The preincubation of NK-92 cells with 1, 2 or 3 ‰ ethanol had a highly significant effect on the detectability of IFN-γ in the cell-free supernatant at 10 and 70 min compared to the supernatant of cells without ethanol treatment (see Table 4-23 and Figure 4-31).

To reevaluate the results, absolute data were transformed into relative data. The absolute IFN-γ concentration at 0 minutes was set to 100 % (see Table 4-24 and Figure 4-32).

Table 4-24. Effect of ethanol on the detectability of IFN-γ in presence of the cell-free supernatant of NK 92 cells. Relative data. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down and cell culture supernatant was replated in 24 well plates and supplemented with 2 ng/ml human recombinant IFN-γ (0 min = 100 %). Samples of the supernatants were collected and IFN-γ was determined via ELISA. Table a) relative mean values ± SD; Table b) [c]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]Tukey's test (p.c. versus 1, 2, or 3 ‰).

a	relative IFN-γ concentration [pg/ml]							
Inc [min]	N	IL-12 0 ‰		IL-12 1 ‰		IL-12 2 ‰		IL-12 3 ‰	
		mean	SD	mean	SD	mean	SD	mean	SD
10	8	76.3	24.9	8.28	3.13	2.6	1.18	2.52	1.31
70	8	79.1	31.4	14.6	7.14	3.31	1.07	9.55	19.4

b				
Inc [min]	significance of ethanol concentration			
	[c]F-test	[#]Tukey's test: p.c. vs. 1 ‰	[#]Tukey's test: p.c. vs. 2 ‰	[#]Tukey's test: p.c. vs. 3 ‰
10	<0.001	<0.001	<0.001	<0.001
70	<0.001	<0.001	<0.001	<0.001

As seen in absolute data, relative data emphasize the rapid changes in the concentration of IFN-γ in cell supernatant of ethanol-treated NK-92 cells compared to the concentration of IFN-γ in the supernatant of untreated NK-92 cells. The results in Table 4-23 and Figure 4-31 document that the loss of IFN-γ in cell-free supernatant of ethanol-treated NK-92 cells already appeared 10 min after addition of human recombinant IFN-γ. Suggesting, that ethanol-treated cells release a substance that is able either to bind or to degrade IFN-γ. The same holds true at 70 min, where the concentration of IFN-γ tended to be slightly higher compared to the 10-min-values (Table 4-23).
This effect is not seen in incubation experiments where ethanol-treated cells were separated from their supernatant and resuspended in new cell culture medium. The results of these experiments are shown in Table 4-25 as absolute and in Table 4-26 as relative data and depicted in Figure 4-31 and 4-32.

Table 4-25. Effect of ethanol on the detectability of IFN-γ in presence of NK 92 cells. Absolute data. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down, resuspended in new medium and replated in 24 well plates and supplemented with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Table a) absolute mean values ± SD; Table b) [e]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]Tukey's test (p.c. versus 1, 2, or 3 ‰).

a	absolute IFN-γ concentration [pg/ml]							
Inc [h]	N	0 ‰		1 ‰		2 ‰		3 ‰	
		mean	SD	mean	SD	mean	SD	mean	SD
10	8	1524	782	1528	746	1557	696	1607	478
70	8	1617	838	1735	806	1633	781	1830	523

b				
Inc [h]	significance of ethanol			
	[e]F-test	[#]Tukey's test: p.c. vs. 1 ‰	[#]Tukey's test: p.c. vs. 2 ‰	[#]Tukey's test: p.c. vs. 3 ‰
10	0.999	1	1	0.9999
70	0.935	0.9999	1	0.9988

Table 4-26. Effect of ethanol on the detectability of IFN-γ in presence of NK 92 cells. Relative data. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down and cell were replated in 24 well plates and supplemented with 2 ng/ml human recombinant IFN-γ (0 min = 100 %). Samples of the supernatants were collected and IFN-γ was determined via ELISA. Table a) relative mean values ± SD; Table b) [e]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]Tukey's test (p.c. versus 1, 2, or 3 ‰).

a	relative IFN-γ concentration [%]							
Inc [min]	N	0 ‰		1 ‰		2 ‰		3 ‰	
		mean	SD	mean	SD	mean	SD	mean	SD
10	8	78.6	45	78.1	42.2	79	37.7	82.6	32.5
70	8	81.7	43.3	87.3	40.4	82.0	38.6	92.8	30.5

b				
Inc [min]	significance of ethanol			
	[e]F-test	[#]Tukey's test: p.c. vs. 1 ‰	[#]Tukey's test: p.c. vs. 2 ‰	[#]Tukey's test: p.c. vs. 3 ‰
10	0.996	0.999	0.999	0.997
70	0.930	0.991	0.999	0.939

In comparison to the results seen in cell-free supernatant, the concentration of IFN-γ remained stable when added to ethanol-treated NK-92 cells, separated from their incubation medium and resuspended in new cell culture medium. The fact that ethanol-treated cells are not the reason for the loss of IFN-γ, negate a mechanism by which the once released IFN-γ is re-uptaken by NK-92 cell as a consequence of ethanol preincubation.

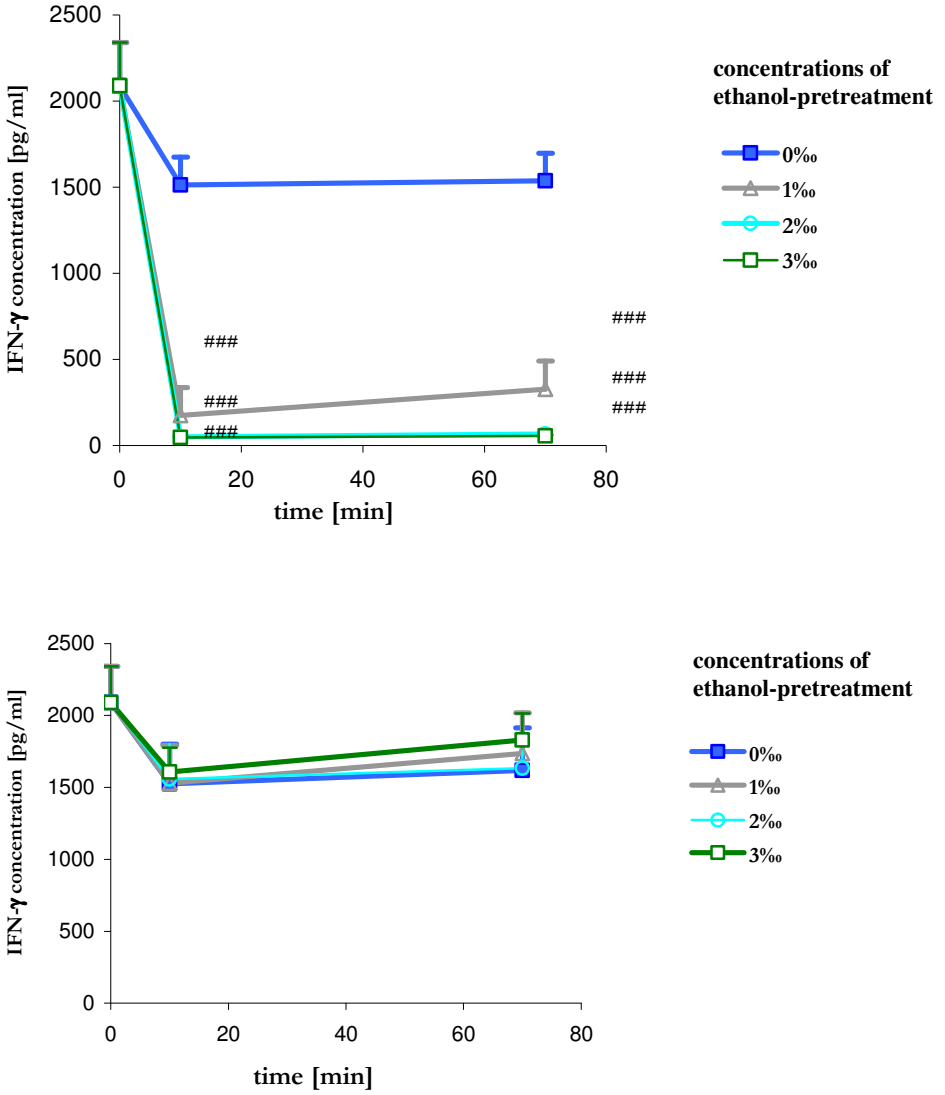


Figure 4-31. Comparison of the ethanol-mediated detectability of IFN-γ in presence of the cell-free supernatant (top) or ethanol-treated NK-92 cells (bottom). After 4 hours of starvation, cells were treated with different ethanol concentrations, spinned down and cell culture supernatant was separated from NK-92 cells. Both cell supernatant and resuspended cells were replated and supplemented with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Absolute mean values ± SEM; [e]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]Tukey's test (p.c. versus 1, 2, or 3 ‰), ### = p-value < 0.001

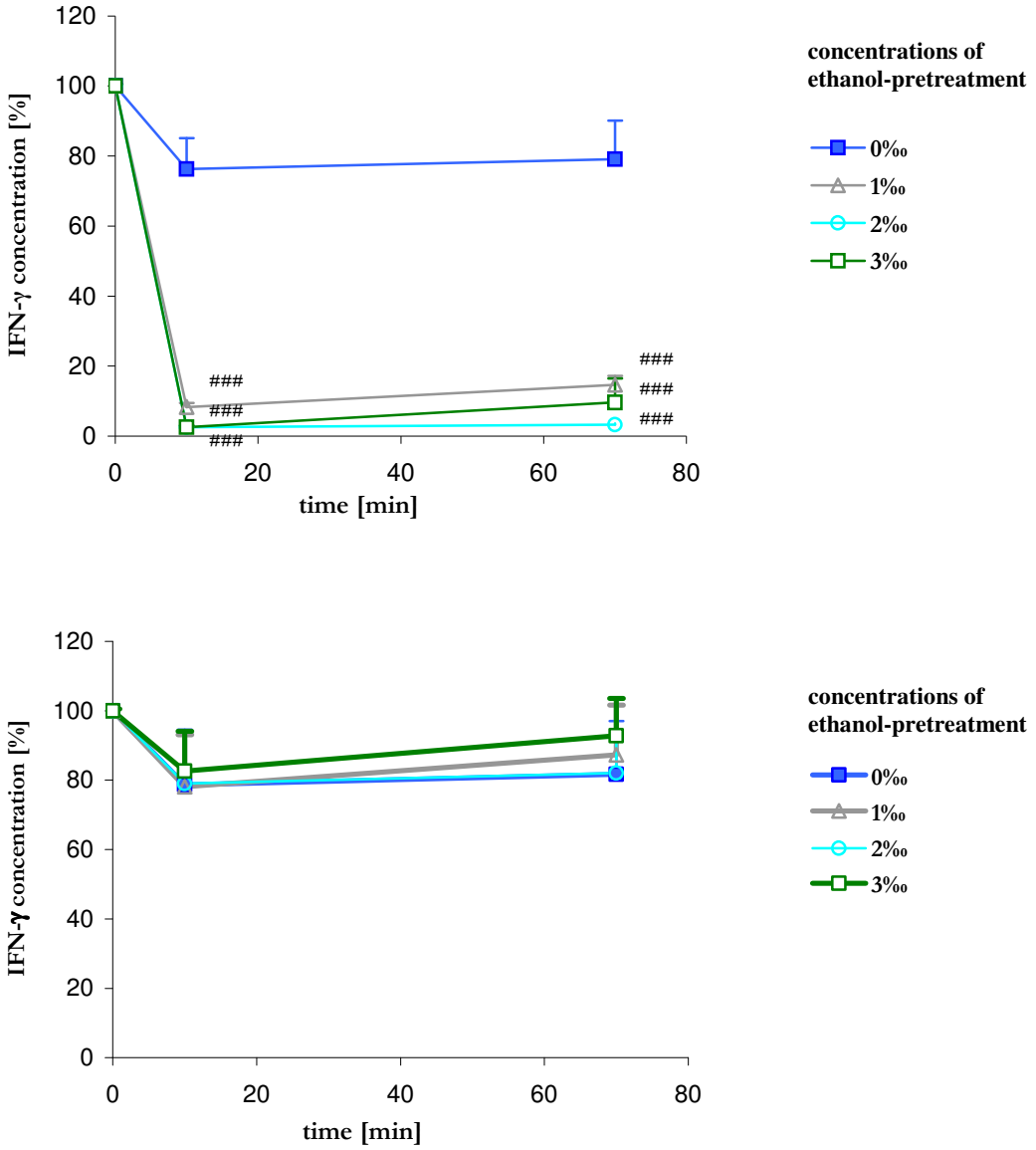


Figure 4-32. Comparison of the ethanol-mediated detectability of IFN-γ in presence of the cell-free supernatant of NK 92 cells (top) or ethanol-treated NK-92 cells (bottom). NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down and cell culture supernatant was separated from NK-92 cells. Both cell supernatant and resuspended cells were replated in 24 well plates and supplemented with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Relative mean values ± SEM; ^{e}F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: $^{\#}$Tukey's test (p.c. versus 1, 2, or 3 ‰), $^{\#\#\#}$ = $p < 0.001$.

4.11 Ethanol-induced secretion of soluble IFN-γ receptor

The result of the former experiment suggests a mechanism, in which once released IFN-γ is either bound or degraded by an unknown soluble protein that is secreted by ethanol-treated NK-92 cells. The fact that soluble cytokine receptors are a possible way to regulate cytokine signaling, we hypothesized that this also may be the case in ethanol-treated NK-92 cells.

In the following, we used two different approaches to investigate the change of soluble cytokine receptors in the supernatant of NK-92 cells incubated with different ethanol concentrations.

First, we used IFN-γ receptor antibody to prevent binding of IFN-γ protein by the soluble receptor. Second, we used a commercially available ELISA kit to measure IFN-γ receptor 1 concentration in NK-92 cell supernatants.

4.11.1 Effect of IFN-γ receptor antibody on the ethanol-mediated change of IFN-γ concentration in NK-92 cell supernatant

The addition of IFN-γ receptor antibody was used to investigate if the ethanol incubation of NK-92 cells leads to an increase in soluble cytokine receptor, which is able specifically to bind IFN-γ released from NK-92 cells.

To address this question, cells were kept in starvation medium containing 2.5 % FCS for four hours at a density of 2.5×10^6 cell/ml. At the end of starvation phase, cells were treated with 3 different ethanol concentrations for 10 min, cells were spinned down and cell culture supernatant was replated in 24-well plates. In a next step, IFN-γ receptor antibody was added at a concentration of 2 ng/ml and incubated for 1 h to allow receptor/antibody binding. To examine whether the addition of IFN-γ receptor antibody changed the amount of IFN-γ detectable in cell culture supernatant of ethanol-treated NK-92 cells, recombinant human IFN-γ was supplemented at a concentration of 2 ng/ml and samples were taken at 10 min upon IFN-γ addition.

The addition of IFN-γ receptor 1 antibody had no effect on the concentration of IFN-γ in cell-supernatants of ethanol-pretreated NK-92 cells. IFN-γ detectability in supernatants of NK-92 cells that underwent a pretreatment with ethanol at concentrations of 44 mM remained low (~12 %) compared to supernatants of untreated NK-92 in the presence or absence of the IFN-γ receptor antibody (see Table 4-27 and Figure 4-33).

Table 4-27. Effect of IFN-γ R1 ab addition on the detectability of IFN-γ in cell supernatants of ethanol-treated NK 92 cells. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down and cell culture supernatant was replated in 24 well plates, 2 ng/ml IFN-γ R1 ab was added. Incubation lasted 1 h, followed by a supplementation with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Absolute mean values ± SD; [e]F-test (ANOVA, monofactorial): significance of ethanol, post hoc test: [#]T-test = [#]Tukey's test (0 versus 2 ‰) [ab]F-test (ANOVA, monofactorial): significance of antibody incubation.

absolute IFN-γ concentration [pg/ml]								
N	ab	0 ‰		2 ‰		significance of ethanol		significance of antibody incubation
		mean	SD	mean	SD	[e]F-test	[#]T-test	[ab]F-test
15	–	633	380	74	33	<0.001	<0.001	0.116
9	+	861	253	112	77		<0.001	

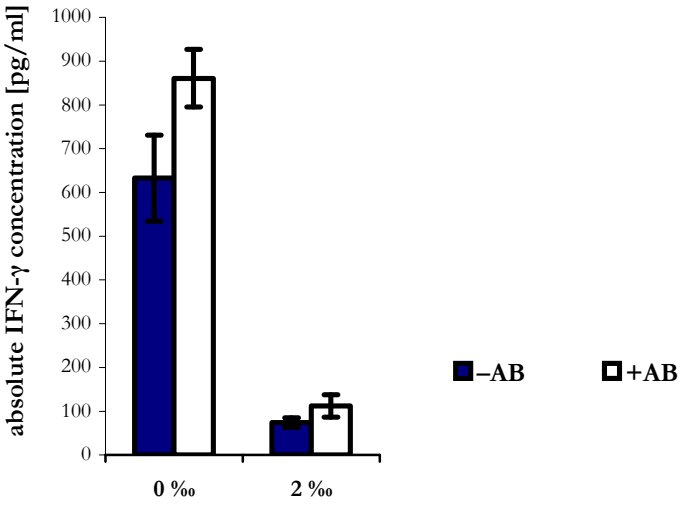


Figure 4-33. Effect of IFN-γ R1 ab addition on the detectability of IFN-γ in cell supernatants of ethanol-treated NK 92 cells. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down and cell culture supernatant was replated in 24 well plates, 2 ng/ml IFN-γ R1 ab was added. Incubation lasted 1 h, followed by a supplementation with 2 ng/ml human recombinant IFN-γ. Samples of the supernatants were collected and IFN-γ was determined via ELISA. Absolute mean values ± SEM.

4.11.2 Effect of ethanol on the IFN-γ receptor 1 concentration in NK-92 cell supernatant

Since the question remained whether the affinity of IFN-γ to the soluble receptor was higher then that of soluble receptor/receptor antibody complexes, the second approach to measure IFN-γ receptor concentrations in cell supernatants of ethanol-treated NK-92 cells was a direct method, using a commercial available ELISA kit for the IFN-γR1 chain.

To investigate the effect of ethanol on the concentration of IFN-γ receptor subunit R1 concentration in cell supernatants, NK-92 cells were kept in starvation medium containing 2.5 % FCS for four hours at a density of 2.5×10^6 cell/ml. At the end of starvation phase, cells were treated with 3 different ethanol concentrations for 10 min, cells were spinned down and cell culture supernatant was collected for ELISA assay.

Table 4-28. Effect of ethanol on the IFN-γ receptor antibody concentration in NK-92 cell supernatant. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down, samples of the supernatants were collected, and IFN-γR1 concentration was determined via ELISA. Absolute mean values ± SD; [e]F-test (ANOVA, monofactorial): significance of ethanol

absolute IFN-γ R1 concentration [pg/ml]				
				significance of ethanol concentration
ethanol concentration [‰]	**N**	**mean**	**SD**	[e]F-test
0	4	40	35	0.279
1	4	85	65	
2	4	115	57	
3	4	99	52	

Direct measurement of IFN-γ receptor 1 concentration in cell supernatants of ethanol-treated NK-92 cells in the presence of ethanol had no significant effect on the secretion of soluble IFN-γ receptor, though there is a tendency to higher receptor subunit 1 concentrations in cell supernatants of NK cells incubated with 3 different ethanol concentrations (Table 4-28 and Figure 4-34).

The results on the IFN-γR1 concentration in the supernatant of NK-92 cells reflect the data of experiments done with IFN-γ receptor antibody (see Table 4-27 and Figure 4-33).

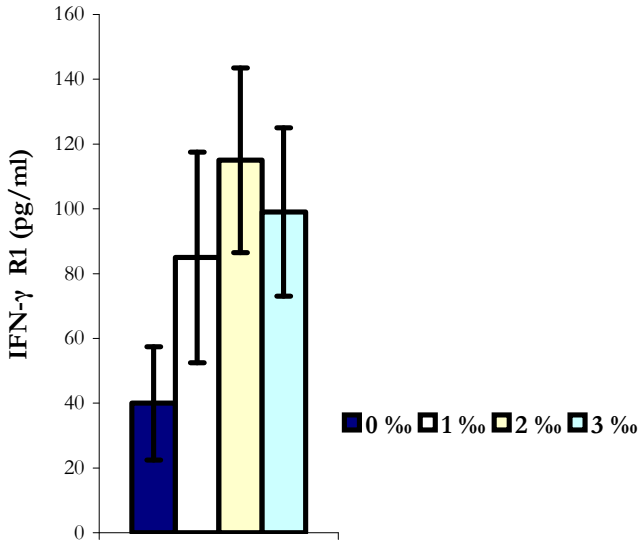


Figure 4-34. Effect of ethanol on the IFN-γ receptor antibody concentration in NK-92 cell supernatant. NK-92 cells were maintained in starvation medium for 4 hours. Cells were treated with different ethanol concentrations, spinned down, samples of the supernatants were collected and IFN-γR1 concentration was determined via ELISA. Absolute mean values ± SEM.

In summary, the concentration of IFN-γR1 concentration was extremely low and the changes of IFN-γR1 cannot serve as an explanation for the fact that the supplementation with 2000 pg/ml human recombinant IFN-γ is practically not longer detectable anymore in the supernatants of ethanol-incubated cells.

4.12 Effect of a protease inhibitor cocktail on the ethanol-mediated change of IFN-γ concentration in NK-92 cell supernatant

Instead of binding to a specific protein, IFN-γ loss in supernatants of ethanol-treated NK-92 cells could be due to extracellular proteolysis. Another posttranslational mechanism by which once released cytokines are modulated. Since human mononuclear cells express an array of serine and metal dependent proteinases that are under complex developmental control and are also highly regulated by physiologic and pharmacologic stimuli, we hypothesized whether an increase in IFN-γ extracellular degradation by proteolytic enzymes is a result of ethanol incubation. If so, inhibition of proteases, followed by IFN-γ addition to cell supernatants of ethanol-treated NK-92 cells should abolish the dramatic decrease of IFN-γ concentration seen in preparations containing ethanol.

To confirm this hypothesis, cells were kept in starvation medium for four hours. At the end of starvation phase, cells were treated with 3 different ethanol concentrations for 10 min and cell culture supernatants were replated in 24-well plates. In a next step, a protease inhibitor cocktail was added. To examine whether the addition of a mixture of inhibitors of proteolytic degradation changed the amount of IFN-γ detectable in cell culture supernatant of ethanol-treated NK-92 cells, recombinant human IFN-γ was supplemented at a concentration of 2 ng/ml. Samples were probed of IFN-γ after 10 min of incubation.

Table 4-29. Effect of a commercially available protease inhibitor cocktail on the IFN-γ concentration in cells supernatants of ethanol-incubated NK-92 cells, supplemented with 2 ng/ml human recombinant IFN-γ. Cells were starved for 4 hours, treated with ethanol, spinned down, protease inhibitor cocktail was added, samples of the supernatants were collected and IFN-γ concentration was determined via ELISA. Absolute mean values ± SD; ^{Inh}F-test (ANOVA) effect of protease inhibitor cocktail; post hoc test hoc test: $^{\#}$t-test (effect of inhibitor cocktail for each ethanol concentration) ### = p< 0.001.

absolute IFN-γ concentration (pg/ml)						
					effect of protease inhibitor cocktail	
ethanol [‰]	N	Inhibitor	mean	SD	^{Inh}F-test	$^{\#}$t-test (Student)
0	8	–	1806	181	<0.001	1.000
	8	+	1841	99		
1	8	–	1618	225		0.868
	8	+	1748	115		
2	8	–	399	273		<0.001
	8	+	1846	241		
3	8	–	149	32		<0.001
	8	+	1889	224		

The addition of protease inhibitor cocktail inhibited the ethanol-induced decrease of IFN-γ concentration compared to preparations without inhibitor when cells were treated with 2 and 3 ‰ (Table 4-29 and Figure 4-35). We therefore assume that ethanol incubation is leading to a rapid release of proteases by NK-92 cells.

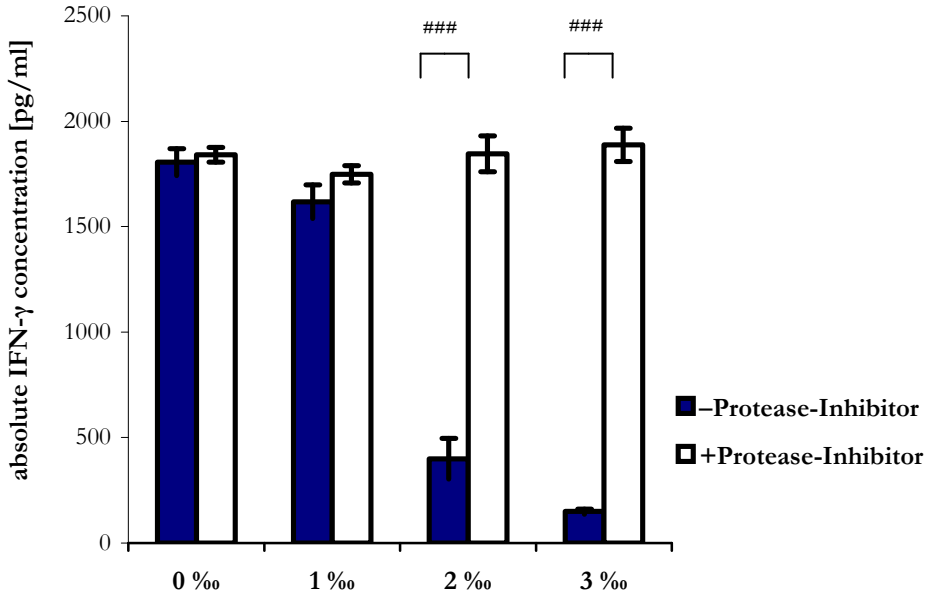


Figure 4-35. Effect of a commercially available protease-inhibitor cocktail on the IFN-γ concentration in cells supernatants of ethanol-pretreated NK-92 cells, supplemented with 2 ng/ml human recombinant IFN-γ. Absolute mean values ± SEM; ^{Inh}F-test (ANOVA, monofactorial): significance of the addition of a commercially available protease inhibitor cocktail; post hoc test: $^{\#}$Tukey's test (effect of inhibitor for each ethanol concentration) $^{\#\#\#} = p < 0.001$.

5 Discussion

5.1 Different cells as a model to investigate IFN-γ expression

5.1.1 Isolated human Peripheral Blood Mononuclear Cells (PBMC)

The model system of activated PBMCs has been well established in clinical immunology for several decades and allows standardization of T cell activation and T cell–macrophage interaction. It is certainly more informative than using cell lines alone and more relevant for *in vivo* testing. Our approach has already been used for testing ethanol effects for more than 5 years with very reproducible results even among cytokine assays of blood from different donors. PBMC preparations provide insight into signaling cascades, especially those initiated by T cells. Moreover, this strategy monitors the net effect of various pro- and antiinflammatory cascades initiated during stimulated immune response *in vitro* and provides data on the influence of tested compounds on the whole cascade of events (Winkler *et al.*, 2006).

The Th1-type cytokine Interferon-γ was predominantly affected by ethanol and besides that is an important proinflammatory mediator, which is pivotal in inflammation and highly relevant in the pathogenesis of alcohol-induced processes. Additionally, seven other cytokines were determined to study alcohol-mediated effects.

Accordingly, we investigated human peripheral blood mononuclear cells (PBMC) freshly isolated from whole blood of healthy donors and stimulated them with a mitogen (PHA) or LPS. We found a concentration of 2 μg PHA or 1 μg LPS per ml was optimal for cell activation, proliferation and/or cytokine production. The concentrations of IFN-γ, TNF-α TGF-β, IL-2, IL-10, IL-12 IL-18 and IP-10, secreted by PHA-activated or LPS-challenged PBMC from healthy individuals were determined and compared with those treated with different ethanol concentrations.

However, it has to be taken into consideration that cytokine production by immunocompetent cells investigated by using multistep procedures to isolate blood cells, may lead to uncontrolled cell activation, a change in the ratio between lymphocytes and monocytes, and deprive cells of exposure to essential cellular and molecular components present in whole blood. In whole blood, natural cell–cell interactions are preserved but also circulating stimulatory and inhibitory mediators, such as soluble receptors, are present at their physiological concentrations (Godoy-Ramirez *et al.*, 2004).

5.1.2 NK-92 cells in culture

Cytotoxicity mediated by NK cells has been hypothesized to play an important role in host's defense. Human NK cells are an immunophenotypically distinguishable subset of lymphocytes that exhibits a striking capacity to destroy tumor cells and virally infected cells. This cytotoxic activity does not require prior sensitization and is not restricted by MHC antigens. The development and functional activity of NK cells are regulated by many cytokines, including IL-2, a cytokine initially identified as T cell growth factor. IL-2 stimulates the proliferation of both T cells and NK cells. However, in NK cells, IL-2 has the additional effect of augmenting cytotoxic function, similar to the effect of IL-12. On the other hand, NK cells are a major source of Interferon γ, whose importance as an immunmodulatory cytokine has already been described (Ahmad *et al.,* 2005).

Both, enriching the NK cell fractions without comprising their biological activity and obtaining pure NK cells without contaminating T cells or other immune-response effector cells, limit studies of the mechanisms whereby NK cells exert their tumoricidal effects and maintain their cytokine production. To avoid these problems, many investigators have used established NK-derived cell lines to explore the biological mechanisms of cytotoxicity and IFN-γ expression.

The NK-92 cell line is a established human NK cell clone, originally derived from a human non-Hodgkin's lymphoma with the morphology of large granular lymphocytes and a $CD56^{+}CD3^{-}CD16^{-}$ immunophenotype. As a rhIL2-dependent cell line, NK-92 cells retain the characteristics of activated human NK cells (Yan *et al.*, 2006).

The NK-92 cell line was used as a model within the current study because of its similarity to primary human NK cells with respect to changes in IFN-γ production in response to IL-2 and IL-12 (Hodge *et al.*, 2002).

The proliferation of NK-92 cells depends on IL-2, and NK-92 cells express the cell surface marker $CD56^{bright}$ indicating the subpopulation of natural killer cells that are specialized in the production of cytokines such as IFN-γ (Cooper *et al.*, 2001).

Since NK-92 cells are IL-2 dependent, cells were maintained in α-modification of Eagle's Medium supplemented with 12.5 % fetal calf serum, 12.5 % horse serum and 10 ng/ml IL-2. Cell viability tests indicated that NK-92 cells depleted of IL-2 die within 72 hours. The addition of 1 ng/ml IL-2 already leads to proliferation of NK-92 cells, which is dose dependent and reaches its maximum at 50 U/ml. In contrast to IL-2, other cytokines such as IL-12, IFN-γ IL-4 and IL-6 exerted no effect on cell proliferation (Gong *et al.*, 1994).

5.2 Stimulation of lymphocytes

5.2.1 Human T cell activation with phytohemagglutinin

Lymphocyte proliferation in response to mitogens such as phytohemagglutinin (PHA), concanavalin A, pokeweed, and/or specific antigens has been the method of choice for *in vitro* diagnosis of cell-mediated immune function. Phytohemagglutinin, the lectin extract from red kidney bean (Phaseolus vulgaris), contains potent cell agglutinating and mitogenic activities (Tyrsted *et al.*, 1977). PHA includes a family of five isolectins of two different types, designated leukocyte reactive (L) and erythrocyte reactive (E). Type L has high affinity for lymphocyte surface receptors and is responsible for the mitogenic properties of the isolectins. PHA is a commonly used high quality tool for the stimulation of cell proliferation in lymphocyte cultures and functional analysis of T lymphocytes. PHA is not processed by proteases like other antigens, but binds to T cell receptors and MHC-II-molecules, acting as a superantigen (Löffler and Petrides, 2003).

This interaction initiates a cascade of biochemical events in the T cell that results in growth and proliferation. This occurs primarily through an increase in IL-2 secretion by the T cell and an increase in IL-2 receptors on the T cell surface. IL-2 is a potent inducer of T cell growth, which, in T cell activation, acts in an autocrine fashion to promote the growth, proliferation and differentiation of the T cell recently stimulated by antigen. Activated Th cells then continue to become effector cells whose role is proliferation and cytokine production (Augustine *et al.*, 2007).

In our study, PHA confirmed its capability as a potent activator of T cell function seen in a strong induction of cytokine production (TNF-alpha, IL-2 IL-12, IP-10 and IFN-γ) and proliferation in comparison to untreated cells that showed negligible cytokine production and proliferation within 4 days of cell cultivation.

Fluorocytometric analysis also demonstrated the change in cell composition in consequence of PHA-activation. PHA-stimulation is causing selective proliferation of certain cell types. Four days of activation led to a fraction of $CD3^+/CD56^-$ cells of more than 90 %. Monocytes instead decreased probably due to adherence. These findings are in agreement with other studies (Gollob *et al.*, 2000, Toyoda *et al.*, 2004) where PHA-activation of freshly isolated PBMC led to more than 95 % $CD3^+/CD56^-$. This activation and differentiation of cells causes an increase in IL-12Rβ1 and IL-12Rβ2 expression on the T cell surface. Thus, our findings are in agreement with studies showing that IL-12 responsiveness, including IFN-γ secretion, is PHA-dependent (Gollob *et al.*, 2000). The coherence between IFN-γ production and IL-12Rβ expression supports previous studies showing that IL-12 increases the IFN-γ expression only in preactivated (PHA-stimulated) T cells (Janefjord and Jenmalm, 2001), whereas non-activated T cells are poor responders for IL-12.

5.2.2 Induction of cytokine production in human T lymphocytes by lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is the main constituent of the outer leaflet of Gram-negative bacteria (Rietschel and Brade, 1992). LPS consists of a saccharide part and a lipid part, termed lipid A. The hydrophobic lipid A portion has been identified as the endotoxic principle of LPS (Galanos *et al.*, 1985). LPS released into the blood stream, as observed during severe Gram-negative bacterial infections or caused by translocation of enterobacteria from the gut, leads to various pathophysiological reactions such as fever, leucopenia, tachycardia, tachypnoe, hypotension, disseminated intravascular coagulation, and multi-organ failure (Rietschel *et al.*, 1994). Many different cell types have been documented to be reactive to LPS, e.g. monocytes/macrophages, vascular cells, polymorphonuclear cells, and even B lymphocytes.

Additionally, LPS is a potent inducer of human T lymphocyte proliferation and cytokine production. The activation of T lymphocytes by LPS requires direct cell-to-cell contact with viable accessory monocytes. This interaction was found to be MHC-unrestricted, but strongly dependent on costimulatory signals provided by B7/CD28 interactions. The frequency of responding T lymphocytes is less than 1:1000 (Ulmer *et al.*, 2000).

Stimulation of T lymphocytes by a classical protein antigen requires accessory cells presenting antigen-derived peptides as well as providing costimulatory signals. Purified T lymphocytes could not be stimulated by LPS neither to proliferate nor to produce cytokines. The response of T lymphocytes to LPS, however, could be restored by the addition of monocytes. Macrophages and dendritic cells, matured from blood monocytes *in vitro*, were found to be less active in providing accessory cell activity than monocytes, whereas B lymphocytes exhibited no accessory cell activity (Mattern *et al.* 1998).

In our study, stimulation of freshly isolated Peripheral Blood Mononuclear Cells with 1 μg/ml LPS led to a highly significant induction of monokine production as seen in TNF-α release. IFN-γ production was also increased significantly by LPS compared to unstimulated cells, supporting the ability of LPS to induce typical T cell-related cytokines, but IFN-γ concentration was still 30-times lower than in supernatants of PHA-stimulated PBMC.

The ability of T cells to responded to LPS was already provided by the findings that human MNC, stimulated with LPS, not only expressed mRNA for the monokines such as IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α), but also mRNA for the Th1-cytokines interferon-gamma (IFN-γ) and IL-2 (Ulmer *et al.* 2000).

We were also able to detect a highly significant increase of the Th2-cytokine IL-10 in consequence of LPS challenge, which is not surprising since TNF-α, is the most important trigger of IL-10 release. The concentration of IL-18 protein secreted into the culture supernatants was close to the detection limit.

In contrast to other studies, cell proliferation was not observed during LPS stimulation (data not shown). This could be due to the isolation method leading to a depletion of $CD34^+$ hematopoietic stem cells that are required for the stimulation of human T lymphocytes by LPS. Since the depletion of $CD34^+$ cells from MNC resulted in an almost total loss of the LPS-induced DNA synthesis (Ulmer *et al.*, 2000).

In conclusion, the activation of T lymphocytes by LPS represents a unique mechanism of T lymphocyte activation differing from that induced by classical mitogens, superantigens or antigens reflected in a different cytokine pattern and in the absence of cell proliferation.

5.2.3 Enhancement of NK-92 cell activity by Interleukin 12

NK cells play a pivotal role in protecting the body against infectious agents and cancers. Thus, cytokine-induced variations in activation in NK cells may have profound effects on NK cell biology and immune response. Cytokine treatment of NK cells results in alterations in multiple cellular responses that include cytotoxicity, cytokine production, proliferation, and chemotaxis.

IFN-γ, a product of NK and T cells, is a key cytokine contributing to innate and adaptive immunity. IFN-γ production is induced via direct cell–cell contacts with APC and IFN-γ-producing cells or by cytokines. During microbial infections, macrophage-derived IL-12 enhances IFN-gamma production and Th1 response.

In NK cells, IL-12 induces proliferation and cytolytic activity as well. IL-12 transmits signals by binding to a receptor complex composed of two subunits, leading to the formation of a high affinity receptor and subsequent signaling. IL-12 receptor subunits do not possess intrinsic kinase activity but rather function by associating with the members of the Janus family of kinases (JAK).

Phosphorylation of JAK2 and TYK 2 induces the phosphorylation of other transcription factors such as STAT4. The translocation of transcription factors to the nucleus then leads to gene regulation (Sudarshan *et al.*, 1999). STAT4 and AP-1 both participate in the regulation of IFN-γ gene expression, consequently, we focused our investigations on these two transcription factors although the IFN-γ gene is known to include consensus sequences for other transcription factors as well(other STATs, NFAT) (Aramburu *et al.*, 1995).

IL-12-induced activation of STAT4. To investigate the kinetics of STAT4 phosphorylation and nucleic translocation, NK-92 cells were stimulated with IL-12 and incubation was stopped at different time points, cell lysates were prepared and underwent a Western-Blot analysis using specific phospho-STAT4-Ab. In this study we have shown that STAT4 is phosphorylated in response to IL-12 stimulation. The addition of 75 ng/ml IL-12 led to a significant increase in STAT4 activation, compared to untreated cells, where no phosphorylated STAT4 was detectable. The IL-12-induced phosphorylation peaks at 120 min. Following IL-12 stimulation for more then two hours results in the down-regulation of phosphorylated STAT4 for which a mechanism of proteosomal degradation has been suggested (Wang *et al.*, 2000).

It has been reported earlier that STAT4 is activated in response to IL-12 in activated NK and T cells as well as NK-92 cells. The kinetics of tyrosine phosphorylation and activation of STAT4 in these studies was similar to our findings. STAT4 phosphorylation was already detected at 30 min, was maximal at 2 hours and remained elevated up to 6 hours (Matikainen *et al.*, 2001).

The observation that IL-12 is able to activate STAT4 30 min upon stimulation suggests that the cytokine receptors and transcription factors are already present in cells without the requirement of ongoing protein synthesis and can directly activate the IFN-γ gene (Matikainen *et al.*, 2001).

The results reported here further demonstrate that IL-12 induces transient STAT4 presence in the nucleus.

IL-12-induced IFN-γ production is not mediated by AP-1. Within the current work, we investigated whether the transcription factor AP-1 is also involved in NK-92 cell activation, leading to IFN-γ production. Consistent with other studies conducted either with T cells or native NK cells AP-1 is not activated in IL-12 stimulated NK-92 cells. Nakahira *et al.* (2002) already showed that upon IL-18, but not upon IL-12 stimulation accumulation of phosphorylated c-Jun occurred in the nucleus of a mouse T cell clone. Additionally, IL-18 confirmed its ability to promote IFN-γ expression via AP-1 in human primary $CD4^+$ T cells. This was not observed by IL-12 alone (Barbulescu *et al.*, 1998).

The same holds true for IL-2 stimulated IFN-γ production where only IL-2 induced IFN-γ gene activation is mediated by AP-1. Consequently, AP-1 transcriptional activity and increased binding to its response element was only present in IL-2 stimulated cells (Azzoni *et al.*, 1996).

c-Jun is phosphorylated by c-Jun N terminal kinase (JNK), a kinase that is not activated following IL-12 stimulation. This may explain why IL-12 fails to induce IFN-γ gene expression via AP-1, whereas other cytokines such as IL-18 exhibit JNK activation capability. Although c-Jun was found to accumulate in the nucleus upon IL-12 stimulation, without prior phosphorylation by JNK, AP-1 binding to the promoter region of IFN-γ remained very low (Sugimoto *et al.*, 2003).

IL-12-induced alterations in IFN-γ gene expression. Control of IFN-γ gene expression is complex and involves both transcriptional and posttranscriptional regulation. The complexity of this regulation is common among many early-response genes that encode proteins for transcriptional modulators, structural proteins, and cytokines (Hodge *et al.*, 2002).

In NK cells the IFN-γ promoter is primed for transcriptional activation, as reflected by constitutive demethylation of the locus and acetylation of histone H3. The short-life of transcripts prevents synthesis of higher protein amounts in the absence of appropriate stimuli. These conditions make the cell exquisitely poised for rapid and efficient induction of IFN-γ biosynthesis in response to signals that activate transcription (Mavropoulos *et al.*, 2005).

The fact that NK cells are able to react rapidly in the presence of transcription-activating proteins is reflected in the results measured, when mRNA expression of IFN-γ was quantified using RT-PCR. Already 2 hours of IL-12 stimulation led to a significant increase of IFN-γ mRNA compared to initial level and unstimulated control. This increase can still be detected 8 hours upon IL-12 activation. Interestingly, long-time incubation for more than 48 hours caused a decrease in mRNA expression below initial values.

With our results we demonstrate IL-12 impacts the transcriptional activity of the IFN-γ gene and the amount of IFN-γ mRNA produced.

IL-12 has no effect on intracellular IFN-γ concentrations. IL-12 stimulation did not significantly affect the intracellular concentration of IFN-γ in NK-92 cells. During 24 hours of incubation and IL-12 stimulation, intracellular concentration remained constant, suggesting that NK-92 cells maintain an intracellular pool of IFN-γ at an average of 350 pg per mg cellular protein. Above this level, NK-92 cells continuously secrete IFN-γ.

IL-12 induced IFN-γ production by NK-92 cells. The results mentioned so far accent the importance of IL-12 in NK cell activation, where STAT4 activation followed by induction of mRNA expression finally leads to IFN-γ production. This increase in protein expression is secreted into cell culture supernatant, when reaching more than 350 pg per mg cellular protein, since intracellular accumulation was not observed.

This significant increase in IFN-γ production induced within current work is in agreement with several previous findings (Kalina *et al.*, 2000, Hodge *et al.*, 2002). To compare the absolute amount of IFN-γ produced in different studies, one must take into consideration, that different cell numbers and IL-12 concentrations were applied. In our case, cells produced 1144 pg IFN-γ per 10^6 cells. The cell density was 2.5×10^6 per ml, the applied IL-12 concentration was 75 ng/ml. Hodge and colleagues used NK-92 cells at a concentration of 1 x 10^6/ml and stimulated them with 10 U/ml IL-12 for 24 h. In this case, cells produced up to 5000 pg/ml IFN-γ.

This was confirmed in several other studies where IL-12 induced IFN-γ production in NK cells freshly purified from PBMCs (Chan *et al.*, 1991, Aste-Amezaga *et al.*, 1994, Wang *et al.*, 2000, Matikainen *et al.*, 2001). Using human NK3.3 cells Ye *et al.* (1995), showed a significant increase in IFN-γ production upon IL-12 stimulation. Additionally, mouse splenocytes were also capable of IL-12 induced IFN-γ production (Thierfelder *et al.*, 1996, Lawless *et al.*, 2000).

It is important to mention, that though cells were depleted of IL-2 and cultivated in serum-free medium for 16 hours previous to IL-12 stimulation produced IFN-γ. This is probably due to IL-2, which is essential for NK-92 cell survival (Gong *et al.*, 1994) but is also able to induce IFN-γ production (Ye *et al.*, 1995, Liebermann and Hunter 2002, Hodge *et al.*, 2002); Concluding that the initial IFN-γ levels produced during 'starvation phase' were still a result of prior IL-2 supplementation.

The kinetics of IL-12-induced IFN-γ production showed an increase within the first 6 hours where cells responded more stronger to IL-12 than in the later phase of incubation. In the delayed phase of IL-12, exposure IFN-γ levels remained static. This supports the findings by Watford *et al.* (2004) where prolonged exposure also resulted in a stagnation of IFN-γ concentrations.

5.3 Ethanol-induced changes in cell activation and cytokine pattern seen in activated PBMC and NK-92 cells

5.3.1 Ethanol in cell culture: maintenance of constant ethanol concentrations

In order to investigate the effect of ethanol exposure, it was important to keep constant alcohol concentrations in the cell culture medium during the whole time of incubation. To avoid evaporation, cells were maintained in ethanol-containing medium of a specified concentration inside of plastic boxes with additional open beakers containing an aqueous solution of the corresponding ethanol concentrations, in this case 1, 2 or 3 ‰.

This model was used in our laboratory for years to guarantee constant conditions concerning ethanol incubation during periods lasting for several days without substantiate decrease in ethanol concentration due to evaporation. It was first described by Rodrigues and colleagues in 1992 where cells culture dishes were kept in 'alcohol vapor chambers' on the one hand or were placed directly in the incubator. On the other hand, in the 'open system' 90 % of the initial concentration of 100 mM evaporated within 24 h. In the 'closed system' with ethanol-containing dishes, the ethanol concentrations in cell culture medium remained constant. They further showed that the number of dishes including ethanol solutions had no effect. Changes in pH-values were not observed (Rodrigues *et al.,* 1992).

Even more important is the fact that cultivating cells in ethanol-free media inside sealed plastic boxes did not change their viability, growth rate and protein or phospholipid composition compared to cultures grown outside the boxes. This model was even improved using a special 'Bio-foil' in the lid of each box allowing a constant exchange of O_2 and CO_2 without any passage of ethanol. With this 'improved alcohol vapor chambers', tests concerning ethanol evaporation were performed by Gesierich in 2002. Cell culture media containing 1 and 2 ‰ ethanol were incubated for more than 4 days, ethanol concentrations were tested using an enzymatic method at 16, 32, 48, 72 and 96 hours upon incubation start. The change in alcohol concentration over incubation period was below 0.03 ‰ and therefore negligible low.

Regarding experiments done within this study, it was suggested that ethanol concentrations remained stable during incubation time. In long-term incubation, ethanol-containing beakers were changed every 24 hours to avoid loss in alcohol concentration. We therefore affirm that all experiments done in 'alcohol vapor chambers' were conducted with constant ethanol concentrations in culture media and that all effects observed were due to the applied concentration.

5.3.2 Influence of ethanol on Peripheral Blood Mononuclear Cells

Effect of ethanol on PHA-induced IFN-γ production. In this work, the effect of acute ethanol administration on PHA-stimulated IFN-γ production was evaluated using freshly isolated Peripheral Blood Mononuclear Cells. In our model we used ethanol levels, which may be on the one hand representative for acute, moderate alcohol intake (1 ‰) and, on the other hand, are seen in binge drinking and chronic abuse in humans (3 ‰).

In the current study PHA-stimulated IFN-γ production was already reduced in a significant manner upon the addition of 1 ‰ Ethanol to the cell culture medium. This decrease in IFN-γ expression was even more predominant, when 3 ‰ of ethanol were applied. IFN-γ concentration in cell culture medium was reduced to 5 % compared to ethanol-free stimulation after 72 hours of incubation.

Ethanol consumption has adverse effects on the host's ability to combat infectious pathogens. Numerous clinical reports have identified alcoholism as a comorbidity with pulmonary infections (Friedman *et al.*, 1998). Additionally ethanol-associated defects in the ability to clear invading microbes have been described in the experimental setting for bacterial pathogens, mycobacteria, Listeria and others (Nelson *et al.*, 1990).

Similar experiments were conducted by Wagner *et al.*, in 1992 where isolated human peripheral blood mononuclear cells were isolated and treated with ethanol. In this case, cells were incubated with ethanol concentrations ranging between 0 and 100 mM for 72 hours, after removement of ethanol-containing medium cells were stimulated with Con A and PHA for additional 24 hours.

As seen in our results, ethanol induced a statistically significant inhibition of Interferon γ secretion. The effect was also dose-dependent and already demonstrable at very low ethanol concentration (6.25 mM). At 50 mM, ethanol-induced reduction ranged from 72 % to almost 90 % (depending on individuals tested). With these findings, Wagner *et al.*, demonstrated for the first time that ethanol inhibits the mitogen-induced IFN-γ secretion by human peripheral blood mononuclear lymphocytes of healthy donors *in vitro*.

Our results are in agreement with Wagner's study. We have also shown that PBMCs isolated from healthy donors were strongly affected by ethanol treatment concerning IFN-γ production and our findings suggest that this down-regulation of IFN-γ expression may be an important element to many immune alterations seen in alcohol abuse.

Does ethanol influence the production of other cytokines? In addition to IFN-γ, the effect of ethanol on seven other cytokines was investigated. The question was, whether changes seen in ethanol-treated Peripheral Blood Mononuclear Cells were exclusively concerning IFN-γ expression or if other cytokines were affected by alcohol as well, and if so, to which extend addressing hereby the exclusive interaction between ethanol and IFN-γ production.

PHA- as well as LPS-stimulated TNF-α production by PBMC was suppressed in a highly significant manner when cells were treated with 3 ‰ ethanol for 12 hours (38 % for PHA-stimulation, 55 % for LPS-stimulation). Remarkably is the fact that the addition of 1 ‰ ethanol to the cell culture medium led to a TNF-α release that tended to be higher but the level of significance was >0.05.

Other researchers that focused on acute exposure also observed an ethanol-induced suppression of TNF-α production in stimulated immunocompetent cells. Using LPS-stimulated isolated human monocytes Bikash *et al.* (1993) and Szabo *et al.* (1996) obtained a TNF-α suppression that was dose-dependent (in contrast to our results) and this effect was already seen in monocytes incubated in medium containing 25 mM (1.1 ‰) ethanol-containing medium.

This suppression of TNF-α expression by acute ethanol exposure can be explained by the interference of ethanol with different intracellular signaling molecules.

Mitogen-activated protein kinases (MAPK) have been demonstrated to play a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli. In particular, p38 plays a role in the LPS (endotoxin)-induced inflammatory response (Nick *et al.* 1996).

LPS-induced TNF-α production was inhibited in a similar pattern by pretreatment with either ethanol or SB202190 (1 μM), a specific inhibitor of p38 kinase. Western blot analysis, using a dual phospho-specific p38 mitogen-activated protein kinase Ab, demonstrated that ethanol pretreatment inhibited LPS-induced p38 activation (Arbrabi *et al.* 1999).

NF-κB, a pivotal transcription factor regulated by various stresses of bacterial or viral stimuli, serves as the central mediator of innate immune responses. The NF-κB transcription factor consists of two subunits of either homodimers or heterodimers of RelA/p65, c-Rel, and p50.

The complexes are present in cytoplasma and prevented from activation by a class of proteins referred to as inhibitors of NF-κB or IκB proteins. Upon stimulation, the IκB proteins are phosphorylated by the IκB kinases (IKK) IKKα, IKKβ, and IKKγ ubiquitinated, and degraded thereby releasing the NF-κB complex for nuclear translocation (Hacker *et al.* 2006).

Using human peripheral blood monocytes and Chinese hamster ovary cells transfected with CD14, Mandrekar *et al.* 2007 showed that acute alcohol treatment *in vitro* exerts NF-κB inhibition by disrupting phosphorylation of p65. Immunoprecipitation of p65 and IκBα revealed that acute

alcohol exposure for 1 h decreased NF-κB-IκBα complexes in the cytoplasm. Phosphorylation of p65 at Ser^{536} is mediated by IκB kinase (IKK)β and is required for NF-κB-dependent cellular responses. Furthermore, nuclear expression of IKKα increased after alcohol treatment, which may contribute to inhibition of NF-κB (Szabo *et al.* 1999).

Some researchers have also shown a significant posttranscriptional and posttranslational component of acute alcohol-mediated TNF-α suppression in both rodent and primate macrophages (Zhao *et al.* 2003). One possible posttranslational effect of acute ethanol results in decreased processing of TNF-α by TNF-α-converting enzyme (TACE), a member of the disintegrin and metalloproteinase (ADAM) family of proteins (Zhang *et al.* 2003).

Using transfected murine fibroblasts, acute alcohol resulted in a dose-dependent suppression of TACE-mediated processing of TNF-α. Additionally, acute ethanol suppresses processing and secretion of TNF-α by altering membrane compartmentalization of the enzyme and substrate (Zhao *et al.* 2003).

TNF-α is also thought to play a particularly critical role in the pathogenesis of ALD. Production of TNF-α is one of the earliest responses of the liver to injury. Circulating TNF-α is increased in the blood of alcoholics and in animals chronically exposed to ethanol. In addition to increasing LPS exposure, chronic ethanol also increases sensitivity to LPS. Moreover, it was shown that LPS-stimulated TNF-α secretion is increased in Kupffer cells isolated from rats fed ethanol in their diet for 4 weeks compared to pair-fed controls (Kishore *et al.* 2005).

In this case chronic ethanol feeding disrupts specific LPS-stimulated signal transduction pathways which regulate both TNF-α transcription and mRNA stability.

Chronic ethanol has also complex effects on the regulation of LPS-stimulated TNF-α mRNA transcription; the transcriptional activity of NF-κB was dramatically decreased, but this was compensated by increased Egr-1 activity (Kishore *et al.*, 2002). Therefore LPS-stimulated TNF-α mRNA accumulation in Kupffer cells isolated from rats chronically exposed to ethanol is due to a stabilization of TNF-α mRNA. These data suggest that regulation of TNF-α mRNA stability mediates increased TNF-α production during ethanol consumption and thus contributes to the progression of inflammation during alcoholic liver disease (Kishore *et al.*, 2002).

In our model system of isolated human PBMC, IL-2 was only induced in cells treated with PHA at a concentration of 2 μg/ml. The addition of ethanol to cell culture medium of PHA activated cells had no effect on IL-2 concentration measured. This is in agreement with other studies, where ethanol did not affect IL-2 expression itself but suppressed mRNA expression of a variety of IL-2-induced genes and the activity of transcription factors (Zhou *et al.*, 2003).

Previous studies clearly show that ethanol consumption decreases innate cytolytic activity of NK cells as well as IL-2 stimulated NK cytolytic activity and lymphokine-activated killer cell activity (Gallucci *et al.*, 1995, 1996; Meadows *et al.*, 1993). It is also known that this inhibition of NK cytolytic activity is associated with decreased protein expression of perforin, granzyme A, and granzyme B in response to IL-2 stimulation (Collier 2000).

Ethanol has shown to modulate significantly the production not only of proinflammatory cytokines such as TNF-α but also the expression of antiinflammatory mediators such as TGF-β or IL-10 (Szabo, 1996). In addition, monocyte-derived TGF-β and IL-10 are both potent inhibitors of T cell proliferation (Wahl *et al.* 1992). Ethanol has shown to induce TGF-β and IL-10 in Kupffer cells and monocytes (Szabo *et al.* 1992). This change in expression of antiinflammatory cytokine production can potentially attenuate host defense in both inflammatory and immune responses.

The use of blood monocytes, treated with a physiologically relevant dose of alcohol (25 mM) resulted in a significantly decreased production of TGF-β and IL-10, and further augmented bacterial (both LPS and SEB) stimulation-induced production of both cytokines (Szabo *et al.* 1996). These results are not supported by our findings, where neither LPS nor PHA had any effect on TGF-β production and the addition of ethanol to cell culture medium did not change TGF-β production in a significant manner. Even more interestingly, in contrast isolated human monocytes, IL-10 production in isolated human PBMC treated with ethanol concentrations of 22 to 66 mM led to a significant and dose-dependent decrease when cells were additionally stimulated with LPS.

Interleukin-12 is an important effector cytokine for defense of the host against a multitude of invading organisms, including intracellular bacterial pathogens. Enhancement of NK cell and T lymphocyte activity by IL-12 is responsible for augmentation of defenses against these pathogens. IFN-γ then plays a key role in the orchestration Th1 lymphocyte cell-mediated immunity directed against intracellular pathogens. Thus, IL-12 exerts its immunostimulatory effects in most parts by activating IFN-γ production (Watford *et al.,* 2004).

Since IL-12 is a potent inducer of IFN-γ production one could ask whether ethanol-mediated IL-12 expression is causing low IFN-γ concentrations. A question we also took into consideration, especially since Mason *et al.* (2000) showed that ethanol suppressed IL-12 expression in LPS-challenged mice and Interleukin 12 therapy restored cell-mediated immunity in ethanol consuming

mice (Peterson *et al.*, 1998). But besides the measurement of IFN-γ levels, we also tested cell culture supernatants of ethanol-treated cells for IL-12 concentration. Surprisingly ethanol-treated PHA-activated PBMCs showed an increase in IL-12 expression compared to untreated cells. This is the reason why low IFN-γ production was not due to low stimulation by IL-12.

IL-18, like IL-12, was discovered initially to be a factor that drives the T cell toward T helper 1 cell subtype and thus was referred as 'IFN-γ-inducing factor' (Okamura *et al.* 1998) and since IL-18 has been shown to enhance IL-12-depedendent IFN-γ production, it appears that IL-18 promotes cell-mediated immunity and thus is essential to host defences against a variety of infections. However, like many other proinflammatory cytokines, IL-18 possesses broad immunomodulatory properties (Leung *et al.* 2001).

In the present study we tested cell culture supernatants for IL-18 to investigate if IL-18 − as IFN-γ-inducing factor − was suppressed in a similar way than IFN-γ expression itself. But in our model using isolated human PBMC IL-18 expression was not induced in PHA or LPS-stimulated cells. Hence it is unlikely that IL-18 is responsible for the IFN-γ-suppressing effect of ethanol.

IP-10 was initially identified as an abundantly induced mRNA in U937 cells upon IFN-γ stimulation, and its expression is predominantly induced by IFN-γ in endothelial cells, monocytes, fibroblasts, astrocytes, keratinocytes, and neutrophils. IP-10 chemoattracts activated T cells and NK cells, but not resting T cells, B cells, or neutrophils (Faber *et al.* 1997). IP-10 expression has been found in various clinical conditions such as psoriasis, tuberculoid leprosy, sarcoidosis, and viral meningitis. All of these diseases are associated with an increased expression of IFN-γ (Th1-type diseases), which may induce IP-10 expression in involved tissues (Mosmann *et al.* 1996).

As expected, high IFN-γ expression measured in PBMC treated with PHA is associated with high IP-10 levels in cell culture supernatant of those cells, whereas lower IFN-γ expression in LPS-stimulated cells is reflected by lower IP-10 expression. However, a significant reduction in IP-10 expression after ethanol treatment as seen for IFN-γ was not observed.

Is T cell proliferation affected by ethanol? Many of the immunosuppressive effects of acute alcohol treatment have been linked to decreased production of proinflammatory cytokines. On the other hand, a decline in cell number and viability was also observed in some *in vivo* studies (Nagy *et al.,* 1994). To find out whether ethanol concentrations administered in this study are responsible for lower cytokine release or if this is simply due to an impaired PHA-driven proliferation, we determined cell proliferation and viability.

The addition of ethanol to cell culture medium of isolated and PHA-activated cells had no significant effect on cell proliferation over 9 days of incubation.

These results are in contrast to other studies, where ethanol was applied *in vivo* to healthy volunteers and peripheral blood mononuclear cells were isolated from blood samples taken before alcohol consumption, 4 hours and 18 hours after alcohol intake. The allostimulatory capacity of blood monocytes was determined by [^{3}H]thymidine incorporation into purified naïve allogeneic T cells in a mixed lymphocyte reaction (MLR) (Mandrekar *et al.,* 2004).

Compared with T cell proliferation in MLR prior to alcohol use, T cell proliferation was reduced in the presence of stimulator cells obtained from the same individual. These results suggest that the reduced alloantigen-induced T cell proliferation is due to suppressed accessory cell function rather than T cell dysfunction after acute ethanol intake (Mandrekar *et al.,* 2004). A report by Peterson *et al.*, (1998) identified reduced accessory cell-derived IL-12 levels as a potential mechanism for diminished antigen-specific T cell proliferation after chronic alcohol feeding.

Results obtained in the same study with PBMCs stimulated with PHA isolated before and 18 hours after alcohol intake support the findings of the current study. In contrast to reduced antigen-induced T cell proliferation, T cell proliferation after direct mitogen stimulation remained unchanged after alcohol intake. Considering that, PHA-induced T cell proliferation is not dependent on costimulatory signals from accessory cell. The current results support the hypothesis that acute alcohol intake rather affects accessory cell function than proliferation of lymphocytes per se (Szabo *et al.*, 2001).

T cell proliferation response in MLR was also decreased in mice after chronic alcohol feeding (Chang and Norman, 1999). In those experiments, allospecific MLR of the responder cells from alcohol-consuming mice was reduced but there was no suppression of the cytotoxic T lymphocytes in the alcohol consuming mice, suggesting involvement of MHC class II rather than MHC class I events (Chang and Norman, 1999).

After acute alcohol intake in humans, there was no significant defect in T cell proliferation when cells were obtained from the alcohol-consuming volunteers, suggesting that acute and chronic alcohol treatment may have different effects on T lymphocytes (Szabo *et al.*, 2001).

In the current work, there was no ethanol-modulated proliferation verifiable, suggesting that the suppression of proinflammatory cytokines such as IFN-γ is due to suppression of molecular mechanisms regulating cytokine production instead of diminished cell numbers caused by acute ethanol treatment.

Fluorocytometric analysis of IL-12 receptor subunits. IL-12-driven Interferon γ production requires signals provided through interleukin 12 receptors. It has been proposed that the magnitude of the immune response in T-lymphocytes may be directly correlated to the cell surface expression of IL-12 receptor subunits (Trinchieri *et al.*, 2003). The biologic activities of IL-12 are mediated through a specific, high affinity IL-12R composed of an IL-12Rbeta1/IL-12Rbeta2 heterodimer, with the IL-12Rbeta2 chain involved in signaling via STAT4. For that reason, we investigated IL-12R expression in human PHA-activated PBMC when treated with ethanol.

To date, two subunits of the IL-12R, IL-12Rβ1 and IL-12Rβ2, have been identified and cloned. When individually expressed in COS-7 cells, human IL-12Rβ1 and β2 each bind IL-12 with low affinity, while coexpression of both IL-12Rβ1 and β2 results in both low and high affinity IL-12 binding and responsiveness. Splenocytes from IL-12Rβ1-deficient mice fail to display IL-12-induced enhancement of NK lytic activity, suggesting that IL-12Rβ1 is an essential component for IL-12 responsiveness *in vitro* and vivo (Zhang *et al.*, 2003).

From previous studies, it has been become increasingly evident that IL-12 is a key regulator of IFN-γ. The ability of IL-12 to stimulate Th1 responses requires the expression of the IL-12R on T cells. However, both receptor chains are differently regulated. The IL-12Rβ1 is constitutively expressed on T cells; in contrast, the IL-12Rβ2 is selectively expressed on Th1 but not on Th2 cells (Kim *et al.*, 2001).

In the current study, PHA-activation led to an increase in IL-12Rβ1 and IL-12Rβ1 expression on T cell surface; suggesting that PHA-stimulation leads to an induction of Th1 cytokine pattern.

The importance of sufficient IL-12 R expression with respect to host resistance against pathogens is highlighted since IL-12Rβ1 deficiency is the most common genetic aetiology of Mendelian susceptibility to mycobacterial disease. The known mutations in the IL12Rβ1 gene are recessive and are associated with the abolition of the response to both IL-12 and IL-23. Patients with IL-12Rβ1 deficiency classically experience clinical disease caused by Bacille Calmette-Guerin (BCG), environmental mycobacteria, and non-typhoid Salmonella species (Rosenzweig and Holland, 2004).

As a result of defective IL-12R signaling, IFN-γ is underproduced by T cells and natural killer cells. For that reason, we asked whether the same held true in alcohol-treated cells. Underproduction of IFN-γ in the presence of sufficient IL-12 concentrations may be caused by changes in IL-12 receptor expression on activated T cells.

The fluorocytometric analysis of PHA-activated T cells did not support this hypothesis of ethanol-mediated changes in IL-12R subunit expression. The addition of 1 and 3 ‰ ethanol to cell culture media did not affect IL-12 receptor expression of both subunits at all.

5.3.3 Ethanol-induced suppression of IFN-γ expression in NK-92 cells

NK cells play a pivotal role in protecting the body against infectious agents and cancers. Thus, alcohol-induced variations in IFN-γ production may have profound effects on NK cell biology and immune response. In this study, we have examined global changes in IFN-γ expression in response to IL-12 alone and in combination with different ethanol concentrations.

As demonstrated within the current study, ethanol treatment *in vitro* resulted in a significant decrease of IL-12-induced IFN-γ production by NK-92 cells, just in the same manner as in PBMC.

Other *in vitro* studies on the effects of ethanol on cytokine production are the continuation of the observed effects of ethanol on circulating cytokine levels *in vivo* (Deaciuc 1997).

In vivo studies in alcohol abusers and in patients with alcohol-induced liver disease are often hard to interpretate since possible comorbidities and malnutrition as well as vitamin deficiency among alcohol abusers may at least in part contribute to immune alterations seen in alcohol-consuming patients (Windle *et al.*, 1993).

Although these observations cannot be directly related to the ethanol intake, the correlation of findings *in vivo* is supported by animal and cell models (Wagner *et al.*, 1992, Chen *et al.*, 1993, Laso *et al.*, 1997, Deaciuc, 1997, Waltenbaugh *et al.*, 1998, Szabo *et al.*, 2001, Starkenburg *et al.*, 2001, Dokur *et al.*, 2003).

The ethanol-mediated changes seen in NK-92 cells are in agreement with several other studies, where different models were used with respect to cell type and applied ethanol concentration as well as different cell stimuli.

A comparable model was used by Wagner *et al.*, (1992) where freshly isolated PBMC were treated with ethanol concentration ranging between 0 and 100 mM *in vitro*. Cells produced significant less IFN-γ either unstimulated or mitogen-activated. This was even seen at as low ethanol concentrations as 6.25 mM.

Ethanol-treatment of isolated mouse splenocytes also resulted in downregulation of IFN-γ production. Splenocytes from healthy mice were challenged with LPS or Con A for 24 hours and additionally incubated with ethanol ranging from 0.1–1.0 % (v/v). The production of IFN-γ in this model was found to be reduced in a dose-dependent manner (Chen *et al.*, 1993).

Other studies focused on *in vivo* exposure to ethanol, followed by cell isolation, direct cytokine measurement or prior *in vitro* stimulation.

Isolated PBMC of healthy volunteers stimulated with PHA showed a reduced IFN-γ production upon *in vivo* alcohol consumption in an acute and moderate manner (Szabo 1998). The same result was obtained when rats were treated with ethanol *in vivo* and isolated splenocytes were activated thereafter with Con A *in vitro* (Dokur *et al.*, 2003).

Furthermore, mice chronically fed a liquid diet with 5 % ethanol for 11 weeks showed a decrease in splenocyte IFN-γ production in the presence of Con A (Wang *et al.*, 1994).

In contrast, splenocytes obtained from mice ethanol-fed for 5 months did not express a significant change in IFN-γ production when stimulated with LPS or Con A. They produced as much IFN-γ as their non-alcohol-fed littermates (Chen *et al.*, 1993).

The comparison of different models to investigate ethanol-mediated changes in IFN-γ is always difficult to do and, in some cases, may lead to inconsistent results. It was already mentioned that ethanol-abuse in humans is correlated with malnutrition and different stages of alcoholic liver disease, making it impossible to distinguish the effect of ethanol alone, but still reflects the actual case in humans depending on alcohol. Animal models of *in vivo* exposure may be advantageous due to the control of nutrient intake, and are superior to cell models cause of existing organ and cell–cell interactions.

Cell isolation either from humans or animals is still a time-consuming method and may cause alterations of cells properties as well may cause non-specific stimulation. It is even questionable whether the changes induced by *in vivo* consumption are still persistent upon cell isolation and *in vitro* stimulation.

A reason we favored the current model were isolated cells or cell lines were challenged simultaneously with ethanol at different concentration and potent inductors of IFN-γ expression. The resulting effects thereby can be directly related to acute ethanol treatment.

It was even more important, that pretreatment with ethanol followed by ethanol-free PHA stimulation had no effect on IFN-γ production (data not shown). The effect of ethanol-induced IFN-γ suppression is therefore only present in coincubation experiments.

Another quite important fact only occurred, when NK-92 cells were incubated with IL-12 and ethanol for a short period of time. First results obtained from isolated PBMCs only reflected long-term incubation for more then 24 hours. This was quite normal since PHA-activation did not lead to rapid and sufficient short-term IFN-γ production within minutes or hours. In contrast, IL-12 stimulation of NK-92 cells induced high amounts of IFN-γ already upon 6 hours of incubation. At first, we did not focus on this incubation time point, but it was quite astonishing, that in short-time incubation IFN-γ concentration in the cell culture medium of ethanol-treated cells was even below values of unstimulated cells without ethanol treatment. It seemed that in this case already produced IFN-γ virtually disappeared and was no longer detectable in cell culture supernatant. This results at first unattended, later changed our focus from intracellular signaling pathways to posttranscriptional mechanism such as the formation/release of a specific binding proteins or extracellular degradation.

5.3.4 Cell viability

Is ethanol-induced suppression of IFN-γ expression due to changes in cell viability? Cell viability was assessed to exclude non-specific toxic effects of the ethanol concentrations applied in our cell culture model. The viability of NK-92 cells was 59 % in control cultures without ethanol addition, compared to 65 % under treatment with 1 ‰ ethanol, 60 % under 2 ‰ and 62 % when 3 ‰ ethanol were applied. We therefore conclude that in all cases ethanol had no significant effect on cell viability.

This is in agreement with several other studies where isolated PBMC were not affected by ethanol concentrations ranging between 0–100 mM for 72 hours (Wagner *et al.*, 1992). Spleen cells treated with 0.1–1.0 % ethanol also showed no change in cell viability (Chen *et al.*, 1993).

Wagner and colleagues tested concentrations up to 100 mM and Chen and colleagues used concentration reaching 220 mM and are therefore not considered as concentrations seen in alcohol consuming humans. But even those high concentrations did not affect cell viability.

The total number of viable cells in our study was lower compared to the studies by Wagner and Chen (95 %) though applied ethanol concentrations in those studies were comparable and even higher than those we used ranging between 0 and 66 mM.

This fact can be explained by different culturing conditions. To avoid any additional effect of IL-2, NK-92 cells were maintained in a 'starvation medium' prior to incubation experiment. Since NK-92 cells depend on IL-2, the number of viable cells upon incubation was lower in general, regardless whether cells were treated with or without ethanol. NK-92 cells are not able to survive for more than 72 hours upon IL-2 depletion (Gong *et al.*, 1994).

In summary, we assume that ethanol in concentrations applied here does not influence cell viability of NK-92 cells and even more important alcohol-suppressed IFN-γ production is not caused by lower cell numbers in experiments where ethanol was implemented. A non-specific toxic effect of ethanol on NK-92 cells can therefore be excluded.

5.3.5 Physiological relevance of applied ethanol concentrations

The previous chapter already dealt with the different alcohol concentrations applied in studies focusing on *in vitro* effects of ethanol in human, animal and cell models; a reason to address the consequences of physiological relevant alcohol blood concentrations.

Many studies (Diamond, 1990) have established that in humans, the evident behavioural changes associated with increases in blood alcohol concentrations are as follows: altered mood, impaired attention (6 to 20 mM); impaired cognition and coordination, and sedation (20–40 mM); intoxication, ataxia (40 to 65 mM); severe stupor, coma, death (65–110 mM). In addition, subjects can achieve what is referred to as acute tolerance, whereby they exhibit intoxication at a blood alcohol concentration of 40 mM. Heavy drinkers and alcoholics can achieve what is referred to as chronic tolerance whereby subjects can maintain blood alcohol concentrations of 30–120 mM ethanol and still appear sober. According to such studies, severe alcoholics can attain blood alcohol concentrations as high as 330 mM and still remain conscious and alert.

Considering the alcohol concentrations mentioned above, the concentrations applied in this study ranging between 0 and 66 mM correspond to relevant blood alcohol levels occurring after acute, moderate alcohol consumption and even binge-drinking, defined within the field of epidemiology as drinking 5 or more drinks for men and 4 or more for women per occasion leading to blood alcohol concentrations of 3 ‰ and more. 66 mM *in vitro* correspond to 0.3 g/dl seen in alcoholics after acute alcohol consumption (Szabo 1997).

Thus, our results are relevant for moderate as well as excessive ethanol intake by healthy subjects. Higher blood alcohol concentrations are only seen in chronic alcoholics in whom tolerance evolved over years of alcohol abuse.

5.3.6 Ethanol effects on transcriptional activation of IFN-γ in NK-92 cell

Are intracellular signaling molecules affected by ethanol? To study IL-12-induced STAT4 activation and phosphorylation, NK-92 cells were 'starved' for two hours and stimulated with 75 ng/ml IL-12 for another 2 hours. Additional cells were treated with ethanol concentrations of 22–66 mM. Western blot analysis showed no difference in IL-12-induced STAT4 phosphorylation whether cells were treated with ethanol of different concentrations or not. In contrast to IL-12 treated cells unstimulated NK-92 cells showed no STAT4 activation at all.

To the best of our knowledge, no other study conducted so far focused on STAT4 activation in case of ethanol-induced modulation. However, Jak/STAT-signaling was already investigated and changes in the activation of other STAT molecules were already observed in other cell culture models. A reason we also hypothesized a mechanism of ethanol-mediated change in intracellular signaling molecules underlying the suppression of IFN-γ expression.

Chen *et al.* (2003) studied the effect of ethanol on the Jak/STAT signaling in freshly isolated, cultured rat hepatocytes. Acute ethanol exposure inhibited IL-6-activated STAT3 in freshly isolated hepatocytes. Interleukin 6-induced activation of STAT3 is a critical step in liver regeneration.

Chronic ethanol consumption is known to increase the plasma concentration of IL-6, yet the ability of the liver to regenerate and regenerative induction of several IL-6 initiated events are impaired in chronic alcoholic liver disease. The inhibitory action of ethanol on the Jak/STAT signaling pathway therefore might be implicated in pathogenesis and progression of alcoholic liver disease (Chen *et al.*, 2001).

Similar results were obtained by Nguyen *et al.*, (2000). They studied mechanism underlying the ineffectiveness of Interferon treatment of viral hepatitis in alcoholics. They report that IFN-α/β and IFN-γ rapidly activate the Jak/STAT1 in freshly isolated rat hepatocytes. Treatment of hepatocytes with 25–100 mM ethanol for 30 min inhibited IFN-induced STAT1 activation and tyrosine phosphorylation. Pretreatment with non-selective tyrosine phosphatase and specific proteasome inhibitors did not reverse the inhibitory effect of ethanol, suggesting that phosphatases and the ubiquitin-proteasome are not involved in the suppressing action of ethanol (Nguyen *et al.*, 2000).

Accumulating evidence suggests that activation of a variety of cytokines and their downstream signals are important in the development and progression of liver injury. TNF-α activation of the NF-κB signal, IFN-γ activation of STAT1 and IL-4 activation of STAT6 have all been shown to play crucial roles in the development of liver injury in Con A-induced hepatitis used as a model by Jaruga *et al.*, in 2004. Results showed that in ethanol-fed mice, Con A activation of STAT1 and STAT3 signaling pathway was inhibited, whereas NF-κB, the expression of various chemokines and adhesion molecules controlled by NF-κB is enhanced in ethanol-fed mice, which may be an important mechanism contributing to the increase in susceptibility of ethanol-fed mice to T cell-mediated hepatitis induced by Con A (Jaruga *et al.*, 2004).

Leptin acts via receptors that are homologous in sequence to class I cytokine receptors. These include receptors for IL-6, leukocyte inhibitory factor (LIF) and granulocyte colony stimulating factor (G-CSF). The leptin receptor is able to induce tyrosine phosphorylation through its association with Jak2. In the following leptin appears to activate STAT1, STAT3, STAT5 and STAT6. A reason the group of Degawa-Yamauchi examined the effects of ethanol on leptin-induced STAT3 activation using human hepatoma cell lines. To determine the dose response of the inhibitory effects of ethanol on leptin-induced STAT3 phosphorylation, cells were incubated with 0–100 mM ethanol for 30 min followed by leptin stimulation for 15 min. Inhibition of 50 % was already observed at a concentration of 0.1 mM and complete inhibition at 100 mM ethanol, indicating a dose-dependent inhibition of STAT3 activation (Degawa-Yamauchi, 2002).

Summarizing these studies, it is quite obvious that ethanol is able to interfere with intracellular signaling molecules such as STATs or NF-κB, but due to our study does not support the idea of

ethanol-mediated inhibition of STAT4 activation at physiologically relevant ethanol concentrations. Once activated by tyrosine phosphorylation, STAT4 forms a homodimer, translocates to the nucleus, binds to specific DNA sequence and activates the transcription of the IFN-γ gene. To evaluate whether phosphorylated STAT4 of ethanol-treated NK-92 cells is able to translocate into the nucleus, we performed Western blotting using nuclear extracts prepared from unstimulated or IL-12 stimulated NK-92 cells with additional ethanol treatment. Phosphorylated STAT4 increased in the nuclear extracts of IL-12 treated cells already 30 min upon stimulation and peaked at 2 hours (data not shown), whereas untreated cells showed no translocation of activated STAT4 into the nucleus. Additional effects of ethanol were not observed.

The mechanism regulating STAT4 cytoplasmatic – nuclear import and export is critical for IFN-γ production. As was shown for STAT1, facilitated transport of STAT1 into the nucleus requires the presence of signal motifs that are recognized by specific soluble shuttling receptors of the importin/karyopherin family. The exact mechanism how cells import STAT4 molecules on cytokine induction as well as the regulation of the export from the nucleus, is still not fully understood (Toyoda *et al.*, 2004).

There are a few cases reported where impaired translocation of STAT4 is causing recurrent infections in humans, which seems to be based on genetic defects (Toyoda *et al.,* 2004). Whether exogenous agents such as alcohol, certain medication or specific inhibitors are able to influence STAT4 translocation has not been studied so far. At least in our cell culture model using ethanol-treated NK-92 cells, effects on the nucleic accumulation of STAT4 on IL-12 stimulation were not detectable.

Another transcription factor important for IFN-γ production is described as being ethanol-sensitive. This was shown in mice administered the TLR3 ligand poly(I:C), causing an immune response similar to those seen in some virus infections. Additionally, 6 g/kg ethanol was given as a 32 % (v/v) ethanol-water solution. In this case, the amount of phosphorylated c-Jun (AP-1 component) in the nucleus of peritoneal macrophages was increased. In the case of simultaneous *in vivo* exposure to ethanol, this increase was suppressed. This was associated with a suppressed induction of mRNA for IFN-γ by ethanol (Pruett *et al.*, 2004).

This effect was also observed in splenic NK cells from mice fed an ethanol containing diet. Enriched NK cells were stimulated ex vivo with IL-2. As seen previously, the ethanol-induced reduction of AP-1 binding activity paralleled reduced mRNA expression of AP-1 related genes (perforin, granzyme A and granzyme B) IFN-γ expression was not investigated in this study (Zhou and Meadows 2003).

Within the current work, it was intended to investigate the effect of ethanol on AP-1 mediated IFN-γ production. However, since no IL-12-induced AP-1 activation was involved in IFN-γ production in NK-92 cells, the investigation of the effect of ethanol on AP-1 transcriptional activity was not preceded.

Effect of ethanol on IFN-γ mRNA expression. The fact that ethanol has no effect on some transcription factors crucial for IFN-γ gene activation is supported by results on the level of IFN-γ mRNA expression. As expected IL-12 treatment of NK-92 cells led to a significant increase in IFN-γ gene expression seen in the relative number of IFN-γ copies compared to 18S rRNA, additional incubation with 1, 2 and 3 ‰ ethanol had no significant effect on mRNA expression compared to IL-12 stimulated NK-92 cells. In summary, activation of transcription factors correlated with mRNA expression and was not affected by ethanol in a significant manner.

In contrast, Dokur *et al.* showed previously that ethanol administration suppresses NK cell cytolytic activity in male Fischer rats. This study analyzed the effects of ethanol on perforin, granzyme B, and the cytokine interferon (IFN)-γ, factors that modulate NK cell cytolytic activity, to understand the molecular mechanism involved in ethanol's suppression of NK cell activity.

Male Fischer rats were fed an ethanol-containing diet (8.7% v/v), whereas a control group was pair-fed an isocaloric diet. After 2 weeks, spleen tissues were immediately removed and used for analysis of NK cell cytolytic activity, the release of perforin, granzyme B, and IFN-γ messenger RNA (mRNA) or protein levels. The mRNA levels of perforin, granzyme B, and IFN-γ were evaluated by quantitative real-time polymerase chain reaction. Results showed that ethanol reduced the NK cell cytolytic activity and decreased the mRNA expression of perforin, granzyme B, and IFN-γ in ethanol-fed animals when compared with pair-fed animals. Ethanol also significantly reduced the protein levels of perforin and IFN-γ and the enzyme activity of granzyme B in alcohol-fed animals as compared with pair-fed animals. These data suggest that chronic ethanol consumption may suppress *ex vivo* NK cell cytolytic activity in male Fischer rats by decreasing the production, activity, or both of granzyme B, perforin, and IFN-γ (Dokur *et al.*, 2003).

Arjona *et al.* (2004) already demonstrated in their previous work that ethanol treatment for 2 weeks did not affect the splenocyte number, total NK cell number, or percentage of NK cells within the spleen in male Fisher rats. However, in their latest study they provide data that suggest the ability of ethanol to disrupt the physiological circadian rhythms of granzyme B, perforin, and IFN-γ, specifically by hampering the occurrence of the peaks in their mRNA and protein levels. This represents a specific alteration of NK cell circadian regulation. Furthermore, they demonstrated that chronic ethanol consumption is able to suppress NK cell activity by directly disrupting the circadian rhythm of granzyme B, perforin, and IFN-γ mRNA expression (Arjona *et al.*, 2004).

5.3.7 Posttranscriptional events influenced by ethanol

Since ethanol did not modulate the activation of IL-12-induced transcription factors and had no effect on IFN-γ mRNA-expression, we focused on posttranscriptional events that might be influenced by ethanol.

Is there an alcohol-induced intracellular accumulation of IFN-γ? IL-12 stimulation did not significantly affect the intracellular IFN-γ concentration of NK-92 cells. The intracellular concentration of IFN-γ remained stable for 24 hours of incubation. Suggesting that NK-92 cells maintain an intracellular pool of IFN-γ at an average of approximately 350 pg per mg cellular protein; above this concentration, NK-92 cells continuously secrete IFN-γ.

This intracellular pool was not affect by ethanol. Thus, the ethanol-induced decrease in IL-12-induced IFN-γ medium concentration in NK-92, cannot be explained by an intracellular accumulation of IFN-γ. Hence, the transport of IFN-γ out of the cells was not affected by ethanol.

These findings are in agreement findings of other workgroups. Mice were fed with drinking water containing 20% (w/v) ethanol for 2 weeks. NK cells isolated and enriched from splenocytes of these mice were cultured in the presence of 20 pg/ml IL-2 for 24 hours. *In vivo* treatment with ethanol did not affect intracellular levels of IFN-γ (Galluci and Meadows 1996). By contrast, in chronic alcoholics cytoplasmatic IFN-γ levels of peripheral blood T cells were found to be increased (Laso *et al.*, 1999). As already mentioned, *in vivo* ethanol treatment and subsequent cell isolation may be associated with alterations of cells that modulate the resulting cytokine concentration. Additionally, the effect of ethanol treatment may not persist during and after cell isolation (Deaciuc 1997). Moreover, ethanol abuse in humans is often associated with malnutrition and comorbidities, which can be an indeterminable component for interpreting modified cytokine production (Chen *et al.*, 1993). Thus, it is impossible to distinguish effects of malnutrition and comorbidities from effects of ethanol alone in human alcohol abusers (Lin *et al.*, 1998). In comparison to *in vivo* ethanol exposure, simultaneous *in vitro* incubation with ethanol, as it was used within the current work, may be advantageous, since the effects on IFN-γ can be directly related to the acute effect of ethanol (Chen *et al.*, 1993). In contrast to the results within the present work, the Dokur *et al.* showed that *in vitro* ethanol treatment resulted in decreased intracellular IFN-γ levels in rat splenocytes enriched for NK cells. Isolated splencocytes enriched for NK cells were cultured for 24 hours prior to incubation with 100 mM ethanol for another 18 hours. Under these conditions, ethanol treatment decreased intracellular protein levels of IFN-γ (Dokur *et al.*, 2005).

The controversial results may be explained by the different cell models applied and the way of ethanol treatment (chronic vs. acute). However, with respect to the applied cell model within the current experiments, an intracellular accumulation of IFN-γ by ethanol can be excluded.

Is there an increased uptake of IFN-γ in ethanol-treated NK-92 cells? If the whole process of NK-92 activation and IFN-γ expression is not affected by ethanol, there might be a mechanism where once released from NK-92 cells, IFN-γ is re-uptaken in NK-92 cells and degraded intracellularly.

To address this question, we used a differential experimental setting than in the former trials. In this case, IFN-γ production was not generated by IL-12 stimulation, but cells were treated with ethanol in the first place to induce intracellular changes or the release of certain proteases or binding proteins, cells and cell culture supernatant were separated and afterward human recombinant IFN-γ was supplemented at a concentration of 2000 pg/ml (a concentration also reached when cells were stimulated to produce IFN-γ and were left ethanol-untreated). If ethanol would induce a more rapid uptake of IFN-γ into NK-92 cells, the supplemented IFN-γ concentration might be lower in ethanol-treated preparations compared to experiments where NK-92 cells were untreated.

In detail, the ethanol-treatment of NK-92 cells did not change the concentration of IFN-γ supplemented cell culture medium. Within one hour of incubation, the amount of IFN-γ in cell supernatants remained stable. For this reason, we do not assume a mechanism in which ethanol-treated cells are induced in IFN-γ uptake. Different results were obtained when cell culture supernatant of ethanol-treated cells were supplemented with 2 ng/ml human recombinant IFN-γ. In this case, the supernatant of previously ethanol-treated cells was capable of a fast degradation of recombinant human IFN-γ.

Unknown protein that binds or degrades IFN-γ upon ethanol incubation: Since no significant difference occurred in mRNA expression and intracellular accumulation of IFN-γ, we asked whether ethanol-treatment is leading to a more rapid degradation of IFN-γ once released from NK-92 cells instead of a lower production by the cells itself. The preincubation of NK-92 cells with 1, 2 or 3 ‰ ethanol had a highly significant effect on the concentration of IFN-γ in the cell-free supernatant at 10 and 70 min compared to the supernatant of cells without ethanol treatment. The detectability of IFN-γ might have been negatively affected, by a decomposing factor, such as a protease, in the presence of ethanol. Additionally, IFN-γ might have been masked by a protein in the presence of ethanol. Hence, no longer it would be possible to detect IFN-γ by ELISA. This *in vitro* observation of the reduced detectability of IFN-γ in the presence of the cell-free supernatants of NK-92 cells preincubated with ethanol is first described within the current work and comparable results were not found in literature.

We would like to point out that all the results obtained so far are consistent with this observation, since no change in transcriptional activation and mRNA-expression was observed and intracellular accumulation of IFN-γ upon ethanol-action was not demonstrated, even more important this mechanism might explain the ‘disappearance’ of IFN-γ in short-term incubation.

This results led us to the conclusion that NK-92 cells treated with different ethanol concentration release an unknown protein that is either able to bind or to disintegrate IFN-γ.

To the best of our knowledge, we are the first being able to demonstrate that changes in IFN-γ levels observed upon ethanol treatment are due to extracellular modification.

The soluble IFN-γ receptor. Soluble cytokine receptors, which either attenuate or promote cytokine signaling, are important regulators of inflammation and immunity. The key role of soluble cytokine receptors is to prevent excessive inflammatory responses is illustrated by the autosomal dominant, autoinflammatory, TNF receptor-associated periodic syndrome (TRAPS), which was initially identified in patients with mutations in the extracellular domain of the 55-kDa, type I TNFR (TNFRSF1A, TNFR1) that impaired receptor shedding (Levine, 2004).

The IL-1 and IL-6 receptor systems are paradigms for soluble cytokine receptors that mediate antagonistic and agonistic effects. Both systems are complex and are regulated by multiple cell-associated and soluble receptors, as well as receptor-associated proteins.

In contrast to the antagonistic effect of sIL-1RII on IL-1 signaling, soluble IL-6 receptors are an important mechanism by which IL-6 signaling is amplified. Soluble IL-6 receptors can be generated by two distinct pathways: proteolytic cleavage that sheds the membrane-bound IL-6R ectodomain or alternative mRNA splicing, with resulting synthesis of an IL-6Rα that lacks the transmembrane domain. Soluble IL-6 receptors bind IL-6 with an affinity similar to the membrane IL-6R, thereby prolonging the IL-6 half-life and preventing IL-6 from starting cellular activation (Rose-John *et al.,* 2006).

Proteolytic cleavage of cell surface receptors is typically catalyzed by zinc metalloproteases of the ADAM (a disintegrin and metalloprotease) family. ADAM17 or TNF-α-converting enzyme (TACE) is the prototypical receptor sheddase that was identified by its ability to cleave membrane-bound TNF to its soluble form (Levine, 2004).

The discovery that soluble forms of cytokine receptors are involved in the endogenous regulation of cytokine activity has prompted substantial interest in their potential application as immunotherapeutic agents (Fernandez-Botran *et al*., 2002).

Subcutaneous administration of increasing doses of IL-12 in cancer patients increased the expression of certain cytokine genes (e.g. IFN-γ) in peripheral blood mononuclear cells. However, a marked decline of IFN-γ was observed. In addition, a constant up-regulation of serum soluble IFN-γ receptor levels was observed after each cycle of IL-12 treatment. The constant rise of soluble IFN-γ receptor during IL-12 therapy may therefore contribute to the inhibition of IFN-γ activity detected after repeated cycles of IL-12 (Haicheur 2000).

The ability of many soluble cytokine receptors to inhibit the binding and biological activity of their ligands makes them very specific cytokine antagonists. The question whether the release of soluble cytokine receptors is also induced by exogenous substances such as ethanol is yet to be elucidated.

For that reason, we used two different approaches to study ethanol-mediated secretion of the IFN-γ receptor. In a first attempt, we used the addition IFN-γ receptor specific antibody to block the binding of NK-92 cell-released IFN-γ binding to the soluble IFN-γ receptor to enable detection of free IFN-γ.

A second attempt to study ethanol-mediated IFN-γ receptor release was to use a commercially available ELISA set designed to detect the IFN-γ receptor 1 concentration. Concentration of IFN-γ receptor subunit 1 in cell supernatants of ethanol-treated cells tended to be slightly higher in comparison to cell supernatants of untreated NK-92 cells. However, this increase was not significant and overall concentrations ranged from 40 to 100 pg/ml and were far to low to explain the 'loss' of almost 2000 pg/ml of supplemented human recombinant IFN-γ protein.

In summary, we conclude that the release of soluble IFN-γ receptor is not responsible for changes in IFN-γ concentration in supernatants of NK-92 cells incubated with ethanol.

Does ethanol induce extracellular proteolysis? The addition of a commercially available protease inhibitor cocktail had a highly significant effect on the change in IFN-γ concentration in cell supernatants of ethanol-treated cells. This fact leads one to suggest that ethanol-incubated NK-92 cells rapidly release proteases capable of degrading IFN-γ in a specific manner and that the addition of a mixture of different protease inhibitors blocked the proteolytic effect, hereby stopping the degradation of IFN-γ, leading to IFN-γ concentrations in ethanol-treated cell's supernatants as high as supplemented.

Proteinases are important regulators in antigen presentation and cytotoxic activity. The most important roles of proteinases in the immune system are found in apoptosis and major histocompatibility complex (MHC) class II-mediated antigen presentation.

A variety of cysteine proteinases, serine proteinases, and aspartic proteinases as well as their inhibitors are involved in the regulation of apoptosis in neutrophils, monocytes, and dendritic cells, in selection of specific B and T lymphocytes, and in killing of target cells by cytotoxic T cells and natural killer cells. In antigen presentation, endocytosed antigens are digested into antigenic peptides by both aspartic and cysteine proteinases.

Proteinase activity in these processes is highly regulated by balance between active proteinases and specific endogenous inhibitors such as cystatins, thyropins, and serpins. (van Eijk 2003).

The possible involvement of cell surface-associated proteolytic enzymes in human NK cell-mediated cytotoxicity and the mechanism by which such enzymes exert their activity have already been studied. Of the membrane-associated enzymes, those engaged in cytotoxicity seem to be concealed from the external environment, as pretreatment of the effector cells with protease inhibitors such as trasylol and PMSF have no effect on the reaction. Immediately upon addition of the target cells and the initiation of cytotoxicity, the reaction becomes highly sensitive to inhibitors. The surface-associated elastase on the other hand maintains a constitutive mode of activity distinctive and unrelated to that of enzymes engaged in cytotoxicity. These findings suggest the existence proteases on the surface of the NK lymphocyte and of a mechanism where resting cytotoxic structures become activated as the receptor attaches to the target cell. Therefore binding to the target cell triggers the exposure of the proteolytic moiety, and initiates the lytic phase of the reaction (Lavie *et al.*, 1985).

Another group of protease expressed in immunocompetent cells are the matrix metalloproteinases. They are members of a family of at least 21 $Zn2^{+}$-dependent endopeptidases, of which 16 are soluble, secreted enzymes, while the other 5 are membrane bound. The expression of most MMPs is highly regulated by several mechanisms: at mRNA level transcriptionally by cytokines, hormones, and growth factors, and at protein level by proteolytic activation of latent enzymes and inhibition of active enzymes by endogenous inhibitors. They play important roles in many normal biological processes such as wound healing, and angiogenesis as well as in pathological processes, including arthritis, emphysema, and cancer metastasis (Nagase *et al.* 1999). The main characteristic of MMPs is the degradation of the extracellular matrix of basement membranes, thus enabling cells to invade into tissues. MMPs are secreted as proenzymes and subsequently activated by proteolytic cleavage. MMP activity is regulated by the naturally occurring inhibitors such as α-macroglobulins and the tissue inhibitors of MMPs (TIMPs) (Parson *et al.* 1997).

MMPs in immune cells serve numerous specialized immunologic functions in addition to extracellular matrix degradation (Goetzl *et al.* 1997). T lymphocytes have been shown to produce MMP-9 constitutively, whereas MMP-2 expression is induced by IL-2 and VCAM-1-dependent adhesion to endothelial cells. These MMPs contribute to the ability of T cells to migrate through model subendothelial basement membranes (Xia *et al.* 1996). Neutrophils have been shown to store MMP-8 and MMP-9 intracellularly in specific granules and to secrete these enzymes upon stimulation. Macrophages express MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MT1-MMP as well as MMP-12. These MMPs mediate secretion of Fas ligand and TNF-α by cleavage of their membrane-bound forms and generation of angiostatin from plasminogen by proteolytic cleavage (Patterson *et al.* 1997).

Natural killer cells are endowed with the ability to express spontaneous cytotoxicity against tumor cells or virus infected cells. Upon several biological responses such as virus infection and carcinogenesis, NK cells encompass movements directed against target cells. They should possess proteinases, which mediate their transmigration through the ECM component for their migration to the target cells (Ishida *et al.,* 2004).

In the animal model, it was already documented that rat IL-2-activated NK cells produce matrix metalloproteinase-2 (MMP-2) and MMP-9. Additionally it was shown that mouse NK cell-derived MMPs, include MT-MMPs, and also TIMPs. RT-PCR analysis from cDNA of mouse NK cells revealed mRNA for MMP-2, MMP-9, MMP-11, MMP-13, MT1-MMP, MT2-MMP, TIMP-1, and TIMP-2. MMP-2 and MMP-9 expression was confirmed by gelatin zymography (Kim *et al.* 2000). Moreover, Kim *et al.* (2000) report MT-MMPs are expressed by NK cells, i.e., large granular lymphocytes as determined by both RT-PCR and Western blots. Taken together, these findings suggest that NK cells may therefore use multiple MMPs in various cellular functions.

Recent findings suggested that ethanol activates matrix metalloproteinases (MMPs) via protein tyrosine kinase (PTK) signaling. Chronic alcohol administration has been shown to activate MMP-2 and -9 in *in vitro* and *in vivo* models (Aye *et al.,* 2004; Lois *et al.,* 1999); however, mechanisms leading to such effects remain undefined. Two *in vivo* studies examined the effect of ethanol on MMPs. Lois *et al.* demonstrated that ethanol exposure increases MMP-2 and MMP-9 activity but not their production in rat lungs. Similarly, ethanol consumption upregulates the enzymatic activity of MMP-2 in rat aortas. Although these studies do not identify the source of MMP-2, they support the fact that MMP-2 is a target of ethanol.

The underlying mechanism of ethanol-mediated IFN-γ expression is still unknown and will be topic of our future investigations. With our results demonstrating a possible proteolytic degradation activation of MMPs by ethanol will be in the centre of our further experiments.

5.4 Outlook

With our results demonstrating a possible proteolytic degradation of IFN-γ as a consequence of ethanol treatment we provide for the first time evidence that alcohol induces the release of specific proteolytic enzymes that are able to disintegrate IFN-γ. Since ethanol is long known to modulate cytokine patterns *in vivo* the question remains whether this mechanism seen in cell culture model is also present in isolated PBMC as well as *in vivo*.

Future research will be the application of more specific inhibitors makes it possible to further characterize the protease responsible for IFN-γ degradation. It would be even more interesting if other cytokines influenced by ethanol are also substrates of ethanol-induced proteolytic enzymes and if the addition of inhibitors in those cases might also lead to the compensation of ethanol-modulated effects.

Additionally, one has to take into consideration that even though the amount of IFN-γ detected in cell culture supernatant of ethanol-treated cells might be restored by the addition of protease inhibitors, the question remains whether biological activity of IFN-γ, leading to STAT1 phosphorylation and expression of IFN-regulated genes is reestablished in effector cells, such as monocytes and activated T cells.

6 Summary

Expose to potential pathogens is followed by inflammation. This reaction of the immune system includes the production of cytokines, a group of multifunctional proteins that play a critical role in cellular communication and activation. Cytokines have been classified as being proinflammatory or anti-inflammatory depending on their effects on the immune system. However, cytokines regulate inflammation, cell death, and cell proliferation and migration as well as healing mechanisms.

Alcohol is known to modulate the immune system in a complex manner. The effects of alcohol on immune responses vary with acute and chronic exposure as well as depending on the history of alcohol consumption and the blood level of alcohol. The presence or absence of alcohol can affect the cytokine cascade in complex ways.

Chronic alcohol consumption increases TNF-α, IL-6 and reactive oxygen release from resident macrophages in liver that underlie early alcohol-induced liver disease. Additionally alcohol use alters immune defenses against infections and results in increased incidence of bacterial pneumonias, a higher rate of chronic hepatitis C infection, and increased susceptibility to HIV infection (Nelson and Kolls, 2002; Prakash *et al.*, 1998; Wiley *et al.*, 1998; Zhang *et al.*, 1997). Alcohol-induced alterations in immune functions extend to both innate and adaptive immune responses. Previous studies demonstrated that alcohol exposure results in impaired adaptive immune responses and antigen-specific T cell activation.

In the current study the immunmodulatory capability of an acute, moderate (1 ‰) to high amount (3 ‰) of alcohol was tested on isolated Peripheral Blood Mononuclear Cells production of several proinflammatory and antiinflammatory cytokines after incubation for 12 to 72 hours.

We found that lipopolysaccharide (LPS)-induced as well as phytohemagglutinin (PHA)-stimulated TNF-α was significantly reduced when PBMC were incubated with high concentrations of ethanol (3 ‰) for 12 hours. The same was seen in IL-10 production of LPS-challenged PBMC, whereas IL-2 concentrations in cell culture supernatants of ethanol-treated (3 ‰) cells was significantly increased compared to standard incubation. This is also true for PHA-stimulated PBMC tested for IL-12 concentration. In contrast, differences in cells proliferation and viability were not observed.

However, the most affected cytokine in our model system of isolated human PBMC treated with two different ethanol concentrations was IFN-γ. Its concentration decreased in a highly significant manner in PHA- as well as in LPS-stimulated PBMC when treated with 66 mM ethanol and in a significant manner in PHA-activated PBMC when treated with 22 mM ethanol.

The fact that ethanol negatively affects IFN-γ production is supported by several *in vivo* and *in vitro* studies by Wagner *et al.,* 1992, Chen *et al.,* 1993, Laso *et al.,* 1997 Waltenbaugh *et al.,* 1998, Starkenburg *et al.*, 2001, Szabo et al., 2001, Dokur *et al.,* 2003.

The reduced IFN-γ level observed might be a key factor in explaining comprised immunity seen after chronic alcohol abuse, since together with IL-12, IFN-γ is crucial for the innate and adaptive immune response to viral and bacterial infection (Vicente-Gutierrez *et al.*, 1991, Windle *et al.,* 1993, Szabo 1997, Szabo *et al.,* 1999).

Since NK cells are acting in the early state of host defense against infection where they serve as main source of IFN-γ and since IL-12 is one of the most efficient inducers of IFN-γ gene expression, we studied ethanol effects on IFN-γ in IL-12 stimulated NK-92 cells.

As seen in isolated human Peripheral Blood Mononuclear Cells IFN-γ production by IL-12 stimulated NK-92 cells is significantly reduced in the presence of ethanol. However, this decrease did not correlate with decreased phosphorylation and nuclear translocation of STAT4, a central regulator of IFN-γ gene expression.

These results indicated that acute alcohol treatment *in vitro* did not affect intracellular pathways leading to IFN-γ gene expression. These findings paralleled results indicating that the amount of mRNA for IFN-γ synthesis in NK-92 cells is not affected by the applied ethanol concentrations as well. Additionally it was shown within the current work, that the reduced IFN-γ production by NK-92 cells in the presence of ethanol might not be explained by an intracellular accumulation of the IFN-γ protein.

The inhibitory action of ethanol on IFN-γ may rather be caused by posttranslational modification once IFN-γ is released by NK-92 cells, since the addition of recombinant human IFN-γ to the cell culture supernatants of ethanol-treated cells led to a decline in the amount of IFN-γ concentration.

We therefore hypothesized that ethanol may cause the release of either an IFN-γ-binding or IFN-γ-degrading protein. An increase in soluble IFN-γ receptor as a result of ethanol treatment was not observed. But the addition of mixture of 5 commercially available protease inhibitors counteracted the effect of ethanol treatment, giving us a first hint of IFN-γ-modulatory mechanism, where IFN-γ released by NK-92 cells may be disintegrated by a protease released as consequence of ethanol incubation.

To our best knowledge we are the first to demonstrate a posttranslational modification of IFN-γ as a consequence of ethanol incubation.

In summary, the present results support the inhibitory role of ethanol on IFN-γ, but are too preliminary to explain the underlying immunmodulatory effect.

7 Zusammenfassung

Eine Entzündung ist die Folge einer Reaktion des Immunsystems auf potentielle Pathogene. Die Zytokine, die hierbei gebildet werden sind Proteine, die eine zentrale Rolle im Zusammenspiel und in der Aktivierung von immunkompetenten Zellen spielen. Die einzelnen Zytokine können in pro- und antiinflammatorisch wirksam eingeordnet werden. Insgesamt sind sie für die Regulation der Entzündung, die Proliferation und Migration, sowie am Heilungsprozess beteiligt.

Die Bildung von Zytokinen und damit die Immunabwehr selbst kann durch exogene Faktoren, wie Alkohol beeinträchtigt werden. Der Einfluss von Alkohol auf die Immunabwehr unterscheidet sich je nach Menge des konsumierten Alkohols und des damit erreichten Blutalkoholspiegels, sowie nach der Dauer des chronischen Missbrauchs. Dabei führt Alkohol zu vielfältigen Veränderungen innerhalb der Zytokin-Kaskade: Chronischer Alkoholmissbrauch führt in der Leber zu einer vermehrten Freisetzung von TNF-α aus Kuppferzellen und damit zu einer Entzündungsreaktion, die schon in einem frühen Stadium der alkoholbedingten Lebererkrankung eine entscheidende Rolle spielt. Zusätzlich kommt es zu weiteren Veränderungen, die in einer erhöhten Inzidenz von bakteriellbedingten Pneumonien sowie in einer erhöhten Anfälligkeit gegenüber viralen Infektionen wie Hepatitis C und HIV zu sehen ist (Nelson and Kolls, 2002; Prakash *et al.,* 1998; Wiley *et al.,* 1998; Zhang *et al.,* 1997). Dabei sind diese Veränderungen sowohl bei Reaktionen des angeborenen, als auch des adaptiven Immunsystems von entscheidender Bedeutung. In früherer Studien konnte bereits eine deutliche Schwächung der adaptiven Immunantwort nachgewiesen werden, sowie eine deutlich geringere Aktivierung der Antigen-spezifischen T-Zellaktivierung.

In der nun vorliegenden Arbeit wurden die immunmodulatorischen Eigenschaften von Alkohol l in Konzentrationen von 1 ‰ und 3 ‰ untersucht. Hierzu wurde die Fähigkeit isolierter humaner PBMC unter dem Einfluss von Ethanol pro- bzw. antiinflammtorische Zytokine zu produzieren untersucht. Dabei konnte ein deutlicher Rückgang der LPS- bzw. PHA-induzierten TNF-α-Ausschüttung gemessen werden, wenn Alkoholkonzentrationen von 3 ‰ zur Inkubation eingesetzt wurde. Für IL-10 gilt das gleiche, während im Gegensatz dazu IL-2 in seiner Produktion erhöht war. Für PHA-stimulierte PBMC wurde diese Erhöhung auch für IL-12 nachgewiesen. Andere Zytokine wie TGF-β, IP-10 und IL-18 wurden in ihrer Produktion nicht verändert. Messungen der Proliferation und Vitalität der Zellen ergaben ebenfalls keine Veränderungen bei Alkoholzusatz.

In unserem Modellsystem wurde IFN-γ in seiner Ausschüttung am deutlichsten gehemmt.
Hohe Alkoholkonzentrationen von 3 ‰ führten zu einem hochsignifikanten Rückgang auf ca. 4 % der IFN-γ Menge, wie sie unter alkoholfreien Bedingungen gemessen werden konnte. Für Alkohol-Konzentrationen von 1 ‰ betrug dieser Rückgang ca. 30 %.
Die Tatsache, dass Alkohol die IFN-γ Ausschüttung negativ beeinflusst wurde bereits in anderen *in vitro* und *in vivo* Studien (Wagner *et al.,* 1992, Chen *et al.,* 1993, Laso *et al.,* 1997 Waltenbaugh *et al.,* 1998, Starkenburg *et al.,* 2001, Szabo *et al.,* 2001, Dokur *et al.,* 2003) gezeigt.
IFN-γ ist ein pleiotropes Zytokin, essentiell für die angeborene und adaptive Immunantwort auf virale und bakterielle Infektionen. Damit könnten die verminderten IFN-γ Spiegel, wie sie auch in Seren von Menschen mit Alkoholabusus gemessen werden ein Schlüssel für die Erklärung der 'Immunschwäche' in dieser Patientengruppe sein.
Bei der Erstreaktion des Immunsystems wird IFN-γ hauptsächlich von NK Zellen gebildet. Dabei ist IL-12 der effektivste Aktivator der IFN-γ Genexpression und damit entscheidend für eine angemessene Reaktion gegen eingedrungene Pathogene viraler oder bakterieller Genese.
Wir konnten belegen, dass die IFN-γ Ausschüttung durch IL-12-stimulierte NK-92 Zellen durch Ethanol in gleicher Weise gehemmt wird, wie bereits für PBMC beschrieben. Diese Hemmung geht jedoch nicht mit einer Veränderung der STAT-4-Phosphorylierung, einem entscheidenden Schritt hin zur IFN-γ-Genexpression, einher. Parallel zu dieser Messung konnte auch keine Verminderung der IFN-γ-mRNA-Menge unter dem Einfluss von Ethanol nachgewiesen werden. Auch eine intrazelluläre Akkumulation kann nach unseren Ergebnissen ausgeschlossen werden.
Damit ist die inhibierende Wirkung von Alkohol auf posttranslatorisch wirksame Mechanismen zurückzuführen. Dabei zeigte sich, dass ein unbekanntes Agens im Zellkulturüberstand von zuvor mit Ethanol behandelten Zellen die Fähigkeit besitzt extern zugesetztes humanes rekombinantes IFN-γ so zu modulieren, dass ein anschließender Nachweis im ELISA nur noch in geringen Mengen möglich war. Damit scheint Alkohol die NK-92 Zellen zur Ausschüttung eines IFN-γ bindenden oder IFN-γ abbauenden Moleküls zu stimulieren. Die Bindung an den löslichen IFN-γ-Rezeptor kann nach den Ergebnissen unserer Untersuchungen ausgeschlossen werden. Durch den Einsatz einer kommerziell erhältlichen Mischung von 5 Protease-Inhibitoren konnte der ethanolbedingte Abbau von IFN-γ in den Zellkulturüberständen gehemmt werden und damit ein möglicher Wirkmechanismus aufgezeigt werden.
Ingesamt unterstützen unsere Resultate eindeutig die inhibierende Wirkung von Ethanol auf IFN-γ. Die bisher vorliegenden Resultate und der fehlende Nachweis einer IFN-γ modulierenden Protease macht es jedoch noch nicht möglich, den Mechanismus dieser hemmenden Wirkung zu erklären.

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Publications

Original work:

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Suppression of TNF-alpha production by S-adenosylmethionine in human mononuclear leukocytes is not mediated by polyamines.

Biol Chem. 2006 Dec;387 (12):1619-27 17132109

Poster presentation:

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Transepithelial Transport of Primary Bile Acids (CPBA) through a IEC Layer

Falk Symposium 155 XIII FALK LIVER WEEK (Part I) XIX International Bile Acid Meeting Bile Acids: Biological Actions and Clinical Relevance

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BERUFSTÄTIGKEIT

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Declaration/Eidesstattliche Erklärung

I hereby certify that this thesis is entirely my own work. All material and references, which were required for this work, are indicated.

Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit von mir selbst, lediglich unter Verwendung der aufgeführten Literatur angefertigt wurde. Wörtlich oder inhaltlich zitierte Stellen wurden als solche kenntlich gemacht.

Stuttgart, im April 2008

(Senja Sauter)