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(Direktor: Prof. Dr. med. Michael P. Manns)

Role of the CD26-ADA-adenosine system in viral and autoimmune chronic liver disease

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To my wife Brigitte who means the world to me! My adorable son Noah for always bringing a smile into my face! My parents for their love, guidance and unconditional support until this very day! My brothers for always looking out for me! And last but not least, my grandparents for being great role models!

Table of Contents

1 Introduction ... 3

1.1 The human immune system ... 3

1.2 Innate and adaptive immunity ... 3

1.3 Regulation of immune responses ... 4

1.4 The CD26-ADA-adenosine system ... 5

1.4.1 CD26 ... 5

1.4.2 Adenosine deaminase ... 6

1.4.3 Adenosine ... 7

1.4.4 Interactions between CD26, ADA and adenosine ... 8

1.5 Inflammatory liver disease ... 8

1.5.1 Hepatitis B Virus (HBV) ... 10

1.5.2 Hepatitis C Virus (HCV) ... 10

1.5.3 Autoimmune Hepatitis (AIH) ... 11

1.6 Effects of adenosine and interferone alpha on effector functions of lymphoid cells ... 12

2 Aim of this study ... 14

3 Materials and Methods ... 15

3.1 Materials ... 15

3.2 Methods ... 18

3.2.1 Patient cohort ... 18

3.2.2 Isolation of peripheral mononuclear blood cells (PBMC) ... 19

3.2.3 Thawing of PBMC samples ... 19

3.2.4 Surface marker staining and flow cytometric analysis ... 19

3.2.5 Antigen specific stimulation of PBMC ... 21

3.2.6 Carboxyflourescein succinimidyl ester-based (CFSE) proliferation assays ... 22

3.2.7 Enzyme-linked immunosorbent spot (ELISpot) assay ... 23

3.2.8 Statistical analysis ... 23

4 Results ... 24

4.1 CD26 expression on PBMC in healthy donors ... 24

4.2 CD26 expression on PBMC of patients with viral hepatitis and autoimmune hepatitis ... 27

4.3 Biochemical activity of liver disease and CD26 expression ... 29

4.4 Viremia and CD26 expression in patients with HCV infection ... 31

4.5 CD26 expression and HBV viremia ... 33

4.6 CD26 expression during interferon alpha (IFN-α) treatment ... 34

4.7 Lymphocyte proliferation and CD26 expression with and without adenosine 36
4.8 Adenosine and antigen-specific T cell function 38
5 Discussion 40
5.1 CD26 expression on lymphoid cells and possible functional implications 40
5.2 CD26 in patients with inflammatory liver disease 42
6 Summary 47
7 References 49
8 Abbreviations 61
9 Acknowledgements 64
10 Curriculum Vitae 65

1 Introduction

1.1 The human immune system

The human organism is confronted with various kinds of pathogens such as bacteria, viruses, fungi and parasites invading the body through the skin, the gastrointestinal and the respiratory tract and sometimes via blood transmission. The role of the immune system is to detect these pathogens, inhibit their spreading in the organism and in an ideal setting to eliminate them. The immune system deletes abnormal cells from the body in order to maintain the general structure of the organism. At the same time, the immune system must be able to distinguish self from foreign without destroying natural and necessary microbial flora within the body or prevent overreaction against harmless substances.

The immune system is composed of different kinds of soluble molecules (e.g. the complement system, cytokines and chemokines) and various immune cells, each with its own specificity and function.

Two key components are distinguished, namely, the innate immunity, which mediates the initial unspecific protection against infections, and the antigen-specific adaptive immunity, which develops more slowly and mediates the later defense against infections.

1.2 Innate and adaptive immunity

The term innate immunity refers to the fact that this type of defense is always present in healthy individuals, whereas the adaptive immunity is stimulated by pathogens evading the innate immune system, meaning, it adapts to the presence of microbial invaders.

The first line of defense in innate immunity is provided by mechanical barriers like epithelial layers and the mucosa, both of which function to block the entry of microbes. In case these barriers are penetrated, microbes are immediately attacked by phagocytes, natural killer (NK) cells and proteins of the complement system. All mechanisms of the innate immunity specifically recognize and react against microbes. In addition to providing early defense against infections, innate immune responses regulate adaptive responses against the infectious agents (Janeway, 2002; Janeway and Medzhitov 2002).

There are two types of adaptive immunity, called humoral and cell-mediated immunity, that are designed to provide defense against extracellular and intracellular microbes. Humoral immunity is mediated by antibodies, which are produced by B lymphocytes. These antibodies are secreted into the circulation and mucosal fluids where they neutralize microbes that are present in the blood and in the lumens of mucosal organs. However, antibodies usually do not have access to microbes that live and divide inside infected cells (Dorner and Radbruch, 2007). Defense against such intracellular microbes is mediated by T lymphocytes. The antigen receptors of T lymphocytes recognize peptide fragments of protein antigens that are bound to major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APC). Among T lymphocytes, $CD4^+$ and $CD8^+$ T cells can be distinguished (Yewdell and Bennink, 1999). Because $CD4^+$ T cells help activating phagocytes to destroy ingested microbes and B lymphocytes to produce antibodies, they are also referred to as T helper cells. $CD8^+$ T cells are called cytotoxic T lymphocytes (CTL) because they kill cells infected by intracellular microbes. Special characteristics of the adaptive immune system, and the ones that distinguish it from innate immunity, are the fine specificity for structurally distinct antigens and memory of prior exposure to antigen (Soroosh and Doherty, 2009).

1.3 Regulation of immune responses

The regulation of immune responses requires a sophisticated network of activating and inhibitory mechanisms, which may act directly on the respective effector cells (e.g. lymphocytes, granulocytes and macrophages) or indirectly via the regulation of specific ligands expressed on APC and target cells (Alexander et al., 2009). Further regulation is mediated through the secretion of soluble factors including various cytokines, which may than act on the effector cells. It is essential for an organism to survive, so that when innate and adaptive immunity have been established and are ongoing, inhibitory mechanisms have to prevent overwhelming immune responses which can eventually lead to pathology. In recent decades several of such inhibitory factors have been identified. For example a whole set of inhibitory receptors is expressed on NK cells, which interact with various class I MHC molecules and other ligands expressed on basically all nuclear cells, leading to downregulation of NK cell function (Biassoni, 2008). Similarly, the adaptive immune response (i.e. of $CD4^+$ and $CD8^+$ T cells) is tightly controlled by the fine balance of multiple activating and inhibitory receptors and their respective ligands, as well as by soluble factors like cytokines. Moreover, special cell populations such as regulatory T cells characterized by

expression of CD25 and Foxp32 (Feuerer et al., 2009) or myeloid derived suppressor cells CD14(+)HLA-DR(-/low) (Hoechst et al., 2008) have recently been shown to be of major importance to regulate the strength and specifity of T cell responses in malignancies, viral infections and autoimmune disorders.

1.4 The CD26-ADA-adenosine system

1.4.1 CD26

CD26, a widely distributed multifunctional type II cell surface glycoprotein, is involved in different biological processes. It was first described as “Glycyl-Prolyl-Naphthylmidase” by Hopsu-Havu in 1966 (Hopsu-Havu and Glenner, 1966). Because of its exopeptidase activity, preferentially cleaving N-terminal dipeptides after proline and alanine residues (Kenny et al., 1976; Mentlein, 1999), it was later described as Dipeptidylaminopeptidase IV (DPP IV) (Callahan et al., 1972). Furthermore, an endopeptidase activity of CD26 has also been reported (Bermpohl et al., 1998). Of note DPPIV has been identified as a key regulator of insulin-stimulating hormones (Gupta et al., 2009). DPP IV inhibitors have been recently launched for the treatment of Diabetes mellitus (Krentz et al., 2008). CD26 is widely expressed on T cells, B cells and NK cells (Buhling et al., 1995; Buhling et al., 1994; Fleischer, 1987; Fleischer et al., 1988; Gorrell et al., 1991), but can also be found on epithelial, endothelial and acinar cells in a variety of tissues (Dinjens et al., 1989; Hartel et al., 1988; Heike et al., 1988; McCaughan et al., 2000; McCaughan et al., 1990). In addition to the integral membrane form, a soluble form of CD26 occurs in serum (Gorrell et al., 2001). Due to its interaction with proteins of the extracellular matrix (ECM) (e.g. collagen and fibronectin), CD26 is also considered a cell adhesion molecule (Reutter, 1995). However, the significance of this interaction for the immune system is still unknown (Hoechst et al., 2008). Furthermore, it has been shown that CD26 plays an important role in T cell activation and immune regulation (De Meester et al., 1999; Fleischer, 1994; Franco et al., 1998; Morimoto and Schlossman, 1998). Thus this molecule can be regarded as an activation marker for B, T and NK cells (Ansorge, 2001). Amongst various substrates of CD26 are immunoregulative hormones and chemokines such as substance P, neuropeptide Y, endomorphin-2, GLP-1, RANTES (regulated on activation normal T-cell expressed and secreted), eotaxin, MDC (monocyte-derived chemokine) and SDF-1α and SDF-1β (stromal derived factor) (De Meester et al., 1999; Hildebrandt et al., 2000; Marguet et al., 2000; Proost et al., 1998). Also of interest is that CD26 serves as a co-stimulator in the antigen-stimulated activation of T

lymphocytes (Dang et al., 1990; Tanaka et al., 1993) and mediates signaling by direct interaction with CD45 (Ishii et al., 2001; von Bonin et al., 1998). In addition, CD26 serves as a receptor for ADA on T lymphocytes and, hereby, may play an important role for the regulation of the immune response (Dong et al., 1997). CD26 expression has also been linked to autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Graves' disease, Hashimoto's thyreoditis and sarcoidosis, in which high numbers of $CD26^{+}$ T cells can be found during active phases (Mizokami et al., 1996). This increased number of $CD26^{+}$ T cells at inflammation sites suggests an important role in chronic inflammation.

1.4.2 Adenosine deaminase

Adenosine deaminase (ADA), an enzyme that metabolizes extracellular adenosine, is a ligand of cell surface and soluble CD26 (De Meester et al., 1994; Kameoka et al., 1993; Martin et al., 1995; Morrison et al., 1993). The enzyme is well known because the hereditary lack of ADA activity causes severe combined immunodeficiency (autosomal SCID), which is characterized by the absence of functional B and T lymphocytes in affected individuals (Apasov et al., 1995; Franco et al., 1998; Hirschhorn, 1995). ADA is involved in the breakdown of purines. Hence, ADA-deficiency leads to the accumalation of toxic purine metabolites in proliferating cells i.e. maturing lymphocytes, thereby resulting in their block of maturation (Stephan et al., 1993). Subsequently, gene therapy introducing ADA into hematopoetic stem cells was successfully applied to patients with hereditary ADA deficiency fifteen years ago (Fischer et al., 2000). A recent study by Aiuti et al. investigated the long-term outcome of ten patients, who were treated by means of nonmyeloablative chemotherapy followed by an infusion of autologous hematopoetic stem cells that had been transduced with a retroviral vector bearing the ADA gene. All patients were alive after a median follow-up of 4 years. Serious adverse effects included prolonged neutropenia, hypertension, central-venous-catheter-related infections, Epstein-Barr virus reactivation and autoimmune hepatitis. But in conclusion gene therapy is a safe and effective treatment for SCID in patients with ADA deficiency (Aiuti et al., 2009). As extracellular adenosine inhibits T-cell proliferation in a dose-dependent manner, it is likely that this inhibition is relieved by the localization of ADA to the cell surface by binding to CD26 (Dong et al., 1996). In addition, ADA has been proposed to have a catalytically independent function as a co-stimulatory molecule (Martin et al., 1995).

1.4.3 Adenosine

Adenosine, an endogenous purine nucleotide, has multiple functions in various tissues (Fredholm et al., 2001). It effects multiple physiological functions in the nervous and cardiovascular system. In fact, adenosines influence on the heart was already described eighty years ago (Bennett, 1931). Adenosine is used therapeutically in the treatment of certain heart rhythm disturbances (Delacretaz, 2006). Other than that it also pays an important role in the central and peripheral nervous system including CNS development and regeneration (Dunwiddie and Masino, 2001; Hasko et al., 2005). Moreover, it is well known that adenosine is a potent regulator of the immune system mainly by displaying an immunosuppressive function (Ohta and Sitkovsky, 2001; Sitkovsky et al., 2004). Inhibitory effects of adenosine have been described for activated T cells (Butler et al., 2003; Hoskin et al., 2002; Zhang et al., 2004), dendritic cells (Panther et al., 2003; Panther et al., 2001), NK cells, eosinophils, monocytes, macrophages, neutrophils and mast cells (Fishman and Bar-Yehuda, 2003; Linden, 2001; Sitkovsky, 2003; Thiel et al., 2003). Its extracellular concentration has been reported to rise due to nonlytic secretion of adenosine during hypoxic conditions and inflammatory processes (Cronstein, 1994) as well as in tumor environments (Blay et al., 1997). In addition, adenosine can be generated extracellularly from ATP, ADP and AMP by 5'-nucleotidase and ectopyrase in the brain and lung with a halftime of about 200 ms (Hasko and Cronstein, 2004). There is also a significant nonlytic secretion of 5'-AMP by different cells including neutrophils and mast cells, which is then converted to adenosine by the ecto-5'-nucleotidase (Kobie et al., 2006).

It is believed that most of the effects of adenosine are mediated through the adenosine receptor A_3 (Gomez and Sitkovsky, 2003), one of four known G protein-coupled receptors, although inhibitory effects have been reported for NK cells and T cells via the A_2 receptor in mice (Bours et al., 2006; Hasko and Pacher, 2008). A_3 adenosine receptor ligation leads to inhibition of the adenylyl cyclase activity and activation of phospholipase C via interaction with $G_{i/o/q}$ proteins (Fredholm et al., 2001; Klinger et al., 2002; Linden, 2001). Next, A_3 agonists have been shown to inhibit degranulation of human eosinophils and neutrophils (Bouma et al., 1997; Ezeamuzie and Philips, 2003; Gessi et al., 2002), chemotaxis and interleukin-12 production of dendritic cells (Fossetta et al., 2003; Panther et al., 2001). Also the release of pro-inflammatory cytokines by monocytes (Hasko et al., 2000; Hasko et al., 1998; Sajjadi et al., 1996; Salvatore et al., 2000) and induction of apoptosis in PBMCs (Barbieri et al., 1998; Brambilla et al., 2000) upon A_3 receptor ligation have been described

1.4.4 Interactions between CD26, ADA and adenosine

Several studies have already investigated the function of either CD26, ADA or adenosine. However, as described above, each molecule is part of a network and alterations in expression of one molecule may alter the function of the respective other players in this network. Figure 1 summarizes in a simplified way possible interactions between CD26, ADA and adenosine. As CD26 binds ADA which then metabolizes adenosine, changes in CD26 expression may indirectly lead to significant alteration of adenosine mediated function of immune cells.

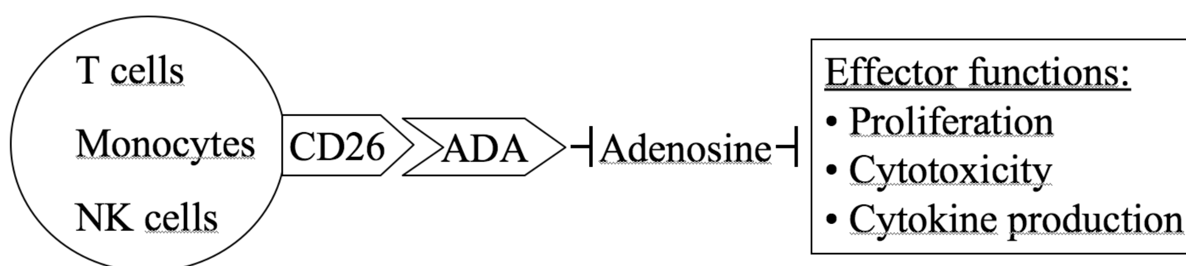

Figure 1. The CD26-ADA-Adenosine system

1.5 Inflammatory liver disease

The liver is the second largest organ in the body and plays a key role in critical metabolic pathways. It also has an important function in maintaining total carbohydrate stores because of its ability to store glycogen and synthesize glucose from precursors. In addition, the liver serves a central role in the synthesis of fatty acid for storage in distal sites and the trafficking of lipids within the body, and it expresses cell surface receptors for circulating lipoproteins and modulates intravascular levels of these macromolecules. Finally, it is the major site for the synthesis of serum proteins involved in coagulation and transport, such as albumin and iron binding, and of protease inhibitors, as well as acute-phase proteins and detoxification.

Additionally, the liver plays a key role in the regulation of the immune system and is known to exert immunoregulatory function (Knolle and Limmer, 2001; Limmer et al., 2000). A great proportion of the immune cells reside in or pass through the liver and about 30% of the total blood volume is filtered by the liver per minute. Therefore this organ is often confronted with toxic substances or pathogens. Accordingly, the liver can be affected by several diseases causing inflammation. These inflammations can be characterized by an inflammatory

infiltrate, which usually involves the portal tracts, and in most severe cases causes lobular inflammation with a widely distributed infiltration of inflammatory cells in the liver.

An inflammatory liver disease is usually characterized biochemically by elevated liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In severe cases liver function can be impaired leading to reduced production of clotting factors and also reduced detoxification capacity which leads to increase of bilirubin as well as in severe cases an increase of ammonia, resulting in encephalopathy (Bailey et al., 2003).

Liver inflammation can be caused by hepatitis virus infections, systemic viral infections such as herpes viruses or HIV, genetic disorders such as hemochromatosis or Wilson's disease, autoimmune liver disease targeting the bile duct such as primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC), or autoimmune disease directly targeting the hepatocyte, the so-called autoimmune hepatitis (AIH) and finally by metabolic disorders such as non-alcoholic steatohepatitis (Argo and Caldwell, 2009).

Hepatitis virus infections
- Hepatitis B
- Hepatitis C
Viral infections
- Herpes viruses
- HIV
Genetic disorders
- Wilson's disease
- Hemochromatosis
Autoimmune liver disease
- PBC
- PSC
- AIH
Metabolic disorders
- Non-alcoholic steatohepatitis

Table 1. Causes of inflammatory liver disease

1.5.1 Hepatitis B Virus (HBV)

HBV infection is considered worldwide the most common cause of viral hepatitis infection. More than 350 million people are chronically infected (Lee, 1997), with an estimated one million deaths each year due to cirrhosis, hepatocellular carcinoma (HCC) and liver failure (Ganem and Prince, 2004; Lok and McMahon, 2007). HBV is a member of the Hepadnaviridae family and consists of partially double-stranded DNA that is surrounded by an outer lipoprotein envelope and an inner core composed of nucleocapsid proteins. In HBV endemic areas, i.e Africa, Asia, Eastern Europe and South America, most people become infected perinatally as infants and most of the adult population (≈ 70 %) have serological evidence of previous contact to HBV (anti-HBc^+). In contrast, in non-endemic parts of the world, most HBV infections occur during adolescence or early adulthood through sexual intercourse, injection-drug use or occupational exposure. The incubation period for acute infection is 45 to 160 days (Deterding et al., 2008), with a mean incubation period of 90 days. The primary reservoir is chronically infected people. HBV infection is preventable by vaccination (Pungpapong et al., 2007). Acute HBV infection can be symptomatic or asymptomatic. The acute phase is either followed by a clearance of the infection or a change into chronicity. The progression from acute to chronic HBV infection is influenced by the patient's age at the time of infection (Dienstag, 2008). Usually, HBV infection does not require treatment because it is spontaneously cleared by most adults (Hollinger and Lau, 2006). Chronically infected patients require antiviral therapy to reduce the risk of cirrhosis, HCC and liver failure. As do patients with high elevated serum ALT and HBV-DNA ≥ 10^4/ml (European Association For The Study Of The Liver, 2009). In immunocompromised patients or patients with fulminant hepatitis early antiviral treatment may help to prevent liver failure and liver transplantation (Tillmann et al., 2006).

1.5.2 Hepatitis C Virus (HCV)

HCV is an enveloped icosahedral RNA virus in the flavivirus family. There are several genotypes (1, 2, 3 and 4, but genotype 1 is the most common in Europe and the United States). These genotypes vary by geographic isolation and they respond variably to antiviral therapy. HCV is transmitted parenterally, with the primary means of infection being through injection-drug use. The incubation period varies between 6-12 weeks with time spans varying from 2-26 weeks (Deterding et al., 2009). HCV accounts for approximately 20% of cases of acute hepatitis, which however is rarely seen clinically, because the vast majority of patients

experience no clinical symptoms during early HCV infection. Occurrence of fulminant hepatitis is extremely rare. Up to 85 % of patients will go on to develop chronic hepatitis. Out of these, 2-40 % will develop cirrhosis within 20 years or less after infection, especially in those with alcohol abuse or coinfection with HIV type 1 or HBV (Lavanchy, 2009). HCV infection is diagnosed by testing for HCV antibodies that are detectable within 6-8 weeks after exposure and remain positive thereafter. HCV-RNA appears in the blood within 1-2 weeks after exposure and is followed by an increase of aminotransferase levels several weeks later. For years, interferon given as monotherapy was the standard treatment in patients with chronic HCV infection (Poynard et al., 1995). The current treatment of choice is pegylated interferon alfa plus ribavirin (Heathcote et al., 2000; Manns et al., 2006; Zeuzem et al., 2000). The primary goal of therapy for HCV infection is to eradicate the infection early in the course of the disease to prevent progression to end-stage liver disease and eventually HCC (Veldt et al., 2007). The duration of therapy is variable and is based on the infecting genotype. Unfortunately, the predominating genotype 1 is the least likely to respond to therapy. Response rates for genotype 1 are in the range of 30-45 % and genotypes 2 or 3 in the range of 70-90 % (Manns et al., 2006).

The mechanisms of immune responses against HCV are still unclear. An early involvement of the innate immune system is assumed to help limiting the spread of the infection. However, the main role for the eradication of the virus seems to be the adaptive immunity, mediated especially by T cells. Previous data suggests that defective functions of HCV-specific $CD8^+$ T cells might contribute to viral persistence in chronically infected patients (Rehermann, 2009).

1.5.3 Autoimmune Hepatitis (AIH)

AIH is a chronic inflammation of the liver of unknown cause. It is characterized by the presence of interface hepatitis on histological examination, hypergammaglobulinemia and autoantibodies in the serum (Bantel et al., 2005; Strassburg et al., 2003). Diagnosis requires the exclusion of other chronic liver diseases that have similar features, including Wilson disease, chronic viral hepatitis, α_1-antitrypsin deficiency, genetic hemochromatosis, drug-induced liver disease, non-alcoholic steatohepatitis and the immune cholangiopathies of PBC, PSC and autoimmune cholangitis. The prevalence of AIH roughly ranges between 50 and 200 cases per million in Western Europe and North America among the Caucasian population (Manns and Vogel, 2006). AIH predominantly affects women at any age (Feld et al., 2005). Prednisone alone or in combination with azathioprine induces a clinical, biochemical and

histological remission in 65% of patients within 3 years (Czaja, 1999). The average treatment interval until remission is 22 months. The 10-year life expectancy for treated patients with and without cirrhosis at the time of the initial medical consultation are 89% and 90%, respectively (Roberts et al., 1996). The overall 10-year survival rate is 93% and is comparable to that of an age- and sex-matched cohort from the general population, if treatment is successful. About 9% of patients become progressively worse despite treatment compliance (Czaja, 1999) and require liver transplantation at the first symptoms of hepatic failure, usually being ascites (Sanchez-Urdazpal et al., 1992).

1.6 Effects of adenosine and interferone alpha on effector functions of lymphoid cells

A previous study performed by F. Jeffe et al. in our laboratory investigated the role of different adenosine receptors in regulating functions of mitogen-activated PBMC and, whether or not, adenosine affects the activation of PBMC by IFN type I (Mirabet et al., 1999). Our interest for IFN resulting from its use in the treatment of viral hepatitis (Manns et al., 2006), showing some efficacy in inhibiting viral replication. However, 50 % of patients with chronic HCV infection and two-thirds of patients with chronic hepatitis B do not respond to IFN-therapy. Thus, there is a pressing need to further improve the efficacy of current therapies available. The inhibitory effect of adenosine was confirmed by inducing apoptosis of activated human PBMC and by inhibiting the proliferation of PBMC. Other findings were also consistent with previous studies showing that adenosine can prevent proliferation of anti-CD3 stimulated T cells and that killer T cells display weaker cytotoxic activity, when generated in the presence of adenosine. As we showed a reduced cytotoxic activity in the presence of adenosine and a dose-dependent reduction of IFN gamma-production of PHA-stimulated PBMC. However and surprisingly, when PBMC were stimulated with IFN alpha, adenosine did not decrease, but synergistically increased the IFN gamma production of NK cells. This synergistic effect of adenosine was mediated via the A3 receptor. As our results show, adenosine contributes differentially to the regulation of immune responses during inflammatory processes by increasing effector functions of NK cells in combination with IFN alpha but also by preventing overwhelming immune responses through inhibition proliferation and induction of apoptosis of activated PBMC.

In summary, previous work of other laboratories as well as our own data clearly suggest a major role of adenosine in regulating immune responses in viral infections. As CD26 is a key player in controlling adenosine levels, we have aimed to study CD26 in more detail in patients with inflammatory liver disease.

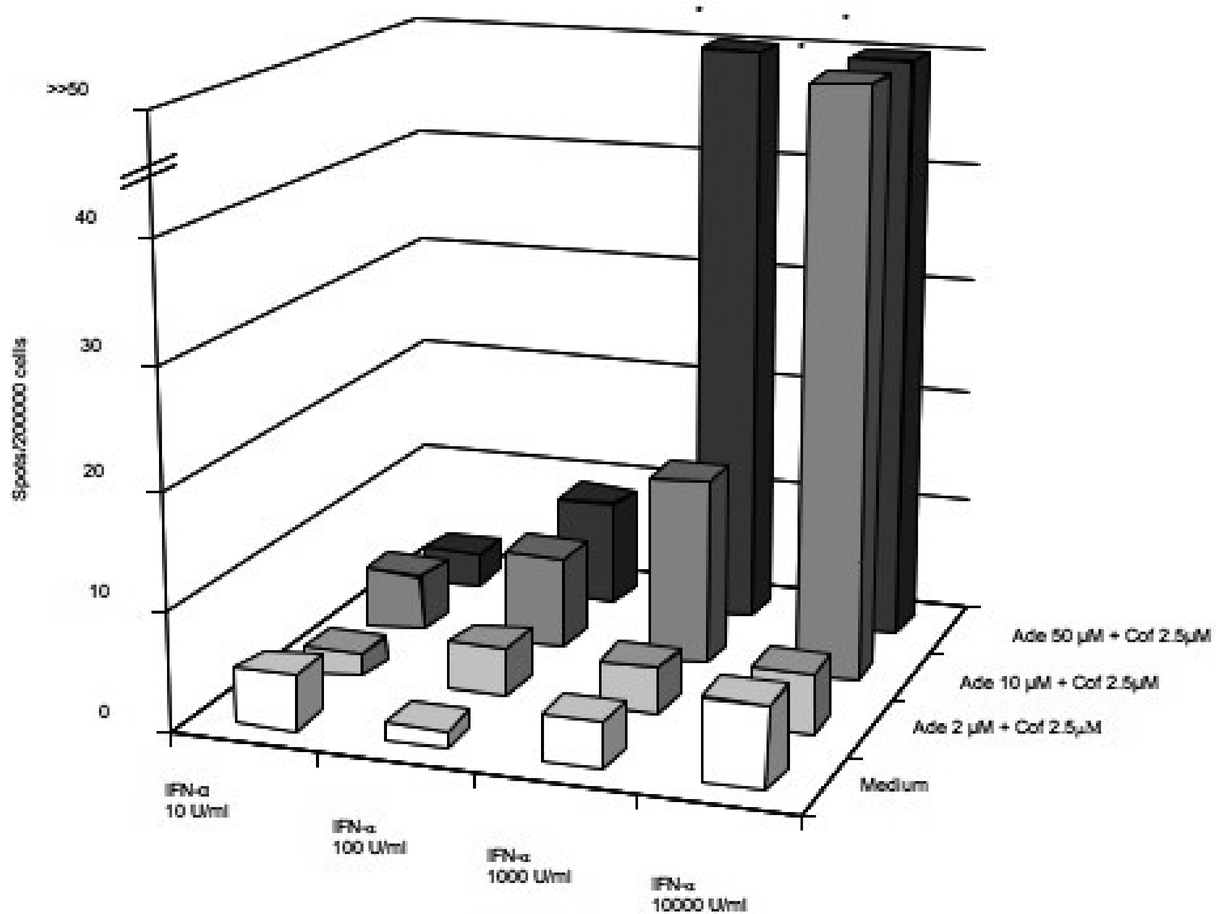

Fig. 2 Adenosine and IFN alpha synergistically increase the IFN gamma production of PBMC via the A3 receptor. This effect was independent from the underlying infection with HCV; ongoing, antiviral treatment, IFN alpha-based therapies; and other chronic inflammatory liver diseases such as PBC. PBMC were incubated for 24h. IFN gamma production was investigated by ELISpot assays. Spot forming units (SFU) of one representative experiment are shown.

2 Aim of this study

The aim of this study was to examine the role of the CD26-ADA-adenosine system in viral and autoimmune chronic liver disease in more detail. Specifically, we addressed the following questions:

1. Which leukocyte subsets express CD26?
2. Is CD26 expression on different lymphocyte subsets altered in patients with HBV, HCV or AIH?
3. Can lymphocyte proliferation be influenced by different levels of CD26 expression in the presence or absence of adenosine?
4. Is adenosine involved in the regulation of antigen specific $CD4^+$ or $CD8^+$ T cell responses?

The overall aim of this study was to investigate to what extent it is possible to modulate the innate or adaptive immune response in patients with viral hepatitis or autoimmune liver disease and explore whether the CD26-ADA-adenosine system could be therapeutically altered for future clinical purposes.

3 Materials and Methods

3.1 Materials

Reagents for PBMC isolation and culture

Biocoll Separating Solution (Biochrom AG, Germany, Cat No. L6155)

RPMI 1640 with L-Glutamax ® (Invitrogen, UK, Cat. No. 61870-010)

Fetal calf serum (FCS) (PAA Laboratories, Austria, Cat. No. A15-101)

Dulbecco's PBS (D-PBS) (s. Buffers and Media)

Antibodies for T cell stimulation

Mouse anti-human CD3, purified (clone HIT3 a, BD Pharmingen, Cat. No. 555336)

Mouse anti-human CD28, purified (clone CD28.2, BD Pharmingen, Cat. No.555725)

Reagents for cell proliferation and cell cycle assay

CFSE (Carboxyflourescein diacetate succinimidyl ester) (Molecular Probes, USA, Cat. No. C34554)

DMSO (Dimethylsulfoxide; sterile, endotoxin-free) (Sigma Aldrich, Germany, Cat. No. D2650)

Host species	Reactivity	Clone	Fluorochrome	Source	Cat No.
Mouse	Anti-human CD3	UCHT1	APC	BD Pharmingen	555332
Mouse	Anti-human CD3	UCHT1	FITC	BD Pharmingen	555335
Mouse	Anti-human CD4	RPA-T4	PE	BD Pharmingen	555347
Mouse	Anti-human CD4	RPA-T4	CY	BD Pharmingen	555348
Mouse	Anti-human CD8	RPA-T8	APC	BD Pharmingen	555369
Mouse	Anti-human CD14	MϕP9	FITC	BD Pharmingen	347493
Mouse	Anti-human CD19	4G7	FITC	BD Pharmingen	345776
Mouse	Anti-human CD26	M-A261	CY	BD Pharmingen	555438
Mouse	Anti-human CD56	B159	PE	BD Pharmingen	55517

Table 2. Antibodies for the analysis of human cell surface antigens

ELISpot Reagents and Antibodies

Mouse anti-human IFNγ monoclonal antibody (Endogen, Woburn, USA)

Mouse anti-human IFNγ monoclonal biotin-labeled antibody (Endogen, Woburn, USA)

Tween 20 (Polyoxyethylene-sorbitan monolaurate)

BCIP/NBT Color Development Substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; Promega Corp., Wisconsin, USA)

Antigens and Peptides

Tetanus toxoid (20 µg/ml; Calbiochem, La Jolla, Ca, USA)

Staphylococcal enterotoxin B (SEB; 1µg/ml, Sigma Aldrich, Germany)

Phytohemagglutinin-M (PHA; 1µg/ml, Calbiochem, La Jolla, Ca, USA)

Influenza-A Matrix protein 58-66, amino acid sequence GILGFVFTL

hCMV-pp65 protein 495-504, amino acid sequence NLVPMVATV

HCV-core protein 132-140, amino acid sequence DLMGYIPLV

HCV-NS3 protein 1073-1081, amino acid sequence CINGVCWTV

Stock solutions

1 mM CFSE stock solution

CFSE (MW: 557.46g)	25 mg
DMSO	4.5 ml

Aliquots were stored at -20° C

1mg/ml anti-human CD3 antibody stock solution

Purified anti-human CD3 antibody	0.5 mg
Sterile PBS	0.5 ml

Stored at 4° C

1mg/ml anti-human CD28 antibody stock solution

Anti-human CD28 antibody	0.5 mg
Sterile PBS	0.5 ml

Stored at 4° C

Buffers and Media

5% AB-Medium

RPMI 1640	141 ml
Human Serum type AB (heat inactivated, Blood bank, Hannover Medical School)	7.5 ml
100 U/l Penicillin + 100 mg/ml Streptomycin (Gibco ®, Invitrogen, Germany)	1.5 ml

10% AB-Medium

RPMI 1640	178 ml
Human Serum type AB (heat inactivated, Blood bank, Hannover Medical School)	20 ml
100 U/l Penicillin + 100 mg/ml Streptomycin (Gibco ®, Invitrogen, Germany)	2 ml

Cell culture Medium

RPMI 1640	500 ml
FCS	50 ml
100 U/l Penicillin + 100 mg/ml Streptomycin (Gibco ®, Invitrogen, Germany)	2.5 ml

FACS staining buffer

PBS	500 ml
FCS	10 ml

Freezing medium

RPMI 1640	25 ml
FCS	50 ml
DMSO	8.3 ml

Dulbecco's PBS

KH_2PO_4	final 2 mM
Na_2HPO_4	final 10 mM
KCl	final 2.7 mM
NaCl	final 137 mM

pH adjusted to 7.4, sterilized by autoclaving

PBS/BSA Buffer

PBS	150 ml
BSA	1.5 g

PBS/BSA/Tween

PBS	500 ml
BSA	5 g
Tween 20 (Polyoxyethylene-sorbitan monolaurate)	250 g

ELISpot Detection Buffer

Tris base	12.11 g
NaCl	5.89 g
$MgCl_2$	1.09 g
Destilled water	to 1 liter

pH adjusted to 9.5 with 1M HCl, sterilized by filtration

3.2 Methods

3.2.1 Patient cohort

CD26 expression was analyzed on PBMC from 101 subjects including 25 healthy donors, 45 chronic HCV infected patients, 16 individuals with chronic HBV infection, and 15 AIH patients (male/female 50/53, age 43 ± 14 years, range 20-74 years). Patients with liver disease and healthy controls were recruited in the outpatient clinic of Hannover Medical School and from colleagues and friends, respectively.

All HCV patients studied were viremic with a mean viral load of 2.6 x 10^6 IU/ml. Biochemical disease activity was minimal (ALT < 1.5 fold upper limit of normal) in 14 %, moderate (1.5-5 fold increased) in 30.5 % and severe in (>5 fold increased) 55.5 % of patients. HBV patients were HBsAg-positive and had variable viral loads. 5 patients had a viremia above 1000 IU/ml and 7 patients showed low HBV-DNA levels of <1000 IU/ml. In two cases, the HBV-DNA levels were not documented. Biochemical disease activity was minimal (ALT < 1.5 fold upper limit of normal) to moderate (1.5-5 fold increased) in seven and severe (>5 fold increased) in five cases.

All Patients with autoimmune hepatitis had compensated liver disease. These received standard immunosuppressive treatment consisting of corticosteroids and/or azathioprine.

	HCV	**HBV**	**AIH**
Sex (male/female)	25/20	10/4	4/11
Age (mean/SD)	47.7/11.2	42/15.3	43.5/13
ALT (mean/SD)	79.3/69.4	338.3/89.4	57.8/42.0
AST (mean/SD)	50.3/37.7	88.1/543.5	39.5/20.5
Bilirubin (mean/SD)	13/8.2	70.1/91.5	13.8/9.9

Table 3. Patient characteristics

3.2.2 Isolation of peripheral mononuclear blood cells (PBMC)

PBMC were isolated from fresh whole blood samples using a standard density gradient centrifugation method with Ficoll separating solution (centrifugation 650 G for 15 min). Mononuclear cells at the interface were collected and washed twice with PBS (centrifugation 435 G for 8 min). Lymphocytes were stored in freezing medium using cryopreservation in liquid nitrogen until further use.

3.2.3 Thawing of PBMC samples

Frozen PBMC samples were removed from the liquid nitrogen and thawed using sterile PBS. PBS was added stepwise to avoid harsh changes of DMSO or FCS concentrations. Cells were washed twice (centrifugation at 435 G for 5 min) and resuspended in the appropriate buffer or medium according to further experimental procedure.

3.2.4 Surface marker staining and flow cytometric analysis

PMBC from frozen samples (see 3.2.3) were used for fluorescence activated cell sorting (FACS) analyses. 3×10^5 cells per sample were washed with FACS staining buffer and labeled with monoclonal flourochrome-conjugated antibodies (Table 4) for 15 min at 4 °C, in protection from the light. The cells were then again washed and resuspended in FACS staining buffer and the expression of each surface marker was measured using a flow cytometer (FACSCalibur, BD Biosciences, Germany) and CellQuest Pro software (BD Biosciences, Germany). Forward and sideward scatter was used to identify the lymphocyte and monocyte population (see Fig. 3 below) and subsequently an analysis was performed on this gated set of cells. 100.000 cells per sample were acquired and results were analyzed by FlowJo Software Version 6.1.1 (Tree Star Inc., Oregon, USA).

	Fluorescein Isothiocyanate (FITC)	Phycoerythrin (PE)	Cyanine (CY)	Allophycocyanin (APC)
1	CD19 (IgG1κ)	CD4 (IgG1κ)	CD26 (IgG1κ)	CD8 (IgG1κ)
2	CD14 (IgG2bκ)	CD56 (IgG1κ)	CD26 (IgG1κ)	CD3 (IgG1κ)

Table 4. Combinations of antibodies for CD26 screening
Isotypes for the antibodies: IgG1κ; IgG2bκ

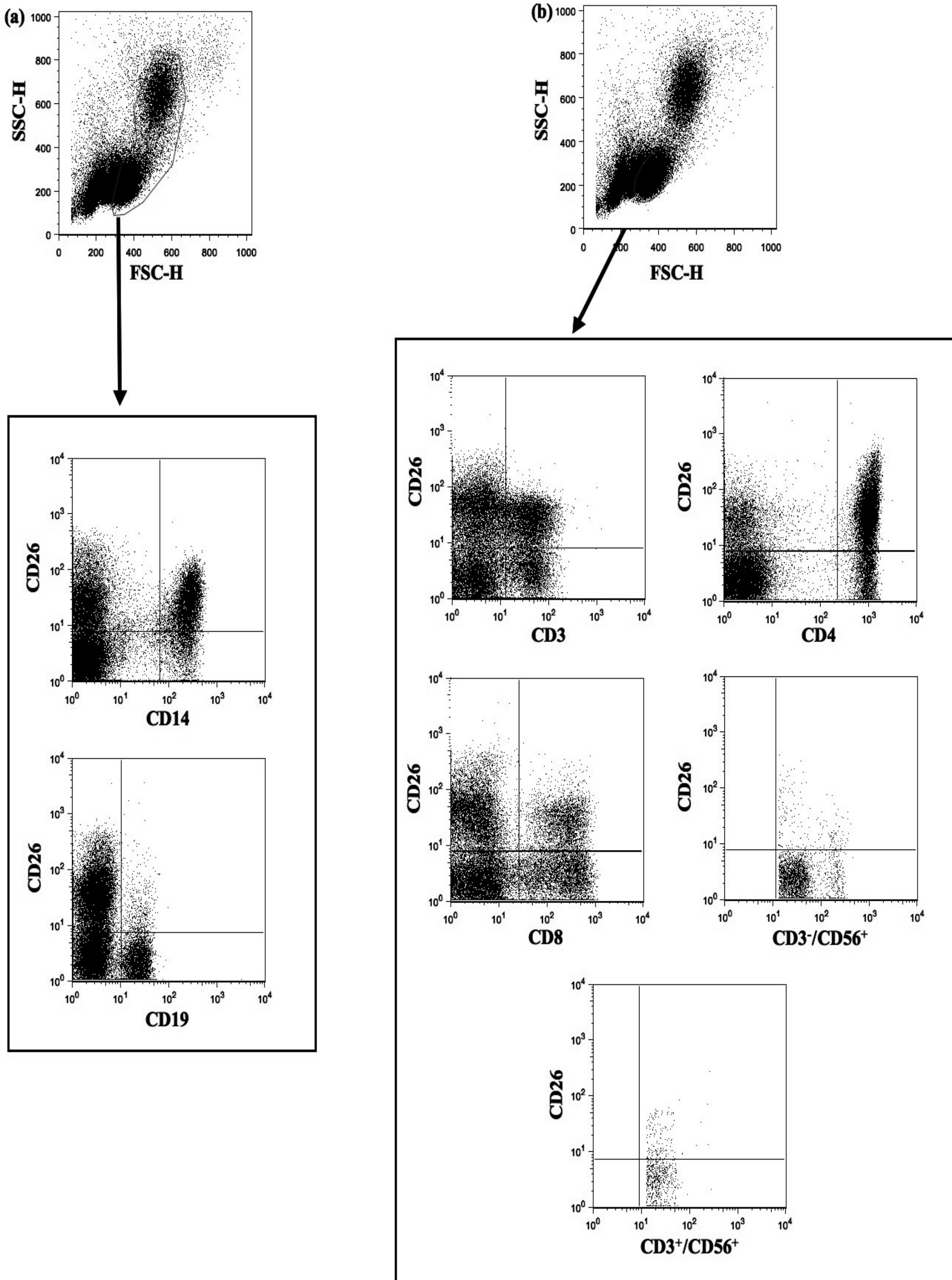

Fig. 3 Gating strategy using the example of cryopreserved PBMC of a healthy individual as shown by FACS dot plots. Forward and sideward scatter was used to identify monocytes (a) and lymphocytes (b), and a subsequent analysis was performed on this gated set of cells.

3.2.5 Antigen specific stimulation of PBMC

After thawing of PBMC (see 3.2.3), the cells were resuspended in 10 % AB medium. 2×10^5 cells per well were incubated on a 96-well round bottom plate (Nunc, Denmark) in a total volume of 200 μl medium with or without different concentrations of adenosine (9-beta-D-Ribofuranosyladenine), CCPA (2-Chloro-N_6-Cyclopentyladenosine, Sigma, St. Louis, USA) a highly selective A_1 adenosine receptor agonist, CGS (CGS-21680 Hydrochloride, Sigma, St. Louis, USA), a A_2 adenosine receptor agonist, IB-MECA (Sigma, St. Louis, USA) a selective A_3 adenosine receptor agonist, PHA and respective peptide antigens. The cells were then incubated overnight at a temperature of 37 °C with 5 % CO_2 and subsequently used for ELISpot assay.

3.2.6 Carboxyflourescein succinimidyl ester-based (CFSE) proliferation assays

This method for analyzing cell division by using serial dilution of the flourescein-based dye CFSE was introduced in 1994 by Lyons and Parish (Lyons and Parish, 1994). It has since then become widely used for investigating cell division-linked differentiation of lymphocytes and for the investigation of kinetics of cell proliferation during immune responses. CFSE consists of a flourescein molecule containing two acetate moieties and a succinimidyl ester functional group. It is membrane permeant and non-fluorescent. The cell division can be tracked by detecting the progressive halving of the fluorescence intensity of the dye in cells after each division (Parish, 1999).

Thawed PBMC were stained prior to *in vitro* culture with 2.5 μM fluorescent dye CFSE for 7 min at 37 °C. Uptake was stopped by adding 10 % FCS and subsequent washing steps (3x) with PBS (Suneetha et al., 2009). CFSE-labelled PBMC were resuspended in AB-medium and set up in three conditions: (1) AB-medium, (2) anti-CD3/anti-CD28 and (3) staphylococcal enterotoxin B (SEB). Now, adenosine was added to each condition at concentrations of 0, 5 and 30 μM. Every condition was set up in triplicates. SEB and medium alone served as positive and negative control respectively. After one week, the cells were washed with FACS staining buffer and labelled with CD4, CD8 and CD56 monoclonal antibodies for FACS analysis.

3.2.7 Enzyme-linked immunosorbent spot (ELISpot) assay

ELISpot was originally developed by Czerkinsky in 1983 (Czerkinsky et al., 1983) to enumerate B cells secreting antigen-specific antibodies. Since then, it has been adapted to identify and enumerate cytokine-producing cells at the single cell level. In other words, it allows the visualization of the secretory product of individual cells.

For this purpose, 96-well plates (Millititer; Millipore, Bedford, USA) were coated with anti-human IFNγ antibody (10 μg/well) at 4 °C overnight in a humidified chamber. Afterwards, the plates were washed four times with sterile PBS and then blocked with PBS/BSA for 1 h at room temperature. 2×10^5 previously thawed PBMC were co-cultivated with individual peptides (10 μg/ml) for 20-30 h. After this time, the plates were washed three times with PBS/Tween and four times with PBS and incubated with 100 μl (0.5 μg/ml) of the secondary biotin-labeled antibody for 2 h. After four washing steps, streptavidin-alkaline phosphatase (1.2 μg/ml) was added. Finally, the plates were washed again four times with PBS and developed with BCIP/NBT alkalinephosphatase substrate. The reaction was stopped by rinsing with distilled water. The results were considered as positive, if at least 10 spots/1×10^6 PBMC were formed and the stimulated control showed three-fold higher numbers of spots as the negative controls. The spots were counted using an automated ELISPOT reader (A-EL-VIS Elispot Reader, Hannover, Germany).

3.2.8 Statistical analysis

All data is expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used to estimate the levels of significance for the differences between more than two groups. Paired T tests were used to detect differences between various groups. For all analyses, a value of $P < 0.05$ was considered to be significant. For the statistical analysis, SPSS version 8.0 (SPSS Corp., Birmingham, AL) was used. All statistical analyses were done by Ms. Jana Prokein from the Department of Biometrics, Hannover Medical School.

4 Results

4.1 CD26 expression on PBMC in healthy donors

CD26 was detected by flowcytometry on the surface of all lymphoid cell subsets which were investigated. Mean CD26 expression showed a high interindividual variability for all cell types. The highest expression levels of CD26 was observed on $CD14^+$ monocytes (69%, range 24-96%). $CD4^+$ T cells also showed a high expression with a mean above 50% of positive cells (61%, range 21-90%) in the large majority of the patients. In contrast, $CD8^+$ T cells showed a weaker expression of CD26, as on average less then half of these cells expressed this surface marker (44%, range 10-78%). $CD19^+$ B cells and $CD3^-/CD56^+$ NK cells showed the weakest expression of CD26, usually not exceeding 10% and 20% ($CD19^+$ B cells 6%, range 1-38%; $CD3^-/CD56^+$ NK cells 14%, range 1-54%). $CD3^+/CD56^+$ NK-like T cells showed intermediate CD26 expression levels (26%, range 2-92%), as approximately one-fourth of this cell subset was positive. As shown in Figure 6, not only the percentage of $CD26^+$ cells differed among the various PBMC subsets, but also the mean fluorescent intensities of CD26 (Figures 4 and 5). For example, $CD14^+$ cells showed weaker expression intensities for CD26 than $CD4^+$ or $CD3^+$ cells. CD26 expression was neither associated with sex nor age for all subsets of the healthy volunteers.

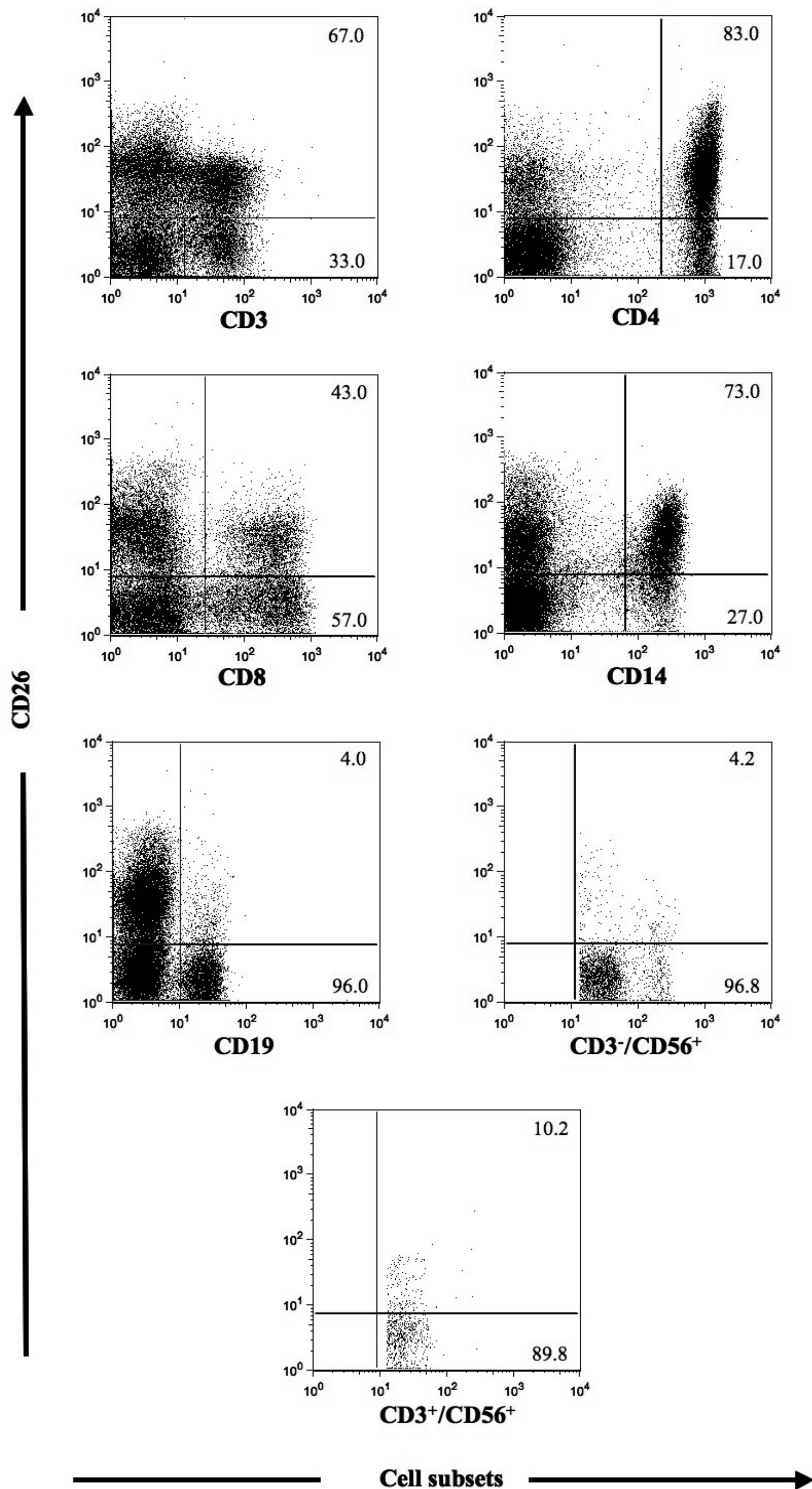

Fig. 4 Expression of CD26 on different leukocyte populations in a healthy control. Analysis of cryopreserved PBMC of one healthy subject for CD26 expression on various lymphocyte cell subsets as shown by FACS dot plots. All cells were gated on total lymphocytes according to size and granularity. Numbers indicated represent percentages of CD26 expression in reference to the respective subpopulation.

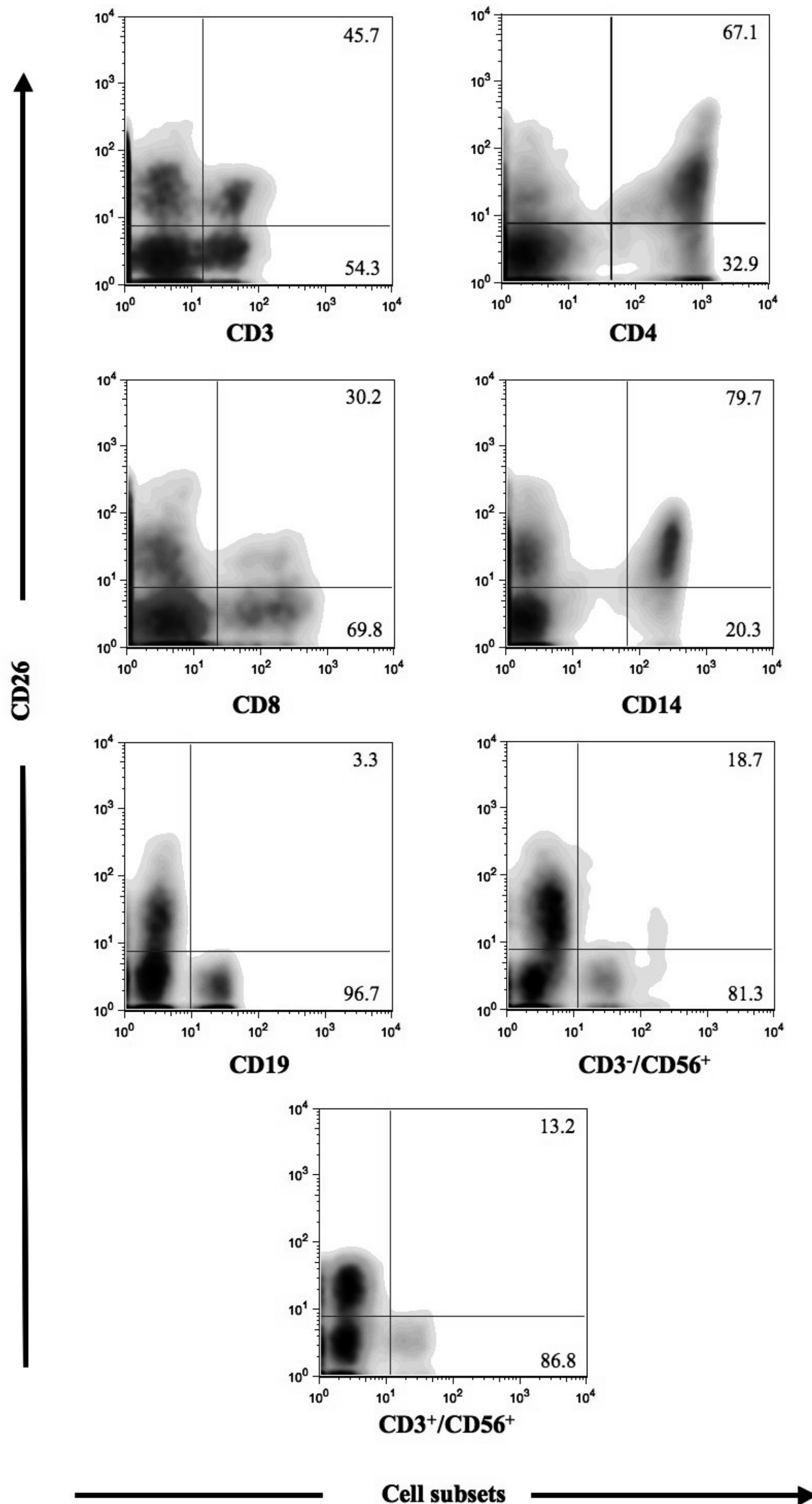

Fig. 5 Expression of CD26 on different leukocyte populations in a HCV patient. Analysis of cryopreserved PBMC of one healthy subject for CD26 expression on various lymphocyte cell subsets as shown by FACS density plots. All cells were gated on total lymphocytes according to size and granularity. Numbers indicated represent percentages of CD26 expression in reference to the respective subpopulation.

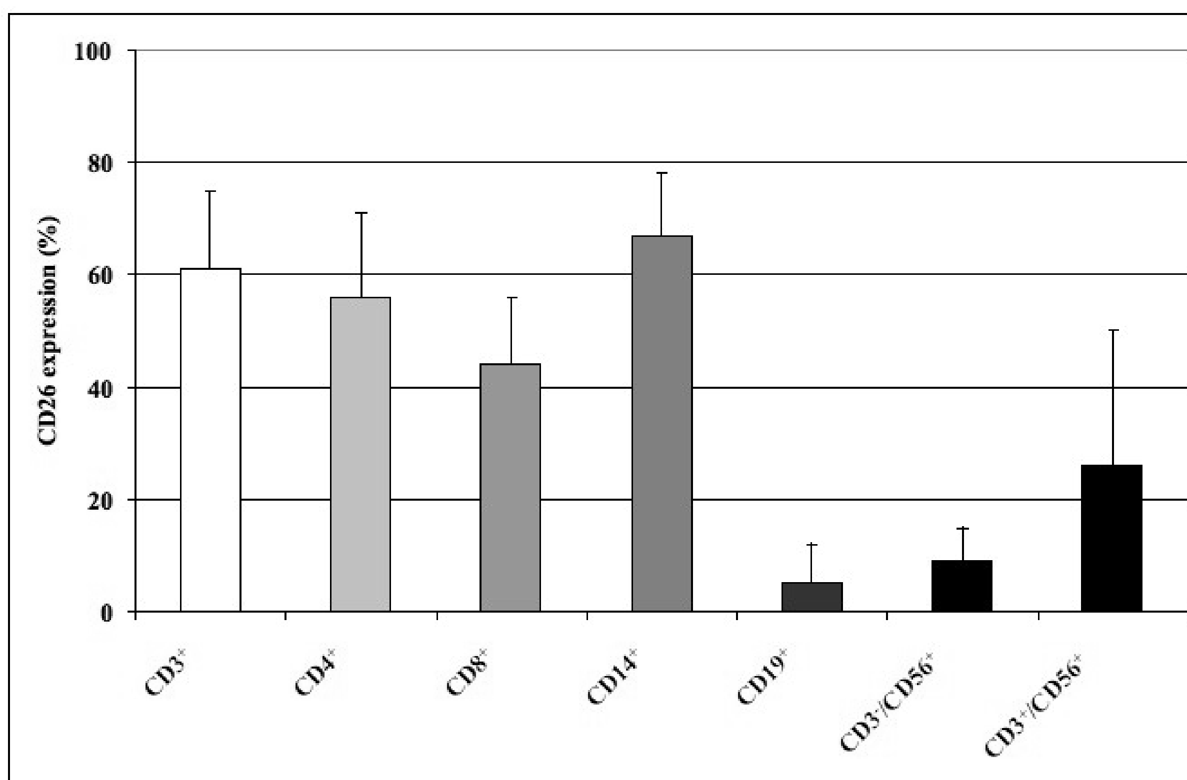

Fig. 6 Percentage of CD26 expression on leukocyte subsets of healthy individuals (n = 25). Fresh and frozen PBMC from healthy subjects were stained for expression of CD26 on various leukocyte subsets. There was no difference in CD26 expression between frozen and fresh PBMC (Figure not shown).

4.2 CD26 expression on PBMC of patients with viral hepatitis and autoimmune hepatitis

Overall, the variable pattern of CD26 expression in patients with liver disease was similar to the pattern found in healthy controls (Figure 6). In every group, the highest levels of CD26 expression were found on $CD14^+$ and $CD4^+$ cells and was comparatively low on $CD3^-/CD56^+$ and $CD19^+$ cells. No significant differences in CD26 expression were seen for $CD14^+$, $CD3^+$ and $CD3^+/CD56^+$ cells in all four groups studied. As for the occurrence of B cells, a significant increase was noticed in patients with AIH compared to patients with HBV infection. Similarly, CD26 expression was elevated on $CD3^-/CD56^+$ cells from AIH patients as compared to both patient groups with HBV or HCV infection. With regard to $CD4^+$ T cells, we only observed a minor increase of CD26 expression in HCV patients as compared to healthy controls and individuals with AIH. Finally, $CD8^+$ T cells showed a significantly lower

expression of CD26 in HBV infected individuals as compared to HCV infected individuals and patients with AIH.

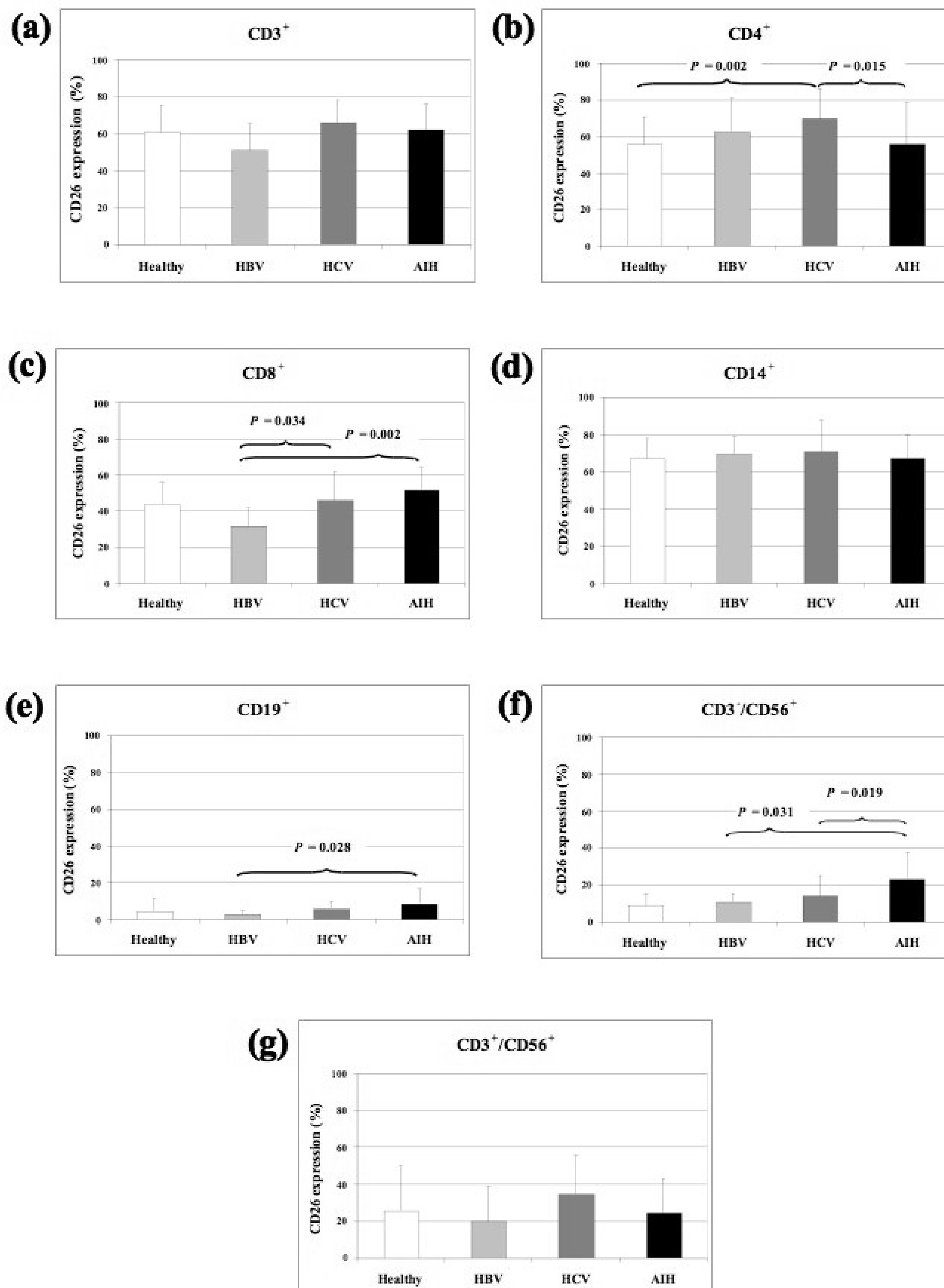

Fig. 7 Comparison of CD26 expression on different leukocyte subsets in healthy subjects and HBV, HCV, and AIH patients. Cryopreserved PBMC from healthy individuals (n = 25) and from patients with HCV (n = 45), HBV infection (n = 16) and autoimmune-induced hepatitis (n = 15) were analyzed for expression of CD26 on various leukocyte subsets. **(a)** Expression of CD26 on $CD3^+$ cells showed no significant differences between all 4 groups. **(b)** $CD4^+$ cells had rather high levels of CD26 expression. In comparison to healthy controls and patients with autoimmune-induced hepatitis, expression of CD26 was increased in HCV-infected patients. **(c)** In HBV infected patients CD26 expression of $CD8^+$ T cells is significantly lower when compared to patients with HCV or autoimmune-induced hepatitis. **(d)** From all cell populations investigated, $CD14^+$ cells showed the

highest expression of CD26 with no significant differences between all four groups. **(e)** $CD19^+$ cells showed a comparatively low expression of CD26. However a significant increase was found in patients with autoimmune-induced hepatitis compared to HBV infection. **(f)** Similar picture as in (e) with the addition of HCV. **(g)** No significant differences in CD26 expression in all four groups.

4.3 Biochemical activity of liver disease and CD26 expression

In a second step, we aimed to determine possible associations between the activity of liver disease and CD26 expression on the respective cell subsets. For this an extensive statistical analysis was performed. The outcome of the analysis is summarized in Table 5, where all *P*- and *R*-values for correlations between CD26 expression on $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD3^-/CD56^+$ and $CD3^+/CD56^+$ cells are shown for correlations with ALT, AST, bilirubine levels, thrombocyte count and sex. As shown in this table, only few biochemical markers of liver disease showed significant associations with CD26 expression. ALT and bilirubine levels were negatively correlated with CD26 expression on $CD4^+$ T cells. Furthermore, female sex was associated with CD26 expression on several PBMC subsets with the exception of $CD4^+$ T cells, $CD19^+$ B cells, $CD14^+$ cells and $CD3^+/CD56^+$ cells.

CD26 % expression of	ALT (*P*-value/*R*-value)	AST (*P*-value/*R*-value)	Bilirubine (*P*-value/*R*-value)	Platelets (*P*-value/*R*-value)	Sex (*P*-value)
$CD3^+$	0.49/-0.81	0.66/0.05	0.46/-0.09	0.84/-0.03	0.04*
$CD4^+$	0.03*/-0.25	0.12/-0.18	0.03*/-0.24	0.46/-0.1	0.55
$CD8^+$	0.39/-0.99	0.97/0.004	0.23/-0.14	0.63/0.07	0.006*
$CD14^+$	0.54/-0.07	0.78/-0.03	0.52/-0.08	0.52/0.09	0.16
$CD19^+$	0.99/-0.001	0.29/0.12	0.44/-0.09	1/0.00	0.57
$CD3^-/CD56^+$	0.65/0.05	0.25/0.13	0.66/0.05	0.08/-0.24	0.00*
$CD3^+/CD56^+$	0.3/-0.13	0.58/-0.07	0.28/-0.14	0.64/0.64	0.23

Table 5. Biochemical parameters and CD26 expression on different PBMC subsets
(* $P < 0.05$). Age was also correlated (not shown), but it is not associated with CD26 expression.

With regard to HCV patients there was no correlation between CD26 expression and biochemical markers of liver disease (see Figure 8 below).

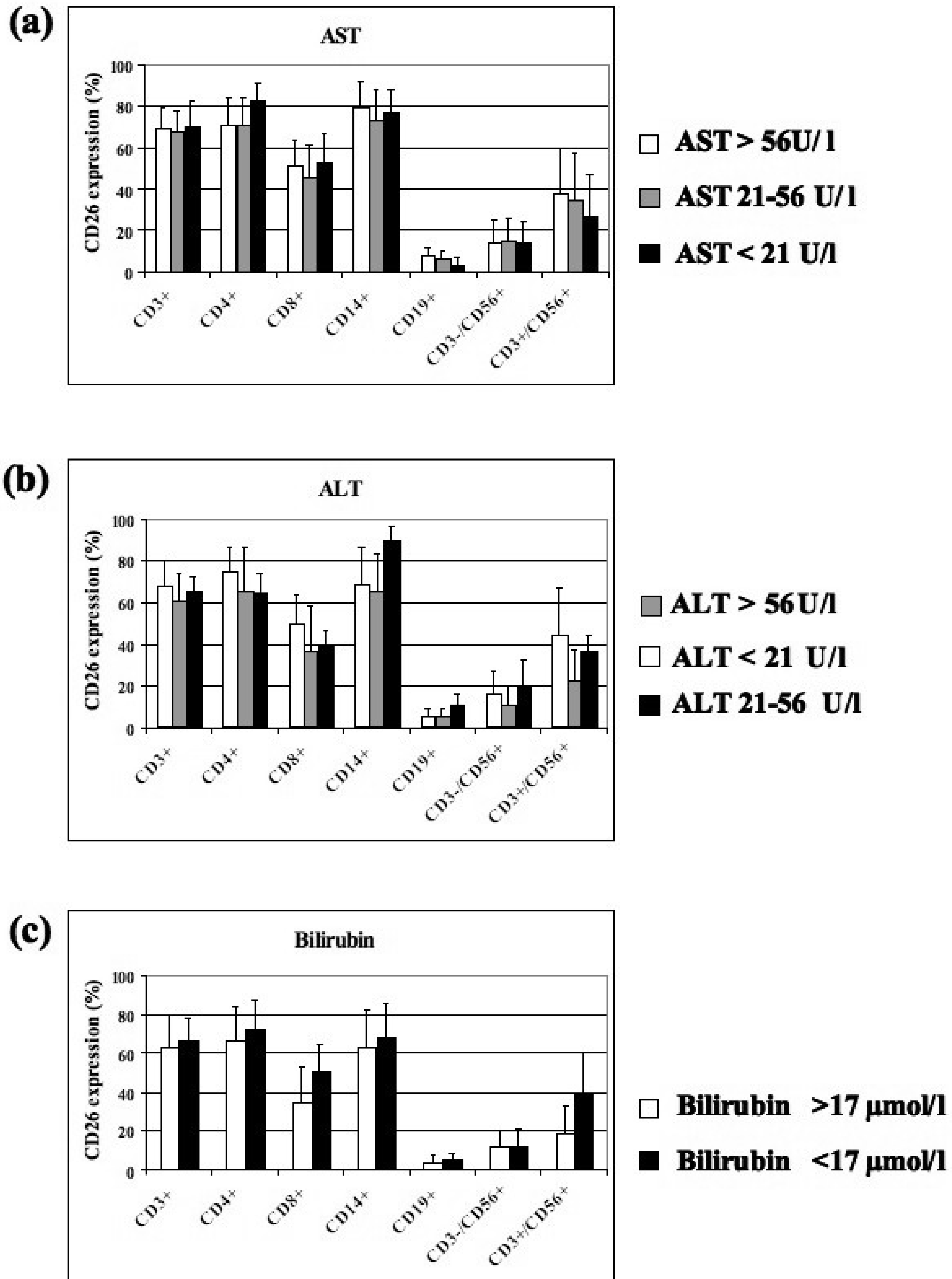

Fig. 8 Correlation of CD26 expression on different leukocyte subsets with clinical parameters of liver disease. 36 HCV patients were grouped according to clinical parameters of liver disease like **(A)** AST, **(B)** ALT, and **(C)** bilirubine into different categories. All factors showed almost no correlation with CD26 expression.

4.4 Viremia and CD26 expression in patients with HCV infection

The level of CD26 expression on the respective PBMC subsets of all HCV infected individuals in correlation to the respective HCV-RNA levels are shown. Again, a wide interindividual variability of CD26 expression became evident. Overall, there was no significant correlation found between CD26 expression and HCV-RNA levels for any of the investigated PBMC subsets. Interestingly, there seems to be a trend for positive correlation between CD26 expression and HCV-RNA levels for $CD19^+$ B cells.

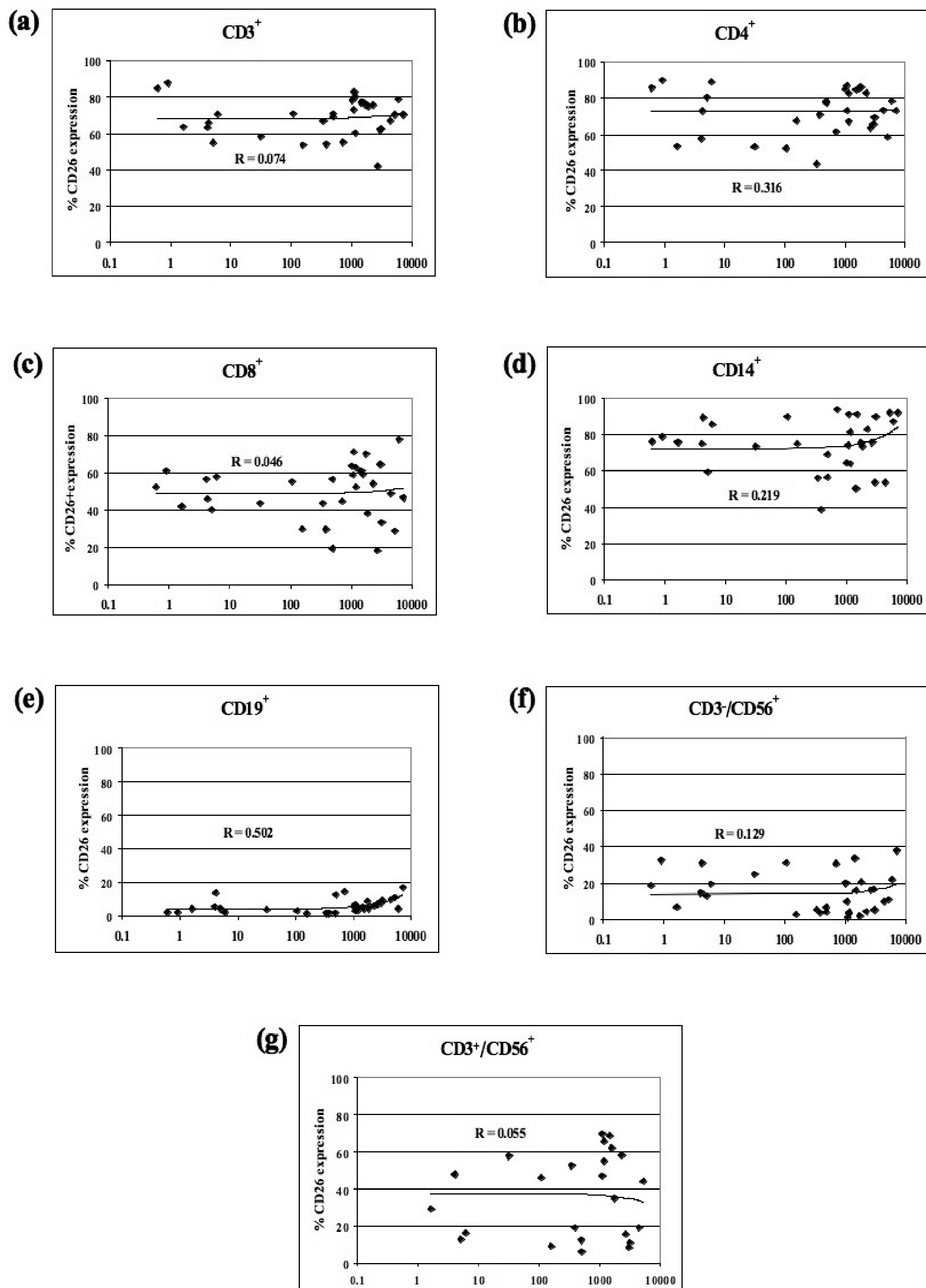

Fig. 9 Correlation of CD26 expression on lymphoid cell subsets with HCV-RNA. No association of serum viral load was seen in case of chronic HCV patients. Note the logarithmic scaling of the x-axis.

4.5 CD26 expression and HBV viremia

Next, we investigated possible associations between CD26 expression and levels of HBV viremia. For this purpose, patients were grouped according to HBV viremia in line with the updated German guidelines on the treatment and management of HBV infection (Cornberg et al., 2007). The only statistically significant correlation was found between CD26 expression on $CD4^+$ T cells and HBV viremia (P = 0.005) as patients with low viremia had generally higher levels of CD26 expression. Importantly, there was no association between HBV viremia and CD26 expression on $CD19^+$ cells.

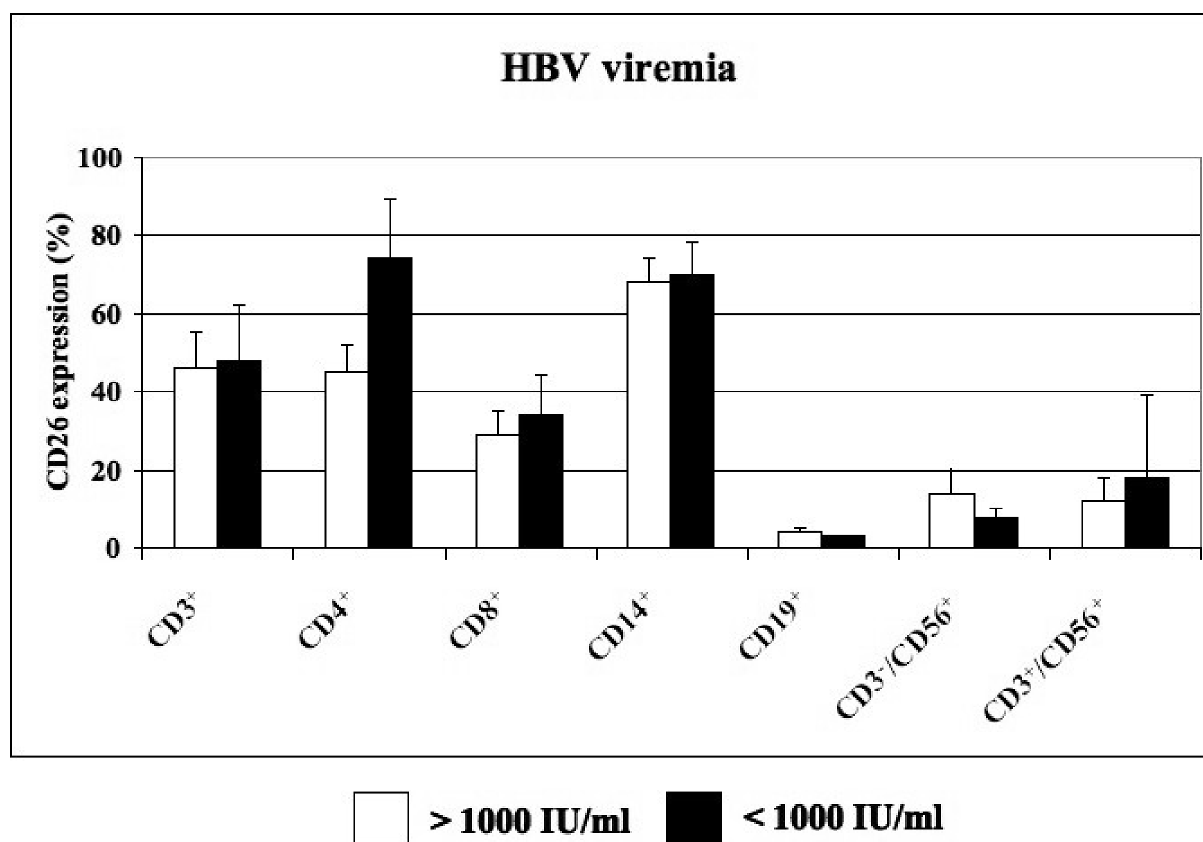

Fig. 10 Correlation of CD26 expression on leukocyte subsets with levels of HBV viremia. Only CD26 expression on $CD4^+$ T cells and HBV viremia was statistically significant (P = 0.005). The cut-off for HBV viremia being low or high was set at 1000 IU/ml. 11 patients were included in this analysis (n = 5, > 1000 IU/ml; n = 6, < 1000 IU/ml).

4.6 CD26 expression during interferon alpha (INF-α) treatment

Since patients with viral hepatitis are frequently treated with interferon alpha, we wanted to investigate to which extent CD26 expression is altered by IFN treatment. For this purpose, five patients undergoing interferon alpha treatment were stained for CD26 expression at baseline and after 3, 6, and 12 months of therapy. All patients responded to antiviral therapy as HCV-RNA became negative during treatment and showed sustained HCV-RNA-negativity 24 weeks after the end of therapy. As shown in Figure 11, there was a wide interindividual variability and CD26 expression fluctuated in single patients. Overall, CD26 expression on $CD4^+$ T cells and $CD14^+$ cells was rather stable, not showing dramatic changes, while in the case of $CD8^+$ T cells it seemed to be more variable over time. However, no clear change of expression pattern could be observed in any of the individuals studied. This also held true for CD26 expression on $CD19^+$ B cells. In case of $CD3^-/CD56^+$ cells, CD26 expression increased significantly in two individuals (pt. 2 and pt. 5), persisting throughout antiviral treatment. However, it could not be observed in the other patients studied. The same two patients also appeared to have a similar CD26 expression pattern in case of the $CD3^+/CD56^+$ subset, although to a lesser degree. Overall, there was no obvious correlation between CD26 expression patterns in the 5 patients studied.

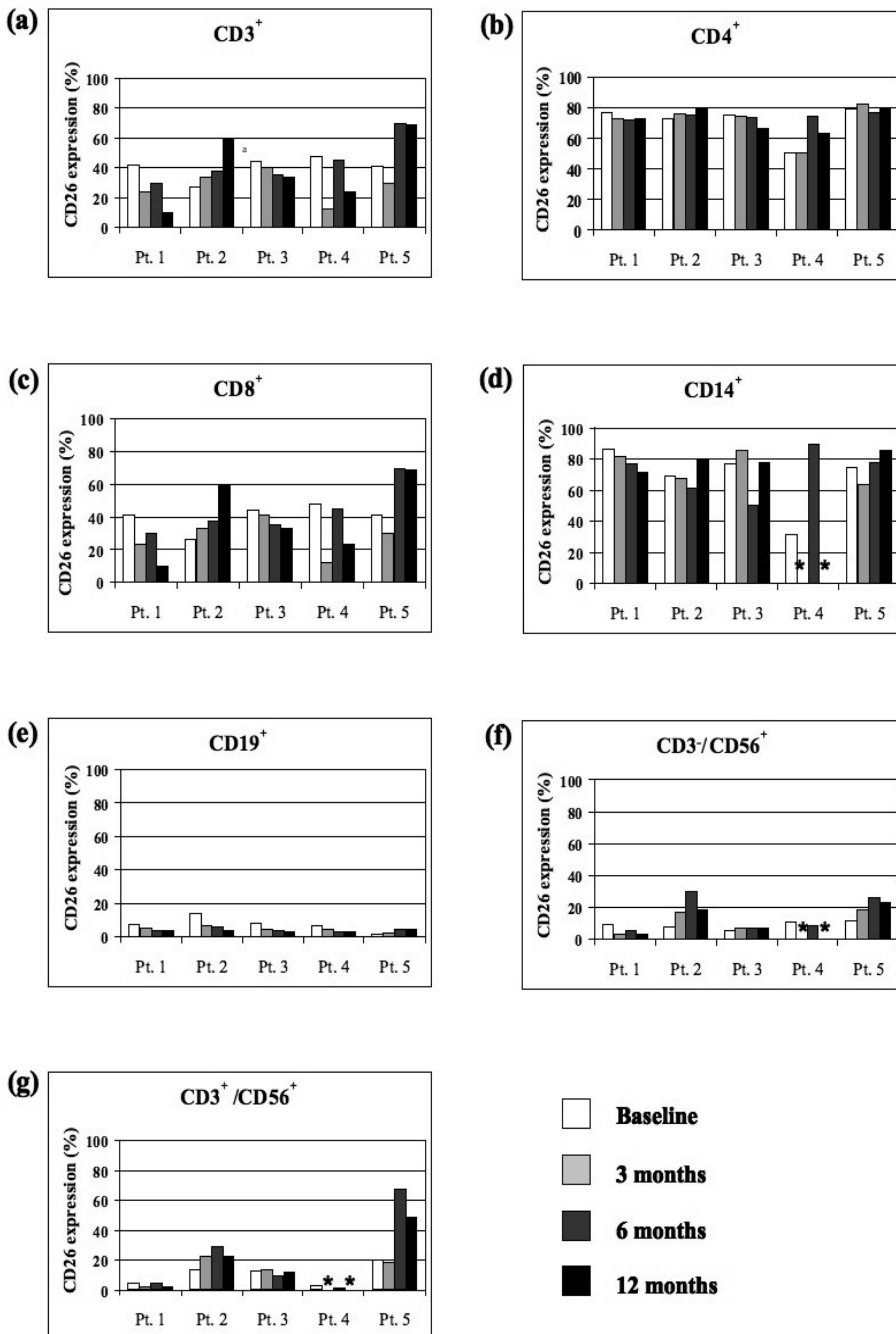

Fig. 11 Correlation of CD26 expression on various PBMC subsets under INF-α treatment. Thawed PBMC of chronic HCV patients were analyzed for CD26 expression on various leukocyte subsets. Overall, no obvious change of expression pattern was observed in any individual. However, in case of NK cells, CD26 expression increased significantly in two patients (2 & 5). * represents missing values.

	Pt. 1	Pt. 2	Pt. 3	Pt. 4	Pt. 5
0 months	> 850.000	3.900.000	3.520.000	2.920.000	164.000
3 months	negative	negative	negative	< 600	negative
6 months	negative	negative	negative	negative	negative
12 months	negative	negative	negative	negative	negative

Table 6. HCV-RNA levels for Patients 1-5 at baseline and after 3, 6, and 12 months of IFN-α therapy.

4.7 Lymphocyte proliferation and CD26 expression with and without adenosine

As shown in Figures 4 and 5, both $CD4^+$ and $CD8^+$ T cells express CD26 on their cell surface. Therefore, we aimed to investigate the functional consequences of CD26 expression in the context of co-culturing with and without the addition of adenosine. As outlined in the introduction, CD26 binds ADA, which then metabolizes adenosine and thereby inhibits its potential effect. We therefore assumed that a weaker CD26 expression would subsequently be associated with lower ADA levels and the inhibitory effect of adenosine would be stronger than in subjects with high CD26 levels where the bound ADA would metabolize adenosine. Proliferation of T cells was studied in six individuals: three healthy individuals and three patients with chronic hepatitis C infection, respectively. PBMC were stimulated with anti-CD3 and anti-CD28 antibodies for 7 days either in medium or together with different concentrations of adenosine. Absolute calculated cell counts after a fixed collection time by a flowcytometer are shown in Figure 12.

In accordance with our hypothesis, the addition of adenosine in all three subjects with low CD26 expression resulted in a 20-50% reduction of surviving $CD4^+$ T cells. In contrast, two out of three subjects with high CD26 expression were resistant to adenosine. In line with the CD26 expression levels, the inhibitory effect of adenosine was mainly evident on $CD4^+$ cells while $CD8^+$ T cells and NK cells were not altered using this readout. Thus, CD26 levels were associated with the effect of adenosine on cell survival and proliferation. Other functional readouts were not investigated.

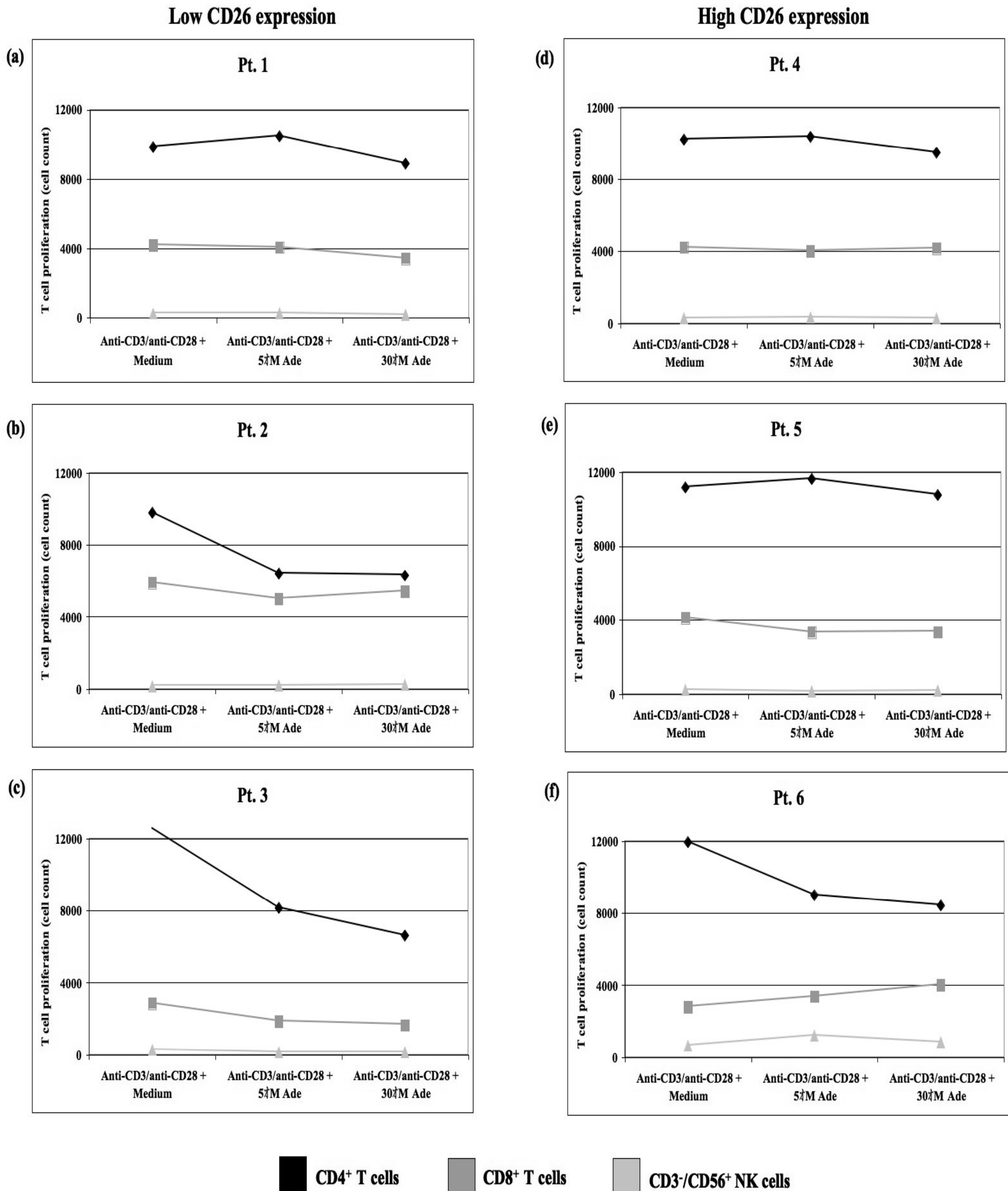

Fig. 12 Cells counted after stimulation with anti-CD3 and anti-CD28 antibodies for 7 days either in medium or together with different concentrations of adenosine. Absolute calculated cell counts after a fixed collection time of 7 days by a flowcytometer are shown. Figures (a), (b), (c) show individuals with a low (< 20 %) and (d), (e), (f) with a high (> 60 %) CD26 expression on $CD4^+$, $CD8^+$ T cells and $CD3^-/CD56^+$ NK cells, respectively.

4.8 Adenosine and antigen-specific T cell function

After having shown that adenosine may effect the proliferation of antiCD3/antiCD28 stimulated T cells, we wanted to investigate whether adenosine also might display inhibitory effects on $CD4^+$ and $CD8^+$ T cells in the context of antigen-specific stimulation of T cells. For this purpose, PBMC from individuals with known responses towards either tetanus toxoid, influenza virus, CMV- or HCV were incubated with the respective total protein stimulating $CD4^+$ T cells or MHC class II restricted peptides known to stimulate $CD8^+$ T cells. Examples of the experiment are shown in Figure 13. For this purpose, we incubated the cells with either antigens or antigens plus IB-MECA, a known adenosine A_3 receptor agonist. The selection of this agonist was based on our previous experience that the inhibitory effects of adenosine on PBMC are mainly regulated by the A_3 receptor (Jeffe et al., 2009). Tetanus- as well as HCV-specific $CD4^+$ T cell responses were inhibited by adding IB-MECA by up to 70-80%. In contrast, influenza- and CMV-specific $CD8^+$ T cell responses were inhibited to a lesser extent of about 40 to 50%. This data confirms that, indeed, adenosine displays inhibitory effects on antigen-specific T cell responses which are mediated via the A_3 adenosine receptor.

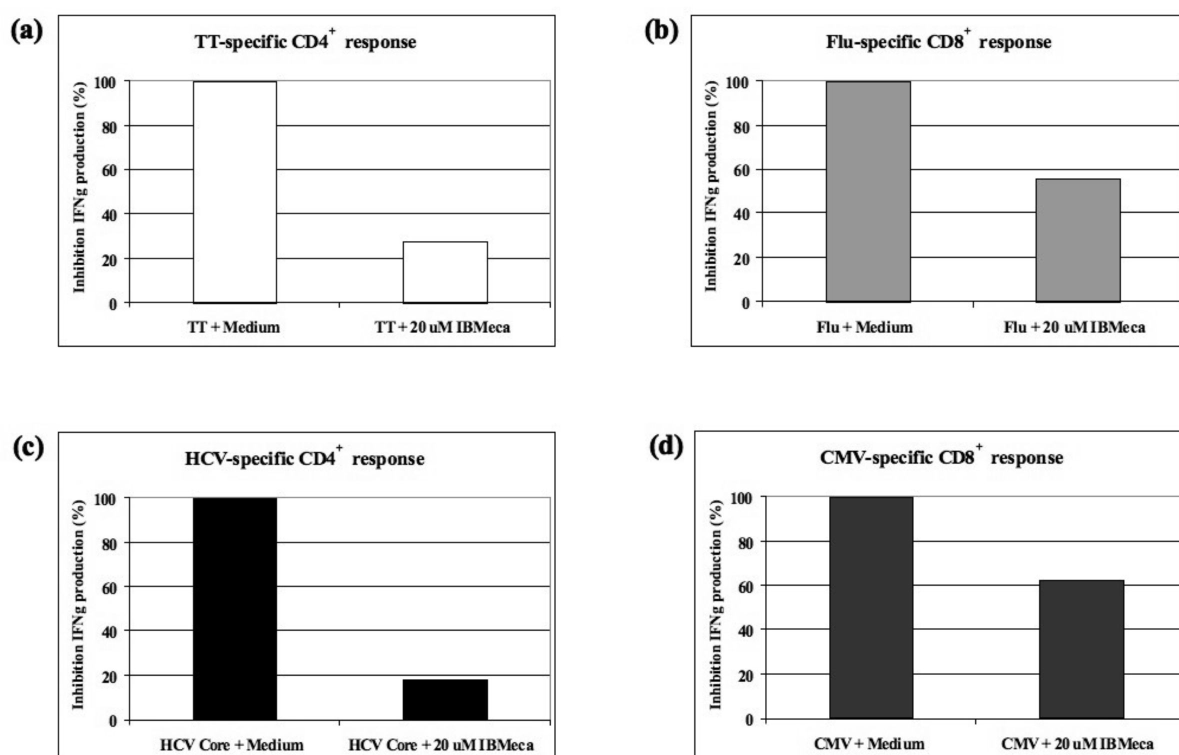

Fig. 13 Inhibition of antigen-specific T cell responses by adenosine via the A3-receptor. Total PBMC from different individuals were incubated with either total protein stimulating $CD4^+$ T cells or MHC class II restricted

peptides stimulating $CD8^+$ T cells. **(a)** Tetanus **(b)** and HCV-specific $CD4^+$ T cell responses were inhibited by 70-80 %. $CD8^+$ specific T cell responses to **(c)** influenza and **(d)** CMV were inhibited to a lesser extent of about 40-50 %. Stimulation without IB-MECA was determined to be 100 %.

5 Discussion

The CD26 expression on various PBMC subsets in different patient cohorts has been subject of various previous studies. This comprehensive analysis is the first study investigating CD26 expression on a wide variety of lymphoid cells. Moreover, CD26 has never been investigated to this extent in patients with inflammatory liver disease.

5.1 CD26 expression on lymphoid cells and possible functional implications

The importance of CD26 for the regulation of activity of various immune cells is well established (Gorrell et al., 2001). In this study, we showed that CD26 is expressed in particular on $CD14^+$ cells and on $CD4^+$ T cells. Anti-CD26 antibodies have been shown to enhance CD3-dependant activation of T cells (Bristol et al., 1992). Moreover, only $CD26^+$ CD4 T cells can provide sufficient helper function to $CD8^+$ T cells (Fleischer, 1987). Using the anti-human CD26 antibody, our findings on expression levels are in line with most previous studies although we found a higher frequency of $CD26^+$ $CD8^+$ cells than some previous studies (Scholz et al., 1985). The rather high expression of CD26 on $CD14^+$ monocytes has rarely been recognized. However, $CD14^+$ monocytes are also highly sensitive to soluble CD26 (Ohnuma et al., 2001) as they take up large amounts of soluble CD26 leading to upregulation of the costimulatory molecule CD86. Subsequently, T cell proliferation can be markedly increased.

CD26 can be considered as an activation marker on T cells. Several previous studies showed that CD26 is co-expressed on cells expressing other markers of activation such as CD25 or CD69 (Gorrell et al., 2001). These results are in line with our findings on higher CD26 expression in patients with high disease activity (discussed in detail below). It is important to note that TCR-expression is obligatory for CD26 mediated signals (Dang et al., 1990) and thus CD26 differs from CD28 which can trigger a separate signal transduction pathway independent from the T cell receptor.

Only a few CD56^{+} NK cells expressed CD26. CD26 was suggested of being involved in cytokine production in natural killer cells (Buhling et al., 1997), however, future studies clearly have to investigate the role of CD26 for NK cell function in more detail.

The lymphoid population with the lowest CD26 expression levels were B cells. CD26 has mainly been described only on B cell lymphoma cells and has therefore been suggested as a potential marker for B-cell chronic lymphatic leukemia (Bauvois et al., 1999). However, some healthy subjects also showed CD26 expression of up to 38% (mean 6 %, range 1-38%) on B cells in our study questioning the role of CD26 as a “tumor” marker for B cell lymphoma.

We could not find any correlation between CD26 expression and age both in healthy subjects as well as in individuals with inflammmatory liver disease. Elderly persons showed significant phenotypical and functional changes of circulating monocytes and leukocytes (De Martinis et al., 2004). However, CD26 expression seems to be rather stable which is in line with at least one other study that has investigated CD26 in younger and older individuals (Stohlawetz et al., 1998).

However, CD26 seems to be of importance to maintaining lymphocyte composition, memory T cell generation and thymic emigration during immunosenescence as old CD26 deficient F344 rats show significant alterations in leukocyte populations (Klemann et al., 2009).

What are the potential ligands for CD26 leading to subsequent activation of downstream signals? ADA-CD26 complexes may play a role in the co-stimulatory function (Franco et al., 1998). Moreover, fibronectin has been suggested as a CD26 binding protein being associated with co-stimulation (Cheng et al., 2003). Recently, caveolin-1 has been described as a putative ligand for CD26 with an important function in T cell costimulation (Ohnuma et al., 2008). All these molecules need more detailed studies in the context of various inflammatory conditions including infections and autoimmune diseases.

5.2 CD26 in patients with inflammatory liver disease

Few studies have investigated so far the CD26 expression in patients with viral hepatitis. Some suggested a role of soluble CD26 in patients with viral hepatitis, as CD26 has two physiological forms in humans (Oravecz et al., 1997): the soluble form that can be found in serum, cerebrospinal- and seminal fluid (De Meester et al., 1999) and a membrane-bound molecule (Hafler et al., 1985). Firneisz et al., published in 2001, suggests that soluble CD26 may be an indicator of HCV induced liver injury and that levels of serum CD26 activity may differ between patients who respond to a subsequent interferon alpha treatment versus those who do not respond to this antiviral treatment of chronic hepatitis C infection. The authors presented different considerations for explanation: (1) the high amount of CD26, partially derived from lymphocytes, present in the liver during chronic inflammation, (2) a strong correlation between serum CD26 activity and liver necrotizing enzymes suggest that the HCV infection generates a higher expression of CD26 on infected hepatocytes and thus, the liver cell death leads to the break off of CD26 from the cell membranes, (3) liver epithelial cell injury due to immune-mediated reactions in chronic hepatitis C or PBC that contributes to the elevation of serum CD26 activity.

Dourado et al (2004) aimed to clarify whether CD26 expression on T cells and CD26 serum activity changes after hepatitis B vaccination. They could not find a significant difference between CD26 expression on T cells in responders (55.9 ± 7.7 %) versus in non-responders (51.9 ± 7.0 %) to hepatitis B vaccine, but a significant difference ($p < 0.05$) in serum CD26 activity between both groups (59.9 ± 8.4 U/L versus 50.3 ± 10.6 U/L).

Other authors (Lee et al., 2002) investigated the potential role of soluble CD26 in relationship with the response to interferon treatment. They studied 31 patients with hepatitis C, who either showed a response or non-response to antiviral treatment. These investigators found a significant difference between CD26 levels in patients with chronic hepatitis C and healthy controls ($P < 0.05$). Although they could not find a highly significant difference between responder and non-responders.

Finally, the role of soluble CD26 was investigated in a group of HIV-HCV-coinfected patients. For these individuals, the authors suggested that an increased hepatotoxicity might be associated with higher serum CD26 activity, which was also associated with increased $CD8^{+}$ T cell counts (Stone et al., 2002).

In our study we did not systematically analyze the potential role of CD26 in interferon responders versus interferon non-responders. However, in our study, the disease activity and membrane-bound CD26 levels did not show a significant correlation with disease activity as reported by the above mentioned study. It is important to emphasize that we only studied membrane-bound CD26 expression, since our interest was based on the direct consequences of ADA binding to CD26 on cell surfaces and the subsequent inhibition of adenosine activity.

As CD26 can display multiple functions on immune cells, our comprehensive analysis of CD26 expression on various PBMC subsets was overall a negative study for several analyses performed in patients with chronic inflammatory liver disease. There were no obvious correlations with any biochemical or hematological disease parameters and CD26 expression (Table 5). Nevertheless, we found that patients with AIH showed significantly elevated CD26 expression on NK cells as compared to age-matched healthy controls and patients with viral hepatitis (Figure 7).

Thus, our study may give first evidence for a potential role of CD26 on NK cells in the regulation of disease activity in autoimmune liver diseases. Overall, our findings are in accordance with several previous reports demonstrating that CD26 expression is associated with autoimmune diseases (De Meester et al., 1999). Patients with autoimmune diseases such as multiple sclerosis, Grave’s disease and rheumatoid arthritis show increased levels of CD26 in their inflamed tissues (Morimoto and Schlossman, 1998). Based on our findings, we have solid ground to assume, that an increased CD26 expression leads to an increased ADA binding on NK cells which subsequently metabolizes adenosine and thereby decreases the activation threshold of NK cells and thus, potentially contributes to autoimmunity.

An additional finding of this study was that CD26 expression on $CD4^+$ T cells was negatively associated with ALT and serum Bilirubin levels in patients with chronic liver disease. This additional observation supports a further hypothesis that increased levels of ADA on $CD4^+$ T cells lead to a decline of adenosine thereby inhibiting the negative impact of adenosine on $CD4^+$ T cell function.

We could not find an obvious association between CD26 expression on NK cells and $CD8^+$ T cells in patients with viral hepatitis (Figure 7). Previous data suggest that defective functions of HCV-specific $CD8^+$ T cells may contribute to viral persistence in chronically infected patients (Wedemeyer et al., 2002). It was therefore concluded that one potential mechanism of $CD8^+$ T cells immunregulation could be promoted by one of the various CD26 functions.

In Figure 13, we clearly demonstrate that adenosine is able to inhibit both tetanus-specific and HCV-specific $CD4^+$ T cell responses. As a result, we suggest that increased levels of CD26 expression might lead to lesser inhibitory effects of adenosine, thereby increasing the potential antiviral effector functions of $CD4^+$ T cells and, subsequently, leading to less severe disease activity. This could explain the lower ALT and serum Bilirubin levels (Figure 8).

However, the suppressive effect of adenosine on $CD8^+$ T cell responses was only minor in our experiments as demonstrated for influenza- and CMV-specific T cell responses (Figure 13).
The investigation of the functional consequences of CD26 expression for T cell and NK cell function in viral hepatitis has several limitations which need to be considered for future studies. For example, we observed correlations to some extent of CD26 expression on NK cells with AIH as an underlying disease. Unfortunately, the clinical observation of a correlation between AIH and CD26 on NK cells was observed after the functional experiments had been performed. Therefore, we did not investigate the functional consequences of CD26 expression specifically on NK cells, but only on $CD4^+$ and $CD8^+$ T cells. Future studies specifically need to address the role of CD26 on NK cell function.

Our data also demonstrated that adenosine inhibits not only anti-CD3/anti-CD28 stimulated $CD4^+$ and $CD8^+$ T cells, but also $CD4^+$ and $CD8^+$ T cells stimulated with specific antigens derived from various pathogens, including tetanus toxoid, CMV, HCV and the influenza virus. Since only the A_3 receptor-specific agonist IB-MECA showed this effect in contrast to the A_1 receptor agonist CCPA or the A_2 receptor agonist CGS, which both did not cause any

changes of T cell activities, we could clearly show that these effects were mediated via the A_3 adenosine receptor. However, no specific agonists against a single adenosine receptor could be found *in vivo*. Adenosine rather plays a role in the regulation of inflammatory processes. Thus, potential other molecules involved in the metabolization of adenosine might be of importance. For this reason, and as outlined in detail in the introduction, we investigated CD26, as it is the binding protein for ADA that catalyzes the deamination of adenosine to inosine.

Importantly, we could collect functional evidence that the level of CD26 expression is correlated with the possibility to further decline proliferation of T cells by exogenously adding adenosine (Figure 12). Although these preliminary data are of interest, we have to emphasize that these experiments were only performed three times and without completely consistent results. Moreover, we primarily focused the readouts on the proliferation of T cells and future studies will have to address additional effector functions of T cells including cytokine production and cytotoxicity. These studies are also currently ongoing in our laboratory.

Another limitation was, that we only studied grouped patients with low and high CD26 expression, then adding adenosine. We could prove that these functional differences are directly related to a weaker or stronger inhibition of adenosine through ADA. It could also hold true that there are some indirect associations through the other effects of CD26, for example the binding of an antibody or the intercellular signalling cascades, which might differ in patients with low and high CD26 expression. Therefore, future experiments will also have to make attempts to inhibit the ADA activity, which, unfortunately, could not be done in this study.

One possible way to achieve this would have been the usage of coformycine, an ADA inhibitor. In the past, this chemotherapeutic agent called Pentostatin (Deoxycoformycine) was used to treat hairy cell leukemia. It was classified as a purine antimetabolite, mimicking the nucleoside adenosine and so inhibiting ADA and a result interfering with the cells ability to process DNA (Aldinucci and Gattei, 2003). In a previous study from our laboratory (Jeffe et al., 2009), we were able to demonstrate that the inhibition by coformycine dramatically influenced the effects of adenosine in our in vitro culture system. However, coformycine was no longer purchasable and despite extensive search in the literature, we were not able to find

an agent similar which would have enabled us to do more extensive experiments in this regard.

Finally, our hypothesis on the potential effects of CD26 did not address the other properties of CD26, e.g. peptidase activity and cell adhesion molecule which were also outlined in detail in the introduction.

Some more recent publications suggest that CD26 could also play a major role in the future treatment of type 2 diabetes. As current type 2 diabetes therapies mainly target at stimulating beta-cell secretion and reducing insulin resistance, alternative therapies are being developed taking advantage of the actions of incretin hormones, e.g. Glucagon-like Peptide-1 (GLP-1). This hormone is released from the intestinal mucosa in response to meal ingestion and stimulates insulin secretion in a glucose-dependent manner (Fehmann et al., 1995; Weir et al., 1989).

In addition, GLP-1 has been demonstrated to have trophic effects on the beta-cells (Zhou et al., 1999) and to promote insulin biosynthesis and insulin gene expression (Fehmann and Habener, 1992). Furthermore glucagon secretion is inhibited (Orskov and Poulsen, 1991). One approach to potentiating the effect of GLP-1 is based on inhibiting CD26, the major enzyme responsible for its degradation *in vivo* (McIntosh, 2008). Subsequently, DPP-IV inhibitors were developed and recently launched for the treatment of type II-diabetes (Krentz et al., 2008). It would now be interesting to study the long-term effects of DPP-IV inhibitors on the functioning of various immune cells in particular in the context of other inflammatory diseases. Of note, non selective DPP-IV inhibitors have been shown to attenuate the severity of autoimmune disease in several animal models (Reinhold et al., 2009).

6 Summary

The cell surface glycoprotein CD26 has multiple functions including exo- and endopeptidase activity, extracellular matrix binding, co-stimulatory effect on T cell activation and adenosine deaminase (ADA) binding. ADA is a ligand of CD26 and displays immunregulatory functions by inhibiting Adenosine, which can deliver negative signals to various immune cells. Since high levels of CD26 can be found on activated T lymphocytes and CD26 expression has been linked to autoimmune diseases, our goal was to investigate the role of the CD26-ADA-adenosine system in the context of viral and autoimmune chronic liver diseases.

In this study, we investigated the CD26 expression on peripheral blood mononuclear cells (PBMC) in 101 subjects, including 25 healthy controls, 45 patients with hepatitis C virus (HCV), 16 hepatitis B virus (HBV) patients and 15 individuals with autoimmune hepatitis (AIH). Moreover, proliferation of T lymphocytes in individuals with low and high CD26 expression was investigated by labeling them with flourescent dye carboxyfluorescein succinimidyl ester (CFSE) and then measured by flow cytometry. Antigen-specific T cell responses were studied by enzyme-linked immunosorbent spot (ELISpot) assay in the presence of the adenosine A_3 receptor agonist IB-MECA.

Overall, the mean CD26 expression showed a high interindividual variability for all PBMC subsets in every group. In patients with AIH, we found a statistically significant increase of CD26 expression in the natural killer (NK) cell population compared to patients with viral hepatitis ($p = 0.019$ for HCV/ $p = 0.031$ for HBV). CD26 expression on $CD4^+$ T cells was negatively associated with alanine aminotransferase (ALT) and serum bilirubin levels ($p = 0.03$ and $p = 0.04$, respectively) in chronic liver disease. No significant role of CD26 could be seen in the impairment of NK cells and $CD8^+$ T cells in chronic viral hepatitis. The stimulation of T cell proliferation with antiCD3 and antiCD28 with the addition of adenosine in individuals with a low CD26 expression resulted in a decrease of proliferation, while this observation could not be made in individuals with a high CD26 expression. Antigen-specific $CD4^+$ and $CD8^+$ T cell responses were inhibited by 70-80 % and 40-50 % respectively by adenosine.

In conclusion, this is the first study that has systematically investigated CD26 expression on different PBMC subsets in the same individual and in a significant number of patients with

liver disease. Despite there being no obvious correlations between CD26 expression and biochemical or hematological disease parameters, there is evidence that CD26 on NK cells might contribute to autoimmunity or lead to lesser disease activity in chronic hepatitis patients through $CD4^+$ T lymphocytes. The physiological relevance of CD26 for T cell function was confirmed by in vitro experiments showing an inhibition of proliferation of $CD4^+$ and $CD8^+$ T cells by adenosine in particular in individuals with low CD26 expression. Finally, our results confirm the fact that adenosine displays most of its inhibitory effects on antigen-specific T cell responses through the adenosine A_3 receptor.

Our results suggest some involvement of CD26-ADA in the pathogenesis of chronic liver disease and of CD26 expression on NK cells for AIH. The potential therapeutic effects of modulating CD26 or ADA activities on the one hand and targeting the adenosine A3 receptor on the other need to be explored in future studies.

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8 Abbreviations

%	percent
°C	degree Celsius
α	alpha
β	beta
γ	gamma
m	milli (10^{-3})
μ	micro (10^{-6})
ADA	Adenosine Deaminase
ADP	Adenosine Diphosphate
ALT	Alanine Aminotransferase
AIH	Autoimmune Hepatitis
AMP	Adenosine Monophosphate
ANOVA	one-way Analysis of Variance
APC	Allophycocyanin
APC	Antigen-Presenting Cells
AST	Aspartate Aminotransferase
ATP	Adenosine Tri-Phosphate
bp	binding protein
BCIP	5-Bromo-4-Chloro-Indolyl-Phosphatase
BSA	Bovine Serum Albumin
CCPA	2-chloro-N^6-cyclopentyladenosine
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CGS	2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine
CMV	Cytomegalovirus
CNS	Central Nervous System
CTL	Cytotoxic T Lymphocytes
CY	Cyanine
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPP	Dipeptidyl-Peptidase

ECM	Extracellular Matrix
e.g.	for example
ELISpot	Enzyme-Linked Immunosorbent Spot
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
Flu-Ma	Influenza-A Matrix
g	gram
G	Gravitational constant
GLP	Glucagon-Like Peptide
h	hour
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IB-MECA	N^6-3-iodobenzyladenosine-5'-*N*-methylcarboxamide
i.e.	that is
IFN	Interferone
IU	International Units
M	Molar
MHC	Major Histocompatibility Complex
min	minute
ml	milliliter
MDC	Monocyte-Derived Chemokine
ms	milliseconds
NBT	Nitroblue Tetrazolium
NK	Natural Killer
PBMC	Peripheral Blood Mononuclear Cells
PBC	Primary Biliary Cirrhosis
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PSC	Primary Sclerosing Cholangitis

RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RNA	Ribonucleic acid
RPMI	Royal Park Memorial Institute (culture medium)
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SDF	Stromal Derived Factor
SEB	Staphylococcal Enterotoxin B
SFU	Spot Forming Units
TCR	T cell receptor
T_{CM}	Central Memory T cells
T_{EM}	Effector Memory T cells
$T_{EM}RA$	RA^+ Effector Memory T cells
TT	Tetanus Toxoid

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Abstracts und Publikationen

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Preise

Young Investigators Bursary Award (EASL 2007, Barcelona, Spanien)

Erklärung nach §2 Abs. 2 Nr. 5 und 6 PromO

Ich erkläre, dass die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel:

„Role of CD26-ADA-Adenosine in viral hepatitis and autoimmune chronic liver disease"

in der Klinik Gastroenterologie, Hepatologie und Endokrinologie unter Betreuung von Professor Dr. Heiner Wedemeyer ohne sonstige Hilfe durchgeführt wurde und ich bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Die Gelegenheit zum vorliegenden Promotionsverfahren ist mir nicht kommerziell vermittelt worden. Insbesondere habe ich keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt haben.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel noch nicht erworben habe.

CH-Suhr, den 11.04.2011