The role of Interleukin-12 in liver inflammation. A study with transgenic mice

Dissertation
zur Erlangung des Grades
"Doktor der Naturwissenschaften"

am Fachbereich Biologie
der Johannes Gutenberg-Universität
in Mainz

Jose Francisco García Lázaro
geboren in Palma de Mallorca

Mainz, Juli 2005

Mündliche Prüfung: Mainz, den 11. November 2005

Table of Contents

1. Introduction

1.1 Hepatic immunity

The liver has a uniquely specialised immune system. On one hand, it can recognise and eliminate a diversity of pathogenic microorganisms, and toxins through inflammatory responses (Doherty and O'Farrelly, 2000). However, inflammation has also been related with liver pathologies. Indeed, exacerbated inflammation is associated with autoimmune hepatitis and lethality due to hepatic failure (Bertolino et al., 2000).

On the other hand, in the liver, induction of immune tolerance is highly favoured over the induction of inflammation (Calne et al., 2000). Immune tolerance is the absence of destructive immunity to harmless self, or dietary antigens and molecules derived from commensal organisms of the gut, which enter the liver via the portal vein. Indeed, the liver is the site of some of the most important persistent viral infections, being chronic hepatic diseases, such chronic active hepatitis and liver cirrhosis, public health problems of worldwide importance and major causes of mortality in certain areas of the world (Kuntz and Kuntz, 2002). Pathological manifestations of chronic hepatitis appear to be mediated by an ongoing immune response that fails to clear the virus. Thus, the high prevalence of chronic infection of the liver seems be related to the hepatic immune tolerance (Bertolino et al., 2000).

Although the mechanisms responsible for tolerance, inflammation, and severe inflammation are not well characterized, they are likely to depend on the nature of the antigen, the way in which an antigen is presented to effector cells of the immune system by antigen presenting cells (APCs), and the presence of different cytokines.

1.2 Th1 (inflammatory) versus Th2 (non-inflammatory) immune responses

Antigen presenting cells process and present antigens to T lymphocytes (T cells). Antigens are presented bound to a surface molecule called major histocompatibility complex (MHC). MHC I-antigen complexes are recognised by CD8+ T cytotoxic cells (CTc), while MHC II-antigen complexes are recognised by CD4+ T helper cells (Th) (Roitt et al., 1996).

Interactions between immature naïve CD4+ T cells and APCs shape the subsequent immune response (Ottenhoff and Bevan, 2004). Thus, during antigen presentation, naïve CD4+ T cells can differentiate into Th1 or Th2 (Abbas et al., 1996). Th1 cells produce IFN-γ, TNF-α and IL-1, (Mosmann et al., 1986), which in turn promotes activation of cytotoxic lymphocytes - natural killer (NK) and CD8+ T cells -, and lymphocytes recruitment and inflammation (Morris and Ley, 2004). Inflammation and cytotoxic lymphocytes are critical in controlling

intracellular pathogens (Mattner et al., 1996; Malmgaard, 2004), and has been associated with chronic inflammation and autoimmune diseases (Strober et al., 1998). Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (Mosmann, 1986), and are involved in protection against extracellular pathogens and in allergic responses (Singh et al, 1999). Immune responses, in which Th1 type cytokines are produced, are called Th1 immune responses, while Th2 immune responses are characterized by Th2 type cytokines.

The induction of Th1 or Th2 responses depends on the pattern of cytokines present during antigen presentation (Mosmann and Coffman, 1989). IL-4 induces the generation of Th2 responses (Kopf et al., 1993), and inhibits Th1 differentiation (Racke et al., 1994). Because of this, Th2 immune responses are also known as non-inflammatory. On the contrary, IL-12 has a central role in inducing Th1 responses and inflammation, inhibiting Th2 differentiation (Seder et al., 1993).

1.3 Antigen-presentation in the liver induces tolerance

In the liver, 70% of the cells are parenchymal cells (hepatocytes), while non-parenchymal cells constitute 30% of the total hepatic cells (Fig. 1). Among the non-parenchymal cells, dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), and Kupffer cells (liver resident macrophages), can present antigens to CD4+ and CD8+ T cells (Fleming, 1999).

Activation of CD4+ T and CD8+ T cells in the liver may induce tolerance under normal circumstances (Mehal et al., 2001):

- Antigen presentation by LSECs can induce: CD4+ T cell proliferation and cytokine production (IL-10 and IL-4), but not Th1 differentiation (Knolle et al., 1999); and activation, but also apoptosis of $CD8^+$ T cytotoxic cells (Knolle and Gerken, 2000).
- KCs seem to induce activation and death by apoptosis of CD8+ T cells (Sun et al., 2003).
- Liver DCs may contribute to tolerance inducing Th2 cell expansion (Khanna et al., 2000), and inactivating self-reactive $CD8^+$ T cells by anergy or apoptosis (Banchereau and Steinman, 1998). Although some in vitro studies have demonstrated that liver DCs are the sole liver APCs with the ability to produce IL-12 and therefore induce inflammatory responses (Pillarisetty et al., 2005), in the healthy liver, DCs show low expression of costimulatory molecules (immature DCs), thus being apparently unable to induce Th1 (Abe et al., 2001). Furthermore, DCs are a very small fraction of the non-parenchimal liver cells, being probably unable to overcome the Th2 milieu during infection (Pillarisetty et al., 2005).
- Constitutively expressing MHC-I, hepatocytes can act as antigen presenting cells. However, hepatocyte-activated CD8+ T cells die by neglect because they fail to receive the

effective co-stimulatory signals necessary for efficient and permanent stimulation (Bertolino et al., 1999).

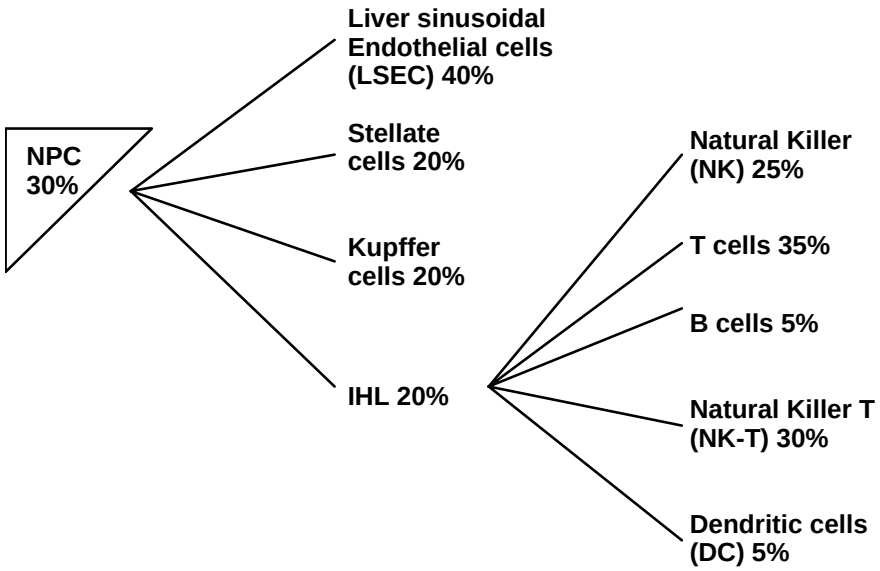


Figure 1. Typical composition of the hepatic nonparenchimal cell (NPC) population in roedents and humans. IHL, intrahepatic lymphocytes. (From Mehal WZ, with modifications)

The Th2 milieu (IL-10, IL-4) present in the liver during immune response may induce tolerance (Chen et al., 2001). Thus, IL-10 seems to influence DCs to induce CD4+ T cell differentiation into Th2 cells, which in turn secrete IL-4, IL-5, IL-10 and/or IL-13 (Steinbrick et al., 1997). IL-10 also appears to down-regulates T cell activation by antigen-presenting LSECs (Knolle et al., 1998), and together with TGF-β1, may influence the efficient presentation of exogenous antigen by LSECs to CD8+ T cells, resulting in tolerance (Limmer et al., 2000).

In summary, tolerance in the liver seems to be induced by apoptosis of autoreactive cytotoxic CD8+ T cells, by differentiation of CD4+ T cells into Th2 cells, which secrete anti-inflammatory cytokines like IL-4, IL-10, and by a Th2 milieu characterized by the presence of Th2 cytokines and the absence of Th1 cytokines like IFN-γ and IL-12.

1.4 Interleukin-12

Interleukin-12 was first identified under the name of natural killer cell stimulatory factor (NKSF), as a product of Epstein-Barr virus (EBV)-transformed human B-cell lines, which was able to activate NK cells, generate lymphokine-activated killer cells (LAKs), and induce IFN-gamma production and T-cell proliferation (Kobayashi et al., 1989).

1.4.1 Structure

Bioactive IL-12 is a heterodimeric protein composed of two disulfide-linked glycoprotein subunits: a 35 kDa light chain (known as p35 or IL-12α) and a 40 kDa heavy chain (known as p40 or IL-12β) (Wolf et al., 1991).

1.4.2 Production

Coexpression of both chains of IL-12 in one cell is required to generate bioactive IL-12 (Gubler et al., 1991). Although the p35 chain is expressed ubiquitously and constitutively at low levels, production of the p40 chain seems to be restricted to activated cells, and its production is highly regulated and induced after cell activation (D'Andrea et al., 1992). IL-12 is mainly produced by activated inflammatory cells: DCs (Macatonia et al., 1995), monocytes, macrophages and neutrophils, (D'Andrea et al., 1992). Inflammatory cells are activated through the binding of products from microorganisms (bacteria, intracellular parasites, fungi, double-stranded RNA, and CpG-containing oligonucleotides) to the toll like receptors present on their surface (TLRs) (Ma and Trinchieri G, 2001).

The production of IL-12 by activated cells can be increased by:

- IL-12 itself and in combination with IL-18, thus presenting an autocrine loop (Grohmann U, et al. 2001).
- IFN-γ, which enhances the transcription not only of the gene p40, but also of the p35, having a marked effect on the production of the heterodimer. This ability of IFN-γ to enhance the production of IL-12 forms a positive feedback mechanism during inflammatory responses (Ma et al., 1996).
- TNF-α (Ma and Trinchieri, 2001).

The production of IL-12 by activated cells can be inhibited by:

- Interleukin-10, which blocks the transcription of p40 and p35 genes (Aste-Amezaga et al., 1998)
- TGF-β1, which can reduce the stability of IL-12p40 mRNA (Du and Sriram, 1998).

1.4.3 Receptor

The activities of IL-12 on different cells are mediated by a high-affinity receptor. The IL-12 receptor (IL-12R) is composed of two type-I-transmembrane-glycoproteins subunits (Fig. 2): IL-12Rβ1, with a molecular size of about 100 kDa and IL-12Rβ2 with a molecular size of about 130 kDa. The IL-12Rβ1-IL-12Rβ2 complex is required for competent signalling of IL-12 (Wilkinson et al., 1997).

IL-12R is expressed mainly by activated T cells, NK cells (Desai et al., 1992), DCs and B-cells (Airoldi et al., 2000). Differential up-regulation of both subunits has been observed: Th2 cells express IL-12Rβ1 but not IL-12Rβ2, whereas expression of IL-12Rβ2 seems to be confined to Th1 cells (Wu et al., 1997). IL-10, and TGF-β seems to down regulated IL-12Rβ2. On the contrary, the IL-12Rβ2 is upregulated by IL-12 itself, IFN-α, IFN-γ, TNF-α (Szabo et al., 1997).

1.4.4 Intracellular signalling pathway

Biological activity of IL-12 requires the initial interaction of p40 with IL-12Rβ1 and p35 with IL-12Rβ2. The IL-12Rβ1 subunit provides the binding site, whereas the IL-12Rβ2 subunit function as the signal-transducing component (Presky et al., 1998). Signalling through IL-12R is mediated by phosphorilation of cytoplasmic TYK2 and JAK2 (Fig. 2).

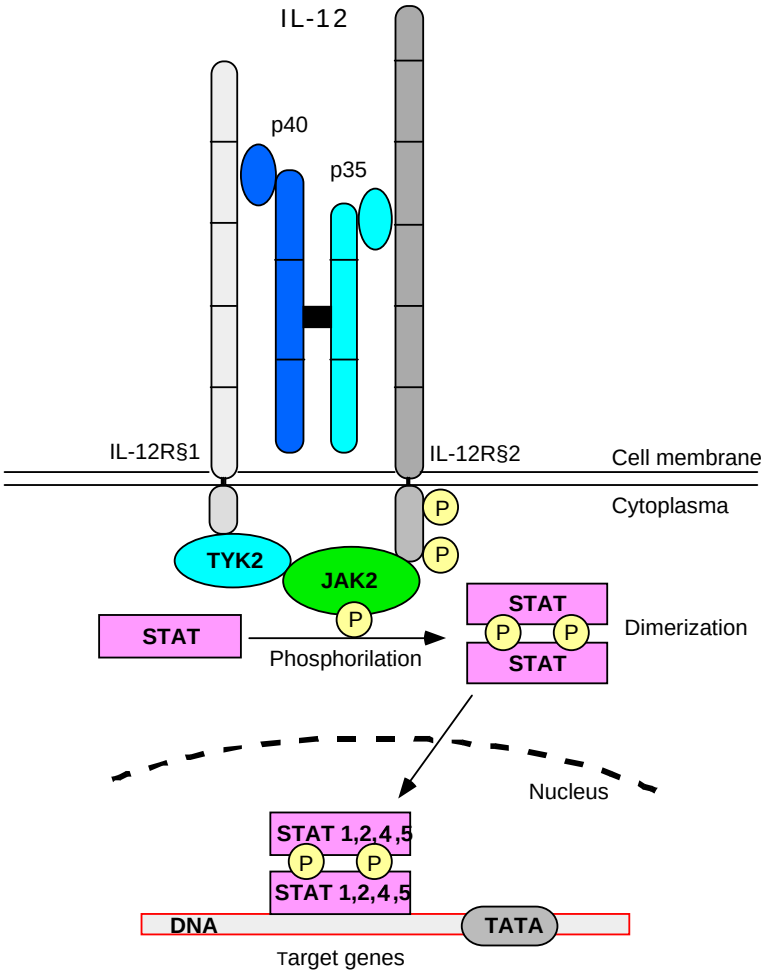


Figure 2. The interleukin-12 receptor and signalling pathway. After binding of IL-12 on the IL-12Rβ1, the IL-12Rβ2 subunit functions as the signal-transducing component. Signal transduction through IL-12R induces tyrosine phosphorylation of the Janus family kinases JAK2 and TYK2, which, in turn, phosphorylate and activate signal transducer and activator of transcription (STAT) 1, 3, 4 and 5. The specific cellular effectors of IL-12 are due mainly to activated STAT4.

While TYK2 directly interacts with IL-12Rβ1, JAK2 interacts with TYK2 and with IL-12Rβ2. However, JAK2 seems to be phosphorylated only by IL-12Rβ2 (Bacon et al., 1995). JAK2 in turn phosphorylates STAT1, STAT3, STAT4 and STAT5 (signal transducer and activator of transcription), although the cellular effectors of IL-12 seems to be mainly mediated by activated STAT4 (Bacon et al., 1995). After phosphorilation, STAT4 homodimerizes, translocates into the nucleus, and binds to its target DNA to activate transcription of IFN-γ and other target genes (Yao et al., 1999).

1.4.5 Biological functions

Interleukin-12 has been described as a key factor for initiation and maintenance of Th1 immune responses (Trinchieri, 1998; Park and Scott, 2001). IL-12 induces development and proliferation of Th1 cells (Hsieh et al., 1993), and enhances the generation, cytolytic activity, and proliferation of NK cells (Trinchieri, 1994). IL-12, IL-12-induced Th1 cells (Airoldi et al., 2000), and IFN-γ (Morris et al., 1994) can directly act on B-cells to enhance IgG1 and decrease IgE production (Fig. 3).

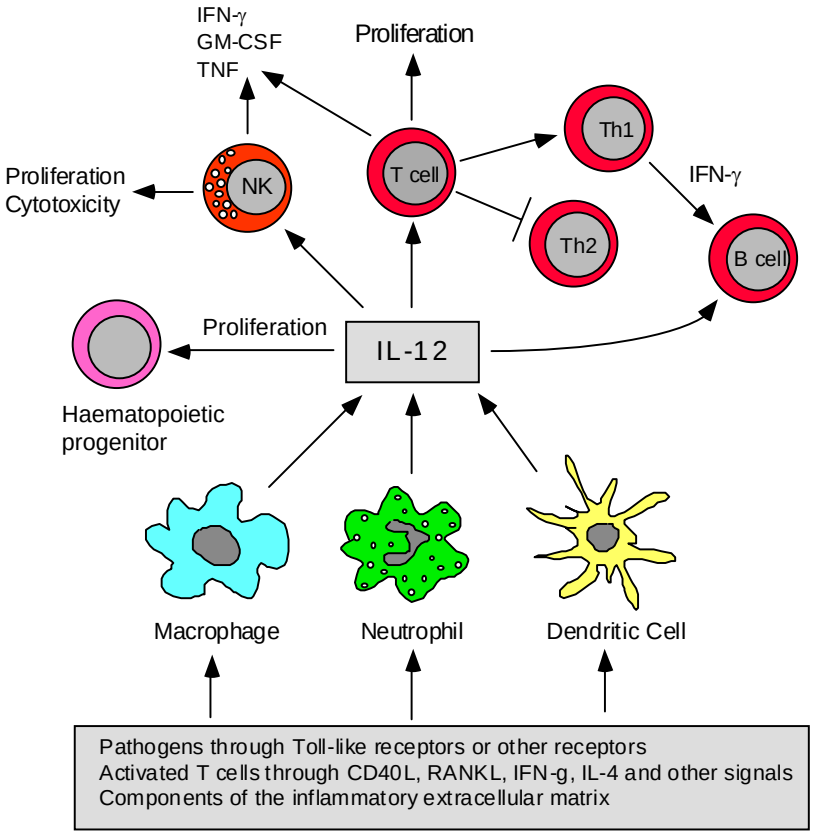


Figure 3. Summary of the biology of IL-12. Interleukin-12 is mainly produced by antigen presenting cells in response to different stimulus. Subsequently, IL-12 acts on: hematopoietic progenitors, inducing proliferation and colony formation proliferation; NK cells, and T cells, inducing proliferation, enhacement of cytotoxicity, and production of cytokines, particularly IFN-γas well as favouring differentiation to cells that produce type-1 cytokines (Th1, and NK cells); and B cells, activating the production of Th1-associated immunoglobulins.

IL-12 irreversibly primes both CD4+ and CD8+ T cells and NK cells to produce several Th1 cytokines (IL-1β, TNF-α), and it is particular efficient and rapid, even a very low concentrations, at inducing the production of IFN-γ (Gazzinelli et al., 1993), which is the most potent effector cytokine during Th1 immune responses (Seder et al., 1993). Furthermore, IL-12 together with IFN-γ can antagonize Th2 differentiation (Seder, 1995). IL-12 can also act synergistically with IL-2 (Chan et al., 1991) and IL-18 for inducing IFN-γ. The synergy with IL-18 lays in the ability of IL-12 to upregulate the expression of the IL-18 receptors (Nakahira et al., 2002), and in the ability of IL-18 to upregulate the expression of IL-12Rβ2 (Chang et al., 2000) (Fig. 4)

Activation of the innate immune system to produce IL-12 seems to be determinant for developing a protective Th1 immune response after infection with intracellular pathogens (Yap et al., 2000). Indeed, mice deficient for interleukin-12 demonstrated impaired Th1 responses (Magram et al., 1996), and severe pathology after infection (Park et al., 2000). Furthermore, IL-12 seems to be required for virus clearance (Orange and Biron, 1996).

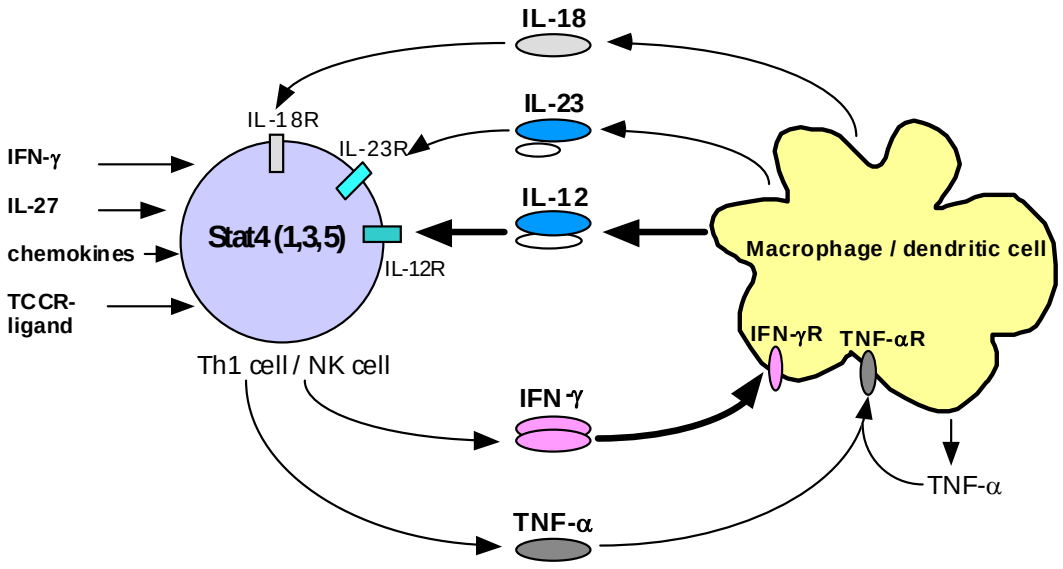


Figure 4. The type-1 cytokine pathway. IL-12 alone or together with IL-23, IL-18, IL-27, TCCR-ligand, IFN-γ and chemokines, induce Th1 and NK cells to produce Th1 cytokines like IFN-γ and TNF-α. Finally, IFN-γ together with TNF-α activates macrophages in a loop system.

1.5 IL-12 related cytokines

Besides IL-12, other p40-dependent molecules have been discovered, with different activities during Th1 immune responses (Trinchieri et al., 2003). These new cytokines are IL-23, IL-27, and the homodimeric p40 (Brombacher et al., 2003).

1.5.1 Interleukin-23

Active IL-23 is a cytokine formed by the p40 subunit of IL-12 linked by a disulfide bridge to a novel protein called p19 (Fig. 5). The p19 protein is similar to the p35 protein and biologically inactive by itself requiring coexpression of p40 for secretion. Microbial products can induce the production of p19 in macrophages, dendritic cells, T cells, and endothelial cells (Oppmann et al., 2000).

IL-23 interacts with IL-12Rβ1 and an additional, novel β2-like receptor subunit designated IL-23R, which is expressed on T cells, NK cells, monocytes, and DCs (Parham et al., 2002). IL-23 binding to the IL-23R leads to activation of JAKs, which in turn phosphorilate STATs (1, 3, 5 and mainly 4). However, the majority of activated STAT4 heterodimerizes with STAT3 before translocation into the nucleus, indicating that IL-12 and IL-23 may act on different target genes (Ihle, 2001).

IL-23, like IL-12, induces production of IFN-γ in T and NK cells (Fig. 4). However, unlike IL-12, IL-23 seems to act on memory T cells inducing proliferation and secretion of IL-17 (Aggarwal et al., 2003). Furthermore, IL-23 has been associated with strong Th1 responses and inflammation (Wiekowski et al., 2001), and can even be more important than IL-12 during inflammatory processes (Langrish et al., 2005; Cua et al., 2003).

1.5.2 Interleukin-27

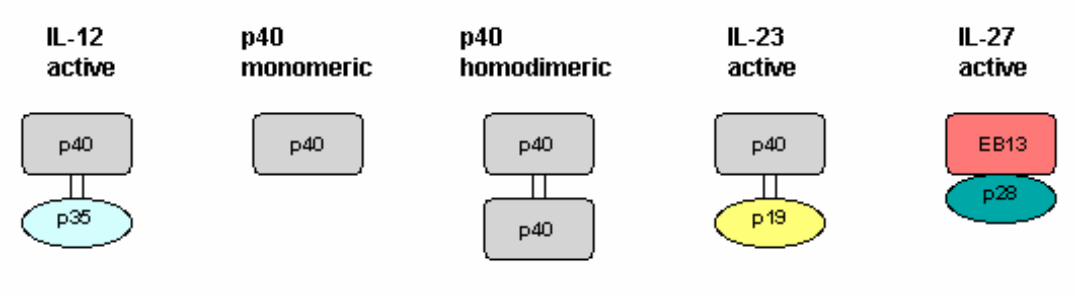
IL-27 is composed of the Epstein Barr virus-induced gene 3 (EBI3) and the novel subunit p28 (Pflanz et al., 2002) (Fig. 5). The p28 subunit is homologous to the p35 subunit of IL-12, whereas EBI3 is related to IL-12 p40, and like IL-12 andIL-23, IL-27 requires expression of both subunits for production of active IL-27 (Devergne et al., 1997).

IL-27 binds to an orphan receptor, designated WSX-1 or TCCR (Chen et al., 2000), inducing clonal proliferation of naïve but not memory T cells, and synergizes with IL-12 to promote IFN-γ production by naïve T cells, and Th1 polarization (Planz et al., 2002) (Fig. 4).

1.5.3 The p40 homodimer

During inflammation, up-regulation of the IL-12 p40 subunit results in secretion of a 10-1000-fold excess of free monomeric and homodimeric p40 (Heinzel et al., 1997) (Fig. 5). The p40 homodimer can, at least in mice, bind to the IL-12Rβ1 with an affinity that is similar to that of the heterodimeric IL-12, but does not lead to competent signalling, thus acting as a natural inhibitor of IL-12 (Gillessen et al., 1995). Indeed, liver specific p40 transgenic mice demonstrated reduced Th1 responses (Yoshimoto et al., 1998), and local delivery of p40 in

mice suppressed Th1 immune responses (Kato et al., 1996). In humans, IL-12 p40 homodimer binds to the IL-12 receptor but does not seem to act as an antagonist for IL-12 (Ling et al., 1995).



Binding	IL-12Rβ1	IL-12Rβ1	IL-12Rβ1	IL-12Rβ1	WSX-1
Signalling	IL-12Rβ2			IL-23R	?

Figure 5. The members of the IL-12 family and their receptors. The IL-12 family is composed of heterodimeric cytokines. IL-12 and IL-23 are composed of the same p40 subunit plus a p35 subunit (IL-12) or a p19 subunit (IL-23). The IL-27 cytokine is composed of an EB13 and a p28 subunit. Finally, the p40 subunit binds to itself to create a p40 homodimer IL-12 binds to the IL-12Rβ1(binding)-β2(signalling)-complex. IL-23 binds to a receptor-complex composed of the same IL-12Rβ1(binding) like IL-12 and a second receptor called IL-23R(signalling). IL-27 binds to the WSX-1 receptor, while the presence of a second receptor for signalling is not clear.

1.6 IL-12 in liver pathology

1.6.1 Viral hepatitis

During viral hepatitis, poor inflammatory responses have been related with chronic hepatitis and virus persistence, while inflammation has been associated with viral elimination during acute hepatitis (Ferrari et al., 1990; Cerny and Chisari, 1999). Since IL-12 is the central cytokine in inducing Th1 development and inflammation (Park and Scott, 2001), IL-12 was suggested to be instrumental in the defence mechanisms during hepatic viral infections. Indeed, IL-12 seems to determine the course of viral hepatitis (Thimme et al., 2002). It has been observed that elevated serum levels of IL-12 in patients with chronic hepatitis were positively correlated with viral clearance (Naoumov and Rossol, 1997; Rossol et al., 1997). Furthermore, other studies attributed the anti-viral effects of IL-12 during hepatic viral infection to its capability to: recruit macrophages and T helper cells into the liver (Park et al., 2001); promote Th1 cell development and production of IFN-γ and TNF-α, which exert their antiviral effects (Guidotti et al., 1999); and increase hepatic inflammation and immune mediated liver cell damage (Myers et al., 1998), which can lead to release of viral antigen and better recognition by the immune system.

However, Th1 immune responses have also been observed during chronic hepatitis and viral persistence (Sobue et al., 2001; Iwata et al., 1995). Indeed, IL-12 was related with progressive liver injury, but not with viral elimination during chronic hepatitis (Nelson et al., 2000; Cecere et al., 2004). Furthermore, while in a phase I/II study of patients infected with hepatitis B virus, treatment with IL-12 appeared to have anti-viral effects (Carreno et al., 2000), a similar study with patients suffering from infection with hepatitis C virus, IL-12 treatment did not show any advantageous effect (Zeuzem et al., 1999).

1.6.2 Fulminant hepatitis

Cytotoxic CD8+ T cell activity and strong inflammatory processes are related with fulminant hepatitis and lethality (Ando et al., 1993). The role of IL-12 in the induction of strong inflammation leading to fulminant hepatitis has been suggested. Thus, in the model of concanavalin A-induced fulminant hepatitis, IL-12 administration was related with exacerbated hepatic damage and inflammation (Nicoletti et al., 2000). Myers also demonstrated that systemic application of IL-12 in mice was associated with hepatocyte damage and necrosis (Myers et al., 1998). Furthermore, IL-12 has been associated with severe liver damage in patients with chronic hepatitis (Quiroga et al., 1998), and with lethality in patients with fulminant hepatitis (Leifeld et al., 2002).

1.6.3 Autoimmune hepatitis (AIH)

The pathogenesis of autoimmune hepatitis (AIH) is still not well understood. A complex interaction between triggering factors, autoantigens, genetic predisposition, and cytokine networks seems to determine the onset of AIH (Czaja, 2001). Triggering factors like viruses (Huppertz et al., 1995; Laskus and Slusarczyk, 1989) and autoantigens (Wesierska-Gadek et al., 1998; Wies et al., 2000) seem to be able to induce AIH only after breaking of liver tolerance. Indeed, AIH is a chronic liver disease characterized by a persistent inflammatory reaction in the liver (Al-Wabel et al., 1993).

Models of autoimmune liver diseases have been developed by immunizing animals with liver antigen (Meyer zum Buschenfelde et al., 1974). However, this model only achieved a short self-limited inflammation (Lohse and Meyer zum Buschenfelde, 1993). Transgenic animals models expressing foreign proteins were also developed (Guidotti et al., 1995). However, all these transgenic mice have the disadvantage that the immune milieu is tolerant to the transgene, which is seen as “self” (Chisari, 1995; Wirth et al., 1995). Indeed, spontaneous inflammation was not observed in any of these mice, and could only be induced after transfer

of specific cytolytic T cells together with IFN-gamma or lipopolysaccharide (Sette et al., 2001). All together, these studies suggest that the presence of autoantigens in the liver is not enough to trigger an autoimmune hepatitis, and that Th1 cytokines are necessary for induction of inflammation against autoantigens.

Although IL-12 seems to be a key cytokine in Th1-mediated autoimmune diseases (Adorini, 1999), nothing is known about the role of IL-12 in autoimmune hepatitis.

1.6.4 Hepatic tumours

Through its pro-inflammatory and immunoregulatory features, IL-12 also appears to have strong anti-tumour properties (Colombo and Trinchieri, 2002). In studies in rats and mice, IL-12 seemed to play an important role in healing hepatic tumours (Sangro et al., 2002). Barajas also demonstrated that intratumoral injection of adenovirus carrying IL-12 (AdCMVIL-12) in animals with a single big tumour nodule implanted in the liver, was related with a significant inhibition of tumour growth. In the same study, animals with multiple hepatic nodules were treated by intra-hepatic artery injection and complete tumour regression was observed in 20% of the animals (Barajas et al., 2001). Furthermore, IL-12 was associated with the inhibition of hepatic metastases (Siders et al., 1998).

1.7 Aim of this work

This work was planned in order to provide a better understanding of inflammatory responses in the liver. While immune tolerance in the liver is related with viral persistence, hepatic inflammation seems to be crucial for viral elimination. However, inflammatory responses have also been observed during viral persistence in chronic hepatitis. The causes leading to viral elimination or viral persistence during hepatic inflammation are not yet clear. In addition, exacerbated inflammation has been related with autoimmune hepatitis and lethality due to hepatic failure.

IL-12 is the key cytokine in inducing inflammatory responses and seems to play an important role in determining the course of hepatic viral infection. However, its role during viral persistence and fulminant hepatitis, is not yet clear (Zeuzem et al., 2001). Thus, we decided to focus our work on this cytokine.

Since during viral hepatitis, hepatocytes can express MHC class II molecules (Dienes et al., 1987), thus having the potential to act as antigen presenting cells (Herkel et al., 2003), for the first part of this work, a hepatic viral infection was simulated through the using of viral infected hepatocytes as APCs.

Thus, part of this work was to learn the influence of IL-12 on the immune response induced by hepatocytes during a viral infection, and in virus elimination.

Until now, all the studies concerning the function of IL-12 in the liver have been achieved either by systemic application or by infection with adenovirus coding for IL-12. However, systematic administration has been related with multiorgan toxicity and lethality (Car et al., 1995). Adenovirus injection allowed IL-12 expression almost only in the liver. However, expression normally lasts for a short time period due to host immune responses against viral proteins, which can mask some of the IL-12 features in the liver. Furthermore, subsequent adenovirus injections led to rapid virus elimination by the immune system (Muruve, 2004). Therefore, an adequate model to deeply understand the role of IL-12 during inflammation in the liver was still necessary.

Thus, the main aim of this work was the generation of transgenic mice expressing active IL-12 in the liver. To avoid the overproduction of the antagonist p40 subunit, the IL-12 transgenic mice were generated with an active IL-12 fusion protein. Part of this work was the creation of this protein. In addition, to avoid constitutive expression of IL-12, which could lead to embryonal lethality (Paradisi et al., 2003), an inducible system as used.

Finally, because IL-23 seems to have an important role in inflammatory processes, an active IL-23 fusion protein was generated, which will be used in future work for the creation of transgenic mice expressing IL-23 in the liver.

1.8 Technical aspects of generating an inducible, liver specific expressing system

To generate transgenic mice with inducible hepatic IL-12 expression, a *Cre-LoxP* expression system (Meyer, 2003; Klopcic, 2002) was used. In the construct, the murine albumin promoter guarantees hepatocyte-specific expression of IL-12 (Gorski et al., 1986), and two *LoxP* sites, found between the promoter and the IL-12 cDNA, allow the induction of the expression at any given moment by Cre recombinase protein. Cre recombinase, an integrase from bacteriophage P1, catalyses site-specific recombination between LoxP sites in the absence of any additional cofactors, excising the DNA between the two LoxP sites along with one LoxP site (Sternberg et al., 1981), thus allowing promoter mediated transcription of the IL-12 cDNA. Albumin promoter activity was reported to begin at about 6^{th} day of embryonal development (Trojan et al., 1995). Taking into account the early activation of the albumin promoter and the multiple inflammatory effects of IL-12, the *Cre-loxP* expression system should avoid possible early embryonic lethality in the transgenic mice.

1.9 Hydrodynamics-based transfection of the liver

Efficient plasmid DNA delivery to the liver can be achieved by rapid tail vein injection of a large volume of DNA solution (Liu et al., 1999). This method, called “hydrodynamics-based transfection”, causes plasmid DNA to enter into hepatocytes through plasma membrane pores generated by the hydrodynamic pressure (Kobayashi et al., 2004). Volume, amount of plamid DNA and speed of injection are very important for expression: optimal transgene expression is obtained by injecting 5 μg plasmid in volumes 1.2, 1.6, and 3.0 ml for animals with body weights of 11-13, 18-20, and 30-32 g, respectively, in 5 to 8 seconds. Decreasing the amount of plasmid and the volume, and increasing the time of injection, was associated with a decrease in transgene expression. Furthermore, liver transfection efficiency was demonstrated, being the liver the organ with the highest level of transgene expression. Finally, although duration of transgene expression is transient, a sustained expression can be achieved by repeated injection (Yang et al., 2001; Liu et al., 1999).

2. Materials and methods

2.1 Materials

2.1.1 Animals

FVB/NHSD mice from the central animal facility "ZVTE" of the Mainz University were used to generate the IL-12 transgenic lines. Homozygotic Actin-Cre mice with FVB/NHSD genetic background were used for breeding with the above mentioned IL-12 transgenic mice to activate the transgene transcription (Sakai and Miyazaki, 1997). OVA323-339-specific T-Cell Receptor transgenic mice (FVB/NHSD), and CIITA-transgenic mice (FVB/NHSD), (Jagemann, 2000) were used for *in vitro* experiments.

2.1.2 Cloning vectors

For different purpose the following vectors were used: PGEM Teasy Vector System (Promega Biosciences, USA), pcDNA3.1(+) (Invitrogen, Carlsbad), the liver specific constitutive expression U3 vector (generated by Dr. Jürgen Henninger), and the liver specific inducible expression IAL vector (Klopcic, 2002; Meyer, 2003)

2.1.3 Primers:

- P40fwd30: 5'- atg tgt cct cag aag cta acc atc tcc tgg –3'
- P40rev30: 5'- cta gga tcg gac cct gca ggg aac aca tgc –3'
- P40fwdSrfI: 5'- tat agc ccg ggc cgc cac cat gtg tcc tca gaa gct aac –3'
- IL-12p35fwd: 5'- atg tgt caa tca cgc tac c –3'
- IL-12p35rev2: 5'- tat agt cga ctc agg cgg agc tca gat agc c –3'
- IL-12p35rev: 5'- tca ggc gga gct cag ata g –3'
- P40SbfIA: 5'- gtt ccc tgc agg gtc cga tcc ggc agt act tcg gg –3'
- P35PflMI: 5'- agg tcc aga gac tgg aat gac cct acc ctt ag –3'
- P19fwd: 5'- atg ctg gat tgc aga gca gta –3'
- P19SalI: 5'- tat agt cga ctt aag ctg ttg gca cta agg g –3'
- P19val: 5'- gtg cct ggc gtg ggc gtg cct ggc gtg ggc cct agg agt agc agt cct gac –3'
- P40SbfIB: 5'- gtt ccc tgc agg gtc cga tcc gtg cct ggc gtg ggc gtg cct ggc –3'
- Crefwd: 5'- atg tcc aat tta ctg acc gta cac –3'
- Crerev: 5'- cta atc gcc atc ttc cag cag gcg –3'

- PArev: 5’- agg aga atc gct tga acc –3’
- LKseqfw: 5’- tgc gtg caa gct cag gat cg –3’
- P40fw3: 5’- gaa gtc caa tgc aaa ggc –3’
- Cre Ia: 5’- gca ctg att tcg acc agg tt –3’
- Cre IIb: 5’- ccc ggc aaa aca ggt agt ta –3’
- RT1: 5’- tca cct ttc cta tca acc cc –3’
- P40seqrev: 5’- ctg aat act tct cat agt ccc –3’
- Beta-actinfwd: 5’- gtg ggc cgc cct agg cac ca –3’
- Beta-actinrev: 5’- tag ccc tcg tag atg ggc aca –3’
- P40fwd2: 5’- atg tgt cct cag aag cta ac –3’
- IL-12revLC: 5’- tcc agt cca cct cta caa c –3’
- B-actinLCI: 5’- cat ggc att gtt acc a-3’
- B-actinLCII: 5’- ggg tgt tga agg tct ca –3’
- Advfw1: 5’- aag cgg gca tga ctt ctg cg –3’
- Advrev1: 5’- gac gag acg cag cta gac cc –3’

2.1.4 Chemicals

All chemicals used were delivered by one of the three following companies: Merck Eurolab GmbH, Frankfurt; Carl Roth GmbH, Karlsruhe; and Sigma-Aldrich Chemie GmbH, Taufkirchen.

2.1.5 Enzymes and DNA ladders

All enzymes and DNA molecular markers were purchased by New England Biolabs, USA

2.1.6 Laboratory instruments

Balance	Sartorius BP 61, Goettingen
Centrifuges	5417R Eppendorf, Hamburg
	Sorvall Du Pont, Bad Homburg
Digital camera Cybertech CS1	Hitachi, Japan
Electrophoresis Horizontal	Hybaid Electro 4, Heidelberg
GeneQuant*pro*	Amersham, Biosciences, UK
Incubator Cellstar	Nunc, Wiesbaden
Kodak BioMax MR-1 films	Integra Biosciences gmbH, Fernwald
Lysing-Matrix-D tubes	BIO 101 inc., CA, USA

Masterflex Perfusionspump	77200-50/7523-25, Cole Parmer, USA
Microplate Reader MRX II	Dynex Technologies, USA
Miniprotean II Electrophoresis	Biorad, Munich
Mini Trans Blot Transfer cell	Biorad, Munich
Photoprinter XLS 8600PS	Kodak, Rochester, USA
PowerPack P25	Biometra, Goettingen
Printer P67E	Mitsubishi
Printer T520	Lexmark, Kentucky, USA
PVDF Membrane 0,45 mm	Biotrace, Pall Corporation
Scan Maker 8700	Microtek, Overath
Shaker-incubator Certomat	B. Braun, Melsungen
Stereomicroscope MZ-APO	Leica, Bensheim
Stereomicroscope SZ 40	Olympus, Hamburg
Thermocycler PTC-200	MJ Research, MC, USA
Waterbath	Memmert D91126, Schwabach

2.1.7 Kits

cDNA synthesis kit	Stratagene, La Jolla, CA, USA
GeneClean II spin kit	Qbiogene, CA, USA
Nucleospin-Extract kit	Macheray & Nagel, Dürren
NucleoSpin Plasmid kit	Macheray & Nagel, Dürren
Plasmid Maxi preparation kit	Qiagen, Hilden
Lipofectamine Reagent 2mg/ml	Invitrogen, Carlsbad
Plus Reagent 3mg/ml	Invitrogen, Carlsbad
First Strand RT-PCR	Stratagene, Heidelberg
Mouse IL-12 (p70) ELISA	BD Biosciences Pharmingen, USA
Mouse IL-17 ELISA	R&D Systems, USA
Western Blot Detection System	ECL plus, Amersham Biosciences, UK
NAP 10 Columns.	Amersham Biosciences, UK

2.1.8 Most frequently used Buffers and Reagents

Proteinase K	Neolab, Heidelberg
Protease Inhibitor Tablets	Roche, Mannheim
Roti-Phenol / C/ I (25:24:1)	Carl Roth

TriReagent	Sigma, Steinheim
ReadyMix REDtaq PCR Reagent	Sigma, Steinheim
Avidin-HRT	DAKO, Denmark
Hydrogen Peroxide (H_2O_2) 30%	Merck
Avertin (2,2,2,Tribromoethanol)	Fluka
Agarose gel (1%):	1,5 g Agarose; 3µl Ethidiumbromid (10mg/ml, Sigma, Cat.Nr.1510); 150 ml 0,5x TBE-Buffer
10x DNA-Loading Buffer:	30% Ficoll; 100 mM Ethylendiaminetetraacetic acid (EDTA); 10% Sodium Dodecyl Sulfat (SDS); 0,25% Bromphenolblue; 0,25% Xylencyanol; in H_2O
PBS (pH 7,2):	8,0 g. NaCl; 1,3 g Na_2HPO_4; 0,2 g. KCl; 0,2 g. KH_2PO_4; q.s. to 1 L H_2O.
TBE-Buffer:	90 mM Tris; 90 mM Boric acid; 20 mM EDTA
10% Polyacrylamid Gel	3,3 ml Acrylamid 30%; 2,5 ml Tris 1,5M pH 8,8; 100 ml 10% SDS; 100 µl 10% Amonium Persulfate (APS); 4 µl TEMED; q.s. to 10 ml H_2O
Western Blot Stacking Gel	830 µl Acrylamid 30%; 630 µl Tris 1M pH 6,8; 50 µl 10% SDS; 50 µl 10% APS; 5 µl TEMED; 3,4 ml H_2O
Electrophoresis Buffer-Western Blot-	3 g Tris; 14,4 g Glycin; 5 ml 20% SDS; 1 L H_2O
Transfer Buffer	3 g Tris; 14,4 g Glycin; 100 ml Methanol; 1 L H_2O
2X Protein Loading Buffer	5 ml 10% SDS; 2,5 ml Glycin; 3,12 ml 0,5 M Tris-HCl pH 6,8; 1 mg Bromphenolblue; 12,5 ml H_2O
ELISA IFN-γ Coating Buffer	0,1 M $Na_2HPO_4.2H_2O$, pH 9,2
ELISA IFN-γAssay Diluent	1% PBS; 1% Gelatine
ELISA IL-4 Coating Buffer	8,4 g $NaHCO_3$; 3,56 g Na_2CO_3; q.s. to 1 L H_2O, adjust to pH 9,6
ELISA IL-4 Blocking Solution	ELISA Wash Buffer with 1 % BSA
ELISA IL-4 Assay Diluent	ELISA Wash Buffer with 0,1 % BSA
ELISA Wash Buffer	1% PBS and 0,05% Tween 20
ELISA Substrate Buffer	6,3 g Citric acid Monohydrat; 1 L H_2O, adjust with KOH to pH 4,1
ELISA Substrate Solution	20 ml Substrate Buffer; 1 ml TMB Solution; 25 µl H_2O_2

TMB Solution	120 mg TMB (Tetramethylbenzidine); 2,5 ml Aceton; 22,5 ml Ethanol 100%
ELISA Stop Solution	28 ml H_2SO_4 (97%); q.s. to 500 ml H_2O
Proteinase K Buffer	100 mM Tris, pH 8.5; 5 mM EDTA; 200 mM NaCl; 0.2% SDS
Protein Lysis Buffer	20 mM Tris; 50 mM EDTA; 0,5% TritonX-100; q.s. to 50 ml, pH 8; 2% Protease Inhibitor before use
Injections Buffer pH 7.5	10 mM Tris and 0.1 mM EDTA
Hepatocyte Buffer I	8,3 g NaCl; 0,5 g KCl; 2,4 g Hepes; q.s. to 1L, pH 7,4
Hepatocyte Buffer II	3,9 g NaCl; 0,5 g KCl; 0,7 g $CaCl_2.2H_2O$; q.s. to 1 L, pH 7,6; 0,05% Collagenase short before use
Hepatocyte Buffer III	8,3 g NaCl; 0,5 g KCl; 0,18 g $CaCl_2.2H_2O$; 2,4 g Hepes; q.s. to 1 L, pH 7,4
Cesium Chloride 1.4, pH 7.9	53 g CsCl and 87 ml of 10 mM Tris-HCl
Cesium Chloride 1.2, pH 7.9	26,8 g CsCl and 92 ml of 10 mM Tris-HCl
NAP-Columns Buffer	10 mM Tris pH 8; 2 mM $MgCl_2$; 5% Sucrose

2.1.9 Mediums and related

LB Medium (pH 7,5):	10 g tryptone; 5 g yeast extract; 10 g NaCl; 1 L H_2O
LB Ampicillin Medium:	1 l LB Medium and 50 mg Amp (50 µg/ml)
LB Ampicillin Agar Medium	20 g agar and 1 L LB Ampicillin Medium
D-MEM Medium	Gibco, Invitrogene
Cell Culture Medium	D-MEM Medium; 1% Penicillin/Streptomycin; 10% FCS
Hepatocytes culture Medium	DMEM; 1% Penicillin/Streptomycin; 20 mM Hepes; 0,05% Insulin; 10^{-6} Lithocol acid; 10% FCS
FCS (Fetal Calf Serum)	Greiner, Frickenhausen
Penicillin / Streptomycin	10.000 U/ml / 10.000 mg/ml, Bio Whittaker.
Human Insulin 40 I.E / ml	Aventis, USA
Collagenase NB 8, 0,93 PZU/mg	Serva, Heidelberg
Trypsin (0,05%)/EDTA (0,02%)	Biochrom AG, Berlin
Covering medium (Aqua polymount)	Polyscience Inc., Warrington, USA
Freezing medium	Jung, Nussloch
Entellan	Merck, Darmstadt

2.2 Methods

2.2.1 DNA extraction and purification

Tissue fragments were digested with proteinase K (2 mg/ml in proteinase K buffer) overnight at 55°C. Ear biopsy digestions were performed in 20 µl whereas tail and liver biopsies in 50 µl total volume. After 1:15 dilution with water, 1-2 µl of digested ear biopsy were used as template for PCR. Total DNA was extracted and purified from liver biopsies digestions by the Phenol-Chloroform Method. Briefly, 450 µl H_2O, 50 µl 3M Na-Acetat, and 500 µl Phenol/Chloroform (Roti-Phenol) was added to the digestions. After 10 min. centrifugation at 14000 rpm, the upper phase was recovered, 500 µl Phenol/Chloroform was added, and centrifuged again. The upper phase was recovered again, 500 µl Chloroform/Isoamylalcohol (50:1) added, and centrifuged again. The upper phase was recovered, and after addition of 1 ml 100% Ethanol and incubation at –80° C for 1 h, DNA was precipitated by 30 min. centrifugation at 14000, 4°C. Pellet was washed twice with 70% Ethanol and dissolved in TE Buffer.

2.2.2 Polymerase chain reaction (PCR)

For PCR amplification 1-2 µl of ear digestion, about 50 ng of purified genomic DNA, or 1-2 µl of cDNA were used as template in a 25 µl volume PCR reaction (ReadyMix REDtaq), with 1pM primers concentration. PCR reaction products were fractioned and visualized by electrophoresis.

Standard protocol was performed as follow:

- After initial denaturation at 95° C during 5 min, 25-35 cycles of :
  - Denaturation 1 min. at 95° C
  - Annealing 30s at 55-65° C (depending on the primers)
  - Elongation 4s /100 base pair at 72° C
- Final elongation for 10 min at 72° C.

2.2.3 DNA Electrophoresis

Samples together with 10X loading buffer were loaded into a 1% Agarose gel and separated according to their size in a horizontal electrophoresis. 0,5X TBE was used as running buffer. The 100bp or 1Kb DNA ladder were used as molecular size standards. Gels were analyzed under UV light.

2.2.4 Cleaning PCR products and cloning in Teasy vector

PCR products were separated by electrophoresis. Target bands were identified under UV light by comparing to a molecular size standard and cut with surgical knife. DNA was recovered out of the gel with a Nucleospin Extract Kit according to the manufactures and measured. PCR products were cloned in a Teasy Vector in a total volume reaction of 10 µl and ratio 3:1 (insert: vector), according to the manufactures. Ligation was performed overnight at 4° C. For bacteria transformation, 5 µl of the ligation product was used.

2.2.5 Teasy vector restriction digestion

Restriction digestions of the Teasy vector were performed in a 10-50 µl total volume with the suitable restriction enzyme (5U pro 1 µg DNA), restriction buffer, and temperature. Digestion products were separated by electrophoresis. DNA was recovered like mentioned above, measured, and cloned into the U3 and IAL plasmids according to the following reaction conditions:

- X µl DNA. Ratio 5:1 insert:vector
- X µl plasmid (50-60 ng)
- 1 µl T4 DNA Ligase (2000 U/ml)
- 1 µl 1X T4 DNA ligase Reaction Buffer
- ad. 10 µl H_2O

Ligation was performed overnight at 16° C. For bacteria transformation, 5 µl of the ligation product was used.

2.2.6 Transformation and Plasmid DNA isolation from bacteria

Fifty microliters Escherichia coli XL10-Gold competent bacteria (Stratagene) were transformed with the ligation reactions according to the instructions manual. After addition of 0,5 ml of preheated (42° C) NZY^+ broth, transformed bacterias were plated on ampicillin agar plates, and incubated overnight at 37° C. Single colonies were selected and grown overnight in 5 ml LB-Ampicillin Medium until saturation. Two mililiters of the saturated LB culture were used for the isolation of the plasmid DNA (NucleoSpin Plasmid), according to manufacturers instructions. Plasmid DNA was submited to restriction digestion to identify the right clones.

2.2.7 Creation of a single-chain murine IL-12 cDNA

For the construction of the single-chain murine IL-12, the p40 and p35 subunits were connected by a 54 bp linker.

2.2.7.1 Synthesis of the p40 cDNA

The p40 subunit cDNA (accession number: M86671) was obtained by PCR using total cDNA from an activated murine macrophage line, and the p40fwd30 and p40rev30 primers. The resulting 1008 bp PCR product was used as template for a second PCR, using the P40fwdSrfI (including a Srf I restriction site and a Kozak sequence: CGCCACC), and the p40rev30 primer. The 1027 bp PCR product, containing a Srf I restriction site at the 5'-end was cloned into Teasy vector and sequenced.

2.2.7.2 Synthesis of the p35 cDNA

The p35 subunit cDNA (accession number: M86672) was obtained by PCR using the IL-12p35fwd and IL-12p35rev primers. The resulting 648 bp PCR product was used as template for a second PCR, using the IL-12p35fwd and IL-12p35rev2 (including a Sal I restriction site). The 658 bp PCR product, containing a Sal I restriction site at the 3'-end was cloned into Teasy vector and sequenced.

2.2.7.3 Synthesis of the linker

The 54 bp linker (GGC AGT ACT TCG GGC AGT GGT AAG CCT GGT AGT GGT GAG GGT AGT ACT AAG GGT, coding for the following aminoacids sequence:Gly Ser Thr Ser Gly Ser Gly Lys Pro Gly Ser Gly Glu Gly Ser Thr Lys Gly) was obtained by PCR using the pscIL-12.2 plasmid (donated by Dr. Lee, University of Taiwan) as template, and the p40SbfI and p35PflMI primers. The 99 bp PCR product contained, in 5'-3' orientation: the last 22 bp of the p40 cDNA, which includes a Sbf I restriction site, and in which the stop codon was omitted; the linker; and the base pair 67 until 90 from the p35 cDNA, which includes a PflM I restriction site. The 99 base pairs PCR product was cloned into Teasy vector and sequenced.

2.2.7.4 Connection of all the components

To construct the single-chain IL-12, the p35 subunit was recovered from Teasy vector by digestion with Pflm I and Sal I, and cleaned by electrophoresis. The Teasy vector containing the linker was opened by digestion with PflM I and Sal I, and the p35 subunit was cloned at the 3'-end of the linker. Subsequently, the linker-p35 subunit was recovered by digestion with Sbf I and Sal I and cleaned by electrophoresis. Finally, after the Teasy vector containing the p40 subunit was opened by digestion with Sal I and Sbf I, the linker-p35 subunit was cloned at the 3'-end of the p40 subunit. A scheme of the cloning procedure is presented in figure 12 (Results).

2.2.8 Creation of a single-chain murine IL-23 cDNA

For construction of the single-chain murine IL-23, the p40 and p19 subunits of the murine IL-23 were connected by a 30 bp linker following this orientation: p40-linker-p19.

2.2.8.1 Synthesis of the linker-p19 subunit

The p19 subunit cDNA (accession number: NM_031252) was obtained by PCR using the primers p19fwd and p19Sal, which introduced a Sal I restriction site at the 3'-end. The 30 base pair linker (GTG CCT GGC GTG GGC GTG CCT GGC GTG GGC, coding for the following aminoacids sequence: Val Pro Gly Val Gly Val Pro Gly Val Gly) was connected to the p19 subunit by sequential PCR like follows. The p19 cDNA was used as template for a PCR using the primers p19SalI and p19val. The p19val primer included, in orientation 5'-3', the 30 bp of the linker and 21 bp of the p19 subunit, starting in codon 23. Therefore, this PCR deleted the first 22 codons of the p19 subunit and connected the linker to the p19 subunit. The 570 bp PCR product was used as template for a second PCR using the primers p40SbfIB and p19SalI. This PCR elongated the linker-p19 subunit at the 5'-end with the last 21 bp of the p40 subunit cDNA, which includes a SbfI restriction site and omits the stop codon. This last 591 bp PCR product was cloned into Teasy vector and sequenced.

2.2.8.2 Connection of the p40 and linker-p19 subunits

To construct the single-chain IL-23, the linker-p19 subunit was recovered from the Teasy vector by digestion with Sbf I and Sal I, and cleaned by electrophoresis. The Teasy vector containing the p40 subunit was opened by digestion with Sal I and Sbf I, and the linker-p35 subunit was cloned at the 3'-end of the p40 subunit. A scheme of the cloning procedure is presented in figure 13 (Results).

2.2.9 Cloning scpIL-12 and scpIL-23 into U3 and IAL vectors

The U3 vector was opened by digestion with Sma I, that created blunt ends, treated with alkaline phosphatase (New England Bio Labs) for 60 min. at 37^{o} C, and cleaned by electrophoresis. Subsequently, the scpIL-12 and scpIL-23 were recovered from the Teasy vector by a double digestion with Sma I and Hinc II (for scpIL-12) and with Srf I and Hinc II (for scpIL-23) and posterior electrophoresis. Finally, scpIL-12 and scpIL-23 were cloned separately into U3 to create U3-IL-12 and U3-IL-23. The IAL vector was opened by double digestion with Srf I and Sal I, and cleaned by electrophoresis. Subsequently, the scpIL-12 and scpIL-23 were recovered from the Teasy vector by a double digestion with Srf I and Sal I, and

cleaned by electrophoresis. Finally, scpIL-12 and scpIL-23 were cloned separately into IAL to create IAL-IL-12 and IAL-IL-23.

2.2.10 Construction of a pcDNA3-Cre-Recombinase plasmid

For generation of a plasmid expressing the Cre Recombinase protein, we decided to clone the cDNA of the Cre Recombinase into an expression plasmid. The Cre Recombinase cDNA (accession number: X03453) was obtained by PCR with the primers Crefwd and Crerev and using genomic DNA from Cre-Recombinase transgenic mice (FVB/Actin-Cre transgenic mice, animal facility of the University Hospital Mainz) as template. The 1031 bp PCR product was cloned into Teasy vector and sequenced. As expression plasmid we chose pcDNA3.1(+) (Invitrogen). This plasmid allows a high-level constitutive expression controlled by a human cytomegalovirus promoter. The cDNA for the Cre-Recombinase was recovered from the Teasy vector by digestion with EcoR I and cloned into the EcoR I-digested pcDNA3.1(+) plasmid in position 5'-3'.

2.2.11 Generation of the IL-12 transgenic mice

2.2.11.1 Preparation of the injection-DNA

All buffers were prepared in sterile non-pyrogenic water (B.Braun, Melsungen). The IAL-IL-12 plasmid was digested with SfiI (5 U/mg DNA) to separate the pBR322*3 bacterial plasmid from the IL-12 expression cassette. Digestions were subjected to ethidium bromide agarose gel (1%) electrophoresis in 1xTAE buffer. Gel slices containing the expression cassettes were cut out under UV light with a sterile surgical knife and transferred into Pyrogen/DNAse/RNAse free tubes (Eppendorf, Hamburg). DNA fragments were isolated out of the gel pieces with GeneClean II. The concentration of the isolated DNA was measured at 260 nm (GeneQuant *Pro*). Final concentration of injection DNAs (2 ng/ml) was achieved by comparison to known quantities of DNA molecular standard, in 1% ethidium bromide stained agarose gels. After centrifugation at 14000 rpm for 30 min, 2/3 of the whole volume from the middle of the tube was preserved, aliquot, and stored at –20^{o}C for pronucleus microinjections.

2.2.11.2 Breeding of transgenic mice

IL-12 transgenic mice were generated according to standard procedures by microinjection of the generated DNA construct into fertilized eggs of FVB/NHSD mice, performed by Dr. Reifenberg (Animal Facility, Uni-Klinik Mainz). The potential founders were identified by

transgenic specific PCRs. Founder animals were bred with FVB/NHSD non-transgenic mice to generate the different transgenic lines. Transgenic offspring from those crossing were bred with homozygotic Actin-Cre +/+ transgenic mice for induction of constitutive IL-12 transgenic expression. Genotyping was performed routinely by specific PCRs.

2.2.11.3 IL-12 transgenic specific PCR

For identification of IL-12 transgenic mice, we used two different transgenic specific PCRs (Fig. 6). The first one was performed with primers PArev (sitting in the polyA sequence situated at the 3'-end of the IL-12 cDNA) and LKfwd (sitting in position 930-945 bp of the p40 subunit), by 60^{o} C annealing temperature and producing a 1060 bp PCR product. The second one was performed with primers IL-12p35rev (sitting in the 3'-end of the p35 subunit) and p40fwd3 (sitting in position 900-915 bp of the p40 subunit), by 58^{o} C annealing temperature and producing a 740 bp PCR product.

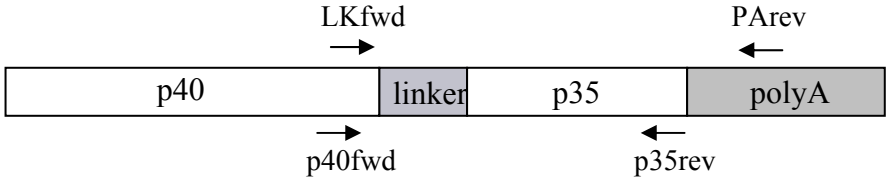


Figure 6. Primers location and PCR products for identification of the IL-12 transgenic mice

2.2.12 Actin-Cre specific PCR

Actin-Cre transgenic mice were identified by a specific PCR, using the primers Cre Ia and Cre IIb at 58^{o} C annealing temperature, and producing a 350 bp band.

2.2.13 RNA isolation

Liver samples (50-100 mg) were homogenized in Lysing-Matrix-D tubes using 1 ml TriReagent according to the manual instructions. RNA was dissolved in RNAse free water, measured in GeneQuantpro and frozen in –80^{o} C. Quality of isolated RNA was determined with the presence of the 18S and 28S RNA bands by electrophoresis in a 1% agarose gels.

2.2.14 Reverse Transcription (RT) and IL-12 mRNA-specific PCR

Reverse transcription of μg RNA was performed with the First Strand RT-PCR kit according to the manufacturers. Oligo-dt-primers were preferentially used. To determine IL-12 mRNA transcripts, the cDNA was used as template for a PCR using the forward primers RT1 (sitting

in the Albumin promoter), and the reverse p40seqrev (sitting in the p40 subunit of the IL-12). PCR was run at 56° C annealing temperature. PCR products corresponding for IL-12 mRNA have a size of 750 bp, while PCR products corresponding to the genomic DNA have a size of 1300 bp -corresponding the difference to the 450 bp of the β-Globulin intron (Fig. 7). A beta-actin specific PCR was performed in parallel as control for total used cDNA. This PCR was performed with the primers beta-actinfwd and beta-actinrev, at a 58° C annealing temperature.

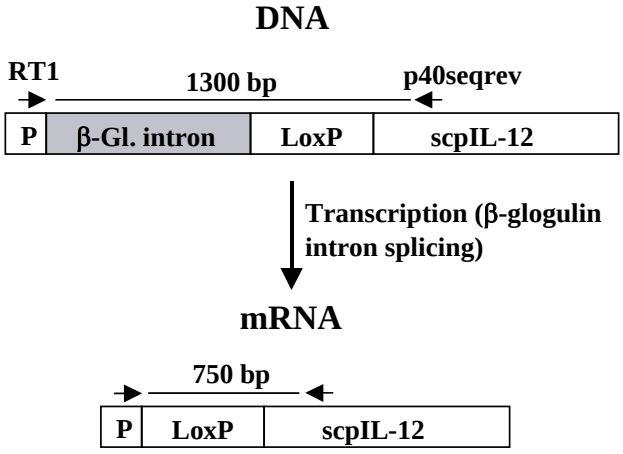


Figure 7. Primer location and PCR products for identification of IL-12 mRNA transcripts

2.2.15 IL-12 mRNA transcripts quantification (real-time PCR)

Total RNA from the liver was isolated using Tri-reagent as described above. For the real-time PCR the cDNA was preferentially synthesized using the 1st-Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, Mannheim) and oligo-p$(dT)_{15}$ primers. To quantify the IL-12 mRNA transcripts a LightCycler instrument (Roche, Mannheim) was used. β-actin house-keeping gene expression served as the reference in relative quantification. Reaction consisted of both 1mM IL-12 specific forward (p40fwd2) and reverse (IL-12revLC) primers, 3 mM $MgCl_2$, 1x LightCycler-FastStart DNA Master SYBR Green I and 2 µl 1/5 distilled water diluted cDNA sample in 20µl reaction volume. Identical reactions were carried out separately for every cDNA with β-actin specific primers forward (B-actinLCI), and reverse (B-actinLCII). All the reactions were run in duplicate. PCR conditions were 95^0 C for 10 min and 45 cycles at 95^0 C for 10 s, 60^0 C for 5 s and 72^0 C for 10 s. Product specificity was confirmed by melting curve analysis and in 1% ethidium bromide agarose gels. LightCycler software calculated crossing point (C_p) values for β-actin and the studied genes were used to compute the relative gene expression ratios according to the DDC method.

2.2.16 Protein isolation from the liver

Liver samples were homogenized with protein lysis buffer in Lysing-Matrix-D tubes. After 30 min incubation at 4° C, cell debris was pellet by centrifugation. Protein concentration in supernatants was measured according to the Bradford method and kept at –80° C until use.

2.2.17 Western Blot

Proteins were first separated according to their size by SDS-Polyacrylamide gel electrophoresis. Briefly, protein samples were loaded together with 2X loading buffer into a stacking gel, and separated through a SDS-10% Polyacrilamid Gel by vertical electrophoresis for 2 h at 150 Volts. Proteins were transferred from gel to a Polyvinylidene fluoride (PVDF) membrane by the semi-dry electrophoresis method. Briefly, membrane was equilibrated first with 100% Methanol for 2 min, and then with transfer buffer for 15 min. Gel and membrane were placed between 4 Whatman-paper sheets (saturated with transfer buffer), with the membrane placed on the side of the gel facing the anode, and transferred during 2 h at 150 mAmp. Membrane was blocked with 2% low-fat dry milk in 1% PBS-0,05% Tween 20 during 2 h at RT, and shaking. For detection of IL-12, membrane was incubated overnight at 4° C with the first antibody (goat anti mouse IL-12p70 polyclonal antibody, R&D), diluted 1:500 in 0,5% low-fat dry milk in 1% PBS-0,05% Tween 20. After washing with 1% PBS-0,05% Tween 20, the bound first antibody was detected by incubating the membrane with 1:2000 diluted rabbit anti goat immunoglobulins HRP-antibody (DAKO) for 1 h at RT. Finally, IL-12 was detected by chemiluminescent (ECL Plus Kit) and autoradiography.

2.2.18 Serum isolation

Mice were anaesthetized with 2,5% Tribromoethanol (Fluka) and blood was collected by heart puncture. After 30 min. coagulation at room temperature, blood was centrifuged at 5.000 rpm for 5 min, and serum was collected and stored at –20° C until use.

2.2.19 ELISA

Mouse IL-12 (p70) and mouse IL-17 ELISAs were performed according to the manufactures. The mouse IFN-γ ELISA was performed according to the following protocol: plates were coated overnight at 4° C with anti-mouse IFN-γcapture antibody (R46A2, generated in the department of Immunology of the Uni-Klinik Mainz), diluted 1:500 in coating buffer. After blocking with assay diluent 30 min at 37° C, IFN-γ standard and samples were diluted in assay diluent, and incubated 1h at 37° C. For detection plates were incubated 1h at 37° C with a

biotinylated anti-mouse IFN-γantibody (AN18.17.2466 generated in the department of Immunology of the Uni-Klinik Mainz), diluted 1:1000 in assay diluent; 30 min at 37° C with avidine-HRT (diluted 1:10000 in assay diluent); and 10-30 min, RT in dark, with Substrate Solution. Reaction was stop with Stop Solution, and read at 450 nm. The mouse IL-4 ELISA was performed according to the following protocol: plates were coated overnight at 4° C with purified rat anti-mouse IL-4 capture antibody (clone 11B11, Pharmingen), diluted 1:500 in coating buffer, and blocked 2h at 37° C with blocking solution. Plates were incubated 2 h at 37° C with detection antibody (1:500, biotinylated rat anti-mouse IL-4 monoclonal antibody, clone BVD6-24G2, Pharmingen), IL-4 standard (recombinant mouse IL-4, Pharmingen), and samples, all together diluted in assay diluent. For detection, plates were incubated 30 min with avidine-HRT (diluted 1:10000 in assay diluent), and 10-30 min, RT in dark, with Substrate Solution. Reaction was stop with Stop Solution and read at 450 nm with correction at 540 nm. For all the ELISAs, plates were washed three times after incubations.

2.2.20 Adenovirus

2.2.20.1 Propagation in 293 Cells

AdIL-12, a type-5 adenovirus encoding a cDNA sequence for murine IL-12, was a gift from Jesus Prieto (University of Pamplona, Spain). AdLacZ, a type-5 adenovirus encoding a cDNA sequence for Escherichia Coli β-gal reporter gene, was a gift from Jürgen Siebler (Uni-Klinik, Mainz). Type-5 adenoviruses are replication-incompetent serotype-5 adenoviral vector, driven by the cytomegalovirus immediate-early promoter. Both viruses were propagated on 293 cells and purified according to the following protocol: 293 cells were propagated on 550 ml Tissue Culture Flasks with 10% FCS-DMEM. When cells were 80% confluence, adenoviral infection was performed with a MOI of 5 in 10 ml 2% FCS-DMEM at 37° C and 5% CO_2. After 30 min incubation, 15 ml 2% FCS-DMEM was added, and flasks were kept in culture for 2-3 days. After mechanical detachment, cells were collected and pellet by centrifugation at 600 g for 5 min and 4° C. Pellets were kept at –80° C until purification.

2.2.20.2 Purification by discontinuous Cesium Chloride Gradient

Cell breakage and virus release was performed by five cycles of freezing (liquid Nitrogen)/thawing (37° C) with vortex. Pellets were resuspended in 20 ml DMEM and centrifuged at maximum speed for 10 min and 4° C. Supernatant was removed for virus purification. Cesium Chloride gradient was performed in polyallomer tubes with 8 ml of CsCl

1.4 overlaid with 6 ml of CsCl 1.2. Supernatant containing viral particles were centrifuged through the Cesium Chloride gradient at 100 000 g for 90 min at 4° C. A bluish white band containing concentrated viral particles was collected and submitted to a second purification through a NAP column. Briefly, the column was first equilibrated three times with buffer and loaded with the concentrated virus. Finally, purified and concentrated virus was recovered from the column with 1,5 ml buffer. Viral particle titration was calculated and viral suspension was kept in –80° C until use.

2.2.20.3 Viral particle titration by the $TCID_{50}$ (tissue culture infectious dose 50) Method

293 cells (10^5 cells/ml) were plated in a cell culture 96 wells plate. Increasing exponential diluted virus in 5% FCS-DMEM were incubated with cells for 10 days. The highest dilution at which a cytopathic effect (CPE) is present, determined the viral titration.

2.2.21 Hepatocytes isolation and adenoviral gene transfer

The abdominal wall of anesthetized (2,5% Avertin) mice was opened under steril conditions. The liver was first perfused with 100 ml (20 ml/min) buffer I (37° C) through the portal vein and was subsequently with 50 ml buffer II (0,05% collagenase). Whole liver was removed from the abdominal cavity and placed in buffer III (37° C). After mechanical dissection and filtration through a gage, the cell suspension was centrifuged at 30g for 5 min. To remove residual nonhepatocytes from the hepatocyte preparation, cells were then incubated with monoclonal antibodies: 3.155 (anti-CD8, against T cells), F4/80.1.15 (against mononuclear phagocytes), B17 (anti-CD13, against Mast cells), RA3-3A1/6.1 (anti B220, against B cells), M1/70.15.11.5.HL (anti-Mac1, against macrophages) and MAR18.5 (against rat immunoglobulin k chains, and rabbit complement for 20 min at 37° C. After centrifugation, hepatocytes were resuspended in DMEM 10% FCS and cultured in 24-well plates, 5 X 10^4 per well. After 1 day of culture, hepatocytes were transfected with AdIL-12 or AdLacz (MOI 10) in 2% FCS-DMEM for 1 h, and after carefully washing with PBS, FCS was replaced by 0,2% bovine serum albumin (BSA) before T-cell stimulation.

2.2.22 CD4 T-cell isolation and stimulation

CD4 T-cells were isolated from the spleen. Briefly, spleens were mechanically dissociated and after centrifugation (400 g for 5 min), primary $CD4^+$ T-cells were separated by MACS (Milteny Biotec, Gladbach, Germany) according to the manufacturer's instructions. For specific stimulation, 10^6 CD4 T-cells from OVA323-339-specific T-cell Receptor transgenic

mice (Murphy et al., 1990) were incubated with 5 X 10^4 hepatocytes from CIITA-transgenic mice, and 5 mg/ml of ovoalbumin peptide (ISQAVHAAHAEINEAGR; Sigma-Genosys, UK). After 5 days of culture, T-cells were recovered, washed, and re-stimulated. For re-stimulation, 24 wells plates were coated with anti-CD3 (1 mg/ml) and 1 X 10^6 T cells were incubated overnight. IFN-γ and IL-4 cytokines were measured in supernatants by ELISA.

2.2.23 Hepatoma Cell Line Transfection

The Hepa 1.6 cell line was platted on a 24 wells plate with an 80% confluence (150000 cells/well), and transfected with one of the following plasmids: U3, U3-IL-12 or U3-IL-23. A green fluorescent plasmid (GFP, donated by Prof. Neurath Uni-Klinik Mainz) was used to check, under a fluorescent microscope, the efficiency of the transfection. Transfection was performed with Lipofectamine-Plus Reagent (Invitrogen) and according to the manufacturer's protocol. The day after transfection, cells were washed, supplied with 10% FCS-DMEM, and incubated for another 3 days. After incubation, supernatant were collected, centrifuged at 10.000 rpm to eliminate cell debris, and stored at –20^o C until use in ELISAs or bioassays. Transfection efficiency was stimated in 60-70%.

2.2.24 IL-12 bioassay

Splenocytes were isolated from the spleens of FVB mice by mechanical dissociation and centrifugation (400 g for 5 min). Splenocytes were cultured in DMEM 10%-FCS in 24-well plates, 2 X 10^6 / well. The culture was supplied with 50 U/ml mrIL-2 (Pharmingen), and/or with the supernatants from U3, U3-IL-12, or non-transfected cells to a 25% final concentration (750 ml DMEM 10%-FCS and 250 ml supernatant). Mouse recombinant IL-12 (Pharmingen), 10 ng/ml, was used as positive control. After 3 days of culture, cell-free supernantants were collected and tested for mouse IFN-γ by ELISA.

2.2.25 IL-23 bioassay

CD4+ T-cells were isolated from the spleen of FVB mice like mentioned before. Cells were cultured at a concentration of 2 X 10^6 / well in DMEM 10%-FCS in 24-well plates previously coated with 1 mg/ml anti-mouse CD28 (Pharmingen). The culture was supplied with 50 U/ml rIL-2, 5mg/ml anti-mouse CD3 (Pharmingen), and/or with the supernatants from U3, U3-IL-23, or non-transfected cells to a 25% final concentration (750 ml DMEM 10%-FCS and 250 ml supernatant). After 3 days of culture, CD4 T-cells were re-stimulated for 2 days with 1

mg/ml anti-mouse CD3 and cell-free supernatants were collected and tested for mouse IL-17 by ELISA.

2.2.26 Tissue preparation and H&E Staining

After mouse sacrifice, liver was fixed in 4% PFA for 3 days. Fixed livers were subjected to the following incubations: 2 h in 30% Ethanol, 2 h in 50% Ethanol, 2 h in 70% Ethanol, 2 h in 90% Ethanol, twice 2 h in 100% Ethanol, twice 2 h in Xylol and overnight in melted Paraffin. Infiltrated tissues were placed into a block with melted paraffin and allowed to cool and solidify before making tissue sections. Before staining, tissue sections were subjected to the following incubation protocol: twice 10 min in Xylol, 5 min in 100% Ethanol, 5 min in 90% Ethanol, 5 min in 70% Ethanol, 5 min in 50% Ethanol, 5 min in 30% Ethanol and 5 min in H_2O. For staining, tissue sections were incubated in hematoxilin during 1-2 min, washed in running water for 10 min, incubated in 0,1% Eosin during 30 sec and again washed in water. Subsequently, tissue sections were subjected to the following incubation protocol: 5 sec in 70% Ethanol, twice 5 min in 96% Ethanol, 5 min in 100% Ethanol and twice 5 min in Xylol. Finally, tissue sections were covered with entellan.

2.2.27 *In vivo* experiments

For *in vivo* infection of mice, AdIL-12 and AdLacZ (both 2 X 10^8 pfu) were injected in the tail vain. At different times after infection, blood was collected from the tail vain, serum gained, and stored at –20^o C until use. The presence of viral particles was determined by PCR. Fifty nanograms of DNA isolated from the liver were used as template and Advfw1 and Adrev1 as adenovirus specific primers. PCR was run at 62^o C and a 450 bp PCR product indicated the presence of viral DNA in the liver.

For *in vivo* induction of the transgenic expression, mice weighted between 20 and 25 grams were pre-warmed, and 1,6 ml steril PBS containing 10 mg of pcDNA3$^+$-Cre Recombinase expression plasmid was injected the tail vain in 5-6 seconds.

3. Results

3.1 IL-12 induces an inflammatory response on CD4$^+$ T-cells stimulated by hepatocytes

To determine, in vitro, the type of immune response after CD4+ T cell stimulation by hepatocytes, hepatocytes from CIITA-transgenic mice, which express MHCII molecules were used as APC. Primary ovalbumin-specific CD4+ T-cells were stimulated, in the presence of OVA peptide, by CIITA hepatocytes infected with Adenovirus-IL-12 (adenovirus coding for murine interleukin-12) or with Adenovirus-LacZ (adenovirus coding for bacterial β-galactosidase) as control. The character of the primary T-cell response was determined by measuring the amount of secreted interferon-gamma and interleukin-4 (Fig. 8). After 5 days of culture and 24 h restimulation, T-cells stimulated by non-infected CIITA hepatocytes and AdLacZ-infected CIITA hepatocytes produced only low amounts of interferon-γ (50 and 100 U/ml respectively) but relatively high amounts of interleukin-4 (600 and 350 U/ml respectively). The presence of interleukin-12 produced by AdIL-12-infected CIITA hepatocytes enhanced the production of interferon-γ (2400 U/ml) and slightly reduced the production of interleukin-4 (250 U/ml) by stimulated T-cells.

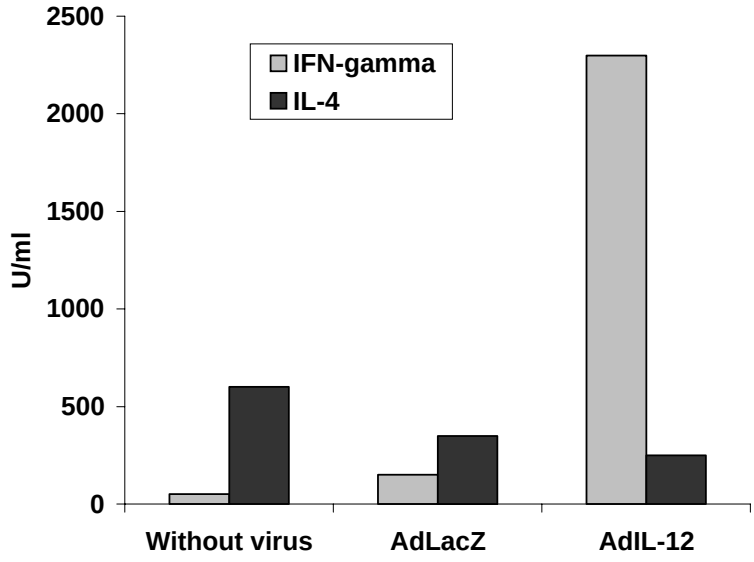


Fig.ure 8. Influence of IL-12 on the type of immune response. Hepatocytes were infected with Adenovirus IL-12 or with Adenovirus-LacZ as control. CD4 T-cells stimulated by non-infected or AdLacZ-infected hepatocytes produced interleukin-4 and negligible amounts of IFN-γ. Enhanced production of IFN-γ and slightly decreased production of interleukin-4 was observed when CD4+ T-cells were stimulated in the presence of IL-12.

3.2 Interleukin-12 induces, *in vivo*, strong inflammation and rapid virus elimination in the liver

To determine the influence of interleukin-12 on the course of viral infection *in vivo*, mice were injected, i.v., with 2 X 10^8 pfu AdIL-12 or AdLacZ as control. The levels of serum transaminases (ALT, Fig.9A; and AST, Fig. 9B) were measured to determine the severity of hepatocyte damage caused by the adenoviral infection in the presence or absence of interleukin-12. Five days after infection, mice infected with AdLacz or AdIL-12 had similar levels of ALT and AST. However, at day 7 after infection, mice infected with AdIL-12 demonstrated significant higher levels of transaminases (AST: 2860 vs. 2000 U/ml; ALT: 2400 vs. 1580 U/ml: p<0,001) than mice infected with AdLacz. At day 10 after infection, the levels of ALT and AST were almost similar again in mice infected with AdLacZ or AdIL-12.

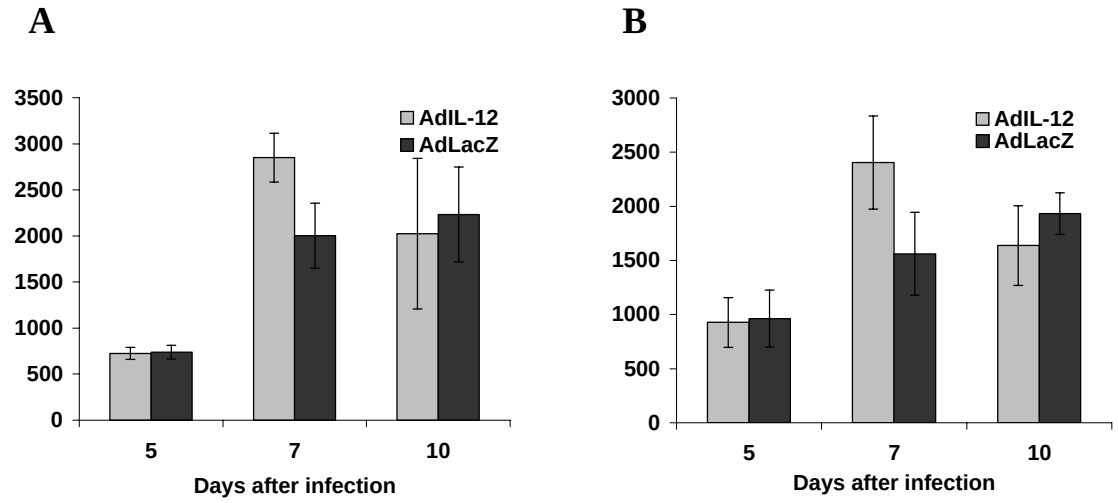


Figure 9. ALT (A) and AST (B) levels after Adenoviral infection. 8-10 weeks old mice were injected in the tail vain with Adenovirus-LacZ or with Adenovirus-IL-12. Serum was gained at days 5, 7 and 10 after infection. Only at day 7 after infection, mice injected with AdIL-12 had higher levels of AST and ALT than mice infected with AdLacZ. Number of mice per group: 5; p<0,001.

When comparing the liver histology at day 7 after infection, mice infected with AdIL-12 (Fig. 10A) showed stronger inflammation with massive periportal cellular infiltrations and more apoptotic hepatocytes than mice infected with AdLacZ (Fig. 10B)

To determine whether the stronger inflammation and cellular infiltrations in mice infected with AdIL-12 had an influence on viral clearance, the presence of viral particles in the liver was assed by adenovirus-specific PCR. Five days after infection, both, LacZ and IL-12 adenovirus were present in the liver (fig. 11A). At day 7 after infection (fig. 11B), AdIL-12 could not be detected by PCR, indicating the successful viral clearance. In contrast, AdLacz

was still detected on day 10 after infection (fig. 11C). These findings indicated that the presence of interleukin-12 induced a more severe hepatitis and faster adenovirus elimination.

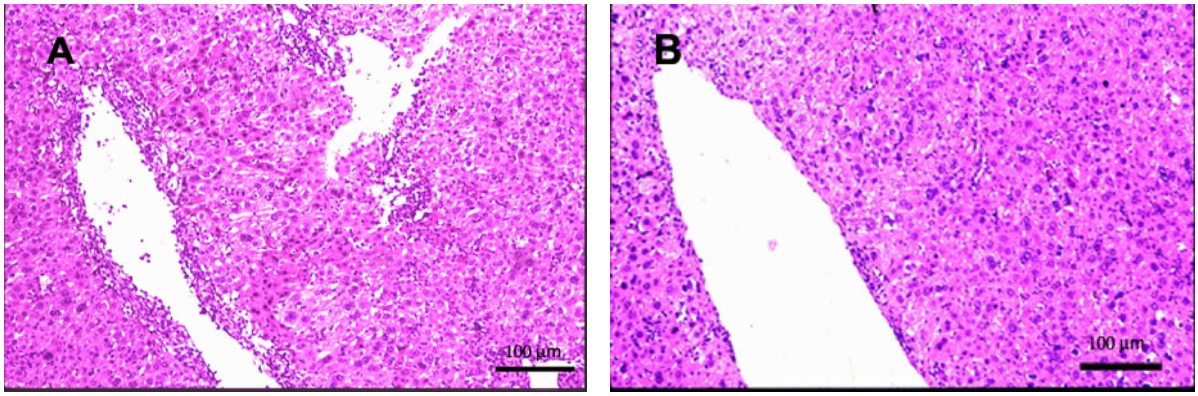


Figure 10: Liver histology 7 days after infection with AdIL-12 (A) or with AdLacZ (B). The livers of mice infected with AdIL-12 showed more apoptotic hepatocytes (black arrow), more periportal infiltrations, and generally a more disturbed liver structure when compared to mice infected with AdLacZ. x100

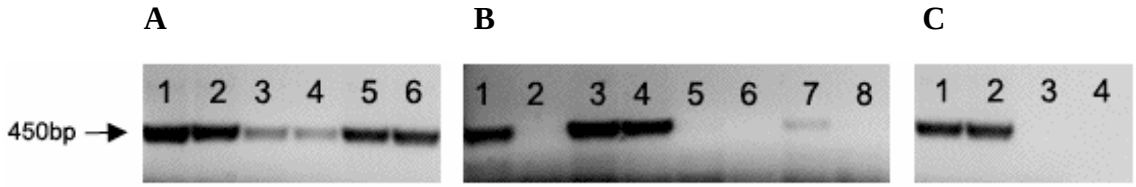


Figure 11. DNA isolated from the livers of mice infected with AdIL-12 or AdLacZ was used for adenoviral-specific PCR. A 450 bp PCR product indicated the presence of viral DNA. Figure A shows the presence of adenoviral DNA in AdLacZ (1, 2, 3) and AdIL-12 (4, 5, 6) infected mice 5 days after infection. Seven days (B) or 10 days (C) after infection, adenoviral particles were still present in mice infected with AdLacZ (B:1,3,4; C:1,2), but not in mice infected with AdIL-12 (B:5,6,7,8; C:3,4).

3.3 Construction of a single-chain murine IL-12 cDNA

In previous experiments, it had been found that vectors containing separate p35 and p40 subunits secreted heterodimeric IL-12 as well as large amounts of free-form p40 protein, which antagonized the activity of IL-12 (Lee et al., 1998). To achieve an equal expression of the p35 and p40 subunits, and prevent the formation of free p40, an IL-12 fusion gene was constructed. For this, the p40 and the p35 subunits were connected by a linker (see material and methods) following this orientation: p40-linker-p35, and with deletion of the stop codon of the p40 subunit and the first 22 codons of the p35 subunit. The p40 and p35 subunits were obtained by PCR. After creation of a Srf I restriction site at the 5'-end by PCR, the p40 subunit was cloned into T-easy vector. After creation of a Sal I restriction site at the 3'-end by

PCR, the p35 subunit was cloned into T easy vector. The 54 bp linker, containing a Sbf I restriction site at the 5'-end and a PflM I restriction site at the 3'-end, was created by PCR and cloned into Teasy vector. For the final construction of the single-chain IL-12, the p35-Teasy vector was digested with PflM I and Sal I, and cloned into the PflM I/Sal I digested Teasy-linker vector. Subsequently, the so generated Teasy-linker-p35 vector was digested with Sbf I and Sal I, and the linker-p35 subunit was cloned into the Sbf I/Sal I digested Teasy-p40 vector. A scheme of the cloning procedure is shown in figure 12.

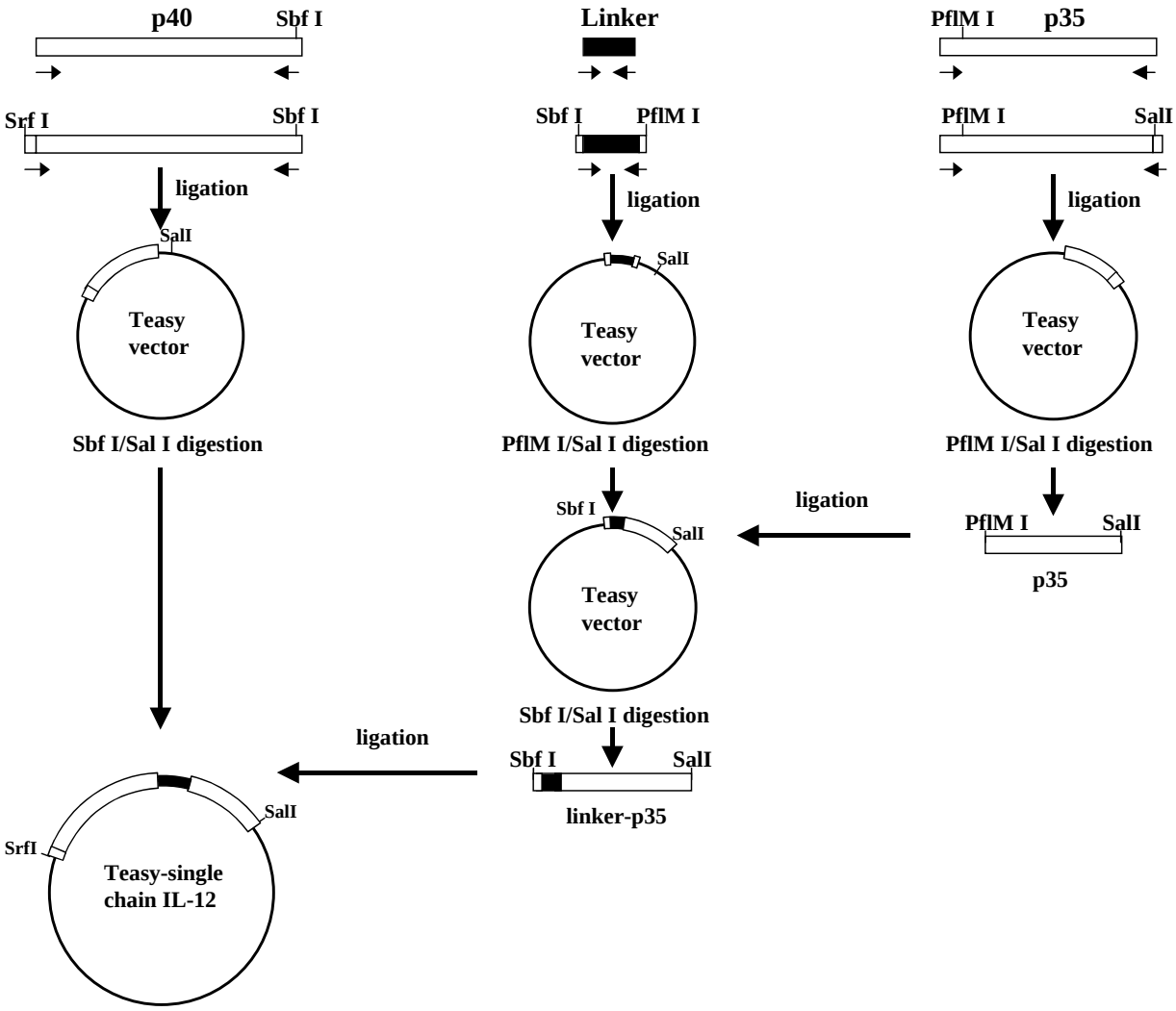


Figure 12. Construction of the murine single-chain IL-12 gene. Each subunit of IL-12 (p40 and p35) and a linker were amplified separately by PCR and, after creation of the different restriction sites, were cloned into Teasy vectors. After digestion with PflM I and Sal I, the p35 subunit was ligated to the 3'-end of the linker. Then, the linker-p35 subunit was recovered from the Teasy vector by digestion with Sbf I and Sal I and ligated to the 3'-end of the p40 subunit.

3.4 Construction of a single-chain murine IL-23 cDNA

According to the possibility that free p40 might also antagonizes the effects of IL-23, an equal expression of the p19 and p40 subunits was desired. Thus, an IL-23 single chain protein was constructed. For that purpose, the p40 and the p19 subunits were connected by a linker (see material and methods). The p19 cDNA was obtained by PCR. After creation of a Sal I restriction site at the 3'-end by PCR, the 30 base pair sequence linker was obtained by sequential PCR from the p19 subunit, starting at the 5'-end and omitting the first 22 codons. After the last 22 base pair of the p40 cDNA (which includes a Sbf I restriction site, and in which the stop codon was omitted) were added at the 5'-end by PCR, the linker-p19 was cloned into Teasy vector. The single-chain IL-23 was generated with the p40 and p19 subunit in this order: p40-linker-p19. For this purpose, the linker-p19-Teasy vector was digested with Sbf I and Sal I and cloned into the Sbf I/Sal I digested Teasy-p40 vector. A scheme of the cloning procedure is shown in figure 13.

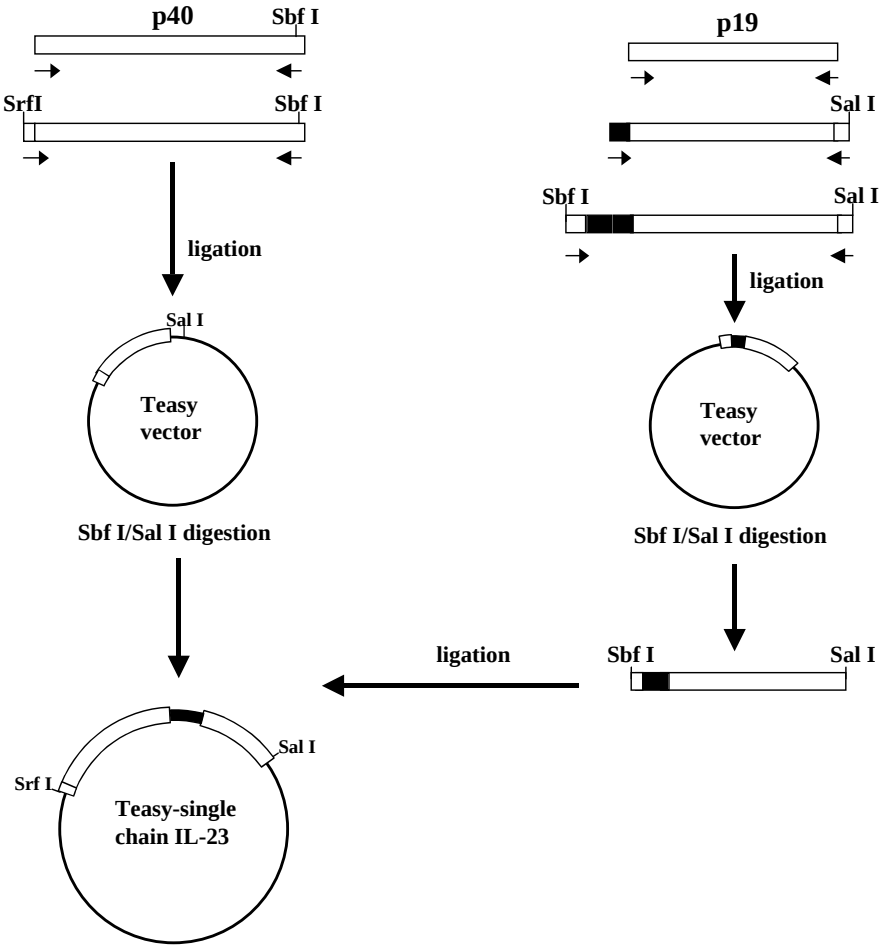


Figure 13. Construction of the murine single-chain IL-23 gene. The linker and p19 subunits were synthesized together by sequential PCR and cloned into Teasy vector. After digestion with Sbf I and Sal I, the linker-p19 subunit was ligated to the 3'-end of the p40 subunit.

3.5 Cloning of the murine scpIL-12 cDNA or scpIL-23 cDNA into the expression vectors U3 and IAL

The scpIL-12 or scpIL-23 were cloned independently in two different vectors. For the constitutive expression of IL-12 or IL-23, the U3 vector was used. U3 vector allows a liver specific gene expression through the Albumin Promoter/Enhancer. IL-12 and IL-23 were digested with Sma I and Hinc II, and cloned separately into the Sma I digested U3 expression vector in the correct open reading frame orientation between the β-globulin intron and the poly-A sequence (Fig. 14) to obtain U3-IL-12 or U3-IL-23. For the inducible expression of IL-12 or IL-23, the IAL expression vector was used. IAL allows a liver specific gene expression through the Albumin Promoter/Enhancer, and an inducible expression through the LoxP sites. Teasy-IL-12 and Teasy-IL-23 were digested with Srf I and Sal I, and cloned separately into the Srf I/Sal I digested IAL expression vector in the correct open reading frame orientation between the second LoxP site and the poly-A sequence (Fig. 15) to obtain IAL-IL-12 or IAL-IL-23.

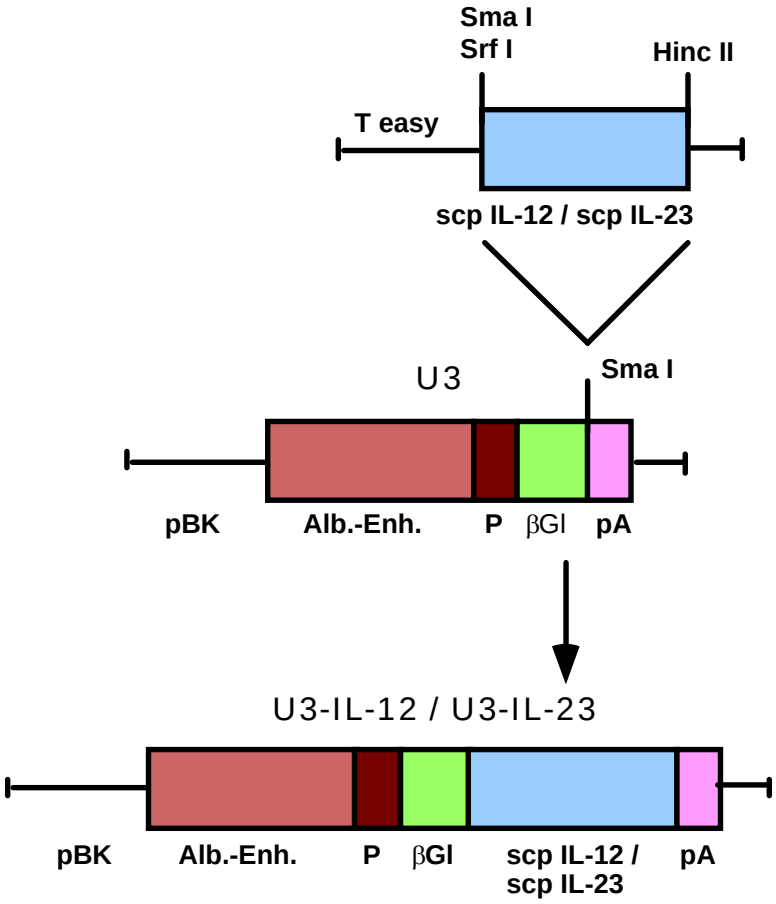


Figure 14. Construction of the U3-IL-12 and U3-IL-23 expression vectors. Single-chain IL-12 or single-chain IL-23 were recovered from the Teasy vector by digestion with Sma I (IL-12) or Srf I (IL-23) and Hinc II (IL-12 and IL-23), and cloned separately into the U3 expression plasmid. P: Albumin Promoter. βGl: β globulin intron.. pA: poly adenosin

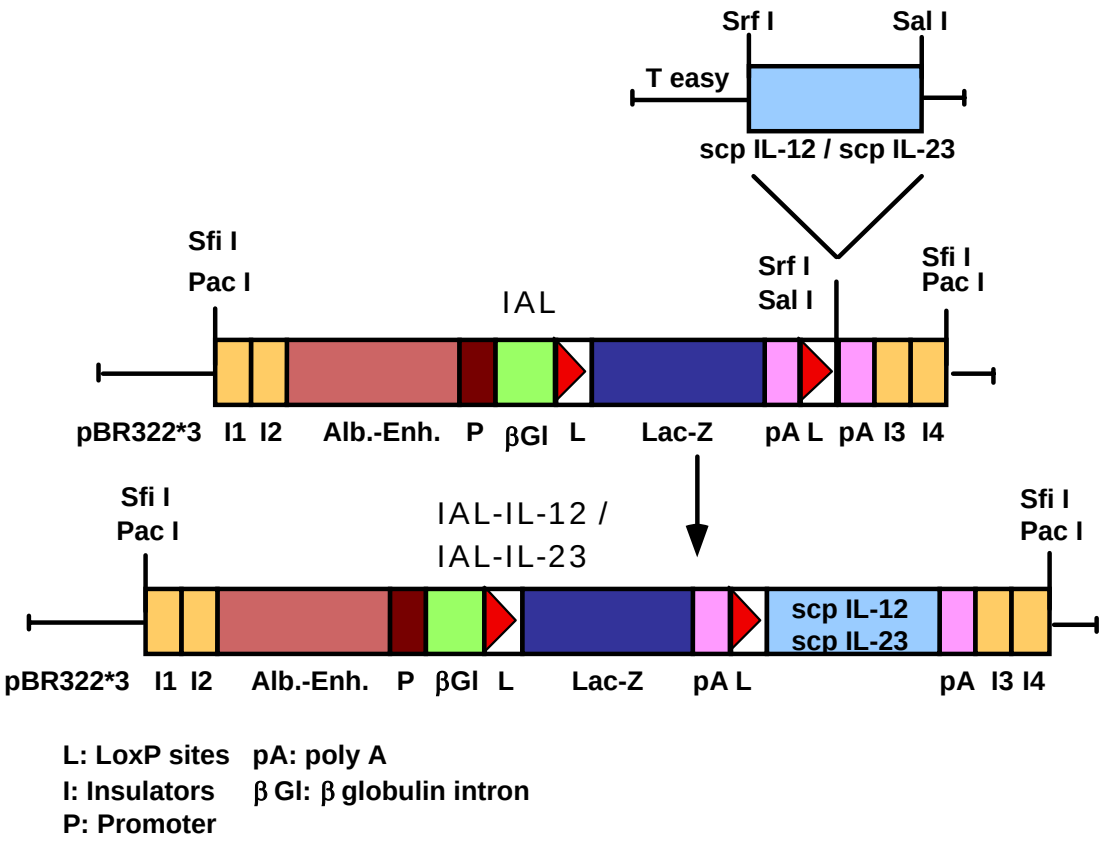


Figure 15. Construction of the IAL-IL-12 and IAL-IL-23 expression vectors. Single-chain IL-12 or IL-23 were recovered from the Teasy vector by digestion with Sal I and Srf I and cloned separately into the IAL expression plasmids.

3.6 Characterization of the single-chain IL-12

To determine whether the single-chain IL-12 was suitable for generation of transgenic mice, it was necessary to know: first, whether the single-chain IL-12 could be synthesized in an active form, and second, whether it was secreted by hepatocytes. To achieve this, the Hepa 1.6 hepatoma cell line was transfected either with the U3-IL-12 plasmid, with U3 as control, or with a GFP plasmid as control for the transfection efficiency. The IL-12 levels from the supernatants of the transfected cells were measured by an IL-12p70 ELISA (Fig.16). At 72 h post-transfection, the IL-12 production was 36,5 ng/ml by the cells transfected with U3-IL-12. Non-transfected cells and cells transfected with U3 or with GFP did not show any IL-12 production.

The ability of IL-12 to stimulate splenocytes to produce IFN-γ was used as bioassay to detect whether the secreted single-chain IL-12 was bioactive. Splenocytes were cultured in the presence of the supernatants from the different Hepa 1.6 transfections. Recombinant IL-12 was used as positive control. As showed in figure 17, splenocytes cultured with the secrected IL-12 present in the supernatant from the cells transfected with U3-IL-12, were stimulated to produce as much IFN-γ (3 ng/ml) as splenocytes cultured with recombinant mouse IL-12 (3,4

ng/ml). Splenocytes alone and cultured with the other supernatants did not produce IL-12. The results in figure 16 and 17 demonstrate that, single-chain IL-12 can be effectively synthesized and secreted by hepatocytes, in a bioactive form as potent as the recombinant protein.

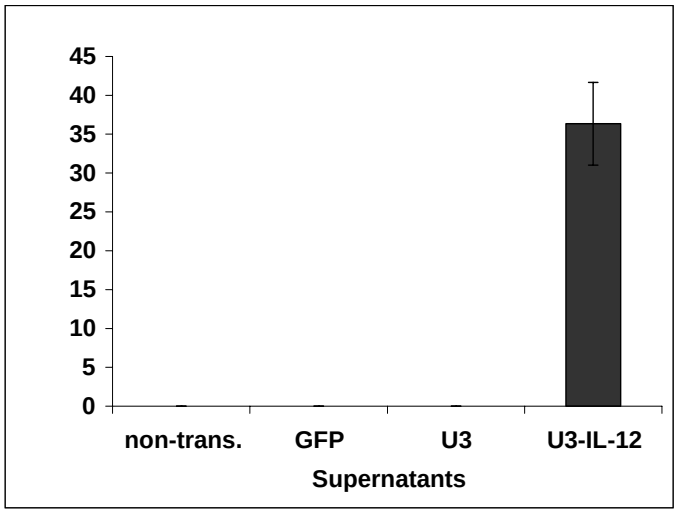


Figure 16. Secretion of scpIL-12. The hepatoma cell line Hepa 1.6 was transfected with U3-IL-12, or GFP for transfection´s efficiency control. Transfection with "empty" U3 and non-tranfected Hepa 1.6 were used as negative control. Supernatants were collected and assayed by IL-12p70 ELISA. High levels of active IL-12 were measured in the supernatant of the Hepa 1.6 transfected with U3-IL-12. The columns and standard deviations are representative of three independent experiments.

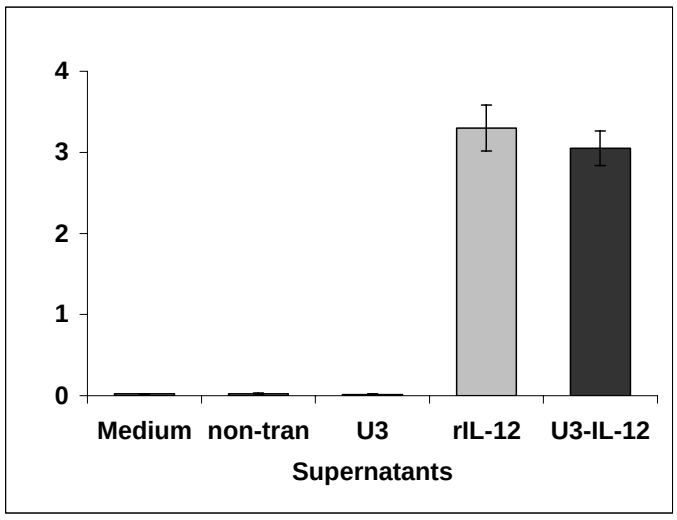


Figure 17. Induction of IFN-γ by scpIL-12. Splenocytes were cultured in medium or in medium supplemented with the supernatants from: non-transfected Hepa 1.6 cell line (non-tran.), or Hepa 1.6 transfected with U3, or with U3-IL-12. Recombinant mouse IL-12 was used as positive control. Supernatants were collected and assayed by ELISA. The columns and standard deviations are representative of two independent experiments.

3.7 Characterization of the single-chain IL-23

To determine whether the single-chain IL-23 was suitable for generation of transgenic mice, it was necessary to know, as with the single-chain IL-12, whether it is synthesized in a bioactive form and secreted by hepatocytes. Since there is neither a commercial mouse IL-23 ELISA nor antibodies for a western blot, the property of IL-23 to stimulate CD4 T-cell to produce IL-17 was used. Therefore, the Hepa 1.6 hepatoma cell line was transfected either with the U3-IL-23 plasmid, with U3 as control, or with a GFP plasmid as control for the transfection efficiency. Subsequently, CD4 T-cells were cultured in the presence of the different

supernatants, and the presence of IL-17 was determined by ELISA. As shown in figure 18, CD4 T-cells cultured with the supernatant of U3-IL-23 transfected Hepa 1.6 were stimulated to produce high levels of IL-17 (1456 pg/ml). CD4 T-cells cultured in the other conditions, even in the presence of IL-12 (U3-IL-12) showed only low levels of IL-17 (243, 340 and 301 pg/ml). This result indicates that IL-23 was present in the supernatant of U3-IL-23 transfected Hepa 1.6 cell line, demonstrating that single-chain IL-23 can be synthesized and secreted by hepatocytes in a bioactive form.

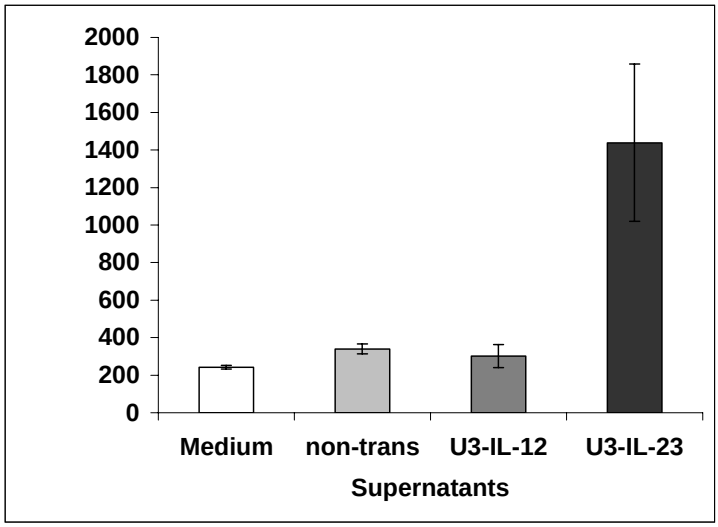


Figure 18. Induction of IL-17 by single-chain IL-23. Purified CD4 T-cells were incubated for 3 days in presence of IL-2 (50 U/ml) and supplemented with the supernatants from: non-transfected Hepa 1.6 cell line (non-trans.), or Hepa 1.6 transfected with U3-IL-12, or with U3-IL-23. After re-stimulation for 2 days with anti-CD3, The columns and standard deviations are representative of two independent experiments.

3.8 Generation of the IL-12 transgenic mice

Because the generation of transgenic mice, in which the expression of IL-12 could be induced was desired, the IAL-IL-12 expression vector was chosen. The IAL-IL-12 expression cassette was separated from the bacterial plasmid (pBR322*3) by digestion with Sfi I and microinjected. The microinjection delivered 8 transgenic mice (Founder), which were identified by specific PCRs (Material and methods), and first crossed with non-transgenic mice to establish the different lines (lines A-H).

3.9 Induction of IL-12 expression

In this inducible IL-12 transgenic mouse, IL-12 expression could be achieved only after elimination of the LacZ cDNA, which is performed by a Cre-Recombinase enzyme. Cre-Recombianse connects both LoxP sites, and eliminates the DNA between them (LacZ-Poly A), leaving a LoxP site and the IL-12 cDNA free for the Albumin Promoter/Enhancer-promoted expression (Fig. 19).

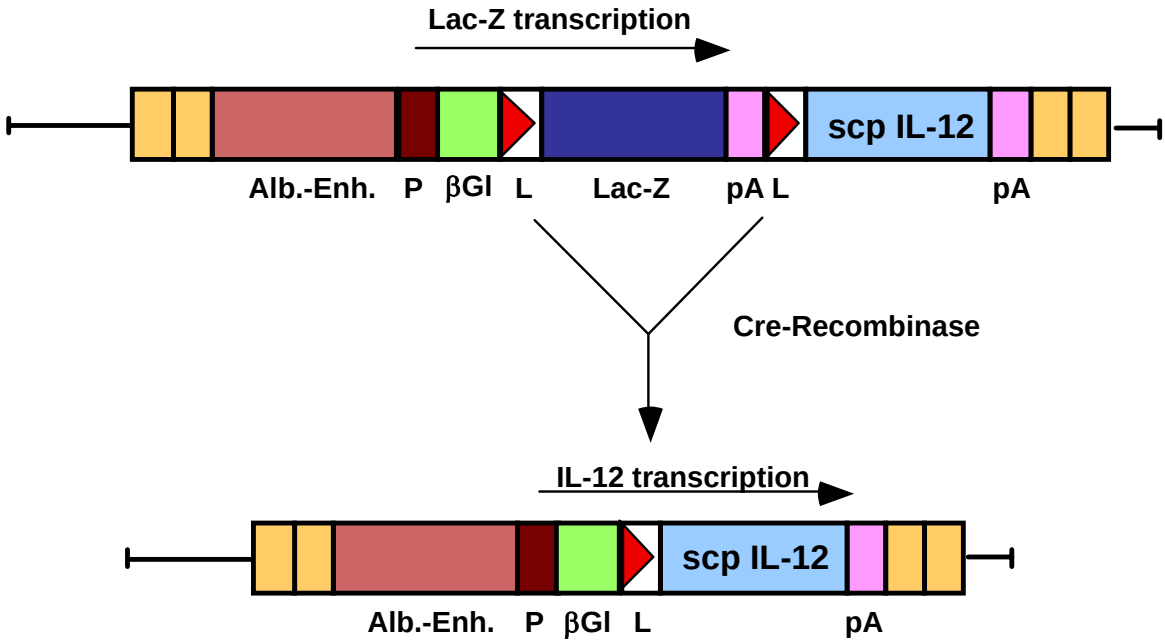


Figure 19. Scheme of the recombination: the IAL expression cassette used to generate the IL-12 transgenic mice leads to a constitutive and Albumin Promoter-induced expression of the LacZ gene. Only after recombination by the Cre-Recombinase can IL-12 been expressed. Alb: Albumin, Enh: Enhancer, P: Albumin Promotor,β: β Globulin intron, pA: poly Adenosine, I: Insulators.

3.10 Perinatal mortality of IL-12/Act-cre transgenic mice

The IL-12 transgenic mice of the different lines were crossed with Actin-Cre++ (homozygotic) transgenic mice, which are characterized by ubiquitous expression of the Cre-Recombinase protein in the embryonic stages. After crossing IL-12 transgenic mice with Act-Cre++ transgenic mice, IL-12/Act-Cre double transgenic and Act-Cre single transgenic mice were delivered. Only in the IL-12/Act-Cre double transgenic mice, the Cre-Recombinase will induce the expression of IL-12.

Table 1 summarizes the results after several crossings. In lines A, C and G we found a 100% mortality rate among the new-borns between 8-12 hours (lines A, C) and 1-5 days (line G) after delivery. We demonstrated by PCR that all dead mice were double transgenic (+/+) for IL-12 and for -Cre (data not shown). By PCR we also checked that all the surving mice were transgenic for -Cre but not for IL-12.

Line F did not show any mortality among the new-borns after delivery. In this line we demonstrated by PCR that all delivered newborns were single transgenic -Cre, indicating that all +/+ transgenic mice died during pregnancy.

Lines B, D and H demonstrated almost normal delivery rates. Single and double transgenic mice were present among the newborns, however, no significant mortality was observed

among the IL-12/Act-cre double transgenic newborns which grew normally. Line E did not deliver any mice, and was therefore not studied. Supported in the observed phenotype, we chose lines A, C, F, and G for further studies.

Table 1. Resume of deliveries of the crossing IL-12 X Actin-Cre transgenic mice. The figure shows the total number of new-borns, how many were double transgenic for IL-12 and for Actin-Cre (+/+), and the lethality among the double transgenic mice.

Lines	Deliveries	+/+ Transgenic offspring	Lethal / Transgenic
A	5+8+3= 16	7	7/7
B	7+8= 15	8	0/8
C	8+8+8+4= 28	11	11/11
D	6+7= 13	7	0/7
E	------	------	------
F	4+4+5= 13	0	------
G	9+7+8= 24	6	6/6
H	7+10+8= 25	12	1/12

3.11 IL-12/Actine-Cre double transgenic mice express IL-12 mRNA in the liver

To determine whether IL-12 was expressed in the liver of the double transgenic mice, total RNA was isolated from the liver of the newborn mice from lines A, C and G, and reverse transcribed to generate cDNA. The cDNA was used as template for an IL-12-specific PCR. A 750 bp band indicated IL-12 mRNA transcripts, while a 1300 bp band was due to genomic DNA (see matherial and methods).

As shown in figure 20, IL-12 transgene-specific transcripts were detected in IL-12/Act-Cre double transgenic mice (1), but not in Act-Cre (2) and IL-12 (3) single transgenic mice. The 1300 bp band corresponding to the genomic DNA was not present, indicating that the cDNA were not contaminated with genomic DNA, and therefore, that the PCR products were specific for IL-12 mRNA. Double transgenic mice from lines B, D, and H did not demonstrate any IL-12 mRNA transcripts by RT-PCR (data not shown)

IL-12 mRNA transcripts were also quantified the by real-time PCR (Fig.21). Using primers specific for the single-chain IL-12, transgene-specific transcripts in the double transgenic mice from lines A, C and G were present, but not in the control mice.

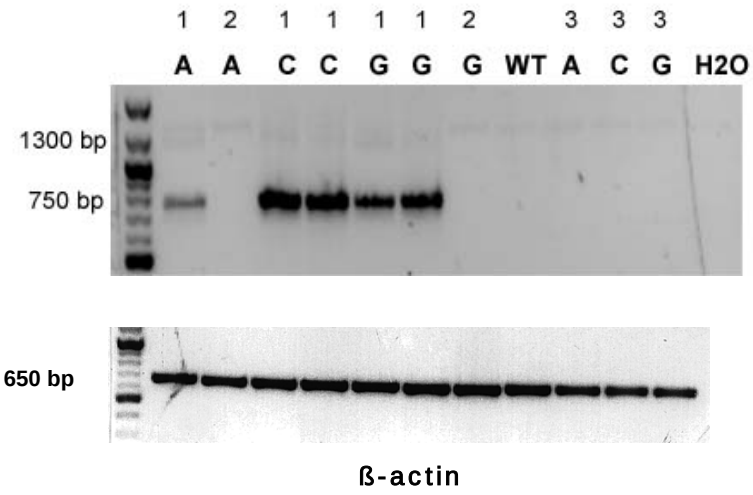


Figure 20. IL-12 mRNA specific RT-PCR 1: IL-12/Actin-Cre transgenic mice; 2: Cre transgenic mice; 3: IL-12 transgenic mice; WT: non-transgenic mice. A 750 bp IL-12 mRNA specific PCR product was present only in the double transgenic mice from lines A, C and G. A β-Actin PCR was performed to control amount of cDNA in each sample.

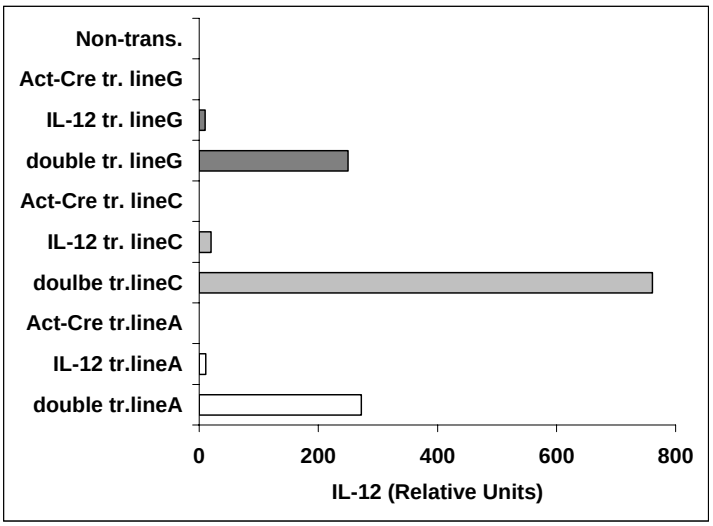


Fig. 21. Single-chain IL-12 mRNA expression levels by real-time PCR. IL-12 mRNA transcripts were demonstrated in IL-12/Act-cre double transgenic mice from lines A, C and G, but not in control animals (IL-12 transgenic, Act-Cre transgenic and non-transgenic mice). Relative Units were calculated comparing the expression levels of the IL-12 with the expression levels of the β-actin house keeping gene.

3.12 Single-chain IL-12 protein expression in the liver

Once the expression of transgene-specific mRNA was determined, the translation of mRNA into protein was assed. For that purpose, the presence of IL-12 protein in the liver of the double transgenic mice was measured. Protein was isolated from the liver of IL-12/Act-cre double transgenic, IL-12 single transgenic and Act-Cre single transgenic mice and analyzed by Western Blot. As shown in Figure 22 IL-12/Act-Cre double transgenic mice (1) from lines A (A), C (B) and G (C), demonstrated IL-12 protein expression. IL-12 transgenic mice (2) and Act-cre transgenic mice (3) did not show any IL-12 expression.

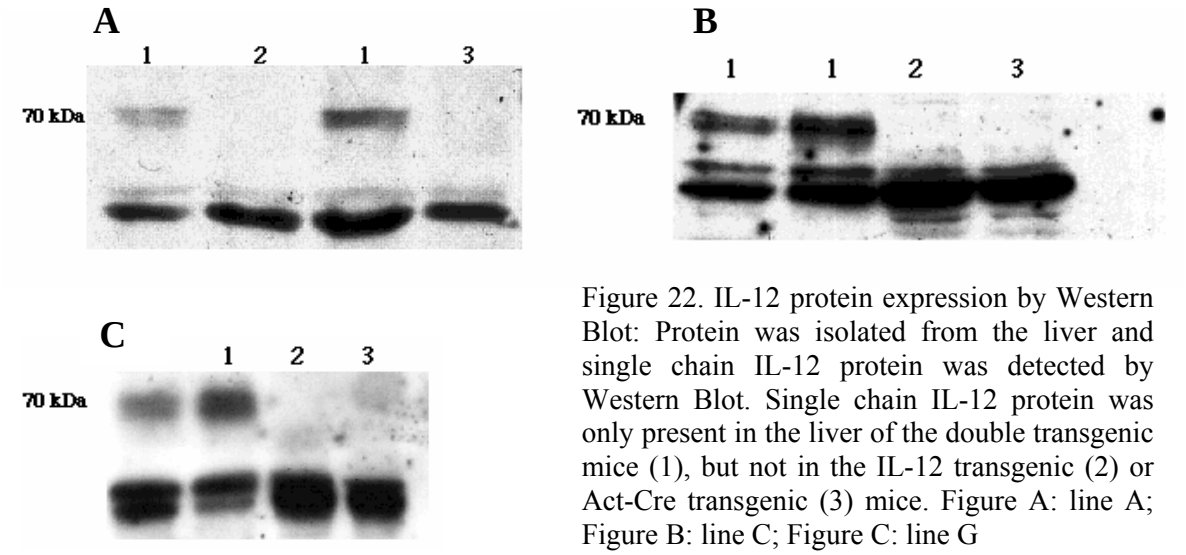


Figure 22. IL-12 protein expression by Western Blot: Protein was isolated from the liver and single chain IL-12 protein was detected by Western Blot. Single chain IL-12 protein was only present in the liver of the double transgenic mice (1), but not in the IL-12 transgenic (2) or Act-Cre transgenic (3) mice. Figure A: line A; Figure B: line C; Figure C: line G

3.13 IL-12 expressing transgenic mice present disturbed phenotype and liver pathology

As mentioned before, after crossing with Act-Cre transgenic mice, IL-12 transgenic mice from lines A and C delivered double transgenic mice which died within 12-24 hours after delivery. Line G delivered IL-12 double transgenic mice, some of them surviving until day 4-5 after delivery. Double transgenic mice from line G showed a disturbed development, with a much smaller size when compared to the non-transgenic littermates (Figure 23).

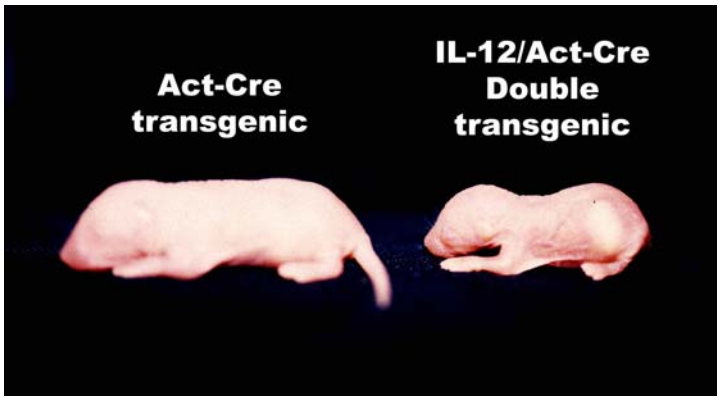


Figure 23: Disturbed development in IL-12/Act-Cre double transgenic mice from line G. This picture shows an IL-12/Act-Cre double transgenic mice and an Act-Cre transgenic littermate 5 days after delivery. The double transgenic mice demonstrated a much smaller size when compared to the littermate.

To analyse the microscopic structure of the liver, cryosections were stained with Hematoxilin-Eosin. As shown in Figure 24, the liver of the IL-12/Act-Cre double transgenic mice from line

G demonstrated a disturbed liver structure and large necrosis areas. Those areas were characterized by the presence of infiltrating lymphocytes and the absence of hepatocytes.

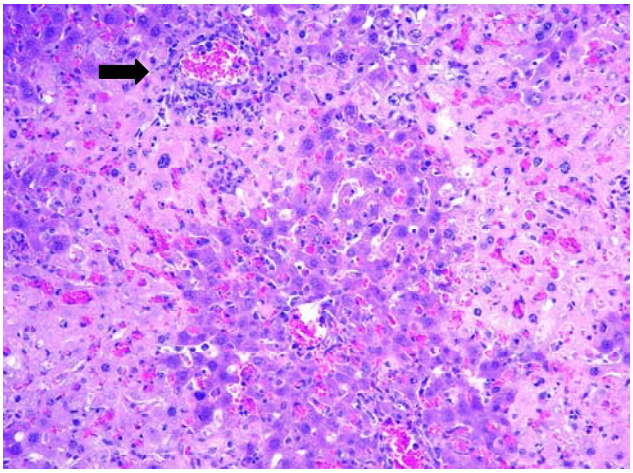
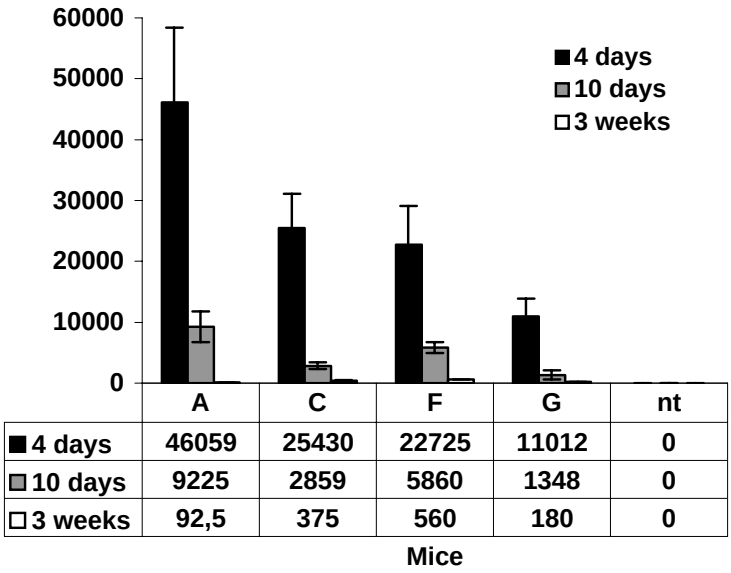


Figure 24: The liver of double transgenic mice demonstrated large necrotic areas (lighter stained areas) and foci of lymphocytes infiltration (black arrow). x100.

3.14 Induction of IL-12 expression in adult IL-12 transgenic mice

To induce the IL-12 expression in adult mice, mice of the lines A, C, F, G, and non-transgenic littermates were intravenously injected with a pcDNA3-Cre-Recombinase plasmid (containing the cDNA for the Cre-Recombinase, see material and methods). As shown in figure 25, IL-12 expression was induced in all four transgenic lines (A, C, F and G). Serum levels of IL-12 were very high at 4 days after induction (line A: 46059 ± 12333 pg/ml; line C: 25430 ± 5698 pg/ml; line F: 22725 ± 6362 pg/ml; and line G: 11012 ± 2908 pg/ml). At day 10, IL-12 levels decreased but were still elevated (line A: 9225 ± 2510 pg/ml; line C: 2859 ± 569 pg/ml; line F: 5860 ± 933 pg/ml and line G: 1348 ± 718 pg/ml). Finally, 3 weeks after induction we still measured IL-12 in sera from all four transgenic lines, although IL-12 levels had much decreased (line A: 92,5 ± 39 pg/ml; line C: 375 ± 100 pg/ml; line F: 560 ± 88 pg/ml; and line G: 180 ± 50 pg/ml). These data demonstrated a potent and fast induction of IL-12 expression in IL-12 transgenic mice. These data also demonstrated that although the levels of IL-12 decreased with the time after induction, IL-12 was still elevated in transgenic mice 3 weeks after induction. Non-transgenic mice did not produce any IL-12 at all (0 pg/ml) at any time after plasmid injection.



	A	C	F	G	nt
4 days	46059	25430	22725	11012	0
10 days	9225	2859	5860	1348	0
3 weeks	92,5	375	560	180	0

Figure 25. Induction of IL-12 expression in adult mice. Mice were injected with a pcDNA3-Cre-Recombinase plasmid.. IL-12 expression was determined in serum by ELISA at 4 and 10 days, and 3 weeks after injection. All four IL-12 transgenic mice lines (A, C, F and G) expressed IL-12. After very high IL-12 expression at day 4, IL-12 levels decreased slowly with time. Three weeks after induction IL-12 was still present in the sera from IL-12 transgenic, but a much lower concentration. IL-12 was not measured in the sera from non-transgenic mice after plasmid injection.

3.15 Expression of IL-12 in transgenic mice was related with inflammation

Related to the expression of IL-12 in the liver, IL-12 transgenic mice demonstrated a strong liver and spleen inflammation three weeks after induction by plasmid injection (Fig. 26). The liver and spleen from non- transgenic mice remained normal after plasmid injection.

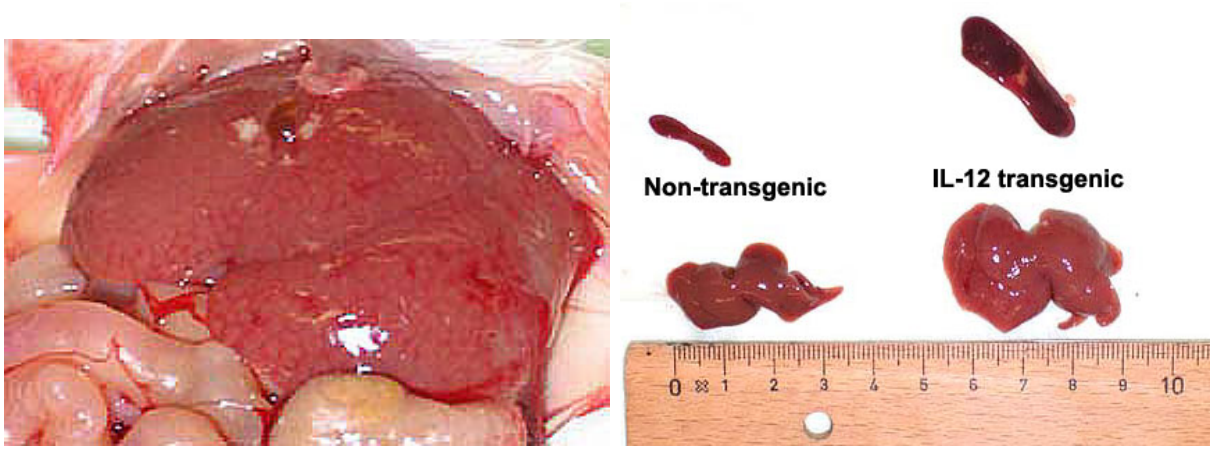


Figure 26. Left, the liver of an IL-12 transgenic mouse 3 weeks after induction of IL-12 expression by plasmid injection. The liver was much larger than normal and presented a rough patched surface, as signs of lymphocyte infiltrations and probably necrosis. Right, liver and spleen of a non-transgenic and an IL-12 transgenic mouse 3 weeks after induction of IL-12 expression by plasmid injection. IL-12 transgenic mice exhibited liver and spleen inflammation. Non-transgenic mice, on the contrary, exhibited normal liver and spleen size after plasmid injection.

The degree of inflammation among lines A, C, F and G at different time points after induction of IL-12 expression was compared calculating, respectively, the ratios between liver and body weight (in normal situations the ratio is 0,046), and between spleen and body weight (in normal situations the ratio is 0,005). Mice were studied until 3 weeks after induction, and as shown in figure 27, the liver (A) and the spleen (B) demonstrated a gradual increase in their size in IL-12 transgenic mice. Although no differences were observed among lines in liver sizes at days 4 and 10, lines C and F demonstrated a stronger liver inflammation 3 weeks after induction (ratio liver/body weight: line C: 0,165; line F: 0,162; line A: 0,11; and line G: 0,105, which corresponds to 3.6, 3.52, 2.17 and 2.18 folds larger than normal liver size). Lines C and F also demonstrated 3 weeks after induction larger spleens than lines A and G (line C: 0,029; line F: 0,034; line A: 0,02; and line G: 0,018, which corresponds to 5.8, 6.8, 4, 3.6 folds larger than normal spleen size). The liver and spleen of non-transgenic mice demonstrated almost normal sizes after plasmid injection.

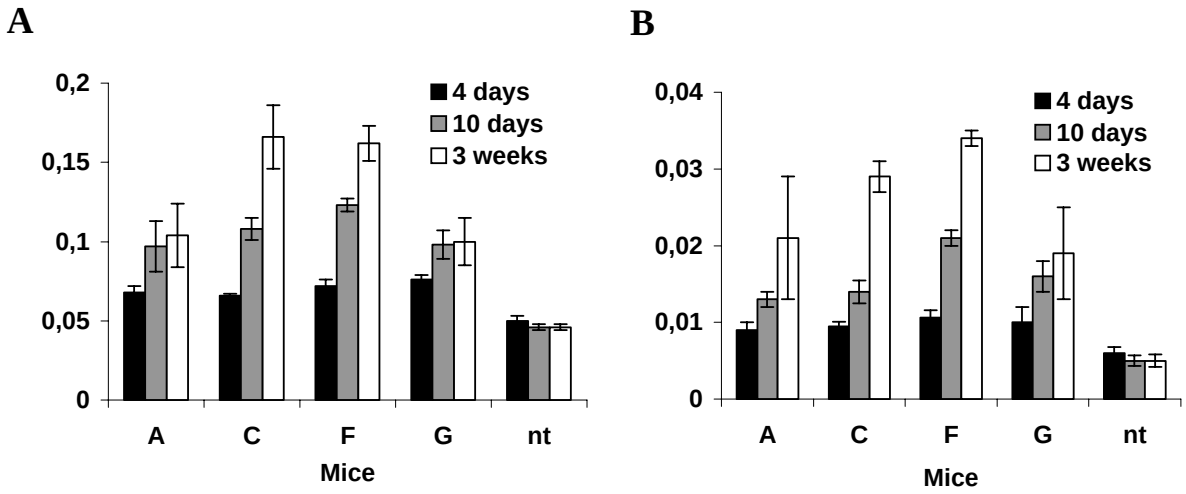


Figure 27. Liver and spleen inflammation after induction of IL-12 expression. (A) Using the ratio liver/body weight we measured a gradual increase in liver size in IL-12 transgenic mice from all four lines (A, C, F and G). No differences were observed among lines at days 4 and 10, but at 3 weeks, livers from lines C and F were larger than those from lines A and G. (B) Using the ratio spleen/body weight we also measured a gradual increase in spleen size in IL-12 transgenic mice. Lines C and F demonstrated larger spleen than lines A and G at 3 weeks after induction. Non-transgenic mice demonstrated normal livers (ratio: 0,046) and spleens (0,005) after plasmid injection.

3.16 Expression of IL-12 in transgenic mice was related with severe hepatitis, liver damage, and 20% lethality

At the time of maximal liver size (3 weeks after plasmid injection), mice of all four transgenic lines demonstrated severe liver infiltration (Fig. 28) around portal tracts, and in the parenchyma. Although no major differences were observed between lines A, C and F, line G

demonstrated less infiltration. The liver of the non-transgenic mice did not show any infiltration. These data demonstrated that expression of IL-12 in transgenic mice after induction was associated with a severe hepatitis.

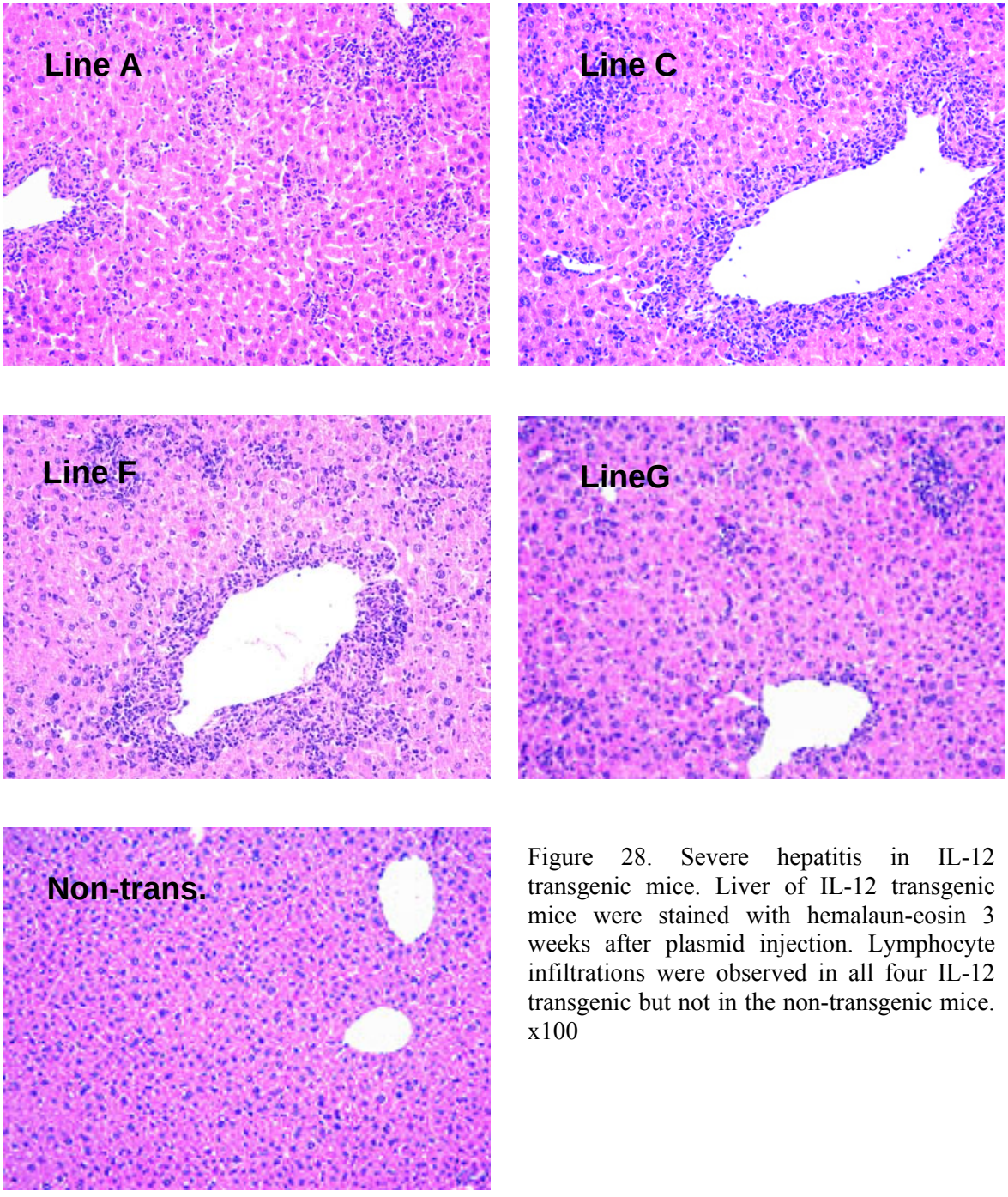


Figure 28. Severe hepatitis in IL-12 transgenic mice. Liver of IL-12 transgenic mice were stained with hemalaun-eosin 3 weeks after plasmid injection. Lymphocyte infiltrations were observed in all four IL-12 transgenic but not in the non-transgenic mice. x100

To examine liver cell injury, serum levels of transaminases were assayed at 4 and 10 days, and 3 weeks after plasmid injection. As shown in Fig. 29 and 30, ASAT and ALAT levels were slightly elevated in all four transgenic lines already 4 days after plasmid injection. Cell injury was not due to the induction method, since non-transgenic mice did not demonstrate increased transaminase levels 4 days after plasmid injection. An increase in ALAT and ASAT

levels was observed at day 10. Three weeks after plasmid injection, we still observed elevated ASAT and ALAT levels, which were higher in lines C and F than in lines A and G. Non-transgenic mice demonstrated normal ASAT and ALAT levels at each time point after induction. These data demonstrated liver injury in IL-12 transgenic mice after induction of IL-12 expression.

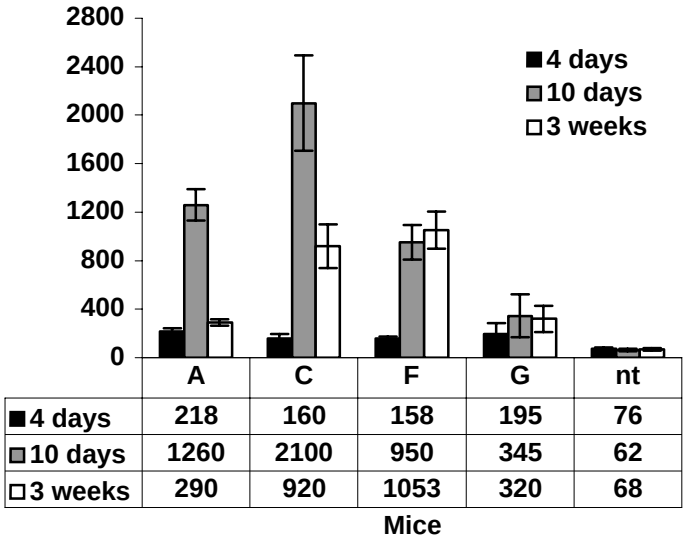


Figure 29. Serum ASAT levels. IL-12 transgenic mice demonstrated elevated serum ASAT and after induction of IL-12 expression. Non-transgenic mice demonstrated normal levels of ASAT after plasmid injection.

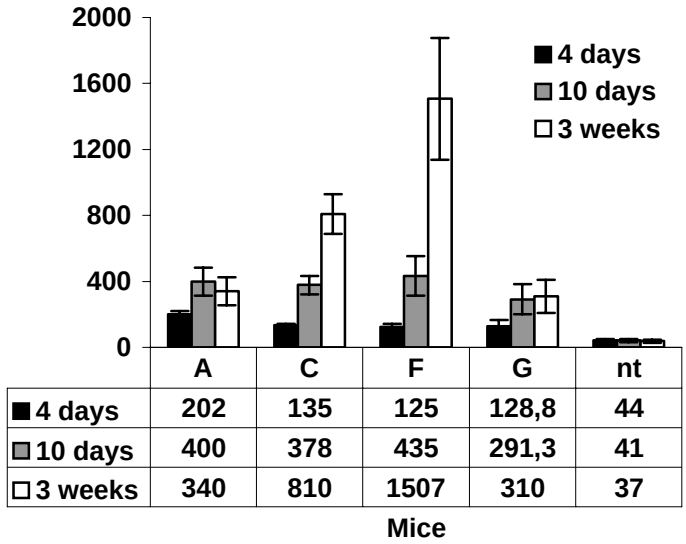


Figure 30. Serum ALAT levels. IL-12 transgenic mice demonstrated elevated serum ALAT after induction of IL-12 expression. Non-transgenic mice demonstrated normal levels of ALAT after plasmid injection.

Hepatic IL-12 expression resulted in a 20% total mortality in mice from lines A, C and F within 8-12 days after plasmid injection. The liver of those mice was characterized by large necrotic areas (Fig. 31).

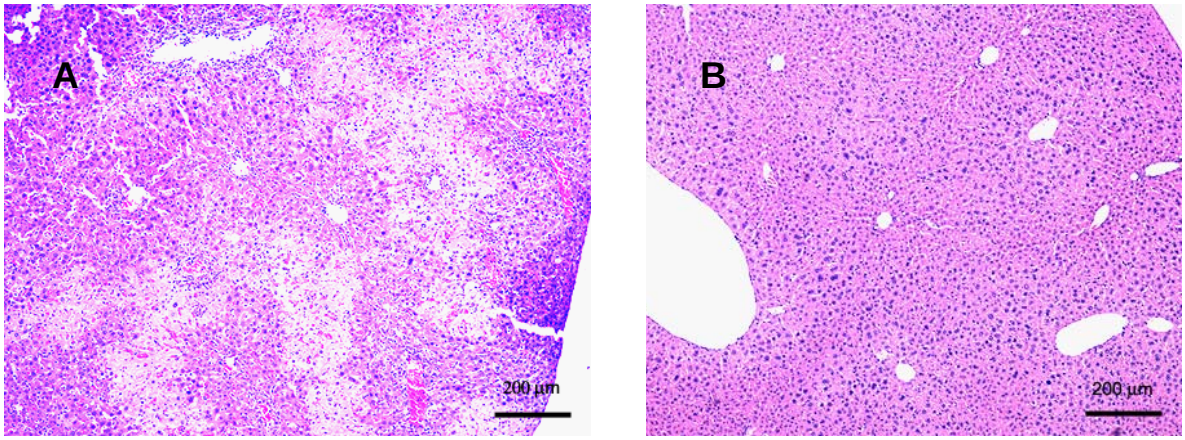


Figure 31. (A) The liver histology of adult mice expressing IL-12, in the moment of casualty, demonstrates large areas of hepatocyte destruction. (B) The liver of a non-transgenic mouse. Hematoxylin and eosin staining. x20

4. Discussion

4.1 IL-12 influences the type of immune response in the liver

The initial immune response to hepatic virus after infection is largely mediated by CD8+ cytotoxic T cells (Lechner et al., 2000). However, viral clearance and the maintenance of effective anti-viral immunity require the help of CD4+ T helper cells (Gerlach et al., 1999). Development of Th cells into Th1 is necessary for inflammatory immune responses and virus elimination (Sun and Ran, 2004). In viral hepatitis, hepatocytes are the main cells infected by hepatic virus, and as a consequence upregulate MHC class II molecules (Senaldi et a., 1991; Chu and Liaw, 1994), thus being probably capable of presenting antigen to CD4+ T lymphocytes. Indeed, Herkel et al. demonstrated that transgenic hepatocytes expressing MHC class II were able to present antigen and to stimulate specific $CD4^+$ T cells. However, until now, it is not known whether CD4+ T cells stimulation by hepatocytes is of type Th1 or Th2. In addition,

To know the type of immune response induced by viral-infected hepatocytes on $CD4^+$ T cells, adenovirus-infected, MHC class II-expressing hepatocytes were used as APCs. This work demonstrated that inexperienced CD4+ T cells stimulated by MHC class II-expressing hepatocytes resulted in a poor inflammatory response with high levels of IL-4 and low levels of IFN-γ. However, when IL-12 was present, a potent inflammatory response was induced, marked by a strong increased in the production of IFN-γ.

Despite hepatocytes are able to express some adhesion molecules during viral infections, the lack of costimulatory molecules necessary for optimal stimulation of CD4+ T cells (Gao, 2000) can be the explanation for the observed poor inflammatory response induced by hepatocytes. According to these results Bertolino et al. also demonstrated that hepatocytes were able to activate CD8+ T cells through MHC class I molecules, but failed to promote T cell survival (Bertolino P, 1999).

Summarizing, this study demonstrates that, hepatocytes, like other liver APCs, induce poor inflammatory immune responses, and underline the importance of IL-12 in switching to Th1 responses. Thus, hepatocytes may initiate an inflammatory immune response only if IL-12 is present: infected hepatocytes may then favour acute hepatitis and viral clearance in a pro-inflammatory setting, or chronic hepatitis and viral persistence in a poorly inflammatory setting.

4.2 IL-12 was related with strong liver inflammation and rapid virus elimination

Since the presence of IL-12 induced a proinflammatory response *in vitro,* the role of endogenous IL-12 during a hepatic viral infection*, in vivo,* was studied.

For this study, a model of liver specific viral infection and liver specific IL-12 production through infection with adenovirus coding for IL-12 was chosen. Replication deficient adenovirus efficiently target hepatocytes when injected into the circulation (Jaffe et al., 1992). In this work, a direct relation between IL-12 and virus elimination was demonstrated. Mice infected with AdIL-12 eliminated viral particles already at day seven after infection, while viral particles were still present in mice injected with AdLacZ. Therefore, IL-12 induced a rapid virus elimination. These findings are in concordance with some previous studies in mice (Coutelier et al., 1995; Cavanaugh et al., 1997).

During viral infection, IL-12 can increase hepatic inflammation and hepatocyte damage (Nicoletti et al., 2000). Interestingly, it has been reported that hepatocytes treated with IL-12 produced T cell-chemo attractant activity (Park et al., 2001), enhancing the recruitment of T lymphocytes. According to this, large infiltration areas around portal tracts and veins, and a high number of apoptotic hepatocytes in mice infected with AdIL-12 were observed. Furthermore, liver pathology was rather related to IL-12 than to the adenoviral infection (Mazzolini et al., 2001) since a non-toxic adenovirus concentration was used.

Thus, and according to this study, IL-12 seems to induce the recruitment of T lymphocytes into the liver, which may induce the elimination of infected hepatocytes by apoptosis, leading to a more effective and faster viral elimination. Although some studies with mice have shown that IL-12 is not necessary for Th1 responses during viral infection (Schijns et al., 1998; Oxenius et al., 1999), and other studies with patients have demonstrated that IL-12 was related with progressive liver injury, but not with virus elimination during chronic hepatitis (Nelson et al., 2000; Cecere et al., 2004), the important role of IL-12 in hepatic virus elimination has been demonstrated here.

4.3 Single chain protein IL-12 (scpIL-12) construction and characterization

Generation of IL-12 expressing transgenic mice is complicated by the nature of this bioactive protein as a heterodimer –p40 and p35 subunits- expressed from two independent genes. The p40 subunit can form a homodimer with antagonist properties against IL-12 activity through competitive inhibition of the IL-12 receptor (Gillessen et al., 1995). To avoid this problem, a single-chain IL-12 cDNA encoding an IL-12 fusion protein, which ensured the equimolar expression of each subunit, was constructed. In some previous studies, IL-12 single chain

proteins were created (Lieschke et al., 1997; Lee Y, et al., 2001). Those studies demonstrated, that the IL-12 fusion protein created by connection of the p40 subunit cDNA in position 5' to the p35 subunit cDNA in position 3', had high levels of bioactivity. Following those results, the p40 and p35 subunits were connected in the order 5'-3' with a linker. Different linkers have been used to create fusion proteins. For this study, the linker 218 published by Lee et al. (2001) was used. The sequence consists primarily of the stretches of glycine and serine residues for conformational flexibility, with charged residues such as glutamic acid and lysine interspersed for solubility (Pantoliano et al, 1991). Again, like Lee, the leader sequence (first 22 amino acids) of the p35 subunit was deleted before connecting to the p40 subunit through the linker. Deletion of the leader sequence of the trailing cDNA might contribute to the relative potency and stability of the fusion protein, possibly by minimizing the bulk of nonessential sequences in this region, and by removing potential protease cleaving sites (Lieschke et al., 1997)

The idea of creating a fusion protein was also based on the features of the LoxP/Cre system vector. After microinjection of the p40 and p35 subunit separately, each cDNAs integrate randomly into genomic DNA in the form of head-to-tail, tail-to-tail, or head-to-head connected tandems up to a few hundred copies long (Richa J, 2001). In this situation, the cre-recombinase mediated splicing can cause loss of all the p40 subunits or all the p34 subunits.

For characterization of the scpIL-12, we cloned it into U3, which allowed a hepatocellular specific constitutive transgenic expression (Dr. Jurgen Henninger, Uni-Klinik Mainz), which was used for transfection of a hepatoma cell line. Transfection of cell lines has been used to expresse proteins, which can be measured in the supernatant by ELISA (Belladonna ML, et al., 2002). In this study, the scpIL-12 was produced and secreted in high amounts as measured by ELISA. Moreover, the scpIL-12 demonstrated a potent bioactivity, inducing IFN-gamma production by splenocytes

4.4 Generation of IL-12 transgenic mice

Transgenic mice expressing murine active IL-12 in hepatocytes were generated. For this purpose, the scpIL-12 was cloned into a liver specific inducible Lox-P system expression cassette (IAL; kindly provided by Dr. Meyer). Through microinjection of the IAL-scpIL-12 eight transgenic lines were created. The use of the Cre-LoxP recombination system allowed circumvent the problem of possible embryonic lethality due to constitutive IL-12 expression. All eight IL-12 transgenic mice lines generated with our LoxP system delivered successfully. Around 50% of the newborn were transgenic and did not demonstrate any pathology.

Furthermore, no IL-12 expression was found in the livers of the IL-12 transgenic mice, which demonstrated the leak-proof of the system.

4.5 Induction of liver specific IL-12 expression

In this work, IL-12 expression in the liver was demonstrated after induction by the Cre-Recombinase protein. Constitutive induction of IL-12 expression was achieved by crossing IL-12 and Act-Cre$^{+/+}$ transgenic mice. Since the actin promoter induces the expression of the *Cre* recombinase in all cells of the embryo by the blastocyst stage of development (Araki et al., 1995), IL-12 expression was induced in the embryos of the IL-12/Act-Cre double transgenic mice. From the eight generated lines, lines A, C, and G delivered double transgenic mice, which demonstrated mRNA and protein expression of IL-12 in the liver. Line F did not deliver any double transgenic mice, which could be due to embryonic lethality. Hypothetically, the lethal embryonic phenotype might be connected with stronger IL-12 expression in this line. Indeed, serum levels of proinflammatory cytokines has been related with abortion (Paradisi et al., 2003). Therefore, it could be possible that strong IL-12 expression in line F during pregnancy led to embryonic death, while weaker IL-12 expression in lines A, C, and G allowed normal pregnancy. This was supported by the observation that the ratio of double transgenic newborn in lines A, C, and G, correspond to the expected 50%, indicating that no IL-12 expressing embryo was dying during pregnancy.

However, since application of IL-12 during pregnancy has also been related with normal foetal development and delivery (Reina et al., 2004), a second possible explanation for the embryonic lethality in line F could be provided by integration mechanisms of the transgene-construct into the genome, and their rearrangement mediated by the Cre-recombinase in double transgenic offspring (Lewandoski and Martin, 1997). The expression vectors injected into the male pronuclei of one-cell stage mouse embryos integrate randomly into genomic DNA, and multiple integration sites for such tandems are theoretically possible (Richa et al., 2001). In this situation, the cre-recombinase mediated splicing can cause loss of genomic DNA sequences lying between the tandem integration sites (Lewandoski and Martin, 1997). Of course, such an event leading to elimination of possibly important genes could produce the above-mentioned embryonic lethality in IL-12/Act-Cre embryos of line F. Future studies using neutralizing anti IL-12 antibodies during pregnancy will help us to clarify this and the influence of IL-12 during embryonic lethality.

IL-12 expression was also induced in adult mice. By hydrodynamic-based transfection with a plasmid coding for the cre-recombinase protein, IL-12 expression in adult mice was induced.

However, only a transient expression was achieved. Although the hydrodynamic-based transfection method has been successfully used for delivery of DNA into hepatocytes (Andrianaivo et al., 2004), the DNA is quickly degraded resulting in a transient gene expression (Liu et al., 1999). However, once the recombination takes place, degradation of the Cre-recombinase-plasmid should not influence the sustained expression of IL-12. Some studies demonstrated that DNA degradation was related with a strong decrease of protein expression as soon as 70 hours (Yang et al., 2001) after plasmid injection. However, high levels of IL-12 were measured at four days after plasmid injection. Even more, IL-12 was still found three weeks after induction. Thus, plasmid degradation is probably not the explanation for the transient expression, but elimination of the IL-12 expressing hepatocytes. Indeed, related to IL-12 expression high levels of transaminases were measured in serum, indicating hepatocyte damage (see point 6.2). Furthermore, it has been demonstrated that IL-12 induces adhesion molecule expression in murine hepatocytes (Myers et al., 1998). Thus, it is likely that IL-12 also induces adhesion molecules in IL-12 expressing hepatocytes, making them target cells for cytolytic T lymphocytes.

However, transient expression of a gene delivered into hepatocytes by a hydrodynamics-based procedure can be overcome. Yang et al. demonstrated that repeated injection of the plasmid-DNA lead to a sustained gene expression (Yang et al., 2001). According to this, repeated injection of Cre-recombinase-plasmid should allow the transfection of new hepatocytes, and therefore a sustained IL-12 expression in IL-12 transgenic mice.

4.6 IL-12 expressing transgenic mice

In this study, constitutive and induced liver-specific production of IL-12 demonstrated to be sufficient for inducing a severe inflammatory response and lethality. A number of autoimmune disease models demonstrate the critical role of IL-12 in this disease (Segal et al., 1997). Although in those models, autoimmunity was initiated by self-antigens, the intrinsic environment of the target organ and different cytokines may further modulate the localized immune response. It has previously been documented that systemic administration of IL-12 (Trembleau et al., 1995), or transient gene therapy with adenoviral vector expressing IL-12 (Parks et al., 1998) or with IL-12-naked DNA (Watanabe et al., 1999) can markedly enhance cell mediated autoimmune responses. Previous studies with transgenic mice expressing IL-12 in pancreas or in the central nervous system demonstrated the important of the intrinsic environment of the target organ. While constitutive expression of IL-12 in the central nervous system induced a potent immune response with cell damage (Pagenstecher et al., 2000), and a

weaker immune response without cell destruction was observed in the pancreas (Holz et al., 2001). However, this study demonstrates that endogenous production of IL-12 was associated with a severe immune response in the liver. Thus, constitutive expression in IL-12/Act-cre mice was related with hepatic infiltration and severe hepatocyte damage. Furthermore, induction of IL-12 expression in the livers of adult transgenic mice was associated with a spontaneous, vigorous, and destructive immunoinflammatory response, characterized by large hepatic infiltration areas, and hepatocyte damage, demonstrated by increased levels of transaminases. Since inflammatory reactions in the liver are associated with liver cell damage during autoimmune hepatitis (Czaja, 2001), these results suggest an important role of IL-12 in this pathology.

However, breaking of tolerogenic antigen presentation in the liver through inflammatory responses is necessary for overcoming chronic hepatic viral persistence, leading to acute hepatitis and virus elimination (Sette et al., 2001). Although it is supposed that the inflammatory and anti viral properties of IL-12 are mainly due to IFN-γ (Novelli and Casanova, 2004), transgenic mice expressing IFN-γ in the liver demonstrated chronic but not acute hepatitis (Toyonaga et al., 1994). Toyonaga et al. demonstrated, that although local production of IFN-γ was sufficient to initiate and maintain a liver-specific inflammatory disease, pathological changes were modest, due to the regenerative capacity of mouse liver cells. Thus, the severe destructive inflammatory reaction observed in the IL-12 transgenic mice suggests IL-12 to be determinant in inducing acute hepatitis.

IL-12 has also been suggested to play a pivotal role during exacerbated inflammatory responses leading to hepatic failure and lethality during fulminant hepatitis. Thus, in the model of concanavalin A-induced (Nicoletti et al., 2000) and LPS-induced (Tanaka et al., 1996) fulminant hepatitis, neutralizing IL-12 antibodies diminished liver cell damage. Furthermore, some studies with patients demonstrated an increase in the expression of IL-12 in hepatic damage and failure (Quiroga et al., 1998; Leifeld et al., 2002). In this work, constitutive expression of IL-12 was associated with 100% lethality within 5 days after delivery, and induced expression of IL-12 in adult mice was associated with 20% lethality within 8 to 12 days after induction. Lethality due to hepatic failure was very likely, since severe hepatocyte damage characterized by large necrosis areas was observed in both cases. Furthermore, the disturbed development observed in the constitutive expressing mice, and the loss of body weight observed in the adult mice after induction, is likely to be related with a liver dysfunction.

Obviously, systemic levels of IL-12 can have an influence on other organs, and mortality due to other pathologies cannot be excluded. Thus, IL-12 has been related with toxicity and mortality during LPS-induced shock in mice (Wysocka et al., 1995). IL-12 was also related with lethality due to pulmonary oedema (Car et al., 1995; Nakamura et al., 2000). Physiologically, mortality was associated with weight loss, diarrhoea, splenomegaly, crouched posture, reluctant to move and rapid respiration. After induction of IL-12 expression in adult transgenic mice, all those symptoms were observed, but not rapid respiration. In addition, lethality was related with large necrosis areas in the liver. Furthermore, IL-12 has also been related with the development of autoimmune myocardial injury, but not with mortality (Okura et al, 1998). Other studies demonstrated that transgenic mice with constitutive expression of IL-12 in pancreas (Holz et al., 2001) and in brain did not show any lethality (Pagenstecher et al., 2000). Obviously, we can hypothesize that in those studies the immune system could have adapted itself to over-expression of IL-12, or just that only mice expressing such amounts of IL-12, allowing pregnancy, were delivered. However, all mice of three IL-12 transgenic lines, which seemed to constitutively express IL-12 low enough to allow pregnancy, died shortly after delivery. Thus, local production of IL-12 in other organs was related with inflammation but not with lethality, while hepatic produced IL-12 is associated with lethality.

IL-12-induced IFN-γ has been supposed to be the effector cytokine inducing toxicity and hepatocyte damage (Ryffel, 1997; Car et al., 1999). Indeed, mice overdosed with IL-12 showed, wasting, lung infiltration and oedema, splenomegaly, foci of hepatic necrosis, and mortality, which were related to IFN-γ (Leonard et al., 1997). However, mortality was observed only in mice deficient for IFN-gamma signalling (IFN-γ -/-), but not in wild-type mice (Car et al., 1995). Furthermore, transgenic mice expressing IFN-γ in the liver demonstrated only modest pathological changes. Those mice died by one year of age of bacteremia, and not of liver cell damage. The role of other cytokines than IFN-γ can be determinant for hepatic failure. Thus, IL-10 and TNF-α have been related with fulminant hepatitis and hepatic failure (Nagaki et al., 2000).

IL-12 has been suggested to function independently of IFN-γ in regulating differential effector response in autoimmune diseases (Seder et al., 1996). IL-12 induces the expression of many other cytokines, like TNF-α, IL-1β, IL-10, and IL-18, (Trinchieri, 1998), which can also play an important role in inflammation. In a toxic shock model induced by combined administration of IL-2 and IL-12, IFN-γ was not necessary, since toxicity occurred in IFN-γ receptor-/- mice (Carson et al. 1999). Indeed, not IL-12 alone but together with IL-18

(Nakamura et al., 2000), or IL-15 (Biber et al., 2002), can induce IFN-γ independent inflammation. Furthermore, IL-12-induced TNF-α was associated with specific cytolytic activity against hepatocytes in an IFN-γ independent manner (Matsushita et al., 1999).

4.7 scpIL-23 construction and characterization

IL-23 is a new cytokine with potent inflammatory properties (Langrish et al., 2005) different from IL-12 (Oppmann et al., 2000). Therefore, the generation of a transgenic mouse expressing IL-23 is very important for studying inflammatory reactions. (Wiekowski et al., 2001) observed multiorgan inflammation and premature death in transgenic mice with ubiquitous expression of the IL-23 subunit p19. However, no pathology was observed in the liver of those mice, due to the lack of coexpression of p40. Based on this observation, transgenic mice expressing IL-23 in the liver was part of this work. However, IL-23 production is complicated by the heterodimeric nature of the bioactive protein. Although it is not known whether p40 can antagonize the activity of IL-23, a cDNA encoding an IL-23 fusion protein, which ensures the equimolar expression of each subunit was generated. For this purpose, the same strategy like the single chain protein IL-12 construction was followed: 5'- p40-linker-p19 -3'. For construction of scpIL-23, the commercial bovine elastin linker (VPGVGVPGVG) already used for the construction of human IL-23 (InvivoGen) was chosen. Again, like the construction of the scpIL-12, the leader sequence (first 22 amino acids) of the p19 subunit was deleted before connecting to the p40 subunit through the linker. The scpIL-23 was cloned into U3 for checking bioactivity. Since no commercial ELISA exists for IL-23, the property of IL-23 in inducing the production of IL-17 was used (Aggarwal et al., 2003). Indeed, we observed that scpIL-23 induced the production of IL-17 by splenocytes, demonstrating be bioactive.

The scpIL-23 was also cloned into the IAL expression vector and is now available for microinjection and generation of transgenic mice with liver specific IL-23 expression.

5. Summary

Chronic liver inflammation during viral hepatitis is a major health problem worldwide. The role of proinflammatory cytokines, like IL-12, in breaking hepatic immune tolerance, and inducing acute liver inflammation and virus clearance is not clear. Nor is clear its role in uncontrolled severe inflammatory response, leading to fulminant hepatitis and hepatic failure.

This work, focused in the study of the role of endogenous produced IL-12 in inducing hepatic inflammatory responses, demonstrates:

In vitro, using adenovirus coding for IL-12, that hepatocytes stimulate CD4+ T cells in a tolerogenic manner, and that endogenous IL-12 is able to switch the immune response into Th1; and *in vivo,* that endogenous IL-12 induces hepatocyte damage and virus elimination in mice infected with adenovirus.

In addition, and in order to study in vivo the relevance of IL-12 in acute inflammation, conditional IL-12 transgenic mice expressing IL-12 in the liver after cre-recombinase mediated induction were generated. For this purpose, an IL-12 fusion protein was created, which demonstrated high levels of bioactivity. Induction of IL-12 expression during embryonic development was achieved by crossbreeding with Act-Cre transgenic mice; induction of IL-12 expression in adult mice was achieved by a plasmid coding for the cre-recombinase.

This study demonstrates that after induction, IL-12 is expressed in the liver of the transgenic mice. It also demonstrates that hepatic expression of IL-12 induces splenomegaly and liver inflammation, characterized by large infiltrations in portal tracts and veins, associated with hepatic damage, necrosis areas and lethality. Furthermore, constitutive hepatic IL-12 expression does not lead to abortion, but to total lethality, short after delivery.

In conclusion, in this study, a transgenic mouse model has been generated, in which the expression of active IL-12 in the liver can be induced at any time; this model will be very helpful for studying hepatic pathologies. This study has also demonstrated that hepatic produced IL-12 is able of breaking liver tolerance inducing inflammation, virus elimination, severe hepatocyte damage, and lethality. These findings suggest IL-12 as a key cytokine in acute liver inflammation and fulminant hepatic failure.

5.1 Future studies

Once the importance of IL-12 in inducing hepatic inflammation and virus elimination was demonstrated in this study, understanding the mechanisms of the IL-12 induced liver damage, and more important, how to avoid it will be the main focus in the future. It is very important

to achieve hepatic inflammation for a more effective and faster viral elimination, but avoiding the toxicity of IL-12, which leads to massive liver injury and lethality is obviously necessary to allow IL-12 as therapy. For that purpose, future studies will be mainly base on three different points:

1. The determination of different cell populations present in the hepatic infiltration, which of them are responsible for liver injury, and as well their state of activation.
2. The measure of other pro- and anti-inflammatory cytokines and chemokines, which can play a role in IL-12-induced liver inflammation and hepatocyte damage. For these purposes, specific blocking antibodies (anti TNF-alpha, anti IL-12, anti IFN-γ) will be used. The study with different transgenic mice: TNF-alpha Receptor knockout, TGF-β, will also help in determining the role of those cytokines during IL-12-induced liver damage and lethality.
3. The establishing of liver pathology models (viral infection, tumours, auto-antigens) in mice. Induction of IL-12 at any time of the pathology development will help in clarifying the role of IL-12 in those models.

Finally, the transgenic mice expressing IL-23 in the liver will be generated.

6. References

Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature. 1996 Oct 31;383(6603):787-93.*

Abe M, Akbar SM, Horiike N, Onji M. Induction of cytokine production and proliferation of memory lymphocytes by murine liver dendritic cell progenitors: role of these progenitors as immunogenic resident antigen-presenting cells in the liver. *J Hepatol. 2001 Jan;34(1):61-7*

Adorini L. Interleukin-12, a key cytokine in Th1-mediated autoimmune diseases. *Cell Mol Life Sci. 1999 Sep;55(12):1610-25*

Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem. 2003 Jan 17;278(3):1910-4. Epub 2002 Nov 3*

Airoldi I, Gri G, Marshall J, Corcione A, Facchetti P, Guglielmino R, Trinchieri G, Pistoia V. Expression and function of IL-12 and IL-18 receptors on human tonsillar B cells. *J Immunol. 2000 Dec 15;165(12):6880-8*

Al-Wabel A, al-Janadi M, Raziuddin S. Cytokine profile of viral and autoimmune chronic active hepatitis. *J Allergy Clin Immunol. 1993 Dec;92(6):902-8*

Ando K, Moriyama T, Guidotti LG, Wirth S, Schreiber RD, Schlicht HJ, Huang SN, Chisari FV. Mechanisms of class I restricted immunopathology. A transgenic mouse model of fulminant hepatitis. *J Exp Med. 1993 Nov 1;178(5):1541-54.*

Andrianaivo F, Lecocq M, Wattiaux-De Coninck S, Wattiaux R, Jadot M. Hydrodynamics-based transfection of the liver: entrance into hepatocytes of DNA that causes expression takes place very early after injection. *J Gene Med. 2004 Aug;6(8):877-83*

Araki K, Araki M, Miyazaki J, Vassalli P. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci U S A. 1995 Jan 3;92(1):160-4*

Aste-Amezaga M, Ma X, Sartori A, Trinchieri G. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. *J Immunol. 1998 Jun 15;160(12):5936-44. Erratum in: J Immunol 2000 May 15;164(10):5330.*

Bacon CM, McVicar DW, Ortaldo JR, Rees RC, O'Shea JJ, Johnston JA. Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. *J Exp Med. 1995 Jan 1;181(1):399-404*

Bacon CM, Petricoin EF 3rd, Ortaldo JR, Rees RC, Larner AC, Johnston JA, O'Shea JJ. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proc Natl Acad Sci U S A. 1995 Aug 1;92(16):7307-11.*

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature. 1998 Mar 19;392(6673):245-52.*

Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, Sangro B, Melero I, Qian C, Prieto J. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology. 2001 Jan;33(1):52-61*

Belladonna ML, Renauld JC, Bianchi R, Vacca C, Fallarino F, Orabona C, Fioretti MC, Grohmann U, Puccetti P. IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. *J Immunol. 2002 Jun 1;168(11):5448-54*.

Bertolino P, Trescol-Biemont MC, Thomas J, Fazekas de St Groth B, Pihlgren M, Marvel J, Rabourdin-Combe C. Death by neglect as a deletional mechanism of peripheral tolerance. *Int Immunol. 1999 Aug;11(8):1225-38*

Bertolino P, Klimpel G, Lemon SM. Hepatic inflammation and immunity: a summary of a conference on the function of the immune system within the liver. *Hepatology. 2000 Jun;31(6):1374-8*

Biber JL, Jabbour S, Parihar R, Dierksheide J, Hu Y, Baumann H, Bouchard P, Caligiuri MA, Carson W. Administration of two macrophage-derived interferon-gamma-inducing factors (IL-12 and IL-15) induces a lethal systemic inflammatory response in mice that is dependent on natural killer cells but does not require interferon-gamma. *Cell Immunol. 2002 Mar-Apr;216(1-2):31-42*

Brombacher F, Kastelein RA, Alber G. Novel IL-12 family members shed light on the orchestration of Th1 responses. *Trends Immunol. 2003 Apr;24(4):207-12*

Calne RY. Immunological tolerance--the liver effect. *Immunol Rev. 2000 Apr;174:280-2*.

Car BD, Eng VM, Schnyder B, LeHir M, Shakhov AN, Woerly G, Huang S, Aguet M, Anderson TD, Ryffel B. Role of interferon-gamma in interleukin 12-induced pathology in mice. *Am J Pathol. 1995 Dec;147(6):1693-707*.

Car BD, Eng VM, Lipman JM, Anderson TD. The toxicology of interleukin-12: a review. *Toxicol Pathol. 1999 Jan-Feb;27(1):58-63*.

Carreno V, Zeuzem S, Hopf U, Marcellin P, Cooksley WG, Fevery J, Diago M, Reddy R, Peters M, Rittweger K, Rakhit A, Pardo M. A phase I/II study of recombinant human interleukin-12 in patients with chronic hepatitis B. *J Hepatol. 2000 Feb;32(2):317-24*.

Carson WE, Yu H, Dierksheide J, Pfeffer K, Bouchard P, Clark R, Durbin J, Baldwin AS, Peschon J, Johnson PR, Ku G, Baumann H, Caligiuri MA. A fatal cytokine-induced systemic inflammatory response reveals a critical role for NK cells. *J Immunol. 1999 Apr 15;162(8):4943-51.*

Cavanaugh VJ, Guidotti LG, Chisari FV. Interleukin-12 inhibits hepatitis B virus replication in transgenic mice. *J Virol. 1997 Apr;71(4):3236-43*.

Cecere A, Marotta F, Vangieri B, Tancredi L, Gattoni A. Progressive liver injury in chronic hepatitis C infection is related to altered cellular immune response and to different citokine profile. *Panminerva Med. 2004 Sep;46(3):171-87*

Cerny A, Chisari FV. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. *Hepatology. 1999 Sep;30(3):595-601*.

Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, Wolf SF, Young D, Clark SC, Trinchieri G. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med. 1991 Apr 1;173(4):869-79.*

Chang JT, Segal BM, Nakanishi K, Okamura H, Shevach EM. The costimulatory effect of IL-18 on the induction of antigen-specific IFN-gamma production by resting T cells is IL-12 dependent and is mediated by up-regulation of the IL-12 receptor beta2 subunit. *Eur J Immunol. 2000 Apr;30(4):1113-9*

Chen Q, Ghilardi N, Wang H, Baker T, Xie MH, Gurney A, Grewal IS, de Sauvage FJ. Development of Th1-type immune responses requires the type I cytokine receptor TCCR. *Nature. 2000 Oct 19;407(6806):916-20*.

Chen Y, Ong CR, McKenna GJ, Mui AL, Smith RM, Chung SW. Induction of immune hyporesponsiveness after portal vein immunization with ovalbumin. *Surgery. 2001 Jan;129(1):66-75*

Chisari FV. Hepatitis B virus transgenic mice: insights into the virus and the disease. *Hepatology. 1995 Oct;22(4 Pt 1):1316-25*.

Chu CM, Liaw YF. Coexpression of intercellular adhesion molecule-1 and class I major histocompatibility complex antigens on hepatocyte membrane in chronic viral hepatitis. *J Clin Pathol. 1993 Nov;46(11):1004-8. Erratum in: J Clin Pathol 1994 Sep;47(9):870*

Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev. 2002 Apr;13(2):155-68.*

Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. *J Exp Med. 1997 Jul 7;186(1):39-45*.

Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature. 2003 Feb 13;421(6924):744-8*.

Czaja AJ. Understanding the pathogenesis of autoimmune hepatitis. *Am J Gastroenterol. 2001 Apr;96(4):1224-31*.

D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste M, Chan SH, Kobayashi M, Young D, Nickbarg E. Production of natural killer cell stimulatory factor (IL-12) by peripheral blood mononuclear cells. J Exp Med. 1992 Nov 1;176(5):1387-98

Desai BB, Quinn PM, Wolitzky AG, Mongini PK, Chizzonite R, Gately MK. IL-12 receptor II. Distribution and regulation of receptor expression. *J Immunol. 1992 May 15;148(10):3125-32*

Devergne O, Birkenbach M, Kieff E. Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin 12 form a novel heterodimeric hematopoietin. *Proc Natl Acad Sci U S A. 1997 Oct 28;94(22):12041-6.*

Dienes HP, Hutteroth T, Hess G, Meuer SC. Immunoelectron microscopic observations on the inflammatory infiltrates and HLA antigens in hepatitis B and non-A, non-B. *Hepatology. 1987 Nov-Dec;7(6):1317-25*

Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. *Immunol Rev. 2000 Apr;174:5-20.*

Du C, Sriram S. Mechanism of inhibition of LPS-induced IL-12p40 production by IL-10 and TGF-beta in ANA-1 cells. *J Leukoc Biol. 1998 Jul;64(1):92-7.*

Ferrari C, Penna A, Bertoletti A, Valli A, Antoni AD, Giuberti T, Cavalli A, Petit MA, Fiaccadori F. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J Immunol. 1990 Nov 15;145(10):3442-9.*

Fleming KA. The anatomy of the normal liver and the hepatic lymphocyte. In: Crispe IN, ed. T lymphocytes in the liver; immunobiology, pathology and host defence. *New York: Wilea-Liss, 1999:1-15.*

Gao D, Li J, Orosz CG, Bumgardner GL. Different costimulation signals used by CD4(+) and CD8(+) cells that independently initiate rejection of allogenic hepatocytes in mice. *Hepatology. 2000 Nov;32(5):1018-28*

Gazzinelli RT, Hieny S, Wynn TA, Wolf S, Sher A. Interleukin 12 is required for the T-lymphocyte-independent induction of interferon gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci U S A. 1993 Jul 1;90(13):6115-9*

Gerlach JT, Diepolder HM, Jung MC, Gruener NH, Schraut WW, Zachoval R, Hoffmann R, Schirren CA, Santantonio T, Pape GR. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. *Gastroenterology. 1999 Oct;117(4):933-41*

Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, Gubler U, Presky DH, Stern AS, Gately MK. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur J Immunol. 1995 Jan;25(1):200-6*

Gorski K, Carneiro M, Schibler U. Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell. 1986 Dec 5;47(5):767-76.*

Grohmann U, Belladonna ML, Vacca C, Bianchi R, Fallarino F, Orabona C, Fioretti MC, Puccetti P. Positive regulatory role of IL-12 in macrophages and modulation by IFN-gamma. *J Immunol. 2001 Jul 1;167(1):221-7.*

Gubler U, Chua AO, Schoenhaut DS, Dwyer CM, McComas W, Motyka R, Nabavi N, Wolitzky AG, Quinn PM, Familletti PC, et al. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A. 1991 May 15;88(10):4143-7.*

Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. *J Virol. 1995 Oct;69(10):6158-69*

Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. *Science. 1999 Apr 30;284(5415):825-9.*

Heinzel FP, Hujer AM, Ahmed FN, Rerko RM. In vivo production and function of IL-12 p40 homodimers. *J Immunol. 1997 May 1;158(9):4381-8*

Herkel J, Jagemann B, Wiegard C, Lazaro JF, Lueth S, Kanzler S, Blessing M, Schmitt E, Lohse AW. MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocyutes. *Hepatology. 2003 May;37(5):1079-85*.

Holz A, Brett K, Oldstone MB. Constitutive beta cell expression of IL-12 does not perturb self-tolerance but intensifies established autoimmune diabetes. *J Clin Invest. 2001 Dec;108(12):1749-58.*

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science. 1993 Apr 23;260(5107):547-9.*

Huppertz HI, Treichel U, Gassel AM, Jeschke R, Meyer z Buschenfelde KH. Autoimmune hepatitis following hepatitis A virus infection. *J Hepatol. 1995 Aug;23(2):204-8*.

Ihle JN. The Stat family in cytokine signaling. *Curr Opin Cell Biol. 2001 Apr;13(2):211-7*.

Iwata K, Wakita T, Okumura A, Yoshioka K, Takayanagi M, Wands JR, Kakumu S. Interferon gamma production by peripheral blood lymphocytes to hepatitis C virus core protein in chronic hepatitis C infection. *Hepatology. 1995 Oct;22(4 Pt 1):1057-64*

Jaffe HA, Danel C, Longenecker G, Metzger M, Setoguchi Y, Rosenfeld MA, Gant TW, Thorgeirsson SS, Stratford-Perricaudet LD, Perricaudet M, et al. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat Genet. 1992 Aug;1(5):372-8*.

Jagemann, B. hepatischen Ueberexpression des MHC Klasse II Transaktivators (CIITA) in transgenen Mäusen und dessen biologische und immunologische Funktion. Dissertation zur Erlangung des Grades “Doktor der Naturwissenschaften”. Johannes Gutenberg-Universität, Mainz. 2000.

Kato K, Shimozato O, Hoshi K, Wakimoto H, Hamada H, Yagita H, Okumura K. Local production of the p40 subunit of interleukin 12 suppresses T-helper 1-mediated immune responses and prevents allogeneic myoblast rejection. *Proc Natl Acad Sci U S A. 1996 Aug 20;93(17):9085-9.*

Khanna A, Morelli AE, Zhong C, Takayama T, Lu L, Thomson AW. Effects of liver-derived dendritic cell progenitors on Th1- and Th2-like cytokine responses in vitro and in vivo. *J Immunol. 2000 Feb 1;164(3):1346-54*

Klopcic B. Untersuchungen zur Funktion des TGF-beta-Signaltransduktionsweges bei der Bildung von Haaren und Zaehnen. Dissertation zur Erlangung des Grades "Doktor der Naturwissenschaften". Johannes Gutenberg-Universität, Mainz. 2002.

Knolle PA, Uhrig A, Hegenbarth S, Loser E, Schmitt E, Gerken G, Lohse AW. IL-10 down-regulates T cell activation by antigen-presenting liver sinusoidal endothelial cells through decreased antigen uptake via the mannose receptor and lowered surface expression of accessory molecules. *Clin Exp Immunol. 1998 Dec;114(3):427-33*

Knolle PA, Schmitt E, Jin S, Germann T, Duchmann R, Hegenbarth S, Gerken G, Lohse AW. Induction of cytokine production in naive CD4(+) T cells by antigen-presenting murine liver sinusoidal endothelial cells but failure to induce differentiation toward Th1 cells. *Gastroenterology. 1999 Jun;116(6):1428-40*.

Knolle PA, Gerken G. Local control of the immune response in the liver. *Immunol Rev. 2000 Apr;174:21-34.*

Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med. 1989 Sep 1;170(3):827-45*

Kobayashi N, Nishikawa M, Hirata K, Takakura. Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. *J Gene Med. 2004 May;6(5):584-92*

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature. 1993 Mar 18;362(6417):245-8*

Kuntz E, H.-D. Kuntz. Hepatology. Principles and Practice. History. Morphology. Biochemistry. Diagnostics. Clinic. Therapy. *Berlin Springer. 2002*.

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med. 2005 Jan 17;201(2):233-40*.

Laskus T, Slusarczyk J. Autoimmune chronic active hepatitis developing after acute type B hepatitis. *Dig Dis Sci. 1989 Aug;34(8):1294-7*.

Lechner F, Gruener NH, Urbani S, Uggeri J, Santantonio T, Kammer AR, Cerny A, Phillips R, Ferrari C, Pape GR, Klenerman P. CD8+ T lymphocyte responses are induced during acute hepatitis C virus infection but are not sustained. *Eur J Immunol. 2000 Sep;30(9):2479-8*

Lee YL, Ye YL, Yu CI, Wu YL, Lai YL, Ku PH, Hong RL, Chiang BL. Construction of single-chain interleukin-12 DNA plasmid to treat airway hyperresponsiveness in an animal model of asthma. *Hum Gene Ther. 2001 Nov 20;12(17):2065-79*.

Leifeld L, Cheng S, Ramakers J, Dumoulin FL, Trautwein C, Sauerbruch T, Spengler U. Imbalanced intrahepatic expression of interleukin 12, interferon gamma, and interleukin 10 in fulminant hepatitis B. *Hepatology. 2002 Oct;36(4 Pt 1):1001-8*.

Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, Sosman JA, Dutcher JP, Vogelzang NJ, Ryan JL. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood. 1997 Oct 1;90(7):2541-8.*

Lewandoski M, Martin GR. Cre-mediated chromosome loss in mice. *Nat Genet. 1997 Oct;17(2):223-5*

Lieschke GJ, Rao PK, Gately MK, Mulligan RC. Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity in vivo. *Nat Biotechnol. 1997 Jan;15(1):35-40*

Limmer A, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, Momburg F, Arnold B, Knolle PA. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. *Nat Med. 2000 Dec;6(12):1348-54*.

Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, Su C, Pan YC, Hakimi J. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J Immunol. 1995 Jan 1;154(1):116-27.*

Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther. 1999 Jul;6(7):1258-66*

Lohse AW, Meyer zum Buschenfelde KH. Remission of experimental autoimmune hepatitis is associated with antigen-specific and non-specific immunosuppression. *Clin Exp Immunol. 1993 Oct;94(1):163-7*

Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, Dzialo R, Trinchieri G. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med. 1996 Jan 1;183(1):147-57*

Ma X, Trinchieri G. Regulation of interleukin-12 production in antigen-presenting cells. *Adv Immunol. 2001;79:55-92.*

Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol. 1995 May 15;154(10):5071-9

Magram J, Sfarra J, Connaughton S, Faherty D, Warrier R, Carvajal D, Wu CY, Stewart C, Sarmiento U, Gately MK. IL-12-deficient mice are defective but not devoid of type 1 cytokine responses. *Ann N Y Acad Sci. 1996 Oct 31;795:60-70*.

Malmgaard L. Induction and regulation of IFNs during viral infections. *J Interferon Cytokine Res. 2004 Aug;24(8):439-54*

Mattner F, Magram J, Ferrante J, Launois P, Di Padova K, Behin R, Gately MK, Louis JA, Alber G. Genetically resistant mice lacking interleukin-12 are susceptible to infection with Leishmania major and mount a polarized Th2 cell response. *Eur J Immunol. 1996 Jul;26(7):1553-9.*

Matsushita T, Ando K, Kimura K, Ohnishi H, Imawari M, Muto Y, Moriwaki H. IL-12 induces specific cytotoxicity against regenerating hepatocytes in vivo. *Int Immunol. 1999 May;11(5):657-65.*

Mazzolini G, Narvaiza I, Perez-Diez A, Rodriguez-Calvillo M, Qian C, Sangro B, Ruiz J, Prieto J, Melero I. Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12. *Gene Ther. 2001 Feb;8(4):259-67*.

Mehal WZ, Azzaroli F, Crispe IN. Immunology of the healthy liver: old questions and new insights. *Gastroenterology. 2001 Jan;120(1):250-60*

Meyer, Erik. Ein transgenes Mausmodell zur Untersuchung des Einflusses von Matrix Metalloproteinase-2 und –14 auf die Leber. Dissertation zur Erlangung des Grades "Doktor der Naturwissenschaften". Johannes Gutenberg-Universität, Mainz. 2003.

Meyer zum Buschenfelde KH, Hopf U. Studies on the pathogenesis of experimental chronic active hepatitis in rabbits. I. Induction of the disease and protective effect of allogeneic liver specific proteins. *Br J Exp Pathol. 1974 Oct;55(5):498-508*

Morris SC, Madden KB, Adamovicz JJ, Gause WC, Hubbard BR, Gately MK, Finkelman FD. Effects of IL-12 on in vivo cytokine gene expression and Ig isotype selection. *J Immunol. 1994 Feb 1;152(3):1047-56*

Morris MA, Ley K. Trafficking of natural killer cells. *Curr Mol Med. 2004 Jun;4(4):431-8*

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol. 1986 Apr 1;136(7):2348-57*

Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol. 1989;7:145-73*.

Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science. 1990 Dec 21;250(4988):1720-3*

Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther. 2004 Dec;15(12):1157-66*

Myers KJ, Eppihimer MJ, Hall L, Wolitzky B. Interleukin-12-induced adhesion molecule expression in murine liver. *Am J Pathol. 1998 Feb;152(2):457-68.*

Nagaki M, Iwai H, Naiki T, Ohnishi H, Muto Y, Moriwaki H. High levels of serum interleukin-10 and tumor necrosis factor-alpha are associated with fatality in fulminant hepatitis. *J Infect Dis. 2000 Oct;182(4):1103-8. Epub 2000 Aug 28*

Nakahira M, Ahn HJ, Park WR, Gao P, Tomura M, Park CS, Hamaoka T, Ohta T, Kurimoto M, Fujiwara H. Synergy of IL-12 and IL-18 for IFN-gamma gene expression: IL-12-induced STAT4 contributes to IFN-gamma promoter activation by up-regulating the binding activity of IL-18-induced activator protein 1. *J Immunol. 2002 Feb 1;168(3):1146-53.*

Nakamura S, Otani T, Ijiri Y, Motoda R, Kurimoto M, Orita K. IFN-gamma-dependent and -independent mechanisms in adverse effects caused by concomitant administration of IL-18 and IL-12. *J Immunol. 2000 Mar 15;164(6):3330-6*

Naoumov NV, Rossol S. Studies of interleukin-12 in chronic hepatitis B virus infection. *J Viral Hepat. 1997;4 Suppl 2:87-91*

Nelson DR, Bhardwaj B, Lau JY. Interleukin-12 production in chronic hepatitis C infection. J *Hepatol. 2000 Jul;33(1):169*

Nicoletti F, Di Marco R, Zaccone P, Salvaggio A, Magro G, Bendtzen K, Meroni P. Murine concanavalin A-induced hepatitis is prevented by interleukin 12 (IL-12) antibody and exacerbated by exogenous IL-12 through an interferon-gamma-dependent mechanism. *Hepatology. 2000 Oct;32(4 Pt 1):728-33*.

Novelli F, Casanova JL. The role of IL-12, IL-23 and IFN-gamma in immunity to viruses. Cytokine Growth Factor Rev. *2004 Oct;15(5):367-7*

Okura Y, Takeda K, Honda S, Hanawa H, Watanabe H, Kodama M, Izumi T, Aizawa Y, Seki S, Abo T. Recombinant murine interleukin-12 facilitates induction of cardiac myosin-specific type 1 helper T cells in rats. *Circ Res. 1998 Jun 1;82(10):1035-42*

Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity. 2000 Nov;13(5):715-25*.

Orange JS, Biron CA. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J Immunol. 1996 Feb 1;156(3):1138-42*

Ottenhoff TH, Bevan MJ. Host-pathogen interactions. *Curr Opin Immunol. 2004 Aug;16(4):439-42*

Oxenius A, Karrer U, Zinkernagel RM, Hengartner H. IL-12 is not required for induction of type 1 cytokine responses in viral infections. *J Immunol. 1999 Jan 15;162(2):965-73.*

Pagenstecher A, Lassmann S, Carson MJ, Kincaid CL, Stalder AK, Campbell IL. Astrocyte-targeted expression of IL-12 induces active cellular immune responses in the central nervous system and modulates experimental allergic encephalomyelitis. *J Immunol. 2000 May 1;164(9):4481-92.*

Pantoliano MW, Bird RE, Johnson S, Asel ED, Dodd SW, Wood JF, Hardman KD. Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in Escherichia coli. *Biochemistry. 1991 Oct 22;30(42):10117-25.*

Paradisi R, Porcu E, Venturoli S, Maldini-Casadei M, Boni P. Maternal serum levels of pro-inflammatory cytokines in missed and threatened abortion. *Am J Reprod Immunol. 2003 Oct;50(4):302-8*

Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, Pflanz S, Zhang R, Singh KP, Vega F, To W, Wagner J, O'Farrell AM, McClanahan T, Zurawski S, Hannum C, Gorman D, Rennick DM, Kastelein RA, de Waal Malefyt R, Moore KW. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol. 2002 Jun 1;168(11):5699-708*.

Park AY, Hondowicz BD, Scott P. IL-12 is required to maintain a Th1 response during Leishmania major infection. *J. Immunol. 2000 Jul 15;165(2):896-902*.

Park AY, Scott P. Il-12: keeping cell-mediated immunity alive. *Scand J Immunol. 2001 Jun;53(6):529-32*

Park JW, Gruys ME, McCormick K, Lee JK, Subleski J, Wigginton JM, Fenton RG, Wang JM, Wiltrout RH. Primary hepatocytes from mice treated with IL-2/IL-12 produce T cell chemoattractant activity that is dependent on monokine induced by IFN-gamma (Mig) and chemokine responsive to gamma-2 (Crg-2). *J Immunol. 2001 Mar 15;166(6):3763-70*.

Parks E, Strieter RM, Lukacs NW, Gauldie J, Hitt M, Graham FL, Kunkel SL. Transient gene transfer of IL-12 regulates chemokine expression and disease severity in experimental arthritis. *J Immunol. 1998 May 1;160(9):4615-9*

Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, Hibbert L, Churakova T, Travis M, Vaisberg E, Blumenschein WM, Mattson JD, Wagner JL, To W, Zurawski S, McClanahan TK, Gorman DM, Bazan JF, de Waal Malefyt R, Rennick D, Kastelein RA. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. *Immunity. 2002 Jun;16(6):779-90*.

Pillarisetty VG, Katz SC, Bleier JI, Shah AB, Dematteo RP. Natural killer dendritic cells have both antigen presenting and lytic function and in response to CpG produce IFN-gamma via autocrine IL-12. *J Immunol. 2005 Mar 1;174(5):2612-8*

Presky DH, Minetti LJ, Gillessen S, Wilkinson VL, Wu CY, Gubler U, Chizzonite R, Gately MK. Analysis of the multiple interactions between IL-12 and the high affinity IL-12 receptor complex. *J Immunol. 1998 Mar 1;160(5):2174-9*

Quiroga JA, Martin J, Navas S, Carreno V. Induction of interleukin-12 production in chronic hepatitis C virus infection correlates with the hepatocellular damage. *J Infect Dis. 1998 Jul;178(1):247-51*

Reina M, Broccia ML, Menegola E, Di Blasio AM, Vigano P, Giavini E. Effects of interleukin-12 administration during the pre- and peri-implantation period on mouse embryofetal development. *Am J Reprod Immunol. 2004 May;51(5):345-51*

Richa J. Production of transgenic mice. *Methods Mol Biol. 2000;136:427-34*

Roitt I, Brostoff J, Male D. Immunology. Fourth edition, 1996. *Mosby and Times Mirror International. London.*

Rossol S, Marinos G, Carucci P, Singer MV, Williams R, Naoumov NV. Interleukin-12 induction of Th1 cytokines is important for viral clearance in chronic hepatitis B. *J Clin Invest. 1997 Jun 15;99(12):3025-33.*

Ryffel B. Interleukin-12: role of interferon-gamma in IL-12 adverse effects. *Clin Immunol Immunopathol. 1997 Apr;83(1):18-20.*

Sakai K, Miyazaki J. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem Biophys Res Commun. 1997 Aug 18;237(2):318-24*

Sangro B, Qian C, Schmitz V, Prieto J. Gene therapy of hepatocellular carcinoma and gastrointestinal tumors. *Ann N Y Acad Sci. 2002 Jun;963:6-12*

Schijns VE, Haagmans BL, Wierda CM, Kruithof B, Heijnen IA, Alber G, Horzinek MC. Mice lacking IL-12 develop polarized Th1 cells during viral infection. *J Immunol. 1998 Apr 15;160(8):3958-64.*

Seder RA, Gazzinelli R, Sher A, Paul WE. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A. 1993 Nov 1;90(21):10188-92*.

Seder RA. The role of IL12 in the regulation of Th1 and Th2 differentiation. *Res Immunol. 1995 Sep-Oct;146(7-8):473-6*

Seder RA, Kelsall BL, Jankovic D. Differential roles for IL-12 in the maintenance of immune responses in infectious versus autoimmune disease. *J Immunol. 1996 Oct 1;157(7):2745-8*

Segal BM, Klinman DM, Shevach EM. Microbial products induce autoimmune disease by an IL-12-dependent pathway. *J Immunol. 1997 Jun 1;158(11):5087-90*

Senaldi G, Lobo-Yeo A, Mowat AP, Mieli-Vergani G, Vergani D. Class I and class II major histocompatibility complex antigens on hepatocytes: importance of the method of detection and expression in histologically normal and diseased livers. *J Clin Pathol. 1991 Feb;44(2):107-14*

Sette AD, Oseroff C, Sidney J, Alexander J, Chesnut RW, Kakimi K, Guidotti LG, Chisari FV. Overcoming T cell tolerance to the hepatitis B virus surface antigen in hepatitis B virus-transgenic mice. *J Immunol. 2001 Jan 15;166(2):1389-97*

Siders WM, Wright PW, Hixon JA, Alvord WG, Back TC, Wiltrout RH, Fenton RG. T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. *J Immunol. 1998 Jun 1;160(11):5465-74*

Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol Res. 1999;20(2):147-6*

Sobue S, Nomura T, Ishikawa T, Ito S, Saso K, Ohara H, Joh T, Itoh M, Kakumu S. Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. *J Gastroenterol. 2001 Aug;36(8):544-51*

Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol. 1997 Nov 15;159(10):4772-80*

Sternberg N, Hamilton D. Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol. 1981 Aug 25;150(4):467-86*

Strober W, Ludviksson BR, Fuss IJ. The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann Intern Med. 1998 May 15;128(10):848-56.*

Sun Z, Wada T, Maemura K, Uchikura K, Diehl AM, Klein AS. Hepatic allograft-derived Kupffer cells regulate T cell response in rats. *Liver Transpl. 2003 May;9(5):489-97*

Sun QL and Ran W. Review of cytokine profiles in patients with hepatitis. *World J Gastroenterol 2004 June 15; 10(12):1709-1715*

Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells. *J Exp Med. 1997 Mar 3;185(5):817-24*

Tanaka Y, Takahashi A, Watanabe K, Takayama K, Yahata T, Habu S, Nishimura T. A pivotal role of IL-12 in Th1-dependent mouse liver injury. *Int Immunol. 1996 Apr;8(4):569-76*

Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, Govindarajan S, Purcell RH, Chisari FV. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. *Proc Natl Acad Sci U S A. 2002 Nov 26;99(24):15661-8. Epub 2002 Nov 19*

Toyonaga T, Hino O, Sugai S, Wakasugi S, Abe K, Shichiri M, Yamamura K. Chronic active hepatitis in transgenic mice expressing interferon-gamma in the liver. *Proc Natl Acad Sci U S A. 1994 Jan 18;91(2):614-8*

Trembleau S, Penna G, Bosi E, Mortara A, Gately MK, Adorini L. Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice. *J Exp Med. 1995 Feb 1;181(2):817-2*

Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood. 1994 Dec 15;84(12):4008-27*

Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol. 1998;70:83-243*

Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity. 2003 Nov;19(5):641-4*

Trojan J, Naval X, Johnson T, Lafarge-Frayssinet C, Hajeri-Germond M, Farges O, Pan Y, Uriel J, Abramasky O, Ilan J. Expression of serum albumin and of alphafetoprotein in murine normal and neoplastic primitive embryonic structures. *Mol Reprod Dev. 1995 Dec;42(4):369-78.*

Watanabe M, Fenton RG, Wigginton JM, McCormick KL, Volker KM, Fogler WE, Roessler PG, Wiltrout RH. Intradermal delivery of IL-12 naked DNA induces systemic NK cell

activation and Th1 response in vivo that is independent of endogenous IL-12 production. *J Immunol. 1999 Aug 15;163(4):1943-50*

Wiekowski MT, Leach MW, Evans EW, Sullivan L, Chen SC, Vassileva G, Bazan JF, Gorman DM, Kastelein RA, Narula S, Lira SA.Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol. 2001 Jun 15;166(12):7563-70*.

Wies I, Brunner S, Henninger J, Herkel J, Kanzler S, Meyer zum Buschenfelde KH, Lohse AW. Identification of target antigen for SLA/LP autoantibodies in autoimmune hepatitis. *Lancet. 2000 Apr 29;355(9214):1510-5*.

Wilkinson VL, Carvajal D, Minetti LJ, Chua AO, Gubler U, Gately MK, Presky DH. Functional characterization of mouse IL-12 receptors. *J. Allergy Clin Immunol. 1997 99:S52*

Wirth S, Guidotti LG, Ando K, Schlicht HJ, Chisari FV. Breaking tolerance leads to autoantibody production but not autoimmune liver disease in hepatitis B virus envelope transgenic mice. *J Immunol. 1995 Mar 1;154(5):2504-15*

Wolf SF, Temple PA, Kobayashi M, Young D, Dicig M, Lowe L, Dzialo R, Fitz L, Ferenz C, Hewick RM, et al. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J Immunol. 1991 May 1;146(9):3074-81*

Wu C, Warrier RR, Wang X, Presky DH, Gately MK. Regulation of interleukin-12 receptor beta1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. *Eur J Immunol. 1997 Jan;27(1):147-54*.

Wysocka M, Kubin M, Vieira LQ, Ozmen L, Garotta G, Scott P, Trinchieri G. Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. *Eur J Immunol. 1995 Mar;25(3):672-6*.

Yang J, Chen S, Huang L, Michalopoulos GK, Liu Y. Sustained expression of naked plasmid DNA encoding hepatocyte growth factor in mice promotes liver and overall body growth. *Hepatology. 2001 Apr;33(4):848-59*

Yao BB, Niu P, Surowy CS, Faltynek CR. Direct interaction of STAT4 with the IL-12 receptor. *Arch Biochem Biophys. 1999 Aug 1;368(1):147-55*.

Yap G, Pesin M, Sher A. Cutting edge: IL-12 is required for the maintenance of IFN-gamma production in T cells mediating chronic resistance to the intracellular pathogen, Toxoplasma gondii. *J Immunol. 2000 Jul 15;165(2):628-3*

Yoshimoto T, Wang CR, Yoneto T, Waki S, Sunaga S, Komagata Y, Mitsuyama M, Miyazaki J, Nariuchi H. Reduced T helper 1 responses in IL-12 p40 transgenic mice. *J Immunol. 1998 Jan 15;160(2):588-94*

Zeuzem S, Hopf U, Carreno V, Diago M, Shiffman M, Grune S, Dudley FJ, Rakhit A, Rittweger K, Yap SH, Koff RS, Thomas HC. A phase I/II study of recombinant human interleukin-12 in patients with chronic hepatitis C. *Hepatology. 1999 Apr;29(4):1280-7*

7. Abbrevations

Ad: Adenovirus
AIH: Autoimmune hepatitis
ALT: Alanine aminotransferase
APC: Antigen presenting cell
AST: Aspartate aminotransferase
□Gl: βGlobulin intron
bp: base pair
CDNA: Coding DNA
CIITA: Class II transactivator
CMV: Cytomegalovirus
CTc: Cytotoxic (cytolytic) T lymphocyte
DC: Dendritic cell
EBI3: Epstein Barr virus induced gene 3
ELISA: Enzyme linked immunosorbent assay
Enh: Enhancer
G-CSF: Granulocyte-colony stimulating factor
GFP: Green fluorescent protein
IAL: Insulator-Albumin-LoxP
IFN: Interferon
IHL: Intrahepatic lymphocyte
IL: Interleukin
JAK: Janus kinase
KDa: Kilodalton
L: LoxP site
LAK: Lymphokine-activated killer cell
LSEC: Liver sinusoidal endothelial cell
MACS: Magnet associated cell separation
MHC: Major histocompatibility complex
MOI: Multiplicity of infection
NKc: Natural killer cell
NKSF: Natural killer cell stimulating factor
NPC: Non-parenchimal cell
OVA: Ovoalbumin
P: Promoter

PA: Poly Adenosine

RT: reverse transcription

Scp: Single chain protein

STAT: Signal transducer and activator of transcription

TCCR: T cell cytokine receptor

T-cell: T lymphocyte

TGF: Transforming growth factor

Th: Helper T lymphocyte

TLR: Toll like receptor

TNF: Tumor necrosis factor

TYK: Tyrosine kinase

UV: Ultraviolet

WSX-1: TCCR