3- Discussion

In the present study, cytokine expression in the liver of mice following adenoviral gene transfer was established. The strategy used in this work was to secrete mouse interferon alpha, beta or gamma from transduced hepatocytes locally in the liver and to characterize the inhibition of hepatic HBV DNA replication, which was previously demonstrated in HBV transgenic mice via both the IFNγ- or IFNα/β-dependent antiviral pathway (Cavanaugh V.J. et al., 1997; McClary H. et al., 2000). To achieve this, two expression systems were used: constitutive expression of cytokines under the control of a CMV promoter and a tetracycline-regulated cytokine and luciferase co-expression. Both systems allowed a strong but only transient secretion of cytokines from transduced hepatocytes.

The following aspects will be discussed in detail:

At first, the experimental mouse models for HBV replication will to be discussed in section 3.1, in which the antiviral effect of cytokine expression on the HBV replication was characterized. *In vitro,* cell culture models were used to prove the antiviral effect of cytokine expression following adenoviral gene transfer. HBV transgenic or HBV transduced mice were employed to characterize the effect *in vivo*.

Secondly, the tetracycline-regulatable gene expression will be discussed in comparison to alternative inducible gene expression systems *in vivo* in section 3.2 .

Third, the effect of constitutive or tet-inducible cytokine expression on HBV replication will be discussed in section 3.3.

Finally, futures perspectives of this work will be discussed.

3.1- Establishment of experimental mouse models

3.1.1- *In vitro* and *in vivo* models for HBV replication

Mouse hepatocytes AML12 cells and primary mouse hepatocytes

In order to characterize the antiviral activity of cytokines on HBV replication following adenoviral transduction, a suitable *in vitro* model using murine hepatocyte lines was needed. The hepatocyte cell line AML12 (Wu j.C. et al., 1993) was selected for this study, because AML12 cells are non transformed hepatocytes, as is the case of the human hepatoma cell lines HuH7 or HepG2, and were immortalized without viral

oncogenes. AML12 cells were obtained from livers of transgenic mice, that over-express the transforming growth factor α (TGFα). The advantage of AML12 cells is that have retained their differentiated features after many passages. Their hepatocytic origin and phenotype were characterized by morphology and high expression of albumin, α-fetoprotein and LDH isozyme 5 exclusively , which proved similar to primary culture of murine hepatocytes (PMH) (Sawada N. et al., 1998). PMH contain various cell types, including 80% of hepatocytes and 20% of nonparenchymal cells as sinusoidal endothelial cells, Kupffer cells, intrahepatic lymphocytes and bile duct epithelial cells (Alpini G. et al., 1994). Because PMH are closed to the *in vivo* situation and allow the interaction between hepatocytes and nonparenchymal cells, they were included in my study. The disadvantage is that primary culture of hepatocytes from perfused HBV transgenic mice become rapidly less well differentiated *in vitro*, and therefore the level of HBV replication is strongly decreased (Pasquetto V. et al., 2002). The PMH preparations could be maintained for seven days in enriched medium for my experiments.

To establish the intracellular HBV replication, AML12 cells or PMH were transduced using an adenoviral vector AdGH1.3 for transfer of the overlength 1.3 HBV genome. The levels of HBV DNA intermediate obtained in transduced AML12 cells was 5-fold lower than the HBV replication in human hepatoma HepG2 (Sprinzl M.F. et al., 2000) or in HuH7 cells (personal communication Klöcker U.). One reason could be the difference of liver-specific transcription factors between mouse and human. Another limitation is the tropism of human adenovirus serotype 5 (Ad5) not optimal for infection of mouse hepatocytes: expression of the coxsackie-adenovirus receptor (CAR), that is necessary for adenoviral infection in combination with the cell surface αvβ3 and αvβ5 integrin coreceptors (Wickhan TJ. et al., 1993; Bai M. et al., 1993), is expected much weaker in mouse cells than in human hepatocytes. Expression levels of both CAR and integrins are important determinants of the transduction efficiency of the adenoviral vectors. Thus, to achieve improved adenoviral gene transfer into AML12 cells or PMH a supplementation of cell culture medium with 1,7% dimethyl sulfoxide (DMSO) was required. DMSO is a solvent known to maintain the differentiated state of cultured cells (Isom I. et al., 1987; Gripon P. et al., 1988). It is retained the competence of the cultured hepatocytes to express duck hepatitis core antigen and duck hepatitis B virus DNA at high levels (Galle et al., 1989). In contrast to the HepaRG cells, where DMSO is

absolutely required for susceptibility to HBV infection (Gripon P., 2002), addition of DMSO on transduced AML12 cells with AdGH1.3 did not support HBV DNA replication, but seemed to facilitate adenovirus binding to cell membrane, clathrin-mediated endocytosis or nucleus import of adenoviral genome. In addition, reduction of the amount of medium was necessary to increase the efficiency of adenoviral infection (Aguilar-Cordova E., 2001).

An alternative *in vitro* mouse model was recently published (Pasquetto V. et al., 2002): HBV-Met cells. HBV-Met cells were obtained from 1.3-HBV/c-Met double transgenic mice and constitute an immortalized hepacytocytes line (HBV-Met). Upon high differentiation, they express and replicate HBV from the integrated transgene at same level as in the liver of HBV 1.3 transgenic mice. In contrast to the above presented *in vitro* models used in this study, HBV-Met cells are a *in vitro* model for a chronic HBV infection and not for acute HBV infection.

Experimental mouse models for HBV replication

To study the immunological response to HBV infection in *in vitro* experiments is not suitable. Infection models with the related viruses DHBV or WHV exist, but the host immune system is not very well defined yet. Because the murine immune system is well characterized, the mouse model were employed in this work to characterize the effect of mouse cytokine expression on HBV replication.

As a first model was the HBV 1.3 fsX(-)3´5´ transgenic mice (Weber O. et al., 2002) were used, which secrete high-levels of HBeAg into the serum and have higher-level of hepatic HBV DNA intermediate forms than the HBV 1.3 transgenic mice (Guidotti L.G et al., 1995). Both HBV transgenic mice are immunotolerant to HBV antigens HBsAg and HBeAg and covalently closed circular (ccc) HBV DNA in the liver is not detectable.

The second mouse model used in this study was C57BL/6 mice, infected with $2{,}5x10^9$ i.u. of adenovirus AdGH1.3 for establishment of HBV replication. This model mimics an acute hepatitis infection with humoral immune response to the secreted antigens HBsAg and HBeAg detectable at 21-28 days p.i., thus the mice were not immunotolerant such as the transgenic mice and transaminase level (ALT) are elevated.. The disadvantage of these mice is the lower lever of hepatic HBV DNA replicate intermediates and a 5-fold lower level of HBeAg in the serum than in HBV transgenic

mice. As in HBV transgenic mice no HBV ccc DNA form was detectable in the liver of transduced mice (personal communication Oberwinkler H. and Protzer U.), and two weeks p.i. a decrease of HBV gene expression and HBV replication was observed.
In summary the two mouse models were very useful for this study, but both showed limitations as expected.

3.1.2- Efficiency of adenoviral-mediated gene transfer into the liver of mice

The first aim of this study was to express cytokines under the control of a CMV promoter in the liver of HBV transgenic mice. As shown by Egelhardt J.F. et al. (1994) and Anderson W.F. (1998) expression of recombinant transgene via gene transfer and transgene expression occur with variable persistence and depends on the vector, route, dose, transgene, species, and/or mouse strain.

Replication-defective adenovirus vectors were used in this study, because they have a high capacity to incorporate a substantial amount of heterologous DNA, infect a wide range of cell types, which is also disadvantage due to no tissue specificity. However adenoviral vectors (95%) preferentially target the liver following post-intravenous injection of rodents (Prevec L. et al., 1989; Jaffe HA. et al., 1992). They are stable *in vivo.* These advantages explain its extensive use in gene therapy studies (Benihoud K. et al., 1999; Russell W.C., 2000). The most severe limitations of adenovirus is its immunogenicity leading to cellular and humoral immune responses. One solution to reduce the immunogenicity and to improve the efficiency and selectivity of adenovirus vectors would be to modulate the adenoviral vector tropism via incorporation of a polypeptide ligand into the fiber protein (Belousova N. et al., 2002), or to eliminate the natural receptor tropism by introduction of a new specific tropism (Kim J. et al., 2002). If viral genes are deleted such as the E2A gene (Gorziglia M.I. et al., 1996), or the DNA polymerase gene (Amalfitano A. et al., 1998), or almost all adenoviral genes (Kochanek S., 1999), the immune response to viral proteins is reduced and it leads indirectly to more efficient transgene expression. All these adenoviral vectors are interesting but more complicated to work with. Other strategies exist to transfer an expression cassette into hepatocytes or tissues, specifically the liver. Naked DNA and liposomes, which reach only a limited number of hepatocytes (Will H. et al., 1985; Takahashi H. et al., 1995), recombinant baculoviruses are effective *in vitro* (Delaney W.E.T. and Isom H.C.,

1998; Delaney W.E.T. et al., 1999), but are inactivated from the complement system *in vivo*.

More important for toxicity of the adenoviral vector is the quality of the vector preparation (personal communication Nelson J.). In this study, the immune responses to adenoviral particles were reduced using low dose of adenovirus with the same efficiency of gene transfer in the liver: a double celsium-chloride gradient of adenovirus preparation was performed followed by a dialysis and reduced 10-fold the part of non-infectious particles in the preparation of high titer of adenovirus. The obtained titer was at least 10^{11} infectious virus particles per ml in 293 cells. This degree of purification of adenovirus stock corresponded to standards applied for the human gene therapy. The administration of low ($<10^9$ P.U.) and intermediate (10^9-10^{11} P.U.) doses of $E1^-/E3^-$ adenovirus gene transfer vectors appears to be well tolerated in patient (Harvey B-G. et al., 2002) as well as in mice in my study.

The strain of HBV transgenic mice used in this study was a good choose in this study, because C57BL/6 mice are more accessible for adenoviral gene transfer (Peng Y. et al., 2001) than BALB/c mice. For example, BALB/c mice produce a higher level of expression of IL-12, which mediated increase of IFNγ. IL-12 and IFNγ activate NK cell, which promote $CD8^+$ T-cell recruitment and priming. The activated CD8+ T cells eliminate then recombinant adenovirus (Yang Y. et al., 1994). Thus, the adenovirus is less persistent in BALB/c mice (Schowalter D.B. et al., 1999) and lower transgene expression than in C57BL/6 mice is reported (Aurisicchio L. et al., 2001).

In this work, the production of high titer of the adenoviruses AdGmIFNβ and AdTTR-rtTA was hampered. Expression of rtTA may be toxic for the cell at high levels. This technical problem was resolved by using the tetracycline-inducible gene expression system of the Adbiluc system, for example the recombinant adenovirus Adbiluc-mIFNβ. However, a low level of basal expression was still observed in 293 cells transduced with Adbiluc vectors.

Take together, the efficiency for gene transfer in mouse cells are low with human recombinant adenovirus. Elaboration of favorable conditions to adenovirus infection on tissue cultures, higher purification degree of viral preparation, which allow injection of low dose of vectors, and the choose to used C57BL/6 mouse strain has made the gene transfer via adenoviral vector enough high-performance in mouse models.

3.2- A liver specific tetracycline-regulated gene expression via adenoviral gene transfer in mice

To avoid side effects provided from high level expression of cytokine in liver and in other organs and to better correlate cytokine expression to inhibition of HBV replication in the liver of HBV transgenic mice, a tetracycline-regulated and liver specific gene expression system was established using adenoviral vectors for the gene transfer.

For liver directed gene transfer, different gene delivery systems have been developed. These include liver-specific nonviral vectors (e.g. asialoglycoprotein receptor-targeting system) and viral vectors (retrovirus-mediated gene transfer system, adenovirual vector, herpes simplex virus vector, etc.) (Ferry N. and Heard J.M., 1998). The adenoviral vector, employed in this work, does not target exclusively hepatocytes *in vivo*. Other possibility is to use a murine leukemia virus (MLV) as pseudotype virus containing HBV surface proteins, which confers strict hepatotropism (Sung V.M.H. and Lai M.M.C., 2002). The hepatitis B virus itself as vector allows targeting of hepatocytes and liver specific gene expression (Protzer U. et al., 1999). However, the capacity to incorporate a substantial amount of heterologous DNA in HBV vector is to restricted for tet-regulated gene expression cassette.

The ideal alternative strategy is to establish a liver specific gene expression following adenoviral gene transfer using a liver specific promoter. In this study, the promoter for expression of the liver enriched activator protein (P_{LAP}) promoter (Talbot D. et al., 1994) was employed in transgenic mice. The promoter-enhancer of transthyretin (P_{TTR}) (Fung w. et al., 1988; Qian X. et al., 1995) was used in the stable expressing cell line HuH7-TTR-rtTA and in adenoviral vectors (AdTTR-rtTA and Adbiluc-rtTA). The liver specificity of transgene expression was assured in tTA and rtTA transgenic mice and in transduced cells with AdTTR-rtTA (data not shown). However, weak expression of tTA is detectable in the kidney of tTA transgenic mice (Kistner A. et al.,1996).

The Tet-On system (Gossen et al., 1995) yields advantage for gene therapy approaches, because target gene expression is only induced if the drug was added into the drinking water. By removing the drug, gene expression ceased relatively fast. The elimination of the remaining drug can be prolonged by retention in fat tissues of the mouse. Thus, at least three days were necessary to wait in this study, before the luciferase activity was assayed in living mice (fig.19). The originally described Tet-On system works well in most tissue culture applications, where cells were transfected with plasmids containing

both components of the system, but it was less effective in transgenic animals (Kistner A. et al., 1996; Mansuy I et al., 1998; Mallet G. et al., 2001). The second generation of synthesized rtTA, rtTA2^s-M2 and rtTA2^s-S2 (Urlinger S. et al, 2000), which were used in the present study, yielded satisfactory results with respect to induction ratio, background level and kinetics of induction in transfected HeLa cells (Urlinger S et al., 2000), in primary neurons, in transgenic mice (Hasan M.T. et al., 2001; Schönig K. et al., 2002) and in transgenic flies (Stebbins M.J. et al., 2001). The potential toxicity associated with the VP16 transactivation domain in the original tTA or rtTA was avoided with rtTA2^S-S2, which consist of fusing the amino acid sequences of wild-type TetR to three minimal activation domains of the "F"-type (Baron U. et al., 1997). This exchange resulted in a drastically increased affinity of the transactivators to tetO in the presence of low concentrations of doxycycline. In this study, a concentration of 1µg/ml in tissue culture and 2 to 0,1 mg/ml of doxycycline in drinking water was used for mice at weight of ~200g.

The background level of reporter gene expression is very low in the Tet-On system and the progesterone antagonist-induced (Prog) gene expression system, both inserted in an adenoviral vector. Confirming the results observed in my study, the induction for the Tet-On system is 3-fold higher than for the Prog system *in vitro* (Molin M. et al., 1998). Even *in vivo* model, the results in the present study were in line with observations in transgenic mice and in mice infected with HD-adenovirus vector, where a 10-fold reduction of the amount of doxycycline resulted in at least 100-fold lower luciferase activity in the liver (Kistner A. et al., 1996; Aurisicchio L. et al., 2002).

In this study, I inserted a tet-responsive bidirectional promoter P_{bi-1} (Baron U. et al., 2000) into an adenoviral vector. Thus, cytokine expression from transduced cells can be monitored indirectly via co-expression of the luciferase reporter gene *in vitro* and in living mice. Recent technical advances made *in vivo* imaging of luciferase reporter proteins in living mice possible by using a cooled charge-coupled device camera. These techniques allow real-time monitoring of the kinetics of gene expression via co-expression of luciferase in the living mouse. For example, the kinetics and the extent of the disease progression in animals infected with recombinant HSV-1 or adenovirus, which expressed firefly luciferase reporter protein, are monitored (Luker G.D. et al., 2002). Bioluminescence measured correlated well with luciferase activity and the amount of luciferase expressed (Wu J.C et al., 2001; Hasan M.T. et al., 2001; Luker

G.D. et al., 2002). In transgenic mice, full induction of tet-controlled gene expression is achieved 8 hours in the liver (Hasan M.T. et al., 2001); transduction with my adenovirus needed less than five hours to establish the episomal adenoviral genome in hepatocytes and to co-express transgene and luciferase in the on-state. In double transgenic mice (Tet-On system), the activity of the tet-responsive bidirectional promoter P_{bi-1} is dependent first on the amount of available tTA or rtTA, and second on the integration site of the transgene (Krestel H.E. et al., 2001).

In my study, the activity of P_{bi-1}, which was inserted into the E1A/E1B deletion of an adenovirus type 5 vector, also depended on the expression level of tTA or rtTA and on the position of P_{bi-1} in the adenoviral genome. The inverted terminal repeat (ITR) of the adenovirus 5 genome contains redundant binding sites for cellular factors that stimulate transcription of E1 and E4 genes (for an overview see fig.1a in (Buvoli M. et al., 2002)). Since some of these elements overlap with the viral origin of replication and the packaging signal, they can not be deleted or inactivated. In the left ITR (34931-35935 bp) the bipartite E1A enhancer is located, which trans-activates E1A genes and controls expression of early genes after infection (Leza MA. and Hearing P., 1988; Miralles VJ. et al., 1989; Hatfield L and Hearing P., 1991). The promoter for the intermediate transcript pIX is situated just downstream of the E1 site (Hearing P and Shenk T., 1983). All these elements can act on any heterologous promoter inserted into the E1 deletion of an adenovirus vector. As shown in fig.14 of this study, enhancer elements had a cis-acting effect on P_{bi-1} as basal expression depended on the orientation of the cassette in Adbiluc or Adbiluc2. As the Adbiluc adenoviral vectors assured a tight expression of the cytokine in transduced hepatocytes, they were used to produce a set of vectors for a therapy approach to hepatitis B infection.

A combination of the Tet-On system with adeno-associated viral vectors (AAV) allows the establishment of a long term transgene tetracycline-regulation due to integration in chromosome, but high basal expression in non-induced state was observed inducing a strong humoral and cellular immune responses to rtTA expression (Favre D. et al., 2001). To efficiently repress such background activities, tetracycline controlled transcriptional silencers, that bind tTA-responsive promoter in the absence of doxycycline, can be employed. In this study, stable HeLa cell line HR5-13S expressing tTS^{Kid} (Freundlieb S. et al., 1999) was used. tTS^{Kid} reduced basal expression by a factor of 5, but decreased to the same extend the induction of transgene expression. Thus,

switching on/off for gene expression was possible but not effective in tissue culture any more. A strong decrease of basal expression levels of luciferase was reported in murine muscle cells following gene transfer mediated by AAV vectors when a plasmid coding for tTS^{Kid} was co-transferred. However, luciferase expression decrease to 70% after two weeks and was not detectable anymore after one month (Perez N. et al., 2001). In contrast, the weak basal expression of luciferase observed in in vitro with Adbiluc vectors was disappeared by application in mice (fig. 18a) especially when a transgene fragment such as GFP was inserted into the multiple cloning site of the Adbiluc vector (forming Adbiluc-GFP). In this case, the 810 bp fragment coding for GFP may insulate P_{bi-1} from adenoviral cis-acting elements. Despite flanking the expression cassette with transcriptional insulator elements such as derived from the γ-globin locus (HS-4) (Steinwaerder DS and Lieber A., 2000) allows the re-establishment of selective expression in adenoviral vector, functional limitations of transgene expression are reported (Buvoli M. et al., 2002) and the lack of cloning facilities in Adbiluc shuttle plasmid did not allow to use this strategy in the present work.

To avoid co-transfection, we wanted to generate a one-vector system that allow tet-regulated gene expression. Bujard H. et al. (1985) and Yin DX. et al. (1996) have recommended not co-transfecting the expression unit of the transactivator and the response element since the minimal promoter might function as an enhancer trap amenable to influence by a close-by integrated CMV enhancer/promoter. However, Johansen J. et al. (2002) investigated this assumption and showed no difference in background expression between native and mutant constructs, when the CMV promoter was deleted. Exploiting the adenoviral cis-acting effect on P_{bi-1} activity in Adbiluc and Adbiluc2, a vector component that contains a “tet-autoregulation” system was generated by insertion of the rtTA gene into the multiple cloning site (fig.13c). By this, liver specific gene expression was lost. Using the autoregulatory vector, luciferase expression was induced in lysates of HuH7 cells transduced with Adbiluc-rtTA (fig.17) in a doxycycline-dependent fashion after 4 days p.i.. A higher basal expression than in experiment with HuH7-TTR-rtTA cells transduced with Adbiluc was observed (fig.15). Since the basal expression of Adbiluc *in vivo* was low, no luciferase induction was detected in livers of C57BL/6 mice infected with $2{,}5.10^9$ i.u. of Adbiluc-rtTA even after seven days treatment with doxycycline (data not shown). Therefore, I generated

Adbiluc2-rtTA and expected higher basal expression to induce rtTA expression as starter for the tet-autoregulation.

An other approach was also investigated in this study unsuccessfully: the rtTA gene under the control of the liver specific promoter P_{TTR} was cloned into the E3 deleted region (480-3534 bp) of the adenoviral vector. Recently, Mizuguchi and Hayakawa (2002) generated such a single adenovirus vectors of the first generation containing a tetracycline-controllable expression system (tet-off and tet-on system). They reported, that the adenoviral vector using the tet-off system allows to induce gene expression 20- to 500-fold. In contrast, the adenoviral vector containing components of the tet-on system induces gene expression by only 2- to 28-fold in cell culture. The latter requires about two-log orders higher concentration of doxycycline to switch on the gene expression in comparison with the vector containing components of the tet-off system (Mizuguchi and Hayakawa, 2001; Mizuguchi and Hayakawa, 2002). Aurisicchio L. et al. (2001) generated a one component helper-dependent adenovirus vector (HD vector). The $rtTA2^S$-S2 is cloned under the TTR promoter/enhancer and in the opposite orientation mIFNα2 gene is inserted under the control of a tet-regulated promoter. Tight expression was observed in cell culture and no IFNα2 is detectable in serum of mice in absence of doxycycline. A single adenoviral vector containing the tet-controllable expression system will facilitate analyses of gene function *in vitro* and *in vivo* and may be useful for gene therapy.

3.3- Novel option to study hepatitis B virus infection

This study demonstrated that local expression of mIFNγ, mIFNβ and mIFNα suppressed HBV replication in livers of the HBV transgenic mice following an adenoviral gene transfer.

3.3.1- Is the cytokine expression a good strategy for antiviral approach on HBV infection ?

To allow an efficient therapy of hepatitis B disease and to suppress viral replication, different steps in the HBV replication cycle can be targeted. For example, HBV transcription is regulated by liver-enriched nuclear factors, e.g. HNF3β (Tang H. and McLachlan A., 2002; Banks K.E. et al., 2002). However disruption of intracellular

signaling pathways by factors as HNF3β, which has been implicated in the nucleosomal organization of the albumin gene, could have a general impact on hepatic specification and differentiation. HNF3β leads to increased HNF4 expression and other liver specific genes whose products are important in the glucose metabolism.

More promising is the intrahepatic induction of interferon alpha/beta (IFNα/β), interferon gamma (IFNγ), or tumor necrosis factor alpha (TNFα) by various stimuli, which can inhibit HBV gene expression and/or HBV replication noncytopathically in the liver of HBV transgenic mice (Guidotti L.G. et al., 1996; Cavanaugh V.J et al., 1998; Guidotti L.G. and Chisari F.V., 1999; McClary H. et al., 2000; Guidotti L.G. and Chisari F.V., 2001). The strong induction of secretion of IFNα, IFNβ or IFNγ causes inflammatory effects in other tissues as the central nervous system as observed in animals and patients. Moreover, the antiviral signaling pathway, which is specific for each cytokine can not be characterized, because a set of cytokines was induced following injection/infection of stimulating mediators onto immune cells. In comparison with the current treatment of patient with chronic hepatitis B with systemic injection of natural cytokines (rIFNα2a) with ribavirin, local expression might reduce the strong site effects.

In the present study a concept was tested in mouse models of HBV infection, that combined cytokine expression and gene transfer based on adenoviral vectors. A defined and locally restricted cytokine expression in the liver allowed to overcome strong side effects and to characterize the antiviral pathway induced by the secreted cytokines. In this study, the cytokine genes were expressed under control of two types of heterologous promoters: one was the cytomegalovirus immediate-early promoter-enhancer (P_{CMV}) and the second a bidirectionnal tet-regulated promoter (P_{bi-1}). Secretion of cytokines under control of both promoters was at comparable levels *in vitro*, but it was shown that the activity of these promoters was downregulated after 5 days in AML12 cells (data not shown) and after 10-14 days p.i. *in vivo* (fig.12 and fig.22). These results were in line with observations in fourteen cell line of many tissues and several species, where gene expression (luciferase) decreases under control of SV40 or CMV promoter in the presence of cytokines, especially IFNγ (Harms JS. and Splitter GA., 1995; Ritter T. et al., 2000). The tumor-suppressor mouse p53 represses transcription from the promoters of viruses, that do not express proteins that complex with p53, such as the human cytomegalovirus early promoter and the Rous sarcoma

virus long terminal repeat (Jackson P. et al., 1993). The secreted cytokines might repress the heterologous promoter activity directly or indirectly via a p53-dependent pathway in addition to elimination of the adenoviral genome when hepatocytes are replaced. The bidirectional promoter P_{bi-1} contains seven tet operator sequences flanked by two minimal promoters. Since these minimal promoters span the sequence of P_{hCMV} from position +75 to –53 and –31 (Baron U. et al., 1995), the direct or indirect inactivation of P_{bi-1} induced by long-term cytokine secretion must target this domain of P_{CMV}. This downregulation occurred simultaneously on the two transcriptional units fused to the P_{bi-1}: luciferase and cytokine co-expression was repressed to the same extent *in vivo*. In comparison, the activity of a tissue-specific promoter, such as the constitutive promoter/enhancer of transthyretin (P_{TTR}) (Fung w. et al., 1988; Qian X. et al., 1995), seems to remain constant in mice for a prolonged period of 10 months, even if IFNα is expressed (Aurisicchio L. et al., 2001).

Since low dose of adenovirus ($2x10^8$ i.u. per mouse) was injected in mice, a low induction of mIFNα/β in liver was expected in this study, so that the independently elimination of adenoviral vectors from the liver may be limited. An alternative would be to co-inject neutralizing antibodies to IFNα/β and/or IFNγ to increase the efficiency of adenovirus entry into the hepatocytes.

3.3.2- Antiviral effects of each cytokine on HBV replication

In this study, less than 10% of hepatocytes transduced with AdmIFNγ or AdGmIFNβ were sufficient to eliminate HBV DNA intermediate forms from the liver and reduced HBV particle secretion into the serum of mice. According dose of adenovirus and gene transfer efficiency, a moi of about 1 i.u. per transduced hepatocytes was acquired for each vector in liver of mice. A comparison with in vitro data, the inhibiting effect on HBV DNA replication was only 2-fold amplified. Intrahepatic macrophages such as Kupffer cells, may be responsible for this increase *in vivo*. Since Kupffer cells similar to sinusoidal endothelial cells are not infected with adenovirus type 5 (Hegenbarth S. et al., 2000), only hepatocytes secreted cytokine following adenoviral gene transfer. These cytokines may activate non-parenchymal cells and indirectly amplify the antiviral effect. In opposition of these in vivo data, no amplification was observed in primary mouse hepatocytes, when mIFNγ or mIFNβ were secreted (fig. 9). Klöcker U. et al. (2000) could show that endotoxin inhibits hepadnavirus replication in primary duck

hepatocytes in an indirect, noncytopathic fashion: it stimulates hepatic nonparenchymal cells, most probably liver macrophages, to secrete soluble antiviral mediators. When present during DHBV infection of duck hepatocytes, the antiviral mediators block intracellular viral protein synthesis and progeny virus production as well as cccDNA amplification (Schultz U. and Chisari F.V., 1999; Klöcker U. et al., 2000). The transcriptional template of the virus, the cccDNA, is required for initiation and maintenance of HBV infection. Whether the cccDNA is susceptible to noncytopathic control of interferon, it must be eliminated for viral clearance to be permanent and complete. In mice, such studies are not possible, because they do not establish HBV cccDNA (Guidotti L.G. et al., 1995; personal communication Protzer U.). However, it has been shown in acutely infected chimpanzees that the viral cccDNA is susceptible to noncytolytic control (Guidotti L.G. et al., 1999).

No changes in the intrahepatic amount of viral protein and HBV RNA were detectable. If a distinction was made between nuclear and cytoplasmatic core protein, the reduction may be more significant (Cavanaugh V.J. et al., 1998; Wieland S. personal communication, 2002).

The intracellular mechanism of inhibition of HBV replication was not investigated, but the inhibiting effect of each cytokine on HBV replication was described.

The expression of mIFNγ induced a strong inhibition of HBV replication: 95% of the intrahepatic HBV DNA was eliminated *in vitro* (fig.8) and *in vivo* (fig.10) and the amount of viral particles containing HBV DNA was reduced 25- to 50-fold in the serum of mice (tab.5). Secretion of mIFNγ *in vivo* usually activates the macrophages in the liver to produce cytokines (e.g. IFNα/β, TNFα and nitric oxide). These cytokines have a direct antiviral activity as well as other cytokines with indirect immunoregulatory functions (IL-1, IL-6, IL-8, IL-10, IL-12 and IL-18). Activated macrophages produce chemokines (IP-10, MIP1α, MCP-1), that recruit cytotoxic T-lymphocytes to the site of infection. When HBV transgenic mice were injected with a high dose of $5x10^8$ i.u. of AdmIFNγ (fig.10), the secretion of mIFNγ repressed intrahepatic HBV DNA replication and induced a recruitment of CTL into the liver. The induction of IP10 transcription from immune cells by secreted mIFNγ demonstrated indirectly a migration of T cells into the liver with high localized concentrations of chemokines such as IP10 or Mig. Thus, the increase of ALT level observed in treated mice (tab.5) reflects resulting hepatocellular necrosis (Feutren G. et al., 1984). The antiviral potential of the activated

CTL is primary mediated by noncytolytic mechanisms that involve the intrahepatic production of IFNγ by the immune cells (Guidotti L.G., 2002). In comparison with the woodchuck animal model, the administration of wIFNγ did not inhibit the WHV replication in primary WHV-infected woodchuck hepatocytes, but induced an enhanced transcription of woodchuck MHC-I heavy-chain mRNA (Lu M. et al., 2002). These results were confirmed in WHV carrier injected with 10^{12} virus particles of the helper-virus dependent adenovirus HD-wIFNγ, in which only a minor reduction of WHV replicative intermediates in the liver is observed. In contrast, after transduction with HD-wIFNα (Salucci V. et al., 2002), a decrease of viremia of one log step and reduction of the amount of replicative forms of WHV in the liver by 80% are shown during four months (Fiedler M. personal communication). In the duck model of hepatitis B, IFNγ was able to inhibit DHBV replication in primary duck hepatocytes, but the formation of cccDNA was unaffected by duck IFNγ (Schultz U. and Chisari F.V., 1999).

In my study, a weaker inhibition of HBV replication *in vitro* as IFNγ resulted from expression of IFNβ, but led to the disappearance of intrahepatic HBV replicative intermediates in mice. The first difficulty was the the level of expression of mIFNβ could not be monitored in mice infected with AdGmIFNβ, because no assays for mouse IFNβ were available. IFNβ has also an extremely short half-life in the blood system after systemic protein administration (Salmon P. et al., 1996). Compared to mIFNγ, expression of mIFNβ was 500-fold lower as measured indirectly via the luciferase activity in the livers of mice (fig.24). As it known, IFNβ down regulates expression of the gene encoding angiogenic factor and inhibits angiogenesis *in vivo* (D´Amore P.A., 1992; Dong Z. et al., 1999). An according observation was made in this study, when lower amounts of β actin in the liver were observed independent from the dose of AdGmIFNβ injected (fig.10c). IFNβ is released from virus-infected cells, thereby conferring antiviral effects on neighboring cells (Watanabe Y. and Kawade Y., 1987). The down regulation of cellular protein biosynthesis could be a mechanism to repress viral replication. However, no significant decrease of HBV particle was detected in sera of mice injected with AdGmIFNβ (tab.5). In addition, no inhibition of translation of viral proteins such as the surface proteins was observed. Direct local IFNβ gene therapy can also lead to an activation of the innate and adoptive immune system (Qin X-Q. et

al., 2001), which reflected in mice infected with dose of $5x10^8$ i.u. of AdGmIFNβ by elevated ALT level in serum (tab. 5).

Expression of luciferase was 5-fold higher mice injected with Adbiluc-mIFNα than in those infected with Adbiluc-mIFNβ or Adbiluc-mIFNγ respectively (fig.24c and d). Accordingly, IP10 and 2´5´OAS was more pronounced following Adbiluc-mIFNα infection. Because a high basal expression was also observed in the off-state following Adbiluc-mIFNα injection, either virus titers were not exactly the same or mice had a variable doxycycline intake (fig.24d). The analysis of the antiviral effect on HBV replication in H1.3/rtTA double transgenic mice could not be completed in my experiment because half of the mice was lost and material was limited. However, 50 to 70% of HBV mRNA´s disappeared in livers of mice , when mIFNγ or mIFNβ were expressed respectively, was observed (fig. 24f). According to decrease of HBV RNA in livers, secretion of HBV virion in sera of these mice was reduced 2,5-fold. This effect on HBV RNA level was not observed in mice infected with AdmIFNγ and AdmIFNβ. Induction of IFNγ and TNFα by MCMV infection of HBV transgenic mice has the above observed effect to suppress the hepatic steady-state content of the 3.5- and 2.1-kb mRNA by day 3 to day 5 p.i. (Cavanaugh V.J. et al., 1998). This is accompanied by strongly suppression of hepatic HBV replicative DNA forms, which was not analyzable in Southern blot in my study, but is expected *in vitro* (Rang A. et al., 1999; Rang A. et al., 2002) and *in vivo* (Protzer U. et al., 1999; Aurisicchio L. et al., 2001). Induction of mIFNα expression in the liver of mice infected with the helper-dependent adenoviral vector HD-Tet-mIFNα was associated with a partial doxycycline-dependent hepatic protection against an acute infection with mouse hepatitis virus type 3 (MHV-3) (Aurisicchio L. et al., 2001). A low expression of mIFNα was sufficient to observe this protective effect. However, results obtained in an acute infection model with MHV-3 do not necessarily correspond to those obtained in a model for HBV infection.

The level of the local expression of interferons is an important safety issue. H1.3/rtTA double transgenic mice were infected with a low dose of Adbiluc-mIFNα or Adbiluc-mIFNβ or Adbiluc-mIFNγ (fig. 24). An i.p. injection of doxycycline induces in few minutes almost all hepatocytes transduced with adenovirus mediating a strong cytokine expression. In my study, this high level of mIFNα, mIFNβ or mIFNγ expression was

toxic. It may have activated the murine immune system and caused that these mice died hours later. The same induction scheme was before tested in mice injected with a 10-fold higher dose of Adbiluc-GFP. The strong expression of luciferase or GFP did not induce this massive immune response and ALT levels in these mice were normal (data not shown). Cytokine induction *in vivo* with the Adbiluc system should be performed by addition of doxycycline to the drinking water, which was non toxic in my experiments.

The mouse model for acute HBV infection was used in my study to determine whether interferons might enhance HBV elimination as described for hepatitis C virus (Jaeckel E. et al., 2001; Guo J.T. et al., 2001; Frese M. et al., 2001; Lanford R.E. et al., 2003) using the bicistronic replicon system (Lohmann V. et al., 1999). During the acute hepatitis the elevation of ALT level in sera reflected an inflammatory response in transduced mice and not in chronically infected patients (Chisari F.V. and Ferrari C., 1995) like by HBV transgenic mice. Two weeks post infection with 10^9 i.u. of AdGH1.3, the HBV replication level in transduced mice decreased strongly. Twenty days p.i. is the earlier time point for detection of humoral response to HBV infection in these mice. However, no difference was found between interferon treated and untreated animals in my experiment.

Gene transfer of cytokine was a comfortable strategy to determine the effect of single cytokines, when the set of adenoviral vectors is present. An alternative strategy is to cross HBV transgenic mice with mice genetically deficient for IFNγ, TNFα receptor or the IFNα/β receptor. Generation of double transgenic mice needs at least six months and lot of work for the screening of mice. Using these transgenic mice, two distinct antiviral mechanisms are determined. The first is an IFNα/β-dependent pathway (Guidotti L.G. et al., 1996b), where IFNα/β mediates the early inhibitory effect on HBV replication in the livers of HBV transgenic mice (McClary H. et al., 2000) by inhibiting the formation and/or promoting the destabilization of immature HBV pregenomic RNA-containing capsids (Wieland S. et al., 2000). One investigation is the processing of the mouse La protein (mLa), an HBV RNA-specific binding protein, which may make more accessible the viral RNA to endoribonucleotytic cleavage (Heise T. et al., 1999a; Heise T. et al., 1999b). The second antiviral mechanism is an IFNγ-dependent antiviral mechanism, which leads to reduction in the intracellular level of HBV transcripts, especially when it acts synergistically with TNFα (Pasquetto V. et al., 2002).

Component of the IFN-induced antiviral response on HBV replication may be the proteasome-dependent degradation of viral or cellular proteins required for HBV replication (Robek M. et al., 2002).

To give an overview over the system employed in my study, fig.26 was designed. It summarizes the action of mIFNγ and mIFNβ on HBV replication observed in my study and the current knowledge of the antiviral pathways induced by cytokines. Expression of IFNγ had a strong antiviral effect on HBV replication. Amplification of the mIFNγ effect on HBV replication by activation of macrophages and T-lymphocytes is expected *in vivo*, but was not evident in primary mouse hepatocytes in my study. Long term secretion of mIFNγ repressed CMV and minimal CMV promoter activity. Expression of mIFNβ also had an inhibiting effect on HBV replication; as a side effect an arrest of the translation machinery was observed. An antiviral effect of mIFNα could not clearly be shown in this study due to experimental problems, but is also expected according to published data. The antiviral mechanisms induced by mIFNα or mIFNβ expression seem differ from IFNγ-dependent pathways according the results in this work. At the molecular level, for both types of interferon, signaling through JAK/STAT pathways is essential: two examples are STAT1 and p48, which are induced by binding of IFNγ and IFNα/β to their specific receptor (Muller M. et al., 1993; Stark G.R. et al., 1998). It is known, that cytokines lead to a post-transcriptional degradation of the viral RNA (Tsui L.V. et al., 1995). If the Adbiluc model will be used, as presented in fig.26, this observation can be studied in more detail

It has been demonstrated, that the antiviral activity of interferon in HBV transgenic mice is not solely mediated by Mx, RNAse L, PKR, or interferon regulatory factor 1 (IRF-1) (Guidotti L.G. et al., 2002), but may contribute to modulate HBV replication. Unfortunately, which signal genes and antiviral pathways were specifically induced by mIFNγ or IFNα/IFNβ was not determined in my study, but needs to be investigated in more detail in future studies.

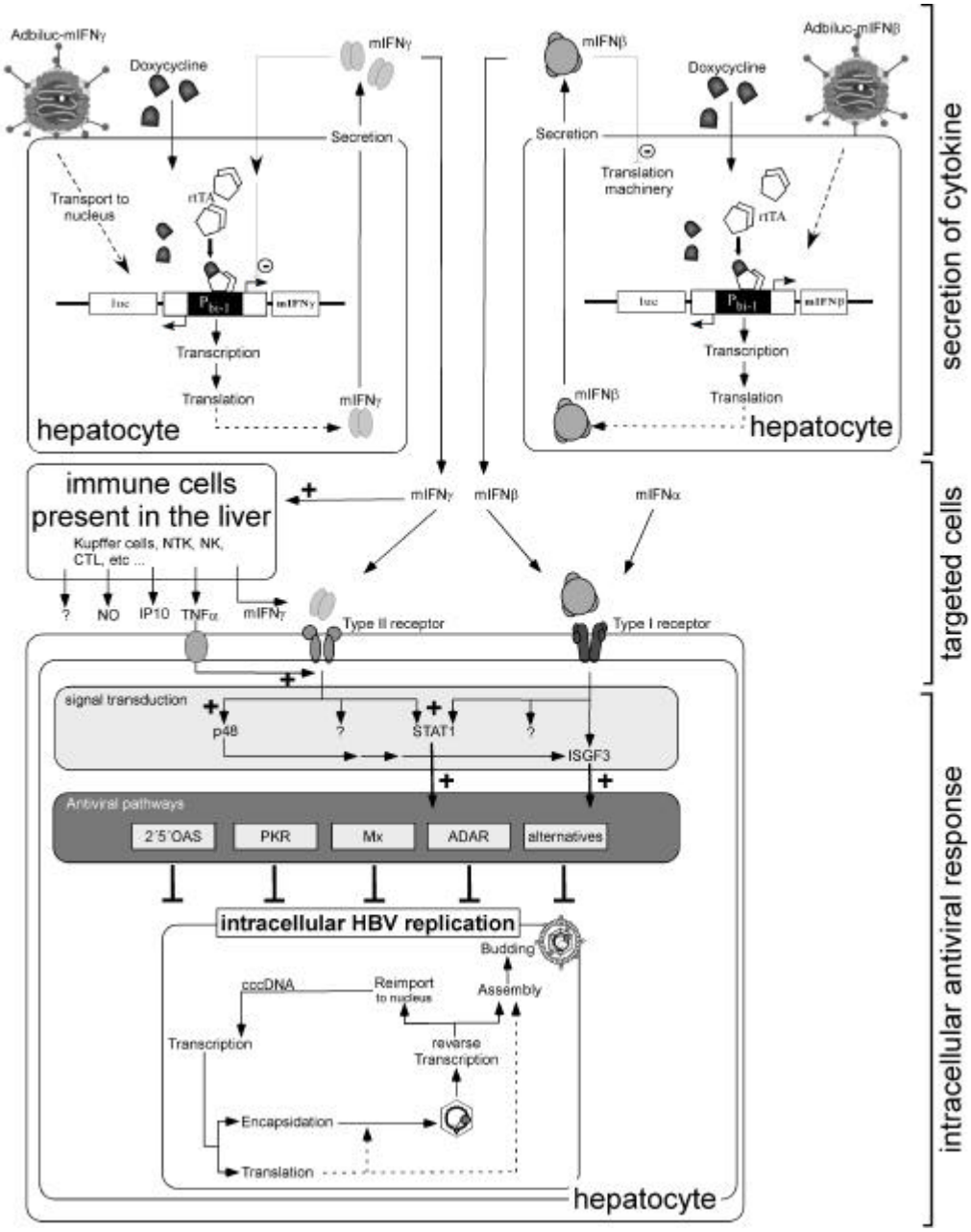

Fig.26: Schematic representation of the antiviral effect of type I and type II interferons expressed from hepatocytes transduced with Adbiluc vectors.

The expression of mIFNγ or mIFNβ, under control of the tet-regulated promoter P_{bi-1}, is induced by the addition of doxycycline. Doxycycline binds to reverse tet-transactivator rtTA, the complex rtTA-Dox binds to P_{bi-1} and induces expression of the luciferase (luc) and mIFNγ or mIFNβ genes. Secreted mIFNγ and mIFNβ bind to specific surface receptors on neighboring hepatocytes and induce transcription of a variety of genes via different signal transduction pathways (STAT1, p48 and ISG3 genes for example). One or more intracellular antiviral pathways (2´5´ oligoadenylate synthetase (2´5´OAS), PKR, Mx proteins, ADAR or unknown pathways, called alternatives) may be activated, which repress HBV replication. Local secretion of mIFNγ may also activate immune cells in the liver, which secrete immune mediators, such as TNFα, this increasing the antiviral activity, or chemokines like IP10 . In an autoregulation fashion, a downregulation of P_{bi-1} controlled gene expression by mIFNγ and an inhibition of the translation machinery by secretion of mIFNβ was observed.

3.3.3- Immune response to the adenoviral vector *in vivo*

Using adenoviral vectors for gene transfer means to activate efficiently the innate immune responses which occurs in the absence of virus gene transcription (McCoy R.D. et al., 1995; Schnell M.A. et al., 2001), a response that leads to acute inflammation of transduced tissues and reduced gene transfer efficiency (Lieber A. et al., 1997; Schnell M.A. et al., 2001; Tao N. et al., 2001) in contrast to infection with AAV vectors, where the response did not result in detectable tissue damage or inflammation (Zaiss A. et al. 2002). The chemokine and cytokine induction following exposure of mice to adenovirus vectors is rapid (6 hours p.i.), marked and sustained (Zaiss A et al., 2002). An inflammation was also observed in HBV transgenic mice infected with high dose of adenoviral vectors (McClary H. et al., 2000).

To resolve this difficulty in the present study, adenovirus vectors were injected at low dose of $2x10^8$ i.u. and a slight elevated ALT level was induced in serum of the mice (tab.6). An anti-adenoviral immune response was detected by a drop of hepatic HBV DNA replicative intermediate during 24 hours p.i. (fig.11b). At the same time cytokine was expressed already 4 hours p.i. (fig.19) in mice infected with AdmIFNγ and AdGmIFNβ and induced an antiviral response (30% decrease of hepatic HBV DNA). Since adenovirus infection induced IFNα/β and TNFα expression during the first 24 hours (Cavanaugh et al., 1998), the observed reduction of replicative HBV DNA intermediates is supported by other published data with markely higher dose of $>10^{11}$ pfu of adenovirus. *In vivo* studies of adenovirus-mediated inflammation show locally increased expression or release of cytokines including TNFα, IL-6, IL-8, Mip-1a, IL-1 and Mip-2 (Otake et al., 1998 Cartmell et al., 1999; Muruve et al., 1999). Adenoviral particles elicit a very brisk release of chemoattractant cytokines *in vitro*. A minimal loss of activity after UV inactivation of virions, and sensitivity to heat inactivation that denatures virion structure leads to the conclusion that the stimulation depends on binding and/or internalization (Higginbotham J.N. et al., 2002).

In summary, the dose of adenoviral particles used to infect mice has an indirect effect on the study during the first three days. After this period these side effects are removed in my *in vivo* experiments. Thus, the given data could be employed for the

characterization of antiviral events from constitutive cytokine expression on HBV replication or the induction of cytokine expression should occur first after 3 days p.i..

3.4- Futures perspectives

Expression of interferon suppressed HBV replication, but the effect was transient. It was indirectly shown that the activity of both heterologous promoters, P_{CMV} and P_{bi-1}, was down regulated after 10 days of cytokine expression. The tetracycline-regulated cytokine expression using the Adbiluc system was successfully established in the livers of mice and monitoring of transgene expression via luciferase activity in living animal proved very useful for divers applications. Using the adenoviral Adbiluc system, the molecular antiviral pathway induced by expression of each cytokine can now be identified and better characterized.

An other pathways involving yet-undefined interferon-regulated genes may mediate the antiviral activity of interferons against HBV. In the future, the identification of the cytokine-induced intracellular molecular events (fig.26) that control viral infections will be an important area of investigation specially with recent advances available in functional genomics and proteomics. Recently, twenty-nine genes were identified as transcriptionally controlled hepatocellular genes, that are tightly associated with the inhibition of HBV replication by indirectly cytokines induction in HBV transgenic mice or incubation of recombinant cytokines on immortalized differentiated hepatocyte cell line, the HBV-Met cells (Wieland S. et al., 2003). Using the technologies of DNA microarrays and high-throughput cDNA differential display method, it could be easier to identify activated hepatocellular genes following expression of one specific cytokine with the Adbiluc system *in vivo.*

The disadvantage of a first generation of adenoviral vector as used in this study for gene transfer into the livers of mice is that it is strongly immunogenic. Application for a second infection in mice or human has to be supplemented by an immuno-suppression treatment. A promising alternative would be to employ helper-dependent adenoviral (HD) vectors, which are less immunogenic because of lack of expression of viral proteins. The second disadvantage of the Adbiluc system is that one has to use two

components for tet-induced gene expression. A one-vector system would be advantageous because no tTA or rtTA transgenic mice and no co-infection with AdTTR-rtTA were needed. Thus, an application in patient would be conceivable. Integration of a tetracycline-sensitive transactivator element into the expression cassette employing the P_{bi-1} promoter was one possibility, which worked in cell culture and should also work with the Adbiluc2 vector in mice. However, tissue-specific gene expression was lost in this model. A cassette containing tTA- or rtTA-gene under the control of tissue-specific promoter could be inserted into the E3-deleted region of the adenoviral backbone plasmid or upstream of the cassette containing luciferase and transgene under the control of P_{bi-1}. In the later vector, insulator sequence such as UMS (McGeady M.L. et al., 1986) should be inserted between transactivator and P_{bi-1} to limit transcriptional interference between the two transcription units (Corti O. et al., 1999).
To study a protective effect of interferon expression for an acute HBV infection, cytokine expression needs to be induced before HBV replication starts.

In summary, in this study an adenoviral system very helpful to study the antiviral effect of cytokine expression on HBV replication was established. Not only the understanding of host-virus interactions will be improved, but also new approaches for the treatment of persistent viruses such as HBV, HCV and HIV can be now investigated using tet-inducible cytokine expression following adenoviral gene transfer.