INTERLEUKIN-12 INHIBITS LIVER-SPECIFIC DRUG-INDUCIBLE SYSTEMS IN VIVO

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ABSTRACT

Drug-inducible systems allow the modulation of duration and intensity of cytokine expression in liver immuno-based gene therapy protocols. However, the biological activity of the transgene may influence their function. We have analyzed the kinetics of IL-12 expression controlled by the doxycycline (Dox)- and the mifepristone (Mif)-dependent systems using two long-term expressing vectors directed to liver: a plasmid administered by hydrodynamic injection and a high capacity adenoviral vector. Daily administration of Dox or Mif was associated with a progressive loss of inducibility and a decrease of murine IL-12 production. This inhibition occurred at the transcriptional level and was probably caused by an IFN-γ-mediated down-modulation of liver-specific promoters that control the expression of transactivators in these systems. Genome-wide expression microarrays studies revealed a parallel down-regulation of liver-specific genes in mice over-expressing murine IL-12. However, a promoter naturally induced by IL-12 was also inhibited by this cytokine when placed on a plasmid vector. Interestingly, treatment with sodium butyrate, a class I/II histone deacetylase inhibitor was able to rescue liver-specific promoter activity solely in the vector. We conclude that biologically active IL-12 can transiently inhibit the function of drug-inducible systems in non-integrative DNA vectors by reducing promoter activity, probably through IFN-γ and protein deacetylation-dependant mechanisms.

INTRODUCTION

The success of gene therapy depends on reaching adequate levels and duration of transgene expression in a target organ or tissue. When the therapeutic gene is toxic, this requirement needs to be coupled with an efficient gene regulatory system. However, the biological effects of the gene product in the organism may affect its own regulation *in vivo* through mechanisms that are difficult to predict *in vitro*. This is especially relevant in case of molecules with immunostimulating properties that have been successfully used in cancer gene therapy.

Interleukin-12 (IL-12) a potent inducer of IFN-γ and has been shown to exert efficient antitumor effects against liver cancer. A strategy based on the intratumor (it) injection of an IL-12-expressing adenoviral vector (Ad-CMVmIL12) resulted in low toxicity, tumor reduction and prolonged survival in different animal models of metastatic and primary liver cancer (reviewed in [1]). However, a limited and transient antitumor effect was evidenced with a similar approach using Ad-CMVhIL-12 vector in a Phase I clinical trial, probably due to low levels and short-term expression of the transgene [2,3]. This prompted the development of new vectors and a strategy based on the long-term expression of IL-12 in the liver. In order to avoid toxicity of the cytokine we placed the two IL-12 chains under the control of the doxycycline (Dox) or the mifepristone (Mif)-inducible systems [4,5] . This allows modulation of IL-12 expression in a dose and time-dependent manner upon administration of the inducer drug (Dox or Mif), as reported for hGH and IFN-α in a similar setting [6,7]. Here we study the influence of the biological effect of IL-12 on the *in vivo* function of these systems. In our study the production of IL-12 is restricted to the liver by placing transactivators (TA) under the control of tissue-specific promoters, and also by virtue of the vectors and delivery methods used.

In the case of the Dox-inducible system, the plasmid pTonT(L2)-IL12 was administered by hydrodynamic injection in mice [4], whereas the Mif-inducible system was delivered by intravenous or intrahepatic injection on a high-capacity adenoviral vector (GL-Ad/RU-IL-12) [5]. In each case, two sets of vectors were constructed to express either human IL-12 (hIL-12) or murine IL-12 (mIL-12). In mice, hIL-12 is not biologically active and it was used as a reporter gene whose production can be easily quantified in the serum of animals. We describe here the kinetics of mIL-12 expression induced from the pTonT(L2)-mIL12 plasmid and the viral vector GL-Ad/RUmIL-12 in the liver of immunocompetent mice. The results evidence a drastic, although reversible silencing of mIL-12 expression following continuous transgene induction. In contrast, the expression of hIL-12 was more stable using these same systems, as reported previously [4,5]. We have also defined the effect of mIL-12 on endogenous liver-specific gene expression and the participation of IFN-γ at least in initial stages of transgene inhibition on plasmid- and adenoviral-based vectors. Finally, we describe that the histone deacetylase inhibitor (HDACi) sodium butyrate (NaB) is able to alleviate transgene silencing *in vivo*.

RESULTS:

1. **Kinetics of IL-12 production using drug-dependant gene regulatory systems in mice.**

In order to study the influence of transgene expression on the function of inducible systems *in vivo*, we have used the immunostimulatory cytokine mIL-12 in the context of a Tet-on system (plasmid pTonL2(T)mIL12) or a Mif-inducible system (adenoviral vector Ad-GL/RUmIL-12). As a control, we used identical vectors expressing hIL-12, which is not biologically active in mice (pTonL2(T)-hIL12 and Ad-GL/RUhIL-12). Schematic representations of the plasmids and the adenoviral vector genomes are shown in figure 1A and 1B, respectively. The plasmid pTonL2(T)-hIL12 was administered to C57BL/6 mice by hydrodynamic (HD) injection, and hIL-12 induction was carried out by administration of Dox for 8 consecutive days. The concentration of hIL-12 in the serum of animals was measured at different time points by ELISA. In the absence of Dox, no hIL-12 was detected (data not shown). As expected, the continuous induction stimulated sustained levels of hIL-12, with only a moderate reduction at day 8 versus day 1 (Fig. 1C). In contrast, when the same procedure was carried out with the pTonL2(T)mIL12 plasmid in a 10 days induction protocol, a progressive reduction of mIL-12 was observed starting at day 3, with more than 100 fold decrease at days 8 and 10 (Fig. 1E). Interestingly, when the same induction protocol was repeated after a resting period of 3 weeks, inducibility was restored (Fig. 1E, dark columns) and expression of mIL-12 followed the same pattern of progressive decline observed in the first round. Apart form a moderate reduction in the mIL-12 levels, the kinetics of both rounds of induction were identical. Similar experiments were performed with the viral vector carrying the Mif-inducible system. Ad-GL/RUhIL-12 or Ad-GL/RUmIL-12 were administered intravenously (i.v) by tail vein injection and IL-12 induction started 2

weeks later by daily intraperitoneal (i.p) injections of Mif. Serum IL-12 was measured 10 h after drug administration. As shown in figure 1D, the inducibility was maintained in mice inoculated with Ad-GL/RUhIL-12, as previously reported [5]. However, as observed with the plasmid vector, a progressive silencing of mIL-12 expression occurred (Fig. 1F). Again, a short period without induction (in this case of 10 days) was sufficient to recover the function of the system almost completely (Fig. 1F, dark columns). These results indicate that the biological activity of a transgene product such as IL-12, can inhibit the function of a drug-inducible system. This effect seems to be independent on the regulatory system and the type of vector used. The recovery of inducibility after a resting period argues against the loss of transgene as the explanation for this phenomenon. In fact, measurement of vector DNA by real time RT-PCR after 8 daily inductions did not reveal a significant reduction compared with the first day of induction (data not shown).

2. Inhibition of IL-12 expression is associated with transactivator down-regulation

In order to study the mechanism of mIL-12 reduction, we analyzed if the amount of protein detected in serum was associated with a parallel decrease of mRNA. To this end, we sacrificed animals that had been treated with pTonL2(T)mIL12 or Ad-GL/RUmIL-12 at the beginning or the end of the induction protocol, and quantified the amount of mIL-12 mRNA in the liver. As shown in figure 2A and B, continuous induction caused a drastic (40-fold) reduction of mIL-12 mRNA independently of the vector used. We next explored whether the system was inhibited at the level of TA production, since transcription of mIL-12 is controlled by inducible promoters that respond to rtTA2^{S}-M2 or GLp65 in the Dox and Mif-dependant systems respectively, and there is a direct

correlation between TA levels and transgene induction [4]. To this end we quantified rtTA2^S-M2 and GLp65 mRNAs in the same liver samples used above. We observed 20 fold decrease expression of both TAs, which was consistent with the reduction of mIL-12 levels (Fig. 2C and D). In contrast, a similar amount of TAs and hIL-12 mRNAs was detected throughout all the induction protocol (data not shown). Interestingly, at day 1 rtTA2^S-M2 expression was enhanced about 2 times with Dox compared to untreated animals (Fig. 2C). This effect was seen when Dox was given either i.p or in drinking water and also when using hIL-12 or luciferase reporter genes under the control of the Dox-inducible system (data not shown). A similar behavior was observed for Mif-treated animals (Fig. 2D). These observations suggest that although Dox and Mif can induce TA expression by themselves, this induction cannot alleviate the repression effect of mIL-12 on gene transcription after several days of drug administration. Together, our data indicate that mIL-12 induces reduction of rtTA2^S-M2 and GLp65 mRNA levels, and therefore a negative feedback is established causing a decrease of mIL-12 expression, unrelated to loss of vector DNA. This suggests that inhibition of TA expression could be the consequence of EalbPα1AT and TTR promoter silencing, since rtTA2^S-M2 and GLp65 are driven by these liver-specific promoters in the Dox- and Mif-dependant systems, respectively (Fig. 1A and B).

3. Effect of mIL-12 on liver-specific gene expression.

In order to obtain a general perspective about the influence of IL-12 on liver-specific promoters, we performed gene expression microarray analysis to identify genes that are differentially expressed in response to this cytokine. To this end, mice were injected with the pTonL2(T)-mIL12 or control plasmid EalbPα1AT-rtTA2 [4], in which the TA is expressed but no IL-12 cassette is present. Both groups were treated with Dox for 3

consecutive days, and then liver samples were collected for RNA extraction and analysis. A total of 2030 genes-probesets (with False Discovery Rate, FDR=0.05) were found altered when mIL-12 was induced. Among them, a number of IFN-γ responsive genes such as Icsbp1, Ifi47, Ifi203, Iigp1, Irf1, Stat1, and Ifn-γ itself were up-regulated (Fig. 3A). Since activation of IFN-γ is a hallmark of IL-12 function *in vivo* [8], this observation demonstrates the biological activity of the transgene and the functional relevance of the results found using the genome-wide expression microarrays. We next focused on a set of 101 liver-specific genes, selected based on a 200 fold higher expression in murine liver compared with other tissues according to the transcriptomic profiles in *Gene Expression Atlas* (http://symatlas.gnf.org/SymAtlas/) [9]. We searched for these liver-specific genes in the microarrays and found a set of 94 gene-probesets, which were used to perform a hierarchical cluster analysis. The resulting gene expression profiles along the 6 sample microarrays are presented as a heatmap (Fig.3B). It shows a clear differential expression between the samples in which mIL12 was induced (TAdIL 1, 2 and 3) and the ones that remained in the basal state (TAd 1, 2 and 3). These sets of genes present a mean difference in expression of 0.35 (in log2) between the two states. Among the genes repressed in the presence of mIL-12 we found albumin (Alb1), α-1antitrypsin (also called Serpina1a) and transthyretin (Ttr) (Fig. 3B). This is important because analogous promoters and/or enhancers of these genes where included in the Dox- and Mif- dependant gene inducible system to direct TA expression (Fig. 1A and B). To confirm these data we performed an independent experiment in which the plasmids pTonL2(T)-mIL12 or pTonL2(T)-hIL12 were administered by hydrodynamic injection and induction was carried out for 8 days. At this time, mRNA corresponding to these genes was quantified from the liver of mice by real time RT-PCR. No differences in albumin, α-1antitrypsin and transthyretin mRNA levels were

found in animals expressing hIL-12 compared to untreated controls (data not shown). In contrast, mIL-12 caused a 3 to 8 fold reduction in the expression of these liver-specific genes (Fig. 3C-E), The results indicate that the biologically active mIL-12 causes a significant repression of endogenous liver-specific promoters that are relevant for the function of our inducible systems. On the other hand, a number of genes like hemopexin (Hpxn) were up-regulated in response to mIL-12 expression, as revealed in the microarray analysis (Fig 3B), and confirmed by real time RT-PCR (Fig. 3F).

4. An IL-12 inducible promoter does not alleviate transgene silencing

In order to analyze the direct influence of mIL-12 on the promoters used to control TA expression, we used a plasmid carrying the hAAT gene driven by the EalbPα1AT promoter (EalbPα1AT-hAAT) [10]. Human AAT is a good reporter protein in Balb/c mice and can be easily monitored in serum by ELISA, unlike intracellular TA proteins. EalbPα1AT-hAAT plasmid was co-administered with either pTonL2(T)mIL12 or pTonL2(T)hIL12 by hydrodynamic injection. As expected, activation of mIL-12 expression by Dox caused a marked decrease in hAAT levels after 8 days of induction, whereas no effect was observed with hIL12 (Fig. 4A). The same result was observed using a plasmid containing the TTR promoter driving the expression of hAAT (data not shown). This result initially suggested that mIL-12 exerts the same influence on endogenous promoters as well as those located in extrachromosomal plasmids. If this were a general feature, we would expect that the promoters of genes up-regulated by mIL-12, such as hemopexin, could be used to drive the expression of TAs and avoid mIL-12-mediated down-regulation in the inducible system. In addition, the hemopexin promoter seems a good candidate because it was shown to respond to IL-6-mediated inflammation signals [11] and to drive stable transgene expression if combined with an EII

HBV liver-specific enhancer [10]. In order to test this strategy, we repeated the experiment described in figure 4A using the EIIPHpx-hAAT plasmid instead of EalbPα1AT-hAAT. Surprisingly, we found down-regulation of hAAT expression in response to mIL-12 induction (Fig. 4B), which could indicate dissociation between the effect on the endogenous and the extrachromosomal vector-derived promoter.

5. Involvement of IFN-γ in the inhibitory effect of IL-12 on gene expression.

IL-12 stimulates the production of IFN-γ in different cell types, and this mediates many of the biological effects of this cytokine. To define whether the inhibition of liver-specific gene regulatory systems was a direct effect of IL-12 on transduced hepatocytes, or if it was mediated by IFN-γ, we analyzed the presence of IL-12 and IFN-γ receptors in murine hepatocytes. The expression of IL-12 receptor β1 and β2 chains and IFN-γ receptor α and β chains were quantified in total mRNA samples isolated from primary murine hepatocytes, whole liver samples and from the well-differentiated murine hepatocellular carcinoma cell line Hepa1-6. Both receptor subunits need to be present in the same cell for efficient signal transduction [12]. We found that only the β2 chain of IL-12 receptor is expressed in hepatocytes and in Hepa1-6 cells (Fig. 5 A and B), indicating that these cells are not able to transmit IL-12-mediated signals. The detection of both chains in whole liver samples (Fig. 5A and B) may be the due to the presence of non-parenchymal cells such as T lymphocytes and NK cells. On the other hand, as previously described for most somatic cells [13], a functional IFN-γ receptor is present also in liver, hepatocytes and Hepa1-6 cells (Fig. 5 C and D). Since IL-12 can activate T and NK cells to produce IFN-γ, and this cytokine is abundantly secreted in IL-12-stimulated organisms [14], these data indicate that the effect of IL-12 on transgene silencing may be mediated by IFN-γ signaling in hepatocytes. In order to demonstrate

this hypothesis we compared the kinetics of mIL12 expression in wild type (wt) and in IFN-γ receptor α ko mice (IFN-γ R-/-) that received the Ad-GL/RUmIL-12 vector and were subjected to 7 daily inductions of mIL-12 with Mif. In contrast to wt mice, a sustained expression of mIL-12 was observed in the IFN-γ-R ko mice during all the induction period despite the high levels of IFN-γ detected in both types of animals (Fig. 6A and B). This indicates an important role of IFN-γ signaling in the down-regulation of liver-specific drug-inducible systems. In order to investigate if this effect could be attributed to the immunostimulatory functions of IFN-γ, we studied if silencing takes place in severely immunosuppressed animals like nod/SCID mice. As shown in figure 6, a parallel decline in mIL-12 expression in wt and nod/SCID mice was observed, indicating that transgene silencing does not require an efficient immune system but only the ability of lymphoid cells to produce IFN-γ in response to IL-12, and the expression of IFN-γ receptors.

6. Sodium butyrate can rescue mIL-12 silencing without affecting IFN-γ signaling

Histone deacetylase inhibitors (HDACi) induce euchromatin conformation and can revert gene silencing in a variety of circumstances [15]. It has been reported that HDACi can increase the expression of endogenous [16] as well as extrachromosomal transgenes *in vitro* and *in vivo* [17]. Therefore, we studied if Sodium Butyrate (NaB), a commonly used HDACi, was able to restore mIL-12 expression when induced from the pTon(L2)-mIL12 vector. The plasmid was administered to mice as previously described, and induction of mIL-12 with Dox was carried out for 8 days in the presence or absence of NaB treatment at 1.25 or 2 g/kg. As shown in figure 7A, NaB partially avoided the reduction of mIL-12 production in a dose-dependent manner. Animals treated with 2

g/kg NaB achieved 10 times higher cytokine levels as compared to untreated mice at the end of the induction period. This dose of NaB has been described to be sufficient to recover the acetylation status of histones in mice, and is not associated with toxic effects [18,19]. Following NaB treatment, the mIL-12 concentration in serum correlated with an increase in rtTA2^{S}-M2 and mIL12 mRNAs in the liver of mice measured at day 8 (Fig. 7B). In order to determine whether NaB was protecting not only the promoters present in vector DNA, but also the corresponding endogenous liver-specific promoters, we measured expression of albumin, α-1 antitrypsin and transthyretin in the same liver samples. We did not see any difference in the expression of these three genes in treated and untreated animals, indicating that NaB is not able to prevent mIL-12-mediated endogenous gene down-regulation in mice (Fig. 7B). These data suggest that the mechanisms responsible for vector promoter silencing may be different from the ones affecting the cellular genome. Since IFN-γ can induce transgene down-regulation [20] (Fig. 6A), and some HDACi suppress synthesis of various cytokines, including IFN-γ [21] [22], we investigated whether the protective role of NaB was due to a blockade of IFN-γ signaling pathways in our assay conditions. To this end, we measured the expression of the downstream genes Stat1 and Ifit2 in the liver of mice that received the pTon(L2)-mIL12 plasmid and were given Dox for 8 days in the presence or absence of NaB. As a negative control for IFN-γ pathway activation we included IFN-γ-R ko mice over-expressing murine IL-12. As expected, the expression of Stat1 and Ifit2 was increased in response to mIL-12 in wt but not in IFN-γ-R ko mice (Fig. 7C). NaB caused no variation in either the basal or the mIL-12-induced expression of Stat1 and Ifit2 (Fig. 7C), and similar amount of IFN-γ was detected in the serum of NaB-treated and untreated mice (data not shown). These data indicate the lack of an obvious interference of NaB on IFN-γ production and signaling.

DISCUSSION

The ability to exogenously modulate the intensity and duration of transgene expression is essential when placing transgenes with toxic potential in long-expressing vectors. The use of drug-inducible systems would allow the design of tailored protocols adapted to the disease and the status of the patient. In the case of IL-12 these features are critical and may determine the safety and efficacy of the treatment because this cytokine causes severe toxicity at high doses. In this study we have characterized the *in vivo* performance of drug-inducible systems based on hepato-specific promoters for the regulation of IL-12 expression within the liver. We provide evidence that the biological activity of IL-12 causes a gradual inhibition of these systems. Their ability to induce transgene expression is reduced after 3 days of drug administration, and it virtually disappears after an 8-10 days induction period. This phenomenon occurs with both regulatory systems tested in the present work: the Tet-on and Mifepristone-dependant systems, and it is not related to the vector (naked DNA or high-capacity adenoviral vector) or delivery method used (hydrodynamics-based injection or intravenous and intrahepatic liver transduction). It also seems to be independent on the strain of mice analyzed, because we observed the same effect on C57BL/6J and Balb/c (Fig. 1), as well as in 129S2/SvHsd mice (manuscript in preparation). Interestingly, the silencing of gene expression is not an intrinsic limitation of these regulatory systems, since continuous activation of gene expression can be maintained with other transgenes, like human IL-12 and luciferase, that do not elicit the biological effects of murine IL-12 in mice [4-6]. This indicates that potential problems regarding the pharmacokinetics of inducer drugs (Dox and Mif) can be discarded.

Due to the complex functions of IL-12 *in vivo*, a number of different mechanisms may account for the rapid silencing of gene expression observed. We have ruled out the possibility that it is due to the elimination of transduced cells, since no specific decrease of transgene content was observed in the liver of induced mice (data not shown). This is in agreement with the ability of the systems to recover maximum inducibility after a resting (drug-free) period of at least 5 days (Fig.1 and our unpublished data). The blockade of IL-12 production was at the level of transcription, because a parallel reduction of its mRNA was observed. The mechanism involves a down-regulation of both Dox- and Mif- associated TA gene expression (rtTA2^{s}-M2 and GLp65 respectively), suggesting an inhibition of the liver-specific chimeric promoters that were used to control the expression of these TA in our systems [4,5]. The promoters were constructed by combining regulatory sequences from the mouse albumin, human α-1 antitrypsin and mouse transthyretin genes, which allow sustainable and high levels of reporter gene expression in mice [6,10]. Interestingly, gene expression microarray analysis (together with real time RT-PCR validations) indicated that endogenous expression of albumin, α-1 antitrypsin and transthyretin was inhibited in animals over-expressing IL-12, which is consistent with changes observed in acute-phase response [23,24]. Therefore it was possible that these elements were also inhibited in the expression cassettes introduced in our vectors. However, although the endogenous expression of hemopexin was increased in response to IL-12, the hemopexin promoter was equally down-regulated by IL-12 when placed on a plasmid vector to control hAAT reporter gene expression. This indicates that IL-12 may influence differently the transcriptional activity of regulatory sequences depending on their localization: chromosomic or extrachromosomic. Therefore, we believe that the performance of tissue-specific

promoters on gene therapy vectors is difficult to predict based just on endogenous gene expression profiles.

The choice of vectors and the design of expression cassettes used in the present study ensure the synthesis of IL-12 in hepatocytes, but the cytokine is rapidly secreted to the circulation, where it can reach specific receptors present in the surface of target cells such as T lymphocytes and NK cells. When activated, these cells will secrete IFN-γ [8]. Based on our observation that murine hepatocytes do not express functional receptors for IL-12, the silencing of gene expression must be mediated by an indirect mechanism, most probably related to the activation of the IFN-γ pathway. This concept is supported by our finding that in knockout mice for the IFN-γ receptor the system does not suffer the loss of inducibility observed in wt animals. IFN-γ plays a pivotal role in the development of adaptive immune responses and antitumor activity mediated by IL-12; however it could diminish gene therapy efficacy through transgene silencing. This is in agreement with previous observations from other groups using plasmid, adenoviral and retroviral vectors *in vitro* and *in vivo* [20,25,26]. In any case, it is worthwhile to stress that this is a reversible transcriptional inhibition and gene expression can be recovered after a cytokine-free resting-period. In addition, we describe here that silencing takes place in animals with severely impaired immune system such as nod/SCID mice. Together, these data indicate that transcriptional inhibition mediated by IFN-γ in hepatocytes is responsible for this phenomenon, independently of the immunological effects of this molecule.

Gene expression can be regulated through chromatin remodeling mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). This is a dynamic process that can be affected by structurally diverse HDACi, including NaB [15,27]. Here we describe that NaB treatment alleviates transgene silencing *in vivo* in our assay conditions. This

is consistent with the recent observation of episomal vector chromatinization in the liver of transfected mice [28]. Enhanced expression of transgenes upon treatment with NaB was previously observed by other groups using adenoviral vectors [29] and naked DNA [17,30]. However the mechanism responsible for this phenomenon is difficult to access since HDACi affect a number of cellular proteins beside histones [15]. The ability of IFN-γ to decrease histone acetylation through activation of HDACs or inhibition of HATs has been described in different systems [31,32] and is believed to be relevant for its biological functions [33,34]. We observed protection from transgene silencing by NaB without major disruption of IFN-γ pathways such as those leading to Stat1 and Ifit2 induction. Thus, it could re-activate transgene expression by unraveling chromatin at relevant vector promoter sequences, by activating transcription factors or by any of the numerous alternative mechanisms defined for HDACi [15,27]. Interestingly, the effect of NaB was predominant on extrachromosomal DNA, since no re-activation of endogenous liver-specific promoters was observed. Although the mechanism is not clear at this moment, the development of methods to counteract IL-12 transgene silencing is relevant because maintenance of sustained levels of this cytokine for a defined period of time may be necessary to achieve an optimal therapeutic response. The use of HDACi as adjuvants in the induction regime would be possible in humans, since several of these agents are being investigated as anti-cancer agents in clinical trials [27]. Moreover, some HDACi have shown to greatly stimulate standard chemotherapy and gene therapy strategies against liver cancer in experimental models [35,36].

In summary, we describe here that Dox- and Mif- dependant regulatory systems suffer a rapid loss of inducibility when they express biologically active IL-12. The inhibition is transient, is mediated by IFN-γ and is partially blocked by HDACi. These observations

have relevance for the design of gene therapy strategies involving controlled expression of immunostimulatory cytokines for the treatment of cancer and other diseases.

MATERIALS AND METHODS

Animal manipulation. 5-6 week-old female C57BL/6J and BALB/c mice were purchased from Harlan Laboratories (Barcelona, Spain). Nod/SCID mice (NOD.CB17-Prkdc scid/J) were obtained from Jackson Laboratory. Interferon-γ receptor-deficient mice (IFN-γ R $^{-/-}$) were provided by Dr. Gonzalez-Aseguinolaza [37]. Animals were maintained under standard conditions and all procedures were approved by the institutional ethical comitee. Each plasmid DNA (50 µg) resuspended in 2 ml of saline (NaCl 0.9%) was injected into mice using the hydrodynamics-based procedure [38]. The viral vector was administered by tail vein injection diluted in 200 µl of saline or by intrahepatic (i.h) injection in 50 µl to increase transduction efficacy. Direct hepatic injection was performed following laparotomy, as described [39]. In the case of the Tet-on system, inductions started one week after plasmid injection. Doxycycline (Sigma) was given in drinking water (2 mg/ml with 5% sucrose) or by i.p injection (50 µg/g). In the case of the Mif-inducible system, Mifepristone (Sigma) was administered by i.p injection starting two weeks after the vector administration at a dose of 250µg/kg dissolved in 60 µl of sesame oil. NaB (Sigma) was daily administered i.p at 1.25 or 2 g/kg after the second day of induction. Blood samples were obtained by retro-orbital bleeding under inhalatory anesthesia (isoflurane, Forane® Abbott Laboratories). When Dox or Mif were administered i.p, blood was collected after 10 hours. When Dox was given orally blood was taken in the morning. Serum was recovered by double centrifugation at 10,000 rpm for 5 min and stored at –20°C until protein measurement.

Vectors. The pTonL2(T)-hIL12 and pTonL2(T)-mIL12 plasmids were generated as described [4]. In both constructs the EalbPα1AT promoter directs the expression of the

Dox-responsive transactivator rtTA2^{S}-M2. GL-Ad/RU-mIL12 adenoviral vector carry the Mif-responsive transactivator (GLp65) under the control of the liver-specific transthyretin (TTR) promoter [6] and was generated as reported [5]. The p35 and p40 of IL12 are linked by IRES. Plasmids EalbPα1AT-hAAT and EIIPHpx-hAAT express the human α1-antitrypsin (AAT) gene under the control of liver-specific promoters described previously [10]. Plasmid DNA was purified from bacteria using Endofree® Plasmid Maxi Kit (Qiagen, Santa Clara, CA, USA) and stored in Tris–EDTA (TE 10:1) buffer solution at −20°C. HC-Ad vector stocks were prepared and stored as described earlier [5].

Hepatocyte isolation. The liver of 8 week-old female C57BL/6J mice were perfused with collagenase solution (Sigma) and hepatocytes were isolated as described [40]. Cells were stored at –80ºC before analysis.

Determination of IL-12, IFN-γ and AAT levels. Serum concentration of IL-12 and IFN-γ were determined by OptE1A human IL-12 (p70), OptE1A mouse IL-12 (p70) and mouse IFN-γ ELISA kits (BD Bioscience PharMingen, San Diego, CA). Serum levels of AAT were measured by ELISA as described previously [10].

RNA isolation, DNA Extraction and RT-PCR. After sacrifice, liver samples were harvested, rapidly frozen in liquid nitrogen and stored at –80ºC. Total RNA was extracted with TRIzol®Reagent (Invitrogen) from a portion of liver (10mg) homogenized with an Ultraturrax Driver T.25 (Janke & Kunkel, Ika-Labortechnik, Germany), following the manufacturer's protocol. The RNA was stored at -80°C before further processing. Genomic DNA was eliminated from the samples by DNase I

(Invitrogen) treatment using 1µ/µg RNA. Total cDNA was generated using 2µg RNA in 50 µl mix containing 0,8 mM dNTP Mix, 5,3 mM $p(dN)_6$ random primers (Roche), 4mM dTT (Invitrogen), 48 units of RNase inhibitor (Invitrogen), 240 units of M-MLV reverse transcriptase (Invitrogen) and 10 µl of buffer provided by the supplier. The tubes were incubated at 37°C for 1 hour and 95°C for 1 min.

Quantitative PCR. cDNA, was quantified using the iQ™ SYBR® green Supermix in a iQ™5 system from Bio-Rad. We designed all primers (Table 1) to distinguish between genomic and cDNA amplification, except for Ifit2 and the plasmid amplicons. In these cases, absence of genomic DNA contamination after DNAse treatment was demonstrated by qPCR. The PCR amplification was performed under the following conditions: one cycle of 95ºC for 3 min; 40 cycles of 95ºC for 15 s, 58-62ºC for 30 s (annealing), 72ºC for 30 s, 79-82ºC (detection) for 10 s; followed by a final extension of 72ºC for 4 min. Immediately following the PCR, a melting curve was undertaken by raising the incubation temperature from 55 to 95 °C to confirm amplification specificity. For the same purpose, we also performed electrophoresis of the final PCR product. Annealing temperature is 60ºC for all the set of oligos except for AAT (Serpina1), which was performed at 58,5ºC, Stat1 at 62ºC and Il12rb2 at 58ºC. Detection temperature was 79ºC for IRES, GLp65, Ifit2 and albumin DNA, and 82ºC for all the rest. Samples were run in duplicate and mRNA levels were normalized using glyceraldehyde-3-phosphate dehydrogenase (gapdh) as an internal control. The amount of each transcript was indicated by the formula: $2^{Ct(gapdh)-Ct(gene)}$, being Ct the point at which the fluorescence rises appreciably above background.

Microarray data analysis: normalization, signal calculation, significant differential expression and sample/gene profiles clustering. We used GeneChip® Mouse Genome

430A 2.0 Array (Affymetrix®) representing approx. 14,000 well-characterized mouse genes. Microarray data analysis was performed using the following strategy and methods. RMA algorithm was used for background correction, intra- and inter-microarray normalization and expression-signal calculation [41,42]. Once the absolute expression signal for each gene (i.e. the signal value for each gene probeset) was calculated for the whole microarray set, SAM algorithm [43] was applied to calculate significant differential expression and find the gene probesets that changed in each type of samples. The method uses permutations to provide robust statistical inference of the most significant probesets and provides p-values adjusted to multiple testing using FDR (False Discovery Rate) [44]. The cut-off of adjusted p-values applied to select significant genes was < 0.05. After the identification of the differentially expressed gene probesets, the corresponding matrix of expression values for the microarray samples studied was analyzed using the "hclust" clustering algorithm [45]. This algorithm performs agglomerative hierarchical cluster analysis with complete linkage to find similarity between gene probesets based on their similar gene expression profiles along the analyzed microarray samples. We applied these methods using R and Bioconductor as main computational and bioinformatic tools (www.bioconductor.org). R is a programming language and software environment for statistical computing (www.r-project.org). The normalized expression signals calculated for the genes in Figure 3A correspond to the average of ratios of log2 expression signals. The ratios were obtained dividing the log2 signal of each gene in each sample by its median expression value along the 6 mice samples. In this way the controls are close to 1 (that indicates no-change) and the changes in log2 scale are comparable for the different genes.

Statistical analysis. Data were analyzed by Mann-Whitney nonparametric tests. We used nonparametric statistics because sample size was less than 10.

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FIGURE LEGENDS

Fig.1. Schematic representation of vectors and kinetics of IL-12 expression. (A) pTonL2(T)-IL12: plasmid containing the Tet-on system in which the rtTA2^{s}-M2 transactivator is controlled by the EalbPα1AT promoter (mouse albumin enhancer fused to human α1-antitrypsin promoter). (B) GL-Ad/RU-IL-12: adenoviral vector carrying the Mif-inducible system in which the GLp65 transactivator is controlled by the transthyretin promoter (TTR). tetO7: rtTA2^{s}-M2 DNA binding site. Palb: minimum albumin promoter; ITR: inverted terminal repeat; Ψ: adenoviral packaging signal; HPRT and C346: stuffer DNA derived from human hypoxanthine phosphoribosyl transferase locus and human cosmid C346, respectively; GAL4BS: GLp65 DNA binding site; E1b-TATA: adenoviral E1b minimal promoter. (C) pTonL2(T)-hIL12 (50μg) was administered to C57BL/6J mice (n=5) by Hydrodynamics injection followed by 8 days-induction period with Dox. (D) GL-Ad/RU-hIL-12 (3x10^{9} IU) was administered to C57BL/6J mice (n=5) by i.v. injection and Mif was administrated for 10 days i.p. (E) pTonL2(T)-mIL12 (50μg) was injected into BALB/c mice (n=5), and Dox was administrated in two rounds separated by a 3 week resting period. (F) GL-Ad/RU-mIL12 (2,5x10^{8} IU) was inoculated to C57BL/6J mice (n=4) followed by two rounds of Mif administration separated by a 10 days resting period. IL-12 was measured on serum samples at the indicated time points.

Fig.2. Quantification of mRNA levels. Murine IL-12 mRNAs (A, B) and the transactivators rtTA2^{S}-M2 (C) and GLp65 (D) were analyzed by real time RT-PCR in liver samples extracted from C57BL/6J mice treated with the indicated vectors. (A, C) pTonL2(T)-mIL12 plasmid was injected by the hydrodynamics procedure (n=20) and

five animals per group were sacrificed 16 hours after given or not Dox in drinking water (day 1) and at day 8. (B, D) GL-Ad/RU-mIL-12 was administered by i.h injection (n=9). Treated and untreated animals with Mif were sacrificed 10 hours after induction (day 1) and day 10.

Fig.3. Effect of IL-12 on endogenous gene expression. Plasmids pTonL2(T)-mIL-12 or Ealbα1AT- rtTA2^{S}-M2 (carrying only the transactivator gene) were injected into BALB/c mice (n=3) and Dox was administered i.p during 3 days before sacrifice. Liver samples of both groups of animals were used for microarray analysis. (A) The normalized expression signal of 8 genes untreated (TAd, grey columns) or IL-12-treated animals (TAdIL, black columns) is represented: interferon gamma (Ifn-γ), interferon gamma inducible protein 47 (Ifi47), Ifi203, interferon activated gene 203 (Ifi203), interferon inducible GTPase 1 (Iigp1), interferon regulatory factor 8 (Irf8, also called Icsbp1), signal transducer and activator of transcription 1 (Stat1), interferon regulatory factor 1 (Irf1). Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) signal was included as a control reference gene that does not change. The normalized expression signal corresponds to the average of ratios of log2 expression signals. The standard error for the 3 samples is also indicated. In this way, a relative increment of 1.1 and 1.6 (in the log2 ratio scale) corresponds to a 2.15 and 3.0 fold induction (in the lineal ratio scale), respectively. **(B)** Heatmap of the hierarchical cluster analysis of a set of the 94 liver-specific gene-probesets selected. Indicated are the down- (green) and up- (red) regulated genes in samples where mIL12 was induced (TAdIL 1, 2 and 3) and the ones that were not (TAd 1, 2 and 3). Arrows points the probesets corresponding to relevant genes. **(C-F)** Differential expression of a subset of liver-specific genes (albumin, α-1 antitrypsin, transthyretin and hemopexin) in mice (n=5) injected with pTonL2(T)-mIL-

12 or pTonL2(T)-hIL-12 and treated for 8 days with Dox. Real time RT-PCR was performed to determine mRNA content in the liver. *significant differences (*P<0.05, **P<0.01).

Fig.4. Effect of IL-12 on an IL-12 induced promoter. pTonL2(T)-hIL12 or pTonL2(T)-mIL12 plasmids were co-injected with EalbPα1AT-hAAT (A) or EIIPHpx-hAAT (B) in C57BL/6J mice (n=5) followed by 8 consecutive days of induction with Dox in drinking water. In both cases concentration of hAAT was determined in serum at the indicated time point. EIIPHpx: enhancer II of the human hepatitis B virus fused to human hemopexin promoter. hAAT: human α1-antitrypsin reporter gene.

Fig.5. Murine hepatocytes do not present a functional IL-12 receptor. The expression of IL-12 receptor β1 and β2 chains (A, B), as well as IFN- γ receptor α and β chains (C, D) was analyzed by real time RT-PCR in livers or isolated hepatocytes from C57BL/6J mice, and the hepatocellular carcinoma cell line Hepa 1-6.

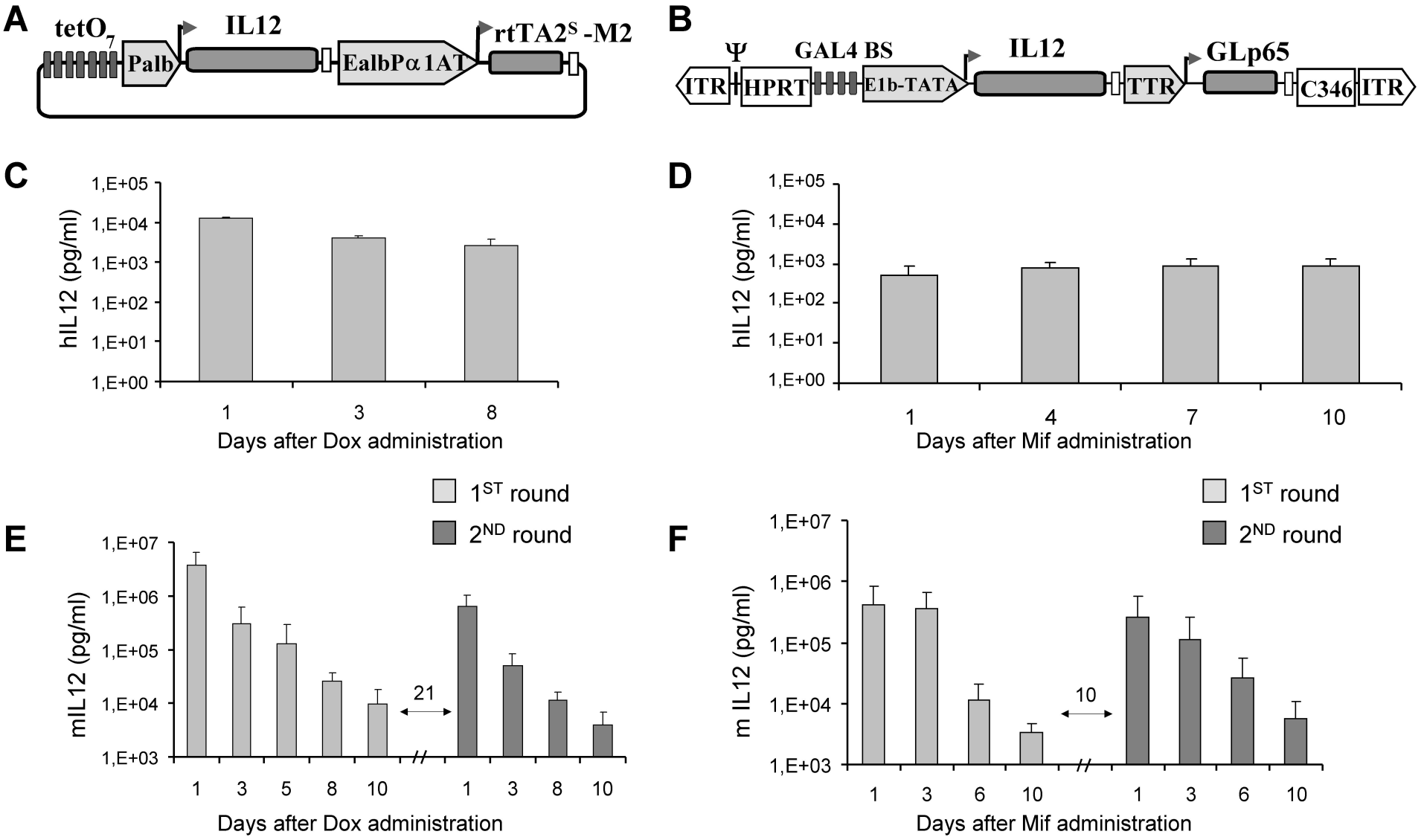
Fig.6. Kinetics of IL-12 and IFN-γ expression in different immunodeficient mice. GL-Ad/RU-mIL-12 ($2x10^8$ IU) was i.v. injected into wt C57BL/6J (n=5) mice, IFN-γ R-/- mice (n=6) or nod/SCID mice (n=3) followed by 8 consecutive days of treatment with Mif. Blood samples were collected 10 hours after the first and last inductions. (A) Serum levels of murine IL-12. (B) Serum levels of murine IFN-γ.

Fig.7. Effect of NaB on the IL-12-mediated inhibition of gene expression. pTonL2(T)-mIL12 was injected into BALB/c mice (n=5) followed by an 8 days-induction period with Dox, in the presence or absence of NaB at the indicated concentrations. (A) Levels

of IL-12 in serum. (B) Plasmid-derived and endogenous liver-specific gene expression determined by real time RT-PCR. The graphic represents the increase of mRNA content for each gene in animals treated with 2 g/kg NaB. (C) Stat-1 and Ifit2 mRNA levels in wt animals expressing IL-12 in the presence or absence of NaB. IFN-γ R-/- mice expressing IL-12 are included as negative control for IFN-γ pathway activation. *significant differences ($P<0.05$). ns: non-significant differences ($P>0.05$).

Table 1. Primers used in this study

Gene symbol	GenBank accession	Primer sequence	Fragment Lenght (bp)
gapdh	BC093508	s.5'-CCAAGGTCATCCATGACAAC-3' as.5'-TGTCATACCAGGAAATGAGC-3'	464
Alb1	NM_009654	s.5'-GCACACAAGAGTGAGATCGC-3' as.5'-CAGCAGTCAGCCAGTTCACC-3'	269
Ttr	NM_013697	s.5'-GCTGGACTGGTATTTGTGTC-3' as.5'-TGCAGCTCTCCAGACTC-3'	195
Serpina1	S70316	s.5'-GCCTTCAGCCTATACCG-3' as.5'-AGCACCCAGCTGGACAG-3'	620
stat1	NM_009283	s.5'-GTGGTTCGAGCTTCAGCAGC-3' as.5'-AGGTCATGGAAGCGGATGGT-3'	171
Ifit2	NM_008332	s.5'-GGAGAGCAATCTGCGACAGC-3' as.5'-CTGGATGAAGCCCTCAGCTT-3'	220
Il12rb1	NM_008353	s.5'-CCATCATTTTCGCGTCTCTGGG-3' as.5'-TACAACACCTCCGGGAAGTCCT-3'	202
Il12rb2	NM_008354	s.5'-TGACAGCTGCTGGTGAAAGT-3' as.5'-ATGTTGGAGGGTAAATAGCC-3'	601
Ifngr1	NM_010511	s.5'-GATTCTGCTGGTGGTCCTGA-3' as.5'-TCCAGGAACCCGAATACACC-3'	208
Ifngr2	NM_008338	s.5'-TCCTCGCCAGACTCGTTTT-3' as.5'-ATGTCCGTACAGTTCGGCTC-3'	191
rtTA2^{s}-M2		s.5'-GTCGGTATCGAAGGCCTGACG-3' as.5'-ACAGGAACGCGAGCTGATTT-3'	304
IRES		s.5'-GCCGTCTTTTGGCAATGTG-3' as.5'-CCCCTAGGAATGCTCGTCAA-3'	75
GLp65		s.5'-TGAACAGCGGATGAAAGAATCA-3' as.5'-TTGACCTCATCTCCTCAAACTG-3'	185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tetO7
Palb
IL12
EalbPα1AT
rtTA2S -M2
B
Ψ
GAL4 BS
IL12
GLp65
ITR
HPRT
E1b-TATA
TTR
C346
ITR
C
hIL12 (pg/ml)
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1
3
8
Days after Dox administration
D
hIL12 (pg/ml)
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1
4
7
10
Days after Mif administration
E
1ST round
2ND round
mIL12 (pg/ml)
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
21
1
3
5
8
10
1
3
8
10
Days after Dox administration
F
1ST round
2ND round
m IL12 (pg/ml)
1,E+07
1,E+06
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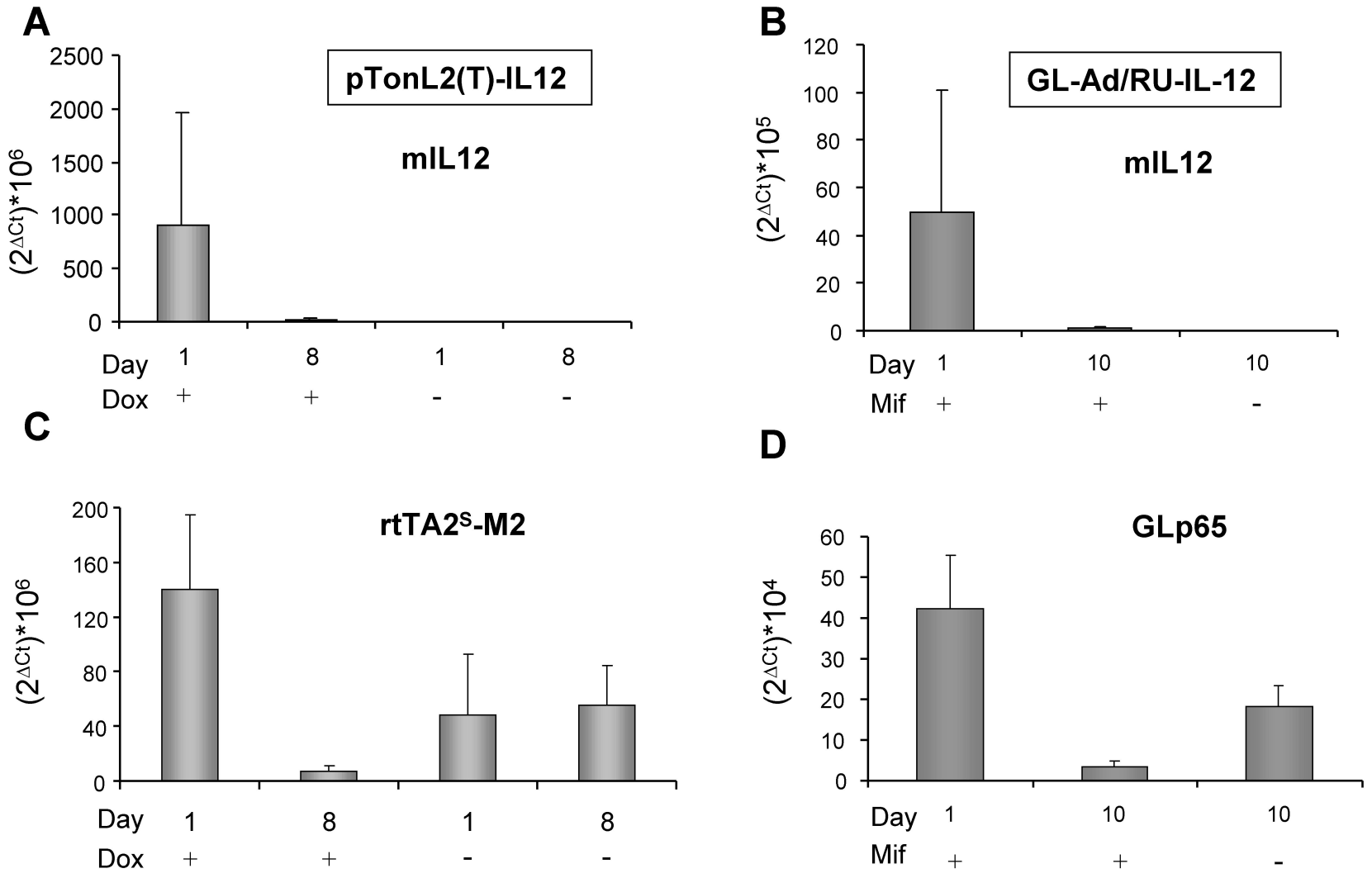
10
1
3
6
10
1
3
6
10
Days after Mif administration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1500
1000
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GL-Ad/RU-IL-12
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rtTA2S-M2
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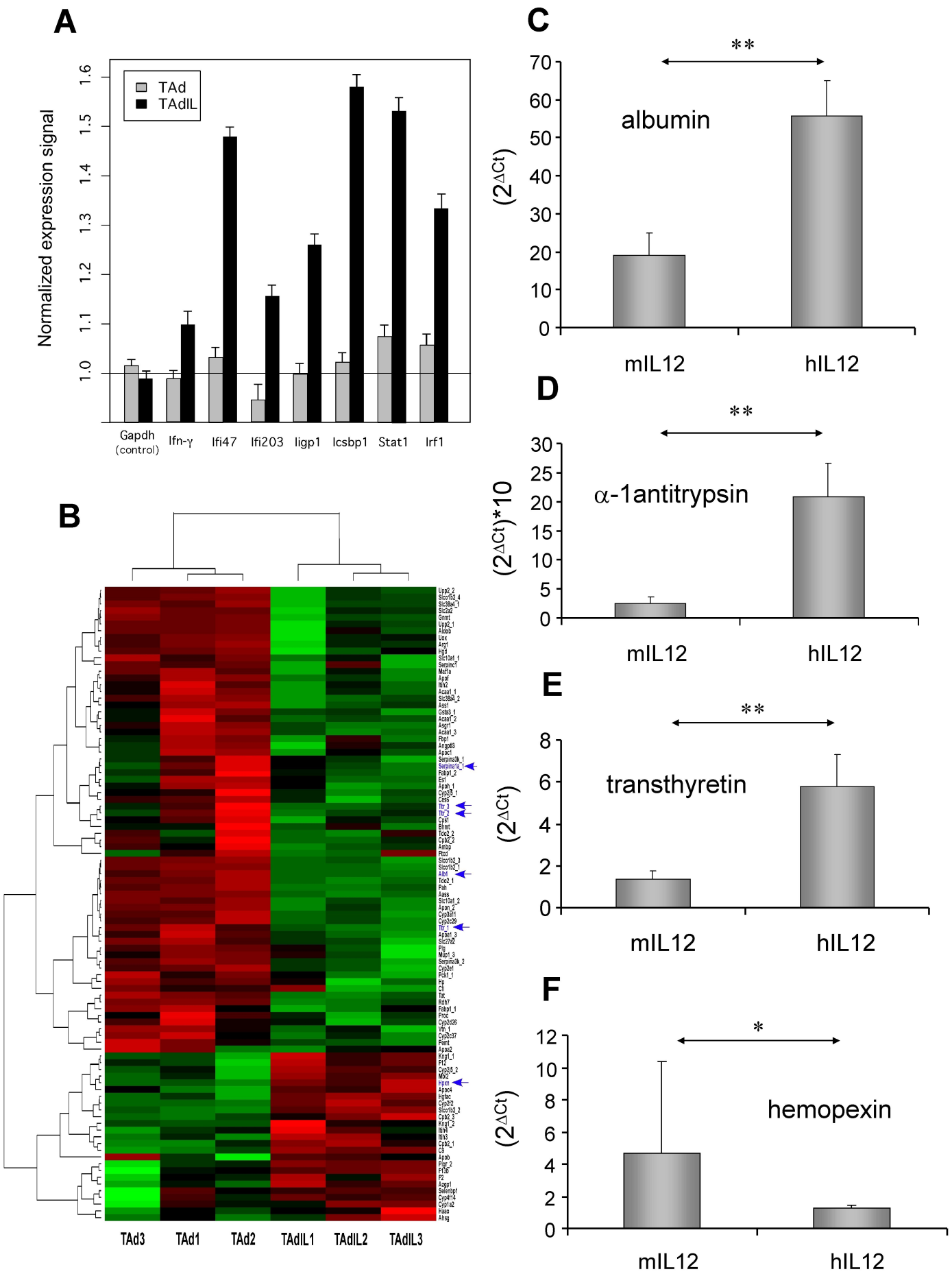
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Normalized expression signal
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1.1
1.2
1.3
1.4
1.5
1.6
TAd
TAdIL
Gapdh (control)
Ifn-γ
Ifi47
Ifi203
Iigp1
Icsbp1
Stat1
Irf1
B
TAd3
TAd1
TAd2
TAdIL1
TAdIL2
TAdIL3
C
albumin
(2ΔCt)
mIL12
hIL12
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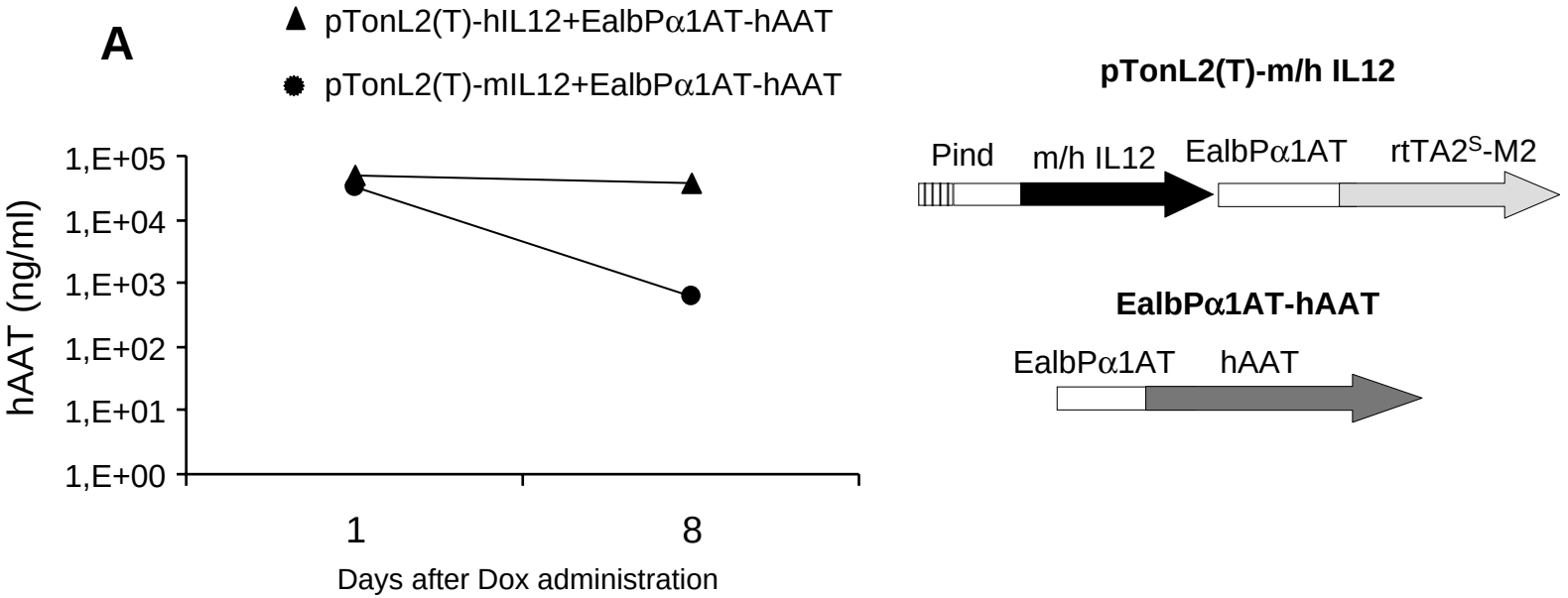
(2ΔCt)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pTonL2(T)-mIL12+EalbPα1AT-hAAT
hAAT (ng/ml)
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
1
8
Days after Dox administration
pTonL2(T)-m/h IL12
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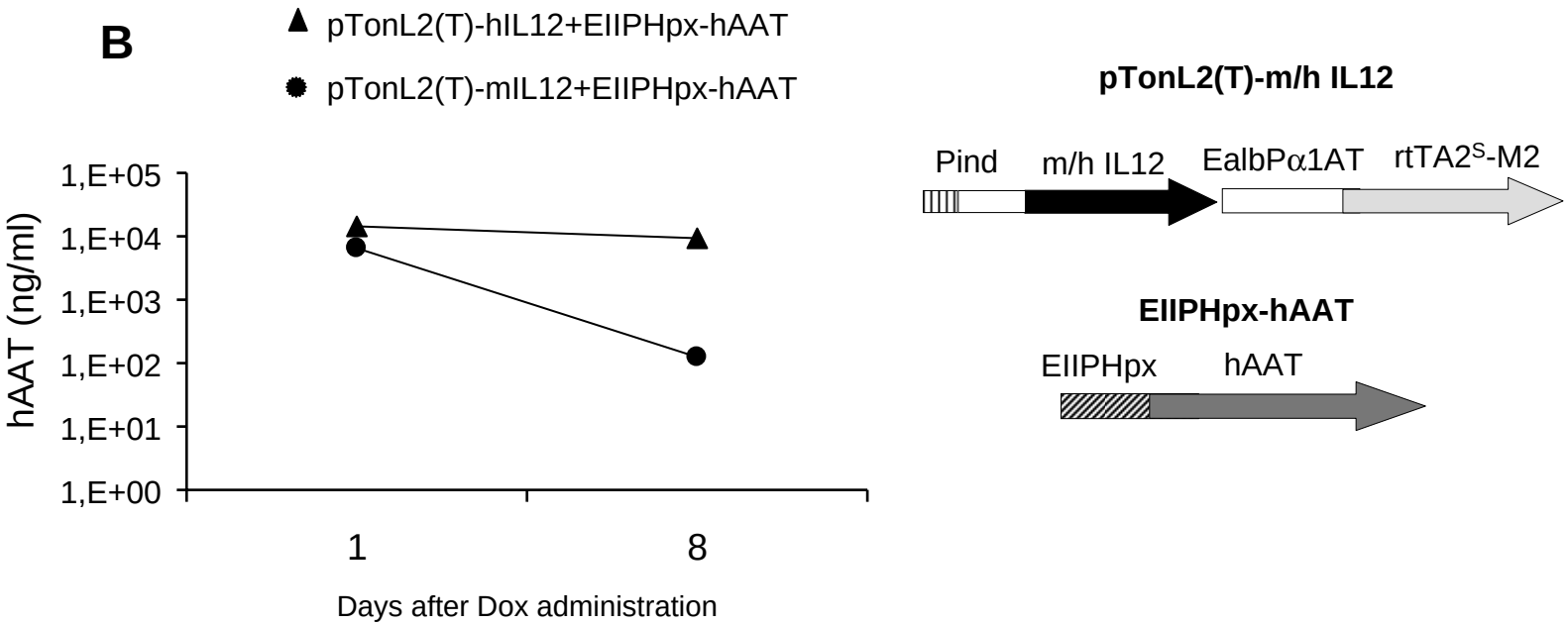
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pTonL2(T)-mIL12+EIIPHpx-hAAT
hAAT (ng/ml)
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
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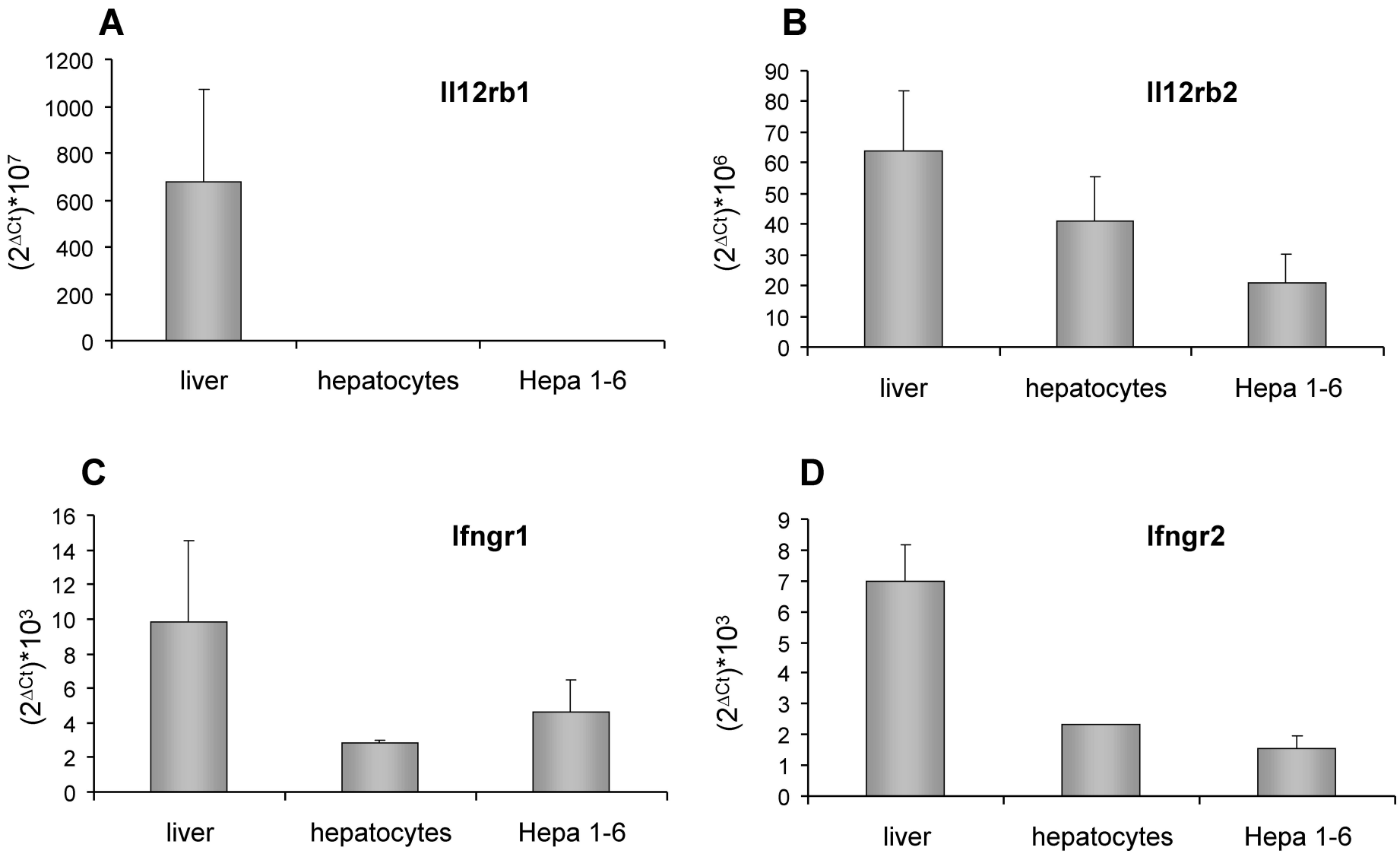
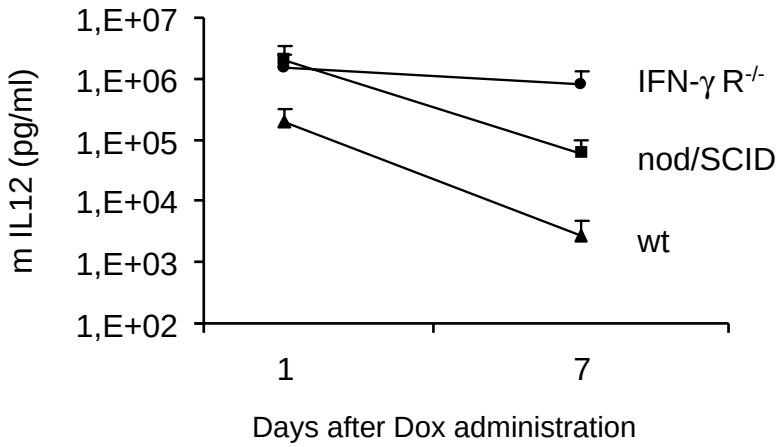
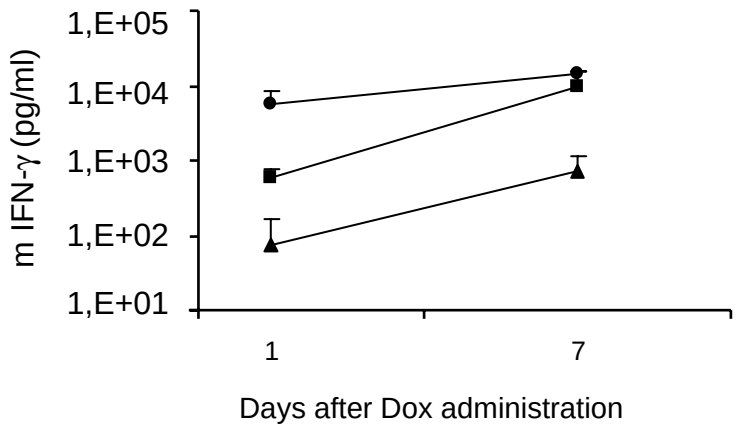
Days after Dox administration
pTonL2(T)-m/h IL12
Pind
m/h IL12
EalbPα1AT
rtTA2S-M2
EIIPHpx-hAAT
EIIPHpx
hAAT

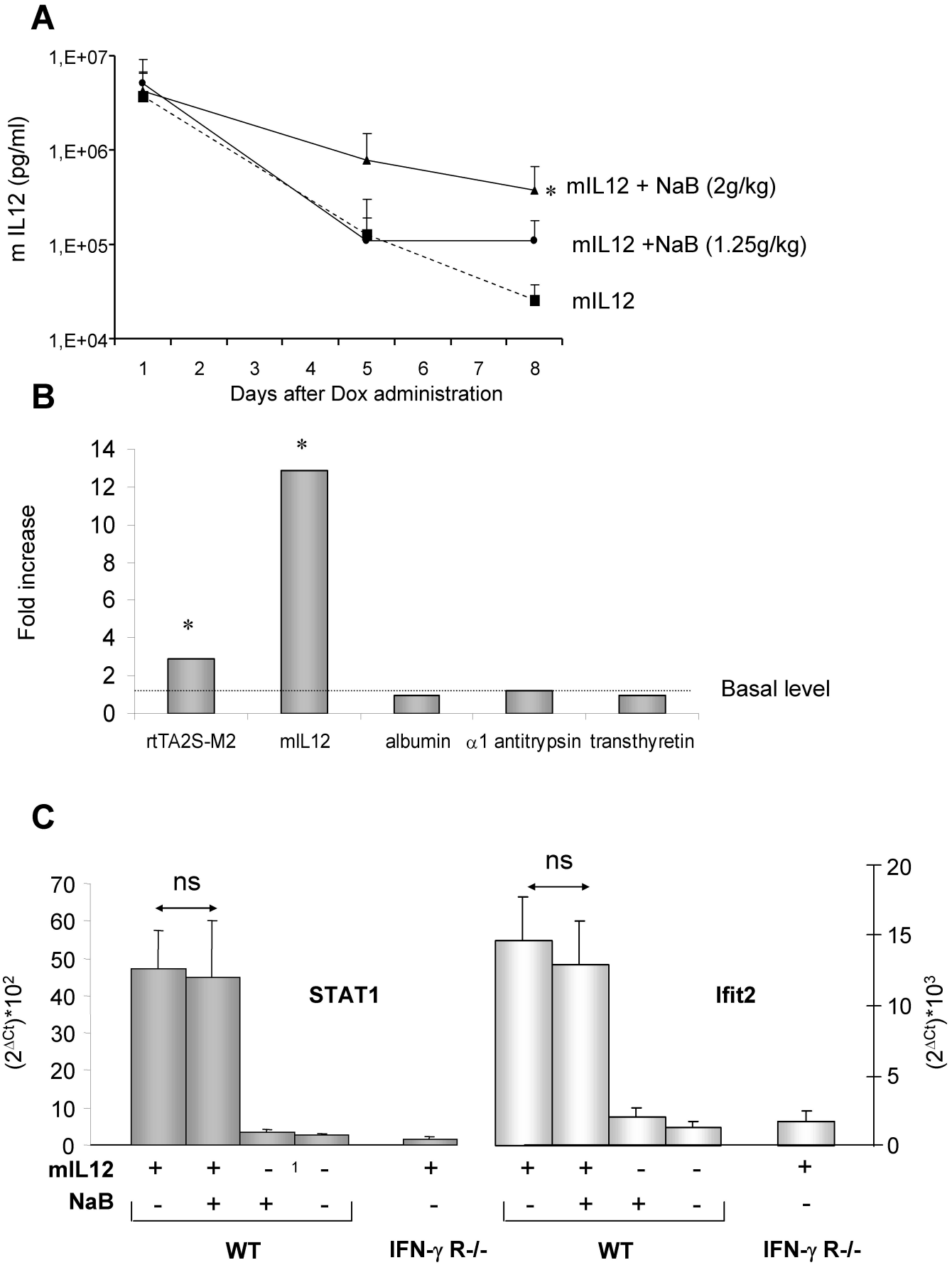

A
Il12rb1
(2^ΔCt)*10^7
1200
1000
800
600
400
200
0
liver
hepatocytes
Hepa 1-6
B
Il12rb2
(2^ΔCt)*10^6
90
80
70
60
50
40
30
20
10
0
liver
hepatocytes
Hepa 1-6
C
Ifngr1
(2^ΔCt)*10^3
16
14
12
10
8
6
4
2
0
liver
hepatocytes
Hepa 1-6
D
Ifngr2
(2^ΔCt)*10^3
9
8
7
6
5
4
3
2
1
0
liver
hepatocytes
Hepa 1-6

A


1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
m IL12 (pg/ml)
IFN-γ R-/-
nod/SCID
wt
1
7
Days after Dox administration

B


1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
m IFN-γ (pg/ml)
1
7
Days after Dox administration


A
1,E+07
1,E+06
1,E+05
1,E+04
m IL12 (pg/ml)
1 2 3 4 5 6 7 8
Days after Dox administration
* mIL12 + NaB (2g/kg)
mIL12 +NaB (1.25g/kg)
mIL12
B
Fold increase
14
12
10
8
6
4
2
0
*
*
Basal level
rtTA2S-M2
mIL12
albumin
α1 antitrypsin
transthyretin
C
ns
ns
70
60
50
40
30
20
10
0
(2^ΔCt)*10^2
STAT1
Ifit2
20
15
10
5
0
(2^ΔCt)*10^3
mIL12 + + - - + + + - -
NaB - + + - - - + + - -
WT
IFN-γ R-/-
WT
IFN-γ R-/-