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RESEARCH ARTICLE

Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12

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Despite the efficacy of IL-12 in cancer experimental models, clinical trials with systemic recombinant IL-12 showed unacceptable toxicity related to endogenous IFNγ production. We report that systemic administration of a recombinant adenovirus encoding IL-12 (AdCMVmIL-12) has a dramatically different survival outcome in a number of mouse pure strains over a wide range of doses. For instance at 2.5×10^9 p.f.u., systemic AdCMVmIL-12 killed all C57BL/6 mice but spared all BALB/c mice. Much higher IFNγ concentrations in serum samples of C57BL/6 than in those from identically treated BALB/c were found. Causes for heterogeneous toxicity can be traced to differences among murine strains in the levels of gene transduction achieved in the liver, as assessed with adenovirus coding for reporter genes. In accordance, IL-12 serum concentrations are higher in susceptible mice. In addition, sera from C57BL/6 mice treated with AdCMVmIL-12 showed higher levels of IL-18, a well-known IFNγ inducer. Interestingly, lethal toxicity in C57BL/6 mice was abolished by administration of blocking anti-IFNγ mAbs and also by simultaneous depletion of T cells, NK cells, and macrophages. These observations together with the great dispersion of IFNγ produced by human PBMCs upon in vitro stimulation with IL-12, or infection with recombinant adenovirus encoding IL-12, suggest that patients might also show heterogeneous degrees of toxicity in response to IL-12 gene transfer. Gene Therapy (2001) **8,** 259–267.

Keywords: *interleukin-12; toxicity; interferon-γ; adenovirus*

Introduction

Interleukin-12 is a heterodimeric soluble cytokine, which has shown antitumor activity in murine tumor models[1,2] due to its ability to promote cellular immune responses[1,3] and to impair tumor vascularization.[4,5] IL-12 mediates its functions through a receptor characterized on T cells and NK cells,[2] but recent functional evidence also shows effects of IL-12 on dendritic cells and macrophages.[6–8] Classical IL-12R consists of two chains (IL-12Rβ1 and IL-12Rβ2) which upon stimulation trigger several biochemical signals with activation of Jak-2, Tyk-2 and subsequent phosphorylation, dimerization and nuclear translocation of the transcription factor Stat-4.[2] Recently, an alternative isoform of IL-12Rβ2 has been identified on dendritic cells which activates NF-κB nuclear translocation.[6] IL-12 has been shown to trigger IFNγ release not only from T cells and NK cells,[9] but also from macrophages and dendritic cells.[7,8]

Both administration of recombinant protein and IL-12 gene transfer approaches with various vectors have successfully eradicated experimental malignancies.[10–14] Such encouraging preclinical data prompted testing clinical safety in a number of cancer patients. After a phase I clinical trial that established a maximal tolerated dose of 500 ng/kg for i.v. injection of rhIL-12 in an intra-patient escalation fashion, a phase II trial was started and eventually stopped because of unacceptable toxicity leading to two fatalities.[15,16] Careful reassessment of patient data and studies in mouse models led investigators to the conclusion that previous low doses of IL-12 in the intra-patient escalation had desensitized the patients to the toxic effects of subsequent doses of IL-12.[17] Most IL-12 toxicity is related to the production of IFNγ, which in turn activates the overproduction of an array of downstream inflammatory mediators.[18,19] The IL-12 toxicity profile in humans frequently involved leukocytopenia and thrombocytopenia with lower gastrointestinal bleeding and signs of liver dysfunction in some cases.[15,17,20] Mice overdosed with IL-12 show alteration in blood counts, extramedullary hematopoiesis, skeletal and cardiac muscle necrosis, wasting, splenomegalia and foci of hepatic necrosis.[15,17,18,20] Lung edema, pleural and peritoneal effusions were also noted. All these effects correlated with IFNγ serum concentrations and all symptoms but the pulmonary edema were absent in IFNγ receptor$^{-/-}$ mice, indicating a key role for this soluble mediator.[20] Indeed, anti-IFNγ blocking mAbs abrogated toxicity in C3H mice overdosed with IL-12.[17] IL-18 has been identified as a potent cofactor of IL-12 to induce IFNγ.[21,22]

IL-12 is instrumental in inducing T_H1 immune responses, enhancing the differentiation of T_H0 naive precursors to produce IFNγ.[2,23] It has been repeatedly observed that certain mouse strains such as BALB/c are prone to mount T_H2 responses in contrast to others such

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as C57BL/6 which tend to respond with T_H1-biased responses. This difference accounts for a dramatically distinct outcome upon infection with a number of intracellular parasites.[24]

When studying the toxicity of a recombinant adenovirus encoding murine IL-12 (AdCMVmIL-12), we noted that certain mouse strains such as C57BL/6 and DBA/2 were killed by doses of adenovirus which left BALB/c mice largely unaffected. It was found that toxicity correlated with the serum levels of IFNγ. Remarkably different levels of gene transduction in the liver among murine pure strains were observed using reporter genes, offering an explanation for the differences in serum IL-12 and IFNγ. IL-12-induced toxicity, which was completely inhibited by neutralizing anti-IFNγ mAb, was dependent on T cells, NK cells, and macrophages. In accordance, parallel genetic or epigenetic variability in humans could cause idiosyncratic adverse effects in response to IL-12 gene therapy.

Results

Differential susceptibility to similar levels of systemic IL-12 gene transfer in distinct murine strains

Back-crosses of mice have standardized the genetic backgrounds of the inbred strains that are routinely used in the laboratory in order to avoid experimental variability attributable to genetic differences. Such mice are therefore homozygous for the possible alleles in most loci and display phenotypic characteristics that clearly differentiate them from other pure strains. It was observed that 5×10^9 p.f.u. i.v of AdCMVmIL-12 killed all C57BL/6 mice, but only two out of five BALB/c mice (Figure 1a). One half of this dose (2.5×10^9 p.f.u.), again killed all C57BL/6 whereas BALB/c mice were largely unaffected (Figure 1b). Such a dramatically different outcome was also observed with 10^9 p.f.u. (data not shown). AdCMVmIL-12-injected C57BL/6 mice appeared ill from day 3 after AdCMVmIL-12 injection. They stopped moving in the cages, displayed unattended fur and ate poorly. At day 4, most animals were critically ill with different degrees of respiratory distress. Such mice had leukopenia, thrombocytopenia and showed elevated serum transaminases (data not shown). No signs of obvious gastrointestinal bleeding were found and death occurred presumably in most cases due to respiratory failure. A control adenovirus encoding for β-galactosidase (AdCMVLacZ) did not affect either BALB/c or C57BL/6 mice at the same doses or higher doses at least up to 10^{10} p.f.u. (data not shown). In contrast, 2.5×10^8 p.f.u. of AdCMVIL-12 i.v. killed three out of five C57BL/6 mice and dose dependence was further confirmed since 2.5×10^7 p.f.u. failed to kill any of 10 thus treated C57BL/6 mice. Serum levels of IFNγ were about three times higher in C57BL/6 mice than in BALB/c mice on days 3 and 5 after i.v treatment with AdCMVIL-12 at 2.5×10^9 p.f.u. (Figure 1c). Interestingly, when 2×10^6 spleen cells/ml were cultured *in vitro* 3 days after AdCMVmIL-12 administration, C57BL/6 splenocytes released into the culture supernatants twice as much IFNγ as BALB/c spleen cells (Figure 1d). However, no significant differences in the amount of IFNγ produced by splenocytes from untreated mice of each strain upon *in vitro* stimulation with recombinant IL-12 could be observed.

In necropsies of moribund C57BL/6 mice upon treatment with 2.5×10^9 p.f.u. of AdCMVmIL-12, the liver appeared pale and also slightly enlarged. No signs of hemorrhage within the peritoneal cavity or intestinal tract were noticed. Lungs appeared clearly congestive. Microscopic examination of H&E-stained tissue sections from lung, liver and spleen from identically treated C57BL/6 and BALB/c disclosed a clear disparity in the severity of lesions (Figure 2). Livers from C57BL/6 mice showed a focal necrosis with an intense inflammatory infiltrate that correlated with increases in serum ALT and bilirubin (data not shown). C57BL/6 lungs showed an intense leukocyte infiltration and signs of exudate. Spleens showed profound disruption of tissue structure and signs of lymphocyte depletion as previously reported.[25] These lesions were in clear contrast to the milder pathological changes observed in BALB/c (Figure 2).

Heterogeneous levels of liver transgene expression after i.v. injection of recombinant adenovirus

In order to discover reasons for the heterogeneity observed, we looked at the level of expression of β-galactosidase in the liver of C57BL/6 and BALB/c mice i.v.-injected with 2.5×10^9 p.f.u. of AdCMVLacZ. X-gal histochemical staining data clearly showed that more cells expressed the transgene in C57BL/6 livers and suggested that the intensity of expression on per cell basis was also higher than in BALB/c livers (Figure 3a and b). These data were confirmed when using a similar adenovirus encoding for the enzyme luciferase (AdCMVLuc) and using chemoluminescence in liver homogenates as a read out. As shown in Figure 3c, levels of transgene expression were 10- to 100-fold higher in C57BL/6 livers than in BALB/c ones. Chemoluminescence was almost undetectable in homogenates from spleen and lungs underscoring the liver tropism of adenoviral vectors. When sera from C57BL/6 mice treated with AdCMVIL-12 were compared with sera from identically treated BALB/c mice for the content of IL-12, we also detected higher levels in C57BL/6, again consistent with a higher level of gene transduction in this strain (Figure 3d). It is noteworthy that differences in gene transduction between strains with reporter genes are greater than those measured with IL-12 serum concentrations, indicating the existence of distinct regulatory mechanisms, in spite of the fact that both genes are under the control of the same promoter.

A panel of mouse strains show variability in AdCMVmIL-12-induced toxicity

Apart from the remarkable contrast in the survival outcome of C57BL/6 mice and BALB/c mice, we analyzed the effect of 2.5×10^9 p.f.u. of AdCMVmIL-12 in other genetic backgrounds to look for variability.

The F1 (C57BL/6 × BALB/c) mice were not killed and remained largely unaffected by AdCMVIL-12 (Figure 4a). The F1 survival outcomes were equal to BALB/c regardless of the gender of parents in the C57BL/6 × BALB/c crosses thus excluding a purely sex-linked inheritance trait (data not shown). Consistent with survival data, F1 (C57BL/6 × BALB/c) mice showed IFNγ concentrations as low as those seen in BALB/c mice (Figure 4b). Looking at the level of gene transduction with AdCMVLuc in the liver of F1 mice, it was of intermediate levels if compared

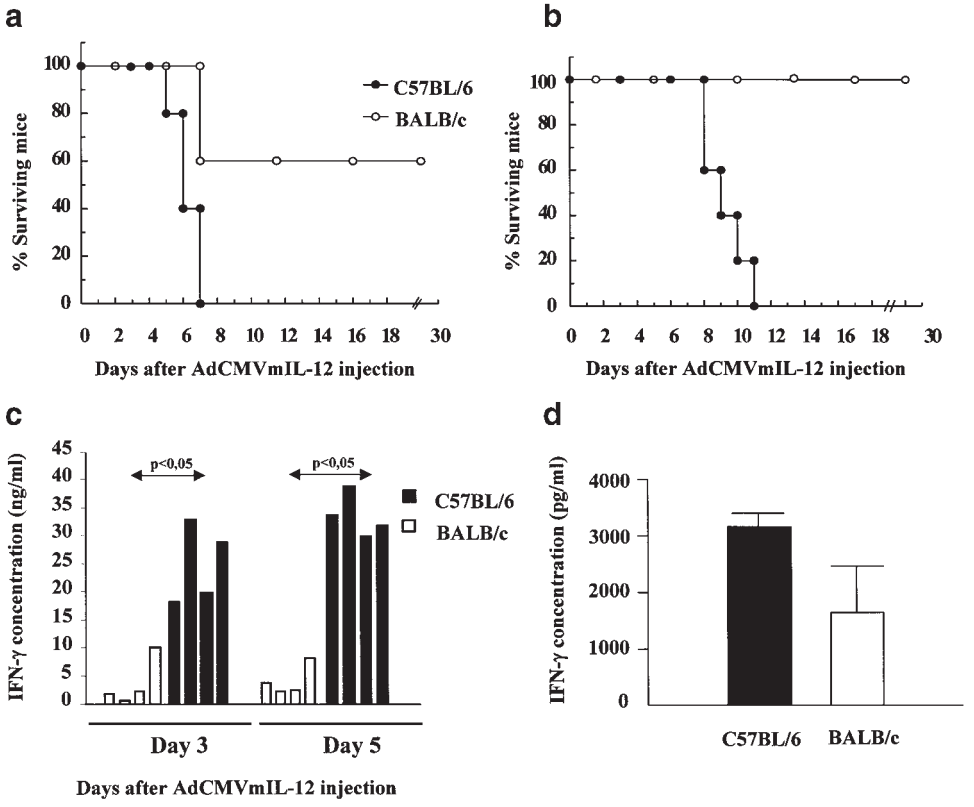

Figure 1 *BALB/c and C57BL/6 mice respond differently to gene transfer of IL-12. (a and b) age-matched male BALB/c and C57BL/6 mice were injected with AdCMVmIL-12 in the tail vein at doses of 5×10^9 p.f.u. (a) and 2.5×10^9 p.f.u. (b). Survival was monitored daily thereafter. (c) Blood samples were drawn from the orbital plexus on days 3 and 5 after AdCMVmIL-12 i.v. injection. IFNγ concentrations were assessed by ELISA and individual data of a representative experiment out of four performed are shown (statistical comparision was by Mann–Whitney test). (d) IFNγ concentration (mean ± s.e.m.) in 24 h supernatants from spleen cell suspensions (1×10^6/ml) derived from four mice per each strain that had been i.v.-treated with 2.5×10^9 p.f.u. of AdCMVmIL-12 3 days before.*

with samples from identically treated BALB/c and C57BL/6 (not shown).

In DBA/2, i.v. AdCMVmIL-12 induced toxicity that was as severe as in C57BL/6 mice. In this case, such mice have a H2 haplotype ($H2^d$) identical to that present in the BALB/c genome suggesting that the character susceptibility/lack of susceptibility to AdCMVmIL-12 is unrelated to this locus. B10.D2 mice also bearing $H2^d$ showed intermediate levels of toxicity with approximately 40% lethality (three out of seven) and a delay of several days in the gap from AdCMVmIL-12 injection to death in comparison with C57BL/6 and DBA/2 (Figure 4a).

Serum IFNγ concentrations in DBA/2 mice at day 3 after AdCMVmIL-12 injection were found to reach higher levels than in identically treated BALB/c, but lower than in C57BL/6 mice (Figure 4b). Such data suggest that different genetic backgrounds could determine adverse effects for IL-12 gene therapy. However, differences in IFNγ serum concentrations between B10.D2 and BALB/c were not found in spite of the different survival outcomes.

Neutralization of IFNγ prevents AdCMVmIL-12 lethal toxicity as well as simultaneous depletion of $CD4^+$, $CD8^+$, NK cells and macrophages

Systemic administration of a lethal dose of AdCMVmIL-12 to C57BL/6 mice was associated with high IFNγ production. Repeated i.p. injection of a blocking anti-murine IFNγ mAb protected all mice and resulted in the same survival as that of identically treated BALB/c mice. Accordingly, IFNγ is critically involved in mechanisms determining toxicity.

IFNγ is mainly secreted by T and NK cells. To study the role of such cell populations in the lethal effect observed on C57BL/6 mice, we depleted $CD4^+$, $CD8^+$ and NK cells both separately or in combination (Figure 5) with specific antibodies. Although such depletions were complete upon FACS analysis of peripheral blood mononuclear cells (data not shown), mice succumbed to AdCMVmIL-12 albeit with some delay if compared with control mice.

In contrast, depletion of NK cells, $CD4^+$, and $CD8^+$ cells plus treatment with macrophage-depleting doses of gadolinium chloride[26] fully protected AdCMVmIL-12-treated C57BL/6 mice from death. Gadolinium treatment by itself did not protect mice indicating that only simultaneous elimination of all such cell populations (T, NK and macrophages) was protective (Figure 5).

Besides the differential gene transduction levels of IL-12, we looked for other reasons that could be enhancing the output of IFNγ. IL-18 levels were twice as high in the sera of C57BL/6 mice treated with AdCMVIL-12 than in equally treated BALB/c 5 days after adenovirus administration (Figure 6). When AdCMVLacZ was used as a control, no IL-18 levels in sera were detected (not shown).

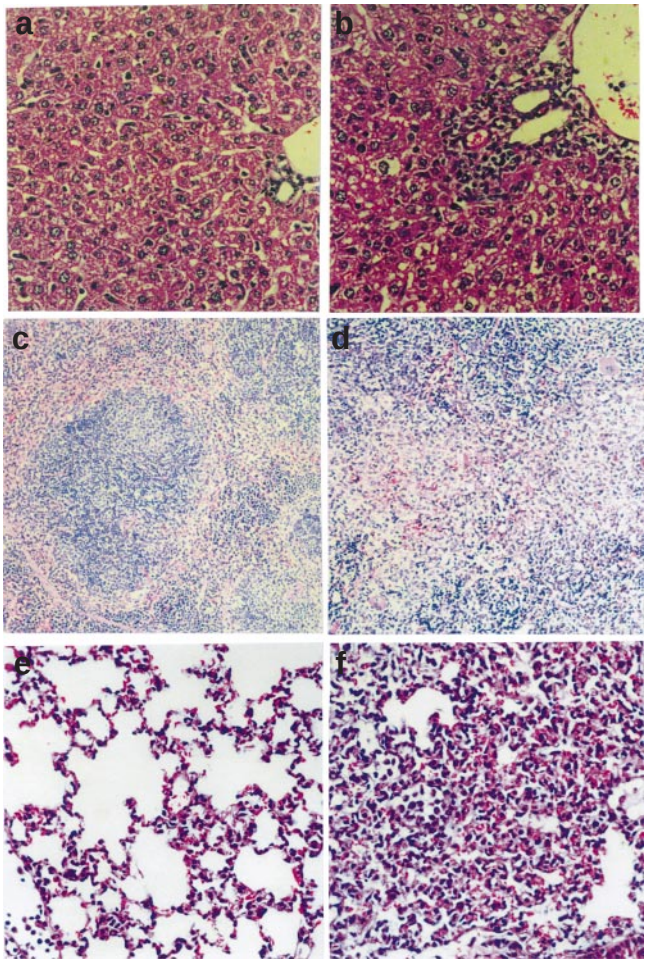

Figure 2 *Pathological lesions in C57BL/6 in comparison to BALB/c. Representative microscopic fields of H&E-stained tissue sections of liver (a, b) (×200), spleen (c, d) (×100), and lung (e, f) (×200) from paraffin-embedded samples obtained from BALB/c (a, c, e) or C57BL/6 (b, d, f) mice 5 days after i.v. injection of 2.5 × 10⁹ p.f.u. of AdCMVmIL-12.*

Since IL-18 is a cofactor for IFNγ production known to contribute in enhancing IL-12 toxicity,[21,22] we speculated that neutralization of IL-18 with specific antibodies would ameliorate toxicity of AdCMVIL-12 in C57BL/6 mice. However, no delay in the onset of symptoms or death time-points was observed in spite of the fact that IL-18 was not detectable in the serum of such animals.

Human subjects show diversity in the IL-12-induced level of secretion of IFNγ from peripheral blood mononuclear cells

In order to investigate whether human subjects quantitatively differed in the capability of their PBMCs to respond to IL-12 in terms of *in vitro* IFNγ production, a sample of healthy volunteers (n = 22) was chosen. PBMCs purified over Ficoll gradients were exposed to 10 ng/ml of rhIL-12, and IFNγ concentrations released into the supernatants were determined by ELISA. A great dispersion was found in the production of IFNγ (CV = 90%) with a range from 150 pg/ml to 13000 pg/ml (Figure 7a).

We could not assess the level of infection by adenovirus in human liver cells from different subjects but our data suggest that other features could account for differential susceptibility to IL-12-mediated adverse effects in humans. We also infected *in vitro* PBMCs of 16 donors with an adenovirus encoding human IL-12 (AdCMVhIL-12) and again great dispersion was observed in the output of IFNγ into the supernatants (Figure 7b). Variability of infection on per cell basis was difficult to assess since recombinant adenovirus only infected a small proportion (less than 5%) of PBMC, mainly among the adherent population. A good correlation was found in the level of IFNγ induced by rIL-12 and AdCMVhIL-12, indicating that the main factor of variability in humans was the response to IL-12. It should be noted that PBMC secreted detectable levels of IFNγ in six of 12 cases also in response to AdCMVLacZ (not shown), probably reflecting previous contact of the subjects with adenoviral antigens, thus adding another potential factor for individual variability.

As a whole these data suggest that several factors could account for individual variability among humans in IFNγ production in response to IL-12 gene therapy, giving rise to a spectrum of differential toxicity.

Discussion

In this study we describe differential susceptibility to systemic gene transfer of IL-12 in various mouse strains which can be accounted for by quantitative variations in IFNγ production. At least for C57BL/6 and BALB/c, the difference seems to be related to a differential level of gene transduction in liver cells by identical doses of first generation recombinant adenovirus. Reasons for differential level of transduction could involve several steps in the process, from viral entry to gene expression. No difference in surface MHC class I or $\alpha_v\beta_3$ were found by flow cytometry on the surface of hepatocyte cell suspensions from C57BL/6 and BALB/c. These proteins are known to be involved in virus attachment and internalization.[27] We are trying to explore a possible differential expression of CAR (Coxackie adenovirus receptor) but no reliable mAbs against mouse CAR are yet available. It should be stressed that differential gene transduction might not be the only mechanism involved in differential susceptibility, and accordingly, we detect more intense differences in transduction levels of luciferase than in serum levels of IL-12 suggesting that compensatory mechanisms have been elicited in the case of IL-12.

Data with gene transduction of luciferase in F1 (BALB/C × C57BL/6) livers show that gene transduction levels are intermediate to those in the parental strains. The genetic trait for this character is currently being explored with analysis of the F2 generation attempting to find the gene or genes involved. Interestingly, IFNγ in sera from F1 mice treated with AdCMVIL-12 is similar to that in sera from BALB/c, again indicating that other mechanisms of regulation beyond gene transduction exist in the case of the cytokines.

IL-12 has proved to be a major hope in the fight against cancer.[1] Both treatment with purified protein and gene transfer with recombinant vectors have displayed unprecedented success in murine experimental tumors. Recent data in cutaneous lymphoma patients undergoing systemic or intralesional injection of rhIL-12 have also been extremely encouraging.[28] Our group as well as others is about to start clinical trials in cancer patients based on intratumoral injection of IL-12 encoding adenoviruses.

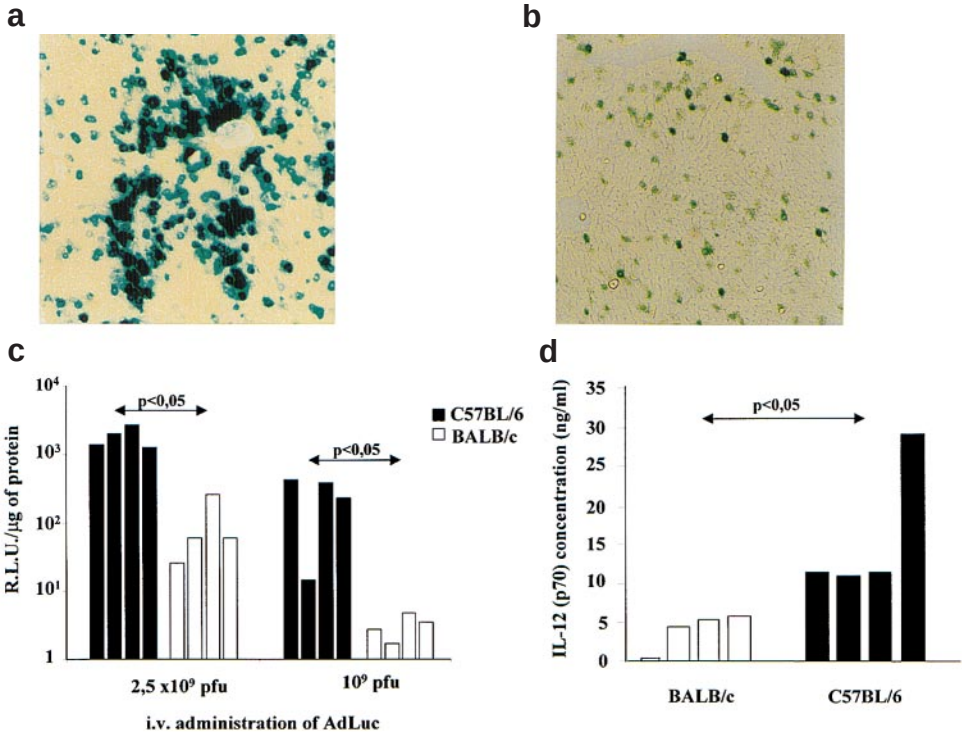

Figure 3 *Murine strain-related differences in liver gene transduction levels by recombinant adenovirus. (a and b) X-gal staining of liver sections from C57BL/6 and BALB/c mice treated with 2.5 × 10⁹ p.f.u. of AdCMVLacZ (×100). (c) Relative light units per µg of total protein in liver homogenates from BALB/c and C57BL/6 mice treated with two doses of AdCMVLuc. Data represent individual mice from a representative experiment. Statistical comparison was by Mann–Whitney test. (d) IL-12 serum concentrations in sera of C57BL/6 o BALB/c mice 3 days after i.v. injection of AdCMVIL-12 (statistical comparison was by Mann–Whitney test).*

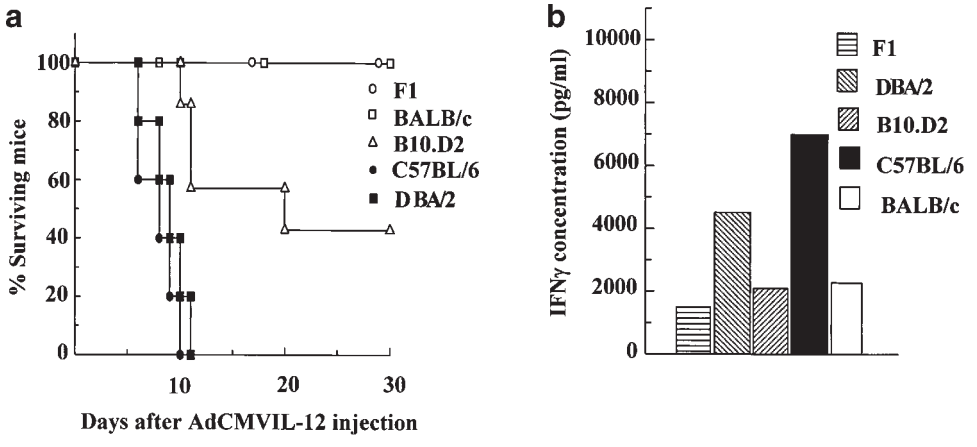

Figure 4 *Strain-related differential survival of mice treated with AdCMVmIL-12. Male age-matched mice from the indicated strains were injected with 2.5 × 10⁹ p.f.u. of AdCMVmIL-12 i.v. Survival was monitored daily thereafter (a) and IFNγ concentrations were determined in serum samples pooled from five mice at day 3 after AdCMVIL-12 injection (b). Data are representative of two independent experiments.*

Although confinement of IL-12 to the lesion should clearly downsize the systemic adverse effects, there is much interest in the assessment of potential risks and adverse reactions.

Previous differential toxicity to IL-12 among mouse strains has been found in a model of glomerulonephritis and genetic background-related differences in the spectrum of IL-12-induced lesions had been noted previously.[29] We report a dramatic difference in survival over a wide range of doses. Importantly, we have found that *in vivo* depletion of $CD4^+$, $CD8^+$ asialoGM-1^+ and gadolinium-sensitive cells was required to achieve the same level of protection as that observed when blocking with anti-IFNγ antibodies. Both CD4 and CD8 T cells and NK cells are known to be major sources of IFNγ. In fact, a crucial role of NK cells in a toxic shock model induced by combined administration of IL-2 and IL-12 has been demonstrated.[30] Curiously, IFNγ was not neccessary in that system since toxicity occurred in IFNγ receptor$^{-/-}$ mice.[30]

The role of macrophages shown in mice treated with gadolinium is difficult to assess. On one hand, they are

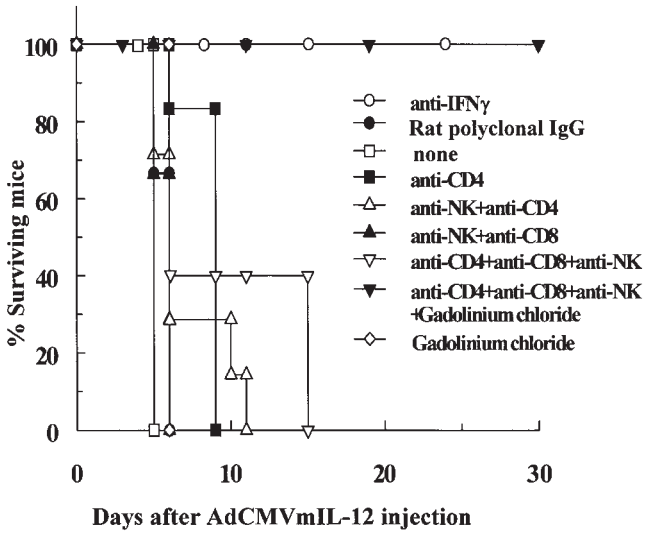

Figure 5 *Simultaneous depletion of T cells, NK cells and macrophages or in vivo neutralization of IFNγ protects C57BL/6 mice from AdCMVmIL-12 toxicity. Mice i.p. treated with the indicated antibodies and/or i.v with 10 μg/kg of gadolinium chloride during the 3 days before AdCMVmIL-12 i.v. injection (2.5 × 10⁹ p.f.u. per mouse) and weekly thereafter as described in Materials and methods, were monitored for survival. Depletion was monitored by FACS analysis of peripheral blood mononuclear cells in identically treated selected mice (data not shown). Data are representative of two independent experiments.*

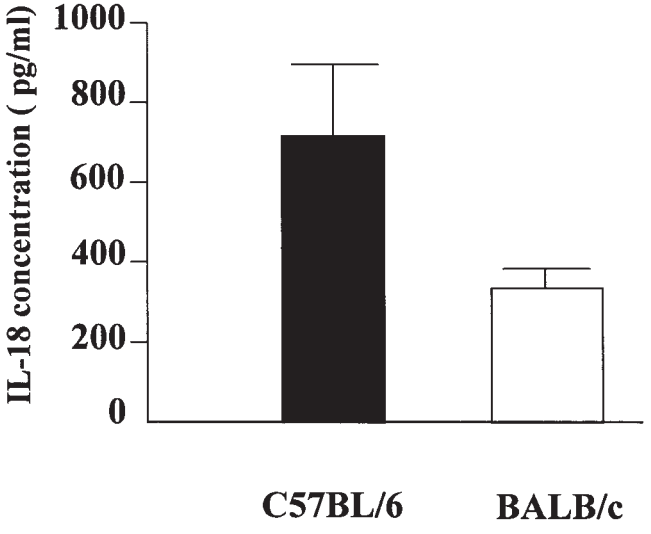

Figure 6 *Different IL-18 serum concentrations in C57BL/6 and BALB/c mice treated with AdCMVmIL-12. Serum concentrations of IL-18 (mean ± s.e.m.) determined by ELISA in BALB/c or C57BL/6 5 days after i.v. injection of 2.5 × 10⁹ p.f.u. of AdCMVmIL-12.*

potential sources of IFNγ as recently reported,[7] and on the other hand, they can secrete many downstream inflammatory mediators in response to IFNγ.[31] Recent information also suggests a major role for lymphoid dendritic cells as a source of IFNγ.[8] It should be noted that depletions with anti-CD8α mAbs also deplete a subset of this cell type.[8] We have found interesting differences in the levels of serum IL-18 among susceptible and non-susceptible mouse strains. IL-18 is a potent promoter of IFNγ[32] production that synergizes with IL-12[22] but we could not inhibit toxicity with anti-IL-18 neutralizing antibodies. Thus, although IL-18 could be involved in the differential toxicity observed, its role does not appear to be critical.

Although highly expressed in macrophages, iNOS (inducible nitric oxide synthetase) activity is not an absolute requirement for IL-12 toxicity since C57BL/6 mice treated with the NOS inhibitor L-NAME also succumb to IL-12 gene transfer as well as iNOS$^{-/-}$ mice (data not shown). None the less, iNOS activity has been found to be instrumental for the immunosupression observed after overdosing IL-12.[25,33]

We present data that suggest human individual variability in the response to IL-12 gene transfer. Although our data are preliminary, this issue is important when considering clinical development of such adenoviruses. It is of note that our AdCMVhIL-12 has been tested in non-human primates and that serious toxicity was found only after injection of very high systemic doses of the recombinant adenovirus (Sangro *et al*, manuscript in preparation). Our plans for the clinic only involve intratumoral injection which has shown remarkable efficacy and safety profiles in mouse tumor models probably related, as mentioned above, to confinement of high concentrations of IL-12 to the local tumor environment only. However, it is noteworthy that even under those conditions some viral vector reaches systemic circulation and infects liver cells.[34] Our data with human PBMCs uncover at least two possible sources of polymorphism in the response to IL-12 gene therapy in humans: (1) differential infectability of liver cells likewise among murine strains, (2) variable sensitivity to IL-12 in IFNγ-secreting cells. *In vitro* laboratory tests assessing IFNγ secretion from PBMCs in response to rhIL-12 or AdCMVhIL-12 might correlate with different clinical outcomes or adverse reactions. As a whole, our results indicate that individual variability might cause shifts in the dose/response curve for IL-12 gene therapy and such information would be important for clinical development.

Materials and methods

Construction of adenovirus

Recombinant adenovirus carrying murine IL-12 (AdCMVmIL-12) has been previously reported.[35] Briefly, an expression cassette of IL-12 under the control of the CMV promoter was constructed encompassing IL-12 p35 cDNA, an internal ribosomal entry site (IRES), IL-12 p40 cDNA and a polyadenylation signal. Recombinant adenovirus encoding the IL-12 cassette of expression was generated by cotransfection of 293 cells according to standard procedures.[36] Adenovirus carrying *lacZ* (AdCMVLacZ) or luciferase reporter genes (AdCMVLuc, a kind gift from Dr David Brenner, University of North Carolina, Chapel Hill, NC, USA) under the control of the CMV promoter were produced similarly. Recombinant adenoviruses were isolated from a single plaque, expanded in 293 cells, and purified by double cesium chloride ultracentrifugation. Purified virus was extensively dialyzed against 10 mM Tris 1 mM $MgCl_2$ and stored in aliquots at –80°C and was carefully titrated by plaque assay.[36] The adenovirus encoding the genes of human IL-12 (AdCMVhIL-12) will be described elsewhere (C Qian *et al*, manuscript in preparation).

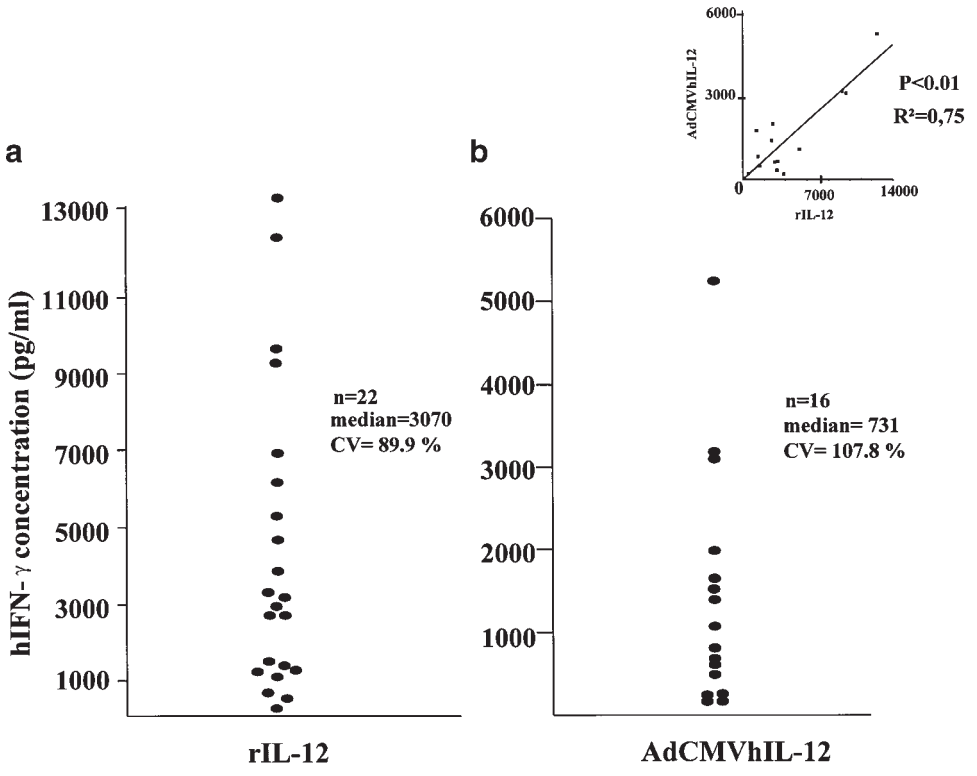

Figure 7 *Individual differences among human PBMCs to produce IFNγ in vitro in response to IL-12. PBMC obtained from healthy volunteers were plated at 2 × 10⁶ cells/ml and stimulated with 10 ng/ml of human rIL-12 (a) or by in vitro infection with AdCMVhIL-12 at MOI of 100 (b). Supernatants collected 48 h (a) or 72 h (b) later were analyzed by ELISA to determine IFNγ concentrations. The small upper panel displays the correlation of IFNγ concentrations induced by rIL-12 and AdCMVhIL-12 in cases when both samples were available. Pearson's correlation coefficient was used for statistical analysis (CV, coefficient of variation (%)).*

Animals, cell culture and reagents

Five- to eight-week-old male BALB/c ($H2^d$), C57BL/6 ($H2^b$), F1 (BALB/c × C57BL/6), B10.D2 ($H2^d$) and DBA/2 ($H2^d$) mice were purchased from Harlan (Barcelona, Spain) and were housed according to institutional guidelines. The 293 cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin. All cell culture reagents were from GIBCO (Basel, Switzerland).

Peripheral blood was obtained from healthy volunteers donors. Fresh peripheral blood mononuclear cells (PBMC) were separated by Ficoll–Hypaque density gradient centrifugation (Vacutainer; Becton Dickinson, Madrid, Spain) and cultured in RPMI 1640 medium with 100 U/ml streptomycin, 100 μg/ml penicillin, 10 μg/ml ciprofloxacin, and supplemented with 10% FCS. Recombinant hIL-12 and mIL-12 were obtained from Peprotech (London, UK).

Animal studies

Mice in groups of five to seven received a single i.v. injection of AdCMVmIL-12 at different doses on day 0. In some cases mice received i.p. administration of mAbs or i.v. gadolinium chloride. Mice were carefully observed for signs of toxicity and followed daily for survival. In some cases mice receiving AdCMVLuc were killed and their livers removed for generation of tissue homogenates.

Serum levels of IFNγ, IL-12 and IL-18

Mouse and human IFNγ concentrations (Endogen, Woburn, MA, USA), IL-12 (Pharmingen, San Diego, CA, USA) and IL-18 (R&D Systems, Abingdon, UK) were assessed by enzyme-linked immunoabsorbent sandwich assay (ELISA) according to the manufacturer's instructions.

Mouse blood samples were drawn from the retro-orbital sinus. Red cell-depleted splenocytes in RPMI 1640 10% FCS for 24 h and tissue culture supernatants were proccessed. Human peripheral mononuclear cells were obtained by Ficoll gradient centrifugation from healthy volunteers.

Luciferase assays on mouse livers

Mice were killed at day 3 after systemic AdLuc administration. Livers were harvested and stored at –80°C. After thawing, 1.5 ml of Reporter Lysis Buffer (Promega, Madison, WI, USA) was added to each liver specimen. Liver tissues were homogenized using Ultraturrax (Ika-Werke, Staufen, Germany) at room temperature, and cell debris was removed by two sequencial high speed centrifugations. Twenty μl of cell-free extracts were added to 100 μl of luciferase assay reagent (Luciferase Assay System; Promega). Relative light units (RLU) were determined using EG&G Berthold Luminometer (Aliquippa, PA, USA). Total amount of liver protein was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

X-gal staining

AdCMVLacZ (2.5×10^9 p.f.u.) in 150 μl of saline was injected intravenously into C57BL/6 and BALB/c mice. Animals were killed 3 days later, and the livers were excised, immediately embedded in optimal cutting tempereture compound (OCT; Tissue Tek, Zoeterwoude, The

Netherlands) and frozen in liquid nitrogen. Sections of 6-μm thickness were fixed with glutaraldehyde (0.5%) and stained with 5-bromo-4-chloro-3-indolyl-β-galactosidase as described previously.[37]

Depletion of lymphocytes, NK cells and macrophages

L3T4 ($CD4^+$)-specific rat anti-mouse hybridoma GK1–5 and ($CD8^+$)-specific rat anti-mouse hybridoma H35.17.2 were used to obtain anti-$CD4^+$ and anti-$CD8^+$ antibodies (ATCC, Rockville, MD, USA). Ascitic fluid was obtained from pristane-primed nude mice injected with 10^6 hybridoma cells. Mice, four to five in each group, were depleted of $CD4^+$ or $CD8^+$ cells by intra-peritoneal injection of 100 μl of anti-$CD4^+$ or anti-$CD8^+$ antibodies five times, on days −3, −2, −1, 7 and 14. Animals received an i.v. injection of AdCMVmIL-12 on day 0. Depletion of NK cells was accomplished via i.v. (100 μl per dose) administration of a rabbit anti-AsialoGM1 antiserum (Wako, Neuss, Germany) on day −2, −1, before AdCMVmIL-12 administration and every 7 days thereafter.[38] Anti-IFNγ mAb (R4–6A2; ATCC) was given i.p. as ascitis fluid (100 μl per dose) starting on days −2, −1 and every 3 days thereafter. Mice were depleted of monocytes/macrophages by i.v. injection of 10 μg/kg per dose gadolinium chloride (Aldrich, Milwaukee, WI, USA) 36 and 3 h before AdCMVmIL-12 injection and every 2 days thereafter.

Pathological studies

Animals were killed 5 days after AdCMVmIL-12 therapy. Liver, spleen and lung were collected, fixed in 10% buffered formalin, sectioned and stained with hematoxylin and eosin (H&E) for histopathological analysis. Blood counts, serum aminotransferases and creatinine were measured by standard clinical laboratory techniques.

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