J. Cell. Mol. Med. Vol 13, No 8B, 2009 pp. 1962-1976

IL-12 immunotherapy of murine leukaemia: comparison of systemic *versus* gene modified cell therapy

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Received: April 4, 2008; Accepted: June 20, 2008

Abstract

The ability of IL-12 to initiate anti-leukaemia immune responses has been well established; however clinical outcomes fail to recapitulate the therapeutic benefits observed in the laboratory. To address this, we compared two systems of IL-12 therapy that elicit protective immune responses against the murine acute lymphoblastic leukaemia (ALL) cell line, 70Z/3. These systems differ in the method of IL-12 administration and ultimately result in leukaemia clearance by distinct mechanisms, emphasizing the importance of treatment vehicle. Injecting low-dose IL-12 was sufficient to elicit long-term protective immunity against an established leukaemia burden, mediated by both $CD4^+$ and $CD8^+$ T cells. These findings agree with the standard model of IL-12 activity. We compared this protocol to a cell-based approach in which a novel lentiviral vector (LV) expressing murine IL-12 was created, 70Z/3 cells transduced, and clones selected that stably secrete different amounts of IL-12. We found that only a small proportion (1%) of IL-12 secreting cells were required for rejection but that the amount of IL-12 produced per cell was critical for successful therapy. Importantly, the levels of IL-12 required were found to be higher than the levels reported to date in the human clinical trial literature. We found that the cell-based approach led to protective immunity that was both long-term and specific but dependent primarily on a $CD4^+$ cellular subset alone. Our results highlight that the mode of IL-12 delivery has a distinct impact on the immune response initiated, leading to leukaemia clearance by disparate mechanisms. We also establish a new and critical parameter, IL-12 production/cell, which may have significant implications for future therapeutic design.

Keywords: B cell • T cell • leukaemia • immunity • cytokines • gene therapy

Introduction

Immunotherapy is based on the premise that cancer cells express antigens that can be targeted by immune mechanisms and recognized by the immune system. However, despite the presence of such antigens, leukaemias are generally not readily recognized and eliminated by the host, as evidenced by the development of disease. The inability of the immune system to protect against leukaemias may be due to mechanisms of evasion, active suppression or sub-optimal activation of a response.

Cytokines are integral to both the innate and acquired immune systems. They can alter the balance of cellular and humoural responses, alter class switching of B lymphocytes and modify innate responses. These traits have made a number of cytokines interesting candidates for cancer immunotherapies [1–3]. Among these, IL-12 has been tested for its ability to promote immune recognition and a response against leukaemias.

Interleukin-12 is a heterodimeric cytokine with multiple biological effects on the immune system. It is composed of two subunits, p35 and p40, both of which are required for the secretion of the active form of IL-12, p70. Interleukin-12 acts on dendritic cells (DC) leading to increased maturation and antigen

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doi:10.1111/j.1582-4934.2008.00412.x

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presentation, which can allow for the initiation of a T-cell response to leukaemia-specific antigens. It also drives the secretion of IL-12 by DCs, creating a positive feedback mechanism to amplify the response. Once a response is initiated, IL-12 plays a fundamental role in directing the immune system towards a Th1 cytokine profile, inducing $CD4^+$ T cells to secrete IFN-γ and leading to a $CD8^+$ cytotoxic T-cell response [4]. However, IL-12 is also a strong pro-inflammatory cytokine that leads to the secretion of other cytokines including TNF-α which, combined with IFN-γ, is a prerequisite for the development of $CD4^+$ CTLs [5]. Furthermore, IL-12 can promote the activation of innate immune cells such as macrophages and eosinophils through its induction of IFN-γ and other cytokines. This activation then leads to IL-12 secretion by these cells and further amplification of both the innate and acquired responses [4]. However, high levels of IL-12, and consequently IFN-γ, have also been associated with induction of antagonistic molecules such as IL-10 and the depletion of signalling molecules downstream of IL-12 such as STAT4 [6–8].

Direct injection of IL-12 has shown effectiveness in some mouse models of leukaemia [9–13]. While initial Phase I human trials employing this approach demonstrated adequate tolerance to treatment to warrant Phase II trials, these were less promising ([14–16] and discussed in [4]). It is well recognized in the literature that IL-12-induced anti-leukaemia activity is largely mediated by the secondary secretion of IFN-γ [13]. Gollob *et al.*, in particular, have suggested that the induction and maintenance of IL-12-induced IFN-γ expression is a key component of effective therapy in patients with metastatic renal cell cancer [17]. However, the concomitant induction of antagonistic effects with elevated IFN-γ levels continues to pose a challenge and is the impetus for a number of groups to continue testing the efficacy of IL-12 following different dose and time protocols [7, 8, 18–20] and to evaluate the therapeutic potential of cell-based IL-12 gene therapy ([21–30] and discussed in [4, 13]) in order to overcome this.

More recent clinical trials have included approaches such as intratumoural injection of IL-12 secreting fibroblasts and dendritic cells, methods that have proven effective in mouse models. To date, although some degree of immune response has been demonstrated, these approaches have not had a significant impact on patient survival [16, 25, 27, 31]. Finding the reason for this disconnect is of paramount importance.

We recently published a model of ALL in which one variant of the 70Z/3 murine pre-B cell leukaemia line, 70Z/3-L, is lethal in syngeneic mice while another variant, 70Z/3-NL, elicits a protective immune response [32]. The 70Z/3-L cells, although unable to initiate immunity, were readily rejected when an immune response was first initiated against 70Z/3-NL cells. Therefore, our model is amenable to testing whether IL-12 can initiate a specific immune response, recognition of 70Z/3-L and survival of challenged animals. 70Z/3 leukaemia is reminiscent of human ALL with neoplastic lesions arising in the liver, spleen, lymph nodes, bone marrow and rarely the central nervous system. Among the most common physical manifestations of the disease are ascites and splenomegaly.

We show here that intraperitoneal (IP) administration of low-dose recombinant IL-12 (rIL-12) elicits a protective response against an established leukaemia burden and that rejection is mediated by a $CD4^+$ and $CD8^+$ T-cell-dependent immune response which leads to long-term immune memory without the induction of antagonistic cytokines. We compared this protocol to a cell therapy approach in which 70Z/3-L cells have been transduced with an LV engineered to express murine IL-12 (both subunits) cDNA. Clones of 70Z/3-L cells producing a wide range of IL-12 were established. Injection of IL-12 producing 70Z/3-L cells provoked long-term and specific immunity without the induction of antagonistic mechanisms. Leukaemia clearance in this instance, however, was mediated by a $CD4^+$ cellular subset alone, suggesting a qualitatively different route to immunity than that seen with systemic therapy. Of critical importance, we found that injection of as few as 1% IL-12 producing 70Z/3-L cells along with 99% untransduced 70Z/3-L cells was sufficient to elicit protective immunity as long as each of these cells produced IL-12 above a necessary threshold. This finding may explain the failure of many human cell therapy-based protocols. In these cases, IL-12 production is measured on bulk populations, making it impossible to know if sufficient IL-12 is being produced in the local environment influenced by the IL-12 producing cell, and normally the average production reported is below our established threshold [24, 25, 27, 31].

Materials and methods

Animals

Female (C57Bl/6xDBA/2)F_1 mice (referred to as BDF_1), 8–12 weeks old, were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were kept under sterile conditions in the specific pathogen-free (SPF) animal facility at the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada. Mice were fed an irradiated diet and autoclaved tap water. Animals were terminated by CO_2 asphyxiation and cervical dislocation. The Animal Care Committee of the Ontario Cancer Institute approved all experimental protocols employed.

Leukaemia cells

70Z/3-L leukaemia cells (described in [33]), derived from BDF_1 mice, were maintained in IMDM with 5% heat inactivated foetal bovine serum (HYCLONE, South Logan, UT, USA), 100 μg/ml penicillin-streptomycin or 100 μg/ml kanamycin (GIBCO-Invitrogen, Burlington, Canada) and 5.5×10^{-5} M β-mercaptoethanol (referred to as complete IMDM) in a humidified atmosphere at 37°C and 5% CO_2. Cell concentrations were kept at $5 - 10 \times 10^5$ cells/ml.

Lentiviral vector construction

Lentiviral vectors expressing IL-12 cDNA were constructed by a method similar to that described by Yoshimitsu *et al.* [34] with modification.

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Plasmid pORF-mIL12 (IL-12elasti(p35::p40) Mouse (p35::p40)) (InvivoGen, San Diego, CA, USA) was modified by creating *Eco*RI and *Bam*HI restriction enzyme sites, upstream and downstream of the IL-12 gene respectively using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). This resulting construct was then digested with *Eco*RI/*Bam*HI (New England Biolabs, Ipswich, MA, USA). Murine IL-12 cDNA was purified after electrophoresis on a 1% agarose gel, and then subcloned into the pHR' LV backbone downstream of the elongation factor 1 alpha (EF1α) promoter. Positive plasmid clones for pHR-cPPT-EF1α-muIL-12-WPRE (*i.e.* LV-muIL-12) were identified by diagnostic restriction enzyme digestion analyses and subsequent DNA sequencing (Innobiotech, Toronto, ON, Canada).

Viral production and transduction of the cells

Concentrated LVs were produced by a transient triple-transfection method using pHR-cPPT-EF1α-muIL-12-WPRE and accessory plasmids onto 293T monolayers by calcium phosphate [35, 36]. An approximate vector titre was estimated based on LV/enGFP [34] production and testing on naïve 293T cells that occurred in parallel. The murine pre-B leukaemia cell line, 70Z3-L, was then transduced with an approximate multiplicity of infection (MOI) of 20. Single cell clones, obtained by limiting dilution in 96 well plates at population densities of less than 0.3 cells/well, were then quantitated for IL-12 production/10^6cells/ml/2 hrs using a commercially available IL-12 ELISA kit (BD Biosciences, San Jose, CA, USA).

In vivo challenge experiments

Leukaemia cells and transduced cells were grown in complete IMDM and were washed 3 times with 30 ml of phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+}. The cells were resuspended at 5 – 10 $\times$ 10^6 cells/ml in PBS and injected into the animals in a volume of 100–200 μl. Mice received IP injections that were performed on the right side of the abdomen using a 1 ml syringe with a 26-gauge needle.

Intraperitoneal administration of rIL-12

Recombinant mouse IL-12 was purchased from R&D Systems, Minneapolis, MN, USA. Mice were injected IP with 10^6 70Z/3-L cells in 100–200 μl PBS on day 0 followed by daily injections of 0.1–20 ng/mouse/day rIL-12 in PBS for a period of 14 days, starting on day 1. A secondary challenge consisted of IP injection of 10^6 70Z/3-L cells 70 days after primary challenge, which was carried out in the manner just described. For the delayed rIL-12 treatments mice received an IP injection of 10^4 70Z/3-L cells in 100–200 μl PBS on day 0. Thereafter, groups of 4 or 5 mice received 14 successive rIL-12 IP injections of 20 ng/mouse/day but the initiation of these injections was delayed by between 0 and 5 days. The animals were monitored daily for the appearance of symptoms both during the injection period and following the end of injections.

Intraperitoneal administration of leukaemia cell-produced IL-12

Interleukin-12 secreting cells were produced as described above. Mice were injected IP with 10^6 transduced cells or a mixture of transduced and naïve cells in various proportions in 100–200 μl PBS. A secondary challenge consisted of IP injection of 10^6 70Z/3-L cells or 10^6 L1210 cells more than 110 days after primary challenge, carried out in the manner just described. The animals were monitored daily for the appearance of symptoms following injection.

Challenge in depleted animals

Mice were depleted of $CD4^+$, $CD8^+$ or both T-cell subsets, as well as NK cells and IFN-γ using specific antibodies. The hybridoma GK1.5 is directed against $CD4^+$ T cells, YTS169 against $CD8^+$ T cells, HB170 (R4–6A2) against IFN-γ and the hybridoma HB9419 was used to produce an isotype control antibody. All hybridomas were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). The lines were grown in 2.5 litres of complete IMDM or OptiMEM in Lifecell culture bags (Lifecell Tissue Culture, Baxter Corporation, Concord, Canada) in a humidified atmosphere at 37°C and 5% CO_2 until a live cell count (using trypan blue exclusion) revealed 30% dead cells in the culture. The media was then centrifuged and filtered to remove cells and cellular debris. Antibodies were purified from the media using an affinity column of packed sepharose beads (Gammabind G, Amersham Biosciences Corp, Piscataway, NJ, USA) and concentrated with Centriprep YM-30 columns (Millipore, Billerica, MA, USA) before dialysis in PBS. NK cells were depleted using an anti-asialoGM1 antibody produced by Wako Bioproducts (Richmond, VA, USA). Rabbit IgG (Sigma-Aldrich, Oakville, Canada) was used as a control for the anti-asialoGM1 antibody. The T-cell subset and IFN-γ antibodies were injected on days –1, 3, 7, 10 and 14. The doses used were 1mg of antibody on day –1 and 500 μg for the remaining injections. The NK cell-depleting antibody was injected on days –1, 4, 9 and 14 using the recommended dilution [37]. Control isotype antibodies were injected following the same dose and schedule as their corresponding depleting antibodies. The depletion potential of the antibodies was demonstrated *in vivo* prior to their use in our experiments by injecting mice with a range of concentrations and subsequently examining tissues by flow cytometry to quantify cellular subsets, or examining serum for the presence of cytokines by ELISA. This experiment was conducted twice to test both model systems. In each case, cells were injected on day 0: either 10^6 70Z/3-L cells followed by 14 daily injections of rIL-12 (10 or 20ng/mouse/day) or 10^6 70Z/3-L vector-transduced cells of the LV12.2 clonal line. Controls included mice injected with 70Z/3-L alone and mice injected with PBS alone according to the appropriate injection schedule.

Serum collection

Serum collection in live mice was achieved by puncturing the saphenous vein with a sterile needle and collecting the blood in a serum separator tube (BD, Franklin Lakes, NJ, USA). These tubes were centrifuged at 10,000 RPM for 5 min. and serum was then transferred to a micro centrifuge tube and frozen at –20°C until use.

Bead assay for cytokine levels in the serum

Mice were injected IP on day 0 with 10^6 70Z/3-L cells in 100 μl PBS and treated daily with 100 μl preparations of PBS alone or containing low doses of rIL-12 (10 or 20 ng/mouse/day) for 14 days. Control groups included mice injected daily for 14 days with PBS in the absence of 70Z/3-L cells and rIL-12, and a group that was left entirely untreated.

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Alternatively, for the leukaemia cell-mediated IL-12 therapy experiment, mice were injected on day 0 with 10^6 70Z/3-L cells in 100 μl PBS containing various proportions of the 70Z/3-L vector-transduced cell line LV12.2 (0.5%, 1% and 10%). Control groups included mice injected with 70Z/3-L cells alone or PBS alone. Serum was non-terminally collected from all groups on days 7, 10 and 20 before their daily injection by puncture of the saphenous vein as described above. Serum samples were diluted 1/5 and stained according to the protocol provided with the Mouse Inflammation Cytometric Bead Array Kit (BD, San Diego, CA, USA). Standards were prepared in triplicate from independent dilutions and flow cytometry was done using a FACScan (Becton Dickinson, Oakville, Canada). Acquisition was performed using CellQuest software version 3.1. Note: due to insufficient blood collection, two mice treated with cell-mediated therapy in the 0.5% and one in the 1% group on day 7 and two mice in the PBS group on day 10 could not be tested. All mice from the group receiving 70Z/3-L cells alone in the leukaemia cell-produced IL-12 therapy model had perished by day 20 such that serum was not collected from this group.

Results

Intraperitoneal administration of rIL-12 protects mice challenged with 70Z/3-L

Interleukin-12 is known to be a potent modulator of the immune response attributed with a number of anti-leukaemia effects including, but not limited to, T-cell-mediated antigen-specific leukaemia clearance. This molecule has been approved for clinical use but optimum delivery programs have yet to be defined. In an attempt to alter the course of 70Z/3-L leukaemia, we began by testing the effect of IP rIL-12 administration on the appearance of morbidity after IP injection of 10^6 70Z/3-L cells. We tested doses of 0.1–20 ng/mouse/day for 14 days, which are at least 20-fold below the maximum tolerated dose in mice. In Figure 1A, we show that doses above 10 ng were sufficient to significantly improve the survival of animals ($P = 0.002$).

Intraperitoneal administration of rIL-12 leads to long-term protective immunity against the 70Z/3-L leukaemia

We next addressed whether the results observed above were due solely to the acute effects of IP-administered rIL-12 on innate responses or to the induction of a long-term adaptive immune response in the mice. To accomplish this, mice received IP injections of 10^6 70Z/3-L cells, were treated for 14 days with 20 ng/mouse/day rIL-12, subsequently challenged 70 days later by IP injection of 10^6 70Z/3-L cells and monitored for the appearance of symptoms. A group of naïve mice was included to control for the efficiency of the cells to cause disease. Figure 1 Bii shows that all animals first treated with IP administration of rIL-12 (Fig. 1Bi) survived a secondary challenge with 70Z/3-L cells in the absence of further IL-12 therapy. Thus, IP administration of rIL-12 not only protected against the primary 70Z/3-L challenge but also established long-term protective immune memory.

Intraperitoneal administration of rIL-12 protects animals with pre-established 70Z/3-L leukaemia

To determine if IP administration of rIL-12 can lead to leukaemia clearance as well as protection from a developing neoplasm, treatment initiation was delayed to allow for dissemination of the disease. We undertook these experiments starting with 10^4 70Z/3-L cells injected IP because of their rapid growth. This dose is still lethal to 100% of mice in approximately 20 days [32]. Initiation of rIL-12 administration was delayed by 0 to 5 days and continued for 14 days following the first injection. We found that we could delay the initiation of rIL-12 therapy by 5 days and still achieve significant protection against the leukaemia (Fig. 1C). The differences between the survival curves of the six treatment groups are not statistically significant and longer delays were not tested.

$CD4^+$ and $CD8^+$ T cells are required for the rIL-12-mediated rejection of 70Z/3-L cells after IP administration

Depleting antibodies were used to determine which cell types mediate the rIL-12-induced rejection of 70Z/3-L leukaemia after IP administration. We show in Figure 1D that both $CD4^+$ and $CD8^+$ T cells are important as depletion of either population eliminates immune protection in all animals. The mean survival was 14 days for mice depleted of $CD8^+$ T cells, 23 days for mice depleted of $CD4^+$ T cells and 13 days for mice depleted of both T cell subsets. The three curves are not statistically different from each other. We also included neutralizing antibodies against IFN-γ to examine its role in the rejection response. We found that this abrogated the protective effects of IP administered rIL-12 demonstrating that IFN-γ plays an essential role in leukaemia rejection. Although the importance of NK cells has been shown in other models of IL-12 therapy [38, 39] we did not observe changes in rejection of the 70Z/3-L leukaemia when NK cells were depleted in this treatment modality (Fig. 1D).

Generation of IL-12 secreting leukaemia cells by implementation of lentiviral transduction

Having established the basic parameters of IL-12 systemic therapy, we next compared this protocol to a cell-mediated approach. Figure 2A shows the lentiviral construct with an IL-12 fusion transgene under control of the EF-1α promoter that we generated. After transducing 70Z3-L cells with an approximate MOI of 20, single cell clones were derived as described in

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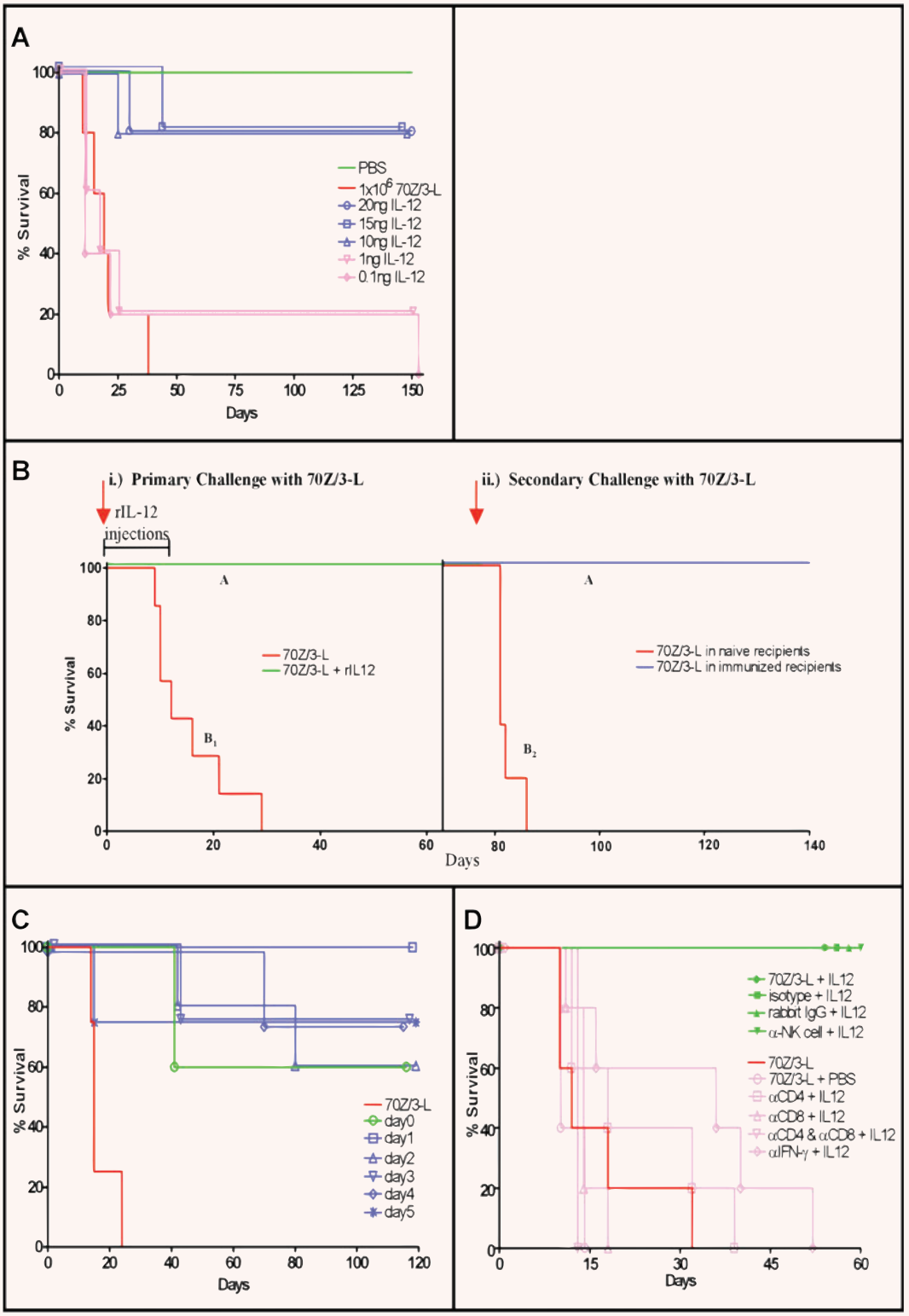

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Fig. 1 (**A**) IP administered rIL-12-mediated protection of mice challenged with 70Z/3-L cells. Mice were challenged with 10^6 cells IP and received either no treatment or injections of 0.1, 1, 10 or 20 ng/mouse/day rIL-12 for 14 days ($n = 5$ mice for each group). (**B**) IP administered rIL-12 therapy leads to long-term protection against challenge with 70Z/3-L. (i) Naïve mice (A, $n = 10$) were challenged with 70Z/3-L cells on day 0 and treated for 14 days with injections of 20 ng rIL-12 /mouse/day. A group of mice (B_1, $n = 7$) were included as controls for the 70Z/3-L cells (comparison of Kaplan–Meier survival curves was performed using Log rank test $P = 0.001$). (ii) After a period of 70 days, five mice from group A that had undergone rIL-12 therapy were secondarily challenged with 10^6 70Z/3-L cells without further rIL-12 treatment. The other five animals were kept to confirm that no toxicity appeared after 70 days. Five naïve mice (B_2) were included to demonstrate the lethality of the 70Z/3-L cells ($P = 0.0015$). (**C**) Delayed IP administration of rIL-12 therapy leads to protection. Mice were injected with 10^4 70Z/3-L cells on day 0. A control group (70Z/3-L) did not receive treatment ($n = 4$). From days 0 through 5, groups of 4 or 5 mice (5 mice for days 0, 1 and 2, 4 mice for days 3, 4 and 5) started receiving injections of 20 ng rIL-12/mouse/day for 14 days. Animals were monitored and euthanized at the appearance of symptoms. Curve comparison was performed using Log rank test. All treatment groups are significantly different from the control group ($P = 0.0029$) but are not significantly different from each other. (**D**) Requirement of T cells and IFN-γ for rIL-12-mediated protection following IP administration. Mice ($n = 5$ mice in each group) were depleted using antibodies as described in Materials and Methods. The mice were challenged with 10^6 70Z/3-L cells IP, injected with 20 ng/mouse/day rIL-12 and monitored for the appearance of symptoms. Comparison of Kaplan–Meier survival curves was performed using Log rank test ($P < 0.0018$).

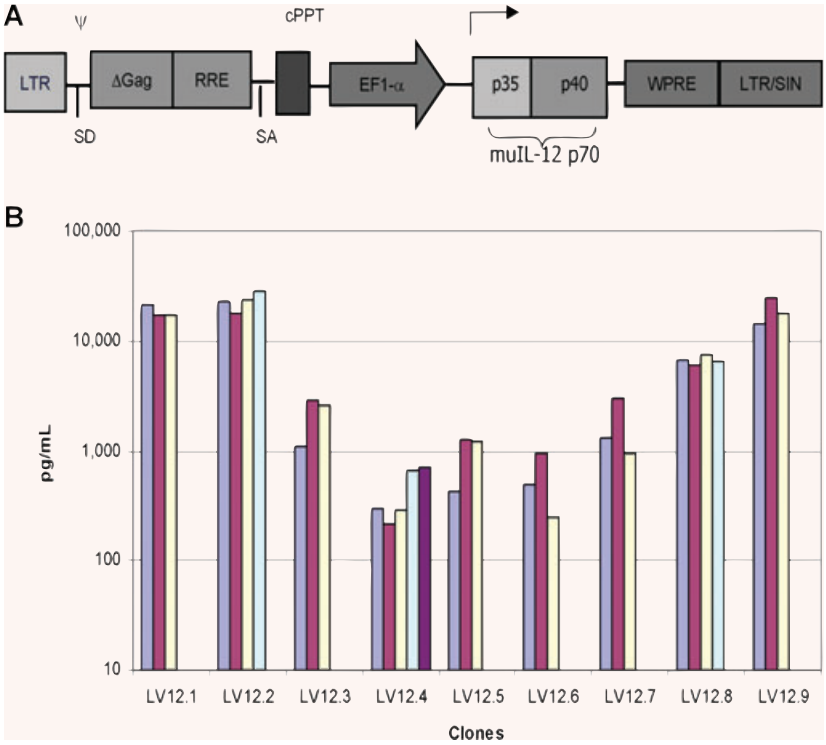

Fig. 2 (**A**) Schematic representation of the LV-muIL-12 (LV-cPPT-EF1α-mIL-12-WPRE) vector. LTR: long-terminal repeat; SD: splice donor; RRE: *rev* response element; SA: splice acceptor; cPPT: central polypurine tract; EF1α: elongation factor 1 alpha; WPRE: woodchuck hepatitis virus posttranscriptional regulatory element; muIL-12: murine interleukin-12; SIN: self-inactivating LTR. (**B**) Interleukin-12 secretion by vector-transduced clones is a stable trait. Levels of IL-12 secretion were measured by ELISA on 2–5 independent occasions (each bar represents an independent measurement) and seen to remain fairly constant; differences are not statistically significant.

Materials and Methods. Supernatants from these clonal cell lines were tested for the production of IL-12. The range of secretion from selected clones varied from approximately 250–40,000 pg/ml/10^6cells/2 hrs and these levels remained stable over time as shown in Figure 2B. Furthermore, the different levels of IL-12 measured did not seem dependent on cell growth kinetics, nor on survival, as the *in vitro* growth properties of the vector-transduced clones were similar as measured by thymidine incorporation and visual inspection. Southern Blot analysis demonstrated that no clone had more than 7 proviral integration events (data not shown).

Only a small proportion of vector-transduced 70Z/3-L cells producing IL-12 are required to confer immunity

We proceeded to determine if the production of IL-12 by vector-transduced 70Z/3-L cells would elicit a protective immune response by injecting 10^6 cells of each of 12 clones, spanning a range of secretion levels, into the abdominal cavity of BDF_1 mice. The three lowest producing clones (range: 200–1000 pg/ml/10^6 cells/2 hrs) failed to elicit an immune response and mice injected

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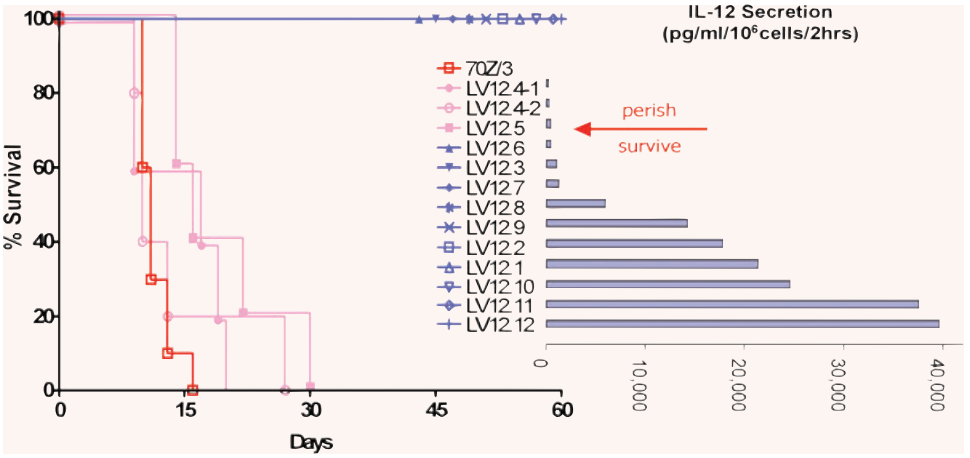

Fig. 3 Leukaemia cell-mediated IL-12 therapy leads to protection of challenged mice. Mice were injected IP with PBS or 10^6 cells of either the parent line, 70Z/3-L, or one of the vector-transduced clones and monitored for the appearance of symptoms. Clones secrete varying levels of IL-12 and a theoretical threshold was established, below which protection is not conferred.

with these cells progressed towards death. In contrast, all mice injected with 10^6 cells of the 10 highest producing clones (range: 1500–40,000 pg/ml/10^6 cells/2 hrs) survived (Fig. 3).

One 70Z/3-L transduced clone, LV12.1 that produces approximately 21,500 pg/ml/10^6cells/2 hrs, was mixed with naïve 70Z/3-L cells to determine if the inclusion of IL-12 producing vector-transduced cells would result in the elimination of non-producing cells also. We initially found that as little as 2% of the vector-transduced cells were sufficient to confer complete protection (Fig. 4A). To further examine the efficacy of producer/non-producer proportions, two other 70Z/3-L-transduced clones were selected that differed in IL-12 production by 10-fold (clone LV12.3: 2000 pg/ml/10^6 cells/2 hrs *versus* clone LV12.2: 20,000 pg/ml/10^6 cells/2 hrs). In this case, as few as 0.5% (*i.e.* 5000 LV12.2 cells in 10^6 total cells) of the higher producing clone was sufficient to confer protection to 80% of the mice but 0.1% failed to protect any mice. However, even 10% (*i.e.* 100,000 LV12.3 cells in 10^6 total cells) of the lower producing clone was insufficient to protect, indicating that a threshold of IL-12 production per vector-transduced cell is required to elicit an effective immune response (Fig. 4B).

Leukaemia cell-mediated IL-12 therapy leads to specific long-term protective immunity against the 70Z/3-L leukaemia

More than 110 days after IP injection with 10^6 LV12.2 cells, mice were challenged with either 10^6 cells of the parental leukaemia line, 70Z/3-L, or another well-characterized B-cell leukaemia, L1210, and monitored for the appearance of symptoms. Groups of naïve mice were included to control for the efficiency of both the 70Z/3-L and L1210 cells to cause disease. Figure 5 shows that all animals to survive the initial insult with LV12.2 were immune to subsequent challenge with 70Z/3-L but not L1210. Thus, cell-mediated IL-12 therapy leads to specific long-term protective immunity.

$CD4^+$ T cells are primarily required for leukaemia cell-mediated rejection of 70Z/3-L cells

Depleting antibodies were used to determine which cell types mediate the IL-12-induced rejection of 70Z/3-L leukaemia. We show in Figure 6 that the $CD4^+$ T-cell subset is of primary importance unlike in the IP administered rIL-12 therapy model above. The mean survival of leukaemia challenged mice was 37 days for animals depleted of $CD4^+$ T cells and 18 days for those depleted of both T-cell subsets. The curves are statistically different (P = 0.003), suggesting an important role for $CD8^+$ T cells but only in the absence of $CD4^+$ T cells. The $CD8^+$ T-cell subset alone is not sufficient to confer protection. Furthermore, the neutralization of IFN-γ did not diminish the protective effect as was seen with IP administered rIL-12 therapy (Fig. 6). This was a surprising result and prompted us to further investigate the regulation of IFN-γ and various other inflammatory cytokines in each model.

In vivo cytokine regulation

Interleukin-12 induces the secretion of other cytokines that can have agonistic, antagonistic or synergistic effects and can influence the specific immune response that is initiated [6–8, 17, 40–43]. It was therefore important to measure the regulation of some of these cytokines *in vivo* to better understand how leukaemia rejection is accomplished and shed some light on the results of our neutralization experiments. For this purpose, we employed a flow cytometry technique that detects a panel of inflammatory cytokines, including IL-12 p70, TNF-α, IFN-γ, MCP-1, IL-10 and IL-6 in serum. Mice received IP injections of 10^6 70Z3-L cells on day 0 and daily IP injections of either 10 or 20ng rIL-12/mouse/day for 14 days. Alternatively, mice were challenged with an IP injection of 10^6 70Z3-L cells on day 0 spiked with various proportions (0.5%, 1% and 10%) of vector-transduced cells and serum samples were collected on days 7,

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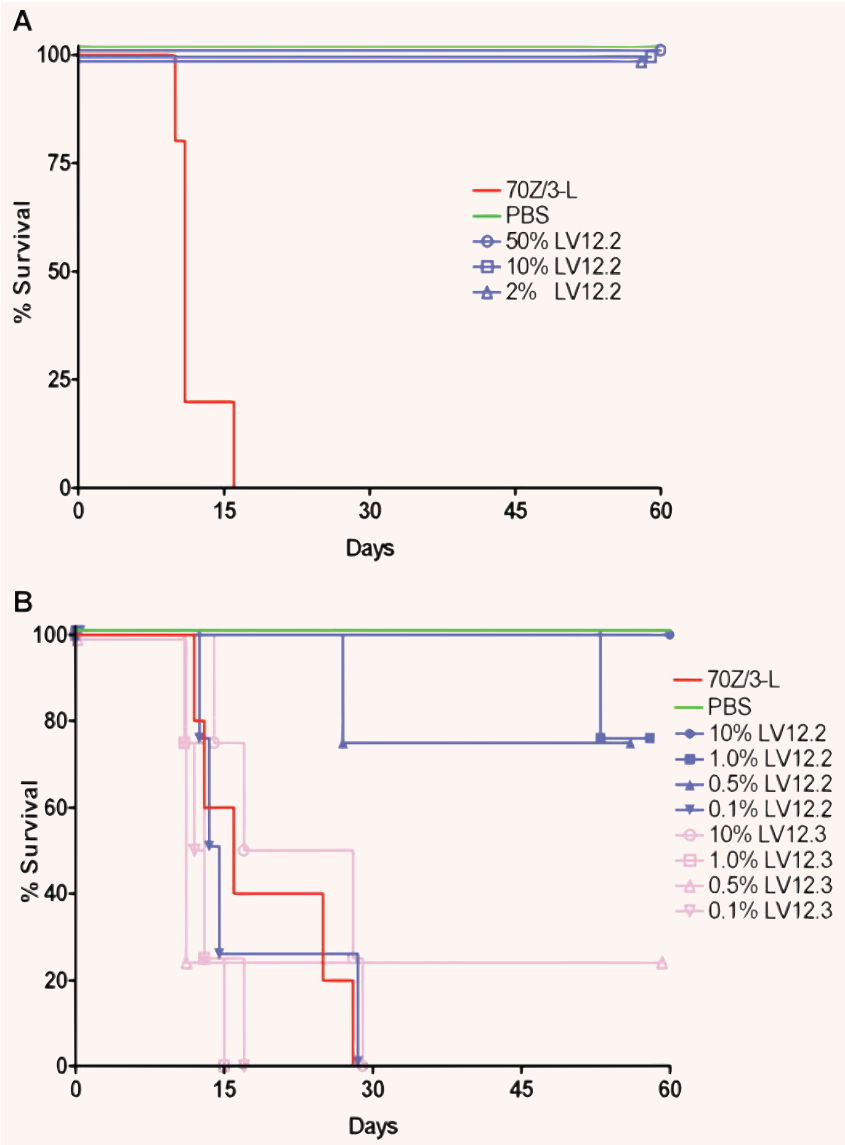

Fig. 4 Leukaemia cell-mediated IL-12 therapy leads to protection of challenged mice when only a portion of the cells are vector-transduced. Mice were injected IP with 10^6 cells of the parent line, 70Z/3-L, and varying proportions (**A**) 2%, 10% and 50% of the LV12.1 secreting clone or (**B**) 0.1%, 0.5%, 1% and 10% of LV12.2 or LV12.3, and monitored for the appearance of symptoms.

10 and 20. The results of these two assays are shown in Figure 7.

The levels of IL-10 induced on day 20 are significantly higher after leukaemia cell-mediated therapy as compared to IP administered rIL-12 therapy ($P < 0.0017$) but are not significantly different between IL-12 treated and control groups for either mode of delivery at any time point. Likewise, the levels of IFN-γ and TNF-α are significantly higher in response to IL-12 secreted from vector-transduced cells ($P < 0.0015$ and 0.0110 respectively). Of note, however, is that leukaemia cell-mediated treatment groups show significantly higher levels of IFN-γ than the control groups on day 7 ($P = 0.0007$) but resolve to near basal level by day 20. Interestingly, the systemic levels of IL-12 measured did not correspond to doses administered.

Discussion

In this manuscript, we demonstrate that IP-administered low-dose rIL-12 therapy can elicit a protective immune response in leukaemia-bearing mice and that an effective approach to deliver IL-12 is *via* the leukaemia cells themselves. We found that remarkably few transduced leukaemia cells are needed to achieve protection provided a sufficient amount of IL-12 is produced per cell, and that protection is achieved in a manner distinct from that with IP administered rIL-12 therapy.

Given the key role that IL-12 plays in the initiation of effective immune responses in various leukaemia models, we re-examined the potential for cytokine therapy using a murine model of ALL.

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Fig. 5 Leukaemia cell-mediated IL-12 therapy leads to long-term and specific protection against challenge with 70Z/3-L. Mice were initially challenged with either 10^6 LV12.2 cells or injected with PBS. More than 110 days following the primary challenge, primed mice ($n = 4$ in each group) were secondarily challenged with either 10^6 70Z/3-L or 10^6 L1210 cells. The PBS injected mice ($n = 5$ in each group) also received either 10^6 70Z/3-L or 10^6 L1210 cells to control for their efficiency to lead to morbidity, or another injection of PBS and monitored for appearance of symptoms. Kaplan–Meier survival curve comparison was performed using Log rank test, $P < 0.0001$.

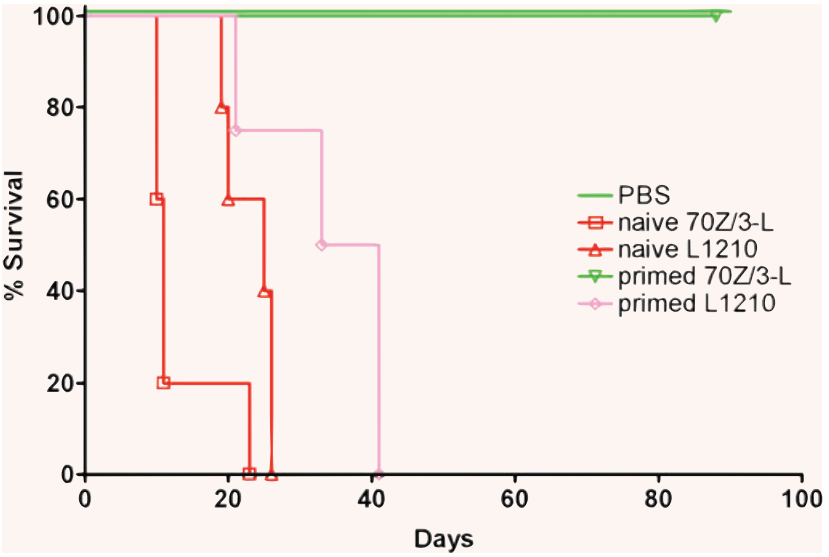
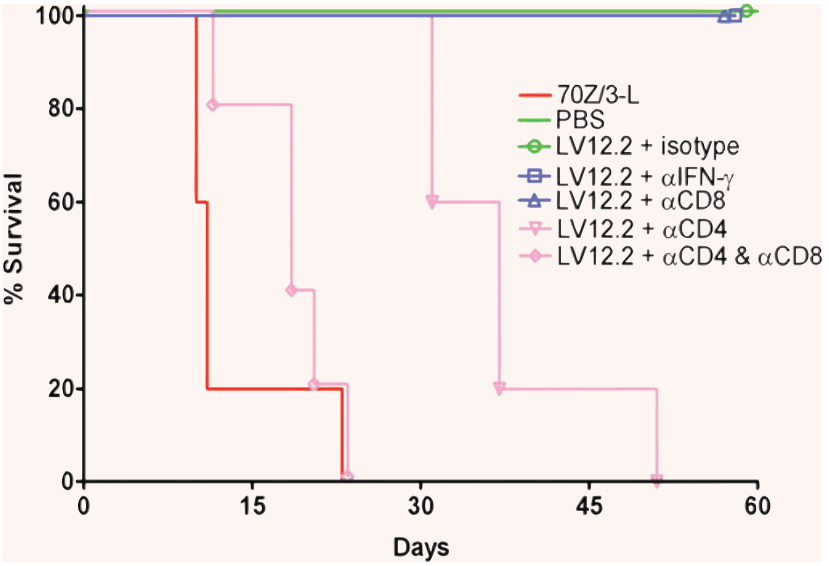


Fig. 6 Requirement of the $CD4^+$ T-cell subset for leukaemia cell-mediated protection of challenged mice. Mice ($n = 5$ in each group) were depleted using antibodies as described in Materials and Methods. The mice were challenged with 10^6 LV12.2 cells IP and monitored for the appearance of symptoms. Kaplan–Meier curve comparison was performed using Log rank test, $P = 0.0084$.



We have previously reported that 70Z/3-L cells lead to the rapid death of mice injected with as few as 10^2 cells. In contrast, we established variants of this line that are recognized by the immune system and subsequently rejected. Mixing as few as 10^5 of these non-leukaemic variants with 10^6 70Z/3-L cells resulted in complete rejection of all 70Z/3 cells [32]. While we have not yet determined why these variants are recognized by the immune system, these experiments revealed that 70Z/3-L cells can be rejected if the immune system is appropriately modulated, making this experimental system amenable to the study of IL-12-induced anti-leukaemia activity.

Interleukin-12-based therapies have not become front line cancer treatments in part because studies often report low response rates among patients [16]. The poor outcomes associated with IL-12 treatment in these clinical studies might be explained by the physiological response to IL-12-induced IFN-γ. For example, high levels of IL-12, and consequently IFN-γ, have been shown to induce IL-10 and lead to down-modulation of IL-12 responsiveness in the host [6]. However, it has also been shown that leukaemia regression occurs only in patients who sustain elevated levels of IFN-γ throughout the course of their treatment [17].

Previous groups have demonstrated that administration of IL-12 at doses significantly below the maximum tolerated dose can avoid the induction of antagonistic mechanisms [19]. Therefore, we first determined that daily IP administration of a dose as low as 10–20 ng of rIL-12 for 14 days, equivalent to 500–1,000 ng/kg, is sufficient to significantly increase the survival of mice injected with 70Z/3-L. Our chosen dose is effective against

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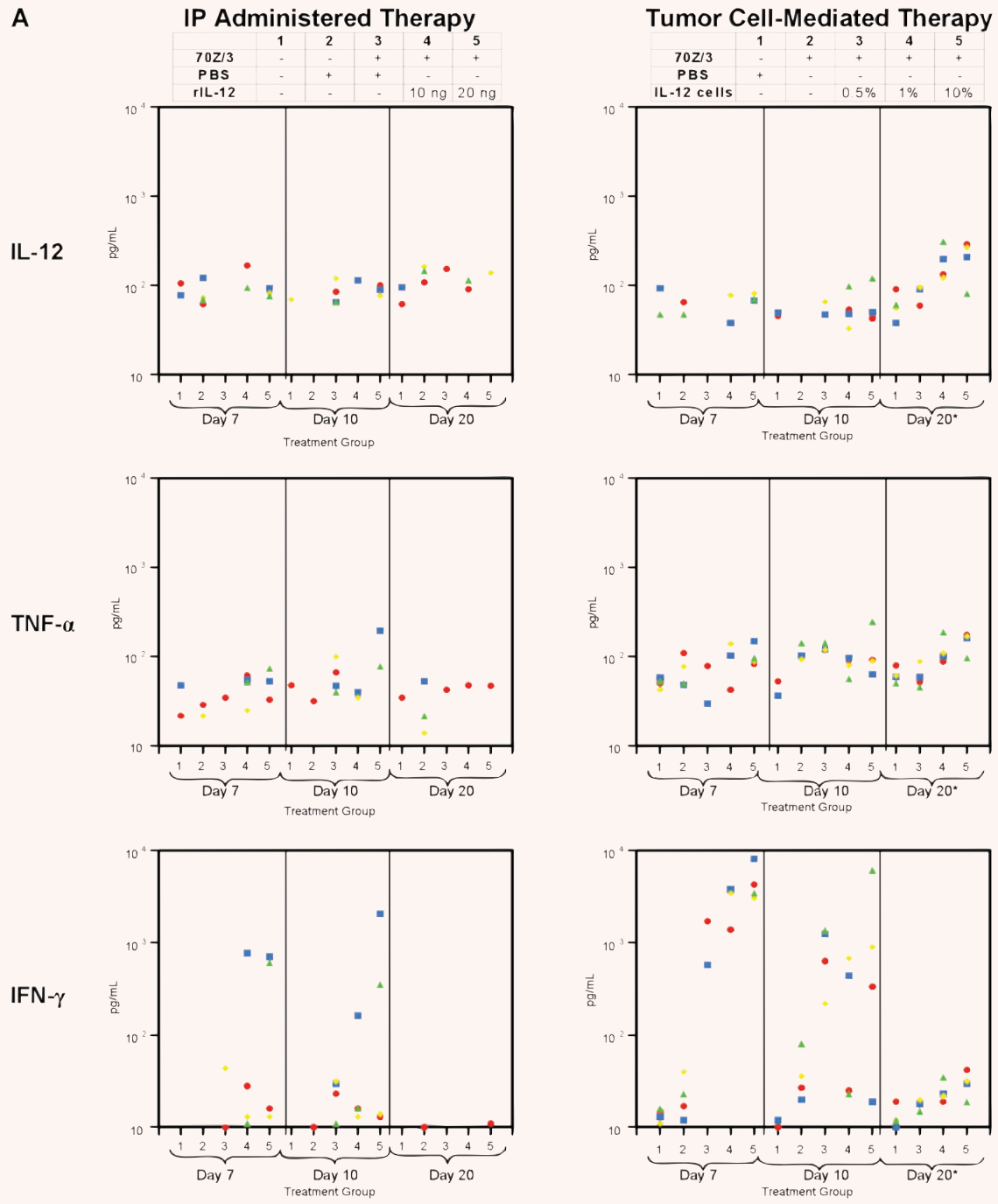

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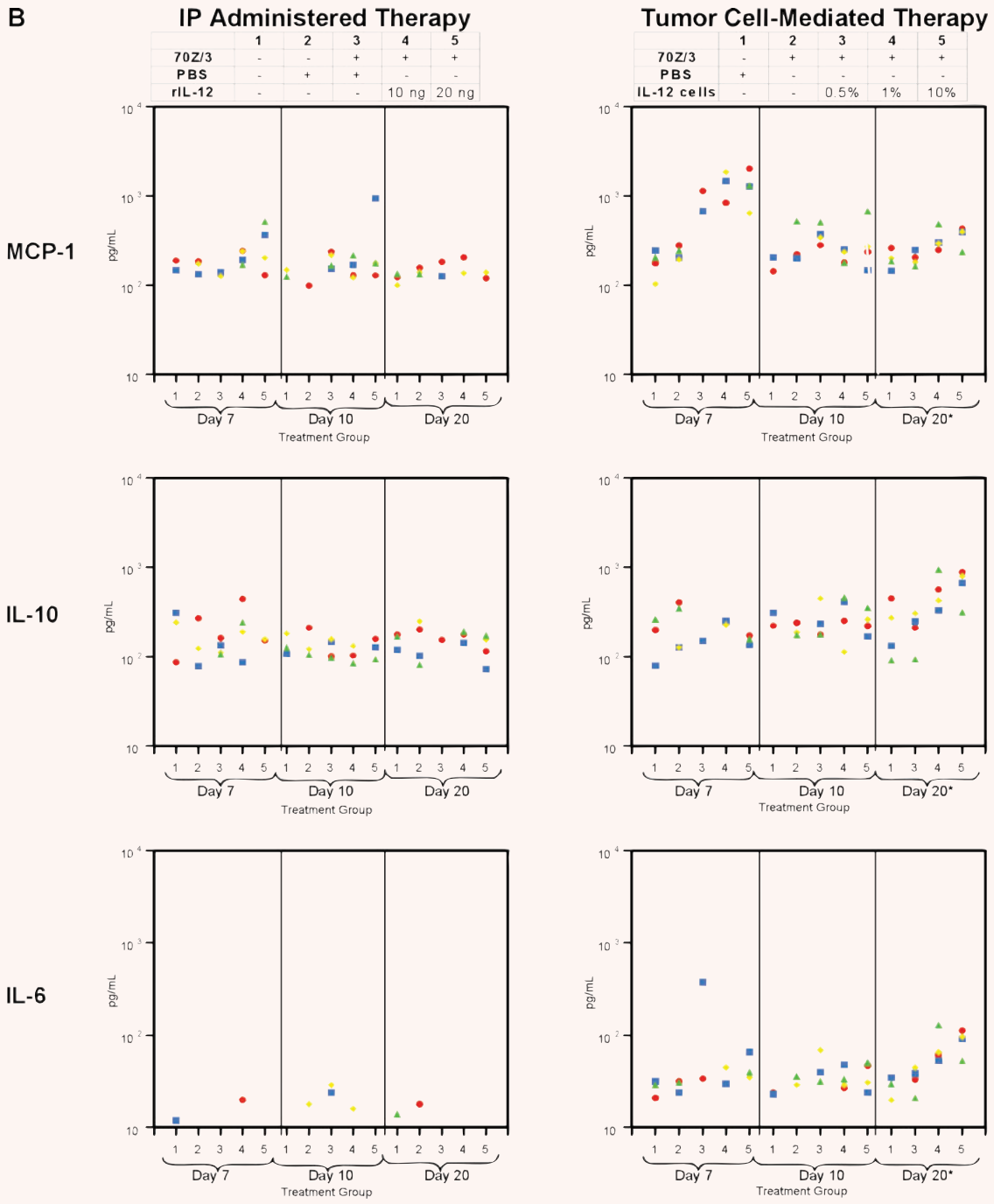

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Fig. 7 Cytokine expression profiles of mice receiving IP administration and leukaemia cell-mediated IL-12 therapies. The mice ($n = 4$ in each group) receiving IP administered rIL-12 therapy were challenged with 10^6 70Z/3-L cells and received either no treatment or injections of 10 or 20 ng/mouse/day rIL-12 for 14 days. A control group of mice receiving neither leukaemia cells nor treatment were included. Mice ($n = 4$ in each group) receiving leukaemia cell-mediated IL-12 therapy were challenged with 10^6 70Z/3-L cells IP and received either no treatment or treatment with various proportions (0.5%, 1% or 10%) of the vector-transduced clone LV12.2. A group of control mice receiving injections of PBS alone, without prior injection of leukaemia cells, was included for both systems. Serum samples were collected and analysed on days 7, 10 and 20 as described in Materials and Methods. Due to insufficient blood collection, two mice treated with cell-mediated therapy in the 0.5% and one in the 1% group on day 7 and two mice in the PBS group on day 10 could not be tested. *All mice in the 70Z/3-L group treated with cell-mediated therapy, as well as two in the 70Z/3-L group of mice receiving IP rIL-12, had perished by day 20 such that serum was not collected. In all other cases, a missing data point is due to serum levels of that particular cytokine that are below the level of detection in this assay.

an established leukaemia burden and rejection leads to long-term immune memory in a T-cell-dependent manner. Similar results have been demonstrated in previous literature. The true value of these experiments lies in the comparisons we were able to draw between our two model systems.

We next investigated a cell-mediated strategy for delivery of IL-12 and found that 70Z/3-L cells could be readily transduced with our novel lentiviral construct. This approach may circumvent the dose-limiting toxicity experienced in human clinical trials of systemically delivered IL-12 that was not foreshadowed by pre-clinical murine studies. We found that different vector-transduced clones produce varied amounts of IL-12. This appears to be a stable trait as we have measured similar levels of secreted IL-12 for each clone on 2–5 independent occasions. The vector copy number in these clones was determined but we found that this alone does not explain the variable secretion levels, nor does their rate of proliferation.

The establishment of clones that produce different levels of IL-12 has allowed us to examine the relationship between IL-12 production and the proportion of IL-12$^+$ vector-transduced *versus* IL-12$^-$ naïve 70Z/3-L cells necessary for immune activation. To date, this potentially critical aspect of cell-mediated cytokine therapy has not been thoroughly examined. We found that a very small proportion of IL-12 producing vector-transduced 70Z/3-L cells are sufficient to trigger a protective immune response. For one clone, LV12.2, 5000 such vector-transduced cells (but not 1000) were sufficient to save 80% of the mice injected with 10^6 70Z/3-L cells. This result could indicate either that a critical number of 'hits' or a sufficient amount of IL-12 is required to trigger an immune response. A reasonable interpretation of 'hit' might be an encounter between an IL-12 producing vector-transduced 70Z/3-L cell and an appropriate APC, such as a DC. The alternative explanation is that these 5000 vector-transduced cells simply deliver a sufficient quantity of IL-12 into the system to trigger an immune response. To determine which of these explanations is correct we employed a different clone, LV12.3, which produces 10-fold less IL-12 per cell and injected titrated numbers of these vector-transduced cells along with 10^6 70Z/3-L naïve cells. We found that even 100,000 LV12.3 cells failed to confer protection. This represents 20-fold more cells and twice the potential IL-12 released into the system. Together, these two experiments suggest that it is the number of 'hits' that matter rather than the absolute amount of IL-12 but that to qualify as a 'hit', the vector-transduced 70Z/3-L cell must produce IL-12 above a certain threshold.

Our findings have important implications for clinical trial design and may partly explain the differences observed between murine studies, in which IL-12 can initiate a curative immune response, and human studies, in which the immune response is modest and patient survival is normally unaffected. The protocols used in mouse studies usually involve selection of clones that secrete relatively high levels of IL-12 and frequently the preparation administered consists of 100% IL-12 secreting cancer cells. In contrast, human studies generally rely on freshly obtained populations of cells that are difficult to clone. Therefore bulk populations are transduced and average amounts of IL-12 produced by these populations are measured. In cases reported to date, these average amounts fall short of what we believe to be necessary to elicit protective immunity and there is no information on the distribution of production levels within these populations. Beyond being insufficient to induce antitumour immunity, lower IL-12 secreting cells may act to inhibit the desired response. The cytokine micromilieu in which a DC matures will define the cytokines that it later releases into the immunological synapse it forms with a T cell. These cytokines form an integral part of the instructive signal that polarizes the T-cells fate and determines the effector function that will be executed [44]. Tumour cells are known to produce cytokines such as IL-10, IL-6 and TGF-β that suppress cellular responses. Because the particular combinations and ratios of cytokines present during DC maturation define their polarizing capacity, we suggest that the amount of IL-12 locally produced by each transduced tumour cell is additionally important as the ratio of IL-12 over such antagonistic cytokines must be sufficiently high to ensure the production of Th1 polarizing DCs and not tolerogenic DCs [45]. A cell-mediated approach may facilitate achieving the necessarily high levels of local expression, without resulting in the toxicity observed in human trials, by confining production to the tumour microenvironment.

The IL-12-induced anti-leukaemia activity in our two models is T-cell-dependent but the subsets that are critical differ depending on the mode of IL-12 delivery. The role of IFN-γ also appeared to differ, prompting us to look at its *in vivo* regulation along with a number of other inflammatory cytokines. This was done using a flow cytometry-based cytokine bead assay. The regulation of IL-10, IFN-γ and TNF-α are of particular interest in our model systems because IL-10 is known to be the most biologically relevant

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antagonist of IL-12 [4], IFN-γ is thought to mediate the effects of IL-12 [4, 13] and a combination of IFN-γ and TNF-α is required for the development of $CD4^+$ CTLs [5].

The fact that IL-10 production was not elevated above background in any of our treatment groups suggests that while local IL-12 production was high, the systemic amount was sufficiently low as to avoid the induction of antagonistic molecules and dampening of the biologic effect. Measured levels of IFN-γ were significantly higher in the treated groups receiving cell-produced IL-12 as compared to controls on day 7 but were not significant by day 10 and returned to near baseline by day 20. Furthermore, IFN-γ production was significantly greater in the cell-mediated model in general. There is ample literature describing how IL-12 leads to the increased maturation of DCs, the production of IFN-γ and more efficient antigen presentation by the IFN-γ-dependent up-regulation of MHC-II and co-stimulatory molecule expression. T-helper lymphocytes are driven by IFN-γ to differentiate with a type-1 functional profile and subsequently promote the strong $CD8^+$ CTL response that we saw with IP administered rIL-12 therapy. This agrees with the classical model of cytotoxicity mediated by the traditional $CD8^+$ CTL. However, there is also literature describing a role for $CD4^+$ CTLs in models of infection [5, 46–48] and more recently in tumour immunology [49–54]. It is possible that the IFN-γ and TNF-α rich environment resulting from cell-mediated therapy led to the development of an effector $CD4^+$ T-Cell population. This could account for the differential importance of T-cell subsets in our two models and explain the distinct results of the neutralization experiments. The major thrust of tumour vaccination research has traditionally focused on targeting $CD8^+$ CTLs, which require stimulation by a $CD4^+$ helper T-cell population, to affect tumour clearance but the clinical response has been limited. An emerging hypothesis in the field is that directly targeting $CD4^+$ effector cells may be important in achieving a more robust anti-tumour response. Although the mechanism of action of the $CD4^+$ population observed in our system has yet to be delineated, the incitement of a potent $CD4^+$ effector response is of clear interest.

Despite the beneficial effects of IFN-γ highlighted above, a dampening of the response with repeated administration is still of concern in models of IL-12 therapy. An important attribute of our cell-mediated model is that a sufficient immune response is stimulated for a period of time corresponding to that required to eliminate the cancer but the signal is self-limiting. The source of IL-12 into the system is the cancer cells that are, themselves, the target of therapy. As the leukaemia cells are rejected, the source of IL-12 is reduced and IFN-γ levels return to baseline without a significant increase in the antagonistic molecule IL-10. Our approach differs from others in the field of immunotherapy in a number of informative ways. In protocols that engineer DCs and fibroblasts to stably secrete IL-12, for example, production would continue even after tumour clearance. In addition, the secretion levels reported to date have not reached what we propose to be therapeutic, possibly explaining the mixed responses observed. Alternatively, methods for stimulating the maturation of autologous tumour antigen-loaded DCs, leading to IL-12 production, have also been described [30]. These DCs are only capable of producing IL-12 for approximately 18 hrs after administration and treatment efficiency may be highly dependent upon the amount of time required for these semi-mature DCs to travel to the LNs where they can stimulate a T-cell response. Another apparent limitation of this approach is the effect the maturation stimulus has on the migratory capacity of these DCs. Our IL-12 transduction protocol does not appear to interfere with the migration of the cell line used in our studies as evidenced by the successful eradication of a widely disseminated leukaemia. Each of these approaches possess merit and may prove to be very valuable and effective therapeutic regimens. We assert that tumour cell-mediated approaches warrant further investigation to round out this growing body of knowledge.

We have demonstrated in this manuscript that IL-12, given at doses below which the resulting systemic levels of IFN-γ lead to the induction of antagonistic mechanisms, is sufficient to launch a protective immune response against 70Z/3-L ALL cells and complete clearance of the leukaemia if individual tumour cells produce IL-12 above a critical threshold. We highlight that the mode of IL-12 delivery can have a profound impact on the nature of the immune response that is mounted and demonstrate a critical role for $CD4^+$ cells in our cell-mediated model that apparently does not exist in our IP administration model. Moreover, a potentially problematic dampening of the immune response was not observed. This is possibly due to the localization of IL-12 secretion by, and self-limiting nature of the cell-mediated therapy approach we employed.

Acknowledgements

We thank Drs. Pam Ohashi, Brian Barber, Mark Minden, Keith Stewart, Craig Milne and Danijela Konforte for reviewing the manuscript and for helpful suggestions, and a special thanks to Paul Boutros for aiding with statistical analysis. We would also like to thank members of the Paige lab for helpful discussions and Dr. Yu Zhang for technical assistance. This work was supported by the National Cancer Institute of Canada/Terry Fox Foundation.
Disclosure: The authors declare no potential conflict of interest.

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