

High Frequency of Specific CD8⁺ T Cells in the Tumor and Blood Is Associated with Efficient Local IL-12 Gene Therapy of Cancer¹

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Cancer immunotherapy often aims at the reactivation and expansion of tumor-specific CTL. In an attempt to correlate in situ and/or systemic tumor-specific T cell expansion with tumor regression, we investigated the effects of adenovirus-mediated IL-12 or IFN- γ gene transfer into established P815 murine tumors. While IFN- γ was no more potent than the vector alone, IL-12 gene transfer promoted tumor eradication. Despite this antitumor effect, no significant cytolytic activity was detectable using classical cytotoxicity assays from in vitro restimulated splenocytes. Since intratumor gene delivery may induce a localized expansion of CTL, the presence of P815-specific CD8⁺ T cells in situ was assessed. Using the Immunoscope approach, we found a dramatic increase in clonotypic T cells at the tumor site following IL-12, but not IFN- γ gene delivery. Antitumor CD8⁺ T cell frequencies were then re-evaluated using this molecular detection technique, which revealed a comparable expansion of specific T cells in the peripheral organs, most strikingly in the blood. These data show that local IL-12 gene transfer, in contrast to IFN- γ , mediates a potent antitumor effect that correlates to clonal tumor-specific T cell expansions in situ and in the periphery. *The Journal of Immunology*, 1999, 162: 609–617.

Since the characterization of tumor-associated Ags recognized by CTL in human tumors such as melanomas (1), one aim of active specific cancer immunotherapy is to induce or stimulate tumor-specific CTL expansion. Many different approaches have been attempted, such as local or systemic administration of cytokines alone or in association with tumor Ags or adoptive transfer of ex vivo propagated tumor-infiltrating lymphocytes (TIL)⁴ (2). The development of optimal immunotherapeutic protocols requires a thorough understanding of the impact of the various treatments on the immune response.

Preclinical studies in rodents have shown that IL-12, a key cytokine for the induction of Th1-oriented immune responses, is one of the most potent molecules in cancer therapy. In initial protocols, administration of IL-12 resulted in severe toxicity in humans (3).

Among other means, this can be circumvented by in situ delivery. The efficiency of local secretion of IL-12 at the tumor site using genetically modified fibroblasts (4) or tumor cells (5–9) or recombinant viral vectors such as retrovirus (10), vaccinia virus (11), or adenovirus (12, 13) has been demonstrated in a variety of tumor models. Analysis of the antitumor mechanism of IL-12 in murine tumor models has shown that T cells are critical immune effectors; however, the involvement of specific T cell subsets (CD8⁺, CD4⁺, or NK T cells) varies with the tumor model studied (14, 15). Other immune effectors, such as NK cells or macrophages, have been implicated in the antitumor effects of IL-12 (5, 16). An antiangiogenic effect has also been ascribed to this cytokine (17). The induction of IFN- γ production is necessary but not sufficient to explain the anti tumor efficiency of IL-12 (18, 19).

The involvement of tumor-specific CTL has been suggested in many systems by in vivo depletion studies showing a requirement for CD8⁺ cells in IL-12-mediated antitumor effects (18, 20). Noguchi and colleagues have further demonstrated that a protocol combining administration of tumor-derived peptide and low doses of IL-12 allows for the induction of specific CTL in naive mice as well as the eradication of established tumors (21). Moreover, the infiltration of IL-12-treated tumors by high numbers of CD8⁺ cells has been observed by immunohistochemistry (18). However, while the induction of IFN- γ production has often been associated with a successful IL-12 treatment, an increase in tumor-specific cytotoxic activity in tumor-bearing mice has rarely been reported. To our knowledge, such an effect has been reported by three groups: in one case, after complete tumor eradication, but compared with mice bearing huge tumors (22); in the second, a weak cytotoxic activity was detectable in the draining lymph node (LN) (23); and recently, IL-12 was shown to enhance the significant cytolytic activity detectable following the intratumoral inoculation of a replication-competent recombinant herpes simplex virus (24). In contrast, in another study the cytolytic activity was not correlated with the antitumor effects of IL-12 used in combination with IL-2-transfected tumor cells (25).

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⁴ Abbreviations used in this paper: TIL, tumor-infiltrating lymphocytes; LN, lymph node(s); RSV, Rous sarcoma virus; LTR, long terminal repeat; AdmIL-12, recombinant adenovirus vector encoding mouse IL-12; pfu, plaque-forming units; AdmIFN- γ , recombinant adenovirus vector encoding mouse IFN- γ ; HPRT, hypoxanthine phosphoribosyltransferase.

We attempted to correlate IL-12-mediated antitumor effects with the presence of tumor-specific CTL in the periphery and in the tumor itself. To do so, we analyzed the effects of adenovirus-mediated IL-12 gene transfer in established P815 tumors. This immunogenic tumor model was chosen because specific CTL have been shown to be involved in its rejection in a number of situations, the clearest proof of this fact being the selection *in vivo* of tumor variants that have lost CTL-recognized Ags (26). Furthermore, the characterization of a public repertoire among P815-specific CTL (27) allows the detection and quantification of clonotypic tumor-specific CD8⁺ T cells directly *ex vivo* from any tissue, using the Immunoscope approach (28).

We treated mice bearing established P815 tumors by direct intratumoral injection of recombinant adenoviral vectors encoding either IL-12 or IFN- γ . Paracrine delivery of IL-12 was found to cure nearly all mice, while IFN- γ was no more efficient than the vector alone. Despite this therapeutic efficacy, we could not detect significant cytolytic activity using classical cytotoxicity assays with *in vitro* restimulated splenocytes. In contrast, the Immunoscope technique revealed a dramatic increase in specific CD8⁺ T cells frequency at the tumor site following IL-12 gene delivery, which was also observed in the peripheral organs, most strikingly in the blood. No significant increase was observed following control virus injection or IFN- γ therapy.

Our data demonstrate that IL-12 gene delivery into established tumors is able to induce a large tumor-specific CD8⁺ T cell expansion that is associated with tumor eradication. Neither of these effects could be attributed only to IFN- γ production.

Materials and Methods

Cell lines

P815 is a mastocytoma of DBA/2 origin (29). The P815 subline used derived from P1.HTR (30) and was grown in RPMI 1640-GlutaMAX (Life Technologies, Cergy-Pontoise, France) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The tumor cell line was maintained *in vitro* for no longer than 1 mo before *in vivo* experiments. P815-B7.1 have been obtained by transfection of P1.HTR by the LL218 vector encoding murine B7.1 (a gift from Dr. L. L. Lanier, DNAX Institute, Palo Alto, CA). L1210 is a DBA/2 leukemia cell line that does not express P815 tumor Ags (31). Cell culture medium and reagents were obtained from Life Technologies.

Mice

Female DBA/2 mice were purchased from the Centre d'Élevage Janvier (Le Genest St. Isle, France) and maintained in the animal facilities of the Institut Gustave Roussy (Villejuif, France). Mice were 8–10 wk of age at the onset of each experiment. Mice were bled (200 μ l) at the orbital sinus after anesthesia with ether.

Replication-defective recombinant adenoviral vectors

The murine IL-12 p35 and p40 cDNAs were obtained from the msp35 and msp40 plasmids, respectively (provided by U. Gubler, Hoffmann-La Roche, Nutley, NJ). The p35 cDNA was first placed under the transcriptional control of the human CMV immediate early gene promoter by subcloning it into the pcDNA3 plasmid (Invitrogen, Leek, The Netherlands). This expression cassette was released and ligated into the pAd.RSV β gal expression plasmid, which contains the left end of the adenovirus type 5 genome, resulting in pCMVp35RSVp40IL-12. The p40 cDNA was subcloned into the plasmid pAd.RSV β gal under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter. The RSVp40 expression cassette was inserted downstream of p35 cDNA into pCMVp35IL-12, resulting in pCMVp35RSVp40IL-12. The recombinant adenovirus deleted for the E1 and E3 regions was constructed by *in vivo* homologous recombination in 293 cells cotransfected with the genome of Ad.RSV β gal (32), left end deleted, and the linearized plasmid pRSV40pCMV35IL-12. The vector AdmIL-12 was rescued, plaque purified, and amplified using 293 cell line. The viral preparations were purified by two CsCl density centrifugations, and viral titer (plaque-forming units (pfu) per milliliter) was determined by plaque assay in 293 cells (33). The recombinant adenovirus encoding murine IFN- γ was constructed following the same procedure as that used for

AdmIL-12 and is described elsewhere.⁵ Expression of IFN- γ cDNA is driven by the LTR RSV promoter. The adenoviruses used as controls were AdCO1, an adenoviral vector identical with AdmIL-12 lacking a transgene (34), and dl324, which contains part of the E1b region of the adenoviral genome not present in AdCO1 or AdmIL-12 (35). Both control vectors were found to behave similarly and are henceforth referred to as empty virus.

In vitro IFN- γ release assays

Spleen and LN of two mice per group were collected 3 days following the *i.v.* injection of 1×10^9 pfu of adenoviral vector diluted in 0.3 ml of PBS. Cells were harvested after mechanical disruption of tissue, depleted from erythrocytes by osmotic lysis, counted, and resuspended in RPMI 1640-GlutaMAX medium containing 5% heat-inactivated FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at a final concentration of 5×10^6 cell/ml. One million cells were plated in 96-well round-bottom plates and incubated for 48 h at 37°C, 5% CO₂. Supernatants were collected, and the amount of IFN- γ was assessed by ELISA (Genzyme, Cambridge, MA). The detection limit of this assay was 5 pg/ml.

In vivo treatment of tumor-bearing mice

Mice were inoculated intradermally into the right flank with the minimal tumorigenic dose of our P815 subline (5×10^5 cells) in a volume of 0.1 ml of PBS. Nine and thirteen days following tumor cell inoculation, 5×10^8 pfu of recombinant adenoviral vector diluted in 0.1 ml of PBS was injected at the tumor site. Mice were scored for tumor growth weekly. The animals were sacrificed when the tumor exceeded a 15 mm mean diameter.

Cytotoxicity assays

Before each experiment, positive control mice (vaccinated mice) were generated by intradermal injection of 10^6 P815-B7.1 cells 15 days before P815 tumor graft. Spleen cells (3×10^7) from mice injected 20 days previously with 5×10^5 P815 cells were stimulated with 10^6 irradiated (100 Gy) P815-B7.1 cells in 20 ml of RPMI 1640 medium containing 10% FCS, 5×10^{-5} M 2-ME, and antibiotics. Cultures were maintained at 37°C for 5 days in upright 25-cm² culture flasks. Cells were then counted and mixed at various ratios with 5000 ⁵¹Cr-labeled P815 or L1210 target cells. One lytic unit is defined as the number of spleen cells that lyse 50% of 10^4 target cells in 4 h (36), calculated using a Microsoft Excel program provided by Dr. B. Van den Eynde (Ludwig Institute, Brussels, Belgium). The number of L1210-specific lytic units was subtracted from the number of P815-specific lytic units to yield the final result.

RNA extraction and cDNA synthesis

Tumor specimens and LN were snap-frozen immediately after removal and were kept at -80°C for no more than 1 mo. Immediately after thawing, they were homogenized and lysed in 4 M guanidium thiocyanate. For spleens and blood samples, pelleted single cell suspensions were lysed in guanidium thiocyanate before being frozen and kept at -80°C . Total RNA was isolated according to the method described by Chirgwin *et al.* (37). cDNA was synthesized from 10 μ g of RNA, using a (dT)₁₇ primer, AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany), and its provided buffer, with addition of RNasin (Promega, Madison, WI).

PCR conditions

All PCR and run-off reactions were performed in a Geneamp 9600 thermocycler (Perkin-Elmer, Foster City, CA). PCR was performed in 20- to 40- μ l volumes; run-off reactions were performed in a 20- μ l volume. The reaction mixture for PCR contained 0.2 mM of each dNTP, 0.5 μ M of each primer, 2.5 mM MgCl₂, variable amounts of cDNA, 25 U/ml of Goldstar Taq DNA polymerase (Eurogentec, Seraing, Belgium), and the provided buffer. The reaction mixture for run-off reactions was the same, except 1) the single primer was added at a 0.1- μ M concentration; 2) Promega Taq polymerase (Madison, WI) was used; and 3) the template was 10 μ l of a PCR product diluted five times with water. Reaction cycles consisted of 25 s at 94°C, 25 s at 60°C (except for run-off reactions with the anti-A clonotypic primer P1AIR, 65°C), and 30 s at 72°C. Cycles were preceded by 3 min of denaturation at 94°C followed by 5 min of elongation at 72°C. The number of PCR cycles depended on the specific application and primers used (see below). Three cycles were used for run-off reactions.

⁵ L. Cordier, N. C. Fernandez, J.-C. Sabourin, P. Opolon, N. Di Falco, M. Perricaudet, J. Bignon, A. Kane, and H. Haddada. Adenoviral mediated cytokine or costimulatory gene transfer into sarcomatous malignant mesothelioma: tumor regression following intratumoral delivery of IFN- γ gene. *Submitted for publication.*

Oligonucleotides

Oligonucleotides were purchased from Eurogentec (Seraing, Belgium). Fluorescent oligonucleotides were directly labeled with FAM fluorophores (Applied Biosystems, Foster City, CA). For IL-12 amplification, the following primers were used. To detect transgenic p40, the RSV-LTR-specific primer CATTACCACATTGGTGTGC was used along with the p40 antisense primer GTCTTCTCTACGAGGAACGC. To detect total p40, the RSV-LTR primer was replaced by the p40 sense primer GAAGCACCAAATACTCCGG. To detect transgenic p35, the CMV promoter-specific primer CACTGCTTACTGGCTTATCG was used with the p35 antisense primer CTAGAATGATCTGCTGATGG. Primer pairs for amplification of endogenous IFN- γ and hypoxanthine phosphoribosyltransferase (HPRT) have been described previously (38) (the 5' and run-off primers have been used). The IFN- γ -specific primers cited above amplify only the endogenous IFN- γ cDNA because they hybridize to the 3' untranslated region, which is not included in the AdmIFN- γ vector. To amplify all IFN- γ messengers (either endogenous or from AdmIFN- γ), the primers CACTGCATCTTGGCTTTG and fam-CCACATCTATGCCACTTGAG, which hybridize to the coding region, were used. An additional primer pair has also been used for HPRT (TTGCTGACCTGCTGATTAC and fam-GTTGAGAGATCATCTCCACC). Primers for TCRBV1 amplification have been described previously (27).

Quantification of HPRT and IFN- γ transcripts

Relative levels of HPRT and IFN- γ transcripts were measured in the different cDNAs by PCR stopped in the exponential phase (39, 40). As one of the two primers used being labeled with a FAM fluorophore, the amount of amplified product could be quantified after loading of 2 μ l of PCR product diluted four times with formamide in a 373A automated DNA sequencer (Applied Biosystems, Foster City, CA). The products were loaded along size standards, and the data were analyzed with the Immunoscope software developed in the laboratory (41). The number of cycles that provided sufficient signal without leaving the exponential phase was first assessed in a preliminary kinetic experiment. Typically, 24 cycles were required for HPRT, and 27 were required for IFN- γ . For each sample, four tubes were amplified in each reaction, containing decreasing volumes of cDNA as initial template. The fact that the final signal decreased proportionally to the initial amount of template was used as a control that the amplification was stopped in the exponential phase. The quantification of HPRT has been double-checked systematically with the two primer pairs. The two quantifications always provided consistent results, and the average value was used for normalization.

Quantification of public P815-specific T cells

In the tumors, the repertoire of infiltrating T cells is oligoclonal, and the public T cells represent the majority of BV1-expressing T cells (27). To compare the levels of infiltration by public P815-specific T cells in the different tumor samples, the levels of transcripts encoding TCRBV1 chains with a 9-amino acid (aa)-long CDR3 were measured by quantitative PCR in the exponential phase (as described above for HPRT or IFN- γ) with the V β S01 and C β 5'-fam primers (27 cycles were usually required). The ratio of the TCRBV1 (9 aa) signal to the HPRT signal was used to obtain a relative level of infiltration. To obtain an estimate of the actual frequency of the public TIL, tumor samples were compared by the same technique with spleen cells from naive mice. The final number was calculated by assuming that 20% of naive splenocytes were T cells, among which 2% used the BV1 segment.

In the peripheral organs, we took advantage of the fact that the clonal expansion of specific public T cells is observed while the repertoire profile of T cells using BV1 with other CDR3 lengths is left undisturbed (27). This allows for a rapid quantification of the frequency of the public T cells that bear a 9-aa-long CDR3 sequence recognized by clonotypic primer by assuming that the frequency of cells using other CDR3 lengths is constant, as described in detail previously (42). The cDNA from the sample of interest was amplified to saturation (40 cycles) in two PCRs, one with the V β S01 and J β 1.2far primers and one with the V β S01 and J β 2.5far primers, to quantitate anti-A and anti-E public cells, respectively. Each of these PCR was subjected to two run-off reactions, one with the corresponding fluorescent BJ-specific primer and one with the appropriate clonotypic primer (P1IR and P2IR, respectively). Equal amounts of run-off products were then electrophoresed in an automated sequencer. The intensity of the unique peak yielded by the clonotypic primer was then compared with the sum of the intensities of the peaks corresponding to CDR3 lengths of 8, 10, and 11 aa (the three major peaks, apart from the variable 9-aa peak). The final frequencies of public anti-A and anti-E cells among T cells was obtained by taking into account the specific activities of the primers and

calculating that the cells represented in 8-, 10-, and 11-aa peaks of the BV1J1S2 and BV1J2S5 subpopulations constitute 1/1700th and 1/600th of the total T cell repertoire, respectively. The frequencies of anti-A and anti-E public cells were then added to obtain the final value.

Statistical analyses of data

Fisher's exact method was performed to interpret the significance of differences between experimental groups. To interpret the survival between different groups, the log-rank test was used. Significances are indicated in the figure legends.

Results

The recombinant AdmIL-12 adenoviral vector induces the release of biologically active IL-12 in vivo

We constructed a replication-defective adenoviral vector harboring the two chains (p35 and p40) of mouse IL-12. Since p40 homodimers are antagonists of heterodimeric IL-12 (43), we devised a system in which more p35 than p40 is expected to be produced. Thus, p35 cDNA was transcribed under control of the CMV promoter, whereas transcription of the p40 subunit was controlled by the LTR RSV promoter, which was shown to be less potent in vivo (44).

The production of biologically active IL-12 by virally infected cells was first assessed using a PHA-blast proliferation assay and ELISA in the supernatant of various cell lines infected in vitro. With a multiplicity of infection allowing the infection of 50–70% of cells, the amount of p70 detected ranged from 15–30 ng/ml (data not shown).

Next, we tested the efficiency of in vivo IL-12 gene transfer. After i.v. injection of 10⁹ pfu of viral particles, mouse IL-12 p70 was detectable from days 1–6, with a peak on day 2 or 3 (1–2 ng/ml) in the sera of AdmIL-12 injected mice (data not shown). Furthermore, since IL-12 systemic administration induces IFN- γ production (18, 45), we measured IFN- γ levels in the supernatants of single cell suspensions obtained from LN and spleens collected 3 days following AdmIL-12 administration. As shown in Fig. 1A, in vivo AdmIL-12 inoculation induced a dramatic increase in IFN- γ secretion from both splenocytes and LN cells. Thus, the recombinant vector AdmIL-12 allowed the production of biologically active IL-12 in vivo.

We then assessed the efficiency of adenovirus-mediated transfer of IL-12 in established P815 tumors. Nine and thirteen days following tumor inoculation, 5 \times 10⁸ pfu of AdmIL-12 or empty virus, or PBS, was injected at the tumor site. The expression of both IL-12 subunits was analyzed at the mRNA level by RT-PCR in tumors collected on day 20. To discriminate between endogenous and transgenic IL-12, we used an upstream primer specific for the transcribed part of the promoter region of the construct along with a downstream primer specific for the appropriate IL-12 subunit. As shown in Fig. 1B, transcription of both IL-12 subunits, driven by the viral vector, was almost systematically observed in the AdmIL-12-treated tumors. Since the last adenoviral injection was on day 13, expression lasts for at least 7 days. Although significantly weaker, some message for the endogenous IL-12 p40 was detected in control animals.

Interestingly, for one AdmIL-12-treated sample (lane 7), no transgenic p40 mRNA could be detected, while some transgene-driven p35 transcription was detectable. Coincidentally, this was the only tumor tested for which no regression had been induced by the treatment.

Similarly, in vivo expression of transgenic mIFN- γ following the injection of AdmIFN- γ into established P815 tumors was detected using RT-PCR (data not shown).

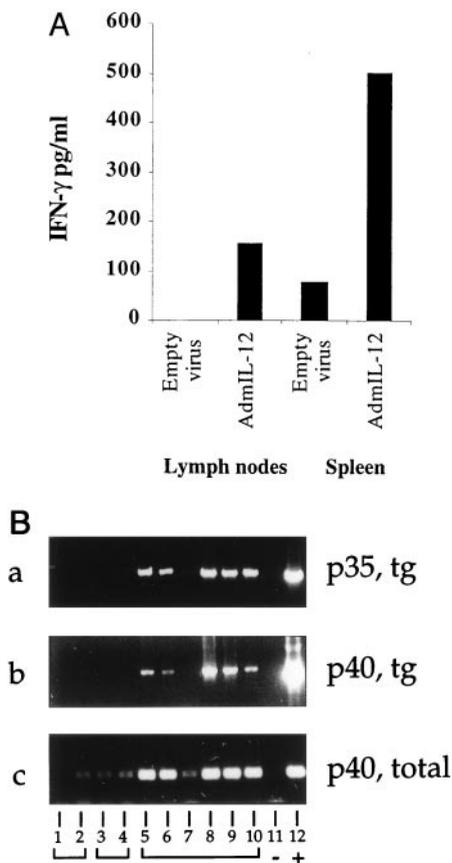


FIGURE 1. In vivo IL-12 expression following AdmIL-12 injection. **A**, Biological activity of the IL-12 secreted following AdmIL-12 administration. Three days following i.v. injection of empty virus or AdmIL-12 (1×10^9 pfu), cells were harvested from spleen and LN. Single-cell suspensions were incubated in vitro for 48 h at a density of 5×10^6 cells/ml, and IFN- γ production in the supernatant was quantified by ELISA. This experiment has been performed twice with similar results. **B**, AdmIL-12 intratumoral transfer induces expression of the transgenes. RT-PCR was performed from PBS-treated (lanes 1 and 2), empty virus-treated (lanes 3 and 4), or AdmIL-12-treated (lanes 5–10) tumors excised on day 20. Lane 11, Negative control (water); lane 12, positive control (1 ng of pCMVp35RSVp40IL-12 plasmid), with primers allowing detection of transgenic p35 (a), transgenic p40 (b), and total p40 (c). HPRT levels had previously been measured by quantitative PCR, and a volume of cDNA corresponding to identical amounts of HPRT has been used in each PCR.

Intratumor AdmIL-12 administration leads to day 9 established P815 tumor eradication

We next evaluated the effects on tumor growth of AdmIL-12 or AdmIFN- γ intratumoral injection into established P815 tumors. As shown in Fig. 2, whereas PBS has no effect on the tumor progression, AdmIL-12 cured most mice. AdmIFN- γ was not more efficient than the empty virus; both induced the regression of about half the tumors or delayed tumor growth. All tumor-free mice were immunized against a second lethal tumor challenge (not shown).

No cytolytic activity is detected in splenocytes

We first investigated the systemic tumor-specific CTL activity in treated mice using a standard chromium release assay. Spleen cells of mice from various treatment groups were removed on day 20 and restimulated for 5 days. As positive controls, we included vaccinated mice rechallenged with tumor cells at the time of tumor establishment in the other mice.

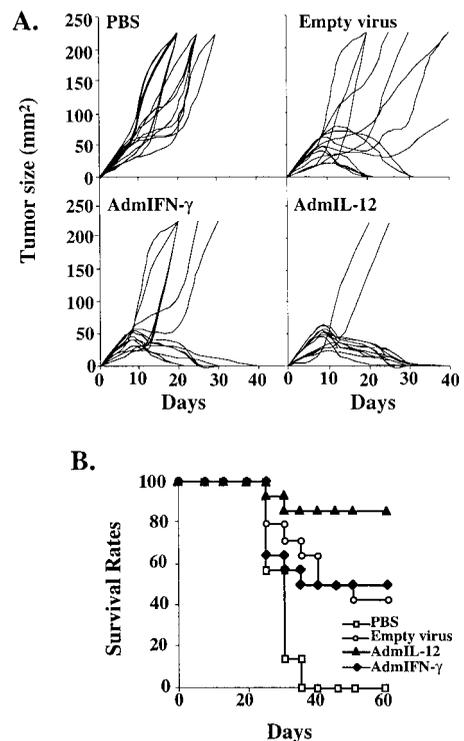


FIGURE 2. Effect of intratumoral administration of AdmIL-12 or AdmIFN- γ on the growth of established P815 tumors. P815 tumors were established by s.c. inoculation of 5×10^5 cells. Eight days later, when tumors reached a diameter of 6–7 mm, the experimental animals were randomly divided into four treatment groups and intratumorally injected on days 9 and 13 with PBS, empty virus, AdmIFN- γ , or AdmIL-12. The results of three pooled experiments are presented. **A**, Tumor size of individual mice plotted as a function of time after tumor cell inoculation. **B**, Percentage of surviving animals in each group plotted as a function of time. All treated groups (including empty virus) differ significantly ($p \leq 0.01$) from the PBS group. Only the AdmIL-12 group exhibits a significant ($p < 0.05$) increase in survival compared with the empty virus group.

Although almost all AdmIL-12-treated mice as well as half of the AdmIFN- γ - or empty virus-treated mice were in the process of rejecting their tumors, specific cytolytic activity against P815 in spleen cells was rarely detectable (Fig. 3).

NK activity was also assessed by chromium release assays performed on day 20 with fresh splenocytes, using YAC-1 cells as targets. We were unable to detect NK activity in either untreated or treated mice (data not shown).

AdmIL-12 treatment induces a dramatic increase in IFN- γ expression and in the frequency of public CD8⁺ T cells at the tumor site

We next decided to measure parameters of immune activity in the tumor, which is the most relevant location to study the effector cells. Among surrogate markers of tumor regression is the expression level of IFN- γ , since 1) it constitutes an indirect assessment of the activity of infiltrating CTL, NK, and/or Th1-type CD4⁺ cells; 2) its expression has been shown to be correlated with prognosis in human cancer (46); and 3) its expression is induced by IL-12 (47).

Quantitative PCR analysis of tumor specimens removed on day 20 revealed that regression of tumors upon AdmIL-12 treatment was associated with a dramatic, nearly 50-fold increase in the levels of endogenous IFN- γ mRNA (Fig. 4A). This group was the only one found to exhibit a significant increase over both control

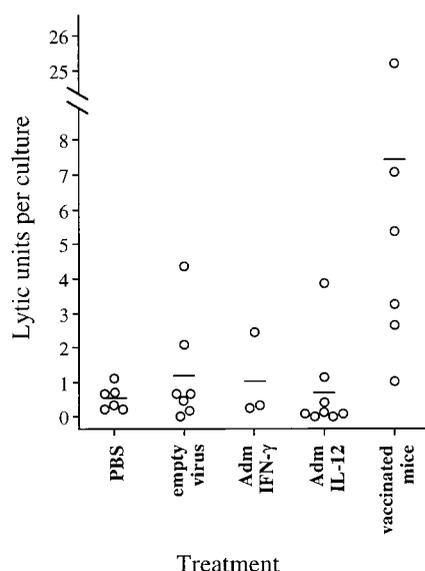


FIGURE 3. No cytolytic activity is detected in splenocytes of treated mice. The cytolytic activity of spleen cells from day 20 tumor-bearing mice treated with PBS, empty virus, AdmIFN- γ , or AdmIL-12 was assessed by ^{51}Cr release assay after a 5-day mixed lymphocyte-tumor culture. As a positive control, the spleens of mice vaccinated by s.c. inoculation of P815-B7.1 cells 15 days before challenge by P815 tumor cells were included. Lytic units were calculated as described in *Materials and Methods*. The results of two pooled experiments are presented. Statistical analysis shows that the vaccinated mice group significantly ($p < 0.02$) differs from any other group, which do not differ from each other.

groups. A weak but nonsignificant increase was observed in the empty virus-treated tumors. The AdmIFN- γ -treated tumors displayed a wide range of individual endogenous as well as total IFN- γ expression. The total amount of IFN- γ expressed in AdmIFN- γ -treated tumors was comparable to that detected in IL-12-treated samples.

We also measured the frequency of tumor-specific CD8 $^{+}$ T cells at the tumor site. This is possible in the P815 system by quantitative PCR, because we previously demonstrated that the CD8 $^{+}$ T cell response to P815 includes a public component, i.e., cells that possess TCR rearrangements similar in different individual mice (27). Thus, by measuring the amount of TCR transcripts harboring such rearrangements in the tumor using the Immunoscope technique, we could obtain an estimate of the frequency of P815-specific CD8 $^{+}$ T cells at the tumor site.

When this analysis was performed on AdmIL-12 treated tumors, a striking increase (~ 30 -fold compared with control samples) in the frequency of tumor-specific public T cells was found (Fig. 4B). Although empty virus or AdmIFN- γ induced regression or delay the growth of most tumors, no significant increase in the frequency of the specific T cells was observed. Thus, tumor regression induced by AdmIL-12 treatment is associated with a dramatic increase in the frequency of P815-specific CD8 $^{+}$ T cells in the tumor, which cannot be attributed to IFN- γ induction alone.

Finally, since NK T cells have been shown to play a major role in IL-12-induced tumor regression in some models (15), we assessed their presence in the P815 tumors. This could be performed with the Immunoscope method, since these cells bear a characteristic TCRAV14AJ15 (the AJ15 segment is better known as J α 281) TCR rearrangement (48). We did detect NK T cells, but AdmIL-12 treatment only led to a twofold increase in the frequency of these cells, which was not statistically significant (data not shown).

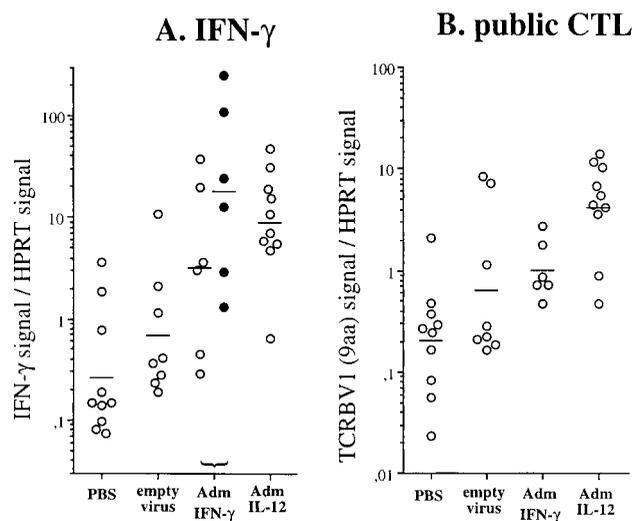


FIGURE 4. AdmIL-12 increases the levels of IFN- γ transcripts and specific CD8 $^{+}$ T cells at the tumor site. Quantitative PCR was performed on cDNA obtained from day 20 tumors treated as indicated in Fig. 3. *A*, Relative amount of endogenous (open circles) or total (closed circles) IFN- γ /HPRT transcripts. Statistical analysis of endogenous IFN- γ levels shows that only the AdmIL-12 group differs significantly from the PBS group ($p < 0.01$) or from the empty virus group ($p < 0.02$). *B*, Relative frequency of public P815-specific cells among total cells, deduced from the measurement of the public TCR cDNA content. The AdmIL-12 group differs significantly from the PBS group ($p < 0.01$), the empty virus group ($p < 0.05$), and the AdmIFN- γ group ($p < 0.02$). Results are presented as the ratio of the fluorescence intensity of IFN- γ or TCR on HPRT. Bars indicate geometric means. The results of three pooled experiments are presented.

A strong specific CD8 $^{+}$ T cell increase is also observed in the blood following AdmIL-12 therapy

Since we were able to detect a high level of specific T cells in the tumor following AdmIL-12 treatment, we decided to search for these cells in the periphery using the Immunoscope technique. Thus, we measured the frequency of public P815-specific T cells in the blood of individual mice treated with PBS, empty virus, or AdmIL-12 at various time points.

In preliminary experiments we established that during the course of untreated tumor progression, public T cells are first detectable in the blood around day 7, peak around days 10–12, then decrease in numbers (J.-P. Levrard, unpublished observations). There are, however, important interindividual variations. For these reasons, the frequencies of P815-specific T cells had to be assessed in all groups before therapy. As shown in Fig. 5A, the three groups did not significantly differ on day 8.

Following immunotherapy, the dynamics of public T cell populations were similar in all groups until day 13 (not shown). At later time points, however, significant differences were observed. As shown in Fig. 5B, the blood frequency of tumor-specific CD8 $^{+}$ T cells was 30-fold higher in AdmIL-12-treated compared with that in PBS-treated mice on day 20. Empty virus-treated mice displayed a low, but not significant, increase in circulating public T cell frequency. This frequency dropped significantly on day 40 (not shown). Thus, the increase in frequency of circulating tumor-specific CD8 $^{+}$ T cells, as assessed by Immunoscope technology, was found to reflect that observed in the tumor following AdmIL-12 treatment.

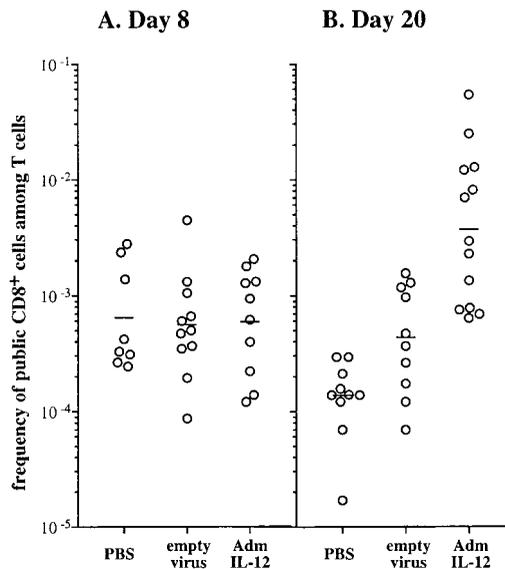


FIGURE 5. Levels of specific CD8⁺ T cells in blood before (day 8; *A*) and following (day 20; *B*) immunotherapy. Levels of public tumor-specific cells were measured among total T cells by quantitative Immunoscope analysis (as described in *Materials and Methods*). Bars indicate geometric means. The results of three pooled experiments are presented. No significant differences are observed on day 8. On day 20, the AdmIL-12 group differs from both the PBS and empty virus groups ($p < 0.05$), while these two groups do not differ significantly.

Comparative study of various sites for the frequency of public T cells

Depending on their activation status, T cells home differently to secondary lymphoid organs. As significant amounts of tumor-specific CD8⁺ T cells were found in the blood, we investigated whether these cells could also accumulate in peripheral sites. We analyzed by Immunoscope the frequency of public T cells in the spleen and draining and contralateral inguinal LN, and compared it with their frequency in the blood of the same mice on day 20 (Fig. 6). Unexpectedly, the levels estimated in the secondary lymphoid organs were much lower than those achieved in the blood. All mice displayed a roughly similar level of tumor-specific CTL in the ipsilateral draining LN. On the contrary, differences were observed between treatment groups in the spleen or contralateral LN, where AdmIL-12-treated mice displayed higher levels. These observations reveal that paracrine delivery of IL-12 into established tumors led to a strong expansion of tumor-specific CD8⁺ T cells that can be detected in the tumor, the blood, and the secondary lymphoid organs using the Immunoscope technique.

Mice with regressing tumors display higher specific cell frequency in the blood

If the population measured by Immunoscope participates in P815 rejection, one should expect to find higher levels of specific CD8⁺ T cells in mice with regressing tumors than in mice with growing tumors, within treatment groups. As can be observed in Fig. 7, mice whose tumor eventually regressed displayed, on the average, a higher level of circulating specific CD8⁺ T cells on day 20 than mice from the same group that had to be sacrificed because of a large tumor burden. This suggests that clonotypic cells detected with the Immunoscope technique mediate a therapeutic role *in vivo*.

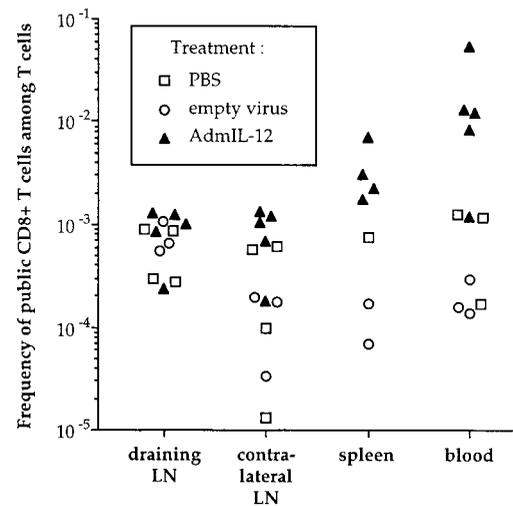


FIGURE 6. Frequency of public P815-specific CTL at various sites of treated tumor-bearing mice. Nine and thirteen days following tumor graft, mice were treated with PBS, empty virus, or AdmIL-12. On day 20, spleen, draining and contralateral inguinal LN and blood were analyzed for each mouse. Frequencies were estimated by quantitative PCR as described in Figs. 4 and 5.

Discussion

IL-12 is known to mediate potent antitumor immune responses in mice, and low doses of IL-12 have been found to allow the induction of protective specific T cell responses in tumor models. We report that local paracrine delivery of IL-12 in established P815 tumors leads to tumor regression (Fig. 2) and to a very significant increase in the frequency of tumor-specific CD8⁺ T cells bearing public T cell rearrangements in tumors (Fig. 4*B*), blood (Fig. 5), and secondary lymphoid organs (Fig. 6). Nevertheless, cytotoxic

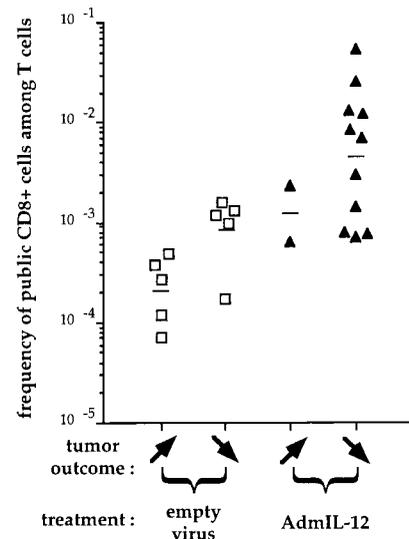


FIGURE 7. Comparison of levels of specific CD8⁺ T cells in blood between mice with growing or regressing tumors within groups. Nine and thirteen days following tumor graft, mice were treated with either empty virus or AdmIL-12. On day 20, a blood sample was taken for Immunoscope analysis. Further growth of the tumor was then monitored. Up-pointing arrows indicate mice that had to be sacrificed because of a large tumor burden; down-pointing arrows show mice that eventually completely rejected their tumors.

assays with *in vitro*-restimulated splenocytes failed to reveal the presence of anti tumor CTL during the course of regression (Fig. 3).

Following AdmIL-12 treatment, tumor regression is associated with a considerable increase in the level of IFN- γ transcripts at the tumor site (Fig. 4A). This is in agreement with results obtained by others with different tumor models (12, 13, 49). Although very high levels of IFN- γ expression were also reached using AdmIFN- γ (Fig. 4A), no improvement over the empty virus has been achieved with this vector (Fig. 2). This is in accordance with results of Brunda and colleagues showing that IFN- γ cannot substitute for IL-12 to induce tumor regression (19).

The very high frequency of tumor-specific CD8⁺ T cells at the tumor site achieved with IL-12, but not with IFN- γ , may explain the difference in therapeutic efficiency between both cytokines. Indeed, our data suggest that the ability of IL-12 to potentiate the proliferation of activated T cells, originally described *in vitro* (50), holds also true *in vivo* in IL-12-treated tumors.

Although a role for the public tumor-specific CD8⁺ T cells in the rejection of the tumors has not been formally established, it is supported by the fact that within treatment groups, their frequency is higher in mice that ultimately reject their tumor (Fig. 7). Unfortunately, since no V β 1-specific mAb is currently available, this question cannot be addressed directly by a depletion experiment *in vivo*. The mechanism by which these cells may exert their antitumor effects is a matter of conjecture. Although no cytolytic activity was revealed with splenocytes, these cells may still be cytolytic at the tumor site. Other potential mechanisms of action would be regulation of effector cells through cytokine secretion. The lack of efficiency of AdmIFN- γ over empty virus argues against a major role for IFN- γ . Whether other cytokines (TNF, for instance) or chemokines are involved remains speculative.

While specific CD8⁺ T cells, presumably cytolytic, may play a major role in the rejection of P815 tumors following AdmIL-12 treatment, it remains possible that other effector cells are involved. No systemic NK cytotoxicity was found in our system (data not shown). However, as a direct intratumoral injection of AdmIL-12 induces a highly localized expression of the transgene (12), this result does not rule out the possibility of an activation of NK cells *in situ*. It has also been shown recently that NK T cells play a major role in IL-12-induced tumor regression in some models (15). Although NK T cells were detected in tumors by Immunoscope, AdmIL-12 treatment only led to a minor increase in the frequency of these cells (data not shown).

Injection of a control, "empty" virus in the tumors, although not as efficient as AdmIL-12, had a clear impact on tumor growth (Fig. 2). Despite this fact, only modest or incidental, but nonsignificant, increases in specific T cells or IFN- γ were observed with the empty virus compared with the vehicle (Fig. 4). The mechanisms that lead to tumor rejection or growth delay following injection of empty virus are also of interest. E1-deleted adenoviral vectors, such as the one we used, are known to induce an inflammatory response at the site of gene transfer (51, 52). This intratumor inflammatory response may induce the activation of non-MHC-restricted effector cells (macrophages, NK cells), leading to the killing of tumor cells without the expansion of specific CTL. Such a mechanism, as previously hypothesized for the effect of IL-2 gene transfer (53), may explain the anti tumor effect of empty virus.

The lack of specific cytolytic activity in the spleen (Fig. 3) despite the high frequency of clonotypic CD8⁺ T cells is an intriguing result. This phenomenon is particularly interesting, as it may be relevant to the paradoxical observation of positive clinical outcomes of melanoma peptide vaccination without detectable increase in specific CTL precursor frequency (54).

We are currently investigating the mechanisms underlying this discrepancy. At any rate, this was not due to a technical failure; it was observed in three independent experiments, and the mice vaccinated before tumor challenge, included as positive controls, displayed strong cytotoxic activities. It seems unlikely that it is due to the low sensitivity of the cytolytic assay, as the frequencies of public P815-specific CD8⁺ cells measured using Immunoscope in the spleens of the positive control mice were lower than those found in the spleens of AdmIL-12-treated mice (data not shown).

It is noteworthy that we performed cytotoxic assays with spleen cells collected 20 days after tumor graft on tumor-bearing hosts, while positive control mice were tumor free at that time. Possibly, the presence of a tumor may influence the outcome of the assay. In accordance with this hypothesis, cytotoxic assays performed on day 40, after total tumor eradication, resulted in strong cytolytic activity in two of three AdmIL-12-treated mice, while the frequency of public CD8⁺ T cells assessed by Immunoscope dropped significantly between days 20 and 40 (data not shown). Similarly, Bramson et al. reported cytotoxic activity following AdmIL-12 treatment of polyoma middle T Ag-expressing tumors in completely cured mice 42 days after the initial tumor graft (22). In contrast, Toda et al. found strong cytolytic activities with spleen cells collected from mice bearing tumors (24); however, the administration of a replicative herpes simplex virus vector to induce tumor necrosis renders the comparison difficult.

Comparison of the numbers of specific T cells by direct physical techniques and functional limiting dilution assays has shown that the activation status of the T cells critically affects the issue of procedures requiring *in vitro* restimulation (55). The lack of cytolytic activity in regressing tumor-bearing hosts might be attributed to the fact that tumor-specific CD8⁺ T cells, having recently encountered their cognate Ag, are activated effector cells prone to apoptosis upon further stimulation. In contrast, in cured mice, tumor-specific cells would be memory cells ready to proliferate *in vitro* in the presence of their Ag.

The observation that P815-specific T cells are more abundant in the blood than in the draining LN (Fig. 6) was unexpected. It should be stressed that our comparative study concerned rather late time points, i.e., 20 days after grafting of tumor cells. Initial expansion of tumor-specific precursors is supposed to take place in the draining LN. Activated T cells, once they lose expression of homing receptors such as CD62L, might leave the node to seed the tumor and the periphery (56). Since the frequency of specific CD8⁺ T cells in the draining LN is not modulated by immunotherapy, in contrast to what is observed in the tumor, it is likely that the major specific T cell expansion occurring under AdmIL-12 treatment takes place at the tumor site itself. The observed differences in public T cell frequencies in the blood, contralateral LN, and spleen are consistent with the re-entry of cells expanded at the tumor site into the bloodstream. More detailed analysis of the kinetics of specific T cell populations in the various organs should clarify this point.

The development of reliable methods to evaluate the biological effects of cancer immunotherapeutic approaches is required for their optimization. The questions of where and how to evaluate the antitumor immune response elicited by immunotherapy are still a matter of debate. The fact that among peripheral organs, the blood contains the highest levels of specific CD8⁺ T cells following AdmIL-12 treatment is an interesting observation, since PBL constitute the most readily accessible source of T cells in humans. In mice, by contrast, it is far easier to obtain large number of T cells from the spleen or LN. However, the higher frequency of precursors among PBL probably makes it worth testing this location more systematically. Furthermore, our data stress the necessity of

direct methods to evaluate the frequency of tumor-specific CTL, rather than systems based on in vitro culture. Techniques must be sensitive and resolutive, and the Immunoscope is clearly well adapted to such applications, as shown in various studies dealing with human cancer (57–61). However, this approach has some intrinsic limitations due to the fact that the TCR rearrangements of the relevant T cells first need to be characterized. The usage of tetrameric MHC-peptide TCR ligands, shown to be able to bind specific T cells for FACS analysis (62), will probably alleviate this problem. Furthermore, it will allow functional analysis of tumor-specific cells. Flow cytometry is limited in sensitivity, but, ultimately, combination of MHC-peptide-tetramers with Immunoscope analysis (63) should provide the advantages of both techniques. It would now be of major interest to test with such technology whether local IL-12 delivery in human tumors is able to induce the expansion of tumor-specific CD8⁺ T cells among the PBL of patients.

To our knowledge, this is the first report of a direct observation of an increase in tumor-specific CD8⁺ T cells frequency following IL-12-mediated therapy of cancer. This study formally demonstrates that IL-12 is able to induce the expansion and possibly the reactivation of specific T cells in hosts bearing progressively growing tumors. Adenovirus-mediated intratumoral delivery of IL-12 is able to induce not only a potent local immune response, but also the recirculation of large numbers of tumor-specific CD8⁺ T cells. Thus, local delivery of IL-12 into tumors should be implemented to induce systemic immune responses and exert effects on distant metastases.

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