

RESEARCH ARTICLE

Biodegradable polymer-based interleukin-12 gene delivery: role of induced cytokines, tumor infiltrating cells and nitric oxide in anti-tumor activity

A Maheshwari, S Han, RI Mahato and SW Kim

Center for Controlled Chemical Delivery, University of Utah, Salt Lake City, UT, USA

The objective of this study was to investigate the role of induced cytokines, tumor infiltrating cells and nitric oxide (NO) in anti-tumor activity upon intratumoral injection of free and condensed plasmid DNA encoding murine interleukin-12 (pmIL-12) into BALB/c mice bearing subcutaneous tumors. Poly[α -(4-aminobutyl)-L-glycolic acid] (PAGA) was used for complex formation with pmIL-12 in presence of 5% (w/v) glucose. Upon characterization, PAGA/pmIL-12 (3/1, \pm) complexes were found to be most effective in gene transfer and were used consistently throughout this study. The levels of mIL-12 p70 and induced cytokines were determined by ELISA in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor bearing BALB/c female mice 48 h after intratumoral injection of PAGA/pmIL-12 complexes

and naked pmIL-12. The levels of IL-12, IFN- γ , TNF- α and NO were higher for the PAGA/pmIL-12 complexes than those for the naked pmIL-12, PAGA alone and 5% glucose injected groups. The relative presence of natural killer (NK) cells, CD4⁺ T cells, and antigen presenting cells, such as macrophages and dendritic cells determined using immunohistochemistry was higher for PAGA/pmIL-12 complexes compared with naked pmIL-12. The presence of CMV promoter in plasmid encoding IL-12 cDNAs did not induce any type I interferon response. There was a significant improvement in the survival rate and the inhibition of tumor growth after repeated injections of PAGA/pmIL-12 complexes. Gene Therapy (2002) 9, 1075–1084. doi:10.1038/sj.gt.3301766

Keywords: biodegradable cationic polymer; interleukin-12; gene expression; tumor regression; tumor-infiltrating cells

Introduction

The primary goal of cytokine gene therapy is to lessen the toxic side-effects associated with the systemic administration of recombinant cytokines, while achieving the same or higher level of therapeutic efficacy in treating various forms of cancer and other diseases. Among the vast family of cytokines comprising interleukins, interferons and tumor necrosis factors, IL-12 has gained prominence due to its multiple roles in inducing potent anti-tumor activity, to eradicate tumors and prevent the development of metastasis.^{1–3} IL-12 induces the production of IFN- γ and TNF- α , which are responsible for antitumor effects of natural killer (NK) cells, CD4⁺ T cells and CD8⁺ T lymphocytes (CTLs). Depending upon the level of stimulation, IFN- γ and TNF- α enhance Th1 type expression, improve antigen presentation on macrophages and dendritic cells, and display potent anti-angiogenic activities. The interdependence of these various cytokines like IL-12, IFN- γ , TNF- α , however, makes it difficult to interpret their roles individually without considering the intricate role of the vast cytokine network.^{4–6} Furthermore, with the latest advances in gene delivery and the additional role of such themes as nitric oxide and

CpG motifs, it is increasingly felt that research must consider the whole array of intertwined factors while analyzing data and making conclusions about their therapeutic effects.

Non-condensing polymeric gene carriers, such as polyvinyl pyrrolidone (PVP), which is water soluble but not biodegradable, was used for intratumoral delivery of pmIL-12 to subcutaneously grown tumors of murine renal carcinoma (RENCA) and CT-26 colon carcinoma.⁷ Although these systems produced mIL-12 and mIFN- γ , the levels of transgene expression and tumor regression were low, possibly due to little protection of plasmid DNA by PVP. Moreover, no comparison was made between naked pmIL-12 in saline and PVP/pmIL-12-based formulations for transgene expression and tumor regression. Recently the role of type I interferons, particularly mIFN- α in IL-12-mediated immune activation has gained attention. For instance, in a subsequent study by the same group, PVP/pCMVhIL-12 complexes were used in combination with PVP/pCMVhIFN- α for investigating a possible synergistic increase in anti-tumor response against RENCA and CT-26 colon carcinoma. However, no comparison was made between combination of naked pHIL-12 and pHIFN- α in saline and PVP/(pHIL-12 + pHIFN- α)-based formulations for gene expression and tumor regression.⁸ Therefore, the therapeutic superiority of PVP/pDNA complexes against naked pDNA could not be justified.

Biodegradable polymers that can protect the pDNA

Correspondence: SW Kim, University of Utah, Center for Controlled Chemical Delivery, 30 S 2000 E RM 201 (SK H), Salt Lake City, UT 84112-5820, USA

Received 21 February 2001; accepted 20 December 2001

from nuclease attack and enhance cytokine gene expression after intratumoral injection into solid tumors will be an exciting option for cancer treatment.⁹ We previously studied the use of poly[α -(4 aminobutyl)-L-glycolic acid] (PAGA), a water soluble, non-toxic, biodegradable cationic polymer for delivery of plasmids encoding firefly luciferase (pLuc) or murine IL-12 (pmIL-12) into cultured CT-26 colon carcinoma cells and into CT-26 subcutaneous tumor-bearing BALB/c mice at mRNA (through RT-PCR) and protein (luciferase assay or ELISA) levels.¹⁰ PAGA/pLuc and PAGA/pmIL-12 complexes were consistently found to be better in terms of gene expression in both *in vitro* and *in vivo* situations. In the present study, we investigated the role of induced cytokines, tumor-infiltrating cells and nitric oxide in anti-tumor activity after intratumoral injection of PAGA/pmIL-12 complexes into BALB/c mice bearing subcutaneous CT-26 colon carcinoma tumor.

Results

Gene expression for induced cytokines

We studied the gene expression levels of mIL-12 and several induced cytokines after intratumoral injections of PAGA/pmIL-12 (3/1, \pm) complexes and pmIL-12 plasmids into CT-26 subcutaneous tumor bearing BALB/c mice. One positive charge per mole of PAGA monomer, L-xylylsine, was used to calculate the total '+' charges, whereas one negative charge per nucleotide of pmIL-12 was used to calculate the total '-' charges. The mice injected with naked pmIL-12, PAGA alone and 5% (w/v) glucose were used to compare the efficacy of the polyplexes. At 48 h after injection, we measured the tumor size and calculated the tumor volume. After excising the tumors, the entire tumors were chopped into small pieces and re-cultured into six-well plates with 1 ml RPMI reconstituted with 10% FBS (v/v) and incubated at 37°C for 24 h. Cytokine measurement was done by ELISA and the amount of cytokines produced were normalized across the tumor volumes for each mouse, respectively and finally plotted as cytokine produced/ml of supernatant from recultured tumors. As shown in Figure 1, IL-12 expression levels for PAGA/pmIL-12 (459 pg/ml) were higher than those for naked pmIL-12 (265 pg/ml), PAGA alone and 5% glucose injected groups. In order to find out the effect of unmethylated CpG motifs present in pmIL-12 on the induction of cytokines, we also administered pCAGGS plasmid without the IL-12 p35 and IL-12 p40 cDNAs to subcutaneous tumor-bearing mice. We found that the levels of naked pCAGGS (104 pg/ml) and PAGA/pCAGGS (158 pg/ml) formulations were approximately 30–35% compared with that of naked pmIL-12 and PAGA/pmIL-12, respectively.

IL-12 primarily exerts its anti-tumor effect via indirect interaction with tumor cells by stimulating potent cytokines such as IFN- γ and TNF- α .¹¹ We, therefore, determined the induced production of IFN- γ and TNF- α at 48 h after injection of PAGA/pmIL-12 complexes into CT-26 subcutaneous tumor-bearing BALB/c mice to see whether IL-12 was able to up-regulate these two cytokines. Similar trends were found in both cases (Figures 2 and 3). In case of IFN- γ , the levels were fairly high for PAGA/pmIL-12 complexes compared with naked pmIL-12 (Figure 2). The empty plasmid pCAGGS used as a con-

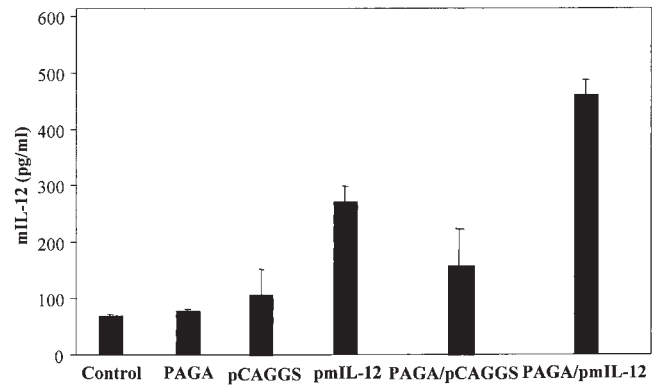


Figure 1 mIL-12 levels in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor-bearing BALB/c mice 48 h after intratumoral injection of PAGA/pmIL-12 (3/1, +/–) complexes and naked pmIL-12. Tumors were isolated, chopped into small pieces and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. Supernatants were assessed by ELISA for mIL-12. PAGA/pCAGGS and naked pCAGGS injected mice were used as negative controls. Five percent (w/v) glucose and PAGA alone were also tested for their mIL-12 inducing capability. mIL-12 protein levels for PAGA/pmIL-12 complexes were significantly ($*P < 0.05$) higher than naked pmIL-12, PAGA/pCAGGS and pCAGGS alone ($n = 4$ mice per group).

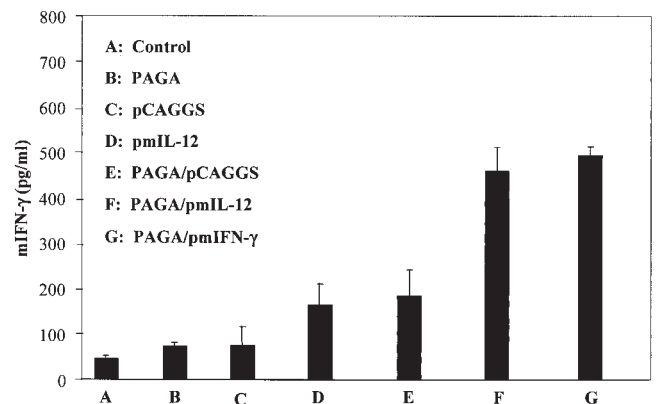


Figure 2 mIFN- γ levels in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor-bearing BALB/c mice 48 h after intratumoral injection of PAGA/pmIL-12 (3/1, +/–) complexes and naked pmIL-12. Tumors were isolated, chopped into small pieces and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. Supernatants were analyzed by ELISA for assessing the levels of mIFN- γ . PAGA/pCAGGS, pCAGGS alone as well as PAGA/pmIFN- γ complexes were tested for their mIFN- γ producing capability. In addition, 5% (w/v) glucose and PAGA alone were also used as additional controls. Induced mIFN- γ levels for PAGA/pmIL-12 complexes are significantly higher ($*P < 0.05$) than naked pmIL-12. Levels of mIFN- γ produced after intratumoral injection of PAGA/pmIL-12 and PAGA/pmIFN- γ complexes were statistically insignificant from each other ($n = 4$ mice per group).

trol did not stimulate any cytokine production and the mIFN- γ levels were similar to those of 5% (w/v) glucose-injected mice (48 pg/ml versus 80 pg/ml, respectively), which might be due to little protection of pCAGGS against nucleases. However, PAGA/pCAGGS complexes did seem to stimulate cytokine production probably due to CpG motifs in the plasmid, leading to increased mIFN- γ levels (176 pg/ml), which were approximately 35% compared with PAGA/pmIL-12-injected mice. We also found that PAGA/pmIFN- γ complexes did not result in any improvement over PAGA/pmIL-12 complexes in

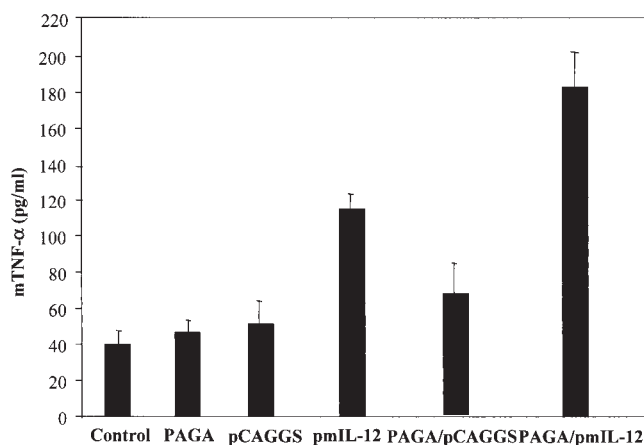


Figure 3 mTNF- α levels in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor-bearing BALB/c mice 48 h after intratumoral injection of PAGA/pmIL-12 (3/1, +/-) complexes and naked pmIL-12. Tumors were isolated, chopped into small pieces and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. Supernatants were analyzed by ELISA for assessing the levels of mTNF- α . PAGA/pCAGGS, pCAGGS alone as well as 5% (w/v) glucose and PAGA alone were also used as additional controls to test for their mTNF- α -producing capability. Induced mTNF- α levels for PAGA/pmIL-12 formulations were significantly higher (* $P < 0.05$) than naked pmIL-12 ($n = 4$ mice per group).

terms of mIFN- γ gene expression. The levels of gene expression were very similar in both cases (471 pg/ml versus 502 pg/ml). IL-12-induced TNF- α production levels were high for PAGA/pmIL-12 compared with naked pmIL-12 (183 pg/ml versus 115 pg/ml) (Figure 3). The empty plasmid pCAGGS did not invoke any TNF- α production, but there was a minor increase in levels of TNF- α upon administration of PAGA/pCAGGS formulations (66 pg/ml), much lower (approximately 30%) compared with the TNF- α levels due to the PAGA/pmIL-12 group. Upon injection of PAGA alone, which is susceptible to rapid hydrolysis, there was no induction of these cytokines, which confirms that the production was solely due to pmIL-12 and not due to any charge or antigenicity associated with PAGA.

The antiproliferative effects of type I interferons such as IFN- α and IFN- β when administered individually are well known.^{12,13} However, their antiproliferative effects in conjunction with IL-12 and other prominent Th1 type cytokines are still controversial. To confirm whether there is any change in the levels of IFN- α after intratumoral injections of PAGA/pCMVmIL-12, PAGA/pmIL-12 complexes and pmIL-12 into the subcutaneous CT-26 tumor-bearing mice, we determined the levels of mIFN- α by ELISA. We found that mIFN- α was minimally stimulated due to IL-12 injections (Figure 4). A careful examination reveals that the levels of IFN- α were similar for 5% glucose, naked pmIL-12, PAGA/pmIL-12 and PAGA/pCMVmIL-12 injected mice. There was no antiviral induction of mIFN- α against CMV promoter in the plasmid constructs, as we did not see any difference in IFN- α levels for any of these groups.

Nitric oxide (NO) is an important cytotoxic molecule against tumor growth and metastasis.¹⁴ Stimulus provided by the induced production of mIFN- γ can lead to the production of NO from macrophages, as well as from NK cells and CD4⁺ T cells. Therefore, NO levels were measured in terms of nitrite (NO₂⁻) using the Griess

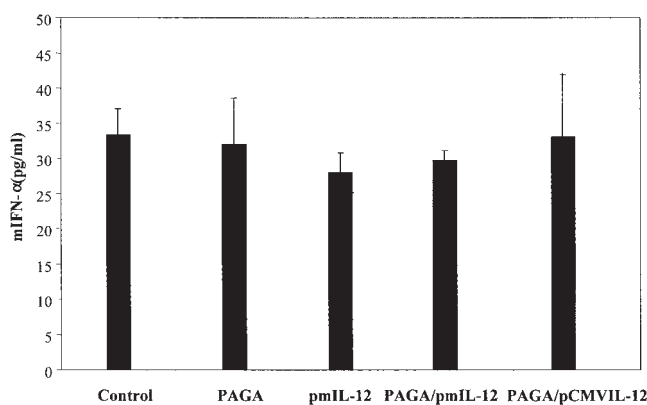


Figure 4 mIFN- α levels in CT-26 subcutaneous tumor-bearing BALB/c mice. PAGA/pmIL-12 (3/1, +/-) complexes, as well as naked pmIL-12 were used for administration. Tumors were isolated, chopped into small pieces and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. Supernatants were analyzed by ELISA for assessing the levels of mIFN- α . Five percent (w/v) glucose-injected mice were used as negative controls. PAGA alone was also tested for its induced mIFN- α producing capability. Induced mIFN- α levels for all the groups were statistically insignificant ($n = 4$ mice per group).

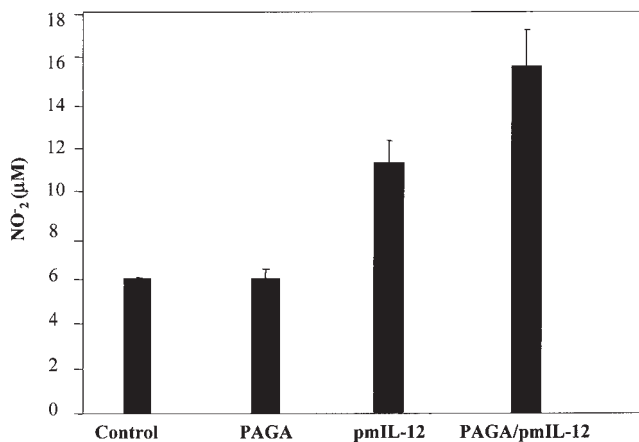


Figure 5 Nitric oxide (NO) levels in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor-bearing BALB/c mice 48 h after intratumoral injection of PAGA/pmIL-12 (3/1, +/-) complexes and naked pmIL-12. Tumors were isolated, chopped into small pieces and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37°C. Supernatants were analyzed by Griess reagent system for assessing the levels of NO in terms of NO₂⁻. Five percent (w/v) glucose-injected mice were used as negative controls. PAGA alone was also tested for its induced NO-producing capability. NO₂⁻ levels for condensed plasmids are higher (* $P < 0.05$) than non-condensed pmIL-12. NO₂⁻ levels produced by the control and PAGA groups were statistically insignificant ($n = 4$ mice per group).

reagent system. At 48 h after injection of PAGA/pmIL-12 complexes into the CT-26 tumor bearing mice, tumors were chopped into small pieces, recultured for 24 h and cultured supernatants were analyzed for NO₂⁻. The nitrite levels were higher in PAGA/pmIL-12 complexes compared with that of naked pmIL-12 injected tumor samples (15.6 μ M versus 11.2 μ M) (Figure 5). In comparison, lower levels of nitrite (6 μ M) were found in 5% glucose and free PAGA injected tumor samples.

Tumor-infiltrating cells

After confirming that there was an active network among various cytokines such as IL-12, IFN- γ , TNF- α along with

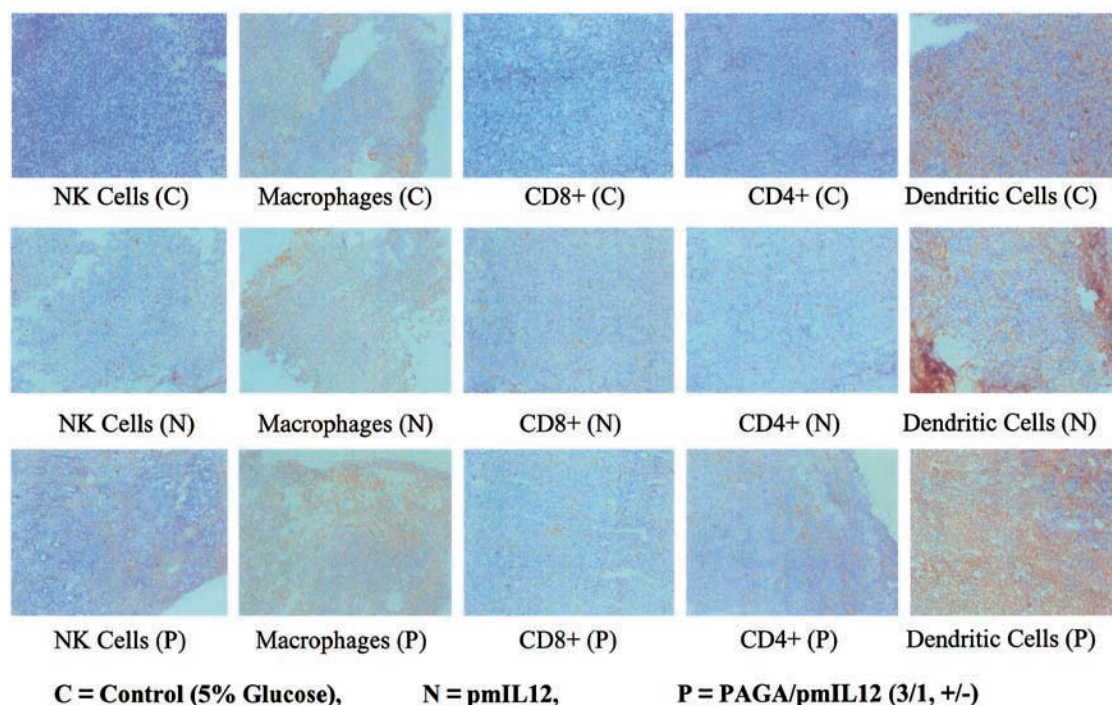


Figure 6 Immunohistochemical staining of infiltrating immune cells present in tumors 24 h after intratumoral injection of PAGA/pmIL-12 (3/1, +/-) complexes into CT-26 subcutaneous tumor-bearing BALB/c mice. Immunohistochemistry was performed on frozen tissue sections with primary antibodies against murine NK cells, CD4⁺ T cells, CD8⁺ T cells, macrophages and dendritic cells. Five percent (w/v) glucose and naked pmIL-12-injected tumors were used as negative and positive controls, respectively. Increase in NK cells, CD4⁺ T cells, macrophages and dendritic cells distribution can be seen at PAGA/pmIL-12 and naked pmIL-12-treated animals, but not in negative controls. There was no significant increase in the CD8⁺ population in either the PAGA/pmIL-12 or the naked pmIL-12 treated group ($n = 4$ mice per group).

the production of cytotoxic molecules such as NO, we wanted to find out the presence of various tumor-infiltrating cells at the tumor site. Therefore, we determined the relative presence of NK cells, CD4⁺ T cells, CD8⁺ T cells and antigen-presenting cells, such as macrophages and dendritic cells in tumor tissue (Figure 6). There was a strong presence of NK cells for PAGA/pmIL-12 complex injected tumors compared with those injected with pmIL-12 and 5% glucose. There was negligible staining for the 5% glucose-injected controls, while for pmIL-12 controls there was substantial staining. PAGA/pmIL-12 complexes were able to produce greater infiltration of NK cells than all other groups. This trend was less pronounced in case of CD4⁺ T cell infiltration at the tumor site. While the presence of CD4⁺ cells was minimal for the 5% glucose-injected controls, it was substantial for the mice treated with pmIL-12 and highest for PAGA/pmIL-12 group. In the case of CD8⁺ T cells, the difference was not much between the PAGA/pmIL-12 and naked pmIL-12 groups. The antigen-presenting cells, such as macrophages and dendritic cells showed rather different trends. In the case of macrophages, there was not much difference between the controls and the PAGA/pmIL-12 complex-treated group. This shows that mere injection of 5% glucose could also stimulate macrophage activity as a second line of defense against any foreign antigen. This does not mean that there was no relevance of PAGA/pmIL-12 complexes for stimulating macrophages, as we saw a modest improvement for these groups against the negative controls (Figure 6). Dendritic cells are known to produce IL-12 in significant amounts and are heavily recruited in the early stages of anti-tumor

immunity for priming of Th1 type cells.¹⁵ We indeed saw heavy staining for dendritic cells (well dispersed brown spots) in case of the PAGA/pmIL-12-treated samples, which was much higher than the pmIL-12-treated samples (Figure 6).

Anti-tumor response

In the present study, we investigated the effect of single and repeated injections of PAGA/pmIL-12 complexes on tumor regression and metastasis. There was a substantial improvement in terms of survival rate, as well as the inhibition of tumor growth after injections of PAGA/pmIL-12 complexes every 3 days (Figures 7 and 8). The mice

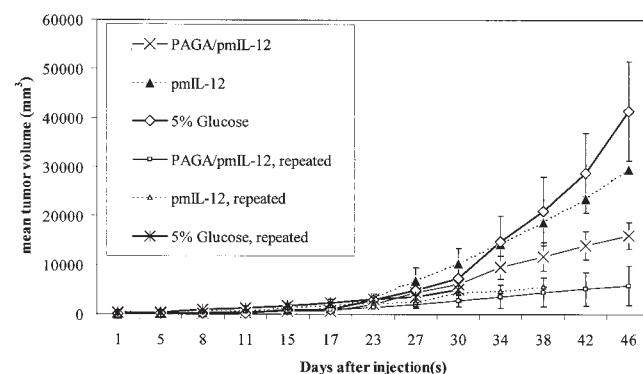


Figure 7 Profile of tumor progression after single and repeated injection of PAGA/pmIL-12 (3/1, +/-) complexes, naked pmIL-12, and 5% glucose (w/v) in CT-26 subcutaneous tumor-bearing BALB/c mice. Mean tumor volumes \pm s.d. were recorded every 3 days until the animals died naturally or were killed due to humane reasons ($n = 4$ mice per group).

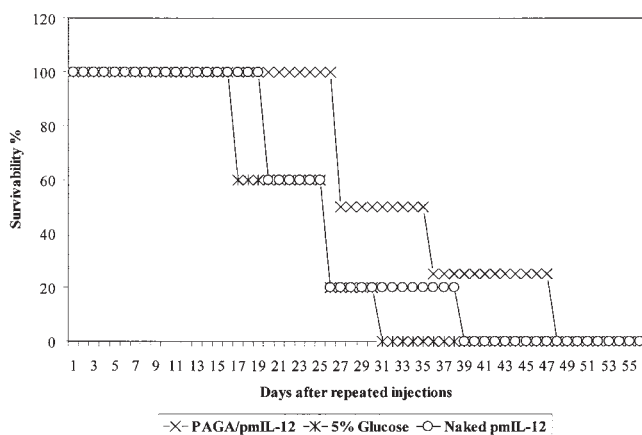


Figure 8 Percentage of surviving animals in each group plotted as a function of time. PAGA/pmIL-12 and naked pmIL-12 survival rates differ significantly from the 5% glucose (w/v)-injected control group. PAGA/pmIL-12 complex group exhibited an increase in survival compared with the naked pmIL-12 group ($n = 4$ mice per group).

treated with PAGA/pmIL-12 complexes had greater physical activity and better response to change in environment compared with the control mice treated with naked pmIL-12 and 5% glucose. The control mice had ruffled skin, showed lethargy, slow movement and lost weight, which might be due to the rapidly growing tumors and low levels of induced mIFN- γ and mTNF- α inside the tumor. It is likely, that, due to continuous replenishment of IL-12 genes, the levels of mIL-12 and induced cytokines, especially mIFN- γ were maintained closer to the therapeutic range required for higher anti-tumor activity.

Five percent glucose-injected mice died earlier compared with the mice injected with PAGA/pmIL-12 complexes. The deaths in case of pmIL-12 injected mice were also quicker compared with PAGA/pmIL-12 complexes (Figure 8). We also saw less prominence of necrosis in the control mice compared with PAGA/pmIL-12. However, the spread of necrosis was almost at the same level for both PAGA/pmIL-12 complexes and pmIL-12 alone. This result correlates well with the induced TNF- α gene expression (Figure 3), as a result of IL-12 gene delivery, where the levels are not very different for naked IL-12 and PAGA/pmIL-12 complexes.

Discussion

The objective of cytokine gene therapy is to deliver therapeutic genes encoding various cytokines selectively into the tumor site, affecting only the local tumor environment for the induction of anti-tumor immune response and subsequent tumor eradication. This would circumvent several problems such as systemic toxicity, rapid degradation and the short half-life associated with systemic delivery of various recombinant cytokines.¹⁶ IL-12 is an immune regulatory cytokine, which plays multiple roles in altering the host-tumor relationship, such as: (1) enhancement of the cellular immune mechanism to favor activation and proliferation of CD4⁺ helper T cells and CD8⁺ cytotoxic T cells leading to potent Th1 type immune response; (2) recruitment of cytotoxic NK and NKT cells to directly participate in early stages of tumor killing; and (3) up-regulation of endogenous IFN- γ and TNF- α , as

well as NO to suppress tumor progression, metastasis and angiogenesis.¹⁷

In the present study, we investigated the role of IL-12 gene delivery with a broader perspective, looking into its role in simultaneous induction of various anti-tumoral cytokines, cytotoxic NO, as well as its role in recruitment of various immune cells, which form a part of innate and adaptive immunity, resulting in anti-tumor activity. Our experimental results demonstrate significant improvement in endogenous expression of IFN- γ , TNF- α , NO as well as infiltration of NK cells, macrophages, dendritic cells, CD4⁺ T cells and CD8⁺ T cells, as a result of intratumoral injection of PAGA/pmIL-12 complexes compared with naked pmIL-12 into BALB/c mice bearing CT-26 subcutaneous tumor (Figures 1–6). We earlier showed that PAGA successfully protected pDNA from degradation by nucleases, enhanced IL-12 gene expression, reduced tumor progression, but did not elicit any antigenic or inflammatory response on its own, by virtue of its rapid biodegradability.¹⁰ The final degradation product of PAGA is its monomer, L-oxylysine, which would be rapidly removed from the cellular compartments followed by metabolism and excretion from the body.¹⁸

The central theme of the present investigation was to see the immunological outcome after intratumoral injection of polymer/pmIL-12 complexes into a weakly immunogenic mouse tumor model. Earlier reports have shown that IL-12 acts independently to promote a Th1 type response against tumor growth and metastasis, as well as by inducing other anti-tumor cytokines such as IFN- γ , TNF- α and granulocyte-macrophage colony-stimulating factor (GM-CSF) which can act synergistically to reduce and in some cases completely eradicate the tumor.^{3,7,19–21} There is evidence that IL-12 inhibits angiogenesis and induces apoptosis, as well as extensive necrosis in murine tumor.²² Our studies show that use of PAGA/pmIL-12 complexes significantly enhances the levels of IL-12 p70 production compared with naked pmIL-12 and 5% (w/v) glucose-injected groups (Figure 1), which would reduce the potential of tumor growth and metastasis. To rule out the influence of bacterial DNA on IL-12 production and tumor regression, empty pCAGGS vector without IL-12 p35 and p40 cDNA sequences was used as a control to compare with pmIL-12, either alone or complexed with PAGA. We found that the levels of mIL-12 due to naked pCAGGS and PAGA/pCAGGS formulations were minor (approximately 30–35%), compared with that of naked pmIL-12 and PAGA/pmIL-12, respectively. While empty pCAGGS plasmid administered in tumor-bearing mice was not able to stimulate substantial cytokine secretion, PAGA/pCAGGS formulations did stimulate mIFN- γ and mTNF- α levels, which was not more than one third, compared with that generated due to PAGA/pmIL-12 formulations. Administration of free cationic lipid or bacterial DNA containing unmethylated CpG motifs has been shown not to elicit an immune response. However, unmethylated CpG motifs present in bacterial DNA have been shown to induce the production of Th1 cytokines, such as IFN- γ and IL-12 after delivery of empty plasmids formulated with cationic liposomes, probably due to the greater protection of DNA in cationic liposome/pDNA complexes.^{23–25} However, this secondary cytokine response was not observed after administration of formulated methylated bacterial DNA or formulated eukaryotic

DNA.²⁶ All these groups have consistently shown that plasmid DNA with therapeutic cDNAs, containing unmethylated CpG motifs invoke substantial amounts of proinflammatory cytokines. However, the production in all cases was less than 40–50% of plasmid with therapeutic cDNAs such as IL-12, IL-2, etc. Our results for IL-12, as well as secondary cytokines such as mIFN- γ and mTNF- α , show a similar trend.

There is strong induction of IFN- γ and weaker induction of TNF- α as a result of rIL-12 administration, which acts synergistically along with IL-12 to generate potent anti-tumor response.²⁰ Low systemic levels of IL-12 have been shown to induce IFN- γ production and, through feedback control mechanisms, IL-12 would activate its own up-regulation through macrophages, dendritic cells and other stimulatory factors, such as NO induced by IFN- γ as a part of adaptive immune response.^{16,19} We, therefore, determined the induced levels of IFN- γ and TNF- α after intratumoral injection of PAGA/pmIL-12 complexes. The production of both induced cytokines and mL-12 was significantly up-regulated compared with control mice injected with 5% glucose (Figures 1–3). The production of IL-12 would cause the virgin T cells to respond by rapidly differentiating to form Th1 type cells, such as CD4⁺ helper T cells and CD8⁺ cytotoxic T cells in addition to NK cells, thereby tilting the balance from Th2 towards the Th1 side.¹⁷ This is highly desirable as it would lead to significant amounts of IFN- γ and TNF- α production along with the activation of macrophages and dendritic cells to produce further IL-12.

Paracrine stimulation with IFN- γ can lead to endogenous synthesis of TNF- α and inducible nitric oxide synthase (iNOS) via macrophages, as well as type I interferons.²⁷ However, it has not been found to be as effective as IL-12 both in terms of its own production *in vivo*, as well as in terms of anti-tumor and anti-metastatic activity.²⁸ In the earlier paper, we reported that *in vitro* transfection of PAGA/pmIFN- γ into cultured CT-26 tumor cells generates similar expression levels of mIFN- γ in comparison with mL-12 induced mIFN- γ .²⁹ In the present study, we found similar levels of mIFN- γ when we administered PAGA/pmIL-12 and PAGA/pmIFN- γ complexes into tumor-bearing BALB/c mice (Figure 2). Since anti-tumor activity of IL-12 depends strongly on IFN- γ induction, it is imperative that use of pDNA encoding IFN- γ should result in similar trends in terms of anti-tumor activity as found via IL-12 gene delivery. However, Rakhmievich *et al*² demonstrated that IL-12 is superior to IFN- γ in terms of tumor regression even at a lower pDNA dose. This could be due to the fact that IL-12 has greater potential to induce a host of cytokines compared with IFN- γ , thereby resulting in a stronger Th1-type immune development.

We studied the effect of IL-12 gene delivery for induction of IFN- α for two reasons. First, type I cytokines, such as IFN- α and IFN- β , have a proven record of anti-viral and anti-tumor activity. For example, administration of retroviruses and herpesviruses has been found to invoke type I interferons, such as IFN- α .³⁰ However, when we used IL-12 plasmid encoding CMV promoter, and compared it with IL-12 plasmid encoding chicken β -actin promoter, we found no difference among them, as well as other control mice, in terms of IFN- α expression levels. This indicates that the presence of CMV promoter as a

viral component in the plasmid construct did not stimulate production of mIFN- α (Figure 4).

Second, the role of induced IFN- α in conjunction with exogenous IL-12 has only begun to be revealed in terms of immune mechanism, compared with other induced cytokines, due to IL-12 delivery such as GM-CSF, IL-1 β and IL-6.^{8,21,31} IFN- α induces the expression of certain tumor-associated antigens, as well as major histocompatibility complex class I proteins, and this effect may account for enhanced anti-tumor response.³¹ IFN- α also shares many biological properties with IL-12, such as activation of signal transducers and transcription protein-4 (STAT4) and enhancement of Th1 cell development. We found little induction of IFN- α due to PAGA/pmIL-12 complexes. PAGA alone also did not induce any mIFN- α response which might be due to its biodegradable characteristics. This is in agreement with Mendiratta *et al*,⁸ who reported that although the combination therapy of pHIL-12 and pHIFN- α significantly regresses tumor growth, intratumoral injection of pHIL-12/PVP did not induce the production of hIFN- α .

Nitric oxide is an important regulator of immune response and is a potent cytotoxic molecule. IL-12 induces IFN- γ , which activates macrophages, NK cells and T cells for NO production and regulation.¹⁹ It has also been shown that the interdependence of IL-12 and iNOS is crucial for recruiting the immune machinery of the host against a significant anti-tumor response. NO levels were significantly up-regulated within a period of 48 h, when the animals were injected with PAGA/pmIL-12 complexes compared with the control animals (Figure 5).

IL-12 stimulates the infiltration of a variety of effector cells, such as NK and NKT cells, antigen presenting cells (APCs), such as macrophages and dendritic cells, and T cells, such as CD4⁺ helper cells and CD8⁺ T cells to the tumor sites.^{15,32–34} To determine the immunological mechanisms involved in mL-12-mediated anti-tumor activity, we performed immunohistochemical analysis of the tumors from the female BALB/c mice 24 h after intratumoral injection of PAGA/pmIL-12 complexes or naked pmIL-12.

We found that 24 h after injection of PAGA/pmIL-12 complexes there was a greater infiltration of macrophages compared to 5% glucose-injected mice. Our results are in agreement with previous reports which have shown that recombinant IL-12 can heavily recruit the macrophages in the early stages of IL-12-induced tumor regression.³³ In addition, it has also been found that induction of IFN- γ can further increase the production of IL-12 and TNF- α through the macrophages.²⁰ However, in terms of tumor infiltration, dendritic cells seemed to be more active in PAGA/pmIL-12 group. Dendritic cells have proven to be one of the most effective APCs in mediating a potent anti-tumor response primarily due to their ability to strongly stimulate NK cells and naive T cells, thereby invoking innate, as well as adaptive immune mechanisms. Dendritic cells genetically engineered to produce IL-12 have been found to be extremely effective against weakly immunogenic tumors and in many cases completely eradicated tumors providing strong resistance against recurrence of tumors at the same site.¹⁵ Dendritic cells also take part in further production of IL-12 upon stimulation by IFN- γ and TNF- α . We found that PAGA/pmIL-12 complexes showed sig-

nificant staining for dendritic cells, with comparatively weak stains for pmIL-12 and 5% glucose injections (Figure 6). It is evident that during early stages of IL-12 gene expression, APCs like dendritic cells and macrophages were one of the most active cells, which in part is due to their close relationship with elevated levels of endogenous IFN- γ and TNF- α .^{15,28,33} Further downstream, events like induction of iNOS from macrophages would result in production of NO.^{14,19} Elevated levels of nitric oxide thus indirectly confirmed the biological activity of IL-12, as well as macrophage infiltration.

We also studied the involvement of NK cells and found them to have significant presence at the tumor site (Figure 6). While CD8⁺ T cells are the most important cells during long term anti-tumor immunity, it is the NK cell which plays the defining role during early stages of tumor regression and inhibition of metastasis.³² NK cells directly kill the tumor cells in initial stages of IL-12 gene expression and are also necessary for activation and priming of CD8⁺ T cells. Brunda *et al*¹ have shown that NK cell involvement in host-tumor interaction does not affect the likelihood of tumor regression and treatment potential of IL-12 as an innate cytokine. However, it was later proven in hepatic metastasis involving weakly immunogenic cancer cells that depletion of NK cells using anti-asialo GM1 antibody significantly impaired the recruitment of CD8⁺ T cells and CD4⁺ helper cell population.³² Trends similar to NK cells were observed for CD4⁺ T cells in terms of tumor infiltration. CD4⁺ T cells interact with dendritic cells upon stimulation by IL-12, leading to IFN- γ and TNF- α secretion which would result in a Th1 phenotype development.³⁴ CD8⁺ T cells were found to be not so prominent at the tumor site, which is in agreement with Pham-Nguyen *et al*³² in that, these cells would be recruited mainly after an initial burst of NK and NKT cells which is essential for long-term immune responses and prevention of metastasis. Future work on host immune response involving immunohistochemical analysis at later time-points, such as 1 or 2 weeks after polymer/pmIL-12 delivery would throw more light on CD8⁺ T cell stimulation and its relative presence at the tumor site.

As can be seen from Figure 6, PAGA/pmIL-12 complexes show a modest improvement over naked pmIL-12 in terms of infiltration of various antigen-presenting and effector cell populations. The immunohistochemistry results were aimed at providing a semi-quantitative scenario after injection of these formulations in CT-26 tumor-bearing BALB/c mice. We plan to perform a comprehensive array of experiments using fluorescence activated cell sorting (FACS), which would quantitate the various immune cells, thereby complementing the results obtained from immunohistochemistry.

To assess the IL-12 treatment outcome, subcutaneous CT-26 tumor bearing BALB/c mice treated with PAGA/pmIL-12 complexes were studied for their long-term survival and tumorigenicity. Mice were monitored at least twice per week for signs of lethargy, subnormal activity and response to light. The tumor size of the animals receiving repeated injections of PAGA/pmIL-12 complexes at 3-day intervals progressed slower compared with the animals receiving single injections comprising PAGA/pmIL-12 and naked pmIL-12 groups (Figure 7). This was probably due to the higher levels of induced immunostimulatory cytokines, such as IFN- α

and TNF- α at all time-points compared with the cytokine levels in the mice administered with only a single dose of these formulations. However, in terms of survivability, we saw PAGA/pmIL-12 groups having a longer life span compared with control groups like that of naked pmIL-12 and 5% glucose-injected groups (Figure 8). In accordance with these findings, it was demonstrated that IL-12 gene therapy using retroviral and adenoviral vectors induced significant tumor regression and prolonged the survival time of the treated animals.^{3,35}

TNF- α modulates the function of tumor-vascular endothelial cells and enhances the immune reaction. An extremely low dose of TNF- α has been shown to be sufficient to induce tumor regression, whereas high concentrations of TNF- α have been reported to cause necrosis and destruction of tumors.³⁶ In the present study, little necrosis of the tumors was seen when the CT-26 tumor-bearing mice received a single injection of PAGA/pmIL-12 complexes. However, repeated administration of PAGA/pmIL-12 complexes every 3 days into the mice caused severe tumor necrosis beginning at the third week of therapy. Extreme tumor necrosis rendered further administration of PAGA/pmIL-12 complexes and naked pmIL-12 progressively impractical. As time proceeded, especially since day 15, we saw a decline in the movement and other physical activities of mice receiving repeated administration of PAGA/pmIL-12 complexes or naked pmIL-12. We envisage that due to the repeated injections of PAGA/pmIL-12 complexes, TNF- α was maintained continuously at the concentrations sufficient enough to cause severe tumor necrosis.

The extent of tumor regression after single intra-tumoral injection of PAGA/pmIL-12 complexes was not good enough to have any clinical significance, whereas the repeated injection every 3 days caused severe tumor necrosis. To minimize tumor necrosis while still maintaining tumor regression, we will be injecting PAGA/pmIL-12 complexes once a week rather than every 3 days. We have previously shown at least 10–15-fold higher production of mIL-12 p40 compared with mIL-12 p70 for the tumor-bearing BALB/c mice treated with PAGA/pmIL-12 complexes.¹⁰ The mIL-12 p40 can act as an antagonist towards mIL-12 p70 by forming (p40)₂ homodimer.³⁷ Moreover, the internal ribosomal entry site (IRES) segment between the p35 and p40 genes of the mIL-12 expression plasmid used in this study may not allow regulated production of p35 and p40 genes to confer increased amounts of IL-12 p70. Rakhmievich and Yang³⁸ demonstrated that the mIL-12 expression plasmid with separate CMV promoters driving p35 and p40 encoding cDNAs produced at least two-fold IL-12 p70 compared with bi-cistronic IRES vector of IL-12 both *in vivo* and *in vitro*. Therefore, we are currently comparing the IL-12 plasmid construct used in the present study with the p2CMVmIL-12 with each subunit-encoding gene under the transcriptional control of a separate CMV promoter. We also plan to synthesize high molecular weight PAGA to slow down its degradation rate due to auto-hydrolysis of ester linkages in PAGA.

In conclusion, we have provided the proof of principle that PAGA/pmIL-12 complexes, when used as the gene delivery system, induce a range of cytokines as well as immune cell infiltration at higher levels compared with naked pmIL-12 gene delivery, resulting in improved inhibition of tumor growth and increase in animal survival.

We envisage biodegradable non-toxic PAGA to be fit for repeated administration to maintain sustained gene expression, thereby offering an exciting incentive for cancer treatment.

Materials and methods

Materials

Polyoxyethylene sorbitan monolaurate (Tween-20) was purchased from BioRad Laboratories (Hercules, CA, USA). Glycerol, terrific broth, ampicillin, hydrogen peroxide, 10 N hydrogen chloride (HCl), and 3-[N-morpholino]propane sulphonic acid (MOPS) were purchased from Sigma Chemical (St Louis, MO, USA). Glucose, sodium dodecyl sulfate (SDS), molecular biology grade isopropyl alcohol, ethyl alcohol and methanol were all purchased from Aldrich (Milwaukee, WI, USA). 1 kb DNA step ladder and Griess reagent system were purchased from Promega (Madison, WI, USA). Library efficiency DH5 α -competent cells, DNA grade high melting agarose, bovine serum albumin (BSA), fetal bovine serum (FBS), phosphate buffered saline (PBS), ethidium bromide, 0.25% (w/v) trypsin-EDTA, Rosewell Park Memorial Institute medium (RPMI 1640), Dulbecco's modified essential medium (DMEM), penicillin, streptomycin and gentamycin were purchased from GIBCO-BRL (Gaithersburg, MD, USA). Qiagen EndoFree Plasmid Maxi Kit was purchased from Qiagen (Boulder, CO, USA). BDOptEIA ELISA sets for mIL-12 p70, mIFN- γ , mTNF- α , 3, 3', 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide substrate reagents were obtained from Pharmingen (San Diego, CA, USA). Bicinchoninic acid (BCA) protein assay reagent kit was purchased from Pierce Chemical (Rockford, IL, USA). Recombinant mouse IFN- α and rabbit anti-mouse polyclonal IFN- α were purchased from PBL Biomedical Laboratories (New Brunswick, NJ, USA). Rat anti-mouse monoclonal IFN- α was purchased from United States Biological (Swampscott, MA, USA). F4/80 and Mac3 antibodies against murine macrophages, CD16/CD32 antibodies against dendritic cells, CD1d antibodies against NK cells, CD4 antibodies against CD4⁺ cells, and CD8 antibodies against CD8⁺ cells were purchased from Serotec (Raleigh, NC, USA). Vectastain ABC-HRP kits for immunohistochemical staining were purchased from Vector Laboratories (Burlingame, CA, USA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG and HRP-conjugated rabbit anti-goat IgG were purchased from Zymed Laboratories (San Francisco, CA, USA).

Mice

Five-week-old female BALB/c mice were purchased from Simonsen Laboratories (Gilroy, CA, USA) and housed in the Animal Care Facility, Biomedical Polymers Research Building, University of Utah. Mice were maintained on *ad libitum* rodent feed and water at room temperature, 40% humidity. All mice were acclimatized for at least 1 week before tumor implantation. All studies were performed in accordance with the approved animal protocol.

Construction and purification of plasmids

Plasmids encoding murine interleukin 12 (mIL-12) and murine interferon γ (mIFN- γ) were obtained as a gift from Dr Jun-ichi Miyajaki of Osaka University Medical

School, Japan. The p35 and p40 subunits of mIL-12 are expressed from two independent transcription units and are inserted into a single plasmid pCAGGS to generate pmIL-12. The expression unit for mIL-12 p35, including CMV immediate-early enhancer-chicken β -actin hybrid promoter, and rabbit β -globin poly(A) signal, is excised from pCAGGS-p35 and is inserted downstream of the mIL-12 p40 expression unit of pCAGGS-p40.³⁹ Another plasmid for mIL-12, pCMVIL-12, was obtained as a kind gift from Dr Steven Dow of the University of Colorado.⁴⁰ The empty plasmid pCAGGS, identical to pmIL-12 while lacking only p35 and p40 transcription units, was used as a control for analysis of pmIL-12 specific effects. The plasmids were amplified, purified and characterized as described previously by Maheshwari *et al.*¹⁰

Formulation and in vitro characterization

Poly[α -(4-aminobutyl)-L-glycolic acid] (PAGA) was synthesized and characterized as described previously by Lim *et al.*⁴¹ Following synthesis, purification and characterization, PAGA was used for complex formation with pmIL-12 in presence of 5% glucose. PAGA/pmIL-12 complexes were characterized in terms of particle size, zeta potential, osmolality and surface morphology as described previously by Maheshwari *et al.*¹⁰ Upon characterization PAGA/pmIL-12 complexes at 3/1 (+/-) charge ratio were found to be most effective in gene delivery and were used consistently thereafter.

Tumor cell lines

CT-26 colon adeno-carcinoma cell lines was a gift from Dr Charles Tannenbaum of Cleveland Clinic Foundation, Cleveland, OH, USA.⁴² Tumor cells were grown and maintained in RPMI 1640 medium, which was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin and 50 μ g/ml gentamycin (all from Gibco-BRL, Gaithersburg, MD, USA) at 37°C and humidified 5% CO₂.

Tumor implantation and treatment

Subcutaneous tumor-bearing BALB/c mice were used for evaluation of PAGA/pmIL-12 complexes for transgene expression. To generate tumors, 5-week-old female BALB/c mice were subcutaneously injected in the middle of the left flank with 100 μ l of a single cell suspension containing 1×10^6 CT-26 cells. Tumor size was measured using a Vernier caliper across its two perpendicular diameters and its depth, and its volume was calculated using the formula $V = (4/3)\pi abc$. Treatment of the tumors was started after about a week when they reached a size of about 100–120 mm³. In all cases, PAGA/pmIL-12 complexes were prepared at 3/1 (+/-) charge ratio and 50 μ l of the complexes were injected directly into the tumors of BALB/c mice at a dose of 25 μ g pDNA/mouse unless otherwise stated. PAGA/pCAGGS formulations prepared under identical conditions were used as control. The treated mice were killed 48 h after injection. Tumors were chopped into small pieces, re-cultured into six-well plates and incubated at 37°C for 24 h. Supernatants were separated from the cells by centrifugation and assayed for mIL-12 p70, mIFN- γ , mTNF- α , mIFN- α and NO.

ELISA for mIL-12 and induced cytokines

Measurement of mIL-12 p70, mIFN- γ and mTNF- α was done using BDOptEIA ELISA sets (Pharmingen, San

Diego, CA, USA) and used according to the manufacturer's instructions. Briefly, ELISA plates (Nunc, Maxi-sorp, Denmark) were coated with capture antibody, sealed and kept overnight for antibody binding. The plates were washed several times followed by incubation with assay diluent to block any non-specific binding for 1 h. After washing several times, the plate was then incubated with samples and standards for 2 h. After incubating with detection antibody solution containing avidin-HRP reagent for 1 h, the substrate solution was added to carry out enzymatic reaction. The reaction was stopped by 2 N H₂SO₄ and the plate was read at 450 nm using BioRad (model 3550) ELISA reader. The mIL-12 p70, mIFN- γ and mTNF- α concentration was reported in terms of pg/ml.

In the case of mIFN- α , rat anti-mouse antibodies were incubated overnight in PBS (pH 7.4), followed by incubation with 100 μ l of standards, as well as samples, for another 2 h. Rabbit anti-mouse IFN- α polyclonal antibodies was then applied to the ELISA plate and incubated for 1 h. The plate was then incubated with detection antibody (HRP-conjugated anti-rabbit IgG) for another 30 min followed by reaction with TMB substrate reagent. The reaction was stopped with 2 N H₂SO₄ and the plate was read as mentioned earlier.

Nitric oxide assay

The levels of nitric oxide (NO) produced in the supernatant of the cultured tumors of the CT-26 subcutaneous tumor-bearing BALB/c were measured in terms of moles of nitrite (NO₂⁻) using the Griess Reagent System (Promega) and used as per the manufacturer's protocol. Briefly, 50 ml of samples were added into a 96-well flat-bottomed enzymatic assay plate along with 100 μ l of 100 μ M nitrite standards in separate wells. Serial dilutions were performed in RPMI 1640 immediately to prepare a standard curve (0–100 μ M) with the bottom-most wells having 50 μ l of RPMI 1640 alone. Thereafter, 50 μ l of 1% (w/v) sulfanilamide solution in 5% (w/v) phosphoric acid was dispensed in all the wells containing samples and standards, and incubated for 5–10 min. Finally, 50 μ l of 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (NED) solution in water was added to the plate and incubated again for 5–10 min. All incubations were carried out at room temperature and protected from light. The absorbance was measured within 30 min at 540 nm using Biorad (model 3550) ELISA reader. The concentration of nitrite was reported in terms of μ M.

Immunohistochemistry

The tumor-infiltrating immune cells, such as NK, CD8⁺, CD4⁺, macrophages and dendritic cells were analyzed by immunohistochemistry to assess the anti-tumor activity of pmIL-12 and PAGA/pmIL-12 complexes. PAGA/pmIL-12 (3/1, +/-) complexes were injected intratumorally into female BALB/c mice bearing subcutaneous CT-26 tumors. Twenty-four hours after injection, the tumors were excised and frozen in 1 \times PBS pre-cooled in liquid nitrogen until further use. Immunohistochemical analysis was carried out as follows: tissues were placed in a cryomold surrounded in OCT and frozen in a dry ice/ethanol (95%) bath. Subsequently, cryostat-cut sections (6 μ m) of tumors were prepared and were picked on Superfrost Plus slides and air dried at room temperature. The slides were fixed in cold acetone (4°C)

for 10 min and the reagent was allowed to evaporate off the cells. The slides were hydrated in phosphate buffered saline, and quenched in 0.01% H₂O₂ in PBS to neutralize any endogenous peroxidase activity for 15 min at room temperature. The slides were then rinsed for 10 min in PBS followed by blocking with 5% normal goat serum in PBS for another 30 min. The fixed sections were then incubated at room temperature for 30 min with 200 μ l/section of primary antibodies as follows: F4/80 and Mac3 antibodies against murine macrophages, CD16/CD32 antibodies against dendritic cells, CD1d antibodies against NK cells, CD4 antibodies against CD4⁺ cells and CD8 antibodies against CD8⁺ cells. All antibodies were used as per manufacturer's protocol (Serotec). The slides were washed again with 1 \times PBS and incubated with biotin conjugated mouse anti-rat secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min at room temperature. Following another washing step, reagents from ABC-HRP kit (Vector) were applied to the tissue sections as per the manufacturer's protocol and incubated for 30 min at room temperature. The slides were then washed in 1 \times PBS followed by the substrate reaction using DAB kit (Vector). The slides were washed again with distilled water for 5 min to stop the reaction and counterstained with Gill's hematoxylin (Vector). The slides were dehydrated and tissue sections were mounted with cytooseal 60 (VWR Scientific, Willard, OH, USA) before analysis under the microscope.

Tumor regression studies

PAGA/pmIL-12 (3/1, +/-) complexes were injected directly into CT-26 subcutaneous tumor-bearing BALB/c mice at a dose of 25 μ g pmIL-12/mouse. Naked pmIL-12 (25 μ g/mouse), as well as 5% glucose, were also injected into separate CT-26 tumor-bearing mice and used as controls. After a single intratumoral injection, mice were closely monitored at an interval of every 3 days for tumor growth. Repeated injections were performed with the same formulations at an interval of every 3 days and tumor growth was monitored simultaneously. Tumor progression was reported in terms of tumor volume over a period of 48 days.

Statistical analysis

Statistical significance of differences between groups was determined by applying one way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$. Results are expressed as the mean \pm s.e.

Acknowledgements

Our special thanks to Kelley Murphy of Huntsman Cancer Institute, University of Utah for immunohistochemistry related experiments. We also thank Alex Zlotnikov for technical assistance and Expression Genetics, for financial support.

References

- 1 Brunda MJ *et al*. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J Exp Med* 1993; **178**: 1223–1230.
- 2 Rakhmievich AL *et al*. Cytokine gene therapy of cancer using gene gun technology: superior antitumor activity of interleukin-12. *Hum Gene Ther* 1997; **8**: 1303–1311.

- 3 Tahara H *et al*. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J Immunol* 1995; **154**: 6466–6474.
- 4 Smyth MJ, Taniguchi M, Street SEA. The anti-tumor activity of IL-12: mechanisms of innate immunity that are model and dose dependent. *J Immunol* 2000; **165**: 2665–2670.
- 5 Fernandez NC *et al*. High frequency of specific CD8⁺ T cells in the tumor and blood is associated with efficient local IL-12 gene therapy of cancer. *J Immunol* 1999; **162**: 609–617.
- 6 Martinotti A *et al*. CD4 T cells inhibits *in vivo* the CD8-mediated immune response against murine colon carcinoma cells transduced with interleukin-12 genes. *Eur J Immunol* 1995; **25**: 137–146.
- 7 Mendiratta SK *et al*. Intratumoral delivery of IL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity. *Gene Therapy* 1999; **6**: 833–839.
- 8 Mendiratta SK *et al*. Combination of interleukin-12 and interferon- α gene therapy induces a synergistic anti-tumor response against colon and renal carcinoma. *Hum Gene Ther* 2000; **11**: 1851–1862.
- 9 Han SO, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. *Mol Ther* 2000; **2**: 302–317.
- 10 Maheshwari A *et al*. Soluble biodegradable polymer-based cytokine gene delivery for cancer treatment. *Mol Ther* 2000; **2**: 121–130.
- 11 Haicheur N *et al*. Cytokine and soluble cytokine receptor induction after IL-12 administration in cancer patients. *Clin Exp Immunol* 2000; **119**: 28–37.
- 12 Coleman M *et al*. Nonviral interferon- α gene therapy inhibits growth of established tumors by eliciting a systemic immune response. *Hum Gene Ther* 1998; **9**: 2223–2230.
- 13 Dong Z *et al*. Suppression of angiogenesis, tumorigenicity, and metastasis by human prostate cancer cells engineered to produce interferon- β . *Cancer Res* 1999; **59**: 872–879.
- 14 Bogdan C, Rollinghoff M, Diefenbach A. The role of nitric oxide in innate immunity. *Immunol Rev* 2000; **173**: 17–26.
- 15 Nishioka Y *et al*. Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin-12. *Cancer Res* 1999; **59**: 4035–4041.
- 16 Rakhmievich AL *et al*. Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy. *J Immunother* 1999; **22**: 135–144.
- 17 Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridges innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol* 1995; **13**: 251–276.
- 18 Lim YB *et al*. Development of a safe gene delivery system using biodegradable polymer, poly[α -(4-aminobutyl)-L-glycolic acid]. *J Am Chem Soc* 2000; **122**: 6524–6525.
- 19 Diefenbach A *et al*. Requirement for type 2 NO-synthase for IL-12 responsiveness in innate immunity. *Science* 1999; **284**: 951–955.
- 20 Nastala CL *et al*. Recombinant IL-12 administration induces tumor regression in association with IFN- γ production. *J Immunol* 1994; **153**: 1697–1706.
- 21 Nagayama H *et al*. IL-12 responsiveness and expression of IL-12 receptor in human peripheral blood monocyte-derived dendritic cells. *J Immunol* 2000; **165**: 59–66.
- 22 Duda DG *et al*. Direct *in vitro* evidence and *in vivo* analysis of the angiogenesis effects of interleukin-12. *Cancer Res* 2000; **60**: 1111–1116.
- 23 Lanuti M *et al*. Cationic lipid: bacterial DNA complexes elicit adaptive cellular immunity in murine intraperitoneal tumor models. *Cancer Res* 2000; **60**: 2955–2963.
- 24 Li D *et al*. Combination surgery and non-viral interleukin-2 gene therapy for head and neck cancer. *Clin Cancer Res* 1999; **5**: 1551–1556.
- 25 Schultz J *et al*. Long lasting anti-metastatic efficiency of interleukin-12 encoding plasmid DNA. *Hum Gene Ther* 1999; **10**: 407–417.
- 26 Freimark BD *et al*. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: cationic lipid complexes. *J Immunol* 1998; **160**: 4580–4586.
- 27 Zhou A *et al*. Exogenous interferon- γ induces endogenous synthesis of interferon- α and - β by murine macrophages for induction of nitric oxide synthase. *J Interfer Cytokine Res* 1995; **15**: 897–904.
- 28 Brunda MJ *et al*. Role of interferon- γ in mediating the anti-tumor efficacy of interleukin-12. *J Immunother* 1995; **17**: 71–77.
- 29 Mahato RI. Polymeric gene delivery for cancer treatment. In: Park KD (ed.). *Biomaterials and Drug Delivery towards New Millennium*. Han Rim Won Publishing; Seoul, 2000, pp 249–280.
- 30 Ghazizadeh S, Carroll JM, Taichman LB. Repression of retrovirus-mediated transgene expression by interferons: implications for gene therapy. *J Virol* 1997; **71**: 9163–9169.
- 31 Sareneva T, Julkunen I, Matikainen S. IFN- α and IL-12 induces IL-18 receptor gene expression in human NK and T cells. *J Immunol* 2000; **165**: 1933–1938.
- 32 Pham-Nguyen KB *et al*. Role of NK and T cells in IL-12-induced anti-tumor response against hepatic colon carcinoma. *Int J Cancer* 1999; **81**: 813–819.
- 33 Ha SJ *et al*. Rapid recruitment of macrophages in interleukin-12 mediated tumor regression. *Immunology* 1998; **95**: 156–163.
- 34 Koch F *et al*. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* 1996; **184**: 741–746.
- 35 Caruso M *et al*. Adenovirus mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc Natl Acad Sci USA* 1996; **93**: 1302–1306.
- 36 Mizuguchi H *et al*. Tumor necrosis factor α -mediated tumor regression by the *in vivo* transfer of genes into the artery that leads to tumor. *Cancer Res* 1998; **58**: 5725–5730.
- 37 Gately MK *et al*. The interleukin-12/interleukin-12 receptor system: role in normal and pathogenic immune responses. *Annu Rev Immunol* 1998; **16**: 495–521.
- 38 Rakhmievich AI, Yang NS. IL-12 gene therapy of tumors. US Patent 1999; 5: 922, 685.
- 39 Okada E *et al*. Intranasal immunization of a DNA vaccine with IL-12- and granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens. *J Immunol* 1997; **159**: 3638–3647.
- 40 Dow SW *et al*. Intravenous cytokine gene delivery by lipid-DNA complexes controls the growth of established lung metastases. *Hum Gene Ther* 1999; **10**: 2961–2972.
- 41 Lim YB *et al*. Biodegradable polyester, poly[α -(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier. *Pharm Res* 2000; **17**: 811–816.
- 42 Tannenbaum CS *et al*. Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. *J Immunol* 1996; **156**: 693–699.