Published in final edited form as:

Cancer Gene Ther. 2012 February ; 19(2): 135–143. doi:10.1038/cgt.2011.78.

The Anti-tumor Effects of Adenoviral-Mediated, Intra-tumoral delivery Interleukin 23 require Endogenous IL-12

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Abstract

IL-23 is a member of the IL-12 family of heterodimeric cytokines, comprised of p19 and p40 subunits, which exhibits immunostimulatory properties similar to IL-12. We have demonstrated previously that adenoviral-mediated, intra-tumoral delivery of IL-23 (Ad.IL-23) was able to induce systemic anti-tumor immunity. Here we demonstrate that Ad.IL-23 requires endogenous IL-12 for conferring an anti-tumor effect after adenoviral-mediated intra-tumoral delivery. In contrast, Ad.IL-12 does not require IL-23 for its anti-tumor effects although endogenous IL-23 appears important for induction of systemic anti-tumor immunity by IL-12. However, despite the requirement for endogenous IL-12, co-delivery of IL-23 and IL-12 does not provide even an additive local or systemic anti-tumor effect, regardless of the dose. We further demonstrate that although the use of a single chain IL-23 (scIL-23) results in higher level of expression and a more pronounced IL-23-mediated anti-tumor effect, there is still no synergy with IL-12. These results demonstrate that whereas significant anti-tumor effects are achieved by intratumoral injection of adenovirus expressing either scIL-23 or IL-12 alone and that IL-23 requires endogenous IL-12 for maximum anti-tumor benefit, the combined use of these cytokines provides no additive or synergistic effect.

Keywords

Interleukin 23; Interleukin 12; adenovirus; cancer; gene therapy

INTRODUCTION

IL-12 is a heterodimeric, proinflammatory cytokine comprised of p40 and p35 subunits. IL-12 enhances proliferation¹ and cytolytic activity^{2–3} of and IFN- γ production from activated NK and T-cells.^{2, 4–5} In response to microbial infection, IL-12 is produced by macrophages, which in turn drives generation of a Th1-type adaptive immune response. Therefore, IL-12 acts as a bridge between both innate and adaptive arms of the immune system.⁶ IFN- γ is responsible for the majority of the inflammatory activities of IL-12, drives Th1 differentiation, and induces IL-12 secretion from DCs, thereby forming a positive feedback loop for Th1 differentiation.⁷ IL-12 activates the JAK/STAT pathway, with STAT4 being preferentially activated.⁸ In accordance with the proinflammatory and immunostimulatory activities of this cytokine, IL-12 possesses potent anti-tumor effects.

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CONFLICT OF INTEREST

The authors declare no competing financial interests

Indeed, recombinant IL-12 has been shown to inhibit tumor establishment, cause regression of established tumors and induce tumor-specific immunity.^{9–12} However, systemically delivered IL-12 is associated with severe toxicity.¹² Gene delivery of IL-12 directly into the tumor microenvironment using adenoviral vectors promotes tumor regression and generation of tumor-specific immunity, while alleviating the toxicity associated with systemic delivery.¹³

IL-23 is a member of the IL-6/IL-12 family of heterodimeric cytokines and is composed of two subunits: p40, which is shared with IL-12, and p19, which is unique to IL-23.¹⁴ Like IL-12, IL-23 can act on both innate and adaptive arms of immunity. IL-23 stimulates production of IFN- γ from NK cells.¹⁵ Furthermore, IL-23 stimulates proliferation of and IFN- γ production from CD4⁺ memory T-cells, suggesting an important role in maintenance of Th1 immunity.¹⁴ IL-23 activates the same panel of signaling molecules as IL-12, differing only in that STAT3, as opposed to STAT4, appears to be the most prominent STAT activated.¹⁶ Due to the structural and functional similarities between IL-12 and IL-23, it is not surprising that IL-23 also acts as a potent anti-cancer agent in various establishment^{17–20} and therapeutic^{21–23} models of cancer. We previously have shown that treatment of established MCA205 fibrosarcoma tumors with adenovirus expressing IL-23 leads to significant enhancement of survival, tumor rejection and establishment of protective immunity using mechanisms similar to IL-12.²³

IL-12 and IL-23 both activate the JAK/STAT pathway,^{8, 16} induce IFN- γ production from NK and T-cells,^{2, 4–5, 14–15} and utilize common mechanisms of tumor eradication.²³ Furthermore, IL-23 acts on DCs to induce IL-12 production and the combination of IL-12 and IL-23 causes DCs to secrete greater levels of IFN- γ than either cytokine alone.²⁴ Here we examined whether endogenous IL-12 and IL-23 are required for the anti-tumor effects of adenovirally delivered IL-12 and IL-23. We further examined whether IL-12 and IL-23 function in an additive or synergistic manner in conferring anti-tumor effects.

METHODS

Adenoviruses

Adenoviruses expressing IL-12 (Ad.IL-12) and IL-23 (Ad.IL-23) have been described previously.^{13, 25} Ad.IL-12, Ad.IL-23 and Ad.Psi5 (empty vector) were prepared as follows: Viruses were propagated on HEK-293 cells and purified by CsCl banding, followed by dialysis in 3% sucrose solution. Particle titer of purified viruses was determined by spectroscopy using the equation, $(OD_{260})(\text{dilution factor})/9.09 \times 10^{-13}$, with the virus being diluted 1:50 prior to measuring OD. The particle titer was used to calculate MOI in all experiments. Infectious titers were determined using quantitative real-time PCR as previously described²⁶ and were approximately 100-fold less than particle titers. Viruses were aliquoted and stored at -80°C until use.

Adenoviruses expressing single-chain (sc) versions of IL-12 and IL-23 were designed as follows: To construct Ad.scIL-12, the IL-12p40 precursor (Met1 to Ser335) was linked to the mature p35 subunit (Arg23 to Ala215) using the previously described 15 amino acid linker (Gly₄Ser)₃.²⁷ To construct Ad.scIL-23, the IL-12 p40 precursor (Met1 to Ser335) was linked to the mature p19 subunit (Leu20 – Ala196) using (Gly₄Ser)₃.

Relative cytokine expression of each adenoviral preparation was analyzed by infecting 4×10^4 MCA205 cells with a 500 MOI of Ad.IL-12 or Ad.IL-23 for 1 hour at $37^{\circ}\text{C}/5\% \text{CO}_2$ in serum free media. Complete media was added and cells were incubated for 72 hours, after which supernatants were harvested. IL-12 and IL-23 content was analyzed using the mouse

IL-12 p70 and mouse IL-23 p19/p40 Ready-Set-Go IL-23 ELISA kits (eBioscience, San Diego, CA), respectively, following manufacturers' instructions.

Biological activity of Ad.scIL-23 was assayed as follows: 4×10^4 MCA205 cells were infected with a MOI 1000 of Ad.scIL-23 or Ad.Psi5 and supernatants harvested 72 hours post-infection. Splenocytes were then harvested from C57BL/6 mice, mechanically dissociated and treated with Red Cell Lysis buffer (Invitrogen, Carlsbad, CA) to remove all red blood cells. Splenocytes were plated at a concentration of 2×10^6 cells per well in a 24 well plate and 24 hours later treated with supernatants from adenovirus-infected MCA205s. Forty-eight hours after treatment, splenocyte supernatants were collected and analyzed for induction of IL-17 expression using the Mouse IL-17 Immunoassay (R&D Systems, Minneapolis, MN).

Animals

Female C57BL/6 and p40-deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-23 (p19) deficient mice have been previously described and were a kind gift of Dr. Jay Kolls.²⁸ Mice were used at 6 to 7 weeks of age. Animals were maintained under pathogen free conditions at the Biotechnology Center Animal facility at the University of Pittsburgh. All procedures performed were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Cell Lines

MCA205 fibrosarcoma cells were maintained in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA) and L-glutamine (Gibco, Carlsbad, CA). Cells were kept in a humidified chamber at 37°C/5% CO₂ and passaged every 2–3 days.

In Vitro Transduction of MCA205 Cells

MCA205s were plated on 24-well plates at a concentration of 4×10^4 cells per well and infected with a MOI 500 of Ad.IL-12 and Ad.IL-23, either virus alone or Ad.Psi5. Seventy-two hours post-infection, supernatants were harvested and analyzed using Quantikine Mouse IL-23, IL-12 p70 and IL-12/IL-23p40 Immunoassays (R&D Systems, Minneapolis, MN).

In Vivo Tumor Studies

For use in *in vivo* tumor experiments, confluent layers of MCA205 cells were dissociated by trypsin, washed 3 times with Hanks Balanced Salt Solution (HBSS) (Gibco, Carlsbad, CA) and counted using trypan blue exclusion. Mice were inoculated with 1×10^5 MCA205 cells in 100uL HBSS subcutaneously in the abdomen. IL-23 (p19) and IL-12/23 (p40) deficient mice were treated on days 7, 9 and 11 post-tumor inoculation by intratumoral injection of 5×10^{10} particles (approximately 5×10^8 PFUs) of either Ad.IL-12, Ad.IL-23 or Ad.Psi5.

To investigate possible synergy by delivering Ad.IL-12 and Ad.IL-23 at separate time points, mice bearing two tumors were treated in one with 5×10^8 particles of Ad.IL-12 or 2.5×10^{10} particles of Ad.IL-23 as follows: Ad.IL-12 on day 7, followed by Ad.IL-23 on day 11; Ad.IL-23 on day 7 followed by Ad.IL-12 on day 11; Ad.IL-12 on days 7 and 11; Ad.IL-23 on days 7 and 11; or Ad.Psi5 on days 7 and 11. Alternatively, to investigate possible anti-tumor synergy between co-delivered Ad.IL-12 and Ad.IL-23, mice bearing two, day 7 tumors were treated simultaneously with 1×10^9 particles of Ad.IL-12 and 5×10^{10} particles of Ad.IL-23 or either virus alone.

To investigate the anti-tumor activity of Ad.scIL-23, mice were injected intratumorally on days 7, 9 and 11 post-tumor inoculation with 5×10^{10} particles of Ad.scIL-12, Ad.scIL-23 or

Ad.Psi5. To explore possible synergy between Ad.IL-12 and Ad.scIL-23, mice bearing day 7 MCA205 tumors were treated once intratumorally with 1×10^8 particles of Ad.IL-12 and 1×10^9 particles of Ad.scIL-23, either virus alone or Ad.Psi5. In all synergy experiments, total quantity of virus injected was kept constant between groups by co-delivery of Ad.Psi5. In all experiments, tumor volume was monitored using a metric caliper until mice were sacrificed due to excessive tumor size or tumor ulceration. Tumor-free or “cured” mice were subject to tumor challenge 1–2 months after initial tumor resolution with 1×10^5 MCA205 cells subcutaneously in the abdomen.

Immunohistochemistry

Tumors of treated mice were harvested on day 15 post-tumor inoculation (day 8 post-treatment), snap frozen in 2-methylbutane and stored at -80°C . Tumors were then cut by cryostat, ten micron sections placed onto charged slides and stained for CD8 and CD31 as follows: For analysis of intratumoral CD8+ T-cell infiltrate from mice treated with Ad.IL-12 and Ad.IL-23, sections were fixed in -20°C acetone and slides blocked in Exogenous Peroxidase Block (DAKO, Carpinteria, CA), followed by block in 10% goat serum. CD8 antibody (1:150 dilution)(BD Biosciences, San Jose, CA) was then added, followed by incubation with biotinylated goat anti-rat secondary antibody (1:250)(BD Biosciences, San Jose, CA) in DAKO Antibody Diluent (DAKO, Carpinteria, CA). Slides were treated with ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and developed using DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) following manufacturers' instructions. Slides were then counterstained with eosin, dehydrated in increasing concentrations of ethanol, followed by xylene, and coverslipped using Permount Media (Fisher, Pittsburgh PA).

Alternatively, for analysis of tumors from mice treated with Ad.IL-12 and Ad.scIL-23, sections were permeabilized using 10% Triton-X (Sigma, St. Louis, MO), blocked in 2% BSA (Sigma, St. Louis, MO) and incubated with rat anti-mouse CD8 antibody (1:100 dilution)(BD Biosciences, San Jose, CA) overnight at room temperature. Slides were then incubated with Alexa-Fluor goat anti-rat secondary (1:500 dilution)(Invitrogen, Eugene, OR) and coverslipped using Fluoromount media (SouthernBiotech, Birmingham, AL).

To investigate tumor angiogenesis, sections were permeabilized with 10% Triton-X (Sigma, St. Louis, MO) followed by block in 2% BSA (Sigma, St. Louis, MO). Sections were then incubated in rat anti-mouse CD31 primary antibody (1:100) (BD Biosciences, San Jose, CA), followed by Alexa-Fluor goat anti-rat secondary (1:500 dilution)(Invitrogen, Eugene, OR).

Statistics

Kaplan-Meier survival curves were plotted using SPSS version 16.0. Mice were monitored until excessive tumor volume or tumor ulceration, at which time they were sacrificed and recorded as occurrence of an event (death). Cured mice or those with tumors that did not warrant sacrifice by the end of the experiment were censored. Log-rank tests of the survival curves provided p-values. An unpaired T-test was used to analyze immunohistochemistry results if variances between data were equal; if variances were unequal, the Mann-Whitney U test was performed. Statistical analyses were 2-tailed, with a p value less than 0.05 considered statistically significant.

RESULTS

Anti-tumor activity of Ad.IL-23 requires endogenous IL-12

Previously, we have shown that adenoviral-mediated, intra-tumoral delivery of IL-23 resulted in efficient and long lasting tumor eradication by generating a Th1 immune response dependent upon CD4⁺ and CD8⁺ T-cells and endogenous IFN- γ .²³ The cellular and cytokine requirements for the anti-tumor effects of IL-23 were similar, but not identical, to those of IL-12. To determine the role of endogenous IL-12 in mediating the anti-tumor activity of IL-23, IL-12/23 p40 deficient mice bearing MCA205 tumors were treated on days 7, 9 and 11 post-tumor inoculation with 5×10^{10} particles of Ad.IL-12, Ad.IL-23 or saline. Lack of endogenous p40 abolished the anti-tumor activity of Ad.IL-23 (Fig. 1a and b), suggesting that IL-23 requires either endogenous IL-12 or IL-23, or both. In contrast, Ad.IL-12 retained its anti-tumor activity in the absence of endogenous IL-12 and IL-23. Interestingly, there was little evidence of Ad.IL-12 mediated toxicity seen in p40-deficient mice.

Anti-tumor activities of neither Ad.IL-12 nor Ad.IL-23 require endogenous IL-23 expression

To determine if there is a requirement for endogenous IL-23 expression in mediating the anti-tumor effects of IL-12 or IL-23, mice deficient in IL-23 p19 were inoculated with MCA205 tumors and treated with Ad.IL-12 or Ad.IL-23. Treatment of tumor bearing, p19 deficient mice with either Ad.IL-23 or Ad.IL-12 resulted in effective tumor eradication (Fig. 1c and d). This result demonstrates that Ad.IL-23 anti-tumor activity is dependent upon endogenous IL-12, not IL-23. However, it is important to note that two tumors from the Ad.IL-12 treated mice recurred at day 35 post-tumor inoculation, suggesting lack of induction of strong protective immunity in the absence of endogenous IL-23.

Treatment with Ad.IL-12 and Ad.IL-23 at separate time points does not lead to enhanced anti-tumor benefit

The results in IL-12/23 p40 and IL-23 p19 deficient mice suggest that Ad.IL-23 confers at least part of its anti-tumor effects by induction of endogenous IL-12. Therefore, we examined whether treatment of tumors with both Ad.IL-12 and Ad.IL-23 would result in an enhanced anti-tumor effect, resulting in more rapid tumor rejection and subsequent generation of long-lasting protective immunity. We first examined whether treatment with Ad.IL-12 followed by Ad.IL-23 would result in synergistic enhancement of anti-tumor effects both locally and systemically. C57BL/6 mice bearing tumors on each flank were treated with two injections of Ad.IL-12 and Ad.IL-23 at suboptimal doses (5×10^8 and 2.5×10^{10} particles, respectively) into one tumor as follows: Ad.IL-12 on day 7, followed by Ad.IL-23 on day 11 (Ad.IL-12,23); Ad.IL-23 on day 7, followed by Ad.IL-12 on day 11 (Ad.IL-23,12); Ad.IL-12 on both days 7 and 11 (Ad.IL-12), Ad.IL-23 on both days 7 and 11 (Ad.IL-23) or Ad.Psi5 on days 7 and 11 (Ad.Psi5). In the injected tumors, treatment with Ad.IL-12 on day 7 followed by Ad.IL-23 on day 11 resulted in a 29 percent tumor rejection rate which was less than the 43 percent rejection rate achieved with Ad.IL-12 treatment alone (Fig. 2a). Similarly, Ad.IL-23 treatment on day 7 followed by Ad.IL-12 on day 11 resulted in rejection of only 11 percent of tumors. A similar trend was observed in the contralateral tumors. Only 14 percent of the contralateral tumors in mice treated with Ad.IL-12 on day 7 followed by Ad.IL-23 on day 11 were rejected, less than the 42 percent rejection rate in mice treated with Ad.IL-12 alone (Fig. 2b). Additionally, when mice bearing only one tumor were treated with Ad.IL-12 and Ad.IL-23 as described above, there was no discernable enhancement of the anti-tumor effects (data not shown).

Co-treatment with Ad.IL-12 and Ad.IL-23 does not enhance anti-tumor benefit

Since delivery of Ad.IL-12 and Ad.IL-23 at separate time points did not yield additive or synergistic anti-tumor benefits, we next examined whether co-delivery of the viruses could enhance local or systemic anti-tumor activity compared to use of either virus alone. C57BL/6 mice were inoculated with two tumors on the flank and treated once on day 7 with 1×10^9 particles of Ad.IL-12 and 5×10^{10} particles of Ad.IL-23. Animals co-treated with Ad.IL-12 and Ad.IL-23 rejected 67 percent of injected and 45 percent of contralateral tumors. Similarly, mice treated with Ad.IL-12 rejected 78 percent of injected and 36 percent of contralateral tumors (14 mice total). Ad.IL-23 treatment alone resulted in no tumor free mice (Fig. 2c and d) at the suboptimal dose used.

In these experiments, all the Ad.IL-12 and Ad.IL-23 co-treated, tumor free mice were resistant to MCA205 tumor challenge. In addition, all of the tumor free, Ad.IL-23 treated mice and 3 out of 4 of the Ad.IL-12 treated mice were resistant to tumor challenge. Taken together, these results suggest that co-treatment with Ad.IL-12 and Ad.IL-23 does not generate enhanced MCA205-protective immunity.

To determine if the dose of each of the viruses was important, mice bearing only one tumor were treated with various suboptimal doses of Ad.IL-12 and Ad.IL-23. Mice were co-treated with Ad.IL-12 and Ad.IL-23 at the following doses: 5×10^{10} particles of Ad.IL-23 and 1×10^9 particles of Ad.IL-12; 2.5×10^{10} particles of Ad.IL-23 and 5×10^8 particles of Ad.IL-12; 1×10^{10} particles of Ad.IL-23 and 1×10^8 particles of Ad.IL-12 or either virus alone. Twenty-five tumor-bearing mice were co-treated with Ad.IL-12 and Ad.IL-23 total, with no enhancement of anti-tumor benefit observed (data not shown). Thus, it is clear that co-treatment with Ad.IL-12 and Ad.IL-23 does not result in enhanced generation of local or systemic anti-tumor immunity compared to treatment with Ad.IL-12 alone.

Generation and characterization of adenovirus expressing single chain IL-23

Co-treatment with Ad.IL-12 and Ad.IL-23 did not result in any additive or synergistic anti-tumor effects over use of either cytokine alone. However, the level of expression of IL-23 from the adenoviral vector used is significantly lower than IL-12 (data not shown). In addition, since IL-12 and IL-23 share the common p40 subunit, it is possible that *in vivo* co-infection of tumors with Ad.IL-12 and Ad.IL-23 leads to preferential expression of one cytokine over another. Therefore, the lack of therapeutic benefit of co-treatment with Ad.IL-12 and Ad.IL-23 may be a result of suboptimal expression of IL-23 compared to IL-12. Therefore, an adenoviral vector expressing a single chain IL-23 (Ad.scIL-23) was constructed in which the p40 subunit is linked to the mature p19 subunit using a 15-amino acid (Gly₄Ser)₃ spacer (Fig. 3a).²⁷

To demonstrate expression of scIL-23 from Ad.scIL-23 transduced cells, MCA205 cells were infected with Ad.scIL-23 and Ad.IL-23 and supernatants analyzed by ELISA. Ad.scIL-23 expressed much higher levels of IL-23 compared to Ad.IL-23 (Fig. 3b). To demonstrate that the scIL-23 expressed by Ad.scIL-23 was biologically active, splenocytes were treated with supernatants from Ad.scIL-23 infected cells for 48 hours and splenocyte supernatants analyzed for induction of IL-17 expression. Treatment of splenocytes with supernatants from Ad.scIL-23 infected cells resulted in induction of IL-17 expression compared to controls (Fig. 3c).

To determine the anti-tumor effect of Ad.scIL-23, tumor bearing C57BL/6 mice were treated on days 7, 9 and 11 post-tumor inoculation with 5×10^{10} particles of Ad.scIL-23 or Ad.Psi5. Treatment with Ad.scIL-23 significantly increased survival compared to Ad.Psi5 treated controls ($p = 0.00$), decreased tumor volume and led to an overall 90 percent tumor rejection rate (Fig. 3d and e). Upon MCA205 tumor challenge, all mice remained tumor free

up to 45 days after challenge (data not shown). These results demonstrate that higher expression of IL-23 results in a significant anti-tumor effect with induction of anti-tumor immunity

Co-treatment with Ad.scIL-23 and Ad.IL-12 does not result in enhanced anti-tumor activity

We next examined whether co-treatment with Ad.IL-12 and Ad.scIL-23 resulted in an additive or synergistic anti-tumor effect. Mice bearing two tumors were co-treated in one tumor with 1×10^9 particles of Ad.scIL-23 and 1×10^8 particles of Ad.IL-12 or either cytokine alone. Co-treatment with suboptimal doses of Ad.IL-12 and Ad.scIL-23 did not result in enhanced tumor rejection in either the injected or contralateral tumors compared to use of Ad.scIL-23 alone (Figures 4a and b). In both injected and contralateral tumors, 40 percent of co-treated animals experienced tumor rejection, identical to mice treated with Ad.scIL-23 alone (Fig. 4a and b). Treatment with the suboptimal dose of Ad.IL-12 alone resulted in only a 20 percent rejection rate in injected tumors and no rejection in the contralateral tumors (Fig 4a and b).

Co-treatment with Ad.IL-12 and Ad.scIL-23 does not alter CD8+ T-cell infiltrate and angiogenesis

Taken together, our results show no indication of additive or synergistic anti-tumor benefits of adenoviral-mediated, intratumoral co-delivery of IL-12 and IL-23. To determine if intratumoral delivery of the combination of IL-12 and IL-23 alters the type of immune cell infiltrate and angiogenesis compared to use of each alone, tumor bearing C57BL/6 mice were treated with suboptimal doses of Ad.IL-12 and Ad.scIL-23 once on day 7. On day 15 post-tumor inoculation, tumors were harvested and snap frozen. Cryosections of tumors were then analyzed for various immune cell infiltrates and angiogenesis. Co-treatment with Ad.IL-12 and Ad.scIL-23 did not significantly alter CD4+ T-cell, Foxp3 and dendritic cell infiltrate compared to treatment with either virus alone or control treated animals (Fig 5a, c and d). In addition, treatment with Ad.IL-12 and Ad.scIL-23 did not significantly alter CD31 expression compared to use of either Ad.IL-12 or Ad.scIL-23 alone or empty vector controls (Fig. 5e). However, Ad.IL-12 and Ad.scIL-23 co-treatment enhanced CD8+ T-cell infiltrate of tumors compared to animals treated with Ad.scIL-23 alone (Fig. 5a).

DISCUSSION

IL-12 is a heterodimeric, proinflammatory cytokine comprised of p40 and p35 subunits. IL-23 is a member of the IL-12 family of heterodimeric cytokines and is comprised of p40, which is shared with IL-12, and p19, which is unique to IL-23. IL-12 and IL-23 can act on both innate and adaptive arms of immunity.^{1-5, 14-15} In addition, adenoviral mediated, intratumoral delivery of IL-12 or IL-23 results in anti-tumor effects.^{13, 23} In this study we have examined the requirement for endogenous IL-12 and IL-23 for conferring the anti-tumor effects of gene delivered IL-12 and IL-23. We demonstrated that endogenous IL-12 is required for the anti-tumor activities of Ad.IL-23, but not for the anti-tumor effects of Ad.IL-12. In particular, Ad.IL-12 was able to generate an anti-tumor effect in both p40 and p19 deficient mouse strains. In contrast, Ad.IL-23 was able to confer an anti-tumor therapeutic effect in p19 deficient, but not in p40 deficient mice. However, it is important to note that in the p19-deficient mouse background, some of the Ad.IL-12 treated, the tumor free animals experienced tumor recurrence at later time points. Thus it is possible that IL-23 plays a role in establishing IL-12-mediated, systemic anti-tumor immunity. These results also are consistent with previous results demonstrating that the anti-tumor effects of systemic IL-23 required endogenous p35²².

Given the fact that Ad.IL-23 requires IL-12 for its anti-tumor effects and that IL-23 could contribute to the induction of anti-tumor immunity by IL-12, it is possible that the two cytokines may synergize to eradicate tumors. However, no enhancement in systemic anti-tumor immunity was observed in mice bearing two tumors treated with Ad.IL-12 and Ad.IL-23, regardless of timing of delivery. When delivered at separate time points, co-treatment with Ad.IL-12 and Ad.IL-23 did not result in synergistic or even additive enhancement of anti-tumor effects. Similarly, co-delivery of Ad.IL-12 and Ad.IL-23 at various doses resulted in no additive or synergistic effects.

One concern regarding the use of an adenoviral vector expressing both subunits from a single transcript via an IRES is that the level of IL-12 expression was consistently higher than that of IL-23, suggesting variable levels of expression between the two subunits. Thus the amount of virus needed to confer an anti-tumor effect with IL-23 was an order of magnitude higher than with Ad.IL-12. Also, even at higher doses of Ad.IL-23, the magnitude of the anti-tumor effect was less than that of Ad.IL-12. To increase the expression of IL-23, we generated an adenoviral vector expressing a single chain IL-23 (scIL-23) that expressed almost 1000-fold greater amounts of biologically active IL-23 than its double-chain counterpart. Intra-tumoral injection of Ad.scIL-23 had more potent anti-tumor activity, with treatment resulting in significant enhancement of survival, complete tumor rejection in 90 percent of animals and establishment of strong protective immunity. In fact, the efficiency of inducing an anti-tumor effect with scIL-23 approached that observed with IL-12. In addition, in spite of high expression levels, Ad.scIL-23 treatment showed no evidence of the toxicity that is observed when using high doses of Ad.IL-12. The fact that potent systemic anti-tumor immunity was observed following intra-tumoral delivery of Ad.scIL-23 supports the further development of a single chain IL-23 vector for therapeutic treatment of cancer.

Despite the higher levels of IL-23 expression obtained with Ad.scIL-23, no synergistic anti-tumor effect was observed when Ad.IL-12 and Ad.IL-23 were co-delivered. Co-treatment resulted in tumor eradication rates that were identical treatment with Ad.scIL-23 alone in both injected and contralateral tumors. To examine possible mechanisms for the lack of synergy, histology was performed on the treated tumors. Co-treatment with Ad.IL-12 and Ad.scIL-23 did not alter CD4 or Foxp3-positive or dendritic cell infiltrate compared to use of either virus alone or control treatment. Co-treatment did, however, significantly enhance CD8⁺ T-cell infiltrate compared to use of Ad.scIL-23 alone. Angiogenesis also was not altered in tumors treated with both Ad.IL-12 and Ad.scIL-23, or either virus alone, compared to controls.

Our observation that there is no evidence of synergy between IL-12 and IL-23 is supported by a previous study. C57BL/6 mice bearing scIL-23-secreting B16 tumors did not show enhanced tumor rejection when co-treated with recombinant IL-12 intraperitoneally.²⁷ Taken together, the results strongly suggest that these two cytokines simply do not synergize due to their contrasting and mutually exclusive functions: IL-12 strongly drives IFN- γ expression and a Th1 response, while IL-23 is capable of driving IL-17 expression and a Th17 response. While in our model it did not appear that IL-23-mediated eradication of tumors was mediated by a Th17 response (data not shown), it is possible that Th17 T-cells were generated. Furthermore, it has been shown that Th17 cells inhibit CD8⁺ T-cell cytotoxicity.^{29–30} Therefore, co-expression of IL-23 with IL-12 may skew the resulting immune response away from a Th1 phenotype and result in lack of enhancement of anti-tumor effects.

Acknowledgments

This work was supported by grants CA100327 and AR051456 from the National Institutes of Health to P.D.R.

References

1. Perussia B. Tumor infiltrating cells. *Lab Invest.* 1992; 67(2):155–7. [PubMed: 1501441]
2. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med.* 1989; 170(3):827–45. [PubMed: 2504877]
3. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol.* 1998; 70:83–243. [PubMed: 9755338]
4. Kubin M, Kamoun M, Trinchieri G. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. *J Exp Med.* 1994; 180(1):211–22. [PubMed: 7516408]
5. Chan SH, Perussia B, Gupta JW, Kobayashi M, Pospisil M, Young HA, et al. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med.* 1991; 173(4):869–79. [PubMed: 1672545]
6. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science.* 1993; 260(5107):547–9. [PubMed: 8097338]
7. Ma X, Chow JM, Gri G, Carra G, Gerosa F, Wolf SF, et al. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med.* 1996; 183(1):147–57. [PubMed: 8551218]
8. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* 2003; 3(2):133–46. [PubMed: 12563297]
9. Brunda MJ, Luistro L, Warriar RR, Wright RB, Hubbard BR, Murphy M, et al. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J Exp Med.* 1993; 178(4):1223–30. [PubMed: 8104230]
10. Noguchi Y, Jungbluth A, Richards EC, Old LJ. Effect of interleukin 12 on tumor induction by 3-methylcholanthrene. *Proc Natl Acad Sci U S A.* 1996; 93(21):11798–801. [PubMed: 8876217]
11. Nanni P, Nicoletti G, De Giovanni C, Landuzzi L, Di Carlo E, Cavallo F, et al. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/neu transgenic mice. *J Exp Med.* 2001; 194(9):1195–205. [PubMed: 11696586]
12. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev.* 2002; 13(2):155–68. [PubMed: 11900991]
13. Gambotto A, Tuting T, McVey DL, Kovesdi I, Tahara H, Lotze MT, et al. Induction of antitumor immunity by direct intratumoral injection of a recombinant adenovirus vector expressing interleukin-12. *Cancer Gene Ther.* 1999; 6(1):45–53. [PubMed: 10078963]
14. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity.* 2000; 13(5):715–25. [PubMed: 11114383]
15. Byrne P, McGuirk P, Todryk S, Mills KH. Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol.* 2004; 34(9):2579–88. [PubMed: 15307190]
16. Parham C, Chirica M, Timans J, Vaisberg E, Travis M, Cheung J, et al. A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol.* 2002; 168(11):5699–708. [PubMed: 12023369]
17. Lo CH, Lee SC, Wu PY, Pan WY, Su J, Cheng CW, et al. Antitumor and antimetastatic activity of IL-23. *J Immunol.* 2003; 171(2):600–7. [PubMed: 12847224]

18. Shan B, Yu L, Shimozato O, Li Q, Tagawa M. Expression of interleukin-21 and -23 in human esophageal tumors produced antitumor effects in nude mice. *Anticancer Res.* 2004; 24 (1):79–82. [PubMed: 15015579]
19. Shan BE, Hao JS, Li QX, Tagawa M. Antitumor activity and immune enhancement of murine interleukin-23 expressed in murine colon carcinoma cells. *Cell Mol Immunol.* 2006; 3(1):47–52. [PubMed: 16549049]
20. Ugai S, Shimozato O, Yu L, Wang YQ, Kawamura K, Yamamoto H, et al. Transduction of the IL-21 and IL-23 genes in human pancreatic carcinoma cells produces natural killer cell-dependent and -independent antitumor effects. *Cancer Gene Ther.* 2003; 10(10):771–8. [PubMed: 14502230]
21. Hu J, Yuan X, Belladonna ML, Ong JM, Wachsmann-Hogiu S, Farkas DL, et al. Induction of potent antitumor immunity by intratumoral injection of interleukin 23-transduced dendritic cells. *Cancer Res.* 2006; 66(17):8887–96. [PubMed: 16951206]
22. Kaiga T, Sato M, Kaneda H, Iwakura Y, Takayama T, Tahara H. Systemic administration of IL-23 induces potent antitumor immunity primarily mediated through Th1-type response in association with the endogenously expressed IL-12. *J Immunol.* 2007; 178(12):7571–80. [PubMed: 17548592]
23. Reay J, Kim SH, Lockhart E, Kolls J, Robbins PD. Adenoviral-mediated, intratumor gene transfer of interleukin 23 induces a therapeutic antitumor response. *Cancer Gene Ther.* 2009; 16(10):776–85. [PubMed: 19390568]
24. Belladonna ML, Renauld JC, Bianchi R, Vacca C, Fallarino F, Orabona C, et al. IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. *J Immunol.* 2002; 168(11):5448–54. [PubMed: 12023338]
25. Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, et al. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J Immunol.* 2003; 170(9):4432–6. [PubMed: 12707317]
26. Bilbao R, Reay DP, Hughes T, Biermann V, Volpers C, Goldberg L, et al. Fetal muscle gene transfer is not enhanced by an RGD capsid modification to high-capacity adenoviral vectors. *Gene Ther.* 2003; 10(21):1821–9. [PubMed: 12960972]
27. Oniki S, Nagai H, Horikawa T, Furukawa J, Belladonna ML, Yoshimoto T, et al. Interleukin-23 and interleukin-27 exert quite different antitumor and vaccine effects on poorly immunogenic melanoma. *Cancer Res.* 2006; 66(12):6395–404. [PubMed: 16778218]
28. Ghilardi N, Kljavin N, Chen Q, Lucas S, Gurney AL, De Sauvage FJ. Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. *J Immunol.* 2004; 172(5):2827–33. [PubMed: 14978083]
29. Hou W, Kang HS, Kim BS. Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. *J Exp Med.* 2009; 206(2):313–28. [PubMed: 19204109]
30. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. IL-23 promotes tumour incidence and growth. *Nature.* 2006; 442(7101):461–5. [PubMed: 16688182]

Figure 1. Ad.IL-23 anti-tumor activity is dependent on endogenous IL-12, but Ad.IL-12 is not
 To determine the roles of endogenous IL-12 and IL-23 in the anti-tumor activities of Ad.IL-12 and Ad.IL-23, MCA205-tumor bearing C57BL/6 mice deficient in IL-12 and IL-23 p40 (a and b) or IL-23 p19 (c and d) were treated on days 7, 9 and 11-post tumor inoculation with 5×10^{10} particles of Ad.IL-12, Ad.IL-23 or saline and survival and tumor volume monitored. Data is represented both as percent animals with tumors and as animal survival using Kaplan-Meier survival curves.

Figure 2. Ad.II-12 and Ad.II-23 co-treatment does not synergistically enhance anti-tumor effects

To determine if Ad.II-12 and Ad.II-23 co-treatment results in synergistic anti-tumor activity either locally or systemically, C57BL/6 mice bearing two MCA205 tumors were treated in one on day 7 with 5×10^8 particles of Ad.II-12 or 2.5×10^{10} particles of Ad.II-23 as follows: Ad.II-12 on day 7, followed by Ad.II-23 on day 11; Ad.II-23 on day 7 followed by Ad.II-12 on day 11; Ad.II-12 on days 7 and 11; Ad.II-23 on days 7 and 11; or Ad.Psi5 on days 7 and 11 (a and b). Alternatively, mice were treated simultaneously with 1×10^9 particles of Ad.II-12 and 5×10^{10} particles of Ad.II-23 or either virus alone (c and d). Data is represented both as percent animals with tumors and as animal survival using Kaplan-Meier survival curves.

Figure 3. Generation and characterization of adenovirus expressing single chain IL-23

Single-chain IL-23 (Ad.scIL-23) was designed to link the p40 and p19 subunits using a 15-amino acid (Gly₄Ser)₃ spacer (a). To ensure cytokine production, MCA205 cells were infected with an MOI 500 of Ad.scIL-23 and supernatants analyzed for IL-23 by ELISA 72 hours post-infection (b). To assess scIL-23 biological activity, Ad.IL-23-infected MCA205 supernatants were incubated with splenocytes for 48 hours and analyzed for IL-17 expression by ELISA (c). To assess the anti-tumor effects of Ad.scIL-23, C57BL/6 mice bearing MCA205 tumors were treated on days 7, 9 and 11 post-tumor inoculation with 5×10^{10} particles of Ad.scIL-23 and tumor volume and survival monitored. Data is represented both as tumor volume (d) and animal survival using Kaplan-Meier survival curves (e).

Figure 4. Ad.IL-12 and Ad.scIL-23 co-treatment does not synergistically enhance anti-tumor effects

To determine if co-delivery of Ad.IL-12 and Ad.scIL-23 results in synergistically enhanced anti-tumor activity, mice bearing two day 7 MCA205 tumors were treated once intratumorally with 1×10^8 particles of Ad.IL-12 and 1×10^9 particles of Ad.scIL-23, either virus alone or Ad.Psi5. Data is represented as percent animals with tumors for both injected (a) and contralateral tumors (b).

Figure 5. Ad.IL-12 and Ad.IL-23 co-treatment does not alter tumor CD8+ T-cell infiltration or angiogenesis

To determine if use of single chain IL-23 in combination with Ad.IL-12 altered patterns of CD8+ T-cell infiltration and angiogenesis compared to use of its double chain counterpart, day 15 injected tumors from mice co-treated with Ad.IL-12 and Ad.scIL-23, or either virus alone, were harvested and analyzed for CD8 (a) or CD31 (b) expression. Images were quantified using MetaMorph software. Statistically significant differences were determined using the T-test.