Adenoviral Mediated, Intra-Tumor Gene Transfer of Interleukin 23 Induces a Therapeutic Anti-tumor Response

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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. IL-23 has been shown to possess potent anti-tumor activities in several establishment models of cancer and a few therapeutic models, but the efficacy of local, adenoviral-mediated expression of IL-23 in established tumors has yet to be investigated. Here we have examined the anti-tumor activity of adenovirally-delivered IL-23 in a day 7 MCA205 murine fibrosarcoma tumor model. Three intratumoral injections of adenovirus expressing IL-23 (Ad.IL-23) significantly increased animal survival and resulted in complete rejection of 40 percent of tumors, with subsequent generation of protective immunity and MCA205-specific cytotoxic T-lymphocytes (CTLs). Additionally, we have shown that the anti-tumor activity of IL-23 is independent of IL-17, perforin and Fas ligand, but dependent on IFN-γ, CD4 and CD8 positive T-cells. These results demonstrate that direct intratumoral injection of adenovirus expressing IL-23 results in enhanced survival, tumor eradication and generation of protective immunity by generation of a Th1-type immune response.

Keywords

Interleukin 23; adenovirus; cancer; gene therapy

Introduction

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. p19 is related to the p35 subunit of IL-12 and is active only when co-expressed with IL-12 p401. The IL-23 receptor is composed of IL-12Rβ1 and the IL-23 receptor2 and is found on human Th0 and Th1 clones and several NK cell lines2. IL-23 is expressed by activated

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dendritic cells and stimulates proliferation of and IFN-γ production from memory Th1 CD4+ T-cells, suggesting an important role for this cytokine in the maintenance of Th1 immunity1. IL-23 also acts on dendritic cells to increase IL-12 production and the combination of IL-12 and IL-23 causes dendritic cells to secrete levels of IFN-γ greater than either cytokine alone3. Furthermore, IL-23-treated dendritic cells pulsed with an otherwise poorly immunogenic peptide induced a CD4+/CD8+ T-cell response *in vivo* 3. Thus, IL-23 is capable of promoting a protective immune response to tumors and, similar to IL-12, may act at the interface of innate and adaptive immunities 3. Additionally, IL-23 plays an important role in chronic inflammation, as IL-23 exposure can induce IL-17 production from activated CD4+ T-cells 4.

Multiple studies previously performed on various murine carcinoma establishment models have shown that stable expression of IL-23 in tumor lines results in decreased tumor growth, increased survival and ultimately tumor rejection with generation of anti-tumor immunity 5-9. When engineered to stably express single chain IL-23, murine colon adenocarcinoma, melanoma, and esophageal tumors exhibited regression and eventual rejection in mice5,6. Tumor rejection due to expression of IL-23 coincided with the generation of a specific adaptive immune response and appears to be dependent on natural killer (NK) and T-cells 7-9. Additionally, treatment with dendritic cells expressing IL-23, as well as systemic expression of this cytokine, resulted in inhibition of growth of pre-established tumors utilizing mechanisms that appear to be T and NK cell dependent10,11. However, the effects of local, intra-tumoral expression of IL-23 in a therapeutic cancer model have yet to be investigated.

Here we have examined the anti-tumor activity of adenovirally-delivered IL-23 (Ad.IL-23) in a day 7 MCA205 murine fibrosarcoma tumor model. Multiple intratumoral injections of Ad.IL-23 resulted in a significant increase in survival time and complete rejection in 40 percent of tumors with subsequent generation of protective immunity and MCA205-specific cytotoxic T-lymphocytes (CTLs). Additionally, we have demonstrated that the anti-tumor activity of IL-23 is independent of IL-17, perforin and Fas ligand, but dependent on IFN-γ and CD4 and CD8 positive T-cells. Taken together, these results demonstrate that direct intratumoral injection of adenovirus expressing IL-23 results in enhanced survival, tumor eradication and generation of protective anti-tumor immunity. Thus, gene transfer of IL-23 could be an effective approach for inducing systemic, anti-tumor immunity following local, intra-tumor delivery.

Materials and Methods

Adenoviruses

Adenovirus expressing IL-23 (Ad.IL-23) has been described previously12. 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 following equation: $(OD_{260})(50)/9.09 \times 10^{-13}$. Infectious titers were determined using quantitative real-time PCR as previously described13 and were approximately 100-fold less than particle titers. Viruses were aliquoted and stored at -80° C until use. Relative IL-23 expression of each adenoviral

preparation was analyzed by infecting 4×10^4 MCA205 cells with increasing MOIs of Ad.IL-23 for 1 hour at 37°C/5% CO_2 in serum free media. Complete media was added and cells were then incubated for 72 hours, after which the supernatants were harvested. IL-23 content was analyzed using the Ready-Set-Go IL-23 ELISA kit (eBioscience, San Diego, CA) following manufacturers instructions.

Animals

Female C57BL/6 and perforin, Fas ligand, IFN-γ, CD4+ and CD8+ T-cell deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The IL-17 receptor knockout mice are also on a C57BL/6 background, have been described previously and were a kind gift of Dr. Jay Kolls 14. Mice were used at 6 to 10 weeks of age. Animals were maintained under pathogen free conditions at the Biotechnology Center Animal facility at the University of Pittsburgh. All procedures preformed were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Cell lines

MCA205 fibrosarcoma and YAC-1 cells (American Type Culture Collection, Manassas, VA) 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% CO_2 and passaged every 2-3 days.

CTL assay

Two to three weeks after tumor resolution, cured mice were sacrificed and spleens harvested. Spleens were mechanically dissociated and treated with Red Cell Lysis buffer (Invitrogen, Carlsbad, CA) to remove all red blood cells. Remaining lymphocytes were cultured with recombinant human IL-2 (R&D Systems, Minneapolis, MN) for 7 days prior to use in CTL assay. CTL assays were performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) following manufacturer's instructions.

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. On days 7, 9 and 11 after tumor inoculation, mice were injected intratumorally with 5×10^{10} particles (approximately 5×10^8 PFUs) of either Ad.Psi 5 or Ad.IL-23. 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.

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. Tick marks on survival curves indicate times at which animals were censored. Log-rank tests of the survival curves provided p-values. Statistical analyses were 2-tailed, with a p value less than 0.05 considered statistically significant.

Results

Production of IL-23 from Ad.IL-23 transduced MCA205 tumor cells

To confirm IL-23 expression following Ad.IL-23-mediated transduction of MCA205 fibrosarcoma tumor cells, 4×10^4 MCA205 cells were infected with increasing MOIs of Ad.IL-23. Supernatants were harvested 72 hours post-adenoviral infection and analyzed for IL-23 expression by ELISA. As shown in Figure 1, transduction of MCA205 cells with Ad.IL-23 resulted in release of IL-23 from the infected cells, but not from Ad.Psi5 or mock infected cells. The biological activity of IL-23 produced by Ad.IL-23 infected cells has been previously confirmed and published [15].

Intratumoral injections of Ad.IL-23 result in eradication of subcutaneous MCA205 tumors

To examine the anti-tumor activity of Ad.IL-23, 6 to 8-week old C57BL/6 mice were inoculated with 1×10^5 MCA205 cells subcutaneously. On days 7, 9 and 11 post-tumor inoculation, mice were injected intratumorally with 5×10^{10} particles of Ad.IL-23 or Ad.Psi 5 in 100 uL of saline. Mice treated with Ad.IL-23 exhibited a statistically significant increase in survival (p=0.000 versus empty vector control) (Figure 2a). In six experiments involving 40 mice treated with three injections of Ad.IL-23, treated mice showed decreased tumor volumes (Figure 2c) compared to empty vector controls and exhibited an overall tumor rejection rate of 40% (Figure 2b), with no evidence of cytokine-mediated toxicity. Additionally, when mice bearing two MCA205 tumors were treated on days 7, 9 and 11 in only one tumor as described above, inhibition of tumor growth was observed in both injected and contralateral tumors, suggesting induction of a systemic anti-tumor response by IL-23 (data not shown). These results demonstrate that direct intratumoral injection of adenovirus expressing IL-23 increases survival, results in tumor rejection and generates specific and protective anti-tumor immunity.

Given that three injections of Ad.IL-23 were highly effective in inducing anti-tumor immunity, we subsequently examined whether two or even one injection of Ad.IL-23 would be sufficient to mount an anti-tumor response. Established MCA205 tumors were injected intra-tumorally either twice (days 7 and 11) or once (day 7) with 5×10^{10} particles of Ad.IL-23. Treatment with two injections of Ad.IL-23 resulted in inhibition of tumor growth and tumor rejection in 2 of 5 mice (data not shown) whereas one treatment, while significantly increasing animal survival (Figure 2d), did not result in tumor rejection in any animals (5 mice total) (Figure 2e). These results suggest that while even one dose of Ad.IL-23 can significantly increase the survival of tumor-bearing mice, multiple injections are necessary for maximal anti-tumor effect.

Ad.IL-23-mediated tumor rejection results in resistance to MCA205 challenge and generation of tumor-specific cytotoxic T-lymphocytes

To determine if Ad.IL-23 mediated tumor rejection led to generation of protective immunity, cured mice were challenged with 1×10^5 MCA205s in the contralateral flank 10 weeks after tumor rejection. Naïve mice were concurrently inoculated with MCA205s to confirm tumor take. Figure 3a shows two representative Ad.IL-23 treated, cured mice, challenged with MCA205s, as well as simultaneously inoculated naïve mice. Overall, "cured" mice remained tumor-free for up to three months and all animals (8 mice total) challenged with a second dose of MCA205 cells were resistant to tumor development.

To determine if treatment of MCA205 tumors with Ad.IL-23 resulted in the generation of tumor-specific CTLs, spleens were harvested from Ad.IL-23-treated, tumor free mice within 2-3 weeks of tumor resolution. Purified lymphocytes were cultured with rhIL-2 for 7 days and re-stimulated with MCA205 cells. CTL activity was measured by LDH release from target cells. Cytotoxic activity was detected against MCA205 tumor cells, but not against YAC-1 (NK cell sensitive) cells (Figure 3b). The cytotoxic activity of splenocytes against MCA205, but not YAC-1, cells suggests that treatment of subcutaneous MCA205 tumors with Ad.IL-23 resulted in generation of anti-tumor immunity by production of MCA205-specific CTLs. This result is consistent with the ability of Ad.IL-23 treated, cured mice to resist MCA205 tumor challenge.

Additionally, angiogenesis in tumors of mice treated with Ad.IL-23 was investigated. Tumors from Ad.IL-23 treated mice were harvested and analyzed for CD31 by immunohistochemistry. Although not significant, a trend towards decreased angiogenesis was observed in Ad.IL-23 treated animals versus controls (data not shown), suggesting that IL-23 mediated inhibition of angiogenesis is at least partially responsible for its anti-tumor activity.

IL-23 anti-tumor activity is independent of endogenous IL-17 production

The results above clearly demonstrate that adenoviral mediated, intra-tumoral delivery of IL-23 results in a significant anti-tumor response. To examine the nature of this immune response, the ability of Ad.IL-23 to induce regression of MCA205 tumors was examined in mice deficient for certain immunoregulatory molecules or cell types. In *vivo*, IL-23 strongly induces production of IL-17[16], which has been previously shown to possess anti-tumor activity[17]. To determine if IL-23-mediated, endogenous IL-17 production may be responsible for the observed anti-tumor effects, IL-17 receptor-deficient mice were inoculated with subcutaneous MCA205 tumors and treated with Ad.IL-23 on days 7, 9 and 11 post-tumor inoculation. IL-23 treatment significantly increased animal survival (p=0.047) compared to empty vector treated mice (Figure 4a). Additionally, 60 percent (3 of 5) of tumor-bearing mice experienced tumor resolution with kinetics similar to wild-type mice (Figure 4b) and were resistant to tumor challenge. Taken together, these data suggest that adenovirally-delivered IL-23 anti-tumor activity is not mediated by endogenous IL-17 production.

IL-23 anti-tumor activity is independent of perforin

Perforin is produced by CD8+ T-cells and NK cells and is released upon contact with the target cell to induce cell death. To determine if the anti-tumor activity of adenovirally delivered IL-23 is dependent on perforin-mediated lysis of tumor cells, perforin-deficient mice with established MCA205 tumors were treated with Ad.IL-23 as described above. Treatment with Ad.IL-23 resulted in a significant increase in survival (p=0.013) (Figure 5a), and led to tumor rejection in 30 percent of mice (3 of 10) (Figure 5b). The cured perforin-deficient mice experienced tumor rejection in a similar time frame as cured, Ad.IL-23 treated wild-type mice and were resistant to tumor challenge (data not shown). These data suggest that adenovirally delivered IL-23 does not utilize perform-mediated killing as an anti-tumor effector mechanism.

IL-23 anti-tumor activity is independent of Fas ligand

Interactions between Fas and Fas ligand (FasL) are used by activated CD8+ T-cells and some CD4+ effector cells to kill target cells via apoptosis. To determine if the anti-tumor activity of adenovirally delivered IL-23 requires Fas-Fas ligand interactions to eradicate tumor cells and generate anti-tumor immunity, FasL-deficient mice were inoculated with tumors and treated as described above. Interestingly, while Ad.IL-23 treatment did not lead to tumor rejection in any treated animals (10 total) (Figure 5d), it did significantly increase survival compared to empty vector controls (p=0.000) (Figure 5c), leading us to conclude that IL-23 is able to function through a Fas-FasL-independent mechanism to exert its anti-tumor effects. However, given that treatment did not result in eradication of tumors in any of the mice, it is also possible that at least part of the anti-tumor effect of IL-23 involves a Fas-FasL-dependent pathway.

Interferon gamma is necessary for IL-23 anti-tumor activity

Cytotoxic T-lymphocytes and a subset of Th1 T-cells secrete IFN-γ upon engagement of the T cell receptor in order to exert their cytotoxic effects on target cells. To determine if IFN-γ is necessary for the anti-tumor activity of adenovirally delivered IL-23, IFN-γ deficient mice with established tumors were treated as described above. Tumor-bearing mice treated with Ad.IL-23 showed no significant increase in survival when compared to empty adenoviral vector controls (Figure 5e). Additionally, none of the treated mice completely rejected their tumor (0 of 10) (Figure 5f), suggesting that adenovirally delivered IL-23 utilizes IFN-γ as an effector mechanism to exert its anti-tumor effects.

Anti-tumor activity of IL-23 is dependent on the presence of CD4+ T-cells

CD4+ T-cells have a variety of functions, including "helping" the activation of CTLs and directly killing target cells, utilizing mechanisms including Fas-FasL interactions and release of IFN-γ. To determine if the anti-tumor activity of adenovirally delivered IL-23 is dependent on the presence of CD4+ T-cells, CD4 deficient mice were inoculated with MCA205 tumors and treated with Ad.IL-23. Again, Ad.IL-23 treated mice showed no significant increase in survival when compared to empty adenoviral vector controls (Figure 6a) and none rejected their tumor (10 animals total) (Figure 6b). These data suggest that

adenovirally delivered IL-23 enhances the activity of CD4+ T-cells to exert optimal anti-tumor effects.

IL-23 utilizes CD8+ T-cells to exert its anti-tumor effects

Since IL-23 anti-tumor activity is dependent on IFN-γ, a mechanism utilized by CD8+ T-cells to exert cytotoxic effects, it is possible that CD8+ T-cells are responsible for the anti-tumor effects of IL-23. Thus CD8+ T-cell deficient mice were inoculated with MCA205 tumors and treated as described above. Interestingly, while treatment with Ad.IL-23 did lead to tumor rejection in 20 percent of animals (2 of 10) (Figure 6d), IL-23 therapy did not significantly increase animal survival (Figure 6c). This suggests that at least part of the anti-tumor effect of adenovirally delivered IL-23 is mediated through CD8+ T-cell activity, in addition to other effector mechanisms that may be important in tumor elimination.

Discussion

The use of gene therapy for the treatment of cancer has been proven effective in many mouse cancer models and comprises the vast majority of human gene therapy clinical trials performed to date18. Here we have investigated the potential anti-tumor activity of the IL-12 family member IL-23. IL-23 is a relatively new member of the IL-12 family of heterodimeric cytokines and shares many immunostimulatory1-3 and anti-tumor activities with IL-1219-22. IL-23 has been shown to possess anti-tumor activity in the context of various establishment models of cancer5-9 and to inhibit established tumor growth when overexpressed systemically or locally via dendritic cells10,11. We chose to investigate the ability of adenovirally delivered IL-23 (Ad.IL-23) to inhibit tumor growth in a therapeutic mouse model of cancer for a number of reasons. First, systemic overexpression of immunostimulatory cytokines may be associated with toxicity, as is the case with systemically delivered IL-1222. Additionally, as IL-23 is associated with a variety of autoimmune inflammatory diseases which are believed to be mediated at least in part by the generation of a specific subset of T-cells that produce IL-1723, we hypothesized that local, rather than systemic, expression would reduce the risk of the unintentional development of any autoimmune pathology. Finally, expression of immunostimulatory cytokines within the tumor cells greatly increases the chances of generating an effective immune response against the tumor itself and is technically much simpler than transduction of any intermediate cell type.

Our results clearly demonstrate that treatment of day 7 MCA205 tumors with repeated intra-tumoral injections of Ad.IL-23 results in a significant increase in animal survival when compared to empty vector treated controls. In a total of 6 experiments involving 40 mice, Ad.IL-23 treated mice overall exhibited decreased tumor volume, with 40 percent of mice rejecting their tumors in about 25 days (Figures 2a-c). The anti-tumor activity of IL-23 is consistent with previously published reports showing that stable expression of IL-23 in tumor cell lines leads to tumor rejection in tumor establishment models5-9. Although the overall cure rate in our experiments was slightly lower than was obtained in previous studies using establishment models (70 percent versus our 40), this disparity is likely due to differences in the model. An established tumor is more difficult to eradicate than a tumor

expressing IL-23 from the onset. Additionally, differences in IL-23 expression levels in therapeutic versus establishment models may also ultimately affect the outcome, as it has been suggested that IL-23 expression levels may dictate whether IL-23 exhibits pro- or anti-tumor effects24.

It is also necessary to note that while this manuscript was in preparation, a paper demonstrating a prophylactic, but not therapeutic, effect of adenovirally delivered IL-23 in variety of murine tumors was published25. This disparity in results may be due to the use of different murine tumor models, use of a mutant form of p4025 or initiating treatment at a different stage of the tumor model. In our studies, tumor bearing mice were treated on days 7, 9 and 11 starting with an average tumor size of 4mm compared to the studies performed in the previously mentioned paper, in which treatment was initiated when the average tumor volume reached 7mm in diameter.

The tumor eradication rate of Ad.IL-23 also was dependent upon the number of treatments, while enhancement of survival was not. Treating the established tumors with two or one injections of Ad.IL-23 (Figures 2d and 2e), the percent of mice with complete tumor regression was reduced, with three injections on alternate days generating the most optimal effects. However, treating mice either once or twice increased survival. We chose three separate injections of Ad.IL-23 as our treatment plan as it yielded both enhanced survival compared to controls and maximum tumor rejection rates. However, increased survival even at the lowest dose has therapeutic implications, as this suggests that use of low dose Ad.IL-23 in combination with other Th1-polarizing cytokines, such as IL-12, will provide even greater therapeutic response with minimal adenoviral or cytokine mediated toxicity.

Interestingly, all cured, Ad.IL-23 treated mice exhibited protective immunity upon tumor challenge up to three months after rejection of the initial tumor (Figure 3a and data not shown). In contrast, only 56 percent (5 of 9) of mice treated with adenovirus-expressing IL-12 were resistant to tumor challenge (data not shown). This suggests that intra-tumoral, adenoviral delivery of IL-23 may activate a robust memory T cell response and generate superior long-term protective immunity. Such results are promising in the context of using IL-23 in conjunction with IL-12, as presumably using IL-23 in combination with IL-12 will decrease the effective dose of IL-12 needed, hence reducing toxicity and increasing the duration of protective anti-tumor immunity.

IL-23 has been shown to induce IL-17 production by aiding the development of Th17 T-cells16. Since IL-17 itself may possess anti-tumor activity17, we investigated whether the anti-tumor activity of IL-23 is mediated by endogenous IL-17 production. Using IL-17 receptor deficient mice, we demonstrated that the anti-tumor activity of IL-23 appears to be independent of endogenous IL-17 expression. IL-17 receptor-deficient mice treated with Ad.IL-23 exhibited increased survival and enhanced tumor rejection compared to animals treated with empty adenoviral vector (Figures 4a and 4b). Thus, although IL-23 has been shown to be a potent activator of IL-17 producing cells4, in the context of adenovirally transduced tumor cells, either Th17 cells do not play a role in the anti-tumor activity or the development of Th1 T-cells is favored.

We next sought to determine the immune cell types involved in enacting IL-23-mediated anti-tumor activity. Tumor-bearing CD4 and CD8 positive T-cell deficient mice treated with Ad.IL-23 exhibited no increase in survival compared to controls (Figures 6a and 6c, respectively), indicating their requirement for IL-23 anti-tumor activity. It must be noted, however, that in two CD8+ T-cell deficient mice, Ad.IL-23 treatment led to tumor rejection, suggesting the involvement of other effector mechanisms in tumor eradication. To determine the effector mechanism(s) employed by these cells, IFN-γ, perforin and Fas ligand deficient, tumor-bearing mice were treated with Ad.IL-23. Mice deficient in IFN-γ were completely refractory to IL-23 treatment (Figures 5e and 5f), whereas perforin and Fas ligand mice exhibited increased survival and tumor rejection compared to controls (Figures 5a, b and 5c, d, respectively). Cytotoxicity of splenocytes harvested from cured, wild-type animals demonstrate that tumor eradication was mediated, at least in part, by MCA205-specific CTLs (Figure 3b), consistent with previous reports[10,11]. Collectively, these data are consistent with previous results suggesting that the primary mechanism of IL-23 anti-tumor activity is CD4+ T-cell-mediated activation of CD8+ T-cells to produce IFN-γ[10].

Overall, our results demonstrate that adenoviral delivery of IL-23 into the tumor microenvironment of early stage tumors is therapeutic and able to generate protective immunity. Furthermore, our results show that the anti-tumor activity of IL-23 is independent of endogenous IL-17, perforin and, in part, FasL, but dependent on CD4 and CD8 positive T-cells and IFN-γ. Together, these data imply that tumor cell expression of IL-23 drives a Th1-type immune response, which is responsible for its anti-tumor activity. Our results indicate that direct adenoviral delivery of IL-23 into the tumor microenvironment to generate protective anti-tumor immunity is feasible and may posses clinical benefit when used either alone or in combination with other cytokines, such as IL-12.

Acknowledgments

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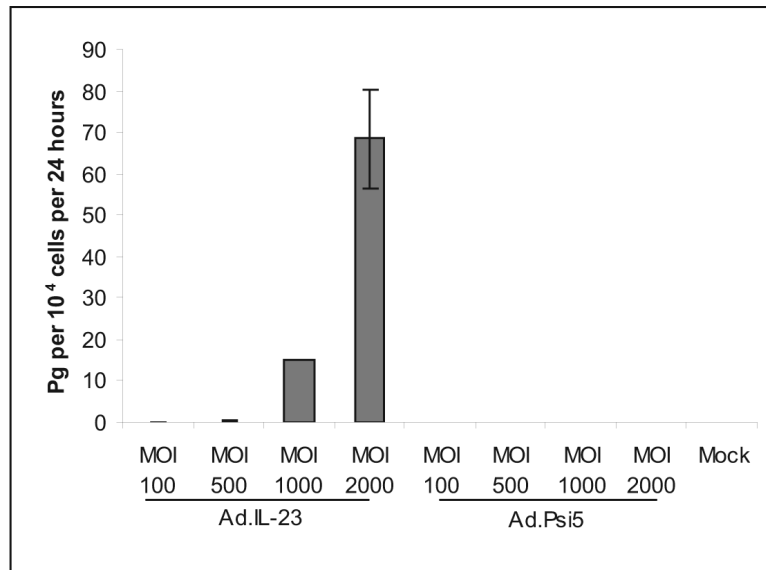
Figure 1.
Ad.IL-23, but not Ad.Psi5, expresses detectable levels of cytokine in MCA205 cell supernatant. To demonstrate that adenoviruses express detectable levels of cytokine, 4×10^4 MCA205 fibrosarcoma cells were infected with increasing MOIs of Ad.IL-23 and Ad.Psi5 for 1 hour at 37°C/5% CO_2 in serum free media. Cell supernatants were harvested 72 hours post-infection and cytokine expression analyzed by ELISA. Data is represented as picograms of cytokine per 10^4 cells per 24 hours.
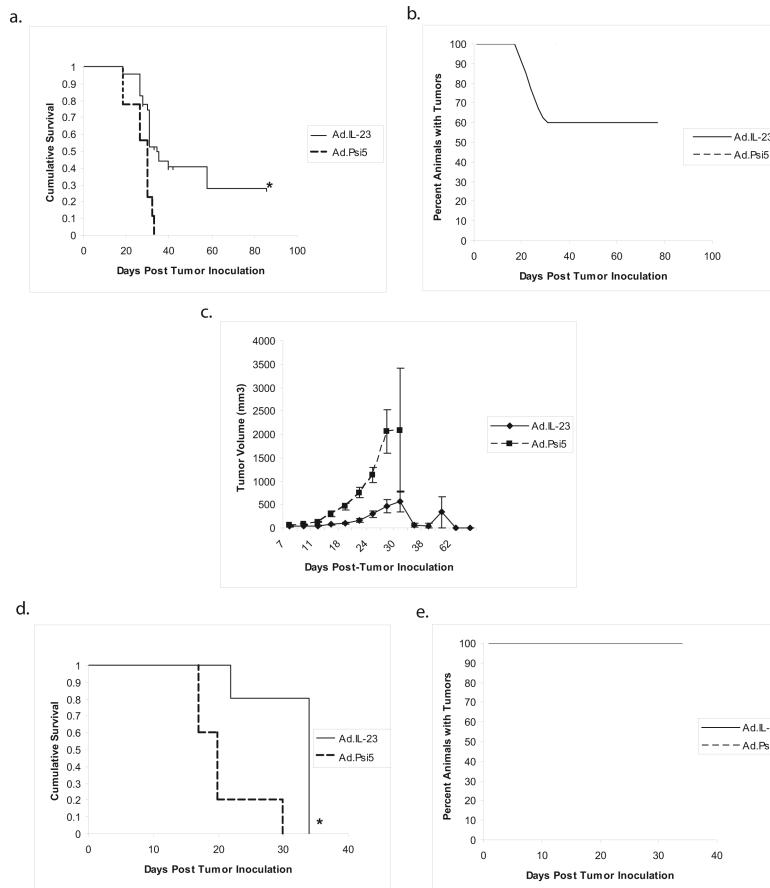
Figure 2.
Ad.IL-23 exhibits potent anti-tumor activity in the 7-day MCA205 fibrosarcoma tumor model. Six to 8 week old C57BL/6 mice were inoculated with 1×10^5 MCA205 cells subcutaneously. Mice were injected intratumorally with 5×10^{10} particles of either Ad.IL-23 or Ad.Psi5 (empty vector) on days 7, 9 and 11 post-tumor inoculation. Tumor size was monitored using a metric caliper until tumor resolution or until animals were sacrificed due to excessive ulceration or tumor size. Data is represented as animal survival using Kaplan-Meier survival curves (a), percent animals with tumors (b) and average tumor volume over time (c). Alternatively, mice were treated with a single injection of 5×10^{10} particles of Ad.IL-23 or Ad.Psi5 on day 7. Data is represented as animal survival and percent animals with tumors (d and e, respectively). Error bars represent ± standard error and * indicates Log-rank tests show that Ad.IL-23 treatment leads to a significant increase in survival compared to empty vector controls in mice treated both 3 times (p=0.000) and only once (p=0.007).
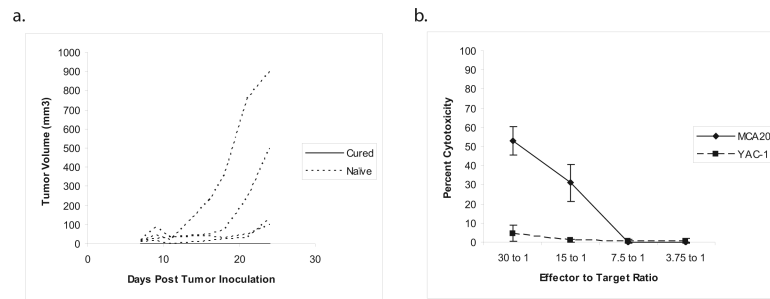
Figure 3.
IL-23-mediated tumor eradication results in generation of protective immunity and tumor-specific cytotoxic T-lymphocytes. (a) To determine if Ad.IL-23 mediated tumor rejection leads to development of MCA205 protective immunity, cured mice were inoculated with 1×10^5 MCA205 cells 10 weeks after tumor rejection. Naïve mice were concurrently inoculated to verify tumor take. Tumor volume was monitored until tumor size or necrosis necessitated sacrifice. (b) To determine if tumor suppression in mice treated with adenovirus expressing IL-23 is mediated by cytotoxic T-cells, splenocytes from cured mice were harvested, incubated with rhIL-2 for 7 days and analyzed for cytotoxicity against MCA205 and YAC-1 cells. Cytotoxicity was observed at various effector to target cell ratios against MCA205 cells.
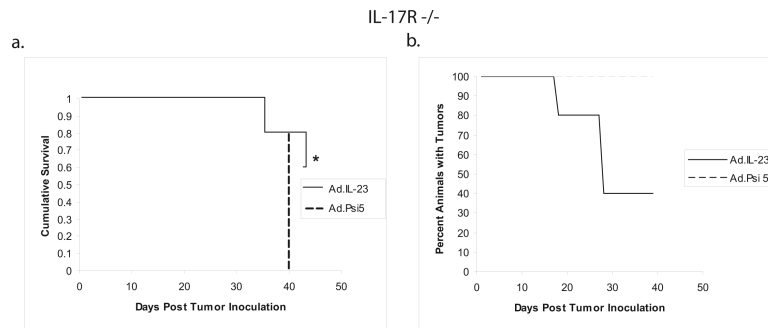
Figure 4.
Anti-tumor activity of IL-23 is independent of IL-17. To determine if the anti-tumor activity of adenovirally delivered IL-23 is dependent on induction of IL-17 production, 8 to 10 week old IL-17 receptor-deficient mice were inoculated with 1×10^5 MCA205 cells subcutaneously and treated on days 7, 9 and 11 post-tumor inoculation with 5×10^{10} particles of either Ad.IL-23 or Ad.Psi5. Data is represented as animal survival using Kaplan-Meier survival curves (a) and as percent animals with tumors (b). * signifies the Log rank test indicates a significant increase in the survival of Ad.IL-23 treated mice compared to controls (p=0.047).
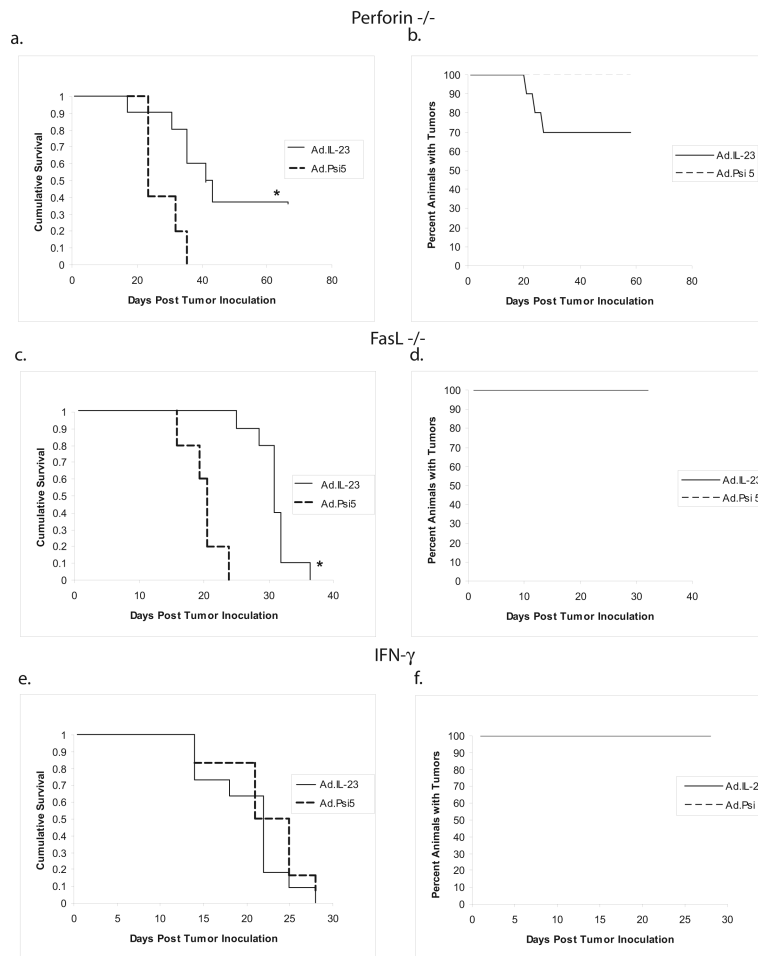
Figure 5.

IFN-γ plays a significant role in the anti-tumor activity of IL-23, while Fas ligand and perforin do not. To determine the primary effector mechanisms involved in the anti-tumor activity of IL-23, mice deficient in perforin (a-b), FasL (c-d) and IFN-γ (e-f) were inoculated with MCA205 tumors and treated with 5×10^{10} particles of Ad.IL-23 or Ad.Psi5 on days 7, 9 and 11 post-tumor inoculation. Data is represented as animal survival using Kaplan-Meier survival curves and as percent animals with tumors. * indicates that Log rank tests of survival curves show a significant increase in survival for perforin and Fas ligand-deficient mice treated with Ad.IL-23 compared to empty vector (p=0.013 and p=0.000, respectively). No significant difference was seen between Ad.IL-23 and control treatment in IFN-γ deficient mice.
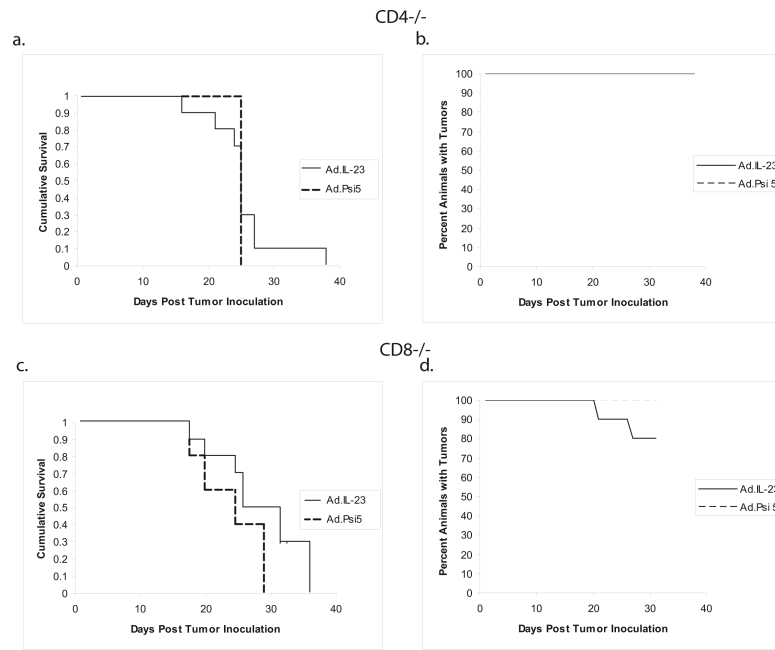
Figure 6.
Anti-tumor activity of IL-23 is dependent on CD4 and CD8 positive T-cells. Mice deficient in either CD4 (a-b) or CD8+ T-cells (c-d) were inoculated with 1×10^5 MCA205 cells and treated with 5×10^{10} particles of Ad.IL-23 or Ad.Psi 5 on days 7, 9 and 11. Data is represented both as animal survival using Kaplan-Meier survival curves and percent animals with tumors. Log-rank tests show no significant increase in survival in either CD4 or CD8-knockout mice treated with Ad.IL-23 compared to controls.