**ORIGINAL ARTICLE**

**Oncolytic adenovirus co-expressing IL-12 and IL-18 improves tumor-specific immunity via differentiation of T cells expressing IL-12Rβ2 or IL-18Rα**

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The oncolytic adenovirus (Ad) is currently being advanced as a promising antitumor remedy as it selectively replicates in tumor cells and can transfer and amplify therapeutic genes. Interleukin (IL)-12 induces a potent antitumor effect by promoting natural killer (NK) cell and cytotoxic T cell activities. IL-18 also augments cytotoxicity of NK cells and proliferation of T cells. This effect further enhances the function of IL-12 in a synergistic manner. Therefore, we investigated for the first time an effective cancer immunogene therapy of syngeneic tumors via intratumoral administration of oncolytic Ad co-expressing IL-12 and IL-18, RdB/IL-12/IL-18. Intratumoral administration of RdB/IL-12/IL-18 improved antitumor effects, as well as increased survival, in B16-F10 murine melanoma model. The ratio of T-helper type 1/2 cytokine as well as the levels of IL-12, IL-18, interferon-γ and granulocyte–macrophage colony-stimulating factor was markedly elevated in RdB/IL-12/IL-18-treated tumors. Mice injected with RdB/IL-12/IL-18 also showed enhanced cytotoxicity of tumor-specific immune cells. Consistent with these results, immense necrosis and infiltration of NK cells, as well as CD4+ and CD8+ T cells, were observed in RdB/IL-12/IL-18-treated tumor tissues. Importantly, tumors treated with RdB/IL-12/IL-18 showed an elevated number of T cells expressing IL-12Rβ2 or IL-18Rα. These results provide a new insight into therapeutic mechanisms of IL-12 plus IL-18 and provide a potential clinical cancer immunotherapeutic agent for improved antitumor immunity.

*Gene Therapy* (2011) **18**, 898–909; doi:10.1038/gt.2011.37; published online 31 March 2011

**Keywords:** cancer immunogene therapy; oncolytic adenovirus; IL-12; IL-18; T cells expressing IL-12Rβ2 or IL-18Rα

**INTRODUCTION**

Over the past decade, tumor immunology has advanced in uncovering the molecular and cellular mechanisms responsible for cancer pathophysiology and for enhancing the immune response against tumors, yet there are still many unresolved problems concerning which tumors can escape, or suppress, the immune response. Therefore, immunotherapy for malignant diseases needs novel, improved clinical protocols that minimize tumor-induced immunological tolerance while simultaneously inducing tumor eradication. One potential strategy is a cytokine immunogene therapy that has a great potential for rejecting cancer cells by activating tumor-specific immune responses in cancer-bearing hosts.

Interleukin (IL)-12 is one of the most effective and promising antitumor cytokines. It is a heterodimeric cytokine composed of two different disulfide-linked subunits, designated p35 and p40, and is mainly produced by activated macrophages and dendritic cells (DCs). IL-12 stimulates interferon-γ (IFN-γ) production by natural killer (NK) cells, T cells, DCs and macrophages. Similarly, it facilitates T helper type 1 (Th1) differentiation and augments the cytolytic effect of NK cells and cytotoxic T lymphocytes (CTLs). Previous preclinical studies of IL-12 reported enhanced antitumor immunity.1,2 More recently, we showed an enhanced antitumor effect of an oncolytic adenovirus (Ad) that expresses both IL-12 and B7-1 in a murine melanoma B16-F10 tumor model.

IL-18, IFN-γ-inducing factor, is secreted mainly by activated macrophages and DCs.4 IL-18 plays a significant role in cell-mediated immune responses and is capable of generating an antitumor immune response via induction of IFN-γ production from,5,6 enhancing the cytotoxicity of,7 and stimulating the proliferation of T and NK cells.6,8 Several studies have shown the antitumor efficacy of IL-18 in murine tumor models. For example, IL-18 exerted antitumor immunity by activating NK cells and establishing cytotoxic CD4+ T cells in Meth A sarcoma-bearing mice.9 In addition, IL-18 was also shown to be a potent adjuvant, enhancing systemic antitumor immune responses and vaccine efficacy.10 Therefore, IL-18 represents a promising therapeutic candidate for cancer immunogene therapy.

Previous studies reported that IL-18 is able to drive the Th2 immune response in an IL-4-dependent manner, but induces a Th1 immune response in the presence IL-12.11,12 These results suggest that IL-18 alone could suppress the Th1 immune response via activation of Th2 immune response. Therefore IL-18, acting in concert with IL-12, may be important in effective immunotherapy. Consistent with this suggestion, an earlier study reported that IL-12 plus IL-18 polarized Th1 immunity, resulting in the generation of potent tumor-reactive T cells.13 This combination also enhanced the cytolytic effect as well as IFN-γ production from T and NK cells, as well as the proliferation in T cells.11,14 Furthermore, previous studies reported that IL-12 in combination with IL-18 inhibited tumor angiogenesis, leading to tumor regression.15

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Received 12 August 2010; revised 23 January 2011; accepted 24 January 2011; published online 31 March 2011
In this study, we show for the first time an effective cancer immunogene therapy of syngeneic tumors via intratumoral administration of oncolytic Ad co-expressing IL-12 and IL-18. We also show the underlying mechanism of oncolytic Ad co-expressing IL-12 and IL-18, showing that antitumor immunity is associated with elevation of Th1/Th2 cytokine ratio and upregulation of IL-12, IL-18, IFN-γ and granulocyte–macrophage colony-stimulating factor (GM-CSF) within the tumor tissues. Moreover, we show that the induction of an optimal tumor milieu, generating effective tumor-specific immunity through the upregulation of Th1 cytokines, enhances the generation of fully differentiated T cells expressing IL-12Rβ2 or IL-18Rα.

RESULTS
Generation of oncolytic Ad-mediated IL-12 and IL-18
Three oncolytic Ads expressing IL-12 alone, IL-18 alone, or IL-12 and IL-18 were generated in the E1B-deleted and E1A-mutated oncolytic Ad, oncolytic Ad vector, Ad-ΔE1Bmt7 (RdB) (Figure 1a). To confirm IL-12 and IL-18 secretion from these Ads, B16-F10 melanoma cells were infected with RdB, RdB/IL-12, RdB/IL-18, or RdB/IL-12/IL-18 at various multiplicities of infection (MOIs), and culture supernatants were obtained 96 h after infection to examine IL-12 and IL-18 production. A dose (MOI)-dependent increase in IL-12 and IL-18 expression was observed in the cells infected with RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 at different MOIs (Figures 1b and c). Interestingly, the addition of IL-12 or IL-18 seemed to adversely affect the expression of either cytokine induced by oncolytic Ad. Both RdB/IL-12 and RdB/IL-18 induced higher IL-12 and IL-18 expression compared with RdB/IL-12/IL-18. Specifically, the expression of IL-12 and IL-18 from RdB/IL-12 (100 MOI) and RdB/IL-18 (200 MOI) were 43,428 ± 797 and 1150 ± 68, respectively, whereas those from RdB/IL-12/IL-18 were 13,468 ± 48 and 445 ± 8, showing about a threefold decrease in cytokine expression.

To further investigate whether IL-12, IL-18, or IL-12 plus IL-18 expression would alter viral replication, RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 were evaluated for their capability to induce viral cytopathic effects in a variety of cell lines. Because murine cells tend to be less sensitive or even resistant to Ad infection compared with human cells, we employed three different types of human cancer cell lines (U343, C33A and A549) from varying histological types. Cells were infected with RdB (cognate oncolytic Ad), RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18, along with Ad-ΔE1 as a negative control. The cells were then treated with crystal violet to show the relative extent of cell lysis. RdB/IL-12, RdB/IL-18 and RdB/IL-12/IL-18 generated cytopathic effects equivalent to RdB alone in all cell lines tested, showing that IL-12, IL-18, or IL-12 plus IL-18 expression does not decrease the viral replication ability of oncolytic Ad (Supplementary Figure 1).

Figure 1 Characterization of adenovirus (Ad) vectors used in this work. (a) Schematic representations of the IL-12 or IL-18 gene inserted into the E3 region of RdB, respectively. RdB/IL-12/IL-18 is comprised of the IL-12 and IL-18 genes in the E1 and E3 region of RdB, respectively (asterisk indicates mutation at Rb binding site of E1A). (b, c) Expression of IL-12 (b) and IL-18 (c). The concentration of cytokines was measured in the culture supernatants 96 h after infection by ELISA. Mean values and s.e.m. of three or more experiments, each performed in triplicate, are shown.
Enhanced antitumor effect of IL-12- and IL-18-expressing oncolytic Ad

To ascertain whether RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 induced the suppression of tumor growth in syngeneic mice, we injected 5×10⁵ cells of B16-F10 melanoma subcutaneously into C57BL/6 mice. When the tumor volume averaged 80 mm³, mice were injected intratumorally with phosphate-buffered saline (PBS), RdB, RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 once every other day for a total of three injections. As presented in Figure 2a, control tumors treated with PBS showed aggressive growth, leading to a tumor volume of 3997.1±412.2 mm³ 14 days after viral treatment. In marked comparison, RdB/IL-12-, RdB/IL-18- and RdB/IL-12/IL-18-treated tumors reached an average volume of 202.4±43, 1009±181.7 and 26.5±10.3 mm³, respectively, showing 95% (RdB/IL-12), 75% (RdB/IL-18) and 99% (RdB/IL-12/IL-18) tumor growth inhibition compared with PBS control (P<0.05). By day 20 following treatment, tumor re-growth was observed in RdB/IL-18- and RdB/IL-12/IL-18-treated tumors, showing 3946.7±817.8 and 960.1±349.3 mm³, respectively, whereas those treated with RdB/IL-12/IL-18 maintained inhibition of tumor growth (145±64.7 mm³) (P<0.01). Moreover, RdB/IL-12/IL-18 elicited more prolonged suppression of tumor growth than RdB/IL-12 (P<0.01) (Supplementary Figure 2). Furthermore, RdB/IL-12/IL-18-treated mice showed higher survival rates compared with either RdB/IL-12 or RdB/IL-18 (P<0.01). All animals treated with RdB/IL-12/IL-18 remained viable 28 days after the beginning of the treatment, whereas only 16.7% of those treated with RdB/IL-18 and 50% of those treated with RdB/IL-12 survived for the same period of time (Figure 2b). Taken together, these results suggest that RdB/IL-12/IL-18 significantly prolonged survival and enhanced antitumor efficacy compared with either RdB/IL-12 or RdB/IL-18 in the B16-F10 murine melanoma model.

In vivo oncolytic Ad treatment increased local expression of IL-12, IL-18, IFN-γ and GM-CSF

To determine the amount of IL-12 and IL-18 produced in RdB/IL-12-, RdB/IL-18- or RdB/IL-12/IL-18-treated mice, tumor tissues were harvested 3 days following the last viral injection. As seen in Figures 3a and b, a minimal amount of IL-12 was observed in the tumors treated with PBS, RdB or RdB/IL-18. In contrast, tumors treated with RdB/IL-12 or RdB/IL-12/IL-18 showed high concentrations of IL-12 (57.3±6.6 pg mg⁻¹ for RdB/IL-12 and 213.6±9.9 pg mg⁻¹ for RdB/IL-12/IL-18, P<0.05) (Figure 3a). Similarly, RdB/IL-18- or RdB/IL-12/IL-18-treated tumors produced significantly higher levels of IL-18 (61±1.8 pg mg⁻¹ for RdB/IL-18 and 213.4±27.1 pg mg⁻¹ for RdB/IL-12/IL-18) than did those treated with PBS, RdB or RdB/IL-12 (18.6±3.3, 14.3±2.9 and 20.4±3.1 pg mg⁻¹, respectively, P<0.05) (Figure 3b). Interestingly, IL-12 and IL-18 levels in tumor tissues treated with RdB/IL-12/IL-18 were synergistically greater than those treated with RdB/IL-12 or RdB/IL-18 in vivo, even though in vitro, IL-12 and IL-18 levels from RdB/IL-12/IL-18-treated cells were much lower than those of either RdB/IL-12- or RdB/IL-18-treated cells.

Given that IL-12 and IL-18 can act synergistically to enhance the production of IFN-γ and GM-CSF from T and NK cells,11,13,14 we examined IFN-γ and GM-CSF production in the RdB/IL-12/IL-18-treated tumor tissue. As shown in Figure 3c and d, PBS-, RdB- and RdB/IL-12/IL-18-injected mice showed relatively low levels of IFN-γ (342.6±54.6, 179.2±80.7 and 391.2±159.7 pg mg⁻¹, respectively). In comparison, RdB/IL-12- or RdB/IL-12/IL-18-injected mice showed relatively low levels of IFN-γ (342.6±54.6, 179.2±80.7 and 391.2±159.7 pg mg⁻¹, respectively). In addition, RdB/IL-12/IL-18-injected mice showed high levels of IFN-γ (2364.4±265.4 pg mg⁻¹ for RdB/IL-12 and 3590.6±231.5 pg mg⁻¹ for RdB/IL-12/IL-18, P<0.05) (Figure 3c). In addition, RdB/IL-12/IL-18-treated tissue showed the highest GM-CSF expression (27.7±4.7 pg g⁻¹) compared with PBS- (2.5±4.7 pg g⁻¹), RdB- (3.9±1.4 pg g⁻¹), RdB/IL-12- (8.5±0.6 pg g⁻¹) and RdB/IL-18-treated tumor tissue (6.8±1.4 pg g⁻¹) (Figure 3d). These results show that RdB/IL-12/IL-18-treated tumors produced significantly higher amounts of IL-12, IL-18, IFN-γ and GM-CSF than any of the other groups.

Increase of Th1/Th2 cytokine ratio

An aberrant Th1/Th2 cytokine balance with elevated Th2 cytokine levels, which favors development and progression of cancers, was found in various tumor-bearing patients, and a correlation between cytokine levels and the efficacy of cancer treatments has also been reported.16,17 Hence, we further investigated whether RdB/IL-12/IL-18, which produced markedly higher amounts of Th1 cytokines (IFN-γ and GM-CSF) than any of the other groups, could converse from Th2 immunity toward Th1 immunity in tumor tissues. Tumor tissues from mice at 3 days following the final viral injection were harvested and analyzed with Th1/Th2/Th17 cytometric bead assay (CBA) kit (BD Biosciences PharMingen, San Diego, CA, USA). As IL-2, IL-4, IL-10 and IL-17 were not detected in the tumor tissues of all groups (data not shown), the ratio of Th1/Th2 cytokine was
estimated from the ratios of IFN-γ/IL-6 and tumor necrosis factor-α (TNF-α)/IL-6. As presented in Figure 4, RdB/IL-12/IL-18-treated tumor tissue exhibited the highest IFN-γ/IL-6 and TNF-α/IL-6 ratio value compared with PBS-, RdB-, RdB/IL-12- or RdB/IL-18-treated tumor tissue (P<0.01), suggesting that RdB/IL-12/IL-18 polarizes T cell responses toward the type 1 pattern by enhancing Th1 cytokine and suppressing Th2 cytokine in the tumor microenvironment.

**Generation of a tumor-specific immune response**

Results from enzyme-linked immunosorbent assay (ELISA) and CBA assay in tumor tissues indicate that RdB/IL-12/IL-18 might generate a tumor microenvironment that is more favorable to activate tumor-specific immune cells than an oncolytic Ad expressing either IL-12 or IL-18 alone. Therefore, we examined whether RdB/IL-12/IL-18 could enhance immune responses in vivo by assessing the number of immune cells expressing IFN-γ, a cytokine secreted by activated T cells. Splenocytes from mice at 3 days following the final viral injection were harvested and co-cultured with irradiated B16-F10 tumor cells for 3 days in the presence of recombinant human IL-2. Co-cultured splenocytes were serially seeded into the IFN-γ ELISPOT plate at concentrations from 2.5×10⁴ to 1×10⁶ cells per well and the number of IFN-γ-secreting cells was determined. As shown in Figure 5, the frequency of IFN-γ-secreting immune cells recovered from mice injected with RdB/IL-12/IL-18 was significantly greater than
Figure 5 Generation of a tumor-specific immune response. Subcutaneous tumors derived from B16-F10 cells were inoculated with RdB, RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 along with PBS as a negative control. Ad-treated mice were killed 3 days following last administration, and IFN-γ ELISPOT and 51Cr-release assays were carried out. (a) Examples of spot-forming cell (SFC) response. (b) ELISPOT assay for IFN-γ. The number of spots counted at a concentration of $2.5 \times 10^4$. Each value represents the mean spot number ± s.e.m. of triplicates of representative of three independent experiments. *$P<0.05$ vs RdB/IL-12. (c) 51Cr-release assay. B16-F10 cells were labeled with 51Cr and incubated with activated T cells isolated from mice treated with Ads at 100:1 E:T ratios. Each data point indicates means ± s.e.m. of triplicates of representative of three independent experiments. **$P<0.01$ vs RdB/IL-12.
cells from mice given RdB/IL-12 or RdB/IL-18. More specifically, the number of IFN-γ-secreting immune cells isolated from RdB/IL-12/IL-18-treated mice at a concentration of $2.5 \times 10^5$ was much higher (380 ± 74) than cells isolated from PBS- (51 ± 18), RdB- (74 ± 15), RdB/IL-12- (147 ± 55) or RdB/IL-18-treated mice (85 ± 13) (Figure 5b).

To further delineate the tumor-specific immune response in vivo, a $^{51}$Cr-release assay was carried out. Splenocytes obtained from RdB/IL-12/IL-18-treated mice showed the most potent B16-F10-specific lytic activity on day 3 after exposure. CTL killing of splenocytes from mice treated with RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 was 32.1 ± 0.5, 10 ± 2.1 and 50.8 ± 0.5, respectively, at an effector-to-target (E:T) ratio of 100:1, respectively, with no measurable background (Figure 5c). The CTL activity of splenocytes from mice treated with RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 was specific to a given tumor, as no CTL activity could be detected when NIH3T3 cells were used as target cells. These results show that RdB/IL-12/IL-18-treated mice had a higher tumor-specific adaptive immune response than did RdB/IL-12- or RdB/IL-18-treated mice.

**Figure 6** Histological and immunohistochemical analysis of tumor sections from mice treated with Ads. Ads were injected on days 0, 2 and 4, and tumors were collected on day 7 for histological analysis. (a) Frozen sections of tumor tissue were then stained by hematoxylin and eosin. Original magnification: ×40 and ×400. Cryosections of tumor tissue were stained with anti-NK-1.1 (b), anti-CD4 (c) and anti-CD8 (d) monoclonal antibodies. Original magnification: ×100 and ×400.
Increased infiltration of CD4\(^+\) T, CD8\(^+\) T and NK cells in RdB/IL-12/IL-18-treated tumors

To analyze histological characteristics of tumor tissues following RdB/IL-12/IL-18 treatment, we performed hematoxylin and eosin staining of tumor tissues. Histological evaluation of tumor sections revealed that large areas of tumors treated with RdB/IL-12/IL-18 were necrotic. In particular, tumors treated with RdB/IL-12 or RdB/IL-12/IL-18 were extensively infiltrated with immune cells, whereas tumors treated with RdB or RdB/IL-18 showed sparse infiltration. Moreover, denser immune cell infiltration was observed not only around, but also inside the remaining tumor tissues treated with RdB/IL-12/IL-18.

To obtain more insight into the molecular mechanisms of cytokine-mediated inhibition of tumor growth, we performed immunohistochemical analysis. Immunohistochemical studies confirmed that the tumor-infiltrating immune cells were CD4\(^+\) T cells, CD8\(^+\) T cells and NK cells. Higher numbers of all three cell types were detected in wider areas of RdB/IL-12/IL-18-treated tumors than in tumors treated with either RdB/IL-12 or RdB/IL-18 (Figure 6).

Increased IL-12RII\(\beta2\) or IL-18R\(\alpha\) expression in RdB/IL-12/IL-18-treated tumors

To examine whether expression of IL-12RII\(\beta2\) or IL-18R\(\alpha\) of T cells could be enhanced in RdB/IL-12/IL-18-treated tumor tissue, we performed immunofluorescence double staining using anti-CD4, anti-CD8, anti-IL-12RII\(\beta2\) and anti-IL-18R\(\alpha\) antibodies. Sections were prepared from C57BL6 mice 3 days after the final virus injection and

![Figure 7](image)

Figure 7 Enhancement of T cells expressing IL-12RII\(\beta2\) or IL-18R\(\alpha\) in tumor tissues. Tumor tissues were harvested from tumor-bearing mice 3 days following final virus treatment. Sections of tumor tissue were incubated with anti-CD4 (Green) plus anti-IL-12RII\(\beta2\) (Red) (a), anti-CD4 (Green) plus anti-IL-18R\(\alpha\) (Red) (b), anti-CD8 (Green) plus anti-IL-12RII\(\beta2\) (Red) (c), and anti-CD8 (Green) plus anti-IL-18R\(\alpha\) (Red) (d). Original magnification: x 400.
were analyzed to determine the population of T cells expressing IL-12Rβ2 or IL-18Rα. The analysis revealed an elevation in the number of CD4+ and CD8+ T cells expressing IL-12Rβ2 or IL-18Rα in the tumor tissues of RdB/IL-12/IL-18-treated mice, compared with those of RdB/IL-12- or RdB/IL-18-treated mice (Figure 7). Effector T cells have to maintain both IL-12Rβ2 and IL-18Rα expression for differentiating T cells. Therefore, these data indicate that RdB/IL-12/IL-18 treatment resulted in an improved generation of optimally differentiated T cells expressing IL-12Rβ2 or IL-18Rα.

DISCUSSION
Therapeutic manipulation of immune responses by the use of cytokines is potent strategy for cancer immunotherapy and tumor growth suppression. However, the systemic administration of cytokines at a therapeutic dose can result in serious toxicity in human cancer-bearing patients as well as in animal cancer models. Some patients given IL-12 have even died following treatment. Therefore, it is important to understand as to how to induce therapeutic levels of cytokines long enough to generate antitumor immunity effectively within the tumor microenvironment while simultaneously attenuating systemic toxicity. To address this issue, we have used an E1B-deleted and E1A-mutated oncolytic Ad, RdB, which exerts an antitumor effect in a cancer cell-specific manner as an intratumoral delivery system of therapeutic cytokines. Moreover, this tumor-selective oncolytic adenoviral vector is competent at killing the tumor cells, as well as amplifying production of transgenes, by continuously replicating in tumor cells until the tumor tissues were eradicated.

Earlier data from our group showed that locally high expression of IL-12 in the tumor tissues by oncolytic Ad elicited potent antitumor effects without the induction of adverse effects by an increase in IL-12

Figure 7 Continued.
or IFN-γ in the serum. However, combination therapies of IL-12 and IL-18 have a more effective antitumor activity than either cytokine alone. Several studies have shown that IL-12 and IL-18 synergistically mediate antitumor immunity in a number of animal tumor models. Oshikawa et al. showed that IL-12-, pro-IL-18- and IL-18-converting enzyme act synergistically to elicit an enhanced antitumor immune response in a murine mammary adenocarcinoma model via induction of cytotoxic CD8+ T cells and IFN-γ. Similarly, recombinant IL-12 and IL-18 proteins were shown to significantly inhibit tumor growth and lymph-node metastasis in hepatoma tumor-bearing guinea-pigs. Furthermore, in DC-based tumor vaccine therapy, DCs co-producing IL-12 and IL-18 induced complete tumor rejection through the generation of an effective Th1 immune response in a BALB/c sarcoma model.

In conclusion, the finding in our report provides a novel underlying mechanism of combination therapies via IL-12 and IL-18, facilitating not only the recruitment, maturation and function of DCs, but also by enhancing the cross-presentation of DCs, which is important for recognizing tumor cells. Recently, we showed that intratumoral delivery of GM-CSF can promote antigen presentation and elevate the tumor-specific CTL response. Therefore, we suggest that upregulation of GM-CSF in the tumor microenvironment may enhance antigen presentation, leading to the generation of a tumor-specific immune response.

Tumor microenvironment has been shown to establish immunosuppressive cytokine networks that favor the suppression of an antitumor immune response and eventually generate tumor proliferation, angiogenesis and metastasis. Therefore, it is critical to understand the immunosuppressive environment at the tumor site to manipulate and generate antitumor immunity effectively within the tumor microenvironment. To this end, our data suggest that RdB/IL-12/IL-18 generated a tumor microenvironment more favorable to activate tumor-specific immune cells via upregulation of IL-12, IL-18, IFN-γ and GM-CSF in tumor tissues. Moreover, the manipulation of a tumor milieu more favorable to activate tumor-specific immune cells by RdB/IL-12/IL-18 was further supported by the observation that Th1/Th2 cytokine ratio was markedly elevated in RdB/IL-12/IL-18-treated tumor tissues (Figures 4a and b).

To activate tumor-specific immune cells, antitumor adaptive immunity in the tumor milieu should be optimally generated. Here, we showed that mice injected with RdB/IL-12/IL-18 had enhanced CTL activity, as well as an increase in tumor-specific IFN-γ-secreting immune cells, than did those given RdB/IL-12 or RdB/IL-18. In accordance with these results, both histological and immunohistochemical results showed immense necrotic regions as well as infiltration of NK, CD4+ T and CD8+ T cells into the tumor tissues of RdB/IL-12/IL-18-treated mice compared with those of RdB/IL-12- or RdB/IL-18-treated mice. NK cells are known to play an important role in the promotion of Th1 responses by producing IFN-γ and modulating DC activity. This observation suggests that IL-12 plus IL-18 activate NK cells that mediate oncolysis and DC maturation that, in turn, enhance antigen presentation to induce tumor-specific T cells to differentiate from naïve T cells, leading to the generation of antitumor immunity.

Biological functionality of RdB/IL-12/IL-18-mediated IL-12 and IL-18 expression in tumor tissues depends on the expression level of IL-12Rβ2 and IL-18Rz. Therefore, upregulation of IL-12Rβ2 and IL-18Rz correlates with the capacity of IL-12 and IL-18 to bind and initiate the development of Th1 immunity. Reports from in vitro studies have shown that IL-12 enhances the production of IL-12Rβ2 on T cells, whereas IL-12 mediates upregulation of IL-18Rz on T cells. Here, we showed that the numbers of T cells expressing IL-12Rβ2 or IL-18Rz were increased in tumor tissues of RdB/IL-12/IL-18-treated tumors (Figure 7). Moreover, animals treated with RdB/IL-12/IL-18 showed an elevated number of CD4+ and CD8+ T cells expressing IL-12Rβ2 or IL-18Rz at the tumor tissues. The expression of IL-12Rβ2 or IL-18Rz receptors are necessary for the differentiation of effector T cells. Therefore, our finding implies that intratumoral delivery of IL-12 and IL-18 by RdB/IL-12/IL-18 resulted in improved generation of optimally differentiated CD4+ and CD8+ T cells expressing IL-12Rβ2 or IL-18Rz. Our results further support the view put forth by Li et al. that IL-12, in combination with IL-18, induces differentiation of the antitumor effector T cells CD4+ and CD8+.

In conclusion, the finding in our report provides a novel underlying mechanism of combination therapies via IL-12 and IL-18 that promotes differentiation of T cells expressing IL-12Rβ2 or IL-18Rz. We also provide the first study where intratumoral expression of both
IL-12 and IL-18 via oncolytic Ad vector results in the creation of more potent tumor-specific immunity. These results provide a new insight into therapeutic mechanisms through IL-12 plus IL-18, as well as a potential clinical cancer immunotherapeutic agent for the generation of improved antitumor immunity.

MATERIALS AND METHODS

Cell lines
All cell lines were maintained in Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco BRL), 1-glutamine (2 mM), penicillin (100 U/ml), streptomycin (50 µg/ml), and amphotericin (1 µg/ml). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Animal studies
Six- to eight-week-old male C57BL/6 mice were obtained from Charles River Laboratories International Inc. (Wilmington, MA, USA). During the experiments, all mice were kept in a laminar air flow cabinet under specific pathogen-free conditions at Yonsei University College of Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility. All animal experiments were conducted under the institutional guidelines established by the Animal Research Committee.

Generation of Ads expressing IL-12 and IL-18
To generate an oncolytic Ad expressing IL-12 at the E3 region, we first constructed a pSP72-E3 Ad shuttle vector4 expressing murine IL-12 comprised of p35 and p40. The IL-12 gene (p35/IRE/p40) was digested from pCA14-IL-12 using BglII and subcloned into pSP72-E3 Ad shuttle vector with BamHI, creating a pSP72-E3-IL-12 E3 shuttle vector. The newly constructed pSP72-E3-IL-12 E3 shuttle vector was then linearized with NdeI digestion, and RdB was linearized with SpeI digestion. The linearized pSP72-E3-IL-12 E3 shuttle vector was then co-transformed into E. coli B75183 along with the SpeI-digested RdB for homologous recombination, resulting in RdB/IL-12 oncolytic Ad. To generate an oncolytic Ad expressing IL-18 at the E3 region, we first constructed an E3 shuttle vector expressing murine IL-18. BglII fragments containing IL-18 expression cassette were excised from pCA14-IL-18, and subcloned into pSP72-E3 Ad shuttle vector predigested with BamHI, resulting in a pSP72-E3-IL-18. The newly constructed pSP72-E3-IL-18 shuttle vector was then linearized with XmnI digestion, and then co-transformed into E. coli B75183 together with the SpeI-digested RdB for homologous recombination, creating an RdB/IL-18 oncolytic Ad. To construct an oncolytic Ad expressing IL-12 and IL-18 at the E1 and E3 region, respectively, pSP72-E3-IL-18 E3 shuttle vector was then linearized with XmnI digestion, and the Ad vector RdB/IL-12 was linearized with SpeI digestion. The linearized pSP72-E3-IL-18 E3 shuttle vector was then co-transformed into E. coli B75183 along with the SpeI-digested RdB/IL-12 for homologous recombination, generating an RdB/IL-12/IL-18 oncolytic Ad. The ratio of plaque-forming unit to viral particle for RdB, RdB/IL-12, RdB/IL-18, and RdB/IL-12/IL-18 used in this study is 1:42, 1:46, 1:39, and 1:47, respectively.

ELISA for IL-12 and IL-18 expression
To assess IL-12 and IL-18 levels, B16-F10 melanoma cells were plated onto six-well plates at 5×104 cells per well, and then infected with RdB, RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 Ad at MOIs of 10–200. At 96h post-infection, the supernatants were harvested. IL-12 and IL-18 expression were determined using an ELISA according to the manufacturer’s specifications (IL-12p70 ELISA kit: Endogen, IL-18 ELISA kit: MBL, Nagoya, Japan).

Antitumor effects of oncolytic Ad co-expressing IL-12 plus IL-18
Tumors were implanted subcutaneously on the left abdomen of C57BL/6 mice by inoculating viable B16-F10 murine melanoma cells (5×105) in 50µl of Hank’s balanced salt solution (Gibco BRL). When the average tumor volume reached 80 mm3, mice were randomly divided into one of five groups (six animals per group) receiving PBS, RdB, RdB/IL-12, RdB/IL-18 or RdB/IL-12/IL-18 mixed with Lipofectamine and plus reagent (Gibco BRL) at a 2:6 ratio. Ads or PBS were injected intratumorally (1×108 plaque-forming unit per tumor in 30µl of PBS) on days 0, 2 and 4. Control mice were injected with PBS only. Tumor volume was evaluated once every 2 days by electronic caliper measure: volume=0.523(W2L)/(L+W)2 (W=the length of tumors; L=the width of tumors). Tumor responses to each treatment were compared using a log-rank test analysis (StatView software; Abacus Concepts Inc., Berkeley, CA, USA). The percentage of surviving mice was determined by monitoring the tumor growth-related events (tumor size >2500 mm3). The survival curve was then plotted against time after treatment. Differences in survival were considered statistically significant when P-values were <0.01.

Quantiﬁcation of cytokines and Th1/Th2/Th17 proﬁle in tumor tissue
Tumors were harvested 3 days after the last viral inoculation of the B16-F10 tumor-bearing mice and snap-frozen in liquid nitrogen. Tumor tissues were homogenized (ART-MFCRA D-8; ART moderne Labotechnik, Munchen, Germany) in ice-cold radio immunoprecipitation assay buffer (Elpis Biotech, Taejon, Seoul, Korea) with a protease inhibitor cocktail (Sigma, St Louis, MO, USA). Homogenates were then centrifuged in a high-speed micro- centrifuge for 10 min and determined for total protein content using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Levels of IL-12, IL-18, IFN-γ and GM-CSF in the tumor tissue extract were determined by ELISA according to the supplier’s recommendations (IL-12 ELISA kit; Endogen, IL-18 ELISA kit; MBL, IFN-γ ELISA kit; Endogen, GM-CSF ELISA kit; Endogen). In addition, Th1/Th2/Th17 type cytokine expression proﬁles in the tumor tissue extract were estimated using Th1/Th2/Th17 CBA kit (BD Biosciences Pharmingen). ELISA and CBA results were calculated normalized to the total protein concentration in each tumor and were calculated as picograms per milligram of total protein.

IFN-γ ELISPOT assay in splenocytes
At 3 days following the last viral treatment, spleens were obtained aseptically from tumor-bearing mice, and unicellular splenocytes were prepared as described previously.3 Preparated spleen cells were co-cultured with irradiated B16-F10 (5000 rad) tumor cells for 3 days in the presence of recombinant human IL-2 (100 U/ml; R&D Systems, Minneapolis, MN, USA). An IFN-γ ELISPOT assay was then carried out as described previously.3 The spots were measured with a computer-based immunospot system (AID ELISPOT Reader System, version 3.4; Autoimmun Diagnostika GmbH, Strassberg, Germany).

Cytotoxicity assay in splenocytes
The cytolytic activity of tumor-specific CTL was evaluated by 4h 51Cr-release assay. Co-cultured spleen cells were prepared as described for the IFN-γ ELISPOT assay. Co-culture, splenocytes as effector cells were recovered and incubated with 51Cr-labeled B16-F10 or NIH3T3 cells as target cells at 100:1 E:T ratios for 4 h at 37°C and 5% CO2. After the supernatant was obtained and 51Cr release was assessed. The percentage of specific lysis was calculated by the following formula: ((c.p.m. experimental release – c.p.m. spontaneous release) / (c.p.m. maximum release – c.p.m. spontaneous release))×100. Spontaneous release was determined by incubation of the labeled target cells without effector cells. For maximum release, labeled target cells were treated with detergent.

Histology and immunohistochemistry for NK-1.1, CD4 and CD8
For histological study, tumor tissues were snap-frozen in OCT compound (Sakura Finetec, Torrance, CA, USA), and 10-µm sections were cut on a cryostat. Representative sections were stained with hematoxylin and eosin, and then investigated using light microscopy. To detect lymphocytes, tumor tissues were snap-frozen in OCT compound (Sakura Finetec), and 10-µm sections were cut, subjected to immunohistochemistry, and then counterstained with Mayer's hematoxylin. The sections were incubated with purified rat anti-mouse CD4 monoclonal antibody (BD Biosciences Pharmingen), purified rat anti-mouse CD8 monoclonal antibody (BD Biosciences Pharmingen) or purified mouse anti-mouse NK-1.1 monoclonal antibody (Biolegend, San Diego, CA, USA) as a primary antibodies, and then with goat anti-rat IgG (BD Biosciences Pharmingen) or goat-anti mouse IgG (Southern Biotech, Birmingham, USA).
**Immunofluorescence double staining for T cells expressing IL-12Rβ2 or IL-18Rα**

For immunofluorescence double staining for CD4+ and CD8+ T cells expressing IL-12Rβ2 or IL-18Rα, cryosections were treated with rat anti-mouse CD4 monoclonal antibody (BD Biosciences Pharmingen) or rat anti-mouse CD8 monoclonal antibody (BD Biosciences Pharmingen) and incubated overnight at 4°C, followed by Alexa flour 488 (Green)-labeled goat anti-rat IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. The cryosections were then incubated with goat anti-IL-12Rβ2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-mouse IL-18Rα polyclonal antibody (Santa Cruz Biotechnology) at 4°C overnight and at room temperature for 1 h with Alexa flour 568 (Red)-labeled rabbit anti-goat IgG (Invitrogen) or Alexa flour 568 (Red)-labeled goat anti-rabbit IgG (Invitrogen), respectively. For counterstaining, the samples were incubated with 4,6-diamidino-2-phenylindole (Sigma), and then observed by a laser scanning confocal imaging system (LSM 510-META; Carl Zeiss, Jena, Germany).

**Statistical analysis**

The data are expressed as mean ± s.e. of the mean (s.e.m.), Statistical comparison was made using Stat View software (Abacus Concepts Inc., Berkeley, CA, USA) and the Mann–Whitney test (non-parametric method). The criterion for significance was P < 0.05.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

This work was supported by grants from the Ministry of Knowledge Economy (10003005, Dr C-O Yoon), the Korea Science and Engineering Foundation (R15-2004-024-02010-0, 2009KO01644, 2010-002920, Dr C-O Yoon), the Korea Food and Drug Administration (KFDA-10172-332 to Dr C-O Yoon), and Yeoung University College of Medicine faculty research grant (6-2010-0052, Dr C-O Yoon). I-K Choi is a graduate student sponsored by KOSEF through National Core Research Center for Nanomedical Technology. J-S Lee and S-N Zhang are graduate students sponsored by the Brain Korea 21 Project for Medical Science, Yeoung University College of Medicine, Seoul, South Korea.

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