IL-12 and IL-27 Sequential Gene Therapy via Intramuscular Electroporation Delivery for Eliminating Distal Aggressive Tumors

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*J Immunol* published online 5 February 2010
http://www.jimmunol.org/content/early/2010/02/05/jimmunol.0902371

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Interleukin-12, IL-23, and IL-27 are the three known members of the IL-12 cytokine family. IL-12 is the first member of this family discovered in the early 1990s (1) and is one of the most effective cytokines for inhibiting tumor growth (2–4) among many other cytokines, including IL-2, IL-4, IL-7, IL-8, TNF-α, and GM-CSF (5–10). Daily systemic administration of recombinant IL-12 protein generates a significant inhibitory effect on the metastatic growth of B16F10 melanoma and the established renal carcinoma and CT26 tumors (11–13). However, because a large dose of IL-12 protein must be used due to the short protein half-life, systemic delivery of IL-12 protein is associated with severe toxicity in several experimental animal studies and in initial early stage human trials (14–16). IL-12 gene therapy has demonstrated greater efficacy and less toxicity than recombinant IL-12 protein treatment in the renal carcinoma tumor model (17), but eradication of aggressive tumors by systemic IL-12 gene therapy alone cannot be achieved (18, 19).

IL-27 is a novel member of the IL-12 cytokine family. Similar to IL-12, IL-27 is a heterodimeric cytokine composed of p28 and Epstein-Barr virus-induced gene 3 subunits (20–22). Epstein-Barr virus-induced gene 3 and p28 are very similar to the p40 and p35 subunits of IL-12, respectively. At the molecular level, IL-27 activates Stat1–5 in naïve CD4 T cells via interaction with the cognate heterodimer receptor composed of wsx1 and gp130 (23–27). In naïve T cells, IL-27 activates Stat1, resulting in the induction of T-bet and IL-12Rβ2, which is crucial for Th1 polarization (25). At the cellular level, IL-12 induces clonal proliferation of naïve and activated T cells but not memory T cells. At the immunological level, IL-27 play a role in inducing Th1 response (21, 28) and inflammatory and anti-inflammatory responses (29–40). Moreover, stable expression of IL-27 in tumor cells inhibits tumor development and induces T cell-dependent antitumor immune memory (35, 41, 42), but it is unknown whether systemic IL-27 treatment can trigger antitumor immune responses and eliminate tumors.

Here, we discovered that sequential administration of IL-12– and IL-27–encoding plasmid DNA into tumor-bearing mice through i.m. electroporation, provides a simple but effective approach for eliminating inaccessible residual tumors. The Journal of Immunology, 2010, 184: 000–000.

\[\text{IL-12 and IL-27 Sequential Gene Therapy via Intramuscular Electroporation Delivery for Eliminating Distal Aggressive Tumors}\]

Shiguo Zhu,* Dean Anthony Lee, † and Shulin Li‡

Eradication of residual malignancies and metastatic tumors via a systemic approach is the key for successfully treating cancer and increasing cancer patient survival. Systemic administration of IL-12 protein in an acute large dose is effective but toxic. Systemic administration of IL-12 gene by persistently expressing a low level of IL-12 protein may reduce the systemic toxicity but only eradicates IL-12-sensitive tumors. Here, we discovered that sequential administration of IL-12– and IL-27–encoding DNA, referred to as sequential IL-12–→IL-27 (IL-12 administration followed by IL-27 administration 10 d after) gene therapy, not only eradicated IL-12–sensitive CT26 tumors from 100% of mice but also eradicated the highly malignant 4T1 tumors from 33% of treated mice in multiple independent experiments. This IL-12→IL-27 sequential gene therapy is not only superior to IL-12–encoding plasmid DNA given a total of two times at a 10-d interval sequential gene therapy for eliminating tumors but also for inducing CTL activity, increasing T cell infiltration into tumors, and yielding a large number of tumor-specific IFN-γ-positive CD8 T cells. Notably, depletion of either T or NK cells during the IL-27 treatment phase reverses tumor eradication, suggesting an NK cell requirement for this sequential gene therapy-mediated tumor eradication. Both reversal of the administration sequence and coadministration of IL-12 and IL-27 impaired tumor eradication in 4T1 tumor-bearing mice. This IL-12→IL-27 sequential gene therapy, via sequential administration of IL-12– and IL-27–encoding plasmid DNA into tumor-bearing mice through i.m. electroporation, provides a simple but effective approach for eliminating inaccessible residual tumors. The Journal of Immunology, 2010, 184: 000–000.

**Materials and Methods**

**Gene constructs**

The gene clones IL-12 and IL-27 were used in this study. The IL-12 gene construct was obtained from Valentis (Birmingham, CA) via a material transfer agreement. IL-12 gene construct consists DNA encoding two protein subunits, p35 and p40. Both p35 and p40 subunits were driven by a CMV
promoter and terminated by a bovine growth hormone polyadenylation signal. This construct was used in our previous study via intratumoral administration via electroporation (43). IL-27 was a generous gift from Dr. Masatoshi Tagawa (Chiba Cancer Center Research Institute, Chiba, Japan) (41). To increase the level of gene expression, we have subcloned the IL-27-encoding fragment into the same vector used previously for IL-12.

Animal procedures

All of the animal procedures including tumor transplantation, tumor volume monitoring, gene administration, bleeding, and mice euthanasia were approved by the Institutional Animal Care and Use Committee at Louisiana State University. Six- to eight-week-old female BALB/c mice, weighing 18–20 g, from the in-house animal breeding facility were used for this study. The s.c. tumor model was generated by s.c. inoculating CT26 colon tumor cells (2 x 10^6 in a 30-μl volume per mouse) into BALB/c mice. The adenocarcinoma 4T1 model was generated by s.c. inoculating 4T1 tumor cells (1 x 10^6 in a 30-μl volume per mouse) into BALB/c mice. Both tumor cell lines were maintained in DMEM containing 10% FBS (Life Technologies, Rockville, MD). Tumor dimensions were measured with calipers, and volume was calculated from the formula V = (π/6)xab^2, where V is tumor volume, a is the maximum tumor diameter, and b is the diameter at 90° to a (44). With the protocols described previously, IL-12 and control plasmid DNA (10 μg in a volume of 30 μl per mouse) were injected into hind limb tibialis muscles via electroporation (45). The electroporation parameters for i.m. injection are 350 V/cm and 20 ms pulse duration for two pulses (45). Both hind limb tibialis muscles were used for the first and the second DNA administration via electroporation. The first administration was initiated on days 4 or 11 to test the effectiveness of this therapy. The second administration was performed 10 d thereafter. Tumor growth and tumor eradication were monitored every 3 d. The indicated rabbit polyclonal Abs or murine mAbs were administered into mice (50 μg per mouse once every 3 d) via i.p. administration for depleting the target cells. The mAbs used for depletion were made commercially available through Taconic Farms (Germantown, NY).

Blood was obtained via a cheek bleeding method (tightly grab the mouse neck and poke the skin on the cheek with a 16 gauge needle; the bleeding will stop immediately upon releasing the fingers from the mouse neck). Blood was collected at the indicated time points displayed in the figures, and serum was separated from the coagulated blood cells by centrifugation at 200 x g. Serum was used for detecting IFN-γ, IFN-α, and IL-27 expression using ELISA kits purchased from R&D Systems (Minneapolis, MN).

Detection of IFN-γ, IL-12, and IL-27 expression using ELISA kits

Each collected serum sample (50 μl per mouse) was transferred into a single well of each cytokine assay plate supplied by the manufacturer (R&D Systems), followed by washing and binding with the HRP-conjugated Abs. The HRP substrate was added to each well to trigger the color change, and the enzyme stop reaction buffer was added 30 min later to stop the HRP reaction. The color intensity representing the level of gene expression was determined in the plate reader at 405 nm. A standardized column for each cytokine (ranging from 0 to 500 pg per well in a 2-fold escalated dilution) was set in the plate, and IL-27 expression was confirmed using ELISA kits purchased from a contracted service. The purified wsx1 Ab was used to block the binding site for wsx1. Once confirmed, blood was collected on day 10 following the final boost to purify the anti-wsx1 Ab from the serum. Ab was purified using a protein A-Sepharose column by a contracted service. The purified wsx1 Ab was used to block IL-27 signaling by binding with wsx1, which is achieved by administering 50 μg Ab per mouse.

Immunostaining of T cell infiltration and CTL activity assay

Immunostaining of T cell infiltration was performed on frozen tumor sections. The procedures for frozen-block preparation, tissue sectioning, and immunostaining were the same as those described previously (43, 45–47). Tumor samples were collected 10 d after the final administration to evaluate the T cell numbers via immunostaining. The primary Ab applied to the sections was anti-CD8 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). The same fluorescence-based CTL assay method as described previously was used to determine tumor-specific T and NK cell cytolytic activities against target tumor cells (48). To determine NK cell cytolytic activity in vitro, T cells were depleted by administering both anti-CD4 (GK1.5) and anti-CD8 (2.43) T cell Abs (50 μg per mouse) to the sequential gene therapy-treated mice via i.p. injection 1 d prior to euthanizing mice. To determine the cytolytic activity of T cells against tumor cells in vitro, NK cell-depleted spleen cells were used.

ELISPOT assay to determine the tumor-specific IFN-γ-positive CD8 T cells from the cured mice

To determine the acute induction of tumor-specific CD8 T cells by tumor Ag stimulation, the IL-12 administration followed by IL-27 administration 10 d after (IL-12→IL-27) sequential gene therapy-cured mice were challenged with tumor cells for 3 d prior to euthanasia for analysis of tumor-reactive IFN-γ-secreting CD8 T cells. To avoid the effect of IFN-γ-secreting NK and CD4 T cells on accurately determining the number of IFN-γ-positive CD8 T cells, mice were administered anti-NK1.1 (PK136) and anti-CD4 (GK1.5) T cell Abs 1 d prior to euthanasia. Lymphocyte cells were isolated from lymph nodes by smearing the tissue and pushing it through a 70-nm strainer. A total of 50 μg per mouse was administered via i.p. injection, and flow cytometry was performed to confirm that the primary collected cells were CD8 T cells the next day when mice were euthanized. The isolated CD8 T cells were incubated without and with mitomycin C-treated target tumor cells (CT26) in the IFN-γ-capturing ELISPOT plate purchased from R&D Systems. After being incubated overnight, the plate was

![Figure 1](http://www.jimmunol.org/DownloadedFrom/April13.2017)
washed, and the IFN-γ spots were detected using immunostaining following the manufacturer’s instructions. The image of IFN-γ–positive spots was captured via a Kodak image station 440 (Rochester, NY).

Statistical analysis

Tumor volume, CD8+ T cell infiltration, and gene expression were the primary outcomes measured. Survival analysis and tumor volumes were performed with the Mann-Whitney U test and a one-way repeated-measures ANOVA, respectively. Others were analyzed by the two-tailed Student t test. p values <0.05 were considered statistically significant.

Results

Elimination of highly aggressive tumors by IL-12 and IL-27 sequential gene therapy is administration sequence-specific

Our laboratory has made a surprise discovery that administration of IL-12–encoding plasmid DNA induces IL-27 expression in mice and depletion of IL-12–induced IL-27 impairs IL-12–mediated antitumor efficacy (data not shown). On the basis of this innovative observation, we hypothesize that increased expression of IL-27 in the IL-12–treated mice may enhance IL-12–mediated antitumor efficacy. To test this hypothesis, plasmid DNA encoding IL-12 and IL-27 was administered sequentially and concomitantly into the hind limb muscles of 4T1 tumor-bearing mice via electroporation, where the therapeutic cytokine protein can be manufactured and secreted into the blood circulation to inhibit tumor growth located in other organs via inhibition of angiogenesis and activation of antitumor immune responses (18, 19, 47). The 4T1 tumor model was used because this model was described as a highly malignant tumor, equaling the clinical grade IV malignancy of breast cancer (49). There is no effective systemic delivery approach available to eliminate this tumor.

Intriguingly, sequential administration of IL-12→IL-27–encoding plasmid DNA with a 10-d interval drastically inhibited tumor growth (Figs. 1, 2A) and prolonged survival (Fig. 2B). This approach is referred to as IL-12→IL-27 sequential gene therapy because the tumor regression is entirely dependent on the administration sequence (IL-12 followed by IL-27 after 10 d). A reversed administration sequence failed to eradicate tumors, as did the coadministration of both gene-encoding plasmids (Fig. 1). Neither IL-12 nor IL-27 treatment alone was successful in eradicating tumors (Fig. 1).

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IL-12→IL-27 sequential gene therapy eliminates tumors at a higher percentage than IL-12 alone in an IL-12–sensitive tumor model

CT26 colon tumors were known to be immunogenic and sensitive to IL-12 therapy. With muscle-based sequential administrations by which IL-12–encoding plasmid DNA alone is given a total of two times at a 10-d interval (IL-12→IL-12), 60–75% of tumors were eradicated in both an early treatment model (Fig. 3A) and a late treatment model (Fig. 3C). Compared to IL-12→IL-12 sequential gene therapy, IL-12→IL-27 sequential gene therapy is more effective because tumors were eradicated in 100% of CT26 tumor-bearing mice in both early and late treatment models (Fig. 3B, 3D) with a trend toward earlier tumor resolution. Neither trend (cure rate nor time to complete response) achieved statistical significance in this sensitive tumor model. However, administration of an Ab that binds to the IL-27 receptor, wxs1, significantly reversed IL-12→IL-27 sequential gene therapy–caused tumor eradication in 80% of the mice (Fig. 3F) when compared with IL-12→IL-27 sequential gene therapy alone (Fig. 3E; p < 0.05). Administration of anti-wxs1 Ab resulted in aggressive tumor growth as observed in the control group receiving control DNA (Fig. 3F), further illustrating the significance of exogenous IL-27 in causing tumor regression.

A higher level of IL-27 expression at the later phase of treatment is associated with the eradication of highly malignant 4T1 tumors by sequential IL-12→IL-27 gene therapy

One of the major mechanisms for IL-12–mediated inhibition of tumor growth is to trigger a high level of IFN-γ expression in T and NK cells (50). It was reported that a synergistic induction of IFN-γ was found when both IL-12 and IL-27 were present in T cells (22, 24, 51). Therefore, one possible mechanism for enhanced tumor eradication by IL-12→IL-27 sequential gene therapy is synergistic induction of IFN-γ. To test this assumption, the expression of IFN-γ on the indicated dates after the first and second administrations was compared between the two treatment groups: IL-12→IL-12 versus IL-12→IL-27 gene therapy. No enhanced expression of IFN-γ was found in the IL-12→IL-27 treatment group (Fig. 4A), and similar levels of IL-12 and IFN-γ expression were found in these two treatment groups (Fig. 4A, 4B). As anticipated, an increased level of IL-27 in the later phase was detected with IL-12→IL-27 gene therapy (Fig. 4C). This result suggests that the increased IL-27 expression by IL-12→IL-27 sequential therapy during the late treatment phase may contribute directly to the observed 4T1 tumor regression, which was not obtained by the IL-12→IL-12 gene therapy in this model (Fig. 1). Early administration of IL-27, either alone or in combination with IL-12, increases the level of IL-27 expression; however, it is detrimental to the IL-12–mediated antitumor effect, suggesting that IL-27 is required mainly in the late phase of treatment.

Sequential IL-12→IL-27 gene therapy induces a higher level of tumor-specific T cell response than IL-12→IL-12 gene therapy

One of the main IL-12 functions is to activate NK cells, inducing NK cell-mediated tumor cell death. Because IL-27 induces IL-12Rβ2 expression and may sensitize IL-12 response (24–26, 35, 41), we were interested in knowing whether IL-27 enhances the NK cell-mediated antitumor effect. Therefore, the cytolytic activities of NK cells, isolated from both IL-12→IL-12– and IL-12→IL-27–treated mice, were compared to those against nonspecific tumor target cells. To avoid the possible effect from T cells, T cells were depleted by using anti-CD8 depletion Ab 1 d prior to euthanizing mice. A similar level of NK cell activity was detected from both IL-12→IL-12 and IL-12→IL-27 therapies (Fig. 5A, 5B), suggesting that the discrepancy in tumor eradication between these two treatments was not due to different levels of NK activity.

Others found that expression of IL-27 in tumor cells induced T cell-dependent antitumor effects (35, 41). However, systemic expression of IL-27 via i.m. administration of IL-27–encoding plasmid DNA through electroporation yielded minimum inhibition of tumor growth (Fig. 1A), suggesting that systemic expression of IL-27 alone is not sufficient to induce any strong antitumor immune response. Likewise, our previous study indicates that systemic expression of IL-27 alone induces a weak T cell response (52). We were interested to know whether the increased expression of IL-27 in the later phase (IL-12→IL-27 treatment phase) potentiated the IL-12–mediated T cell-dependent antitumor immune response. To test the T cell response induced by sequential IL-12→IL-27 gene therapy, the tumor-specific CTL activity was determined. A much higher level of CTL activity was detected from the IL-12→IL-27–treated mice compared with that from the IL-12→IL-12–treated mice (Fig. 5C). Moreover, IL-12→IL-27 sequential gene therapy also induced more infiltration of CD8 T cells into tumors than IL-12→IL-12 treatment (Fig. 5D, p < 0.05).

To confirm that cytotoxic T cells did truly contribute to the increased antitumor efficacy, CD8 T cells were depleted or reduced from the sequential gene therapy–treated mice using depletion anti-CD8 Ab 2.43. The depletion Ab was administered concomitantly with IL-27 plasmid DNA during the late phase of treatment. As expected, administration of anti-CD8 Ab abrogated the sequential
IL-12→IL-27 gene therapy-mediated tumor eradication, illustrating the significant role of cytotoxic T cells in tumor eradication (Fig. 5E). Anti-CD4 T cell Ab was not included in this experiment because a preliminary study concludes that depletion of CD4 T cells has no effect on IL-12→IL-27 sequential gene therapy-mediated antitumor efficacy (data not shown). Unexpectedly, administration of anti-NK Ab (PK137, American Type Culture Collection, Manassas, VA) during the late phase also reversed tumor eradication, indicating that NK or NKT cells also contribute to the sequential gene therapy-mediated antitumor effect (Fig. 5E).

The identical NK cytolytic activities derived from both IL-12→IL-12 and IL-12→IL-27 sequential gene therapies suggest that NK cells are critical; however, they are not the key elements engendering the enhanced antitumor activity (Fig. 1).

To determine whether the strong IL-12→IL-27 immune gene therapy also induces a strong antitumor immune memory, the cured mice from both treatment groups, IL-12→IL-12 and IL-12→IL-27, were challenged with the tumor cells 3 mo following tumor disappearance. Interestingly, no tumor incidence was detected from the IL-12→IL-27–cured mice, suggesting the presence of a strong immune response. Tumor incidence was found in IL-12→IL-12–cured mice, but these tumors were regressed slowly 3 wk after the challenge, suggesting that IL-12→IL-12 gene therapy also induced relatively weak antitumor immune responses (Fig. 5F). The same challenge study also was performed in the mice 6 mo after eradicating the aggressive 4T1 tumors by IL-12→IL-27 gene therapy. Tumors developed in two of five challenged mice, but those tumors disappeared after 3 wk, illustrating that this treatment yielded a long duration of antitumor memory against highly malignant 4T1 tumor cells. Because IL-12→IL-12 gene therapy could not eradicate the aggressive 4T1 tumors, this challenge study could not be performed in the IL-12→IL-12 treatment group.

The above result suggests that IL-12→IL-27–cured mice may produce an acute induction of activated CD8 T cells to immediately

**FIGURE 5.** Comparison of the antitumor immune responses between IL-12→IL-12 and IL-12→IL-27 gene therapy. The same dose, treatment schedule, and sequence described in the caption to Fig. 1 applied to this figure. Ten days after the final (second) administration, antitumor immune responses between these two treatments were analyzed. The first administration was performed on day 11 after inoculation of tumor cells. A, NK cell cytolytic activity using homologous tumor cells and NK cell-depleted spleen cells. B, NK cell cytolytic activity using Yac1 cells. C, Differences in T cell cytolytic activities (CTL) between IL-12→IL-12– and IL-12→IL-27–treated mice. CT26 tumor cells were used as target cells. *p < 0.01 representing a significant difference. D, Difference in infiltration of T cells in CT26 tumors between IL-12→IL-12 and IL-12→IL-27 sequential gene therapy (p = 0.036). E, Effect of depletion of CD8 T and NK cells on IL-12→IL-27 sequential gene therapy-mediated antitumor efficacy. *p < 0.001 between injection of depletion Abs against CD8 T cells or NK cells and injection of murine IgG on the indicated days. Abs were administered twice a week at a dose of 50 μg per mouse for each administration. Arrows indicate the plasmid DNA administration time. F, Kinetics of challenged tumor growth between IL-12→IL-12– and IL-12→IL-27–cured mice.

**FIGURE 6.** Induction of antitumor immune memory by IL-12→IL-27 sequential gene therapy. CT26 tumor model was used for this study in vivo, and the spleen cells collected from the treated mice were used for the ELISPOT assay, detecting the differences in induction of acute tumor-specific IFN-γ–positive CD8 cells between IL-12→IL-12 and IL-12→IL-27 sequential gene therapy. To ensure that the effector cells were primarily CD8 T cells, both NK and CD4 T cells were depleted using neutralization Ab 1 d prior to euthanizing mice. Two treatments were performed (see details in the caption to Fig. 1, with the first treatment on day 11 after inoculation of tumor cells). A, IFN-γ–positive spots from different incubations. B, Spot number as counted under dissecting microscope. ***p < 0.001, representing a significant difference. E, effector cells; T, target cells.
remove the challenged tumor cells but the IL-12→IL-12–cured mice may have only a slow response. To test this possibility, the IFN-γ–secreting CD8 T cells were identified from these two groups of mice following stimulation of mice with tumor cells. To avoid detection of the IFN-γ–positive CD4 and NK cells, the cognate neutralization Abs were administered 2 d prior to euthanizing mice. A much higher number of IFN-γ–positive CD8 T cells were detected from IL-12→IL-27–cured mice than IL-12→IL-12–cured mice (Fig. 6). The presence of a larger number of IFN-γ–positive CD8 T cells was a good indicator for the acute induction of tumor-specific CD8 T cells by IL-12→IL-27 gene therapy.

Discussion

Microscopic tumors are the primary cause of tumor recurrence after surgery, chemotherapy, radiation, or a combination of treatments. Metastatic tumors are the primary cause of tumor patient mortality. Developing a therapeutic approach for treating tumor recurrence and metastatic tumors is crucial for reducing mortality of cancer patients and controlling tumors. One ideal approach from an immune therapy point of view is to induce antitumor immunity that inhibits tumor metastasis and recurrence. Previous studies performed by ourselves and others have applied electroporation to administer IL-12 plasmid DNA into tumors, completely eradicating tumors from 40–80% of mice (19, 43), but this approach cannot be used to treat the residual microscopic tumors after the standard treatments because tumors are too small to be detected to perform intratumoral gene administration. Therefore, administration of IL-12 plasmid DNA into muscle via electroporation was performed, in which IL-12 is expressed in the injected muscles but circulated into other organs via blood circulation. This approach, referred to as a systemic approach, does not need the detection of the residual tumor location, and we have demonstrated that two sequential administrations of IL-12–encoding plasmid DNA can eliminate immunogenic CT26 tumors in 60–75% of treated mice (Fig. 3). However, this approach failed to eradicate aggressive tumors (18, 52).

In this study, we illustrated that sequentially systemic expression of IL-12 and IL-27 via i.m. DNA electroporation completely eradicates highly malignant 4T1 tumors from 33% of treated mice. The 4T1 tumors in the non-tumor–eradicated mice are inhibited severely (Figs. 1, 2). Eradication of 4T1 tumor is significant because this mammary-derived tumor model is known to equal clinical grade IV tumors (49) and no other known systemic treatments can completely regress this highly aggressive tumor, and even the aggressive intratumoral treatment with a combination of IL-12 and bleomycin can only lead to tumor eradication from 40–60% of the treated mice (53). Therefore, muscle-based IL-12→IL-27 sequential gene therapy described here is an important approach to treat microscopic tumors.

Very intriguingly, this simple IL-12→IL-27 sequential gene therapy-mediated tumor eradication is administration sequence–specific because all of the other possible combinations, including the reversed administration sequence and coadministration, failed to eradicate tumors (Fig. 1). The fact that IL-27 has to be administered at a later point after administering IL-12 to induce a greater anti-tumor T cell response may challenge the current view that IL-27 is essential for the initial Th1 responses (21, 54–56). Of course, one possible explanation for the late requirement of IL-27 as shown in this study is that IL-12–induced expression of endogenous IL-27 is sufficient for the initial enhancement of the IL-12–mediated anti-tumor immune response. This is most likely true because depletion of IL-27 in the initial phase prior to IL-27 administration did impair tumor eradication by the IL-12→IL-12 sequential gene therapy in a CT26 tumor model (data not shown). Likewise, overexpression of IL-27 in the early phase of treatment may inhibit the antitumor program initiated by the early phase IL-12 treatment, because concomitant administration of IL-27 and IL-12 in the early phase abrogated tumor eradication (Fig. 2). Therefore, the level of IL-27 required at different treatment stages varies, providing the proper levels of IL-27 and IL-12 at different stages can greatly enhance the antitumor immune response and cause tumor eradication. The underlying mechanism by which IL-27 enhances IL-12–mediated tumor eradication is characterized by a compounding enhancement of the T cell response, including an acute induction of a larger number of tumor-specific CD8 T cells upon exposure to tumor Ag (Fig. 6), a higher level of CTL activity, and a larger amount of CD8 T cell infiltration into the tumors (Figs. 5, 6). The augmented T cell accumulation in tumor sites by IL-12→IL-27 gene therapy, as compared with IL-12→IL-12 gene therapy, explained why both treatments yield similar amounts of IFN-γ in the blood but a higher level of IFN-γ in the tumor Ag-stimulated T cells.

In regard to the mechanism, NK cells are also critical because depletion of NK cells reverses the tumor eradication exhibited by the IL-12→IL-27 treatment. This NK cell effect is induced most likely by IL-12 but not by IL-27, because similar NK lytic activity was obtained between IL-12→IL-12 and IL-12→IL-27 treatments (Fig. 5). IL-12 induces a Th1 response and inhibits a Th2 response. In theory, the B cell role may not be critical for IL-12 treatment. However, IL-12–secreting allogeneic HER2-positive cell vaccine induces anti-HER2 Ab response, which plays a role in inhibiting spontaneous tumor development in the NeuT mice because the absence of B cells reverses the effect (57). In a clinical study, intratumoral injection of IL-12 correlates to B cell infiltration (58). In a HER2/neu transgenic mouse model, IL-12 enhanced Ab production via HER2 vaccination (59). The effect of IL-27 on B cells, however, remains unknown. For this reason, B cell function, such as induction of antitumor Ab, was examined by IL-12→IL-12, IL-12→IL-27, and control DNA treatments. To our surprise, control DNA via electroporation alone induces a strong tumor-specific Ab response, which is greater than or commensurate with the IL-12→mediated Ab response (data not shown). This result excludes the possible role of B cells as the primary cells for IL-12→IL-27 sequential gene therapy-mediated tumor eradication. Regardless, this work will inspire a thorough study of this simple therapeutic approach and the underlying mechanism to make this approach more effective for treating systemic malignancy.

Disclosures

The authors have no financial conflicts of interest.

References

2. Burke, F. 1999. Cytokines (IFNs, TNF-α, IL-2 and IL-12) and animal models of cancer. Cytokines Cell. Mol. Ther. 5: 51–61.

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18. Hanna, E., X. Zhang, R. Breau, J. Suen, and S. Li. 2001. Inhibition of interleukin-12 production by recombinant human interleukin-12 in squirrel monkeys (Saimiri sciureus) by guest on April 13, 2017 from http://www.jimmunol.org/Downloaded from