Gene Therapy (2001) 8, 542–550

www.nature.com/gt

RESEARCH ARTICLE

Intratumoral IL-18 gene transfer improves therapeutic efficacy of antibody-targeted superantigen in established murine melanoma

Q Wang[1], H Yu[2], DW Ju[3], L He[3], JP Pan[1], DJ Xia[1], LH Zhang[1] and X Cao[1,3]
[1]*Institute of Immunology,* [2]*Cancer Institute, Zhejiang University, Hangzhou; and* [3]*Department of Immunology, Second Military Medical University, Shanghai, PR China*

Antibody-targeted superantigen C215Fab-SEA is a fusion protein of staphylococcal enterotoxin A (SEA) and the Fab region of the tumor-reactive C215 mAb. It can trigger CTL against C215 antigen-positive tumor cells and induce tumor-suppressive cytokines. However, the antitumor effect of C215Fab-SEA is not satisfactory because of suboptimal production of Th1 cytokines after repeated administration. Interleukin 18 (IL-18) is a novel cytokine with profound effects on Th1 cellular response. In this study, we showed that adenovirus-mediated intratumoral IL-18 gene transfer strongly improved the therapeutic efficacy of C215Fab-SEA in the pre-established C215 antigen-expressing B16 melanoma murine model. More significant tumor inhibition and prolonged survival time were observed in tumor-bearing mice received combined therapy of C215Fab-SEA and Ad IL-18 than those of mice treated with C215Fab-SEA or AdIL-18 alone. Combination therapy augmented NK and CTL activities of tumor-bearing mice more markedly. The production of IL-2 and IFN-γ also increased more significantly. More potent antitumor effect of combined therapy was observed in IL-10 KO mice with enhanced Th1 response. Our data demonstrated that the antitumor effect of C215Fab-SEA immunotherapy could be potentiated significantly by combination with intratumoral IL-18 gene transfer through more efficient activation of Th1 immune responses. Gene Therapy (2001) **8,** 542–550.

Keywords: *antibody-targeted superantigen; interleukin-18; adenovirus; gene therapy; antitumor effect; melanoma*

Introduction

The existence of T cell-specific tumor immunity has been demonstrated in several human malignancies, eg malignant melanoma and renal carcinoma.[1–3] The frequency of these tumor-specific T cells is generally too low to interfere with progressive tumor growth. Recruitment and activation of tumor-specific T lymphocytes is a major goal for immunotherapy of malignant tumors.

Superantigens (SAgs) are a collection of bacterial and viral proteins which bind to MHC class II molecules without the need for processing and subsequently activate a large number of T cells expressing particular T cell receptor (TCR) Vβ-families.[4] The biologic functions of SAgs make them attractive to use for targeted T cell activation and elimination of tumor cells expressing MHC class II. Staphylococcal enterotoxin A (SEA) belonging to the family of bacterial SAgs is capable of activating a large number of T lymphocytes which possess various potential antitumor functions, such as perforin-mediated or Fas–FasL-induced apoptosis and secretion of growth suppressive cytokines.[5–7] However, MHC class II products are expressed on normal B cells and monocytes and only to a limited degree on most tumors. To develop a tumor-specific superantigen for cancer therapy, SEA was genetically fused to the Fab region of C215 or C242 monoclonal antibody (mAb) which was specific for human colon carcinoma.[8] Recombinant fusion protein of the tumor-reactive mAb and SEA (Fab-SEA) expressed a 100-fold stronger affinity for the tumor antigen than MHC class II molecules.[9] *In vitro* and *in vivo* studies have shown that Fab-SEA could target cytotoxic T cells against MHC class II$^-$ tumor cells bearing the proper tumor antigen in the absence of obvious systemic side-effects,[10–17] which demonstrated functional substitution of the MHC class II-dependent presentation of SEA with tumor specificity.

However, the antitumor effect of Fab-SEA is still hampered by some limitations. Targeting of superantigenicity evoked a pseudo-specific immune attack but not a very specific immune response against the tumor.[8] Furthermore, a state of hyporesponsiveness characterized by sub-optimal production of IFN-γ, IL-2 and marginal CTL activity was induced after repeated treatment with Fab-SEA.[18] This hyporesponsiveness state may due to the functional anergy and the ultimate death of a large number of the responding T cells, which directly limited the therapeutic efficacy, and long-term survival was noted only in a small fraction of the tumor-bearing animals.[18]

How to circumvent these problems and enhance the antitumor effect of Fab-SEA immunotherapy more efficiently? Rosendahl *et al*[16] reported that antitumor effect of Fab-SEA immunotherapy was potentiated by co-

Correspondence: X Cao, Institute of Immunology, Zhejiang University, 353 Yan'an Road, Hangzhou, 310031, PR China; or H Yu, Cancer Institute, Zhejiang University, 68 Jiefang Road, Hangzhou, 310009, PR China
Received 17 August 2000; accepted 15 January 2001

administration of fusion protein of Fab and IL-2 (Fab-IL-2). Administration of Fab-IL-2 and Fab-SEA strongly augmented the production of IFN-γ, restored responsiveness of T cells and prolonged the immune response *in vivo*. IFN-γ plays an important role in the innate immunity. It was demonstrated that IFN-γ is an important mediator in the overall cytostatic antitumor response during Fab-SEA therapy.[16] The presence of IFN-γ producing T lymphocytes is highly relevant to the therapeutic effect since the B16 melanoma is extremely sensitive to IFN-γ *in vitro*.[19,20] Based on these findings, we tried a new strategy using adenovirus-mediated interleukin 18 (IL-18) gene transfer to potentiate the antitumor effect of Fab-SEA immunotherapy.

IL-18 is a newly identified cytokine that mediates many important biological functions including induction of IFN-γ, enhancement of NK cell activity, stimulation of proliferation of activated T cells, regulation of FasL-dependent apoptosis and promotion of Th1 response.[21–24] IL-18 was shown to induce the immune defense against tumors in various animal models.[25–27] We hypothesized that transfection of IL-18 gene into the tumor may augment the production of tumor-suppressive cytokines (IFN-γ and IL-2), rescue the CTL against the tumor cells, enhance NK cell activity, and thus profoundly potentiate Fab-SEA-based cancer immunotherapy.

In order to investigate the *in vivo* antitumor immune response elicited by C215Fab-SEA and intratumoral IL-18 gene transfer, in the present study, we used murine B16 melanoma cells transfected with the gene encoding for human colon carcinoma-associated C215 antigen as a model. A B16 cell clone stably expressing C215 antigen (B16-C215) was established which could be specifically recognized by C215Fab-SEA fusion protein and then was used for the following study. C57BL/6 IL-10 gene knock out (KO)[28] and wild-type C57BL/6 mice were inoculated with B16-C215 melanoma cells to observe the therapeutic effects of combination of C215Fab-SEA with adenovirus-mediated intratumoral IL-18 gene transfer and their related mechanisms. Our data illustrated that combination therapy elicited more significant antitumor effects through efficient activation of Th1 immune responses.

Results

Establishment of murine melanoma cell clone expressing human colon carcinoma-associated C215 antigen

The expression vector pKGE839 containing the GA733–2 cDNA (encoding the C215 antigen)[29] and the neomycin-resistance gene was transfected into B16 melanoma cells. G418-resistant clones were harvested. The expression of C215 antigen was analyzed by FACS. A C215 highly expressing clone was selected from B16-C215 cell clones and used in the following experiments. The C215 antigen expression on B16-C215 cells was stable. The *in vitro* and *in vivo* growth pattern of B16-C215 cells was similar to that of the wild-type B16 cells (data not shown).

More potent inhibition of tumor growth in melanoma-bearing mice by combined therapy of C215Fab-SEA and Ad IL-18

The construction of adenoviral vector encoding murine IL-18 (Ad IL-18) was described previously by us.[30] The production of IL-18 in the supernatants of B16-C215 cells after Ad IL-18 transfection was detected by ELISA kit. Two hours after Ad IL-18 infection, about 64.0 pg/ml IL-18 could be detected and it reached the highest level of 660.0 pg/ml at 24 h (data not shown). One week after Ad IL-18 injection the intratumoral IL-18 mRNA expression was analyzed by semiquantitative RT-PCR. As shown in Figure 1, the intratumoral expression of IL-18 mRNA in mice after Ad IL-18 injection was significantly higher than that in mice injected with AdLacZ or PBS. It indicated that IL-18 gene could be efficiently transduced into B16-C215 melanomas *in situ* after Ad IL-18 was injected intratumorally and then expressed to secret IL-18.

Three days after inoculation with 1×10^5 B16-C215 cells, tumor-bearing mice were treated with PBS, AdLacZ, C242Fab-SEA, C215Fab-SEA, Ad IL-18, C215Fab-SEA in combination with AdlacZ (C215Fab-SEA/Ad LacZ), C215Fab-SEA in combination with Ad IL-18 (C215Fab-SEA/Ad IL-18). The results in Figure 2a demonstrated that C215Fab-SEA or Ad IL-18 administration alone showed a marked inhibitory effect on tumor growth when compared with PBS, AdLacZ, C242Fab-SEA control groups ($P < 0.01$). Tumor inhibition in mice treated with C215Fab-SEA was more significant than that in mice treated with Ad IL-18 ($P < 0.01$). The inhibition of melanoma growth could be observed most significantly in mice after combined treatment with C215Fab-SEA/Ad IL-18 when compared with that in mice treated with C215Fab-SEA, Ad IL-18 alone, C215Fab-SEA/AdLacZ, AdLacZ, C242Fab-SEA, or PBS ($P < 0.01$).

C57BL/6 IL-10 KO mice with enhanced Th1 response were used to investigate the roles of endogenous Th1, Th2 cytokines during C215Fab-SEA and Ad IL-18 therapy. The data in Figure 2b showed that tumor growth in IL-10 KO mice treated with C215Fab-SEA was sup-

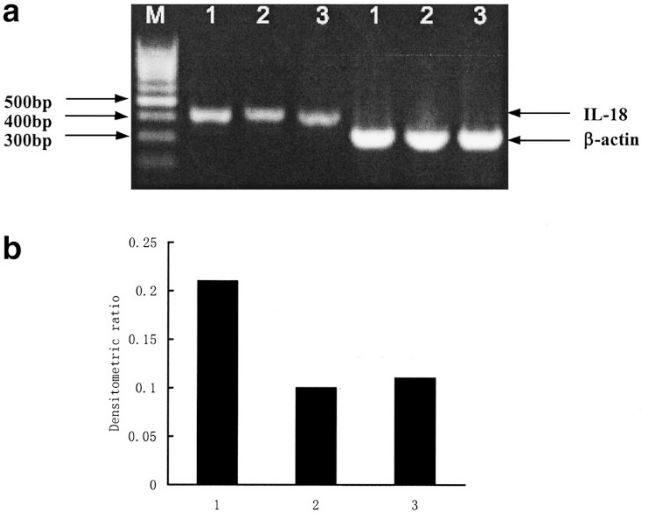

Figure 1 *Intratumoral IL-18 mRNA expression after Ad IL-18 transfection. One week after the mice were intratumorally injected with Ad IL-18, AdLacZ or PBS, total RNA was extracted from subcutaneous tumor tissues by TRIzol Reagent. The intratumoral IL-18 mRNA expression was analyzed by semiquantitative RT-PCR. (a) PCR products of β-actin and IL-18 were visualized by electrophoresis in a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Lane 1, Ad IL-18; lane 2, Ad LacZ; lane 3, PBS. (b) Densitometric analyses of the gel were made and results were expressed as a ratio of quantified IL-18 product over β-actin product. One of three independent experiments with similar results was shown.*

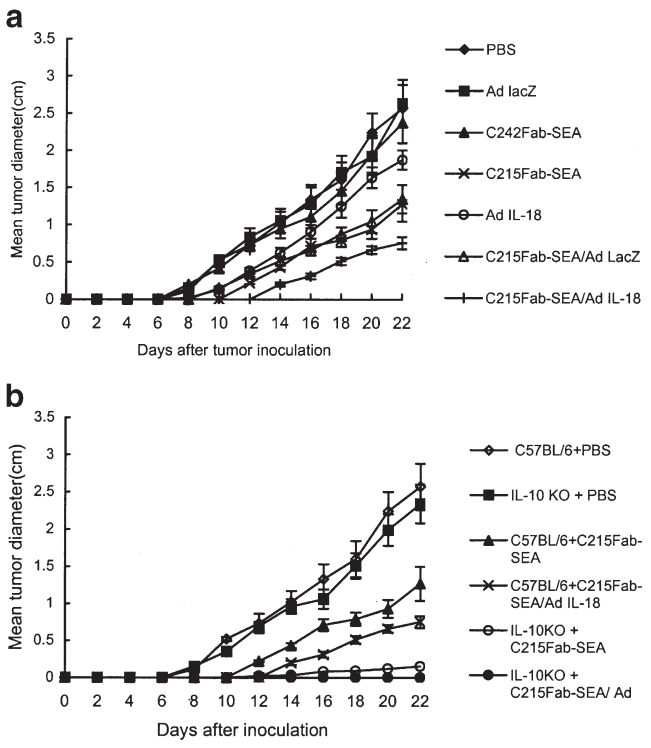

Figure 2 *More potent inhibition of tumor growth in melanoma-bearing mice after combined therapy of C215Fab-SEA and Ad IL-18. C57BL/6 IL-10 KO and wild-type mice were inoculated s.c with 1×10^5 B16-C215 cells on day 0. C57BL/6 mice were divided into seven groups (each group contained eight mice) and injected intratumorally with PBS, AdlacZ, C215Fab-SEA, C242Fab-SEA, Ad IL-18, C215Fab-SEA/AdlacZ, C215Fab-SEA/Ad IL-18, respectively. IL-10 KO mice were divided into three groups (each group contained eight mice) and treated with PBS, C215Fab-SEA, C215Fab-SEA/Ad IL-18. Injection of 10^8 p.f.u. virus/0.1 ml was performed on days 3 and 11. C215Fab-SEA 15 μg per animal was injected intratumorally on days 4, 6, 8 and 10. The length and width of the tumor mass were measured with calibers every other day. (a) Inhibition of tumor growth in melanoma-bearing C57BL/6 mice by combined therapy of C215Fab-SEA and Ad IL-18. (b) Comparison of tumor growth inhibition after treatment with C215Fab-SEA and Ad IL-18 in C57BL/6 IL-10 KO mice and wild-type C57BL/6 mice.*

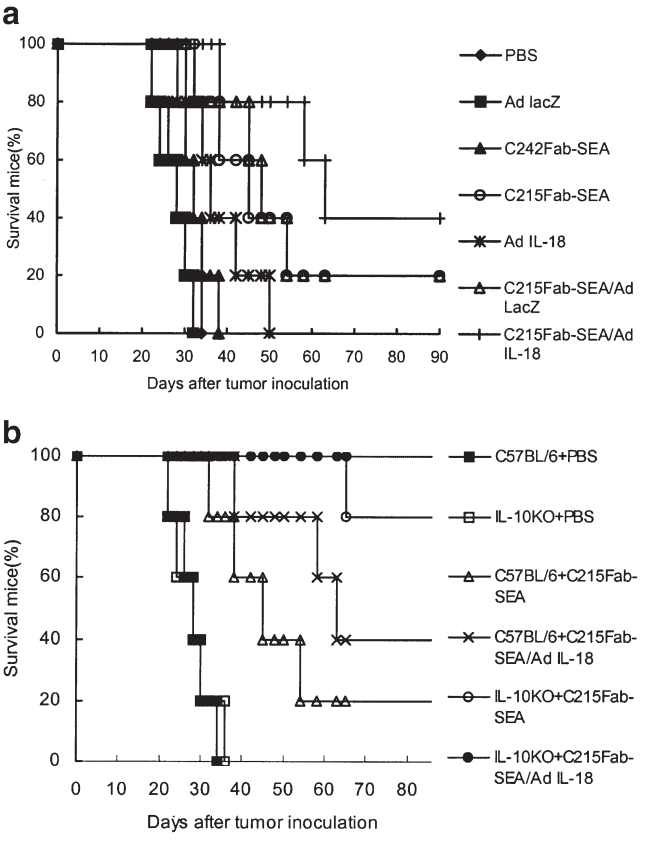

Figure 3 *Survival period of melanoma-bearing mice after combined therapy of C215Fab-SEA and Ad IL-18. C57BL/6 IL-10 KO and wild-type mice were inoculated s.c. with 1×10^5 B16-C215 cells. Three days later C57BL/6 mice received intratumoral injections of PBS, AdlacZ, C215Fab-SEA, C242Fab-SEA, Ad IL-18, C215Fab-SEA/AdlacZ, C215Fab-SEA/Ad IL-18. IL-10 KO mice received treatment with PBS, C215Fab-SEA, C215Fab-SEA/Ad IL-18. Five tumor-bearing mice in each group were observed for their survival time. (a) Survival period of melanoma-bearing C57BL/6 mice after combined therapy of C215Fab-SEA and Ad IL-18. (b) Comparison of survival period of melanoma-bearing C57BL/6 IL-10 KO mice and wild-type mice after combined therapy.*

pressed more significantly than that in wild-type C57BL/6 mice ($P < 0.01$). Tumor growth in IL-10 KO mice receiving combination therapy of C215Fab-SEA/Ad IL-18 was completely inhibited. All mice were tumor-free.

Increased survival period of melanoma-bearing mice after combined therapy of C215Fab-SEA and Ad IL-18

Five mice in each group were observed for their survival period. The results in Figure 3a showed that tumor-bearing C57BL/6 mice treated with Ad IL-18, C215Fab-SEA, C215Fab-SEA/AdLacZ survived much longer than mice treated with PBS, AdLacZ, C242Fab-SEA ($P < 0.01$). Mice of the C215Fab-SEA-treated group survived longer than mice of the Ad IL-18-treated group ($P < 0.01$). The survival time was further prolonged in mice received combined therapy as compared with that in mice treated with Ad IL-18 or C215Fab-SEA alone ($P < 0.01$). As shown in Figure 3b, tumor-bearing IL-10 KO mice treated with C215Fab-SEA or C215Fab-SEA/Ad IL-18 survived significantly longer than tumor-bearing wild-type C57BL/6 mice that received the same therapy ($P < 0.01$). Four of five IL-10 KO mice treated with C215Fab-SEA survived more than 90 days, while only one of five wild-type C57BL/6 mice treated with C215Fab-SEA and two of five mice receiving combined therapy were tumor-free and lived more than 90 days. All of the tumor-bearing IL-10 KO mice that received combined therapy were found to be tumor-free and lived more than 90 days. These data, together with the above data about the tumor growth, suggested that combined therapy of C215Fab-SEA administration and IL-18 gene transfer could elicit a more potent antitumor effect *in vivo* if Th1 response was enhanced.

More massive infiltration of inflammatory cells into the tumor mass after combined therapy of C215Fab-SEA and Ad IL-18

Subcutaneous tumor nodules were taken from tumor-bearing mice that had been killed 5 days after the last injection. Histological examination of tumor mass showed that the most obvious infiltration of inflammatory cells (neutrophils, lymphocytes, monocytes) was present inside and around the tumors of the tumor-bearing mice that received combined therapy of C215Fab-SEA

Table 1 Pathological analysis of tumor mass in melanoma-bearing C57BL/6 mice after combined therapy with C215Fab-SEA and Ad IL-18

Groups	*Tumor necrosis*[a]	*Infiltration of inflammatory cells inside the tumors*[b]	*Infiltration of inflammatory cells around the tumors*[b]
PBS	–	–	–
AdlacZ	–	–	–
c242Fab-SEA	–	–	–
AdIL-18	–	+	+
C215Fab-SEA	+	++	++
AdlacZ/C215Fab-SEA	+	++	++
AdIL-18/C215Fab-SEA	++	+++	+++

[a]Tumor necrosis: –, no necrosis; +, less than 1/3 of the tumor size; ++, 1/3~2/3 of the tumor size; +++, more than 2/3 of the tumor size. [b]Infiltration of inflammatory cells: –, no infiltration; +, minimal infiltration; ++, medium infiltration; +++, intense infiltration.

and Ad IL-18. Inflammatory cell infiltration was markedly observed in mice treated with C215Fab-SEA or AdlacZ/C215Fab-SEA, whereas relatively less infiltration was present in the tumors of Ad IL-18-treated mice. However, little infiltration of inflammatory cells was found inside and around tumors of PBS, AdLacZ and C242Fab-SEA control groups (as shown in Table 1).

Induction of splenic NK activity after combined therapy of C215Fab-SEA and Ad IL-18

Splenocytes derived from the tumor-bearing mice that had been killed after various therapies were used in cytolytic assays against YAC-1 cells at effector:target (E:T) ratios of 25:1, 50:1, 100:1 by a 4-h ^{51}Cr released assay. It was demonstrated in Figure 4 that the NK activity of lymphocytes from the tumor-bearing mice was very low, but the NK activity increased significantly after the mice were treated with C215Fab-SEA or Ad IL-18 ($P < 0.01$). NK activity of Ad IL-18-treated mice was higher than that of C215Fab-SEA or AdLacZ/C215Fab-SEA-treated mice ($P < 0.05$). Lymphocytes from the mice that received C215Fab-SEA/AdIL-18 therapy showed the highest NK activity when compared with lymphocytes from other groups ($P < 0.01$). There was no significant difference in NK activity between the IL-10 KO mice and wild-type C57BL/6 mice that received the same therapy. These data suggested that the augmentation of nonspecific immunity might be involved in the antitumor response of the combined therapy of C215Fab-SEA and Ad IL-18.

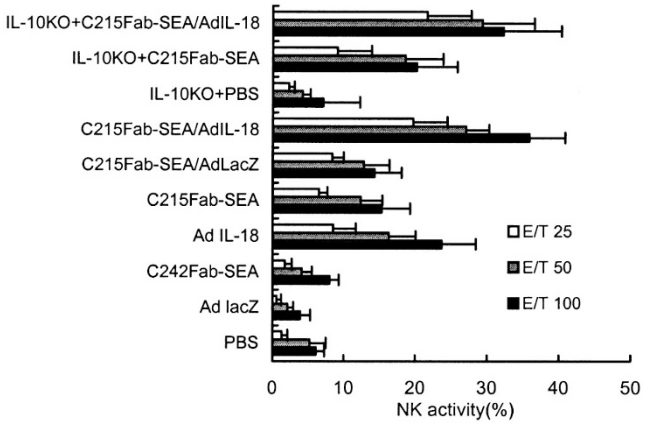

Figure 4 *Induction of NK activity after combined therapy of C215Fab-SEA and Ad IL-18. Splenic lymphocytes isolated from dead tumor-bearing mice of different groups 5 days after the last injection were used in cytolytic assays against YAC-1 cells at effector: target (E:T) ratios of 25:1, 50:1, 100:1 by a 4-h ^{51}Cr release assay.*

Augmentation of CTL activity after combined therapy of C215Fab-SEA and Ad IL-18

Lymphocytes isolated from tumor-bearing mice of different groups that had been killed were cocultured with inactivated B16-C215 (5000 rad) for 7 days in the presence of recombinant murine IL-2 (20 U/ml), then collected as CTL effector cells and B16-C215 melanoma cells were used as target cells. The CTL activity was determined at E:T ratios of 12.5:1, 25:1, 50:1 by a standard 4-h ^{51}Cr release assay. As shown in Figure 5, splenic CTL activity of Ad IL-18, C215Fab-SEA or AdLacZ/C215Fab-SEA-treated mice increased significantly when compared with that of control groups ($P < 0.05$), and CTL activity of C215Fab-SEA or AdLacZ/C215Fab-SEA-treated mice was higher than that of Ad IL-18-treated mice ($P < 0.05$). The highest CTL activity was induced in mice treated with C215Fab-SEA/Ad IL-18 compared with that in mice of other groups ($P < 0.01$). IL-10 KO mice exhibited increased CTL activity than wild-type C57BL/6 mice that received the same therapy ($P < 0.05$). It suggested that tumor-specific immunity was induced most significantly by the combined therapy of IL-18 gene transfer and C215Fab-SEA, especially when Th1 response was enhanced.

Enhancement of cytokine production after combined therapy of C215Fab-SEA and Ad IL-18

The Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cytokines secreted by splenocytes derived from tumor-bearing mice after various therapies were detected by ELISA kits. As shown in Figure 6, the production of IL-2 and IFN-γ from lymphocytes derived from mice treated with Ad IL-18,

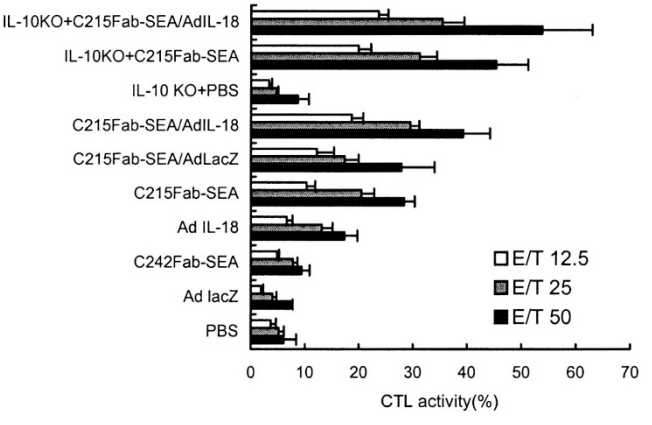

Figure 5 *Elevated splenic CTL activity of tumor-bearing mice after combined therapy of C215Fab-SEA and Ad IL-18. Lymphocytes isolated from tumor-bearing mice after various therapies were cocultured with inactivated B16-C215 (5000 rad) for 7 days in the presence of recombinant murine IL-2 (20 U/ml) and then collected as CTL effector cells. The B16-C215 melanoma cells were used as target cells. The CTL activity was determined at E:T ratios of 12.5:1, 25:1, 50:1 by a standard 4-h ^{51}Cr release assay.*

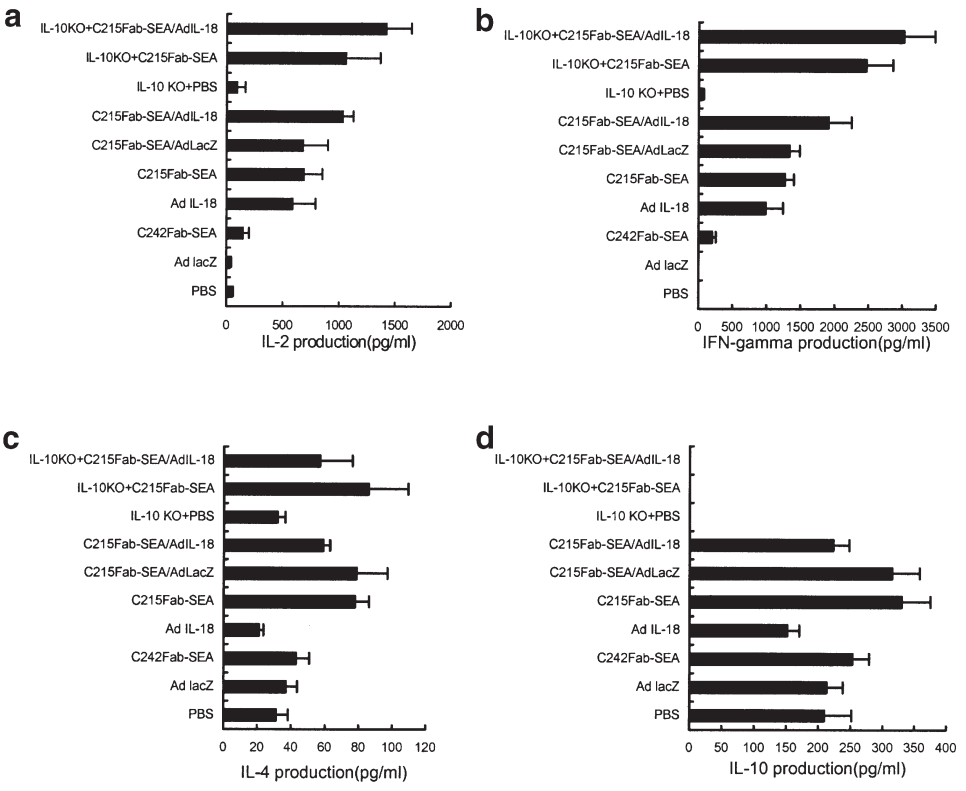

Figure 6 *Production of cytokines by lymphocytes derived from tumor-bearing mice (C57BL/6 mice and IL-10 KO mice) after combined therapy of C215Fab-SEA and Ad IL-18. (a) IL-2 production; (b) IFN-γ production; (c) IL-4 production; (d) IL-10 production. The cytokine contents in the supernatants of lymphocytes after stimulation of irradiated B16-C215 cells were determined by ELISA kits.*

C215Fab-SEA or C215Fab-SEA/AdLacZ were significantly higher than those from control groups ($P < 0.01$). After combined treatment with C215Fab-SEA and Ad IL-18, the production of IL-2 and IFN-γ in the mice increased to the highest levels when compared with those of the three groups above ($P < 0.05$). It was noted that the levels of IL-2 and IFN-γ were more markedly elevated in IL-10 KO mice treated with C215Fab-SEA or C215Fab-SEA/Ad IL-18 than in wild-type C57BL/6 mice receiving the same therapy ($P < 0.05$). The production of IL-4 in the tumor-bearing C57BL/6 mice treated with C215Fab-SEA or Ad LacZ/C215Fab-SEA increased as compared with that of the mice treated with PBS, AdLacZ, C242Fab-SEA ($P < 0.05$). The level of IL-4 in mice treated with AdIL-18 alone decreased as compared with that in mice of the control groups ($P < 0.05$). The IL-4 level in mice treated with C215Fab-SEA/Ad IL-18 was lower than that in mice treated with C215Fab-SEA alone ($P < 0.05$). The production of IL-10 was not detectable in IL-10 KO mice. The level of IL-10 in the wild-type C57BL/6 mice treated with Ad IL-18 alone decreased as compared with that in mice of the control groups ($P < 0.05$), while the IL-10 level in C215Fab-SEA or C215Fab-SEA/AdLacZ-treated mice was higher than that in controls ($P < 0.05$). After the tumor-bearing C57BL/6 mice were treated with C215Fab-SEA/Ad IL-18, the production of IL-10 was down-regulated as compared with that in mice treated with C215Fab-SEA alone ($P < 0.05$). These data indicated that enhanced antitumor effect of Fab-SEA therapy may be related to the increased production of Th1 cytokines (IL-2, IFN-γ) and decreased production of Th2 cytokines (IL-4, IL-10).

Discussion

A variety of immunologic strategies have been used to elicit strong antitumor responses to cause regression of established tumors.[31–35] Studies have illustrated that Fab-SEA therapy induced recruitment of tumor-infiltrating lymphocytes (TILs), cytokine production and apoptosis of tumor cells *in vitro* and *in vivo*.[10–17] However, the antitumor effect of C215Fab-SEA therapy is often not satisfactory, especially in pre-established tumors. In this study, it was shown that tumor growth in mice treated with C215Fab-SEA was significantly inhibited compared with that in control groups, but the antitumor effect was not strong enough; complete tumor remission was seen in only 20% of the treated mice. SEA activates the T cells which express particular T cell receptor Vβ chains but not tumor-specific T cells, so Fab-SEA evoked a pseudo-specific immune attack but not a very specific antitumor immune response. Furthermore, a phase of immune exhaustion including failure to produce IL-2 and the tumor-suppressive cytokines IFN-γ and TNF-α as well as failure to induce potent CTL activity followed after the Fab-SEA treatment.[18] This hyporesponsiveness directly limited the therapeutic efficacy of Fab-SEA immunotherapy. Hyporesponsive T cells have a defect in the activation of $P21^{ras}$ following TCR occupancy, which translates to a down-regulation of the AP-1 transcription factor. Early studies have shown that SAg-induced IL-2 hyporesponsiveness *in vivo* correlates with down-regulation of the AP-1 and p50/p65 Rel transcription factors, suggesting an intrinsic defect in anergic T cells. Exogenous IL-2 has been demonstrated to restore responsiveness

in anergic T cells *in vitro* and *in vivo*.[36] Rosendahl *et al*[18] reported that antitumor effect of Fab-SEA immunotherapy was potentiated by Fab-IL-2 co-administration. Fab-SEA combined with Fab-IL-2 augmented the production of IFN-γ, restored the responsiveness of T cells, prolonged the immune response *in vivo*, so limiting the development of immunological hyporesponsiveness. The presence of IFN-γ-producing T lymphocytes is highly relevant to antitumor effect since the B16 melanoma is extremely sensitive to IFN-γ *in vitro*.[20,21]

IL-18 is a newly discovered cytokine, structurally similar to IL-1, with profound effects on T cell activation. It plays an important role in the Th1 response, primarily by its ability to induce IFN-γ production in T cells and NK cells and regulation of FasL-dependent apoptosis.[22–25] IL-18 exhibits antitumor activity in various tumor models. IL-18 induced the sequential activation of NK cells and CTL to protect syngeneic mice from transplantation with Meth A sarcoma.[25] Protective immunity was induced by the immunization with colon carcinoma cells genetically modified to express IL-18. Colon 26 cells transduced with the IL-18 gene (Colon 26/IL-18) could not form subcutaneous tumors in immunocompetent mice, and the mice became resistant to rechallenge with wild-type colon 26 cells.[26] IL-18 can enhance the antitumor effects on mouse melanoma elicited by local IL-12 secretion.[37] Intratumoral injection of a single dose of recombinant IL-18 substantially delayed the growth of subcutaneously inoculated gliomas.[27] IL-18 also acts as an angiogenesis suppressor *in vitro* and *in vivo*, sufficiently potent to suppress the fibroblast growth factor-induced corneal neovascularization and inhibit embryonic angiogenesis in the chick chorioallantoic membrane assay.[38] The biologic functions of IL-18 mentioned above make it attractive to be used in potentiating the antitumor effects of Fab-SEA.

In this study, we demonstrated that combination of intratumoral IL-18 gene transfer and C215Fab-SEA administration elicited antitumor immune response most potently. It might be a promising new approach for cancer immunogenetherapy. Inhibition of subcutaneous tumor growth was observed most significantly in mice after combined therapy of C215Fab-SEA and Ad IL-18 when compared with that in mice treated with Ad IL-18 or C215Fab-SEA alone. More inflammatory cells were present around and in the tumor mass after combined therapy. Spleen cells from mice that received combined therapy showed the highest CTL activity as compared with those from the mice treated with C215Fab-SEA, Ad IL-18 alone. NK activity is also efficiently induced after combined therapy, suggesting that augmentation of tumor-specific and nonspecific immunity both participated in the enhanced antitumor response induced by C215Fab-SEA and Ad IL-18 combined therapy. Therefore, combination of antibody-targeted superantigen and adenovirus-mediated IL-18 gene transfer might be a potential therapy to be utilized in cancer intervention.

In the murine immune system, $CD4^+$ helper T cells can be distinguished into two types: 'Th1' cells that facilitate cell-mediated cytotoxicity by the secretion of IL-2, IFN-γ, TNF-α and 'Th2' cells that stimulate B cell growth, differentiation and antibody production by the production of IL-4, IL-5, IL-6, IL-10. To investigate the importance of Th1 and Th2 cytokines, antitumor effect of combined therapy in IL-10 KO mice was investigated in our study, but IFN-γ receptor knockout mice were not used because no such kind of mice with the C57BL/6 background were available.

Tumor growth in IL-10 KO mice treated with C215Fab-SEA was suppressed more significantly and these mice survived significantly longer than wild-type C57BL/6 mice. All tumor-bearing IL-10 KO mice receiving combined therapy were tumor-free and survived more than 90 days, but only 40% of tumor-bearing C57BL/6 mice receiving combined therapy were tumor-free. The production of IL-2 and IFN-γ from lymphocytes increased after the mice were treated with C215Fab-SEA, Ad IL-18 alone. The levels of IL-2 and IFN-γ in mice with combined treatment were the highest compared with those of mice in other groups. The levels of IL-2 and IFN-γ in IL-10 KO mice after treatment with C215Fab-SEA or C215Fab-SEA/Ad IL-18 were more markedly elevated than those in wild-type C57BL/6 mice. The level of IL-10 in the wild-type C57BL/6 mice treated with Ad IL-18 alone decreased as compared with that in mice of control groups, while the IL-10 level in C215Fab-SEA-treated mice was higher than that in controls. After being treated with C215Fab-SEA/Ad IL-18, the production of IL-10 in the tumor-bearing C57BL/6 mice decreased as compared with that in mice treated with C215Fab-SEA alone. These results suggested that Th1 cytokines (IL-2, IFN-γ) play important roles in Fab-SEA and IL-18 immunotherapy and *in vivo* expression of IL-18 can augment the Th1 responses and enhance the therapeutic effect of C215Fab-SEA targeted immunotherapy of cancer. The antitumor effect of Fab-SEA-based cancer immunotherapy could be impaired due to the release of the regulatory Th2-type cytokine IL-10. IL-10 has been known to down-regulate T cell immune responses by various mechanisms. It was shown to suppress both IFN-γ production and the antitumor effector phase of IFN-γ.[39–41] It may inhibit and down-regulate MHC-II molecule expression of monocytes as well as interfere with antigen presentation,[42] inhibit the up-regulation of co-stimulatory molecules[43] and suppress the cytotoxicity of macrophages.[44] In addition, it was recently demonstrated that T cell receptor triggering in the presence of IL-10 inhibited IFN-γ production in freshly isolated $CD4^+$ T cells[45] and down-regulated the expression of granzyme B.[40,46] The production of IL-4 in the tumor-bearing C57BL/6 mice treated with C215Fab-SEA alone increased as compared with that of mice in control groups, while the level of IL-4 in mice treated with AdIL-18 alone was lower that in mice of control groups. After the combined treatment with C215Fab-SEA/Ad IL-18, the IL-4 level of tumor-bearing C57BL/6 mice decreased as compared with that in mice treated with C215Fab-SEA alone. IL-4 may act as an immunoregulatory cytokine during C215Fab-SEA treatment and Ad IL-18 therapy may down-regulate the production of IL-4. The mechanism remains to be elucidated.

However, the direct role of SAgs in tumor development remains to be addressed. Bacterial SAgs are often used as immunostimulants for infection and tumor immunity because of their strong T cell stimulation activity. On the other hand, SAg from endogenous mouse mammary tumor virus (MMTV) functions as an immunogenic factor in tumor development. Masayuki U *et al*[47] reported that expression of viral SAg enhances the tumorigenicity of a myeloma cell line through the stimulation of SAg-reactive T cells. SAg expression from inherited

provirus usually leads to depletion of immature T cells expressing reactive TCR β chains during intrathymic T cell development. The characteristic of SAg for strong T cell stimulation is critical in successful infection of the mammary gland for exogenous MMTV and in skewing the T cell repertoire via clonal deletion for endogenous MMTV.

Materials and methods

Animals

Male or female wild-type C57BL/6 mice (H-2K^b), C57BL/6 IL-10 gene knockout mice (IL-10 KO), 6~8 weeks of age, purchased from Joint Ventures Sipper BK Experimental Animal Co (Shanghai, China), were housed in a specific pathogen-free condition for all experiments.

Cell lines

B16, a melanoma cell line derived from C57BL/6 mice, YAC-1, an NK-sensitive lymphoma cell line of A/S (H-2a) origin, and 293, a continuous cell line derived from human embryonic kidney were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA), maintained in RPMI-1640 medium supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, 2-mercaptoethanol 50 mmol/l and 10% fetal calf serum (FCS). All culture media were purchased from Gibco-BRL (Gaithersburg, MD, USA) and fetal calf serum (FCS) was provided by Shanghai Institute of Biological Products (Shanghai, China).

Reagents

C215 and C242 mAbs, reacting with human colon cancer, were produced and provided by Active Biotech Research (Sweden). Recombinant fusion proteins of the Fab regions of C215, C242 mAb and the superantigen SEA (C215Fab-SEA, C242Fab-SEA) were constructed as described by Dohlsten *et al*[8] and kindly provided by Active Biotech Research (Lund, Sweden). The fusion protein was expressed in *Escherichia coli* K-12 UL635 (ara-14, xyl-7, ΔompT, T4^R) and purified using a protein G sepharose column and fractions containing Fab-SEA were passed through a PD-10 column (Pharmacia LKB Biotechnology, Lund, Sweden).

The expressing vector pKGE839 containing the GA733–2 cDNA (encoding the C215 antigen)[29] and the neomycin resistance gene were also gifts from Active Biotech Research AB.

The murine IL-2, murine IFN-γ, murine IL-4, murine IL-10 were purchased from Endogen (Woburn, MA, USA). The murine IL-18 ELISA kit was purchased from R & D Systems (Minneapolis, MN, USA).

Transfection of B16 melanoma cells with cDNA encoding the C215 antigen

The expression vector pKGE839 encoding the C215 antigen was transfected into B16 melanoma cells using lipofectamine (Gibco-BRL, Grand Island, NY, USA). After transfection, the B16 cells were selected in RPMI 1640 medium containing 800 μg/ml G418 (Sigma, St Louis, MO, USA). G418-resistant clones were harvested and analyzed for C215 antigen expression. The C215 highly expressing clone 5.B7 was selected. The expression of C215 antigen was analyzed by FACS. The B16-C215 cells were incubated with an optimal concentration of mouse anti-human C215 monoclonal antibody (Active Biotech) for 30 min at 4°C followed by washing with cold PBS for three times. The cells were then incubated with FITC-conjugated goat anti-mouse IgG followed by washing three times with PBS and resuspended in PBS containing 1% formaldehyde. Flow cytometry was analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Preparation of recombinant adenoviruses

Replication-defective recombinant adenoviruses AdLacZ harboring the β-galactosidase gene and Ad IL-18 harboring the murine IL-18 gene were constructed from human adenovirus serotype 5 using homologous recombination. The procedures were described previously by us.[30] The expression of these genes was driven by a CAG promoter. The recombinant adenoviruses were released from 293 cells by three freeze/thaw cycles and subsequently propagated with 293 cells. The titers of the adenoviral preparations were determined by plaque-forming assay on 293 cells. Briefly, serial 10-fold dilutions of adenovirus were added to 24-well plates (Corning, NY, USA), containing confluent 293 cell monolayers. After 48 h of incubation in a humidified atmosphere, the end point of 50% infectivity was determined according to cytopathic effect. The recombinant adenoviruses produced were diluted to a titer of 10^{10} p.f.u./ml in PBS and stored at –70°C for experiments.

Immunotherapy with C215Fab-SEA in combination with IL-18 gene transfer

C215 antigen-expressing B16 melanoma cells (B16-C215) were taken from continuous culture, washed three times and resuspended in PBS without FCS for inoculation into mice. C57BL/6 IL-10 KO and wild-type mice were inoculated s.c. with 1×10^5 B16-C215 cells in a back leg. Three days after tumor cell inoculation, the tumor-bearing C57BL/6 mice were divided into seven groups and were injected intratumorally with any of the following preparations: PBS, AdlacZ, C242Fab-SEA (non-related antibody control), C215Fab-SEA, Ad IL-18, AdlacZ in combination with C215Fab-SEA (C215Fab-SEA/Ad LacZ), Ad IL-18 in combination with C215Fab-SEA (C215Fab-SEA/Ad IL-18). Each group contained eight mice. Tumor-bearing IL-10 KO mice were divided into three groups and received therapy of the following preparations: PBS, C215Fab-SEA, C215Fab-SEA/Ad IL-18. Each group also contained eight IL-10 KO mice.

Three days after the tumor inoculation 10^8 p.f.u. virus/0.1 ml was injected intratumorally and a booster of the same dose was given after 8 days. From 4 days after the tumor inoculation, C215Fab-SEA 15 μg per animal was injected intratumorally every other day for four times. The length and width of the tumor mass were measured with calibers every other day after tumor inoculation. Tumor size was expressed as 1/2 (length + width). Five mice in each group were observed for their survival period.

Determination of intratumoral IL-18 mRNA expression by semiquantitative RT-PCR

One week after intratumoral injection with Ad IL-18, AdLacZ or PBS, total RNA was extracted from subcutaneous tumor tissues by TRIzol Reagent (Gibco-BRL),

according to the instructions of the manufacturer. One μg total RNA from each sample was reverse transcribed using a Reverse Transcription System kit (MBI Fermentas, Vilnius, Lithuania) in a total volume of 20 μl. cDNA as readout of the mRNA was quantified in a competitive PCR using specific primers for IL-18 and β-actin. Primers for IL-18 amplification were 5′-ACTGTA CAACCGCAGTAATACGC-3′ (sense) and 5′-AGTGAA CATTACAGATTTATCCC-3′ (anti-sense), with an expected PCR product of 434 bp. Primers for amplification of β-actin were 5′-TGGAATCCTGTGGCATCCAT GAAAC-3′ (sense) and 5′-TAAAACGCAGCTCAGTAA CAGTCCG-3′ (anti-sense), with an expected PCR product of 348 bp. Cycling conditions were 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 45 s of extension at 72°C during 25 cycles for β-actin and 30 cycles for IL-18.

PCR products of β-actin and IL-18 were visualized by electrophoresis in a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Densities of the amplified β-actin and IL-18 were analyzed using the Kodak EDAS120 digital imaging system version 3 (Gibco). Results were expressed as a ratio of quantified IL-18 product over β-actin product.

Histologic examination

Subcutaneous tumor nodules were taken from dead tumor-bearing mice 5 days after the last injection. The tumor samples were fixed in 10% formalin solution, dehydrated and embedded in paraffin. Thin-sliced sections were stained with hematoxylin and eosin. The evaluation of tumor necrosis was: −, no necrosis; +, less than 1/3 of the tumor size; ++, 1/3~2/3 of the tumor size; +++, more than 2/3 of the tumor size. The evaluation of the infiltration of inflammatory cell both in and around the tumor was: −, no inflammatory cell infiltration; +, minimal inflammatory cell infiltration; ++, medium inflammatory cell infiltration; +++, intense inflammatory cell infiltration.

Cytotoxic assay of CTL and NK cells

Splenic lymphocytes were isolated from dead tumor-bearing mice 5 days after the last injection. The erythrocytes were depleted with 0.83% ammonium chloride and macrophages were removed by adherence of splenocytes on plastic plates for 2 h. The non-adherent lymphocytes were directly used as NK effector cells. The lymphocytes were cocultured with inactivated B16-C215 (5000 rad) for 7 days in the presence of recombinant murine IL-2 (20 U/ml) and then collected as CTL effector cells. The NK activity and CTL activity were determined by a standard 4-h ^{51}Cr release assay as described in our previous work.[48] YAC-1 or B16-C215 melanoma cells (2×10^6) in 0.5 ml RPMI-1640 with 20% FCS were labeled with 200 μCi $Na^{51}CrO_4$ (Amersham, Arlington Heights, IL, USA) for 2 h. The labeled cells were washed three times in serum-free medium. Target cells (1×10^4) were then mixed with different ratios of effector cells for 4 h. For the maximal ^{51}Cr release control, 0.1 ml of 10% SDS was added to the target cells, and for the spontaneous ^{51}Cr release control, 0.1 ml medium was added to the labeled cells. The amount of ^{51}Cr released was determined by γ-counting on a Minigamma Counter (LKB-Wallac, Turku, Finland). Calculations were carried out according to the formula % of specific lysis = 100 × (experimental c.p.m. – spontaneous c.p.m.)/(maximal c.p.m. – spontaneous c.p.m.).

Cytokine release assay

The non-adherent splenocytes derived from dead tumor-bearing mice at a concentration of 1×10^7 cells/ml were stimulated with 1×10^6 irradiated B16-C215 (5000 rad). Supernatants were harvested after 24 h (for IL-2 assay), 48 h (for IL-4 and IL-10 assay), or 72 h (for IFN-γ assay). The supernatants of B16-C215 cells at a concentration of 1×10^6 cells/ml after Ad IL-18 transfection were harvested at different times to detect the production of IL-18. The cytokines were measured using a standard sandwich ELISA technique with corresponding kits.

Statistics

All the experiments were run in triplicate and the results are means ± s.d. of triplicate determinations (or representation data from one or two independent experiments). Statistical analysis was performed using the Student's *t* test and log-rank test (for survival analysis). The difference was considered statistically significant when the *P* value was less than 0.05.

Acknowledgements

We would like to acknowledge the gifts of C215Fab-SEA, C242Fab-SEA, C215Mab, SEA, plasmid PKGE839 from Active Biotech Research AB (Sweden). This work was supported by grants from the National Natural Science Foundation of China (No. 39770837 and No. 39730420).

References

1 Klarnet JP *et al*. FBL-reactive $CD8^+$ cytotoxic and $CD4^+$ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigen. *J Exp Med* 1989; **169**: 457–467.
2 Kann M *et al*. $CD4^+$ T cells clones specific for the human p97 melanoma-associated antigen can eradicate pulmonary metastases from a murine tumor expressing the p97 antigen. *J Immunol* 1991; **146**: 3235–3241.
3 Hom SS *et al*. Common expression of melanoma tumor-associated antigens recognized by human tumor infiltrating lymphocytes: analysis by human lymphocyte antigen restriction. *J Immunother* 1991; **10**: 153–164.
4 White J *et al*. The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* 1989; **56**: 27–35.
5 Fischer H *et al*. Production of TNF-alpha and TNF-beta by staphylococcal enterotoxin A activated human T cells. *J Immunol* 1990; **144**: 4663–4669.
6 Dohlsten M *et al*. Superantigen-induced cytokines suppress growth of human colon-carcinoma cells. *Int J Cancer* 1993; **54**: 482–488.
7 Dohlsten M *et al*. Immunopharcology of the superantigen staphylococcal enterotoxin A in T cell receptor V beta 3 trangenic mice. *Immunology* 1993; **79**: 520–527.
8 Dohlsten M *et al*. Monoclonal antibody-superantigen fusion proteins: tumor-specific agents for T-cell-based tumor therapy. *Proc Natl Acad Sci USA* 1994; **91**: 8945–8949.
9 Ihle J *et al*. Antibody-targeted superantigens induce lysis of major histocompatibility complex classII-negative T-cell leukemia lines. *Cancer Res* 1995; **55**: 623–628.
10 Lando PA *et al*. Tumor-reactive superantigens suppress tumor growth in humanized SCID mice. *Int J Cancer* 1995; **62**: 466–471.
11 Dohlsten M *et al*. Immunotherapy of human colon cancer by antibody-targeted superantigens. *Cancer Immunol Immunother* 1995; **41**: 162–168.

12 Dohlsten M *et al*. Antibody-targeted superantigens are potent inducers of tumor-infiltrating T lymphocytes *in vivo*. *Proc Natl Acad Sci USA* 1995; **92**: 9791–9795.
13 Litton MJ *et al*. Antibody-targeted superantigen therapy induces tumor-infiltrating lymphocytes, excessive cytokine production, and apoptosis in human colon carcinoma. *Eur J Immunol* 1996; **26**: 1–9.
14 Rosendahl A *et al*. Immune response during tumor therapy with antibody-superantigen fusion proteins. *Int J Cancer* 1996; **68**: 109–113.
15 Giantonio BJ *et al*. Superantigen-based immunotherapy: a phase I trial of PNU-214565, a monoclonal antibody-staphylococcal enterotoxin A recombinant fusion protein, in advanced pancreaticand colorectal cancer. *J Clin Oncol* 1997; **15**: 1994–2007.
16 Rosendahl A *et al*. Perforin and IFN-gamma are involved in the antitumor effects of antibody-targeted superantigens. *J Immunol* 1998; **160**: 5309–5313.
17 Nielsen SE *et al*. Phase I study of single, escalating doses of a superantigen-antibody fusion protein (PNU-214565) in patients with advanced colorectal or pancreatic carcinoma. *J Immunother* 2000; **23**: 146–153.
18 Rosendahl A *et al*. Long-term survival and complete cures of B16 melanoma-carrying animals after therapy with tumor-targeted IL-2 and SEA. *Int J Cancer* 1999; **81**: 156–163.
19 Sakurai M *et al*. Comparative study of the antitumor effect of two typrs of murine recombinant interferons (beta) and (gamma), against B16-F10 melanoma. *Cancer Immunol Immunother* 1988; **26**: 109–113.
20 Mareel M, Dragonetti C, Tavernier J, Fiers W. Tumor-selective cytotoxic effects of murine tumor necrosis factor (TNF) and interferon-gamma (IFN-gamma) in organ culture of B16 melanoma cells and heart tissue. *Int J Cancer* 1988; **42**: 470–473.
21 Okamura H *et al*. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 1995; **378**: 88–91.
22 Micallef MJ *et al*. Interferon-gamma-inducing factor enhances T helper 1cytokine production by stimulated human T cells: synergism with interleukin-12 for interferon-gamma production. *Eur J Immunol* 1996; **26**: 1647–1651.
23 Dao T *et al*. Interferon-gamma-inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity murine T helper 1 cells. *Cell Immunol* 1996; **173**: 230–235.
24 Micallef MJ *et al*. *In vivo* antitumor effects of murine interferon-gamma-inducing factor/interleukin 18 in mice bearing syngeneic Meth A sarcoma malignant ascites. *Cancer Immunol Immunother* 1997; **43**: 361–367.
25 Micallef MJ *et al*. Interleukin 18 induces the sequential activation of natural killer cells and cytotoxic T lymphocytes to protect syngeneic mice from transplantation with MethA sarcoma. *Cancer Res* 1997; **57**: 4557–4563.
26 Tasaki K *et al*. Protective immunity is induced in murine colon carcinoma cells by the expression of interleukin-12 or interleukin-18, which activate type 1 helper T cells. *Cancer Gene Ther* 2000; **7**: 247–254.
27 Kikuchi T *et al*. Antitumor activity of interleukin-18 on mouse glioma cells. *J Immunother* 2000; **23**: 184–189.
28 Yang X *et al*. IL-10 gene knock out mice show enhanced Th1-like protective immunity and absent granuloma formation following chlamydia trachomatis lung infection. *J Immunol* 1999; **162**: 1010–1017.
29 Bjork P *et al*. Isolation, partial characterization, and molecular cloning of a human colon adenocarcinoma cell-surface glycoprotein recognized by the C215 mouse monoclonal antibody. *J Biol Chem* 1993; **268**: 24232–24241.
30 Ju DW *et al*. Interleukin-18 gene transfer increases antitumor effects of suicide gene therapy through efficient induction of antitumor immunity. *Gene Therapy* 2000; **7**: 1672–1679.
31 Pardoll DM. Cancer vaccines. *Nature Med* 1999; **4**: 525–531.
32 Li Y *et al*. Costimulation of tumo-reactive $CD4^+$ and $CD8^+$ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. *J Immunol* 1994; **153**: 421–427.
33 Lotze MT *et al*. Cytokine gene therapy of cancer. *Cancer* 1996; **2**: 63–72.
34 Bubenik J. Granulocyte-macrophage colony-stimulating factor gene-modified vaccines for immunotherapy of cancer. *Folia Biol (praha)* 1999; **45**: 115–119.
35 Blankenstein T, Qin Z. Cancer vaccine. *Gene Therapy* 1996; **3**: 95–96.
36 Belfrage H, Dohlsten M, Hedlund G, Kalland T. Prevention of superantigen-induced tolerance *in vivo* by interleukin-2 treatment. *Cancer Immunol Immunother* 1997; **44**: 77–82.
37 Nagai H *et al*. Antitumor effects on mouse melanoma elicited by local secretion of interleukin-12 and their enhancement by treatment with interleukin-18. *Cancer Invest* 2000; **18**: 206–213.
38 Cao R, Farnebo J, Kurimoto M, Cao Y. Interleukin-18 acts as an angiogenesis and tumor suppressor. *FASEB J* 1999; **13**: 2195–2202.
39 Aruga A *et al*. Type 1 versus type 2 cytokine release by V beta T cell subpopulations determines *in vivo* antitumor reactivity:IL-10 mediates a suppressive one. *J Immunol* 1997; **159**: 664–673.
40 Litton MJ *et al*. The distinct role of $CD4^+$ and $CD8^+$ T-cells during the anti-tumor effects of targeted superantigens. *Br J Cancer* 1999; **81**: 359–366.
41 Sundstedt A *et al*. Immunoregulatory role of IL-10 during superantigen-induced hyporesponsiveness *in vivo*. *J Immunol* 1997; **158**: 180–186.
42 Fiorentino DF *et al*. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; **146**: 3444–3451.
43 Ding L *et al*. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* 1993; **151**: 1224–1234.
44 Oswald IP, Gazzinelli RT, Sher A, James SL. IL-10 synergizes with IL-4 and transforming growth factor-β to inhibit macrophage cytotoxic activity. *J Immunol* 1992; **148**: 3578–3582.
45 Groux H, Bigler M, de Vries JE, Roncarolo MG. Interleukin-10 induces a long-term antigen-specific anergic state in human $CD4^+$ T cells. *J Exp Med* 1996; **184**: 19–29.
46 Fitzpatrick L, Makrigiannis AP, Kaiser M, Hoskin D. Anti-CD3-activated killer T cells: interferon-gamma and interleukin-10 cross-regulate granzyme B expression and the induction of major histocompatibity complex-unrestricted cytotoxicity. *J Interferon Cytokine Res* 1996; **16**: 537–546.
47 Masayuki U *et al*. Expression of mouse mammary tumor virus superantigen accelerates tumorigenicity of myeloma cells. *J Virol* 2000; **74**: 8226–8233.
48 Lei H *et al*. Induction of potent antitumor response by vaccination with tumor lysate-pulsed macrophages engineered to secret macrophage colony-stimulating factor and interferon-γ. *Gene Therapy* 2000; **7**: 707–713.