Article

Antitumor Activity and Immune Enhancement of Murine Interleukin-23 Expressed in Murine Colon Carcinoma Cells

Baoen Shan[1, 3], Jingsheng Hao[1], Qiaoxia Li[1] and Masatoshi Tagawa[2]

Interleukin (IL)-23, a cytokine composed of p19 and the p40 subunit of IL-12, can enhance the proliferation of memory T cells and production of IFN-γ from activated T cells. It can also induce antitumor effects in murine model. To further evaluate the antitumor activity and immune enhancement of IL-23 *in vivo*, murine colon carcinoma cells retrovirally transduced with mIL-23 gene were injected subcutaneously (*s.c.*) into BALB/c mice. Survival time and tumor volume were observed. LDH release assay, [^{3}H]-TdR incorporation assay and ELISA were used to determine CTL activity, proliferation of splenocytes and level of cytokines, respectively. Number of dendritic cells (DCs) was analyzed by flow cytometry (FCM). IL-23 secreted by Colon26/IL-23 cells suppressed the growth of tumor and prolonged the survival time of mice, enhanced proliferation of splenocytes, CTL activity, and number of DCs. IL-23 also promoted the production of Th1 cytokines such as IFN-γ, IL-12 and TNF-α. However, the level of IL-4 was not enhanced significantly. These data suggested that IL-23 secreted by tumor cells can induce antitumor activity by enhancing immune response. *Cellular & Molecular Immunology*. 2006;3(1):47-52.

Key Words: IL-23, antitumor, proliferation, cytokine, CTL, dendritic cell

Introduction

Despite dramatic cancer therapeutic advances, many cancers will recur, metastasize or remain resistant to conventional therapies. Immunotherapy including using cytokines, and cytokine gene therapy might represent further advances in the therapy of cancer. Systemic administrations of some cytokines have resulted in clinical responses in several types of cancers. However, severe toxicities limit them in clinical applications. Therefore, studies have looked for more effective and less toxic cytokines and novel routes of administration. Cytokine gene therapy is an alternative approach of immunotherapy, in which cytokine genes are transduced into vehicles such as tumor cells, dendritic cells (DCs) or fibroblasts. The vehicles carrying cytokine genes in the host can produce cytokines that induce immune responses against tumors and overcome the drawbacks of systemic administration of the cytokines (1, 2). Besides, tumor cells and DCs loaded with tumor antigens can provide the necessary tumor antigens to induce specific anti-tumor responses.

IL-23 is composed of the IL-12p40 subunit and the p19 subunit, which is a novel protein related to the p35 subunit of IL-12 (3). IL-23 is structually related to IL-12 and shares similar biological activities but distinct from IL-12 (3). IL-23 is normally secreted by activated macrophages and DCs and induces proliferation of memory T cells and production of interferon-γ (IFN-γ) from activated T cells, whereas the effects of IL-12 mainly restrict to naïve T cells (3). The receptor for IL-23 is composed of IL-12Rβı and a novel subunit, IL-23R (4). IL-23 has antitumor activity in murine tumor models, causing regression of established tumors (5-7). In this study, we further evaluated the antitumor activities and the possible mechanisms of the mIL-23 secreted from murine carcinoma cells retrovirally trasduced with mIL-23 gene (Colon26/IL-23).

Materials and Methods

[1]Research Center, the Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, Hebei, China;

[2]Division of Pathology, Chiba Cancer Research Institute, 666-2 Nitona, Chuo-ku, Chiba, 260-6717 Japan;

[3]Corresponding to: Dr. Baoen Shan, Research Center, the Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, Hebei, China. Tel: +86-311-8603-3941-283, Fax: +86-311-8699-2004, E-mail: baoenshan@yahoo.com.cn.

Received Oct 10, 2005. Accepted Jan 20, 2006.

Abbreviations: mIL-23, murine interleukin-23; Colon26, murine colon carcinoma cell line; DC, dendritic cell; IFN-γ, interferon γ; Con A, concanavalin A; LPS, lipopolysaccharide; FCS, fetal calf serum; TNF, tumor necrosis factor; MMC, mitomycin C; CTL, cytotoxic T lymphocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipyenyltetrazolium bromide; NK, natural killer; FCM, flow cytometry; *s.c,* subcutaneous.

Mice and cell lines

BALB/c mice (6 to 8 week-old, females) were purchased from Laboratory Animal Center of Hebei (Shijiazhuang, China). All mice were housed under pathogen-free condition. Colon26 is a carcinogen-induced undifferentiated murine colon adenocarcinoma cell line of BALB/c origin. Colon26/IL-23 cells and Colon26/LXSN cells retrovirally transfected with mIL-23 gene and mock vector alone respectively were established as described previously (5). These cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) at 37°C in a 5% CO_2 humidified incubator. Murine heptoma cell line (H22) were culture in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) at 37°C in a 5% CO_2 humidified incubator.

Animal experiment

Exponentially growing Colon26/IL-23 cells, Colon26 cells, and Colon26/LXSN cells were harvested and their viability exceeded 95%, which was determined by trypan blue staining. Wild-type cells or transduced cells (1×10^6) were injected subcutaneously (*s.c.*) into BALB/c mice. Tumor volume was measured with callipers three times per week and calculated according to the formula [length × width^2 × 0.5] (8, 9). In some experiments, the mice which had rejected Colon26/IL-23 tumors were rechallenged with wild-type Colon26 cells (1×10^6) or irrelevant H22 cells (1×10^6) on day 60.

RNA extraction and RT-PCR

Tumor RNA was extracted by TRIzol Reagent (Invitrogen) according to the protocol recommended by the manufacturer. Total RNA was assessed by the ultraviolet spectrophotometer. Primers for IL-23p19 subunit, 5'-ATG CTG GAT TGC AGA GCA GT-3' (as a 5' primer) and 5'-GGC ACT AAG GGC TCA GTC AG-3' (as a 3' primer) were used. One-step RT-PCR kit was obtained from Huamei Biotechnology (Beijing, China). RT-PCR was performed under the following condition: 50 min at 37°C for cDNA synthesis, denaturation at 94°C for 5 min; denaturation for 30 s at 94°C, 30 s at 60°C for primer annealing, 45 s at 72°C for primer extension (35 cycles); and a final extension at 72°C for 5 min. The PCR product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet radiation.

Cytotoxicity assays

Spleens were removed from mice that were tumor free on day 60 after inoculation with Colon26/IL-23 cells or PBS. Splenocytes were harvested by pressed spleens through sterile gauze, rinsed, treated with RBC lysis solution (0.83% NH_4Cl), and washed twice with PBS and pooled from three mice per group. These cells (2×10^6) were restimulated *in vitro* with mitomycin C (MMC) treated Colon26 cells (2×10^5) in the presence of 50 IU/ml of rhIL-2 (CYTOLAB, US). Five days later, cytotoxicity of the restimulated cells against Colon26 cells was determined by using CytoTox96 (Promega, USA) according to the manufacturer's manual. CytoTox96 quantitatively measures the lactate dehydrogenase (LDH) that is released upon cell lysis. The percentage of specific lysis was calculated as [(experimental - effector spontaneous - target spontaneous)/(target maximum - target spontaneous)] × 100.

In vitro cytokine release assay

As described above, splenocytes were harvested from mice on day 10 or 30 after injection with Colon26/IL-23 cells, Colon26/LXSN cells, Colon26 cells or PBS. The splenocytes (2×10^6) were cocultured with MMC treated Colon26 cells (2×10^5) in 1 ml RPMI1640 medium containing 10% FCS in 24-well tissue culture plates (Costar). After 48 h, cell-free supernatants were collected for measurement of murine IFN-γ, TNF-α, IL-4 and IL-12 release by standard ELISA according to the manufacturer's recommendations (Jingmei, China), which is reported as mean ± SD of triplicate samples.

Proliferation assay

Splenocytes (2×10^5) were loaded in 96-well culture plate with or without 5 μg/ml Con A or LPS (Sigma, US) and cultured at 37°C, 5% CO_2 atmosphere in RPMI 1640 for 72 h, with 1 μCi of [^{3}H]thymidine ([^{3}H]-TdR, Institute of Radionuelide of China, Beijing) being added for the last 18 h. The cells were harvested with micromate 96-well harvester and radioactivity was measured by liquid scintillation counter (Beckmen). All proliferation assays were performed in triplicate.

Flow cytometric analysis

Splenocytes were incubated with fluorescein-isothiocyanate (FITC)-conjugated monoclonal anti-CD11c antibody (Santa Cruz) at room temperature for 30 min. These cells were also reacted with FITC-conjugated sheep anti-hamaster immuno-globulin antibody as a control. The stained cells were analyzed by flow cytometry (Beckman Coulter).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to evaluate the significance of differences between the experimental groups. Survival time was compared with the Kaplan-meier method, and significance was determined by the log-rank test. Differences were considered significant when p is less than 0.05. Data were presented as means ± SD.

Results

Antitumor effects of IL-23 in vivo

To determine the antitumor response induced by IL-23 secreted in site, BALB/c mice (n = 8) were injected *s.c.* with live Colon/IL-23 cells, Colon26 cells or Colon26/LXSN cells (1×10^6) and monitored for tumor growth and survival. The tumor growth of the mice injected with Colon26/IL-23 cells was similar to that of the mice injected with Colon26 cells or Colon26/LXSN cells before day 10 after inoculation with tumor cells. From then, the tumor growth of the mice injected

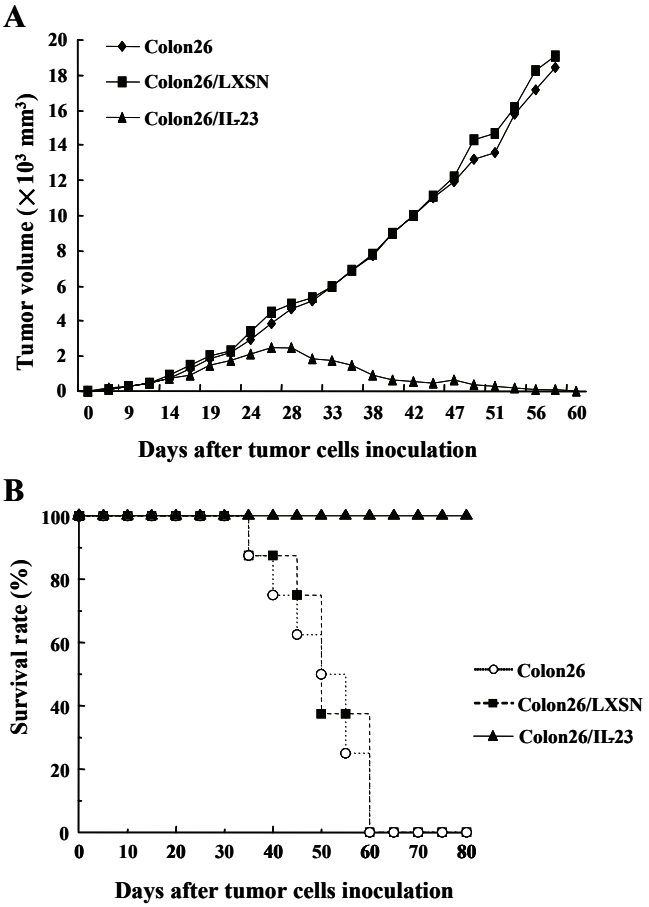

Figure 1. Antitumor activity induced by IL-23. BALB/c mice were inoculated *s.c.* with 1 × 10^6 Colon26/IL-23 cells, Colon26 cells or Colon26/LXSN cells. (A) Tumor growth after inoculation of tumor cells; (B) Survival rate of the mice after inoculation of tumor cells.

with Colon26/IL-23 cells slowed down and all mice displayed complete tumor rejection by day 60. All the mice survived until the end of observation (> 250 days). However, the tumors of the mice injected with Colon26 cells or Colon26/LXSN cells grew progressively, and finally all the mice died before day 60 after injection with the tumor cells (Figure 1).

The mice that had been rejected Colon26/IL-23 tumors were rechallenged with Colon26 cells (1 × 10^6) or irrelevant H22 cells (1 × 10^6). All mice injected with Colon26 cells rejected them, but the mice injected H22 cells developed tumors and died. These data suggested that the mice rejected Colon26/IL-23 tumors developed immune memory for Colon26 cells.

Expression of IL-23 mRNA in tumor tissue

To investigate whether Colon26/IL-23 cells can express IL-23 *in vivo*, tumor mass was harvested from mice on day 10 after inoculation with Colon26/IL-23 cells, Colon26 cells, and Colon26/LXSN cells respectively, and extracted total

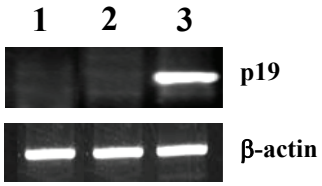

Figure 2. Expression of IL-23p19 subunit in Colon26/IL-23 tumor, but not in Colon26 tumor or Colon26/LXSN tumor. Lane 1, Colon26 tumor; Lane 2, Colon26/LXSN tumor; Lane 3, Colon26/IL-23 tumor.

RNA. The results of RT-PCR indicated that IL-23 was expressed in Colon26/IL-23 tumors, but not in Colon26 tumors or Colon26/LXSN tumors (Figure 2).

Proliferation of splenocytes

There were no differences of splenocyte proliferation stimulated by Con A or LPS among the mice injected with Colon26/IL-23 cells, Colon26 cells, or Colon26/LXSN cells on day 10 after inoculation with tumor cells, at which time all tumors of the mice were growing progressively (Figure 3). On day 40, when tumors of the mice injected with Colon26/IL-23 cells regressed, the splenocyte proliferation stimulated by Con A or LPS dramatically increased compared with the mice injected with Colon26 cells or Colon26/LXSN cells, and there was no difference between the latter two (Figure 3). The splenocyte proliferation of the mice rejected Colon26/IL-23 tumors induced by Con A was higher and that induced by LPS was lower compared with that of the mice on day 40 after inoculated with Colon26/IL-23 cells ($p < 0.01$).

Production of cytokines and CTL activity

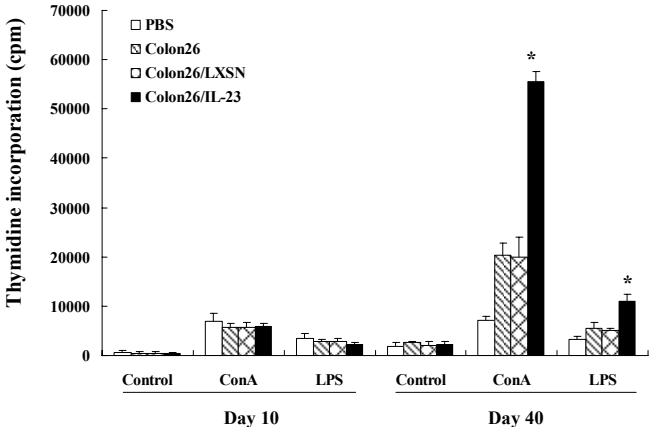

Figure 3. Proliferation of splenocytes. In the phase of tumor regression (on day 40), the proliferation of the Con A or LPS activated splenocytes from the mice inoculated with Colon26/IL-23 cells was significantly enhanced. *$p < 0.01$ compared with that from the mice inoculated with Colon26 cells or Colon26/LXS cells.

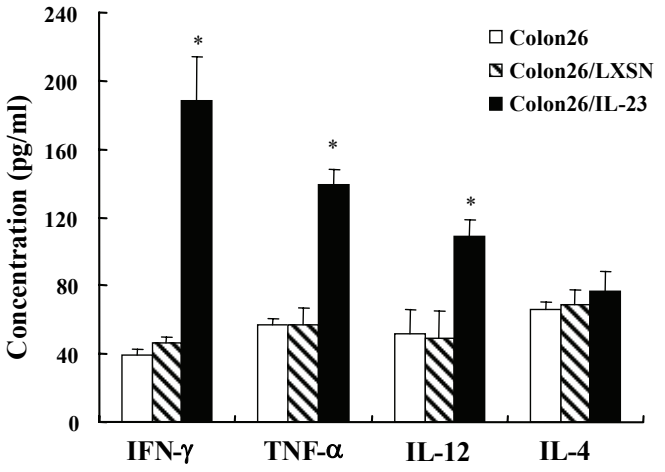

Figure 4. Production of cytokines. The levels of IFN-γ, TNF-α, and IL-12, but not IL-4 secreted by splenocytes from the mice inoculated with Colon26/IL-23 cells were enhanced. *$p < 0.01$ compared with that from the mice inoculated with Colon26 cells or Colon26/LXSN cells.

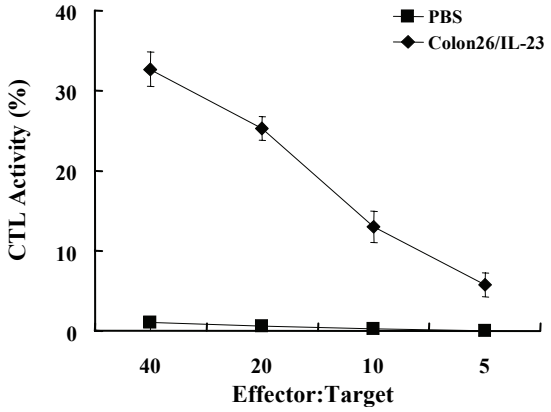

Figure 5. CTL activity was induced by Colon26/IL-23 cells. The splenocytes were collected from the mice that had rejected Colon26/IL-23 tumors or age-matched PBS injection mice, and were induced *in vitro* for 5 days with MMC-Colon26 cells. CTL activity of the mice rejected Colon26/IL-23 tumor was higher than that of control mice ($p < 0.01$).

We examined the level of cytokines (IFN-γ, TNF-α, IL-12 and IL-4) produced by splenocytes of the mice inoculated with tumor cells. On day 30, at which time Colon23/IL-23 tumors were regressing, the amounts of IFN-γ, TNF-α, and IL-12 secreted by splenocytes from the mice inoculated with Colon26/IL-23 cells were higher than those of the mice inoculated with Colon26 cells or Colon26/LXSN cells (Figure 4). There was no significant difference of the level of IL-4 from the splenocytes between the mice inoculated with Colon26/IL-23 cells and the mice inoculated with Colon26 cells or Colon26/LXSN cells (Figure 4). We also evaluated the CTL activity of splenocytes from the mice rejected Colon26/IL-23 tumors. As shown in Figure 5, Colon26/IL-23 cells induced significantly CTL activity compared with PBS-injected mice.

Expression of CD11c

To investigate the effect of IL-23 secreted from Colon26/IL-23 cells on DC *in vivo*, splenocytes were harvested from the mice on day 10 after inoculation with Colon26/IL-23 cells, Colon26 cells or Colon26/LXSN cells, stained with FITC-conjugated monoclonal anti-CD11c antibody and analyzed by flow cytometry. The percentage of CD11c-positive cells in the spleens from the mice inoculated with Colon26/IL-23 cells was higher than that of the mice inoculated with Colon26 cells or Colon26/LXSN cells (Figure 6).

Discussion

Preclinical and clinical studies had demonstrated that IL-12 has potent antitumor activity (10-12), but IL-12 administration leads to severe toxicity that is associated with extremely high level of IFN-γ induced by IL-12 (13). IL-23 has similar structure and bioactivities to IL-12, but induces lower level of IFN-γ than that induced by IL-12, so IL-23 might be a potential antitumor agent with lower toxicity (4). Some reports had demonstrated that expression of IL-23 gene in murine and human tumor cells could induce antitumor activity and cause tumor regression in animal tumor models (5-7, 14). In present study, we showed that the growth of Colon26/IL-23 tumors was similar to that of Colon26/LXSN tumors or Colon26 tumors in the mice before day 10 after inoculation with tumor cells, thereby the Colon26/IL-23 tumors growth slowed down and finally was rejected. The mice rejected Colon26/IL-23 tumors could resist rechallenged Colon26 cells, but not irrelevant H22 cells. These data

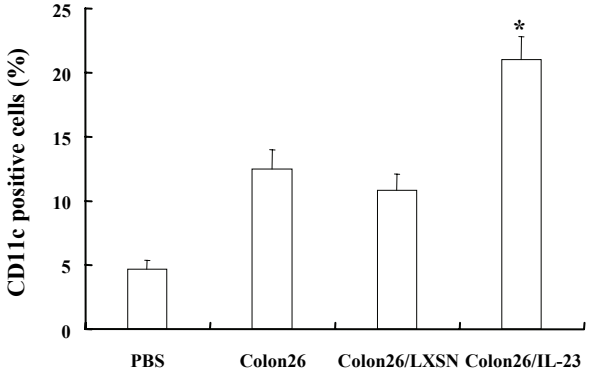

Figure 6. Expression of CD11c. The percentage of CD11c-positive cells (DC) in splenocytes from the mice inoculated with Colon26/IL-23 cells was increased. *$p < 0.01$ compared with that of the mice inoculated with Colon26 cells or Colon26/LXSN cells and PBS-injection mice.

suggest that IL-23 has potent antitumor activity and induced specific immunological memory to Colon26 cells.

In our previous study, we showed that IL-23 did not inhibit growth of Colon26/IL-23 cells *in vitro*, but suppressed tumor growth *in vivo*, which suggested that IL-23 had no direct effect on tumor cells, and IL-23 induced antitumor activity *in vivo via* other pathways. IL-23 possesses ability of inducing immune response that may be associated with its antitumor activity (4, 5, 15-17). Lo et al. demonstrated that immune-deficient mice SCID (deficient in B and T cells) and SCID/beige (deficient in B cells, T cells, and NK cells) injected with CT26 cells expressed IL-23 had no antitumor activity (6). Experiments in immunocomponent animals selectively depleted of various lymphocyte populations suggested that $CD8^+$ T cells, not $CD4^+$ T or NK cells played a crucial role in IL-23-mediated antitumor activity (5, 6). IL-23 could induce high level of CTL response (6), which played key role in antitumor activity (18, 19). Our present study showed that the splenocyte proliferation of the mice inoculated with Colon26/IL-23 cells induced by Con A (T cell inducer) or LPS (B cell inducer) was similar to that of the mice inoculated with Colon26 cells or Colon26/LXSN cells when their tumors grew progressively. When the Colon26/IL-23 tumors were in the phase of regression, the splenocyte proliferation of the mice inoculated with Colon26/IL-23 cells induced by Con A or LPS was higher than that of the control mice. The mice rejected Colon26/IL-23 tumors achieved further enhancement of splenocyte proliferation induced by Con A, but not by LPS. Together these data suggested that T cells play a major role and B cells may play a partial role in antitumor response induced by IL-23.

It has been confirmed that the antitumor mechanisms of IL-12 are involved in both innate and adaptive immunity and high level of IFN-γ induced by IL-12 plays an important role in the antitumor activity of IL-12. IL-23 expression from Colon26/IL-23 cells induced tumor-specific protective immunity and production of IFN-γ, depletion of $CD8^+$ cells markedly decreased IFN-γ production (5). The other study showed that the lack of an early antitumor response of IL-23 compared with IL-12 may be due to its intrinsic lower ability to stimulate IFN-γ production (4, 6, 20), and antitumor activity of IL-23 was not affected in mice depleted of IFN-γ (6). Our present study showed that IL-23 induced production of not only IFN-γ, but also IL-12, and TNF-α by the splenocytes from the mice inoculated with Colon26/IL-23 cells in the phase of tumor regression, and IL-4 production was not significantly affected compared with control groups. Immune balance is controlled by the balance of cytokines produced by two distinct helper T (Th) cell subsets, Th1 and Th2 cell (21). Th1 cells produce IL-2 and IFN-γ, while Th2 cells produce IL-4, IL-5, and IL-6. The former plays a critical role for cellular immunity, while the latter is involved in humoral immunity. IL-12 prompts Th1 cytokines production (22). IL-12 and TNF-α also possess antitumor effect demonstrated in preclinical and clinical studies (10-12, 23, 24). All the data above suggest that IFN-γ may not play a key role in the antitumor activity of IL-23, and the other cytokines such as IL-12 and TNF-α are mainly associated with antitumor activity of IL-23.

DCs are the most important antigen presenting cells (APCs) and plays important role in antitumor immunity (25-27). CD11c, a murine DC marker, is used for isolation and identification of murine DCs (28, 29). IL-23 acts on both $CD8\alpha^-$ and $CD8\alpha^+$ splenic DCs and enhances both DC subsets to present P815AB tumor peptide, while IL-12 only binds to $CD8\alpha^-$ splenic DCs to present tumor peptide (30). IL-23 induces IL-12 and IFN-γ production by DCs, and together with IL-12 further promotes higher levels of IL-12 and IFN-γ production compared with that induced by either IL-12 or IL-23 alone (30). In present study, we only confirmed that IL-23 expression from Colon26/IL-23 cells increased the percentage of DCs in the splenocytes from the mice inoculated with tumor cells, and the effects of IL-23 on the activity of DCs were not investigated. This result suggests that DC is also involved in the antitumor activity of IL-23.

In summary, we concluded that IL-23 possesses potent antitumor activity and T cell-mediated immunity and DCs contribute to the antitumor activity of IL-23.

Acknowledgements

This work was supported by the Doctor Foundation of the Science and Technology Department of Hebei Province (No. 0254700) and the Doctor Foundation of the Education Department of Hebei Province (No.2001245).

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