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ORIGINAL ARTICLE

Early exposure of high-dose interleukin-4 to tumor stroma reverses myeloid cell-mediated T-cell suppression

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Myeloid-derived suppressor cells (MDSCs) inhibit T-cell activity and promote tumor growth in tumor-bearing hosts. We sought to determine how to prevent the generation of these cells and modulate anti-tumor immunity at different times during tumor growth. Interleukin-4 (IL-4), a cytokine closely associated with the differentiation of myeloid cells, was expressed locally at the tumor site with its dose and expression time tightly regulated by a tet-off *system. Early exposure of high-dose IL-4 to the tumor stromal cells effectively prevented the generation of myeloid suppressor cells and led to a T-cell-mediated tumor rejection. However, IL-4 had no effect a few days after tumor growth, when myeloid suppressor cells had been generated and T cells were tolerized. Importantly, coinoculation of IL-4 receptor (IL-4R)-deficient tumor cells with IL-4R competent, but not IL-4R-deficient myeloid cells led to IL-4-mediated tumor regression in IL-4R-deficient mice, indicating that IL-4 acts directly on myeloid cells. These results show a novel way to prevent T cells from MDSC-induced suppression, with important indications for cancer therapy.*

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Keywords: *interleukin-4; myeloid cells; tumor stroma;* tet-off*; anti-tumor immunity*

Introduction

In tumor-bearing animals or cancer patients, large amounts of myeloid ($CD11b^+Gr\text{-}1^+$) cells with immune suppressive character accumulate in the bone marrow, blood, spleen and at the tumor site.[1,2] They facilitate tumor growth by suppressing T-cell activity[3] and/or promoting angiogenesis.[4] They have recently been named as myeloid-derived suppressor cells (MDSCs).[5] Strategies to reduce MDSC accumulation by direct depletion of these cells using a specific monoclonal antibody (mAb)[6,7] have retarded tumor development and partly restored anti-tumor immunity in mice. However, it is difficult to define a distinct phenotype for these immunoregulatory cells because they are considered to be immature myeloid cells.[5] The total population includes several heterogeneous sub-populations, including granulocyte and macrophage precursors, myeloid bone marrow-derived dendritic cells (DCs), as well as other early myeloid precursors. Whether and how the protumorigenic function of MDSCs can be reversed is of special clinical interest and currently not well analyzed.

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Many cytokines, especially interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor, are involved in the proliferation and differentiation of myeloid progenitors.[8,9] IL-4 supports DC maturation and promotes IL-12p70 secretion, and is widely used together with granulocyte-macrophage colony-stimulating factor to obtain mature DCs *in vitro*.[10,11] Phenotypically mature DCs are generated from culturing mouse bone marrow progenitors in a low concentration of granulocyte-macrophage colony-stimulating factor plus IL-4.[12] A more direct indication is that $CD11b^+Gr\text{-}1^+$ splenocytes isolated from tumor-bearing mice differentiate into a nonadherent population of fully mature and highly activated DCs when cultured in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor.[13] However, the effect of exogenous IL-4 on the development of myeloid cells *in vivo* has not been analyzed.

IL-4-gene-modified tumor cells are rejected in immune-competent mice; however, the exact mechanism is still unclear. As a heavy infiltration of $Gr\text{-}1^+$ cells is observed in the tumor sites, the anti-tumor effect of IL-4 is firstly believed to result from an increased inflammation.[14–16] $CD8^+$ T cells are necessary for complete rejection of IL-4-secreting J558L plasmacytoma.[17,18] T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in IL-4-deficient mice.[19] Local IL-4 production at the site of a tumor vaccine also leads to the activation of tumor-infiltrating DCs and these activated DCs induce anti-tumor T-cell responses.[20]

These evidence strongly suggests that IL-4 could be used as a myeloid cell-modifier for promoting the anti-tumor immunity.

In this study, we generated tumor cells that express exogenous IL-4 under the strict control of a *tet-off* system.[21] Stable transfectants were evaluated for their tumorigenic capacity in the absence or presence of different doses of tetracycline (Tc) during different periods of tumor growth. We found that exposure of myeloid cells in tumor stroma to a high dose of IL-4 during the early stage of tumor growth prevented T-cell tolerance and led to tumor rejection. These data provide evidence that both the dose and the expression time are important for the anti-tumor effect of exogenous IL-4, and suggest a novel approach to relieve cancer patients from immunosuppression mediated by myeloid cells.

Results

IL-4-mediated tumor rejection is strictly dose and time dependent

To assess the efficacy of Tc in suppressing IL-4 secretion *in vivo*, C57BL/6 mice were inoculated with MCA205-TrmIL4 cells and supplied with different concentrations of Tc in drinking water throughout the entire experimental period. As shown in Figure 1a, mice given 0 or with 0.2 mg ml^{-1} Tc in drinking water rejected tumor cells completely, while providing 1 mg ml^{-1} Tc led to tumor formation in 20% of mice. When Tc concentration was increased to 2 mg ml^{-1}, all mice developed a tumor within 48 days.

To analyze the time-dependent effect of IL-4 on tumor growth, C57BL/6 mice were injected with MCA205-TrmIL4 cells and given 0 or 2 mg ml^{-1} Tc at different time periods, so IL-4 was expressed as indicated in Figure 1b. If IL-4 secretion was suppressed (no), all tumors grew over 10 mm in diameter at approximately 40 days. If IL-4 was expressed throughout the experiment (day 0), no tumors grew (Figure 1b). Interestingly, if IL-4 secretion was started from day 2 or 5 after tumor cell inoculation, tumors grew progressively for the first 14 days, similar to tumors without IL-4 secretion. However, their growth slowed down during days 15–18, and ultimately all tumors were rejected. If IL-4 was expressed from day 11, three out of five mice got tumors, whereas the other two mice rejected the tumor in the end (40 days). IL-4 expression from day 14 had almost no effect on tumor growth. All tumors grew out (Figure 1b). These data show that early but not late expression of high-dose IL-4 in the tumor site leads to tumor regression.

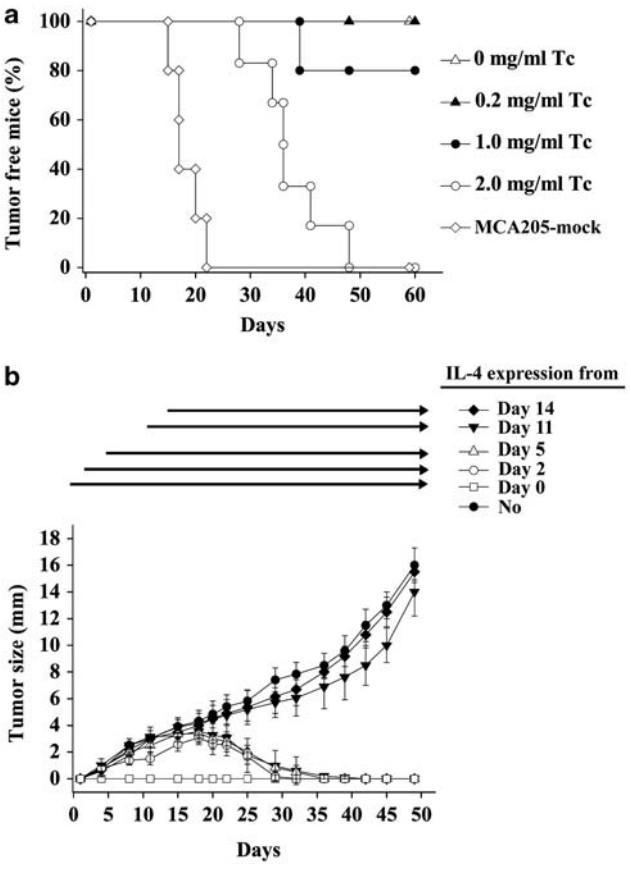

Figure 1 The anti-tumor effect of IL-4 is strictly dose and time dependent. (**a**) Groups of C57BL/6 mice ($n = 5$–6) were subcutaneously injected with 1×10^5 MCA205-TrmIL4 cells and supplied with water containing different concentrations of Tc throughout the entire experimental period. Mice that received MCA205-mock cells served as controls. (**b**) Mice were subcutaneously injected with MCA205-TrmIL4 cells, and IL-4 secretion at the tumor site was either suppressed throughout the experiment with 2 mg ml^{-1} Tc (●) or enabled at day 0 (□), day 2 (○), day 5 (△), day 11 (▼) or day 14 (♦) after tumor cell inoculation. Mice in the day 11 group (▼) were divided into growing tumor and regressing tumor subgroups. Tumor growth was monitored as described in the 'Materials and methods' section. Data are representative of two independent experiments.

IL-4 responsiveness of tumor cells is not necessary for exogenous IL-4-mediated tumor rejection

IL-4 receptor (IL-4R) is expressed on almost all nucleated cells[22] and IL-4 has a modest but direct inhibitory effect on the growth of several tumor cell lines.[23,24] Paradoxically, tumor cells of various histological origins often express increased levels of IL-4R compared with their counterparts.[25,26] We have recently shown that the endogenous IL-4 can promote tumor growth by increasing tumor cell resistance to apoptosis.[27] Nevertheless, exogenous cytokines (artificially expressed at the tumor site) represent an effective method of tumor therapy. To exclude the direct effect of exogenous IL-4 on tumor cells *in vivo*, IL-4R-deficient fibrosarcoma FA10-IL4 cells and control FA10-mock cells were subcutaneously injected into the syngeneic BALB/c mice. As shown in Figure 2, FA10-mock tumors grew progressively after tumor cell injection. Local production of IL-4 by FA10-IL4 cells led to tumor rejection in all mice, although the tumor cells could not respond to IL-4. Therefore, the data suggest that the action of exogenous IL-4 on tumor stromal cells is sufficient for IL-4-mediated tumor regression.

IL-4 influences the distribution and differentiation of myeloid cells

Several groups, including ours, have observed a massive accumulation of Gr-1$^+$ cells at the sites of various IL-4-secreting tumors.[14–16] These cells are recognized as either macrophages,[14] eosinophils[14,15] or neutrophils[16] according to morphology and/or cell surface markers. To understand the effect of local delivery of exogenous IL-4

on the generation of these types of cells, we analyzed the changes of Gr-1$^+$ and/or CD11b$^+$ cells in different tissues, including tumors, draining lymph nodes (DLNs) and spleens in tumor-bearing mice with exogenous IL-4 expressed from day 5 (early IL-4) or from day 14 (late IL-4) after tumor cell inoculation.

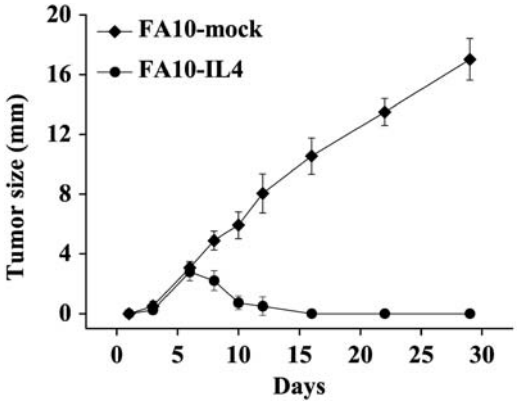

Figure 2 Loss of IL-4R expression on tumor cells does not affect IL-4-mediated tumor regression. Syngeneic BALB/c mice were subcutaneously injected with 1×10^5 FA10-IL4 cells (genetically modified to secret IL-4) in the right abdominal region (●, $n=6$) and FA10-mock cells in the left abdominal region (◆, $n=6$). Tumor growth of these IL-4R-deficient tumor cells was monitored. A similar result was obtained when the experiment was repeated.

Tumors with early and late IL-4 expression were isolated at day 17, when the mean tumor size of these two groups was still very similar (Figure 1b). Tissue cryosections were then stained with anti-Gr-1 or anti-CD11b mAb. As shown in Figure 3a, significantly more Gr-1$^+$ cells infiltrated early IL-4-secreting tumors than late IL-4-secreting tumors, while CD11b$^+$ cells were easily detected in the late, but not early IL-4-secreting tumors. These results suggest that at this time point, single Gr-1$^+$ cells are not the same to typical MDSCs, which coexpress Gr-1 and CD11b. These two populations show distinct roles in anti-tumor immunity. As we reported previously, Gr-1$^+$ cells in early IL-4-secreting tumors were primary effector cells during the inhibition of tumor growth.[16] In contrast, our preliminary data showed that these cells could differentiate gradually into CD11b$^+$Gr-1$^+$ cells in late IL-4-secreting tumors. MDSCs could already be easily detected at day 30 in the late IL-4-secreting tumors (data not shown).

In DLNs, single Gr-1$^+$ cells were more intensively accumulated in the early IL-4 group than in the late IL-4 group at day 17, but no significant difference was detected between the two groups in the total number of CD11b+Gr-1$^+$ cells (Figure 3b). In spleens, a significant

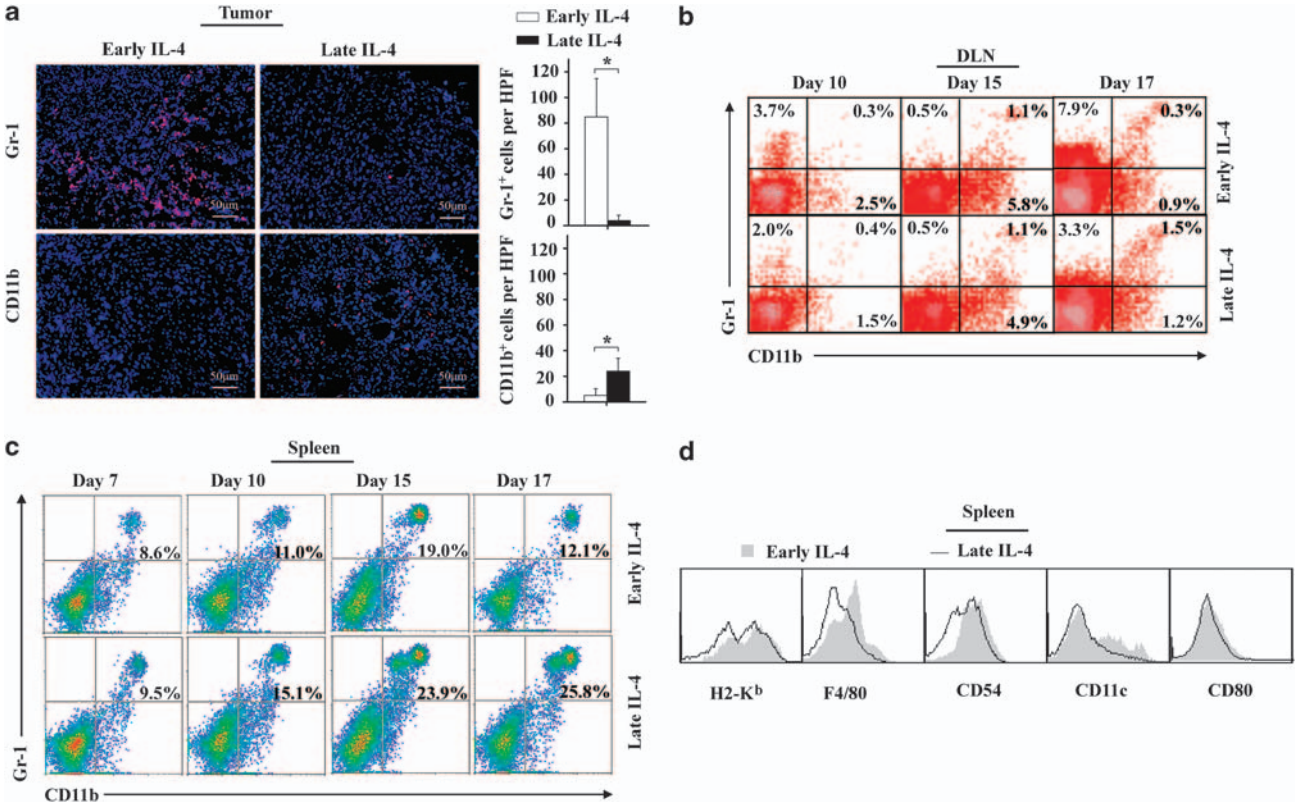

Figure 3 IL-4 influences the distribution and differentiation of myeloid cells in tumor-bearing mice. Mice were subcutaneously injected with MCA205-TrmIL4 cells, and IL-4 was enabled to secrete at day 5 (early IL-4) or 14 (late IL-4) after tumor cell inoculation. At the indicated days, tumors, DLNs and spleens were collected for further study. (**a**) Myeloid cells at the tumor site. The representative staining results and the mean numbers of CD11b$^+$ or Gr-1$^+$ cells per high-power field (HPF, 200 ×) are shown. *$P<0.05$. (**b, c**) The temporal change of CD11b$^+$Gr$-$1$^+$ cells in DLNs (**b**) and spleens (**c**) during tumor growth. (**d**) Phenotypic comparison of splenic CD11b$^+$ cells in mice of early (shaded histograms) and late (solid black lines) IL-4 groups. Two experiments were performed with similar results, and one typical experiment is shown (**a–d**).

difference in CD11b$^+$Gr-1$^+$ cell numbers was detected at days 15 and 17. It is noteworthy that a new cell population of CD11b^{low} and Gr-1^{low} appeared in the late IL-4 group but not in the early IL-4 group (Figure 3c). Further study showed that the expression levels of major histocompatibility complex class I molecule H-2K^b, the mature macrophage marker F4/80 and intercellular adhesion molecule-1 (CD54) on splenic CD11b$^+$ cells were also increased in the early IL-4 group in comparison with the late IL-4 group (Figure 3d), indicating that this population of cells shows a more mature phenotype. In summary, the results above indicate that large amounts of IL-4 produced rapidly at the tumor site influence not only the distribution, but also the differentiation of myeloid cells *in vivo*.

Early IL-4 expression activates CD8$^+$ T-cell-dependent anti-tumor immunity

Tumor-specific T regulatory cells have recently been identified and characterized, providing compelling evidence that such antigen-specific T regulatory cells could induce tumor-specific immune tolerance.[28] MDSCs induce the development of Foxp3$^+$ T regulatory cells.[29,30] As DLNs are the target to which antigen-specific T regulatory cells migrate for activation,[31] we analyzed changes in CD4$^+$Foxp3$^+$ cells in DLNs. However, no difference was detected between the early and late IL-4 groups (Figure 4a). Whether this is also the case at the tumor site remains to be analyzed.

Next, we analyzed T-cell activation at day 17 after tumor cell transplantation. Spleen or DLN-derived CD8$^+$ T cells in mice with early IL-4 expression group were stained more intensely by a mAb against a T-cell activation maker CD69 (Figure 4b) and produced more intracellular interferon (IFN)γ, relative to those cells in mice with late IL-4 expression group (Figure 4c). No difference in the expression level of CD69 or IFNγ was found between early and late IL-4 groups when CD4$^+$ T cells were analyzed.

The importance of CD8$^+$ T cells in early IL-4-mediated tumor rejection was further shown by a T-cell depletion experiment. C57BL/6 mice were inoculated with MCA205-TrmIL4 cells, IL-4 expression was allowed from day 2 and T cells were depleted from day 11 after tumor cell inoculation. As shown in Figure 4d, tumors in CD4$^+$ T-cell-depleted and control mice were rejected similarly, while tumors in CD8$^+$ T-cell-depleted mice grew progressively and reached a size of 10 mm in diameter within 35 days.

In addition, our previous work shows that CD8$^+$ T lymphocytes can inhibit tumor growth by inhibiting

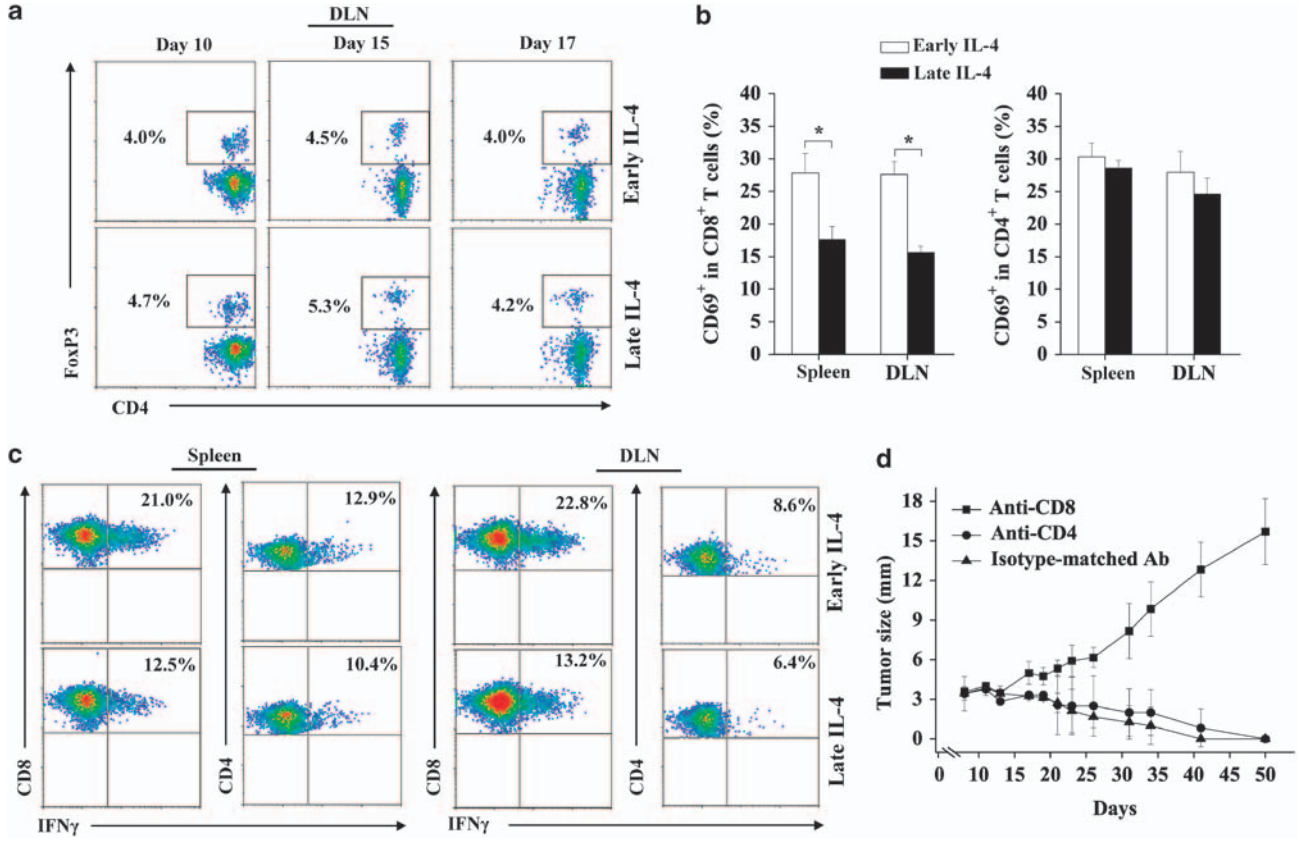

Figure 4 Early IL-4 prevents CD8$^+$ T-cell tolerance. (**a**) T regulatory (Treg) cells contribute little to CD8$^+$ T-cell tolerance. DLN cells were isolated from mice in early or late IL-4 group and costained with anti-CD4 and anti-FoxP3 mAbs at the indicated days. (**b, c**) The activation status of CD8$^+$ and CD4$^+$ T cells at day 17. Spleen and DLN cells were isolated from mice in the early or late IL-4 group and costained with either anti-CD4, anti-CD8, anti-CD69 (**b**) or anti-CD4, anti-CD8 and anti-IFNγ mAbs (**c**). *$P<0.05$. (**d**) CD8$^+$ T cells are required for early IL-4-mediated tumor rejection. MCA205-TrmIL4 tumor cells were injected subcutaneously into C57BL/6 mice and IL-4 was enabled to express at day 2. At day 11, mice were treated with anti-CD8 (■, $n=3$), anti-CD4 (●, $n=4$) antibody or isotype-matched antibody (▲, $n=3$). Mice were treated with the same antibody once per week thereafter to deplete the respective T-cell sub-population. Tumor growth was monitored as described above.

tumor cell-induced angiogenesis.[32,33] We compared angiogenesis between tumors that expressed IL-4 early and tumors in which IL-4 expression was delayed. The results showed that angiostasis was involved in early IL-4-mediated tumor rejection (data not shown). Together, these data indicate that early, but not late exposure of tumor stromal cells to IL-4 prevents the tolerance of $CD8^+$ T cells in tumor-bearing mice.

Early IL-4 expression prevents T-cell tolerance through modulation of CD11b$^+$Gr-1$^+$ cells

To analyze whether there is a causal relationship between the altered suppression status of myeloid cells in response to early IL-4 and triggering of $CD8^+$ T-cell-dependent anti-tumor immunity, we compared the inhibition activity of $CD11b^+Gr\text{-}1^+$ cells between mice with early IL-4 production and mice with late IL-4 production *in vivo* and *in vitro*. As shown in Figure 5a, adoptive transfer of tumor-specific T cells prevented 60% of mice from developing tumors. Addition of $CD11b^+Gr\text{-}1^+$ cells from spleens of mice with early IL-4 production did not inhibit the function of immune cells, while addition of these cells from mice with late IL-4 production impaired immune protection significantly ($P<0.05$), leading to tumor growth in 100% of mice within 30 days.

Next, we compared the arginase activity in $CD11b^+Gr\text{-}1^+$ cells in the early and late IL-4 groups. As shown in Figure 5b, arginase activity was almost unchanged during the observation period in mice with early IL-4 expression (3–4 mU per 10^6 cells), while it gradually increased in mice with late IL-4 expression (from 3.8 mU per 10^6 cells at day 7 to 12 mU per 10^6 cells at day 17). Statistical analysis showed that the difference between these two groups was significant at both days 15 and 17 ($P<0.05$). To assess the involvement of arginase in T-cell suppression, the IFNγ secretion by immune cells (2×10^6) stimulated *in vitro* with irradiated MCA205 cells (5×10^4), with or without $CD11b^+Gr\text{-}1^+$ cells (2×10^5), was analyzed. As shown in Figure 5c, splenocytes from MCA205-immunized mice secreted a large amount of IFNγ at day 3, and this secretion was not obviously influenced by $CD11b^+Gr\text{-}1^+$ cells from mice with early IL-4-production. However, $CD11b^+Gr\text{-}1^+$ cells from mice with late IL-4-production significantly inhibited the IFNγ secretion by immune cells. This inhibition was largely eliminated when a physiologic inhibitor of arginase (N^G-hydroxy-L-arginine, NOHA) was added to the culture (Figure 5c). Together, the above data suggest that the early expression of IL-4 affects anti-tumor immunity by preventing myeloid cell from suppressing T cells.

IL-4R expression on myeloid cells is sufficient for IL-4-mediated tumor rejection

We sought to determine why IL-4 had no effect when tumors had grown for a few days, and we considered three possibilities. (1) Tumor size: it is possible that small

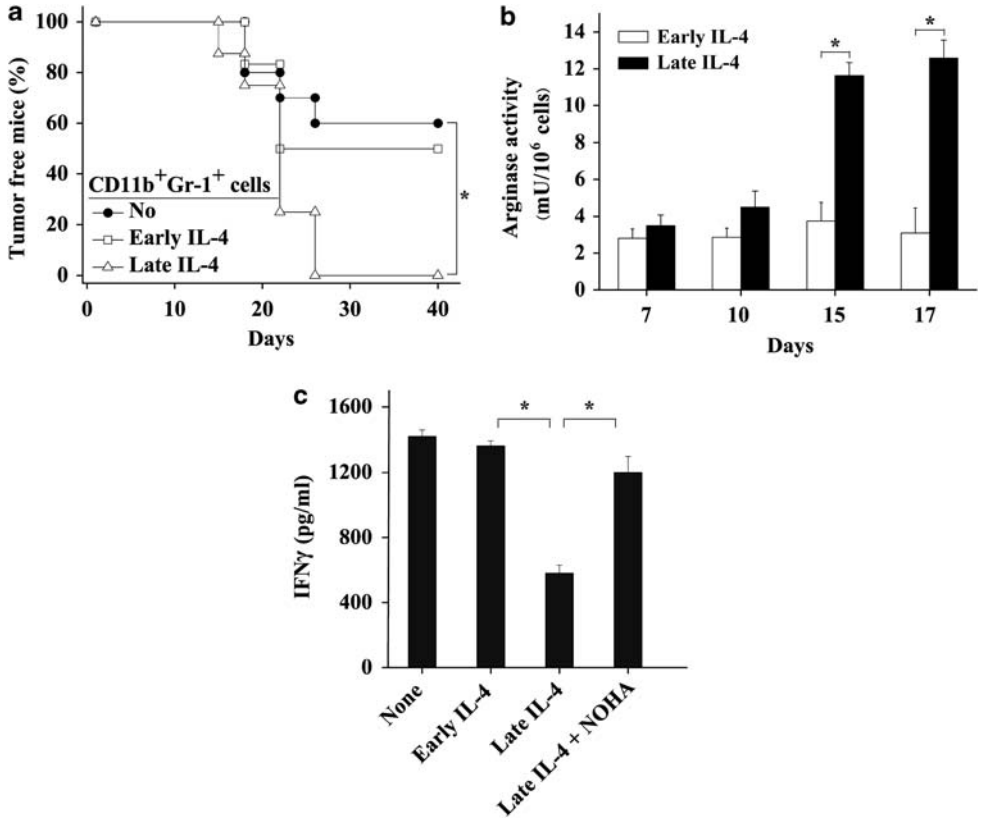

Figure 5 Early IL-4 prevents T-cell tolerance by inhibition of myeloid cell-derived arginase. (**a**) Reversal of myeloid cell-mediated immune inhibition by early IL-4. Mice were subcutaneously injected with viable MCA205 cells; 24 h later, the mice received (through tail vein) immune splenocytes without (●, $n=10$) or with $CD11b^+Gr\text{-}1^+$ cells isolated from mice with early IL-4 production (□, $n=6$) or mice with late IL-4 production (△, $n=8$). Tumor growth was monitored as described above. *$P<0.05$. (**b**) Reduced arginase activity produced by $CD11b^+Gr\text{-}1^+$ myeloid cells from spleens of mice with early IL-4 expression. *$P<0.05$. (**c**) Splenic $CD11b^+Gr\text{-}1^+$ cells in late, but not early IL-4-expressing mice inhibited IFNγ production by immune splenocytes *in vitro*. *$P<0.05$. Data are representative of two experiments.

tumors are more easily rejected than large ones. However, tumors in most of the groups with either early or late IL-4 expression reached a similar size of approximately 3.5 mm in diameter before they were rejected (Figure 1b). (2) Local IL-4 concentration: the IL-4 secretion of MCA205-TrmIL4 cells from mice with late IL-4 expression was determined *in vitro* at day 40 after tumor cell inoculation. As shown in Figure 6a, all cells from different tumors still secreted equivalent amounts of IL-4 in comparison with the original cells that were not passaged *in vivo*. (3) The IL-4 responsiveness of myeloid cells: the expression of IL-4R on splenic CD11b$^+$ cells was detected by flow cytometry at day 17. IL-4R expression on this population in the late IL-4 expression group was slightly lower than that in the early IL-4 expression group (Figure 6b middle panel). Further analysis showed that a major difference in IL-4R expression level existed in CD11b^{low} sub-population (Figure 6b bottom panel). The mean channel fluorescence of IL-4R expression for CD11b^{low} cells from mice in the early IL-4 group was 14 ± 2, while it was only 8.32 ± 0.6 in the late IL-4 group. Further investigations will elucidate whether there is a difference in the function of CD11b^{high} and CD11b^{low} sub-populations and whether the decrease in IL-4R expression on CD11b^{low} cells could explain the altered responsiveness of myeloid cells to late IL-4, as well as the ineffectiveness of the anti-tumor effect of late IL-4.

To further analyze the importance of IL-4 responsiveness of CD11b$^+$Gr-1$^+$ cells, we isolated this population of cells from spleens of both wild-type and IL-4R-deficient tumor-bearing mice. The isolated cells were then coinjected subcutaneously with FA10-IL4 tumor cells into IL-4R-deficient mice. As shown in Figure 6c, 80% of IL-4R-deficient mice rejected tumors when IL-4R-competent myeloid cells were coinjected with the tumor cells. In contrast, one out of four mice did so when myeloid cells derived from IL-4R-deficient mice were coinjected. These data strongly support the hypothesis that myeloid cells respond to IL-4 and cause tumor rejection *in vivo*.

Discussion

The most significant finding in this study is that exposure of tumor stromal cells to an adequate IL-4 dose at an early stage of tumor development can prevent the generation of MDSCs and T-cell tolerance in tumor-bearing animals. The tumor stroma is composed of a variety of normal cell types, including bone marrow-derived circulating endothelia and hematopoietic precursor

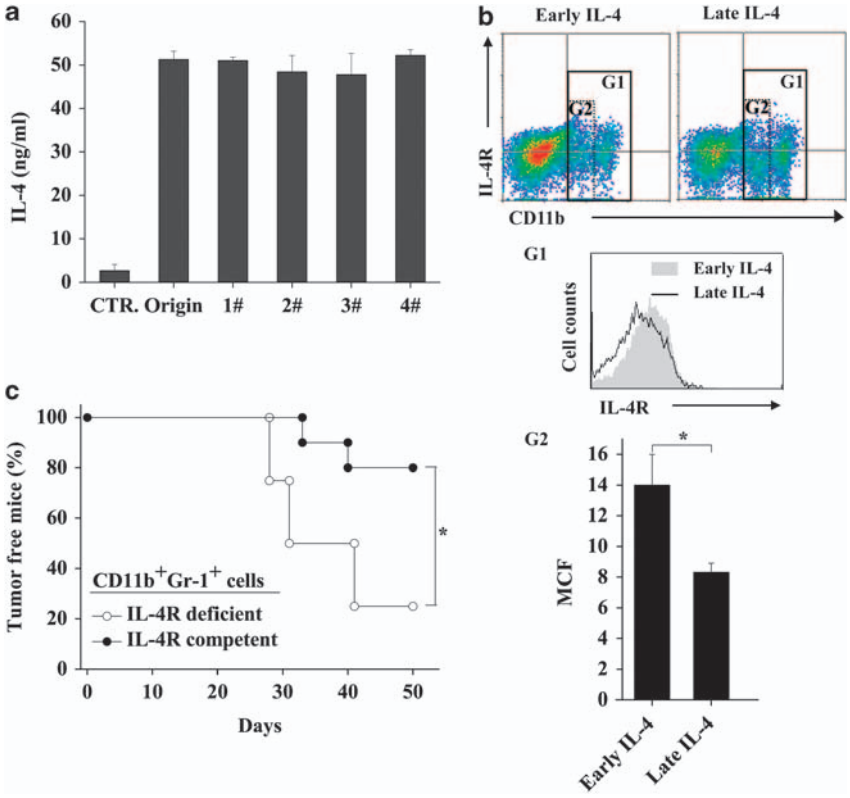

Figure 6 IL-4 responsiveness of myeloid cells is sufficient for IL-4-mediated tumor rejection. (**a**) No alteration in production of IL-4 by tumor cells after *in vivo* passage. Four tumors were isolated from MCA205-TrmIL4 tumor-bearing mice and single-cell suspensions were then prepared. The isolated or original MCA205-TrmIL4 cells were cultured for 48 h, supernatants were then harvested, and IL-4 levels were determined using an ELISA kit. (**b**) Decreased IL-4R expression on splenic CD11b^{low} cells in late comparing to early IL-4 group. Top IL-4R expression on splenic CD11b$^+$ cells in early (left) and late IL-4 (right) group. Middle, overlaid histogram of IL-4R expression on total CD11b$^+$ cells (in gate G1 on top panel). Bottom, mean channel fluorescence of IL-4R staining on CD11b^{low} cells (G2 on top panel). *$P <$ 0.05. (**c**) Necessity of IL-4R expression on myeloid cells for tumor rejection; 1×10^5 CD11b$^+$Gr-1$^+$ cells from wild-type (WT) (●, $n = 10$) or IL-4R-deficient mice (○, $n = 4$) were mixed with 1×10^6 IL-4R-deficient FA10-IL4 cells and then injected subcutaneously into IL-4R-deficient mice. Tumor growth was then measured. *$P < 0.05$. Data are representative of two independent experiments.

cells,[34] which appear to be actively recruited by the tumor to promote angiogenesis.[35] The data here suggest that early exposure of tumor stroma to a high concentration of exogenous IL-4 both recruits and also promotes the differentiation of infiltrating myeloid cells into mature Gr-1$^+$ cells. These cells are involved in early growth suppression of IL-4-secreting tumors.[16] However, the complete eradication of tumor cells often requires T-cell activation. The present work shows that, in the absence of early IL-4, the generation of MDSCs cannot be prevented and CD8$^+$ T cells are functionally suppressed.

Elimination of MDSCs by mAb has been proven to be unreliable. For example, application of anti-Gr-1 mAb slows tumor progression in tumor-bearing mice in one model.[36] However, application of the same mAb inhibits rejection of IL-4-transfected tumors in other models.[15,16] One explanation for this contradiction is that the depleted CD11b$^+$Gr-1$^+$ myeloid cells are in different stages of maturation. Therefore, modulation of this cell population using cytokines *in vivo* seems promising. Pak *et al*.[37] have reported that treatment of tumor-bearing mice with a low dose of IFNγ plus tumor necrosis factor can reduce the number of MDSCs by forcing their differentiation into mature macrophages. IL-4 is a representative cytokine of Th2 cells,[38] and it is unknown whether IL-4 can overcome MDSC-mediated immune suppression *in vivo*. To our knowledge, this is the first evidence that early exposure of high-dose IL-4 in the tumor site could reverse MDSC-mediated immune suppression.

Schuler *et al*.[39] show that IL-4 contributes to tumor rejection by targeted modulation of tumor-associated fibroblasts. The fibroblasts are obtained by culturing adherent cells from the lungs of mice. After eight passages, 85–95% of the cultured cells are positive for fibronectin and collagen I, suggesting that the majority of these cells are fibroblasts. However, it is still difficult to exclude the possibility that the cell culture also contains CD11b$^+$Gr-1$^+$ cells because these cells are also adherent and can survive a few passages.

Recently, MDSCs have been found to infiltrate tumors and promote neovascularization in tumors by directly incorporating into tumor endothelium.[4] MDSCs have also been implicated in tumor refractoriness to anti-vascular endothelial growth factor treatment.[7] IL-4 is reported to inhibit angiogenesis by blocking endothelial cell migration.[40] Thus, we postulate that once tumor angiogenesis is established, it is difficult for the immune system to destroy the tumor. Whether IL-4 acts directly on endothelial cells or indirectly through MDSCs, needs to be further analyzed.

IL-4 artificially delivered in the tumor shows a strong anti-tumor function in animal models. However, when this approach was tested in the clinic as a treatment for hematopoietic and solid malignancies, it showed very limited anti-tumor activity.[41,42] Reasons accounting for this difference are various, including tumor models used, types of effector cells involved, and the difference between human and mouse. However, both dose and time, two important factors for the study of a given cytokine *in vivo*, are often neglected. Using tumor cells transfected with a *tet*-regulatory system, we show here that the role of IL-4 with regard to anti-tumor immunity is strictly dose and time dependent. Therefore, both a suitable dose and an appropriate application time are critical for exogenous IL-4 to be effective. Considering the fact that a big tumor is often accompanied by a high number of MDSCs, while small tumors are associated with low number of MDSCs in tumor-bearing host,[43] as well as the fact that IL-4R is expressed in both mouse and human MDSCs,[44,45] it will be very important to analyze whether IL-4 should be used for tumor therapy shortly after the removal of the original tumor.

Materials and methods

Mice

C57BL/6 and BALB/c mice were purchased from Experimental Animal Center of Weitonglihua (Beijing, China). IL-4R-deficient mice on BALB/c background were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were bred in a specific pathogen-free barrier facility in Institute of Biophysics (Beijing, China). In all experiments described here, female mice at the age of 6–8 weeks were used. All animal studies were conducted with the approval of the corresponding authorities.

Cell lines

MCA205 fibrosarcoma is of C57BL/6 origin.[46] MCA205-TrmIL4, the tumor cell line with *tet*-regulated IL-4 production, was established by co-transfection of MCA205 cells with the responsive plasmid pTRE2-mIL4, which carries mouse IL-4 complementary DNA under the control of a mutant cytomegalovirus promoter, and the regulatory plasmid pTet-off (BD Biosciences, San Jose, CA, USA) at a molar ratio of 10:1. As control, MCA205 cells were co-transfected with the plasmid pTet-off and empty plasmid pTRE2 to obtain mock transfectant (MCA205-mock). Cells were selected in 200 μg ml^{-1} G418 for 5 days and subjected to cloning. Three MCA205-TrmIL4 clones and 10 mock clones were obtained, and one clone of each type was used in this study.

An IL-4R-deficient FA10 fibrosarcoma cell line was previously generated from an IL-4R-deficient mouse.[27] FA10-IL4 and FA10-mock cells were obtained by gene modification of FA10 cells with HyTk-EF1α-mIL-4 and the empty plasmid, HyTk-EF1α, respectively. Stable cells were selected in 40 μg ml^{-1} hygromycin for 6 days. All transfection experiments were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Analysis of tumor growth

Exponentially growing tumor cells were harvested, washed and injected subcutaneously into the left and/or right abdomen region of mice at a volume of 0.2 ml in phosphate-buffered saline. Tumor growth was monitored by a caliper every 2–4 days and expressed as the mean of the largest diameter and the diameter perpendicular to this line. Mice bearing tumors with $\geqslant$10 mm in size were recorded as tumor positive.

T-cell depletion

For *in vivo* depletion of different T-cell subtypes, mice were injected intraperitoneally with 0.5 mg rat anti-mouse mAbs GK1.5 (anti-CD4), 2.43 (anti-CD8) or isotype-matched mAb at day 11 after tumor cell inoculation.

Antibody treatment was performed once per week thereafter, four more times.

Adoptive transfer of immune cells

To collect $CD11b^+Gr\text{-}1^+$ cells, single-spleen cell suspensions were prepared. The cells were then fractionated by centrifugation on a Percoll (Amersham Biosciences, GE Healthcare, Uppsala, Sweden) density gradient and collected as described previously.[47,29] For adoptive transfer, splenocytes from immunized mice were isolated and reactivated as previously described[48] with irradiated MCA205 cells (40:1) and 10 U ml^{-1} recombinant human IL-2 (BD Biosciences) for 3 days. Mice were subcutaneously inoculated with 1×10^5 viable MCA205 tumor cells. After 24 h, 1×10^7 reactivated splenocytes were injected through tail vein alone or together with 1×10^6 $CD11b^+Gr\text{-}1^+$ cells collected from MCA205-TrmIL4 tumor-bearing mice in early and late IL-4 groups at day 17.

Flow cytometry

Single spleen or lymph node cells were washed in fluorescence-activated cell sorting medium (1 × phosphate-buffered saline supplemented with 0.1% bovine serum albumin) and stained with specific mAbs according to the standard procedure. The mAbs used were: fluorescein isothiocyanate-labeled anti-mouse CD11b, F4/80, and FoxP3; PE-labeled Gr-1, CD54, CD69, CD80, IL-4R, major histocompatibility complex class I and IFNγ; Allophycocyanin-labeled CD4, and CD11c, and PerCP-labeled CD8 (all from BD Pharmingen, San Jose, CA, USA). Data were acquired with a FACSCalibur cytometer (BD Biosciences) and analyzed with Cell Quest-Pro software (BD Biosciences) or WinMDI 2.9 software (J Trotter, the Scripps Research Institute, La Jolla, CA, USA).

Immunohistochemistry

Isolation of tumor tissues, preparation of cryostat sections and immunostaining were performed as described previously.[49] The mAbs used for staining were anti-Gr-1, anti-CD11b and isotype-matched control mAbs (all from BD Biosciences). As a secondary reagent, biotin-conjugated rabbit anti-rat IgG (H+L) was used (Jackson Immunoresearch, West Grove, PA, USA). Rhodamine (TRITC)-conjugated streptavidin was used as the third reagent (Sigma, St Louis, MO, USA). All tissue sections were counterstained with 4,6-diamidino-2-phenylindole, a fluorescent stain that binds to DNA. Photos were taken with a digital camera (DP71, OLYMPUS, Center Valley, PA, USA).

Arginase activity

Arginase activity of $CD11b^+Gr\text{-}1^+$ cells was assayed as described by Munder *et al*.[50]

Statistical analysis

Data were analyzed with a two-tailed unpaired Student's *t*-test. *P*-values <0.05 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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