Article

Antisense-Induced Blockade of GATA-3 Expression Could Inhibit Th2 Excursion of Tumor Cells *in vitro* and *in vivo*

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Previous studies have shown that tumor cells predominantly express Th2 type cytokines and transcription factors. GATA-3, as a Th2-specific transcription factor, plays a central role in positive-regulating Th2 development. So whether the expression of GATA-3 in tumor cells has any effect on tumor development is a question of interest. In the present study, we inhibited the expression of GATA-3 in tumor cells through antisense RNA blockade technique, and observed its effects on tumor *in vitro* and *in vivo*. Our results showed that antisense GATA-3 treatment could inhibit the expression of TNF-α and Th2 cytokines in tumor cells, and antisense-induced blockade of GATA-3 could also depress tumor growth in tumor-bearing mice. We suggest that the ratio of T-bet/GATA-3 can be evaluated as a more important marker of the status of Th1/Th2 type. And our results might provide some evidence about the molecular regulatory mechanisms in tumor cell development. *Cellular & Molecular Immunology*. 2005;2(3):189-196.

Key Words: GATA-3, antisense RNA, tumor cell, Th1/Th2 cytokine and transcription factor

Introduction

T lymphocytes, especially $CD4^+$ T helper cells, are critical regulators in mammalian immune responses. T helper cells can be classified into two distinct subsets, Th1 and Th2, based on their profiles of cytokine production. Th1 cells mainly secrete IFN-γ, IL-2, TNF-α and TNF-β, which chiefly mediate cellular immune responses and delayed hypersensitivity. Th2 cells mainly produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, which chiefly mediate humoral immune responses (1-4). The Th1/Th2 balance is closely related to many kinds of diseases (5, 6). In the case of cancer, it has been shown that tumor cells express high levels of IL-4, IL-6 and IL-10 and low levels of IFN-γ and IL-2 (7, 8). The predominant expression of Th2 type cytokines in tumor cells may be one of the reasons for their escape from immune surveillance, given the fact that the cellular immune responses mediated by Th1 cells play major role in anti-tumor immunity (9-13). So it is indicated that treatment which can reverse Th2 condition to Th1 condition in tumor-bearing body could be an effective therapy for cancer.

GATA-3 is a Th2-specific transcription factor and is essential for the Th2 development (14). It is a pleiotropic factor of the C4 zinc-finger family expressed in T cells, mast cells, eosinophils, embryonic brain, basophils, and embryonic kidney. GATA-3, a member of GATA family transcription factors, binds to the consensus DNA sequence, 5'-WGATAR-3' (W = A/T, R = A/G) (15-17). Studies showed that antisense-induced blockade of GATA-3 in Th2 cells correlated with depressed expression of all the Th2 cytokine genes, and forced expression of GATA-3 by retroviral transfection could reverse the differentiation of developing Th1 cells to Th2 type with production of all Th2 cytokines (18). Furthermore, it's considered that in T cells gene expression of GATA-3 is activated through two pathways: one is dependent on STAT6 which is activated by IL-4 co-stimulated with TCR (19); another is STAT6-independent autoactivation (20). And current study showed that retroviral expression of GATA-3 in developing Th1 cells suppressed Th1 development through down-regulation of STAT4, rather than effects on IL-12Rβ2 chain or T-bet, which is the key transcription factor in the differentiation of Th1 cells (21).

Our previous research demonstrated that in patients with lung cancer, interference with traditional Chinese herbal medicine could increase the expression of T-bet and Th1 type cytokines, suggesting that we could reverse the Th2 condition in cancer patients to Th1 condition on transcriptional level and stop the evasion of tumor cells from immune surveillance. Based on the study above, we consider that GATA-3 might have similar functions in tumor cells as it

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Table 1. Specific primer sequences used in semi-quantitative RT-PCR assay

Genes	Species	Primers	bp
β-actin	Mouse	5'-ATGGATGACGATATCGCT-3' 5'-ATGAGGTAGTCTGTCAGGT-3'	569
IFN-γ	Mouse	5'-AACGCTACACACTGCATCT-3' 5'-GAGCTCATTGAATGCTTGG-3'	399
IL-2	Mouse	5'-AACAGCGCACCCACTTCAA-3' 5'-TTGAGAATGATGCTTTGACA-3'	453
TNF-α	Mouse	5'-ACTGGCAGAAGAGGCACTC-3' 5'-CTGGCACCACTAGTTGGTTG-3'	359
IL-4	Mouse	5'-TAGTTGTCATCCTGCTCTT-3' 5'-GTCTTTCAGTGATGTGGAC-3'	359
IL-5	Mouse	5'-CTTCAGAGTCATGAGAAGGA-3' 5'-AATTGTGAAGTCCTGTCACC-3'	448
IL-6	Mouse	5'-AGAGACTTCCATCCAGTTGCC-3' 5'-TCTGAAGGACTCTGGCTTTGTC-3'	412
IL-10	Mouse	5'-AGCTGGACAACATACTGCTAAC-3' 5'-TCATTCATGGCCTTGTAGACAC-3'	301
IL-13	Mouse	5'-GCTTGCCTTGGTGGTCTCG-3' 5'-TCAACCCTCCTCCCTGCCTC-3'	455
T-bet	Mouse	5'-GGAGCGGACCAACAGCATC-3' 5'-CCACGGTGAAGGACAGGAAT-3'	442
GATA-3	Mouse	5'-TCTGGAGGAGGAACGCTAATGG-3' 5'-GAACTCTTCGCACACTTGGAGACTC-3'	408
β-actin	Human	5'-CAACTGGGACGACATGGAGAAAAT-3' 5'-ATTGCCAATGGTGATGACCT-3'	525
IFN-γ	Human	5'-TATCTTGGCTTTTCAGCTCTGCATCGT-3' 5'-ACAGTTCAGCCATCACTTGGATGAGTT-3'	412
IL-2	Human	5'-ATGTACAGGATGCAACTCCTGTCTT-3' 5'-ATTGCCAATGGTGATGACCT-3'	458
IL-4	Human	5'-ATGGGTCTCACCTCCCAACTGCT-3' 5'-CGAACACTTTGAATCTTTCTCTCTCTCAT-3'	456
IL-10	Human	5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3' 5'-GTTTCGTATCTTCATTGTCAT-3'	249
IL-13	Human	5'-CAGGATCCCCTCCCTCTACAGCCCTCAGG-3' 5'-CGGAATTCTTAGTTGAACCGTCCCTCGCG-3'	300

does in T helper cells. We observed the relationship between transcription factors and Th1/Th2 cytokines in tumor cells. Our results showed that local treatment with antisense GATA-3 RNA, which was produced by its antisense RNA eukaryotic expression vector, could inhibit Th2 excursion in tumor cells and in tumor-bearing mice.

Materials and Methods

Construction of GATA-3 antisense RNA eukaryotic expression vector pcDNA3/ASGATA3

Briefly, total RNA was extracted from normal B16 cells (mouse melanoma), and RT-PCR was used to obtain DNA fragments of GATA-3. The fragment overlapped the translation start code ATG (from +188 to +512, and +208 is ATG). The sequence of the selected GATA-3 fragment is homologous between mice and human. Specific primers were designed and synthesized according to mouse GATA-3 mRNA (NCBI GenBank, NM_008091.1, GI:6679950), human GATA-3 mRNA (NCBI GenBank, NM_002051, GI:4503928), and mouse GATA-3 cDNA (NCBI GenBank, AK090089, GI:26105726), as following: P1: 5'-TAG AAT TCC GCG AGC ACA GCC GAG GAC A-3', P2: 5'-TAG GAT CCA GGG CTT GCC GCC ATC CAG-3'. P1 and P2 had the sites of restrict endonucleases EcoR I and BamH I respectively. The target fragments were cut and inserted reversely into eukaryotic expression plasmid pcDNA3, in which the site for EcoR I is downstream of the site for BamH I, so the fragment will be transcribed to an mRNA strand complementary to GATA-3 gene sequence in transfected cells. Binding of the complementary mRNA to the GATA-3 mRNA

will block out the initiation site for translation, and then GATA-3 translation will be inhibited.

Cell culture and transfection

B16 cells were stably transfected with the mixture of 5 μg purified pcDNA3/ASGATA3 plasmid and 5 μl Lipofectamine™ 2000, and cultured in RPMI 1640 medium containing 600 μg/ml G418 to screen the positive clones. The screened clones were maintained in 1640 medium with 250 μg/ml G418. Other tumor cells, such as Karpas (human T cell lymphoma) and YAC-1 (mouse lymphoma), were transfected as described above. PHA was added at a final concentration of 1 μg/ml 4 hours later to enhance CMV promoter activity and gene expression. The cells were examined for transfection efficiency 48 hours after transient transfection.

Mice treatment

The 6-8-week-old male C57BL/6 (hereafter termed B6) mice were purchased from Shanghai Experimental Animal Center, Chinese Science Academy (Shanghai, China). Animals were maintained under controlled conditions (pathogen-free, 22°C, 55% humidity, and 12-hour day/night rhythm). Handling of mice and experimental procedures were conducted in accordance with experimental animal's guidelines. B6 mice were injected intravenously with 3×10^5 B16-ASGATA3 transductants. Lung metastases were counted one, two and three weeks after the tumor challenge.

Preparation of mononuclear cells (MNCs) from livers and spleens

Under deep ether anesthesia, the mice were euthanized by exsanguinations from the subclavian artery and vein. Liver mononuclear cells (MNCs) were obtained as previously described (22). In brief, the liver was removed after sacrificing the animal and then was dissected and passed through a 200-gauge stainless steel mesh. Cell suspension was collected, and kept on ice for 5 to 10 min to precipitate the debris. The supernatant was obtained by centrifugation at 1,500 rpm for 5 min. The pellet was resuspended in 40% percoll solution and placed gently onto the 70% percoll layer, followed by centrifugation at 2,400 rpm for 30 min. The cells layered in the interface between 40% and 70% percoll solution were harvested as liver lymphocytes and washed twice with PBS. Splenocytes were passed through a 200-gauge stainless steel mesh, and were treated with RBC lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, and 170 mM Tris, pH 7.3) and washed twice in HBSS containing 2% FBS.

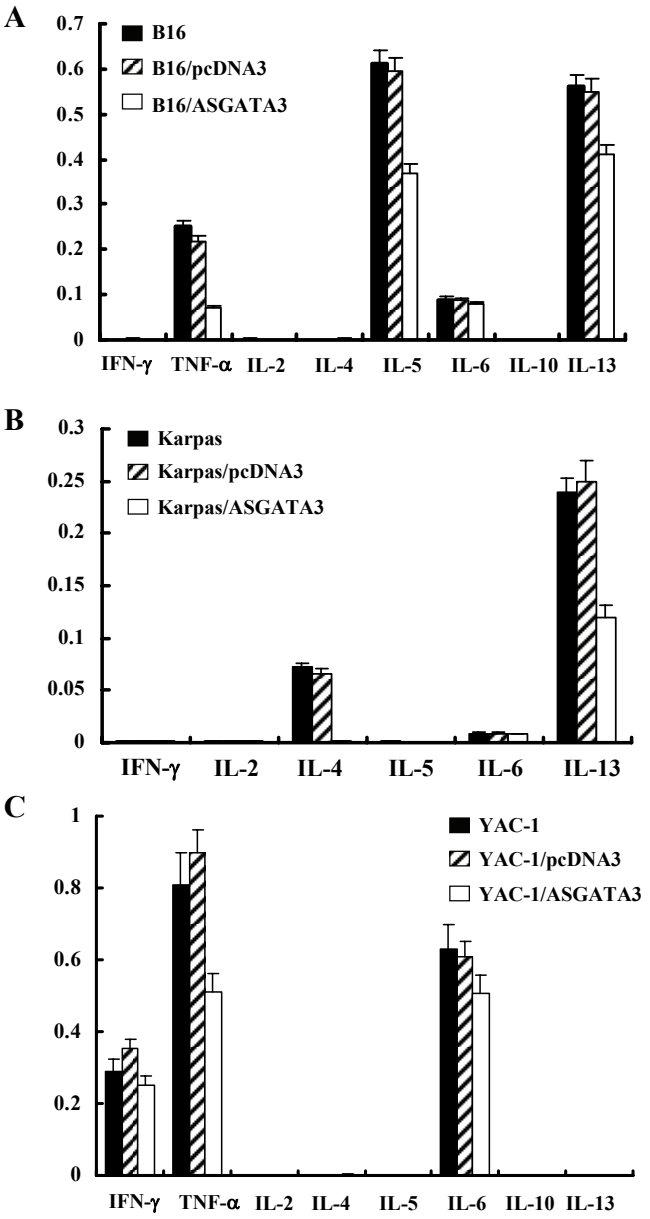

Figure 1. Cytokine profiles of Th1/Th2 polarized cells. B16 (mouse melanoma, A), Karpas (human T cell lymphoma, B) and YAC-1 (mouse lymphoma, C) cells were harvested for total RNA preparation. Gene expression was measured by RT-PCR and gene expression data were normalized to β-actin expression. The results were depicted as relative light intensities of detected cytokines against β-actin, assuming the light intensity of β-actin was 1. Data are expressed as mean ± SD of at least five independent experiments.

Semi-quantitative RT-PCR assay

Total RNA of cells was extracted according to RNA isolation method using acid guanidinium thiocyanate phenol chloroform extraction. Concentration and quality of the extracted RNA were determined by measuring light absorbance at 260 nm (A_{260}) and the ratio of A_{260}/A_{280}. Reverse transcription (RT) was carried out with 4.5 μg total RNA using random hexamers as primers and M-MLV reverse transcriptase with 5× first strand buffer and 0.1 M DTT. The reaction system was incubated at 37°C for 50 min, and M-MLV was inactivated by heating at 70°C for 15 min. PCR was performed using 4× dNTP mixture and the Taq DNA polymerase with 10× reaction buffer and $MgCl_2$. The cytokines, transcription factors' primers and PCR products' sizes were as follows (Table 1). The PCR reaction programs were optimized for

each cytokine. Semi-quantitative RT-PCR was performed using β-actin as an internal control to normalize gene expression for the PCR templates. Results were obtained by electrophoresis and the relative light intensities of bands were analyzed by Scion Image (Release Beta 4.0.2 edition) software.

Statistical analysis

Differences between the groups were analyzed by Student's *t*-test and $p < 0.05$ was taken to imply statistically significant.

Results

Antisense-induced blockade of GATA-3 expression inhibited Th2 cytokine expressions in tumor cells

DNA sequencing identified that the GATA-3 antisense RNA eukaryotic expression vectors were successfully constructed (data not shown). We chose three tumor cell lines to detect the effects of antisense GATA-3 treatment on tumor cells. Both B16 and Karpas cells predominantly express Th2 cytokines, while YAC-1 cells express Th1 and Th2 cytokines equally. The expressions of Th2 cytokines in these cell lines were down-regulated by antisense GATA-3 treatment.

B16 cells mainly express Th2 type cytokines such as IL-5, IL-6 and IL-13. Their normal gene expressions were significantly inhibited by antisense GATA-3 treatment, with distinct decreases of 39.3%, 16%, and 23.6% respectively (Figure 1A). On the other hand, the treatment had no effect on the expressions of IFN-γ and IL-4, which were hardly detected on the level of protein as well (data not shown). The result with Karpas cells was similar (Figure 1B): IFN-γ expression was not detected before or after treatment. The expression of IL-4 and IL-13 decreased about 100% and 46.7%, respectively.

Additionally, YAC-1 cells express Th1 cytokine IFN-γ and Th2 cytokine IL-6. Similar to B16 and Karpas, IFN-γ gene expression had no change after antisense GATA-3 treatment in YAC-1 cells. But, IL-6 had a decrease of 23.1% (Figure 1C).

Antisense-induced blockade of GATA-3 inhibited TNF-α expression in tumor cells

Reports have shown that although TNF-α, a non-classical Th1 type cytokine, was considered to be a factor against tumor, in tumor-bearing body it had a high level in serum instead and might promote tumor growth (23). Interestingly, we found that TNF-α gene expression was very high in tumor cells, especially in B16 and YAC-1 cells. After antisense GATA-3 treatment, it decreased about 74.6% and 38.0%, respectively (Figure 1). This implied that tumor growth might be depressed. So we performed experiments *in vivo*.

Antisense-induced blockade of GATA-3 depressed tumor growth in tumor-bearing mice

B6 mice were injected intravenously with 3×10^5 antisense GATA-3 transfected B16 cells. To serve as control, two groups of mice were injected intravenously with normal B16 cells and empty pcDNA3 transfected B16 cells with the same amount, respectively. We observed that, compared with control groups, the frequency of lung metastases in antisense GATA-3 treated mice reduced till 14 days after cell injection. However, the frequency restored to the same level as that of control groups on day 21 (Figure 2A).

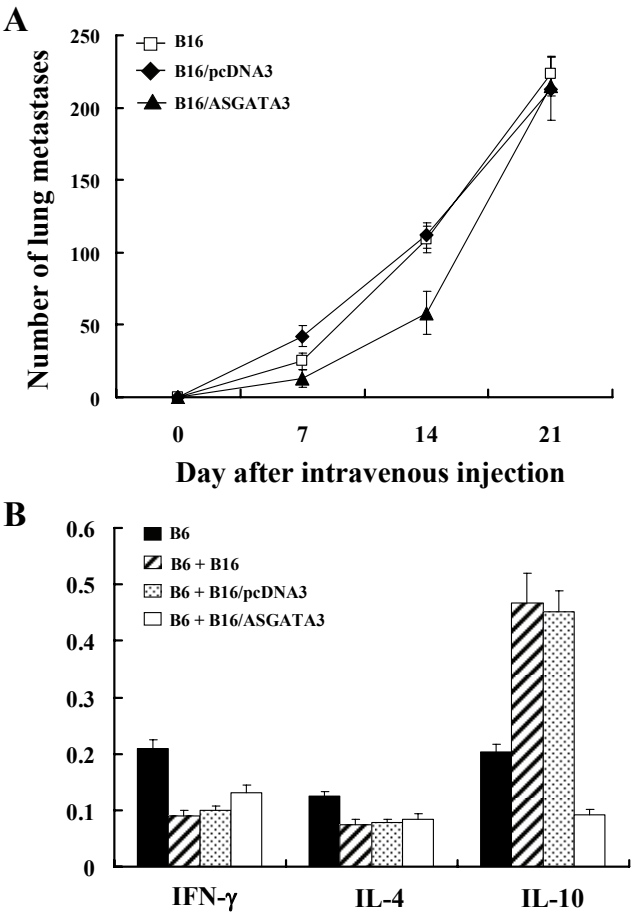

Figure 2. Tumor growth and cytokine profile of splenocytes in B6 mice injected with antisense GATA-3 treated B16 cells. Six to eight-week-old male B6 mice were injected intravenously with 3×10^5 B16-ASGATA3 transductants. As control, two groups of mice were injected intravenously respectively with normal B16 cells and empty pcDNA3 transfected B16 cells with the same amount. Lung metastases were counted one, two and three weeks after the tumor challenge (A). Splenocytes were harvested on day 14, and Th1/Th2 cytokine gene expressions were detected as described above (B). Data are expressed as mean ± SD of at least three independent experiments.

Then we measured major Th1 and Th2 cytokines gene expressions in spleen and liver lymphocytes 14 days after injection. Compared with normal B6 mice, the expressions of IFN-γ and IL-4 decreased respectively by 56.8% and 41.3% in mice injected with normal B16 cells, and IL-10 had a significant increase of 124.1%. In mice injected with antisense GATA-3 transfected B16 cells, gene expressions of all the three cytokines were much lower than those of normal

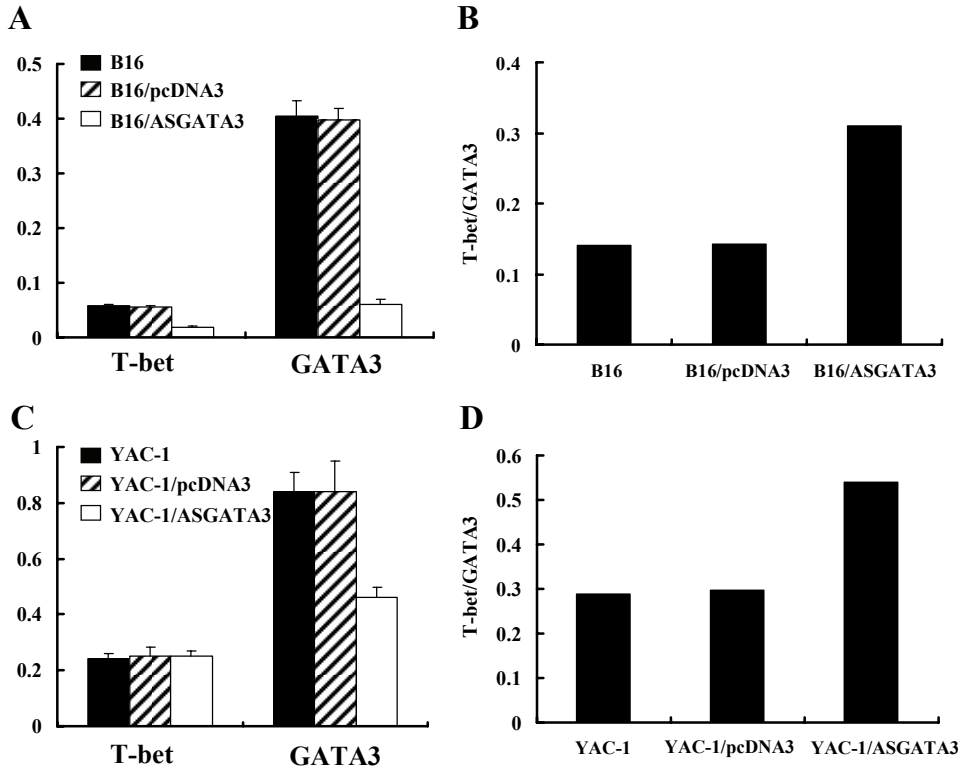

Figure 3. T-bet and GATA-3 gene expression of Th1/Th2 polarized cells. B16 (A, B) and YAC-1 (C, D) cells were harvested for total RNA preparation. Gene expression was measured by RT-PCR. Gene expression data were normalized to β-actin expression. Data are shown as relative light intensities of detected cytokines against β-actin, assuming the light intensity of β-actin was 1. Results are representative of at least five independent experiments.

B6 mice. Importantly, compared with injection with normal B16 cells, IFN-γ had an increase of 42.1% and IL-10 significantly decreased by 79.5% in antisense GATA-3 treated mice (Figure 2B). In liver lymphocytes the results were similar (data not shown).

The ratio of T-bet/GATA-3 could reflect the Th1/Th2 status of tumor cells

It has been reported that, as the essential transcription factors, the ratio of T-bet/GATA-3 might reflect the Th1 and Th2 status in mixed populations (24). So we measured the expressions of the two transcription factors. We found that B16 cells normally expressed much higher GATA-3 than T-bet (Figure 3A). Their gene expressions decreased by 85.4% and 67.2% respectively after antisense-GATA-3 treatment. Additionally, the ratio of T-bet/GATA-3 changed from 0.152 to 0.323 (Figure 3B), which showed an excursion to Th1 type to a certain extent.

We got similar results in YAC-1 (Figure 3C). After antisense treatment, GATA-3 had a decrease of 46.9% and no change with T-bet. The ratio of GATA-3/T-bet changed from 3.678 to 1.907 (Figure 3D), which also showed an excursion to Th1 type.

Similarly, T-bet was expressed more highly than GATA-3 in splenocytes of normal B6 mice. GATA-3 gene expression had no change in B16 tumor-bearing mice, while T-bet decreased about 14.5% (Figure 4A). After injection of antisense GATA-3 treated B16 cells, the expressions of GATA-3 and T-bet decreased, and the T-bet/GATA-3 ratio increased from 1.390 to 1.764, the same level as in normal B6 mice. Moreover, the change of T-bet/GATA-3 ratio showed the same trend as those of IFN-γ/IL-4 and IFN-γ/IL-10. In normal B6 mice, IFN-γ/IL-4 and IFN-γ/IL-10 were 1.735 and 1.054, and then reduced to 1.277 and 0.203 respectively after tumor cell injection. Antisense GATA-3 treatment made them increase to 1.529 and 1.412, which were the same as what in normal B6 mice (Figure 4B).

Discussion

According to the immunosurveillance hypothesis, immune system can keep watch on the generation of tumors and cellular immunity plays major role in anti-tumor immunity. The central regulators are T cells, NK cells and macrophages (25). But the precise relationship between evasion of tumor cells and Th1/Th2 status remains a question.

Many kinds of cytokines, which are secreted by mostly activated Th1 type cells, also play their roles. For example, IL-2 could mediate proliferation of CTL, anti-tumor function,

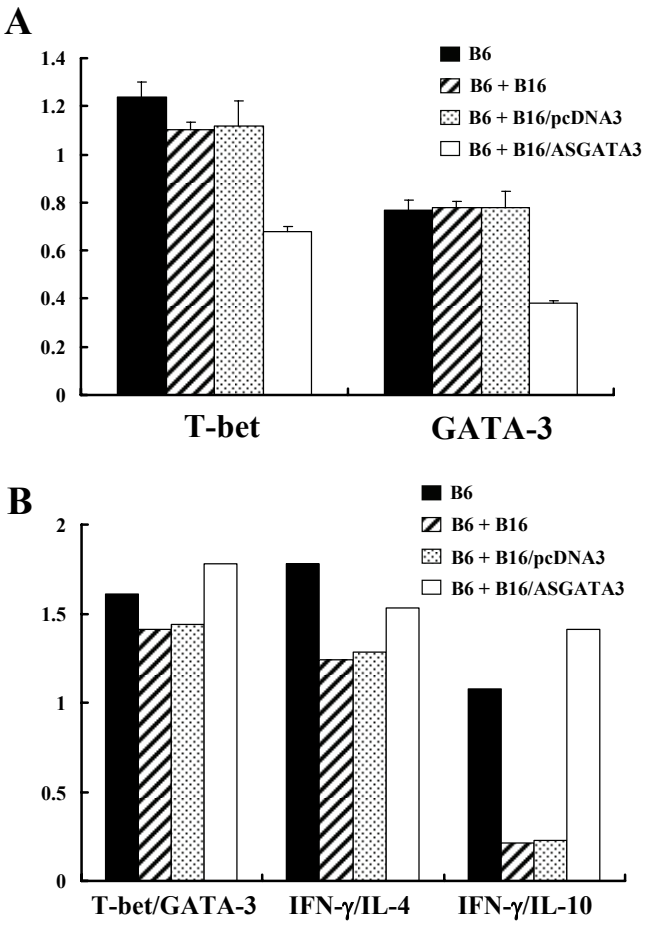

Figure 4. T-bet and GATA-3 gene expressions in mouse splenocytes (A), and the ratios of Th1/Th2 transcription factors and cytokines (B). Splenocytes were harvested on day 14, and T-bet and GATA-3 gene expressions were detected as described above. Data are expressed as mean ± SD of at least three mice.

and activation of NK cells (26, 27). IFN-γ could activate macrophages and NK cells (28), and so on. Moreover, IL-12, a cytokine that could induce Th1 differentiation and development, has the anti-tumor function (29). On the other hand, Th2 cytokines such as IL-10 and IL-4 have functions that inhibit anti-tumor immunity (30). Based on these, we consider that the Th1 condition of body could provide favourable surroundings for anti-tumor effects. When the condition inclines to Th2, immune inhibition will form so that anti-tumor immunity will be seriously interfered. Excursion to Th2 condition has been observed in tumor-bearing patients (7-13). In our researches, we also found normal tumor cells and tumor-bearing mice predominantly expressed Th2 cytokines (Figures 1 and 2B).

Although relative researches are not extensive and systemic, it is reasonable for us to explore a new potential treatment for cancer by reversing the Th2 condition to Th1. By now, inspiring results and foregrounds have shown in existing therapies. For instance, Th1 cytokines such as IL-2, IFN-γ and TNF-α have been used in clinical trials. Notice that changes of Th1/Th2 equilibrium in body by directly introducing cytokines and the reverse of $CD4^+$ T cells differentiation by the same way are mostly the same. Furthermore, similar results will be got by introducing or inhibiting transcription factors which are essential in Th1/Th2 differentiation. Especially, researches in an asthma model have been successful, in which blockade of GATA-3 could inhibit airway inflammation (31-34).

In our researches we got some expected results. For B16 and Karpas, which both typically express Th2 cytokines predominantly, antisense GATA-3 treatment made their major Th2 cytokines decrease more or less. The same change was observed in YAC-1 cells (Figure 1). The results were accordant with the molecular mechanisms of GATA-3. Studies have demonstrated that there are GATA-3 binding sites located in -70 to -59 of the IL-5 promoter (35), -95 of the IL-13 promoter (36), and antisense of GATA-3 can inhibit expression of IL-5 and IL-13 (37). Although there are also GATA-3 binding sites found in IL-4 promoter and enhancer, GATA-3 has less activity in the IL-4 promoter and it may be not sufficient for full initiation of IL-4 gene expression (38).

It was reported that TNF-α could promote tumor growth (39-41). We detected high level of TNF-α mRNA in tumor cells, and its gene expression decreased notably after antisense GATA-3 treatment (Figure 1). So we considered that tumor cell growth had been depressed. Indeed, when B6 mice were intravenously injected with antisense GATA-3 treated B16 cells, the frequency of lung metastases decreased for about two weeks (Figure 2A). Moreover, cytokine production also restored to normal level (Figure 2B).

On the other hand, we got some interesting results on the side of transcription factors. It has been reported that GATA-3 suppresses Th1 development by down-regulation of STAT4 but not through its effects on T-bet (21). And in cell line itself, no anti-tumor function exists, so the outcome of inhibition of Th2 condition might drive to equilibrium that both Th1 and Th2 cytokines express at a low level by auto-regulation. This may be the reason that both essential transcription factors T-bet and GATA-3 decreased after antisense GATA-3 treatment (Figures 3A and 3C). And in tumor-bearing mice, similar trend was observed on the transcription factors with that in B16 cells (Figures 3A and 4A). Recent researches showed that the ratio of T-bet/GATA-3 gene expression was more informative than the level of either transcription factor alone (24). Indeed, we found that the ratio of T-bet/GATA-3 could also be used to estimate the condition of Th1/Th2 in tumor cells, and it is accordant to the ratio of Th1/Th2 cytokine expressions (Figures 3B, 3D and 4B).

But, as we have discussed above, the expressions of Th1 and Th2 cytokines and transcription factors are interactional, and the generation and growth of tumor are more complex processes. So it needs more researches in the effects of inhibition of a single transcription factor GATA-3 on tumor cells. And we also could see that the antisense blockade method has limits. However, these results may provide some evidence about the molecular regulatory mechanisms in

tumor cells.

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