Article

The *ex vivo* Microenviroments in MLTC of Poorly Immunogenic Tumor Cells Facilitate Polarization of $CD4^+CD25^+$ Regulatory T Cells

Yan Wang[1], Le Zhou[1], Hongyan Wang[1], Juxiang Xiao[1], Lusheng Si[1] and Yili Wang[1, 2]

$CD4^+CD25^+$ regulatory T (T_R) cells play an important role in maintaining a balanced peripheral immune system. Recent studies have shown that T_R cells may also play a key role in suppressing anti-tumor immune response. In order to investigate the tumor immune microenvironment and its influence on T_R polarization, poorly immunogenic tumor cell line D_5 (C57BL/6, H-2^b), immunogenic tumor cell lines FBL_3 (C57BL/6, H-2^b) and H_{22} BALB/c, H-2^d) were used to establish the syngeneic/allogeneic, poorly immunogenic/immunogenic mixed lymphocytes-tumor cell culture (MLTC). Our results revealed that the proportion of $CD4^+CD25^+$ T cells in MLTC of syngeneic primed splenocytes stimulated with D_5 tumor cells was higher than that with H_{22} cells (0.43% *vs* 0.044%, and the similar results appeared in allogeneic splenocytes stimulated with D_5 tumor cells (0.39% *vs* 0.04%). The splenocytes stimulated with supernatant from syngeneic MLTC of D_5 tumor cells demonstrated higher proportion of $CD4^+CD25^+$ cells than that from allogeneic MLTC of D_5 tumor cells, and the splenocytes stimulated with supernatant from syngeneic or allogeneic MLTC of H_{22} tumor cells generated lower proportion of $CD4^+CD25^+$ T cells than that of D_5 tumor cells. The TGF-β1 and Th2-oriented cytokines (IL-4 and IL-10) were dominated in supernatants of syngeneic MLTC of poorly immunogenic tumor cells. Our results provided useful information for studying the mechanisms underlying tumor immune surveillance as well as for the tumor immunotherapy. *Cellular & Molecular Immunology*. 2006;3(2):123-129.

Key Words: immunogenic tumor, immunoescape, T_R cell

Introduction

Cancer immunotherapy attempts to harness the exquisite power and specificity of the immune system for the treatment of cancer. Although tumor cells are less immunogenic than foreign pathogens, accumulated evidence has demonstrated that the host immune system is clearly capable of recognizing tumor cells and data from clinical trials provide the promising hope for immune-management of cancer in some patients. However, tumors frequently interfere with the development and function of anti-tumor immune responses (1). The strategies that effectively and safely augment anti-tumor immunity will contribute to the efficacy of cancer immunotherapy. Cancer has been identified to be heterogeneous which is comprised of antigens with various immunogenicities, in which may evoke different immune responses (2). In animal experiments, strong immunogenic tumors can give rise to a relatively extensive immuno-response and destined by the regression of cancer. However, poorly immunogenic tumors often grow despite immuno-surveillance (3).

$CD4^+CD25^+$ regulatory T (T_R) cells play an important role in maintaining the homeostasis under the normal conditions, and currently the role of T_R cells in suppressing T cell immunity to tumors has been attracted much attention (4). It has been demonstrated that these cells make up the majority of tumor-infiltrating lymphocytes at the late stage of tumor progression, and their depletion during the effector phase, rather than priming phase, successfully enhances anti-tumor immunity (4, 5). The predominance of T_R cells in the tumor site raises the question that whether the immune

[1]The Key Laboratory of Biomedical Information Engineering of Ministry of Education, Institute for Cancer Research at Medical Campus, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an 710061, China;

[2]Corresponding to: Dr. Yili Wang, Institute for Cancer Research at Medical Campus, School of Life Science and Technology, Xi'an Jiao Tong University, Xi'an, Shaanxi, 710061, China. Tel/Fax: +86-29-8265-5499, E-mail: wangyili @mail.xjtu.edu.cn.

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Abbreviations: T_R cell, $CD4^+CD25^+$ regulatory T cell; MLTC, mixed lymphocyte-tumor cell culture; DC, dendritic cell; APC, antigen presenting cell; SI, stimulation index.

microenvironment of tumor favor the polarization of T_R cells. The results have demonstrated that the spontaneous human cancer and "poorly immunogenic" animal cancer are capable of developing immune deviation, i.e., the microenvironment of the cancer are dominant of nontherapeutic Type 2 immune response (6-8).

In the present study, using the model of *ex vivo* tumor cell lymphocyte interaction, we investigated the tumor immune microenvironment and its influence on the T_R cell polarization by comparing the poorly immunogenic and immunogenic tumor cell lines with different genetic background under syngeneic and allogeneic status.

Materials and Methods

Mice

BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from experimental animal center of Xi'an JiaoTong University. Female mice of 6 to 8 weeks of age were used. All mice were housed under special pathogen-free condition.

Cell lines and culture medium

D_5 was a poorly immunogenic subclone of the B16BL6 spontaneously arising melanoma derived from C57BL/6 mice. D_5 exhibited low to undetectable class I (H-2D^b and K^b) expression and no class II expression (9-12). FBL_3 (H-2^b) cell line induced with murine leukemia virus was derived from C57BL/6 mice. It has been demonstrated that immunization with FBL_3 cells could protect immunized mice from rechallenging with the same tumor, and spontaneous regression was occasionally observed in non-immuno-compromised mice (13). A hepatocarcinoma cell line H_{22} (H-2^d) derived from BALB/c mice was immunogenic for immunization with H_{22} also protected mice from challenging with same tumor (14). The above cell lines were cultured in complete tissue culture medium consisting of RPMI-1640 (Gibco) supplemented with 10% heat-inactivated NBS, 1 M sodium pyrurate, 20 μM L-glutamine, 50 μM 2-mercapto-ethanol, 12.5 mM HEPES buffer solution, 100 μg/ml penicilin, 100 μg/ml streptomycin. The cultures were incubated in 5% CO_2 at 37°C, and maintained in exponential state by splitting periodically.

Vaccination of mice and preparation of splenocytes

Tumor cells in exponential state were harvested and treated with 40 μg/ml mitomycin C for 1 h, then washed with Hank's balanced salt solution for three times, counted, and adjusted to the desired concentration in PBS. Six to eight-week-old C57BL/6 mice and BALB/c mice received 1×10^7 syngeneic or allogeneic inactivated D_5, FBL_3 or H_{22} cells subcutaneous on the left flank according to the tumor cell origin. All mice were vaccinated once a week for four times. The mice were killed on the fourth day after the last vaccination. The spleens were minced with scalpels into pieces smaller than 1 mm^3 and passed through stainless steel sieve of 200 gauges for preparation of single cell suspensions. The removal of erythrocytes was accomplished with the treatment of ammonium chloride-potassium-lysing buffer (0.155 M NH_4Cl, 0.1 mM Na_2EDTA and 10 mM $KHCO_3$) for 5 minutes at 4°C. After washing with Hank's balanced salt solution, the cell pellets were resuspended in complete tissue culture medium.

Generation of tumor immunity model in vitro by MLTC

Mixed lymphocyte-tumor cell culture (MLTC) is the classic method to generate tumor specific lymphocytes, including $CD4^+$ and $CD8^+$ cells (15). In this study, the responding cells were splenocytes obtained from pre-immunized BALB/c (H-2^d) or C57BL/6 (H-2^b) mice. And the stimulators were inactivated D_5 (H-2^b), FBL_3 (H-2^b), or H_{22} (H-2^d) tumor cells. The principle of responder/stimulator matching was according to the tumor cell origin, i.e., splenocytes from BALB/c (H-2^d) mice matched with the tumor cells of same origin (syngeneic status), when the origin of responder/stimulator cells were different, that implied the mismatch of the MHC molecules (allogeneic status), which referred to mimicry of transplant immunity model. The ratio of responder/stimulator is 10:1 according to predetermind results. Each models had tumor cell control and splenocyte control. All the cultures were incubated in 5% CO_2 at 37°C for 7 days, the immunological parameters were assayed afterwards.

^{3}H-thymidine uptake assay for proliferative response of splenocytes in MLTC

The settings of MLTC were as described above. About 2×10^5 primed splenocytes were mixed with 2×10^4 tumor cells in 96 plates. These mixtures were incubated at 37°C in 5% CO_2, and 1 μCi/well of ^{3}H-thymidine incorporation was measured in a liquid scintillation counter during the final 18 h of the 7 day culture, and data were expressed as mean counts per minute (cpm) of triplicate determination. The stimulation index (SI) was calculated from the following formula: SI = (mean cpm of stimulated cultures - mean cpm of tumor cells alone) / (mean cpm of splenocytes alone). The SI of MLTC equal to or greater than 2.0 was expressed as a positive blastogenic response to tumor cells.

ELISA

The settings of MLTC were as described above, 2×10^7 primed splenocytes were mixed with 2×10^6 tumor cells. Tumor cells and splenocytes alone were taken as control. After 7 days incubation, culture supernatants were collected and stored at -80°C until being used. TGF-β1, IFN-γ, IL-4 (Jingmei BioTech) and IL-10 (Shanghai Senxiong) were assayed by ELISA. The absorbance of each well was measured by using an ELISA microplate reader at 490 nm. All the procedures were performed according to manu-facturer's instruction. Supernatants of tumor cells alone and splenocytes from immunized mice were as controls.

Flow cytometric analysis of T_R cells

CD4 and CD25 markers have been accepted as the T_R cell phenotypes. The cells collected from MLTC were adjusted to 0.5×10^6/ml in phosphate-buffered saline-0.1% sodium azade buffer and stained with PE-conjugated anti-CD4 mAb

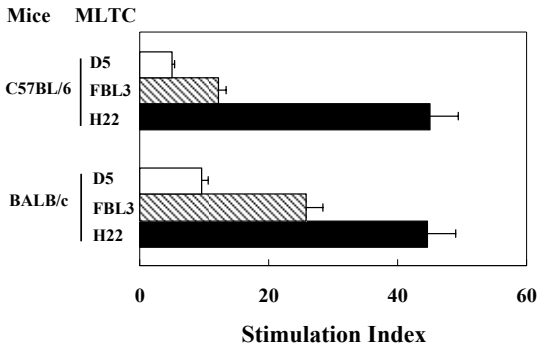

Figure 1. The stimulation index (SI) of splenocytes in MLTC models. About 2×10^5 primed splenocytes were mixed with 2×10^4 D_5, FBL_3 or H_{22} tumor cells in 96 well plates. Cultures were incubated in 5% CO_2 at 37°C for 7 days and pulsed with 1 μCi/well of ^{3}H-thymidine 18 h before harvest. The stimulation index (SI), which represented the fold increase in the cell proliferation of the stimulated spleen cells as compared to the non-stimulated spleen cells, was calculated as described in "Materials and Methods". Results were shown as mean ± SD.

(Beckman) and FITC-conjugated anti-CD25 mAb (Jingmei BioTech), meanwhile, isotype and negative controls were set. The cells were incubated with monoclonal antibodies, which were pre-titered to give optimal staining, for 30 min at 4°C. The stained cells washed twice with PBS-azide buffer and resuspended in 200 μl of 4% paraformaldehyde in the same buffer for two color flow cytometry analysis in EPICS ELITE flow cytometer (Coulter). In order to clarify whether the culture supernatants influence the T_R cell polarization, the supernatants from various MLTCs were added to 4×10^5 splenocytes isolated from unimmunized normal BALB/c mice in 24-well flat-bottom culture plates. Anti-CD3 was added to activate T cells. The cultures were incubated for 3 days at 37°C in CO_2, and then $CD4^+CD25^+$ T cells were determined by flow cytometry (Coulter) as described above.

Intracellular cytokine expression of splenocytes

For the detection of analysis of T helper functional subsets, the approach of intracellular cytokine secretion pattern combined with CD4 phenotype marker were carried out. Splenocytes of primed *in vivo* and stimulated in MLTC were treated with monensin (Sigma) to block cytokine secretion, and first stained with PE-conjugated anti-CD4 mAb, fixed with 4% paraformaldehyde, then treated with 5% permealizing saponion (Sigma), and finally stained with FITC-conjugated anti-IFNγ (Beckman) or FITC-conjugated anti-IL-10 (Beckman) for 45 min on ice. The percentage of $CD4^+$ $IFN\gamma^+$ and $CD4^+IL\text{-}10^+$ cells was determined by flow cytometry (Coulter).

Statistics

Differences between experimental groups were determined by the Student's *t* test. In all experiments, $p < 0.05$ was considered significant.

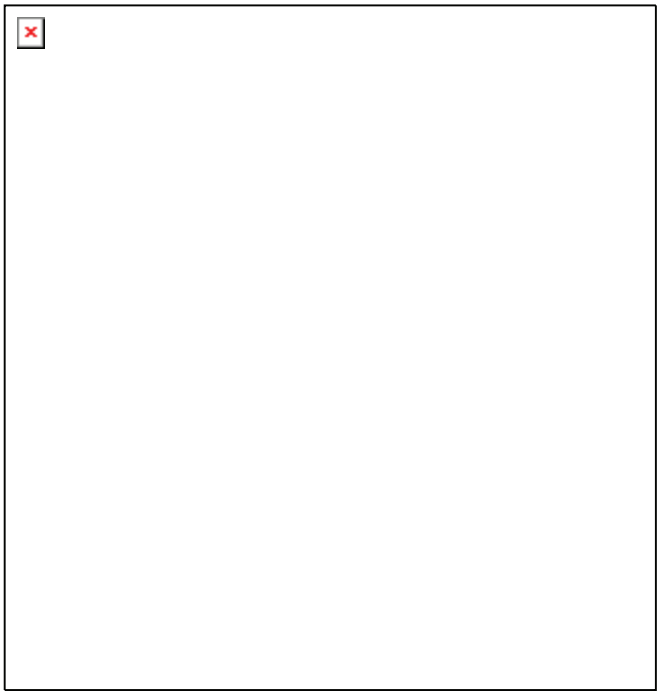

Figure 2. Percentages of $CD4^+CD25^+$ T cells in MLTC were analyzed by flow cytometry. About 2×10^7 primed splenocytes were mixed with 2×10^6 D_5 or H_{22} tumor cells. After 7 day incubation, the cells were stained by PE-conjugated anti-CD4 and FITC-conjugated anti-CD25 mAbs, negative and isotype controls were set and evaluated by flow cytometry. (A) The percentages of the $CD4^+CD25^+$ T cells were shown. Four independent experiments were performed and data were shown as mean ± SD. (B) One representative experiment was shown from four independent experiments.

Results

SI of splenocytes in MLTC of poorly immunogenic tumor was lower than that of highly immunogenic tumor

The proliferation of splenocytes in models was analyzed by ^{3}H-thymidine incorporation assay. As shown in Figure 1, the proliferation of splenocytes stimulated by syngeneic FBL_3 and H_{22} was significantly higher than that of D_5 (3 and 10 folds). The proliferation of splenocytes stimulated by allogeneic FBL_3 or H_{22} was significantly higher than that of D_5 too (3 and 5 folds). The results coincided with the fact that the immunoresponse to immunogenic tumor was higher than that to poorly immunogenic tumor. For D_5 and FBL_3 tumors, SI of MHC unmatched model was higher than that in MHC matched model ($p < 0.05$, Figure 1), although there was no significant difference for H_{22} between two models.

Poorly immunogenic tumor could induce proliferation of T_R cells

$CD4^+CD25^+$ T cells in all MLTC settings were analyzed by flow cytometry. Proportion of $CD4^+CD25^+$ T cells in MLTC of syngeneic splenocytes stimulated with D_5 tumor cells was

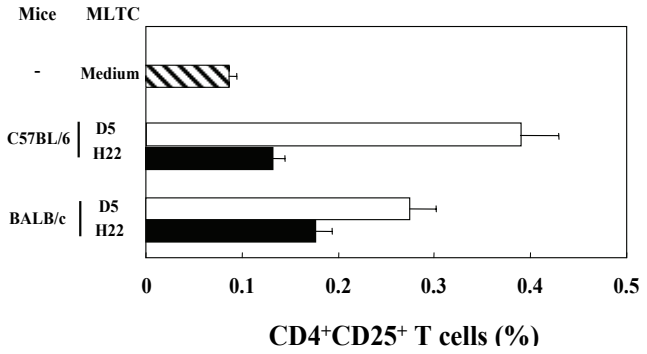

Figure 3. The effect of the MLTC supernatants on $CD4^+CD25^+$ T cells subset of splenocytes from normal BALB/c mice. The supernatants from various MLTCs settings were added to 4×10^5 splenocytes from unimmunized normal BALB/c mice in 24-well flat-bottom culture plates. Anti-CD3 mAb was added to activate T cells. The cultures were incubated in 5% CO_2 at 37°C for 3 days and then $CD4^+CD25^+$ T cells were determined by flow cytometry as described in "Materials and Methods". Four independent experiments were performed and data were shown as mean ± SD (n=6).

higher than that with H_{22} cells ($p < 0.05$) and the similar results appeared in allogeneic splenocytes stimulated with D_5 tumor cells ($p < 0.05$, Figure 2).

The splenocytes stimulated with supernatant from syngeneic MLTC of D_5 tumor cells demonstrated higher proportion of $CD4^+CD25^+$ T cells than that from allogeneic MLTC of D_5 tumor cells ($p < 0.001$). The splenocytes stimulated with supernatant from syngeneic and allogeneic MLTC of H_{22} tumor cells generated lower proportion of $CD4^+CD25^+$ T cells than that of D_5 tumor cells ($p < 0.05$) and there was no significant difference between the syngeneic and allogeneic MLTC of H_{22} tumor cells (Figure 3).

$CD4^+IL\text{-}10^+$ T cells were increased while $CD4^+IFN\text{-}\gamma^+$ T cells were decreased in MLTC of poorly immunogenic tumor D_5

$CD4^+IFN\text{-}\gamma^+$ T cells were increased dramatically in syngeneic MLTC of FBL_3 and H_{22} tumor cells compared with that of D_5 (Figure 4A, $p < 0.05$, $p < 0.0001$). The same results were appeared in allogeneic MLTC of FBL_3 and H_{22} ($p < 0.0001$) and even higher than that in syngeneic setting ($p < 0.05$). As expectedly, the $CD4^+IL\text{-}10^+$ T cell subset in allogeneic MLTC settings were lower compared with that in syngeneic MLTC settings (Figure 4B; D_5, $p < 0.05$; FBL_3, $p < 0.05$; H_{22}, 0.3 *vs* 0.35).

TGF-β1 and Th2-oriented cytokines (IL-4 and IL-10) were dominated in supernatants of syngeneic MLTC of poorly immunogenic tumor cell

The supernatant from MLTC culture were assayed by ELISA for the TGF-β1, IFN-γ, IL-4 and IL-10 level. As shown in Figure 5A, TGF-β1 production was higher in syngeneic MLTC of poorly immunogenic D_5 compared to control ($p < 0.02$). It was also higher in allogeneic MLTC of D_5 ($p < 0.02$) but slightly lower than that of former (15.41 *vs* 9.512). While

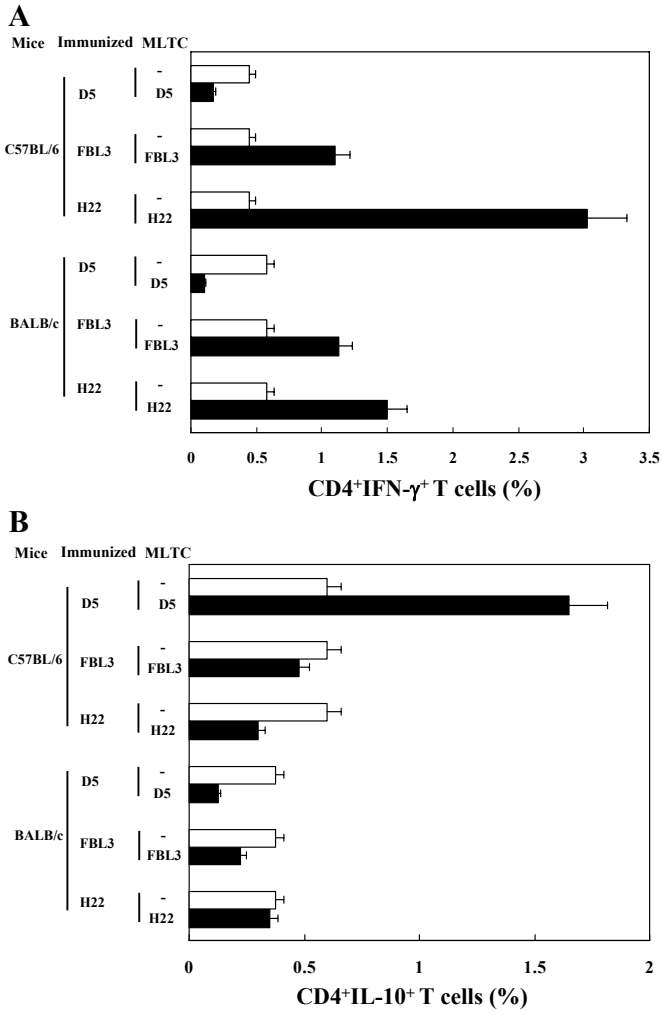

Figure 4. Intracellular cytokine detection by flow cytometry analysis of $CD4^+IFN\text{-}\gamma^+$ T cells (A) and $CD4^+IL\text{-}10^+$ T cells (B) in various MLTC settings. About 2×10^7 primed splenocytes were mixed with 2×10^6 D_5, FBL_3 or H_{22} tumor cells. After 7-day incubation in 5% CO_2 at 37°C, the splenocytes were collected and analyzed for $CD4^+IFN\text{-}\gamma^+$ T cells and $CD4^+IL\text{-}10^+$ T cells as described in "Materials and Methods". Four independent experiments were performed and data were shown as mean ± SD (n = 6).

there was no significant difference in the amount of this cytokine produced in H_{22} and FBL_3 group (Figure 5A). As show in Figure 5B, IFN-γ levels were elevated in supernatants of syngeneic or allogeneic MLTC of FBL_3 and H_{22} compared with that of D_5 ($p < 0.0001$, $p < 0.0001$) (Figure 5B). The IFN-γ levels in supernatants of allogeneic MLTC of FBL_3 and H_{22} were even higher than that of in syngeneic MLTC, although there was no significant difference. The IL-4 and IL-10 levels were elevated in supernatant of syngeneic MLTC of D_5 compared with that of FBL_3 and H_{22} ($p < 0.05$, $p < 0.0001$). Moreover, the IL-4 and IL-10 levels in supernatants of allogeneic MLTC of D_5 were much lower than that in syngeneic MLTC of the same tumor ($p < 0.05$, $p < 0.0001$) (Figures 5C and 5D).

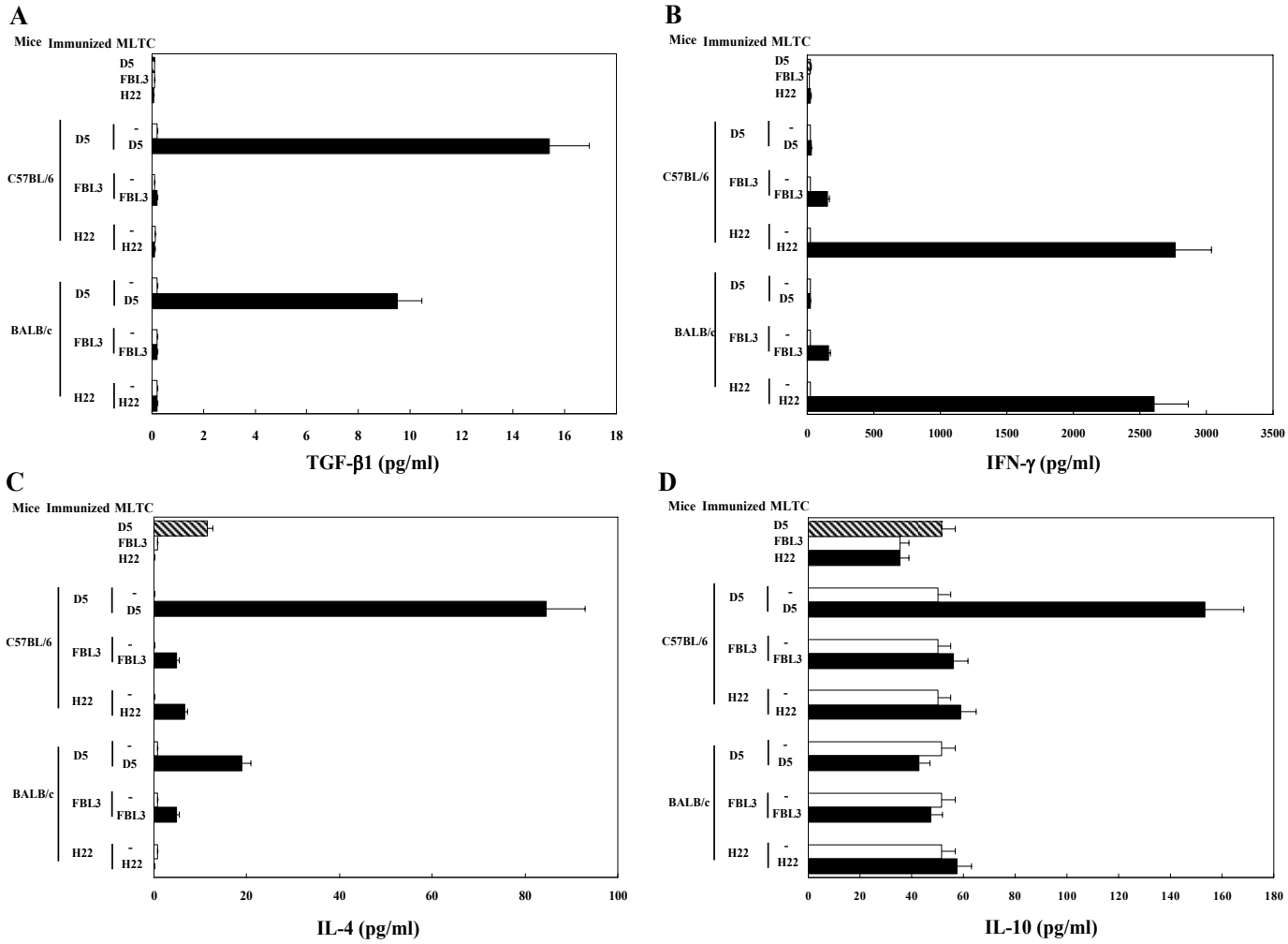

Figure 5. TGF-β1, IFN-γ, IL-4 and IL-10 levels in supernatants from MLTCs of D_5, FBL_3 or H_{22} tumor cells by ELISA. About 2×10^7 primed splenocytes were mixed with 2×10^6 corresponding tumor cells. After 7-day incubation in 5% CO_2 at 37°C, culture supernatants were collected and TGF-β1, IFN-γ, IL-4 and IL-10 were assayed. Data were analyzed from six separate experiments and shown as mean ± SD.

Discussion

In the present study, we generated an *ex vivo* tumor immunity model by using the MLTC method with primed syngeneic splenocytes and the corresponding tumor cells to demonstrate the interaction between tumor cells and tumor-associated lymphocytes. The mimicking transplant model was setting up by MLTC with primed allogeneic splenocytes and the corresponding tumor cells. We first analyzed the proliferation of primed splenocytes and found the proliferation index of syngeneic MLTC model of D_5 was lower than that of FBL_3 and H_{22}. The stimulating index (SI) of latter was 3-fold and 10-fold higher respectively (FBL_3 *vs* D_5 = 12.2:4.94; H_{22} *vs* D_5 = 44.6:4.94) than the former one. The results were in accordance with the wildly accepted fact that the anti-tumor response of poorly immunogenic tumor- afflicted mice was down-regulated and the SI of allogeneic MLTC with D_5 and FBL_3 tumor cell line was higher than that in corresponding syngeneic settings. All these implied the acceptability of our models to certain extent.

The *ex vivo* model of tumor immunology in this experiment mimicked the tumor microenvironment and revealed that splenocytes from MLTC of poorly immunogenic tumor cells (D_5 melanoma cell line) in syngeneic settings were polarized to secrete type 2 cytokines, such as IL-4 and IL-10 and immunoinhibitory factor TGF-β1. Conversely, splenocytes from MLTC of immunogenic tumors (H_{22} and FBL_3 tumor cell lines) induced predominant type 1 immune responses, exhibiting polarized IFN-γ secretion. Intracellular cytokine detection assay displayed that higher $CD4^+IL\text{-}10^+$ T cells were observed in syngeneic poorly immunogenic MLTC setting and higher $CD4^+IFN\text{-}\gamma^+$ T cells in syngeneic immunogenic and allogeneic MLTC settings. It is well known that cancer immunity is mediated mainly by cellular immunity. Tumors that elicit Th1 type response can be eradicated, whereas tumors induce Th2 type response can

continue to grow despite that immune response. And these results indicated that the *ex vivo* models reflect the *in vivo* situation to some extent (6-8).

More significantly, under these conditions, the $CD4^+$ $CD25^+$ T_R cell subsets in the syngeneic poorly immunogenic MLTC of D_5 were ten-fold higher than that in the syngeneic immunogenic MLTC of H_{22}, and the $CD4^+$ $CD25^+$ T cells were also higher than that in allogeneic poorly immunogenic MLTC of D_5. T_R cells are recently identified T cell subsets that exhibit effect on inhibiting autoreactive immune attack for maintaining the homeostasis. It has been recognized recently that besides the expression of CD4 and CD25, they also express CTLA-4, GITR, and Foxp3 etc (16, 17), $Foxp3^+$ are considered the unique T_R cells phenotype. Although in this study we only analyzed $CD4^+CD25^+$ T cells, the splenocytes proliferative states from the MLTC and the cytokine profile indicated that these $CD4^+CD25^+$ T cells were overwhelmingly suppressive T_R cells in nature.

In the study, in addition to stimulatory tumor cells the splenocytes stimulated in the syngeneic MLTC of D_5 were mixture of lymphocytes, dendritic cells and B cells, etc., which involved direct cell-cell contact. Therefore, the $CD4^+$ $CD25^+$ T cells dominance from this culture system seemed to be antigen specific. More interestingly, for further investigation of the effect of supernatants from all types of MLTCs on the generation of T_R cells, we found that the supernatants of syngeneic poorly immunogenic model of D_5 could induce more T_R cells than that of H_{22}. Although the supernatant from allogeneic poorly immunogenic MLTC of D_5 generated higher $CD4^+CD25^+$ T cells too, the magnitude is much lower than that in syngeneic model (0.39 *vs* 0.274, $p < 0.001$). Several studies have verified TGF-β1 could induce the increase of T_R cells (18), though there are still debating on this issue, considering that the TGF-β1 and Th2 cytokine (IL-4 and IL-10) containing supernatants enhance the population of T_R cells in the experimental system, which motivates us to reach the assumption that the antigen specific activation of poorly immunogenic tumor associated lymphocytes is capable of producing the T_R-favoring cytokines (TGF-β1) and creates a special microenvironment, i.e., the initiation of the microenvironment is antigen specific, once it is initiated the generation of T_R are in a non-specific fashion.

Richard showed how T_R cells migrated to tumors under the influence of the chemokine CCL_{22} and provided evidence that T_R cells created a favorable environment for tumor initiation and tumor growth in humans (19). It has been reported that removing such T cells from a cancer-afflicted mouse can cause the rodent to reject a tumor (18). There are similar findings in human tumor, high levels of T_R cells have also been found in several types of human tumors such as lung cancer, pancreatic cancer and breast cancer (20). Curiel's team isolated tumor infiltrating lymphocytes (TILs) from 104 ovarian cancer patients and found that the higher the ratio of T_R cells was the farther the cancer had progressed (21). Also the more tumor associated T_R cells there were, the worse the prognosis was. Because T_R cells play an important role in maintaining immune tolerance and immune equilibration, this function of T_R cell has been considered "policing of the immune system" (22).

There is little information available in literature about the relationship between immunogenicity and T_R cells. This study presented a new approach to analyze the tumor microenvironment and illustrate the discrepancy of immune response stimulated by tumors with different immunogenecity, which might explain why poorly immunogenic tumor can escape immunosurveillance more easily and motivate us to develop new strategies to deplete T_R cells or vaccine to overcome the immune deviation, i.e., they can skew the T cell response from type 2 to a type 1 cytokine profile (23). Since this is a preliminary report, the mechanism of the increase of T_R cells induced by poorly immunogenic tumor remains investigation.

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