Tumor Induces the Expansion of Foxp3+CD25^{high} and CD11b+Gr-1+ Cell Population in the Early Phase of Tumor Progression

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It is well reported that tumor cells can regulate host immune systems. To identify the detailed changes of immune cells between tumor bearing mice and normal mice, we evaluated the systemic immune cell phenotype of B16F10 tumor bearing mice in a time dependent manner. The lymphocytic population (CD4+ and CD8+ T cells) of tumor bearing mice significantly decreased compared to that of normal mice. We found that the Foxp3+CD25+ CD4 T cell decreased, but the Foxp3+CD25^{high} CD4 T cell significantly increased. All subpopulations of CD8 T cells decreased, except the CD62L-CD44+ CD8 T cell subpopulation. The myeloid cell population (CD11b+ and Gr-1+ cells) of tumor bearing mice significantly increased. Specifically, Foxp3+CD25^{high} CD4 T cell and CD11b+Gr-1+ cells significantly increased in early phase of tumor progression. These results are helpful to understand the change of the systemic immune cell subpopulation of tumor bearing mice in a time-dependent manner.

Key Words: Systemic immune cell, Regulatory T cells, Effector T cells, MDSCs

INTRODUCTION

There are many studies showing the association of tumor progression and host immune system (Lambert et al., 2005; Rosenberg, 2001). The host immune system has a dual effect on tumor progression; immune cells such as cytotoxic CD8 T cell (Kilinc et al., 2009; Speiser et al., 2005) and NK cells (Cheng et al., 2013; Gotthardt et al., 2014) are related to tumor growth inhibition, and immune cells such as regulatory T cells (Nishikawa and Sakaguchi, 2010) and MDSCs (Gabrilovich and Nagaraj, 2009) are related to tumor growth promotion. Tumor cells alleviate or evade the host immune

system by inducing immune suppressive environment locally and systemically.

It is well reported that T cells responding to tumor-associated antigens are abundant in tumor tissues. However, tolerance to tumor associated antigen inhibits effective anti-tumor immune response (Zou, 2005). There are many types of tumor infiltrating lymphocyte (TIL) related to tumor inhibition or tumor progression. Specifically, regulatory T cells (Treg) is considered as crucial immune cells to down-regulate the anti-tumor response (Hadaschik et al., 2012; Savage et al., 2013). The population of regulatory T cell is increased in peripheral blood and tumor microenvironment of patients in many cancer types (Mittal 2008; Liyanage 2002). Previous studies showed that characterization of regulatory T cell population is defined as CD25 high and Foxp3 expression on CD4+ T cells (Chatenoud, 2011).

Much of our current information on the development and characterization of CD8+ T cell memory has derived from the research of acute viral infections. Memory CD8 T

*Received: August 7, 2015 / Revised: October 19, 2015

Accepted: October 26, 2015

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cells are divided into two categories; central memory T cells and effector memory T cells (Sallusto et al., 1999). Central memory T cells are antigen-experienced cells that constitutively express CD62L and CD44. By contrast, effector memory T cells are antigen-experienced T cells that have significantly down-regulated CD62L, and express CD44. Naïve CD8 T cell have CD62L expression, and down-regulated CD44 (Foster et al., 2011). Adoptively transferred tumor-reactive CD8⁺ TCM cells are superior mediators of therapeutic antitumor immunity to an established tumor compared with TEM cells (Klebanoff et al., 2005).

Typical features to all MDSCs (myeloid-derived suppressor cells) are their myeloid origin and a remarkable capacity to suppress T cell responses (Kusmartsev et al., 2004). In normal condition, immature myeloid cells immediately differentiated into mature granulocyte, macrophage, or dendritic cells. On the other hand, MDSCs significantly expanded in tumor bearing condition (Li et al., 2004). Recently, MDSCs is used by a therapeutic target in cancer treatment. Several MDSC inhibitors is utilized many murine cancer vaccine studies (Wesolowski et al., 2013). In mice models, MDSCs are broadly characterized as CD11b⁺ Gr-1⁺ cells (Bronte et al., 2000).

However, the systemic change of T cell population and MDSCs between the early phase (day 13) and late phase (day 21) of tumor progression is not well characterized. In this report, we analyzed the change patterns of systemic immune cells containing T cells and MDSC subpopulation between B16F10 tumor bearing mice and normal mice in a time dependent manner.

MATERIALS AND METHODS

Mice

6~8 week old female C57BL/6 mice were purchased from OrientBio (Korea). Mice were housed and maintained under pathogen-free conditions and treated according to approved institutional protocols for animal care.

Cell lines

B16F10 cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were

grown in MEM containing 10% FBS, 10 mM L-glutamine, penicillin/streptomycin, and 0.1% gentamicin.

Tumor studies

B16F10 melanoma cells (3×10^5) in 100 μ l PBS were implanted subcutaneously on the right flank of C57BL/6 mice. Tumors were measured in two dimensions in a blinded fashion using calipers as follows: tumor area (mm^2) = length $\times$ width. All experiments were repeated two or three times.

Evaluation of immune cell phenotype

Mice were sacrificed at the indicated times, and spleens were harvested aseptically and homogenized and pass through a mesh to obtain single-cell suspensions. After the cells were washed twice with PBS, erythrocytes were removed by treating RBC lysis buffer (20 mM Tris-HCl, 140 mM ammonium chloride) at 37°C for 5 min and cells were labeled with the following mAbs: APC-conjugated anti-CD4, F4/80, and PE-conjugated CD8, CD25, CD44, Gr-1, and FITC-conjugated CD11b, CD62L, FoxP3. All antibodies were purchased from eBiosciences. For FoxP3 staining, splenocytes were treated by using Foxp3 staining Buffer kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. Samples were acquired using a FACScaliburTM flow cytometer and CELLquestTM software.

Statistical analysis

The data are described as the means $\pm$ SEM. The values were calculated by the Student's *t*-test. Each assay was performed by three independent experiments. *P* value ($P < 0.05$) was defined as statistically significant.

RESULTS

Systemic immune cell phenotype between tumor bearing mice and normal mice on day 13 and day 21

To identify the tumor area on day 31 and day 21, we inoculated B16F10 mouse melanoma cells on the right flank of C57BL/6 mice. Tumors were measured with caliper at the indicated times. A palpable tumor was seen on days 5~7 after the tumor cell inoculation. The average tumor areas on day 13 and day 21 were $90 \pm 20 \text{ mm}^2$ and $318 \pm 54 \text{ mm}^2$,

respectively. In this study, we define "day 13" as the early phase of tumor progression, and "day 21" as the late phase of tumor progression to figure the immunological difference between tumor bearing mice and normal mice.

To identify the systemic immune effect of tumor progression, we analyzed the lymphocytic (CD4, CD8) and myeloid cell (CD11b, Gr-1, F4/80) populations from the spleens of tumor bearing mice. We prepared splenocytes from B16F10 tumor bearing mice and normal C57/BL6 mice on 13 and 21 days after the tumor cell inoculation and performed FACS analysis. The percentage of CD4+ and CD8+ T cell population of tumor bearing mice decreased 14% and 11% on day 13, and 39% and 39% on day 21 compare to that of normal mice, respectively. The percentage of CD11b+ and Gr-1+ cell populations in tumor bearing mice increased 18% and 34% on day 13, and 47% and 25% on day 21 compared to that of normal mice, respectively (Fig. 1). The percentage of F4/80+ cell populations in tumor bearing mice was no different that of normal mice. These data shows that the lymphocyte (CD4+, CD8+) population is significantly decreased while the myeloid cell (CD11b+, Gr-1+) population is significantly increased in a time-dependent manner.

Systemic regulatory T cell population between tumor bearing mice and normal mice on day 13 and day 21

Since tumor bearing mice have a decreased percentage of lymphocytic cell population, we wanted to identify the subpopulation of regulatory T cells related to tumor progression. We collected splenocytes from tumor bearing mice and normal mice. On 13 and 21 days after the tumor inoculation, the overall population of CD4+ T cells significantly increased compared to that of normal mice. But, in the CD4+ T cell population, the percentage of Foxp3+CD25^{low} CD4 T cells decreased, while the percentage of Foxp3+CD25^{high} CD4 T cells increased (Fig. 2A). On 13 and 21 days after the tumor inoculation, the percentage of Foxp3+CD25+ CD4 T cells of tumor bearing mice decreased 12% and 52% compared to that of normal mice, respectively. On 21 days after the tumor inoculation, the percentage of Foxp3+CD25- CD4 T cells decreased 41%. On 13 and 21 days after the tumor inoculation, the percentage of Foxp3+

CD25^{high} CD4 T cells of tumor bearing mice increased 100% and 65% compared to that of normal mice, respectively, although the percentage of total CD4+ and CD4+Foxp3+CD25+ T cells decreased (Fig. 2B). These data shows that Foxp3+CD25^{high} CD4+ T cells may be related to tumor progression in early stage.

Systemic CD8 T cell subpopulation between tumor bearing mice and normal mice

Since tumor bearing mice had a decrease in the percentage of CD8 T cell population, we wanted to identify which subpopulation of CD8 T cells is related to tumor progression. On 13 days after the tumor cell inoculation, the total percentage of CD8 T cells decreased, but the overall distribution of CD8 T cell subpopulation (CD62L and CD44 expression pattern) was not different between tumor bearing mice and normal mice. On day 21, in the CD8+ T cells, the percentage of CD62L-CD44+ (effector memory) CD8 T cell subpopulation of tumor bearing mice increased, while the percentage of CD62L+CD44- (naïve) CD8 T cell subpopulation of tumor bearing mice decreased compared to that of normal mice (Fig. 3A). On 13 days after the tumor inoculation, the percentage of CD62L-CD44+ (effector memory), CD62L+CD44+ (activated or central memory), and CD62L+CD44- (naïve) CD8 T cells of tumor bearing mice were decreased 12%, 24%, and 8% compared to that of normal mice, respectively. On 21 days after the tumor inoculation, the percentage of CD62L+CD44+ (activated or central memory), and CD62L+CD44- (naïve) CD8 T cells of tumor bearing mice decreased 31%, and 35% compared to that of normal mice, respectively. But, the percentage of CD62L-CD44+ (effector memory) CD8 T cells increased 19% in the same group (Fig. 3B). These data shows that tumor induce the decline of all CD8+ T cell subpopulation.

Systemic CD11bGr-1 myeloid cell population between tumor bearing mice and normal mice

Since tumor bearing mice change the percentage of myeloid cell population, we wanted to identify the subpopulation of CD11bGr-1 cells related to tumor progression. On 13 and 21 days after the tumor inoculation, the percentage of CD11b+Gr-1+ cells of tumor bearing mice increased 43%

Fig. 1. Immune cell phenotype between tumor bearing mice and normal mice. B16F10 melanoma cells were implanted subcutaneously on the right flank of C57BL/6 mice. The splenocytes were collected on 13 and 21 days after tumor inoculation, and the percentage of individual immune cell population (CD4, CD8, CD11b, Gr-1, F4/80 positive population) was analyzed by FACS analysis. Tumor bearing mice versus normal mice; *: $P < 0.05$, **: $P < 0.01$.

and 100% compared to that of normal mice, respectively. In the case of CD11b+Gr-1- cells, the percentage of the subpopulation was not changed on day 13, but increased 30% on day 21. The percentage of CD11b+Gr-1^{high} and CD11b+Gr-1^{low} cells of tumor bearing mice increased 130% and 91% on day 13, and 93% and 94.9% on day 21 compared to that of normal mice, respectively (Fig. 4A and 4B).

These data shows that all CD11b+Gr-1+ subpopulation may be related to tumor progression in early stage.

DISCUSSION

In this report, we demonstrated the change of the systemic immune cell phenotypes in tumor bearing mice and deter-

Fig. 2. The change of Foxp3 and CD25 expression on CD4 T cell of tumor bearing mice. Mice were treated as described Fig. 1. (A) The representative dot plot of CD4+ cell population, and Foxp3 and CD25 expression of CD4+ subgated cell population. (B) The splenocytes were collected on 13 and 21 days after tumor cell inoculation, and the percentage of individual immune cells was analyzed by FACS analysis. Tumor bearing mice versus normal mice; *: $P < 0.05$.

Fig. 3. The change of CD62L and CD44 expression on CD8 T cell of tumor bearing mice. (A) The representative dot plot of CD8+ cell population, and CD62L and CD44 expression of CD8+ subpopulated cell population. (B) The splenocytes were collected on 13 and 21 days after tumor cell inoculation, and the percentage of individual immune cells was analyzed by FACS analysis. Tumor bearing mice versus normal mice; *, $P < 0.05$, **, $P < 0.01$.

mined the subpopulation of immune cells related to tumor progression in a time-dependent manner. In our study, the lymphocytic cell (CD4+ and CD8+ T cell) population of tumor bearing mice decreased about 12% on day 13, and

decreased about 40% on day 21. The pattern of decline in both CD4+ and CD8+ T cells were similar on day 13 and on day 21. But, there was no remarkable decline of CD4 and CD8 T cells on day 13, the relatively early phase of

Fig. 4. The change of Gr-1 expression on CD11b+Gr-1⁺ cell of tumor bearing mice. (A) The representative dot plot of CD11b+Gr-1^{high} and CD11b+Gr-1^{low} cell population. (B) The splenocytes were collected on 13 and 21 days after tumor cell inoculation, and the percentage of individual immune cells was analyzed by FACS analysis. Tumor bearing mice versus normal mice; *: $P < 0.05$.

tumor progression. The myeloid cell population showed various pattern of change. The percentage of CD11b+ cells of tumor bearing mice increased about 18% on day 13, and increased about 47% on day 21. The percentage of Gr-1+ cells of tumor bearing mice were markedly increased by about 34% on day 13, and the population was sustained on day 21. The percentage of F4/80+ cells of tumor bearing mice showed no difference on day 13 and a little increase (about 8%) on day 21 compared to that of normal mice (Fig. 1). The decrease of lymphocytic cell population and the increase of myeloid cell population on day 13 mean that tumor may affect the immune cell proliferation before day 13 of tumor progression. These data suggest that the tumor's microenvironment induces the inhibition of lymphocytic proliferation and the expansion of myeloid cell proliferation.

We speculate that Gr-1 + cells play an important role in tumor progression and the change of immune cells on early phase of tumor environment. In particular, the subpopulation of CD11b+Gr-1+ cells has been determined more detail based on Gr-1 expression level. The percentage of CD11b+Gr-1+ cells remarkably increased on day 13, and further increased on day 21. In our data, CD11b+Gr-1+ cells were subcategorized as CD11b+Gr-1^{high} and CD11b+Gr-1^{low}. The percentage of both subpopulations in tumor bearing mice showed a significant increase compared to that of normal mice on day 13. It has been reported that different subsets of MDSCs might use different mechanisms to suppress T cell proliferation (Movahedi et al., 2008). Two main subsets of MDSCs have been identified: a granulocytic subset and a monocytic subset (Youn et al., 2008). These data show that all CD11b+Gr-1+ subpopulation are related to the early phase of tumor progression.

We checked the systemic change of Foxp3+CD25+ CD4 T cells of tumor bearing mice on day 13 and day 21. The percentage of Foxp3+CD25+ CD4 T cells of tumor bearing mice decreased about 12% and further decreased about 52% on day 21, contrary to our expectations. Our FACS data show that Foxp3+CD25+ CD4 T cells were divided into two subpopulation; Foxp3+CD25^{high} and Foxp3+CD25^{low}. The decline in the total percentage of Foxp3+CD25+ CD4 T cells comes from the Foxp3+CD25^{low} subpopulation. Only the percentage of Foxp3+CD25^{high} CD4 T cell subpopulation

of tumor bearing mice was significantly increased on day 13 and sustained through day 21 (Fig. 2A and 2B). These data shows that the increase of systemic CD4+ T cell with Foxp3+CD25^{high} expression in the mouse tumor model correlate with early tumor development.

The representative FACS data of percentage CD8+ T cells show that the total CD8+ T cell population decreased significantly on day 21. But, the CD8+ T cell subpopulation with CD62L and CD44 expression shows a different pattern. The percentage of CD62L-CD44+ (effector memory) T cell subpopulation increased, while the percentage of CD62L+CD44- (naïve) T cell subpopulation decreased in the CD8+ T cell population (Fig. 3A). The percentage of all CD8+ T cell subpopulations decreased on day 13. But, on day 21, the percentage of CD62L+CD44+ (central memory) and CD62L+CD44- (naïve) T cells remarkably decreased, but interestingly, the percentage of CD62L-CD44+ (effector memory) T cells increased by about 19%. It has been reported that chronic antigen expression can induce relatively naïve CD8+ T cells to strongly proliferate and differentiate into effector memory T cells, and ultimately exhaust the T cell population (den Boer et al., 2004; Lee et al., 1999). The increase of CD62L-CD44+ T cells on day 21 was considered to be the expansion of exhausted T cells. CD8+ T cell memory formation and function can either be altered or impaired in the case of persistent antigen stimulation, such as in the presence of a tumor. The exact role of effector memory T cells and central memory T cells in the case of tumor bearing mice is still controversial. In our study, the CD62L-CD44- CD8 T cell subpopulation decreased 29% on day 13, but increased 10% on day 21, like CD62L-CD44+ CD8+ T cell subpopulation. But, this cell subpopulation was not mentioned earlier, so future studies are needed. In this study, our data shows that Foxp3+CD25^{high} CD4+ T cells and CD11b+Gr-1+ cells may be major suppressive immune cells involved in the early phase of tumor progression.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), which is funded by the Ministry of Education, Science and Technology (2010005446), and by

Conflict of interest

The authors declare that they have no conflict of interests.

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