B16 melanoma expressing EGFP as a self antigen is differentially immunoedited by tolerogenic thymic epithelial and dendritic cells

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ABSTRACT

To investigate how the immune system responds to tumor self antigens, we used enhanced green fluorescent protein (EGFP) in B16 melanoma cells (B16-EGFP) and tested in the mouse lines expressing EGFP in thymic epithelial cells (3.1T-EGFP) or in antigen presenting cells (Get40), in comparison to the wild-type mouse. B16-EGFP cells were distinctively immunoedited in three mouse lines at the early phase, and the cells were completely eliminated only in the wild-type at the late phase, suggesting EGFP-specific tolerance is present in 3.1T-EGFP and Get40. The numbers of tumor-infiltrating T cells in all mouse lines were reversely correlated with the tumor sizes, suggesting dominant T cell mediated tumor elimination. When a soluble EGFP was immunized, surprisingly, the growth of B16-EGFP in Get40 mouse was promoted, while reduced in B6. Immunization did not make significant difference in the growth of tumors in 3.1T-EGFP. Detailed analyses showed the opposite directional changes in the numbers of B and CD8+T cells in B6 and Get40. In Get40 mice, the immunization significantly reduced the percentage of Gr1−CD11b+ cells, indicating that tolerance induction and breaking involve both adaptive and innate cells differentially. Therefore, the strategy for a cancer vaccine should be carefully considered on the types of antigen expressing cell.

Introduction

Antigens in tumor tissues are self antigens unless they are expressed by virus infection. Therefore, host’s adaptive antigen specific B and T cells do not usually respond to the specific self antigens in the growing tumors unless tolerance is broken (Schreurs et al. 2000; Bos et al. 2012).

In general, antigen specific immunological tolerance is maintained by two classifiable categories. The first mechanism is called central tolerance which is exerted by negatively selecting self-reactive T and B cells during the development of the thymus and bone marrow, respectively (Goodnow et al. 1989; Klein et al. 2009). The developing antigen specific T and B cells are deleted from the pool when they interact with antigens presented from the stromal antigen presenting cells. It is well known that dendritic cells, and cortical and medullary thymic epithelial cells (cTECs and mTECs, respectively) are the major critical cells for thymic negative selection (Ahn et al. 2008; Klein et al. 2009). Second, peripheral tolerance is induced in the tissues through mechanisms of anergy, ignorance, or the suppression by regulatory cells (Hori 2003; Kurtz 2004; Parish and Heath 2008).

Besides antigen specific responses, immune responses to tumors are also managed by the innate immune system including NK cells, macrophages and more (Taffet and Russell 1981; Mishra et al. 2010). Antigen presenting cells like dendritic cells, which are an important part of innate immunity, also participate in the adaptive immune system by presenting specific antigens to T cells for specific responses (Gajewski et al. 2013).

The process that the complex immune system controls and/or promotes tumor growth is viewed as cancer immunoediting (Schreiber et al. 2011). This dynamic process consists of three phases: elimination, equilibrium, and escape. Among these phases, the elimination phase is representative of the tumor immunosurveillance phase where the innate and adaptive immune cells attack tumor cells. After the elimination phase, tumor cells have balance with the host immune system, and enter equilibrium phase that the host’s adaptive immune cells prevent tumor cells from outgrowing and keep them dormant. In the escape phase, surviving tumor cells carry different mutations and get the ability to evade immunity and begin to not only expand rapidly but also induce
metastasis. It is well known that tumor cells are heterogeneous and change phenotypically and functionally resulting from the unstable cancer cell nature either from genetic variation (Burrell et al. 2013) or from the effect of immunoediting (Junttila and de Sauvage 2013). Currently it is not easy to chase individual tumor cell variation that results from antigen specific immune responses.

In this study, we report the differential maintenance and breaking of immune tolerances to tumor specific antigen in the mouse model systems with thymic epithelial cells and antigen presenting cells expressing GFP as self antigen.

Materials and methods

Mice

3.1T-EGFP and Get40 mouse lines were described previously (Reinhardt et al. 2006; Park et al. 2013) and maintained in the Laboratory of Molecular and Cellular Immunology Animal Facility at Inha University. All animal studies are in compliance with the Use of Laboratory Animals under proper LMCI protocols. C57BL/6 mice were purchased from Daehan Biolink (DBL; www.dbl.co.kr).

Cells

EGFP expressing B16 cell line, B16-EGFP, was generated by transfecting the construct (pCAGGS-EGFP) that was obtained from the laboratory of Dr. Sungho Jeon, Hallym University. Transfection was performed with Lipofectamine™ 2000 (Invitrogen, Cat #. 11668–019) as described in manufacture’s protocol. Transfected cells were selected with 0.6 mg/ml of G418 (Duchefa Biochemie, Cat #. G0175.0005). Cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; Welgene, Cat #. LM001–01), 0.2 mg/ml Collagenase/Dispase, and 0.8 mg/ml DNase and incubated at 37°C for 1 h. The single cell suspension was filtered with 40 μm nylon mesh after adding FBS into 10% and analysed by flow cytometry.

Flow cytometric analysis

Single cell suspensions from the tumor or spleen (1 × 10^5 to 1 × 10^6 cells) were placed in round bottom 96-well plate (SPL, Cat #. 34096) with FACS buffer (0.1 mg/ml bovine serum albumin in PBS). For the blocking of Fc receptors, we used ultra blocking buffer (monoclonal antibody 2.4G2 and 10% of each normal mouse, hamster, and rat serum). Fixable Viability dye eFluor 455UV (Invitrogen, Cat #. 65–5068) was treated for 30 min at 4°C in dark in order to separate live cells prior to staining with antibodies. All samples were filtered through 40 μm nylon mesh before reading by BD FACS:Aria II and analyzed with the Flowjo 10.0.7 program.

Enzyme-linked immunosorbent assay (ELISA)

For ELISA, 500 ng of recombinant EGFP (rEGFP; Clontech, Cat #. 8365–1) was coated on the wells of 96-well plates in coating buffer (32 mM Na2CO3, 68 mM NaHCO3, 0.1% NaN3, pH9.6) (SPL, Cat #. 38196). After washing three times with 0.1 M Tris buffered saline (pH7.4) containing 0.1% Tween 20, the sera from mice were added as primary antibodies. After washing three times, anti-mouse horseradish peroxidase conjugated antibody was added. After four times

In vivo tumor formation

Mice of eight to sixteen-week-old age were subcutaneously injected with 2 × 10^5 cells of B16-EGFP. Body weight and tumor size were monitored every 2–3 days. The tumor size was measured with a caliper and calculated by an equation (Tomayko and Reynolds 1989):

\[ \text{Tumor size} = \pi/6 \times \text{Length} \times \text{Width} \times \text{Height} \]

For immunization with EGFP, mice were subcutaneously injected with 20 μg of his-tagging EGFP and adjuvant MF59 four times with intervals of two weeks. The sera collected before immunization and 3 days after 4th immunization were analyzed by enzyme linked immunosorbent assay (ELISA).
of wash, Super AquaBlue ELISA substrate (eBioscience, Cat #: 00–4203–58) was added. For isotyping antibodies, the HRP conjugated IgM (SouthernBiotech, Cat #: 1020–05), IgG1 (SouthernBiotech, Cat #: 1070–05), IgG2a (SouthernBiotech, Cat #: 1080–05), IgG2b (SouthernBiotech, Cat #: 1090–05), IgG2c (SouthernBiotech, Cat #: 1079–05), and IgG3 (SouthernBiotech, Cat #: 1100–05) were used as secondary antibody. Optical density (OD) was read at 405 nm using ELISA Reader (TECAN, Sunrise™).

Results

**mTEC and APC based EGFP tolerant mouse lines**

In order to study the status of immunological or tolerant response to a tumor self antigen, we selected three mouse lines derived from C57BL/6 with syngeneic tumor B16 cells. As shown in Figure 1(A), 3.1T-EGFP mouse line is transgenic with EGFP expressed in tolerogenic medullary thymic epithelial cells (mTECs) by 3.1 kb TSCOT promoter (Lee et al. 2012; Park et al. 2013). Get40 mouse expressing EGFP in the IL12p40-expressing cells (Reinhardt et al. 2006). EGFP expression was verified in this mouse model by flow cytometry 16 h after LPS (Figure 1(B)). The subpopulation of MHCII⁺ cells in CD11c⁺CD8α⁺ gated showed EGFP expression (7.58%).

EGFP-expressing B16-F10 melanoma cells were heterogeneous even after selection with G418 by limiting dilution format. Therefore, we sorted B16-EGFP to more than 90% by flow cytometry and used as a stock in this experiment (Figure 1(C,D)).

**Three mouse models showed distinct B16-EGFP growth profiles in early immunoediting stage**

To examine the patterns of immune responses against a self antigen, EGFP, we injected subcutaneously 2 x 10⁵ cells of B16-EGFP into B6, 3.1T-EGFP, and Get40, and monitored their tumor size every 2–3 days (Figure 2(A,B)). It is apparent that tumor in the early immunoediting phase (at 10–19 day) grew differently. Tumors in B6 grew slowest without any clear peak of equilibrium phase. In contrast, the early peak of tumor sizes was clearly distinguishable in other two genetically modified hosts with EGFP expression, later in 3.1T-EGFP (at day19) and earlier in Get40 (at day13–15). These results suggested that, during the early immunoediting phase, tumors were efficiently eliminated in B6, but not in 3.1T-EGFP and Get40. It was unexpected, however, that Get40 hosts showed early phase tolerance to the EGFP tumor antigen even though they express EGFP in APCs. All the host groups eventually showed similar tumor overgrowth in late escape phases (at 19–24 day).

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**Figure 1.** Mouse and cell line model used in this study. (A) Three mouse models used in this study. (B) Flow cytometric plots show I-Aᵇ and EGFP expression of CD11c⁺CD8α⁺ cells from spleen of Get40 mouse. (C) B16-EGFP melanoma tumor cell line before and after EGFP high expressing sorting by Flow cytometry. (D) Images of sorted B16-EGFP cells under bright, green, and red fields. Pseudocolored in Adobe Photoshop CS6.
Figure 2. Immunological response to EGFP⁺ tumor in 3 mouse models. (A) Tumor growth in B6, 3.1T-EGFP, and Get40 mice inoculated with B16-EGFP melanoma. (B) Tumor size at 10, 13, 15, 17, 19, and 21 days after injection in three mouse models. (C) Proportion of GFP⁺ to CD45⁻ in tumor. *p = 0.0390; ***p = 0.0001; unpaired t-test. (D) Tumor size normalized with proportion of GFP⁺ in tumor. Filled bar indicates GFP⁺ population of tumor and blank bar represents the rest of tumor. And dotted line and solid line indicate the mean of GFP⁺ population and the rest of tumor respectively. (E) Representative images of the expression of GFP and G8.8 (EpCAM) on CD45⁻ cells from B16-EGFP cell line and tumors of 3 mouse models. The polygon represents GFP⁺ population in tumors. (F) Correlation between tumor weight and proportion of B220⁺ to CD45⁺ in tumors. (G) Correlation proportion of Thy1.2⁺ to CD45⁺ in tumors with tumor weight. The R² and P value are indicated on each graph.
**EGFP⁺ tumor cells in heterogeneous population were specifically eliminated in B6 host, but not in the host expressing self antigen EGFP**

We analysed the tumors in the late escape phases by flow cytometry for the destiny of EGFP expressing tumor cells. EGFP⁺ population in heterogeneous CD45⁻ tumor cells in B6 mouse line was completely eliminated while there were still significant EGFP expressing tumor cells remaining in 3.1T-EGFP and Get40 hosts (Figure 2(C–E)). In order to show better resolution of EGFP population, we chose to show panepithelial marker, EpCAM as well as EGFP (Figure 2(E)). These results suggest the role of EGFP specific tolerance mechanisms in these hosts. The most survived EGFP⁺ tumor cells were in Get40 hosts (Figure 2(C,D)). This is consistent to the fact that Get40 showed faster self antigen expressing tumor growth in the early phase, suggesting tolerance to EGFP in 3.1T-EGFP and Get40 may be mediated via different pathways. Representative EGFP plots of tumors from the hosts of each group to the original cell line is shown in Figure 2(E). In the expression of EGFP and EpCAM, B16-EGFP cell line showed 49.6% of EGFP⁺ population. The EGFP expressing cells recovered from tumors are far less than the original cells, indicating there are overall selective pressures against the self antigen expressing tumors.

**Adaptive immune responses are involved**

To examine what immune cells are involved in the responses, we analysed the numbers of various immune cells in the dissociated tumor cell suspension. The percentage of tumor infiltrating B cells was in good correlation with tumor weight (Figure 2(F)) while that of Thy1.2⁺ tumor infiltrating T cell was in reverse correlation with tumor weight regardless of mouse strains (Figure 2(G)). This result suggested that T cells in tumors may play major roles in inhibition of tumor growth. Other immune cells in the tumors were not significantly relevant to the tumor sizes (data not shown).

**Breaking the tolerance by immunization in tolerant hosts**

In order to examine responses in the tolerogenic hosts, we immunized soluble EGFP and monitored the injected B16-EGFP tumor growth in three mouse hosts (Figure 3(A)). The patterns of either inhibition or enhancing of tumor growth is not simple as shown in Figure 3(B,C). In addition, in contrast to Figure 2(A), tumor growth patterns did not show the same kinetics with the early peak, possibly we missed the early phase in our monitoring. B6 host, immunization might have increased the ability of tumor immunity in the early phase (dotted line vs solid line in Figures 2, and day 8 and 12 in 3(B)), although it is not statistically significant probably due to the lack of sufficient data points. In 3.1T-EGFP initially tolerant hosts, immunization induced tumor responses, toward the reducing tumor size in the early phase (day 8). In Get40 hosts, immunization accelerated tumor growth, suggesting that interesting unknown mechanisms participate in the immunoediting process.

Next, specific antibody isotypes were examined for the EGFP response at the B cell and T cell help levels. B6 host generated high titer of Th2 type IgG1 antibodies, while initially tolerant mouse hosts also raised specific antibodies, suggesting tolerance to EGFP can be broken by immunization (Figure 3(D)). It appears that the presence of high titer anti-EGFP antibodies did not contribute to the reduction in tumors in B6 animals.

To investigate what types of immune responses were involved, we analysed the proportion of each immune cells at the time of sacrifice (late immunoeediting stage). In the spleen, the proportion of B cells was reduced in B6 but increased in Get40 mice by immunization (Figure 4(A,C)). In contrast, proportion of T cells in the spleen was increased in B6 but reduced in Get40 (Figure 4(B,C)). Among T cell subpopulations, the proportion of CD8⁺T cells shows the same profile as total T cells (Figure 4(D,E)). The profiles of post immunization in B6 were still consistent to the profiles of prior immunization shown in Figure 2. On the contrary, the immunization did not significantly change the proportion of B and T cells in the spleen. These results suggest that there are distinct mechanisms present in the maintenance and breaking of tolerance among three groups.

Tumor infiltrating CD45⁺ cells were also investigated in the late phase (Figure 5(A–E)). Total numbers of CD45⁺ cells in the tumor tissues were overall similar among the different hosts except in the case of unimmunized Get40 hosts (statistically significant, Figure 5(A)). Tumor infiltrating immune cells in the tolerant Get40 unimmunized host uniquely show the elevated level. However, immunized Get40 host showed similar levels to the other types of hosts. Among these CD45⁺ cells, Gr-1⁻CD11b⁻ cells (neutrophil) showed overall similar pattern of total CD45⁺ cells (Figure 5(B)). However, Gr-1⁻CD11b⁺ cells (macrophages) are also similar except for the cases of Get40 (Figure 5(C)). In Get40 animals, relative macrophage proportion is higher than the other animals, but reduced upon EGFP immunization. It is interesting that the proportion of Gr-1⁻CD11b⁺ cells are in good correlation with tumor sizes regardless of host types and immunization status (Figure 5(D)). Representative flow cytometric profiles of myeloid cell analysis are shown in Figure 5(E).
Figure 3. B16-EGFP tumor growth after immunization of his-tagging EGFP in 3 mouse models. (A) The experimental scheme of immunization of his-tagging EGFP (hisEGFP) in 3 mouse models. 3 mouse models were subcutaneously immunized with 20 μg of hisEGFP and adjuvant MF59 four times at intervals of two weeks. 3 days after 4th hisEGFP injection, these mice were inoculated with $2 \times 10^5$ cells of B16-EGFP. (B) Tumor growth of B16-EGFP after four times of hisEGFP immunization. Solid lines indicate hisEGFP-immunized mice and dotted lines indicate non-immunized mice. (C) Tumor growth at 8, 12, 15, and 17 days after injection in each mouse models. (D) Analysis of EGFP specific T\textsubscript{H} type. The dilution rate of the sera was 1:30. Filled and blank dots each indicate immunized mice and non-immunized mice.
Discussion

We studied the actions of tumor immunoediting using a trackable tumor self antigen and mouse host models with the self antigen expressed in mTEC and APC. We show that complex immunoediting pathways are present at the levels of adaptive and innate immune systems in order to control tumor growth.

Tumor immunoediting kinetics in the hosts with different self antigen distribution

The kinetics of tumor growth can be viewed as results of immunoediting (Schreiber et al. 2011). To visualize the portion of immune elimination in the early stage of tumor growth, the only way is to compare with the system without elimination. 3.1T-EGFP mouse system is ideal in this situation since EGFP is expressed in mTEC which is the critical cell for the deletion of antigen specific T cells (Hinterberger et al. 2010; Park et al. 2013). Get40 mouse line was included with our original assumption that it may show enhanced immunity, therefore slower tumor growth since EGFP antigen is expressed in APCs. To our surprise, in addition to our expectation with 3.1T-EGFP that showed faster tumor growth than B6, Get40 mouse showed faster kinetics in B16-EGFP tumor growth in equilibrium, and eventually a similar escape phase (Figure 2(A,B)). The remaining portion of EGFP+ B16-EGFP at the immune escape stage was even higher in Get40 than tolerant 3.1T-EGFP. These results lead us to conclude that Get40 is able to tolerize EGFP specific cells.

Figure 4. B and T cell responses in hisEGFP-immunized 3 mouse models. Proportion of B220+ cells (A), Thy1.2+ cells (B), CD8+T cells (D) to CD45+ cells from spleen. Filled dots indicate immunized mice and blank dots indicate non-immunized mice. All samples were gated with CD45+DAPI−. *p = 0.0319; *p = 0.0410; **p = 0.0054; unpaired t-test. (C) and (E) Shown are representative plots with B220, Thy1.2, CD4, and CD8 expressions.

The proportion of EGFP+ cells in the recovered tumor is far less than original B16-EGFP (Figure 2(C–E)). B16-EGFP cells although they drifted in the percentage of expressing cells from over 90% during culture (Figure 1(C)), showed nearly 50% EGFP+ of whole cells. The isolated tumor cells from immunogenic B6 host which were in various sizes, did not have any remaining EGFP+ cells. In the analyses of the numbers of tumor infiltrating B and T cells, results showed statistically significant correlations to the fact that they can play roles in maintaining the final tumor sizes (Figure 2(F,G)).

Immunity or tolerance governed by central and peripheral mechanism

To understand the tolerance to the EGFP tumor self antigen in Get40, we tried to test thymic EGFP expression in Get40, but failed to identify specific EGFP expressing cells in the thymic stromal cell preparation (data not shown). Only false positive stroma cells were present since the same proportion was found in normal B6 thymus. At this point, it is not clear how the antigen expressed in APC induces antigen specific tolerance rather than immunity. We can easily assume that EGFP expressed in APC in the periphery can induce tolerance to tumor antigen through non-central deletional mechanism. More detailed analysis with conditional APC depletion will help to make a better conclusion in mechanistic aspects.
Breaking tolerance in the hosts with different self antigen distribution

The immune response to a self antigen is generally not present in healthy conditions mostly because self-reactive B and T cells were eliminated during their development. Even if they escaped from the negative selection, they are quiescent or suppressed unless activation signals are given. Antigen specific immune responses can be provoked by providing an alarm signal. To achieve this goal, we immunized EGFP in the presence of an adjuvant and monitored the pattern of tumor growth in our three different hosts. We found that immunization can enhance tumor response in immunogenic B6 host, but two different behaviors (enhancing and eliminating response) are present in 3.1T-EGFP while enhancing tumor escaping process in Get40 (Figure 3(B,C)). In these responses, directionality of B and T (CD8) cells was retained in all three mice. The more B cells and the less T cells at the final immune escape stages are associated with the bigger and faster tumor growth. Besides adaptive B and T cells, myeloid lineage cells, especially CD11b+ macrophages also appeared to play roles in the...

Figure 5. Macrophage responses in hisEGFP-immunized 3 mouse models. (A) Proportion of CD45+ cells. *p = 0.0489; unpaired t-test. (B) and (C) Proportion of ratio of tumor infiltrating cells (RIC; Gr-1+CD11b+, Gr-1−CD11b+). (D) Correlation of ratio of tumor infiltrating Gr-1−CD11b+ cells with tumor weight. The R² and P value are indicated on each graphs. (E) Representative plots of Gr-1 and CD11b expression on CD45+DAPI− cells are shown.
tumor maintenance and breaking of tolerance to the tumor self antigen (Figure 5(A–E)). The implication of this result is very important for the application of cancer immunotherapy against specific target molecules. Therefore, in conclusion, it is important to include information on the expression of specific antigen, not only in the tumor but also in tolerizing thymic epithelium and APCs, in order to expect successful immunotherapy.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work is supported by the Inha University Research Grant award to MGK. SGK is supported by the graduate student scholarship from the Inha University during this work.

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