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COMMUNICATION

The tumor immunosuppressive microenvironment impairs the therapy of anti-HER2/neu antibody

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ABSTRACT

It has been well established that immune surveillance plays critical roles in preventing the occurrence and progression of tumor. More and more evidence in recent years showed the host anti-tumor immune responses also play important roles in the chemotherapy and radiotherapy of cancers. Our previous study found that tumor- targeting therapy of anti-HER2/neu mAb is mediated by $CD8^+$ T cell responses. However, we found here that enhancement of $CD8^+$ T cell responses by combination therapy with IL-15R/IL-15 fusion protein or anti-CD40, which are strong stimultors for T cell responses, failed to promote the tumor therapeutic effects of anti-HER2/neu mAb. Analysis of tumor microenviornment showed that tumor tissues were heavily infiltrated with the immunosuppressive macrophages and most tumor infiltrating T cells, especially $CD8^+$ T cells, expressed high level of inhibitory co-signaling receptor PD-1. These data suggest that tumor microenvironment is dominated by the immunosuppressive strategies, which thwart anti-tumor immune responses. Therefore, the successful tumor therapy should be the removal of inhibitory signals in the tumor microenvironment in combination with other therapeutic strategies.

KEYWORDS anti-HER2/neu antibody, $CD8^+$ T cells, tumor microenvironment, tumor therapy, immune suppression

INTRODUCTION

Understanding how the immune system affects cancer development and progression has been one of the most challenging questions in tumor immunology. Over the past two decades, the immune surveillance concept has been well established and accepted (Schreiber et al., 2011). Various trials aimed to improve host immune response have been applied in clinic for cancer treatments (Lipson and Drake, 2011; Tartour et al., 2011).

Conventional cancer treatments such as chemotherapy and localized radiotherapy (RT) are supposed to mediate their effects via direct elimination of tumor cells. However, it has been recently shown that these therapies are also dependent on innate and adaptive immune responses (Sistigu et al., 2011; Zitvogel et al., 2011). During chemotherapy or RT, dying tumor cells release danger signal molecules such as HMGB1 and calreticulin. These danger signals license DCs for antigen uptake and TLR4-depedent antigen processing, which is required for the priming of antigen-specific $CD8^+$ T cells (Apetoh et al., 2007).

Although anti-tumor immune responses play critical roles in preventing the occurrence and development of tumor, as well as in mediating the therapeutic effects of the conventional tumor therapies, it is documented that immunotherapy has limited efficacy in controlling tumor growth. Some studies suggested that tumor cells induce local immune-tolerance during tumor progression (Willimsky et al., 2008). The major determinant of the poor outcome of immunotherapy may be the immune suppression within the tumor microenvironment (Gabrilovich et al., 2012). Of the two general types of cells within tumor microenvironment, hematopoietic and mesenchymal had been considered as the major constituents. The former includes myeloid-derived suppressor cells (Mantovani

et al., 2008; Kim et al., 2012), M2 macrophages (Mantovani et al., 2008), and regulatory T cells (Yu et al., 2005). These cells not only provide pro-angiogenic factors to growing tumors, but also inhibit $CD8^+$ T cell-mediated anti-tumor immunity.

Based on our previous finding that tumor-targeting therapy of anti-HER2/neu monoclonal antibody (mAb) is mediated by $CD8^+$ T cell responses (Park et al., 2010), we tried to improve the therapeutic effect of HER2/neu mAb by stimulating T cell responses. However, this therapeutic strategy was not efficacious. We further explore the potential mechanisms for the invalid combinational therapy by analyzing the tumor microenvironment.

RESULTS

Combinational therapy of HER2/neu antibody and immunotherapies failed to retard tumor progression.

With our previous discovery that anti-HER2/neu mAb therapy is mediated by $CD8^+$ T cell responses (Park et al., 2010), we hypothesize that the approaches that promote and sustain $CD8^+$ T cell responses can improve the therapeutic efficacy of HER2/neu antibody. It is reported that the complex of IL-15Rα and IL-15 is a strong stimulator for the responses of NK cells and $CD8^+$ T cells (Dubois et al., 2008; Epardaud et al., 2008; Han et al., 2011). We prepared IL-15R/IL-15 fusion protein by fusing IL-15 receptor α sushi domain with IL-15 (Mortier et al., 2006). To measure the activity of IL-15R/IL-15 fusion protein *in vivo*, mice were hydrodynamically injected with IL-15R/IL-15 plasmid or control plasmid. As shown in Fig. 1A and 1B, the percentage of $DX5^+$ NK cells and $CD3^+CD8^+$ T cells increased 2–3 folds in the spleens of IL-15R/IL-15 plasmid-injected mice compared with that of control mice three days after treatment. As reported (Han et al., 2011), IL-15R/IL-15 stimulated the expansion of the memory phenotype $CD44^+$ $CD8^+$ T cells (Fig. 1C). These data proved that our fusion protein is a strong stimulator of expansion of $CD44^+CD8^+$ T cells and NK cells *in vivo*.

We then investigated whether administration of IL-15R/IL-15 protein could improve anti-HER2/neu antibody treatment *in vivo*. We expressed IL-15R/IL-15 protein in mice by hydrodynamic injection. Two weeks after tumor inoculation, the mice were treated with 100 μg of anti-HER2/neu mAb and injected with IL-15R/IL-15 plasmid or control plasmid. As shown in Fig. 2A, IL-15R/IL-15 fusion protein did not improve the therapeutic effect of HER2/neu antibody. Unexpectedly, IL-15R/IL-15 fusion protein seemed to impair the anti-HER2/neu mAb treatment.

Anti-CD40 is an attractive immunomodulatory antibody that can improve antigen presentation and increase the function of $CD8^+$ T cells by binding to DC cells and T cells. Its performance in clinic is impressive, particularly in pancreatic cancer (Beatty et al., 2011). We tested the therapeutic activity of anti-HER2/neu mAb in combination with agonistic anti-CD40. As shown in Fig. 2B, combinational treatment of anti-CD40 did not improve the therapeutic effect of anti-HER2/neu mAb on tumor growth. These data indicate that simply enhancing T cell responses is not efficient to retard tumor progression. We hypothesized whether tumor microenvironment constrain the anti-tumor effects of the immune responses.

Myeloid-derived immunosuppressive cells accumulated in tumor microenvironment accompanying the tumor growth.

The anti-HER2/neu mAb therapy induces the anti-tumor $CD8^+$ T cell responses, which play a critical role in therapeutic effects of the antibody. However, why could the stimulation of $CD8^+$ T cell responses not enhance the anti-HER2/neu mAb therapy? To explore the clues of this question, we analyzed the tumor microenvironment during the tumor growth. We subcutaneously inoculated the HER2/neu dependent cell line TUBO into mice and analyzed tumor-infiltrating cells twenty days after tumor cell injection. The different cell populations in tumor-infiltrating myeloid-derived $CD45^+$ cells were gated as Fig. 3A. By twenty days after tumor inoculation, a lot of myeloid-derived cells had infiltrated into the tumor. The immunosuppressive tumor-associated macrophages, granulocytic and monocytic MDSCs were respectively 22.8%, 8.1% and 7.43% of the myeloid-derived $CD45^+$ cells in the tumor tissues (Fig. 3A and 3B). In contrast, the spleen from tumor bearing mice has much less MDSCs and macrophages (Fig. 3B). These data showed that the tumor is heavily infiltrated with immunosuppressive myeloid-derived cells.

Next we analyzed the functional phenotypes of these tumor-infiltrating macrophages and MDSCs by flow cytometry, and compared them with the cells from spleens of tumor bearing mice or wild-type mice. As shown in Fig. 4, the tumor-associated macrophages, granulocytic and monocytic MDSCs expressed similar levels of CD86, CCR7 and MHCII, except that more tumor-associated macrophage expressed high level of MHCII compared with the macrophages from the spleens. However, all the cells expressed high level of inhibitory co-signaling molecule of B7-H1. Tumor-associated macrophages and monocytic MDSCs expressed higher level of inhibitory co-signaling molecule of B7-H1 than the macrophages and monocytic MDSCs from the spleens. These data suggested that tumor-associated macrophages and monocytic MDSCs showed more immunosuppressive phenotypes.

Tumor-infiltrating T cells (TIL) expressed high level of inhibitory co-signaling receptor PD-1 (Programmed Death-1).

To explore whether the inhibitory tumor microenvironment influence the functions of tumor-infiltrating T cells, we analyzed the phenotypes of T cells in tumors, spleens and lymph

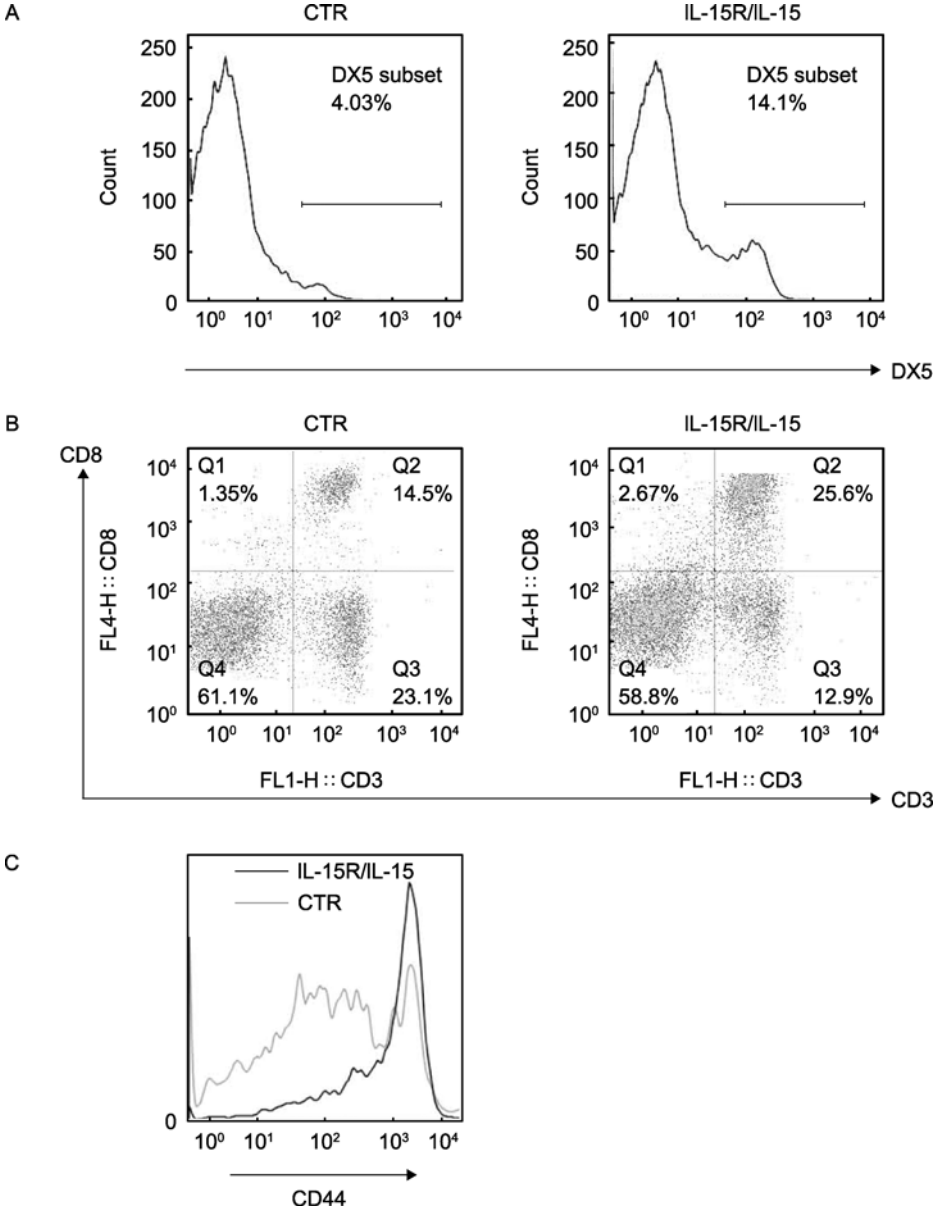

Figure 1. Hydrodynamic injection of IL-15R/IL-15 expands CD8$^+$ T cells and NK cells *in vivo*. BALB/c mice were hydrodynamic injected with IL-15R/IL-15 plasmid or control plasmid. Three days after IL-15R/IL-15 injection, spleen cells were analyzed by flow cytometry. (A) The percentage of NK cells (CD3$^-$DX5$^+$) in spleen. (B) The percentage of CD8$^+$ T cell in spleen. (C) CD44 expression on CD8$^+$ T cell from control group (gray line) and IL-15R/IL-15 group (black line).

nodes of tumor-bearing mice. As shown in Fig. 5A and 5B, more than 50% of CD8$^+$ TILs expressed high level of PD-1, while few CD8$^+$ T cells in spleen and lymph nodes were PD-1$^+$. Nearly half of tumor infiltrating CD4$^+$ T cells also expressed high level of PD-1 (Fig. 5C). Furthermore, the treatment of high dose anti-HER2/neu mab did not change the expression of PD-1 on the TILs (Fig. 5D). In addition, tumor cells also expressed higher level of B7-H1 *in vivo* (Fig. 5E). These data demonstrated that the tumor microenvironment expressed high level of immune inhibitory B7-H1/PD-1 pathway.

DISCUSSION

Trastuzamab has been widely used to effectively control HER2/neu$^+$ breast cancer and prolong survival in some patients. However, most HER2$^+$ patients who initially respond to the antibody treatment are at high risk in relapse, due to dormancy and/or development of resistance to trastuzamab. The high relapse rate after prolonged treatment has been a major clinical problem since these relapsed tumors are often resistant to antibody and chemotherapy treatment. We and

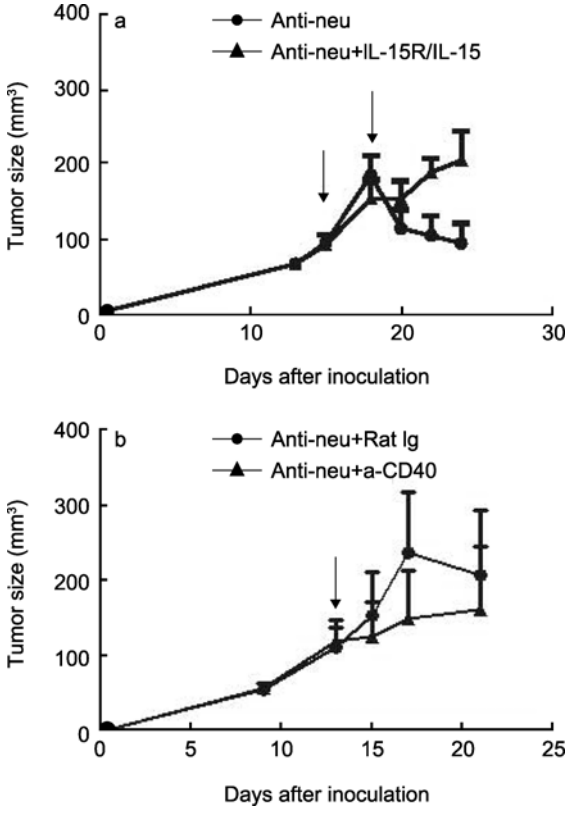

Figure 2. Combinational therapy of IL-15R/IL-15 or CD40 antibody with HER2/neu antibody cannot retard the tumor growth. Growth curves illustrate *in vivo* growth rates of tumors associated with diverse treatments. (A) 8 × 10^5 TUBO tumor cells were implanted into the right flank of wild-type BALB/c mice. Mice were treated with 100 μg of anti-HER2/neu mAb (clone 7.16.4) i.p. on day 17, and hydrodynamic injected with IL-15R/IL-15 plasmid or control plasmid on day 15. (B) For anti-CD40 antibody therapy, the tumor-bearing mice were treated with 100 μg of anti-HER2/neu mAb (clone 7.16.4) and 200 μg anti-CD40 agonist antibody i.p. on day 13. Tumor volume was measured in two dimensions using a caliper.

others have recently demonstrated that antibody mediated tumor control depends on $CD8^+$ T cells (Lee et al., 2009; Park et al., 2010). Therefore, combination of HER2/neu antibody with an immunotherapy, which can improve immune response, may have great impact on the efficacy of HER2/neu antibody treatment.

IL-15 has substantial potential as an immunotherapeutic molecule for increasing immune responses. We constructed a fusion protein IL-15R/IL-15, which can successfully expand $CD44^+CD8^+$ T cells and NK cells *in vivo*. However, we found that combination of IL-15R/IL-15 and anti-HER2/neu mAb could not efficiently eradicate the tumor cells, which indicated that tumor cells might evade from the immune response. We then used the other classic immunotherapy agent, anti-CD40, which not only activates antigen presenting cell (APC), but also has impact on T cells. Nevertheless, we did not observe the synergy effect of anti-CD40 and anti-HER2/neu mAb. These data indicated that there may be a barrier in the tumor that can limit the immunotherapy, even though tumor-specific T cell response was generated. Several lines of evidence suggested that the mechanisms of unsuccessful immunotherapy were classified as three forms (1) recruitment of Treg cells; (2) shape the microenvironment into an immune-suppressive state; (3) intrinsic regulation of T-cell function by co-inhibitory and co-stimulatory receptor–ligand pairs (Ferris et al., 2010; Donkor et al., 2011; Gabrilovich et al., 2012). In this paper, we presented that myeloid-derived cell accumulated in tumor tissues during the tumor progression. It was reported that MDSCs (Myeloid-derived Suppressor Cells) and TAMs (Tumor-associtated macrophages) can suppress T cell responses and induce T cell tolerance. And the accumulation of these myeloid cells in the tumor may account in large part for the limited effectiveness of cancer vaccines and immunotherapies (Ma et al., 2011). Accumulation evidence suggests that, upon entering tumor mass, MDSCs might differentiate into macrophages, resulting in elevated IL-10 production, T cell tolerance and promotion of angiogenesis (Gabrilovich et al., 2012). In our model, we found that macrophages constituted the majority of tumor infiltration leukocytes. We then demonstrated that these myeloid cells in the tumor microenvironment had higher expression of immunosuppressive marker B7H1 and diminished immune supportive marker CCR7 and CD86, indicating that they would convert the microenvironment into an immune-suppressive state (Marigo et al., 2008; Qian and Pollard, 2010).

In addition to editing the tumor microenvironment, tumor cells can evade immunosurveillance through up-regulation immunosuppressive molecules on T cells. Noticed that B7H1 is highly expressed on myeloid cells and tumor cells, we further analyzed the expression of its receptor PD-1 on tumor infiltrated T cells. A large amount of studies indicate that PD-1 functions as a negative regulator of immune responses in chronic infection (Keir et al., 2008). Recently, further studies identified that in cancer, exhausted T cell expressed PD-1 and that blockade of PD-1 and B7-H1 interactions can reverse T cell exhaustion and restore antigen-specific T cell responses (Sakuishi et al., 2010; Zhou et al., 2011). In this paper, we found that PD-1 mainly expressed on $CD8^+$ TILs before or after anti-HER2/neu therapy.

In conclusion, we revealed that in HER2/neu positive breast cancer, tumor microenvironment could alter myeloid cells and convert them into potent immunosuppressive cells. These myeloid cells highly express inhibitory molecule B7-H1. Moreover, we found that $CD8^+$ T cells highly expressed PD-1 in tumor. Such barrier might result in unsuccessful immune-mediated tumor destruction without altering the inhibitory microenvironment. Understanding the details of immune

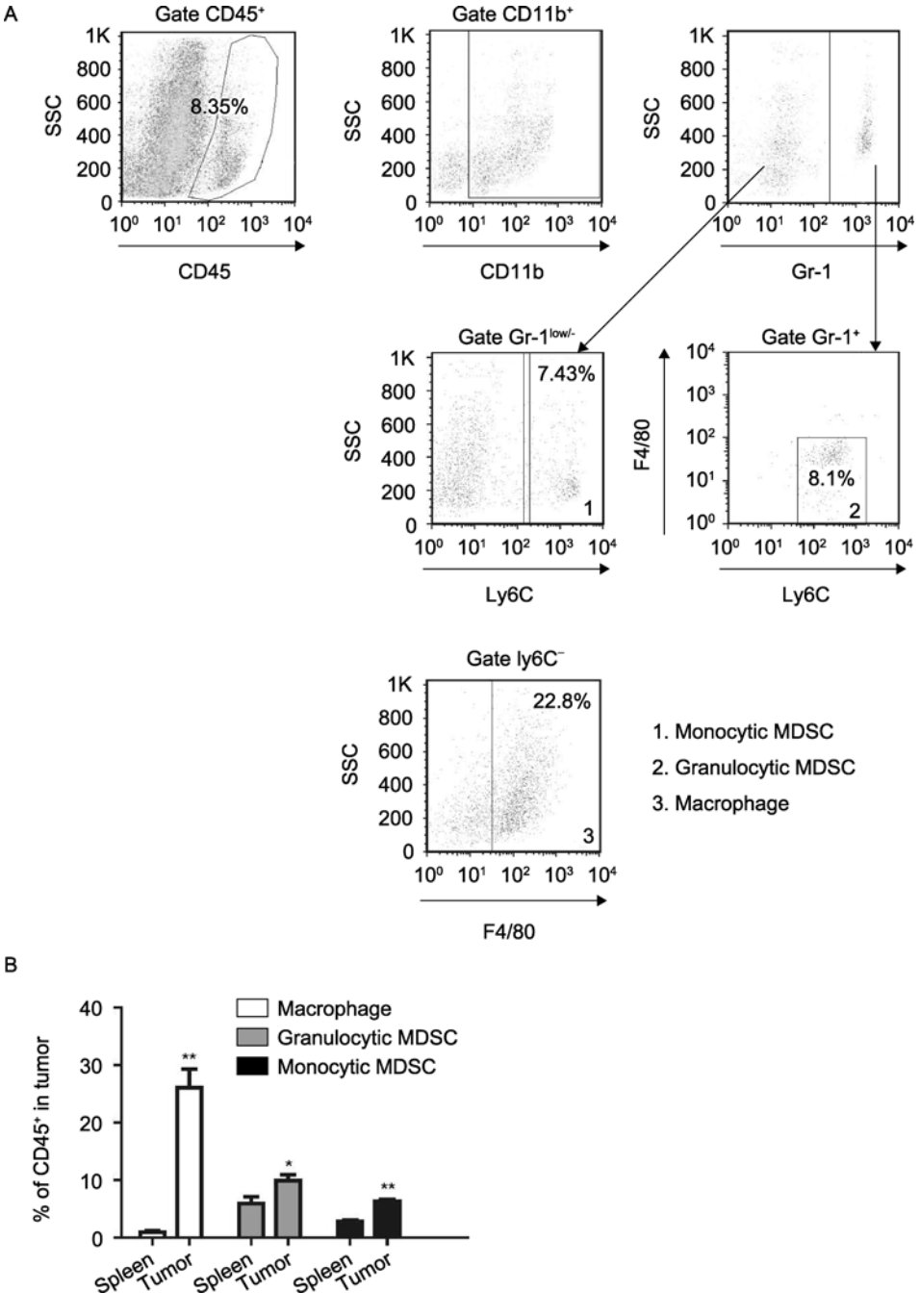

Figure 3. Myeloid cells accumulate in the tumor microenvironment. BALB/c mice were implanted with 8×10^5 TUBO cell line. The infiltrating myeloid cells and spleen cells were analyzed twenty days after tumor cell injection. (A) Representative FACS analysis showing the gating strategy to define tumor infiltrating myeloid-derived cells subsets. After gating on $CD45^+$ cells, myeloid-derived cells were subdivided into three different populations (monocytic MDSCs subsets 1, granulocytic MDSCs subsets 2 and macrophages subsets 3) based on their CD11b, Gr-1, Ly6c and F4/80 expression. The percentage of each population among $CD45^+$ is shown. (B) The statistics analysis of the percentage of each subsets among $CD45^+$ cells from the spleens or tumor tissues of tumor bearing mice. (* $P < 0.05$, ** $P < 0.01$, Student's test.)

evasion in HER2/neu positive breast cancer is critical because it will ultimately enable the development of therapies to specifically overcome these mechanisms and improve the efficiency of anti-HER2/neu therapy.

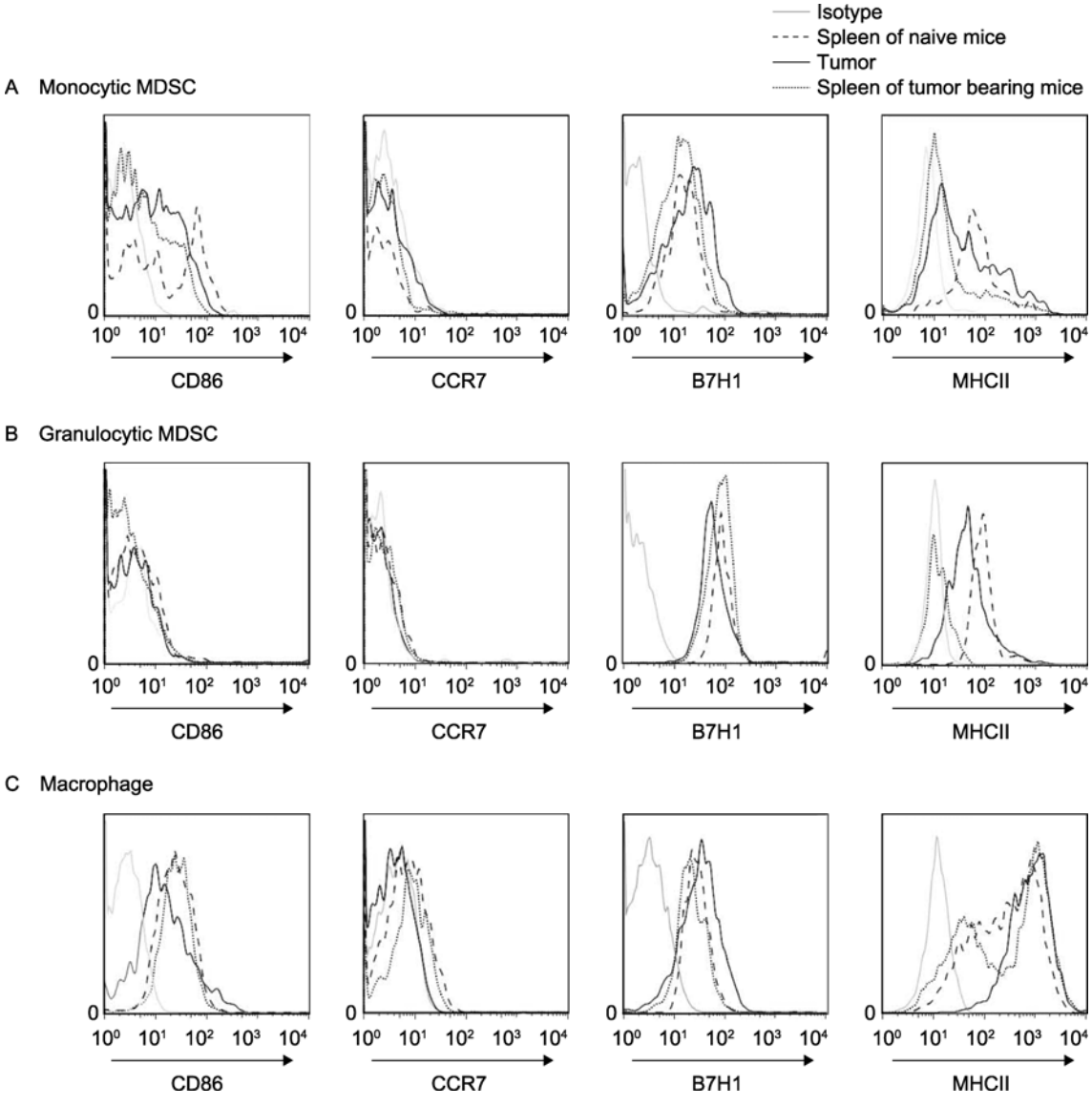

Figure 4. Tumor microenvironment exhibits immunosuppressive state. Spleen and tumor-infiltrating cells were gated by the same strategy as Fig. 3. Monocytic MDSCs (A) granulocytic MDSCs (B) and macrophages (C) were stained for CD86, CCR7, B7H1 and MHCII. Lines correspond to the following: gray line, isotype control; dotted line, splenic myeloid cells from tumor bearing mice; dashed line, splenic myeloid cells from naive mice; and solid line, tumor infiltrating myeloid cells.

MATERIALS AND METHODS

Animals

6–8-week-old female BALB/c mice were used in all experiments. Housing and animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Institute.

Cell lines

TUBO tumor cell line from a spontaneous mammary tumor in a BALB Neu Tg mouse was cultured with 5% CO_2 in DMEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 2 mmol/L L-glutamine (HyClone), 0.1 mmol/L MEM nonessential amino acids (HyClone), 100 units/mL penicillin, and 100 mg/mL streptomycin. TUBO-GFP stable cell line was prepared by transfecting TUBO with lenti-virus encoded GFP.

Construction of ptt3-IL-15R/IL-15/hFc plasmid

The cDNA encoding mouse IL-15Ra-sushi domain (amino acids 1–78) and mouse IL-15 mature sequence were fused by a 24-amino acid linker (MGVLLTQRTLLSLVLALLFPSMAS). The signal peptide of human oncostatin M (OSM) was used.

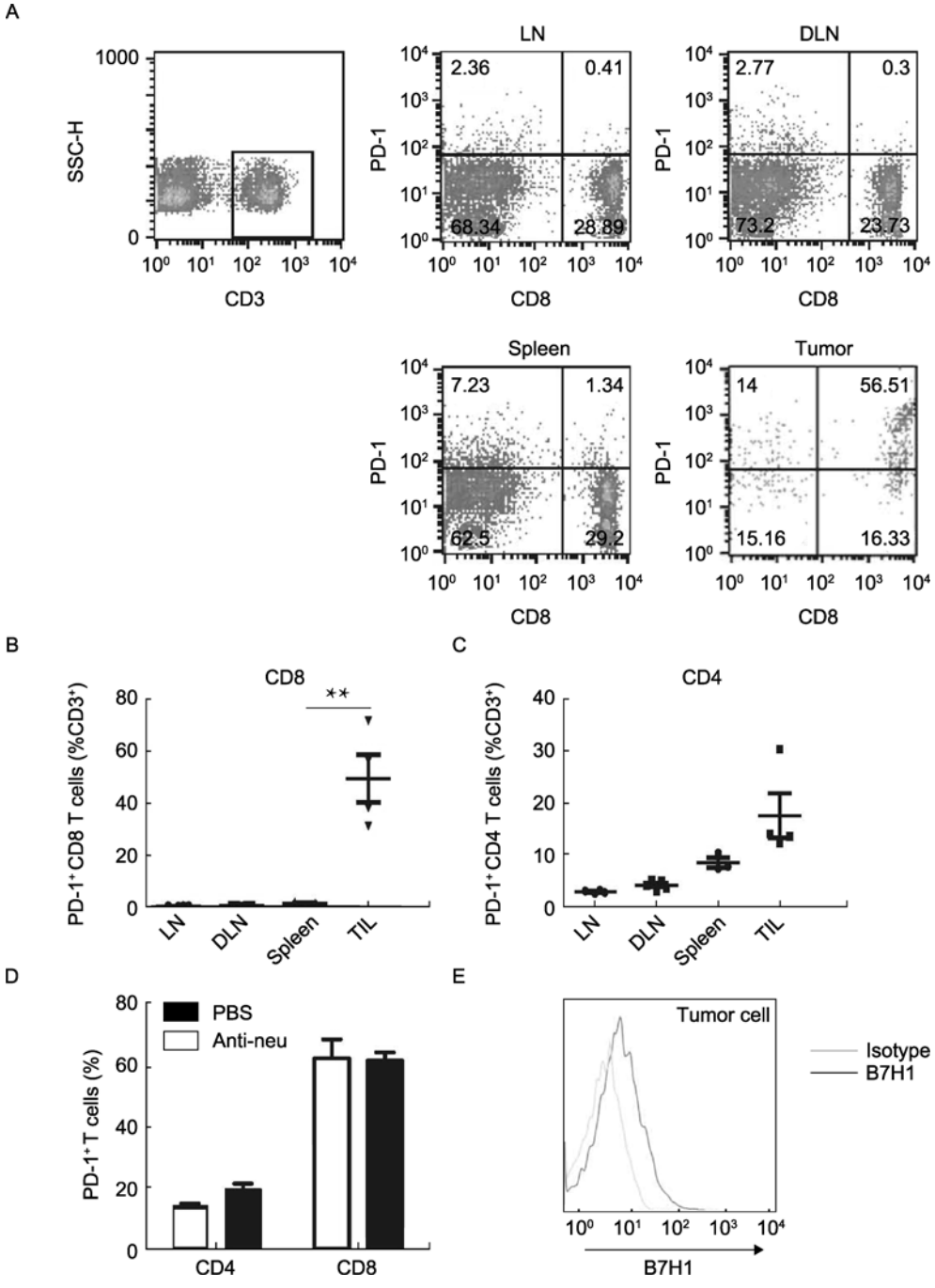

Figure 5. Tumor infiltrating T cells express high level of PD-1. BALB/c mice were implanted with 8 × 10^5 TUBO cell line. Spleen, non-draining lymph node, tumor draining lymph node (DLN) and TILs were harvested at day 26, and the cells were stained against CD3, CD8 and PD-1. (A) Representative FACS analysis of PD-1 expression on T cells; (B and C) Frequency of $CD8^+PD\text{-}1^+$ and $CD4^+PD\text{-}1^+$ cells in spleen, non-draining lymph node, tumor draining lymph node and TILs (**, $P < 0.01$ Student's test). (D) The tumor-bearing BALB/c mice were treated with anti-HER2/neu antibody or PBS at day 14 after tumor inoculation. Three days later, TILs were harvested and stained with antibody against CD45, CD4, CD8 and PD-1. Frequency of $PD\text{-}1^+$ in $CD8^+$ TILs or $CD4^+$ TILs is shown. (E) 1 × 10^6 TUBO-GFP cells were implanted into the right flank of wild-type BALB/c mice. Two weeks after inoculation, tumor was removed and stained with anti-B7H1 (black line) or isotype antibody (gray line). GFP positive tumor cells were gated and evaluated the B7H1 expression on tumor cell.

The function of IL-15R/IL-15 *in vivo*

Balb/c wild-type mice were hydrodynamic injected with plasmid ptt3-IL-15R/IL-15 or control plasmid through tail vein. Three days after injection, splenocytes were stained with anti-CD44, CD8 or DX5 antibody and detected by flow cytometry.

Isolation of TILs

TILs were isolated by dissociating tumor tissue in the presence of 2.5 mg/mL collagenase D for 20 min before centrifugation. The digested tissue was filtered using a 100 μm pore sized mesh and cells were centrifuged 5 min at 1000 rpm. Isolated cells were then used in FACS analysis.

Flow cytometry

Single cell suspensions were blocked with anti-FcR (5 μg/mL) for 10 min and then incubated with antibodies against PE-CD4, APC-CD8, FITC-PD-1, FITC-CD11b, APC-F4/80, FITC-Ly6c, Percpcy5.5-Gr-1, PE-B7H1, PE-CCR7, FITC-MHC class II and PE-CD86 for 30 min at 4°C. All data were collected on Calibur (BD) and analyzed with FlowJo software.

***In vivo* tumor experiment**

8×10^5 TUBO tumor cells were implanted into the right flank of wild-type BALB/c mice. The mice were treated with 100 μg of anti–HER2/neu mAb (clone 7.16.4) i.p. on day 17, and hydrodynamic injected with IL-15R/IL-15 plasmid or control plasmid on day 15. For anti-CD40 antibody therapy, the mice were treated with 100 μg of anti-HER2/neu mAb (clone 7.16.4) and 200 μg anti-CD40 agonist antibody i.p. on day 13. Tumor volume was measured in two dimensions using a caliper.

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ABBREVIATIONS

APC, antigen presenting cell; mAb, monoclonal antibody; PD-1, Programmed Death-1; RT, radiotherapy; TIL, Tumor-infiltrating T cells

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