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Tumor-infiltrating regulatory T cells delineated by upregulation of PD-1 and inhibitory receptors

Hyo Jin Park [a], Anthony Kusnadi [a,b], Eun-Jung Lee [c,d], Won Woo Kim [c], Byoung Chul Cho [e,f], Ik Jae Lee [c], Jinsil Seong [c,d], Sang-Jun Ha [a,*]

[a] Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, Seoul 120-749, Republic of Korea
[b] Underwood International College, Yonsei University, Seoul 120-749, Republic of Korea
[c] Department of Radiation Oncology, Yonsei University Health System, Seoul 120-749, Republic of Korea
[d] Institute for Cancer Research, Yonsei University College of Medicine, Seoul 120-749, Republic of Korea
[e] Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-749, Republic of Korea
[f] Yonsei Cancer Center, Yonsei University College of Medicine, Seoul 120-749, Republic of Korea

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ABSTRACT

Foxp3$^+$ regulatory T (T_{reg}) cells are dominant suppressor cells which regulate conventional T (T_{conv}) cells. Inside tumor microenvironment, T_{reg} cells have been known to become potent in suppressing T_{conv} cell responses, thereby enabling tumor cells to circumvent immune response. However, the underlying mechanism by which tumor-infiltrating T_{reg} cells display enhanced suppressive function is still unresolved. To understand characteristics and function of tumor-infiltrating T_{reg} cells as well as T_{conv} cells in the tumor site, we analyzed their phenotypes either within tumor burden or at distant site of tumor using both heterotopic and orthotopic mouse cancer models. Compared to CD8$^+$ T cells at distant site of tumor, tumor-infiltrating CD8$^+$ T cells dramatically upregulated programmed death 1 (PD-1) and other inhibitory receptors, thereby being more exhausted functionally. Tumor-infiltrating CD4$^+$ T cells also expressed higher level of PD-1 than CD4$^+$ T cells at distant site of tumor but very surprisingly, upregulation of PD-1 occurred in CD4$^+$Foxp3$^+$ T_{reg} as well as CD4$^+$Foxp3$^-$ T_{conv} cells. Moreover, tumor infiltrating T_{reg} cells upregulated other inhibitory receptors such as T cell immunoglobulin mucin 3 (TIM-3), cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), and lymphocyte activation gene-3 (LAG-3). These results suggest that upregulation of PD-1 and other inhibitory receptors on tumor-infiltrating T_{reg} cells is related with their enhanced suppressive function.

1. Introduction

Evasion from cancer immune surveillance is common in many types of tumor. The ability of tumor cells to avoid the immune system's detection gives them not only proliferative and survival advantages, but also its development and progression into metastatic stage. In order to escape the destruction response by the immune system, one mechanism that tumor cells employ is the suppression of antitumor immune response in the tumor microenvironment [1]. The prevalence characteristic of local immunosuppression is the display of effector T cells exhaustion which is constrained in a specific physiological area [2].

Effector T cells exhaustion was first observed in the mice that were chronically infected with lymphocytic choriomeningitis virus (LCMV) [3]. During chronic infection of mice with LCMV, effector T cells such as CD8$^+$ cytotoxic and CD4$^+$ helper T cells have shown severe defects in their canonical effector functions including cytotoxicity and cytokine secretion as a response to stimulation by antigen presenting cells (APCs). Several studies suggested that the sustained expression of inhibitory receptors on the surface of these cells might be the underlying reason behind the effector T cells exhaustion phenotype [4]. Some evidences implied that effector T cells, especially CD8$^+$ cytotoxic T cells upregulate the expression of programmed death-1 (PD-1) receptors in multiple solid tumor microenvironment and the blockage of the interaction between PD-1 with its ligands, PD-L1 and PD-L2 inhibits tumor growth and progression [5–7]. Although there is significant tumor regression observed, blocking the interaction between PD-1 and its ligands alone is not sufficient to restore the effector T cells functions and proliferation [8]. Thus, it is likely that multiple suppressive receptors are involved in shaping effector T cells exhaustion phenotype in tumor microenvironment.

Abbreviations: TILs, tumor-infiltrating lymphocytes; T_{reg} cells, regulatory T cells; MDSCs, myeloid-derived suppressor cells; PD-1, programmed death-1.

* Corresponding author. Address: 134 Shinchon-dong, Seodaemun-gu, Science Research Center Bldg, Room S423, Seoul 120-749, Republic of Korea. Fax: +82 2 362 9897.

E-mail address: sjha@yonsei.ac.kr (S.-J. Ha).

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Indeed, a recent study elucidated that another suppressive receptor, T cell immunoglobulin mucin 3 (TIM-3) is also being co-expressed with PD-1 in the majority of dysfunctional tumor infiltrating lymphocytes (TILs) population [4]. Another report also found that negative co-stimulatory receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4) is being highly expressed in TILs [9]. A growing number of literatures also have observed that multiple inhibitory receptors such as T cell immunoglobulin mucin 3 (TIM-3) [4,10,11], lymphocyte activation gene 3 (LAG-3) [12,13], and glucocorticoid-induced tumor necrosis factor receptor (GITR) [14,15] are being expressed on elevated level in immunosuppressive environment. These data suggested that it is highly probable that these receptors synchronically suppress the function and proliferation of effector T cells, leading to the differentiation into exhausted T cells inside tumor microenvironment.

Recently, a number of studies showed the involvement of regulatory T (T_{reg}) cells on the cancer immunosuppression modulation in multiple murine tumor models [16]. These data demonstrated that there is significant increase of T_{reg} cells recruitment into these solid tumor tissues, compared to tumorigenic peripheral tissues. Thereby, they proposed that effector T cells exhaustion is the consequence of the recruitment of suppressive T_{reg} cells to the primary tumor site. Despite the strong indication that TILs play major roles in immunosuppression response inside tumor microenvironment, the phenotypes of these T_{reg} cells are still an enigma.

In this study, we analyzed tumor-infiltrating T_{reg} cells in tumor burden from different murine tumor model. We demonstrated that there are remarkable distinctions between T_{reg} cells from primary tumor site and those from periphery tissues. We observed that T_{reg} cells within tumor burden substantially elevate the expression of multiple suppressive receptors such as PD-1, CTLA-4, TIM-3, LAG-3, and GITR, which of receptors were even co-expressed on T_{reg} cells in TILs. In addition, our study revealed that inside primary tumor tissue, together with $CD4^+Foxp3^+$ T_{reg} cells, there was also dramatic accumulation of myeloid derived suppressor cells (MDSCs) which are known to control the effector T cells.

2. Materials and methods

2.1. Mice

Six- to seven-week-old male C3H/HeN mice were purchased from Central Lab, Japan (Seoul, Korea). All mice were maintained in a specific pathogen-free facility of the Yonsei University Medical College guidelines, the Guide for the Care and Use of Laboratory Animals and the Guidelines and Policies for Rodent experiment provided by the Assessment and Accreditation of laboratory Animal Care (AAALAC).

2.2. Tumor model

For preparation of heterotopic tumor-bearing model, 1×10^6 MIH-2 cells were implanted into the right tigh of wild-type C3H/HeN mice using intramuscular injection method. For establishing orthotopic tumor-bearing model, 1×10^6 Hca-I cells were surgically implanted into liver of wild-type C3H/HeN mice, respectively. Heterotopic and orthotopic tumor-bearing mice were sacrificed at day 45 and day 18 post-implantation of each tumor.

2.3. Isolation of splenocytes and TILs

Lymphocytes were isolated from spleens as previously described [17]. For the isolation of TILs, the liver and tumor burden were perfused with ice-cold PBS before tissues were removed for lymphocyte isolations. Separated liver and tumor burden were chopped by clipper then incubated in the presence of 1 mg/ml collagenase type IV (Worthington) solution with 0.01 mg/ml DNase I (sigma) at 37 for 25 min. TILs were isolated by Percoll gradient (sigma) after dissociated tissues washing by ice chilled RPMI.

2.4. Flow cytometry and antibodies

Flow cytometry was performed by FACS Calibur and FACS CantoII (BD Biosciences). The data was analyzed using the FlowJo software (Tree Star). The antibodies used in this study were purchased as outlined below. BD Biosciences: CD4 (RM4-5)-PerCP, CD4 (RM4-5)-Pacific Blue, and CD8 (53-6.7)-PerCP; eBioscience: CD25 (PC61.5)-APC eFlour 780, CD103 (2E7)-FITC, CTLA-4 (UC10-4B9)-PE, CD8a (53-6.7)-PerCP-Cy5.5, Foxp3 (FJK-16s)-PE-Cy7, and Foxp3 (FJK-16s)-APC; Biolegend: PD-1(RMP1-30)-PE and GITR (YGITR765)-PE-Cy7; R&D: TIM-3 (215008)-APC; Invitrogen: granzyme B (GB12)-PE.

2.5. Intracellular molecules staining with T_{reg} cells

For T_{reg} cell staining, the cells were stained with various antibodies except Foxp3 and CTLA-4 antibodies were fixed and permeabilized with Foxp3 fixation/permeabilization solution (eBioscience). Foxp3, CTLA-4, and GzmB antibodies were administered after permeabilization for intracellular staining of T_{reg} cells.

2.6. Statistical analysis

Statistical analysis was performed using two-tailed unpaired Student's *t* tests using the Prism 5.0 software (GraphPad).

3. Results

3.1. Functionally impaired phenotypes of T cells among TILs from heterotopic tumor model

To investigate the distribution of T cells in various tissues, we performed FACS analysis using lymphocytes which were isolated from spleen and tumor burden site of naïve and heterotopic tumor-bearing mice model. Mice were heterotopically transplanted with a hepatocellular carcinoma cell line, MIH-2, at the right thigh and sacrificed on day 45 after tumor transplantation. In terms of frequency, $CD4^+$ and $CD8^+$ T cells in splenocytes showed no significant difference between naïve and tumor-bearing mice. In comparison with the frequency of $CD4^+$ or $CD8^+$ T cells, the frequency of $CD4^+$ T cells in TILs was slightly lower, but $CD8^+$ T cells in TILs displayed subtle frequency increase even though it was not statistically meaningful (Fig. 1a and b). To compare the phenotypical characterization of lymphocytes between lymphoid tissue and tumor burden, we examined CD44 and PD-1 expression level on $CD4^+$ and $CD8^+$ T cells. $CD44^+$ populations were increasing in tumor burden sites and spleens of tumor-bearing mice, since more than 90% of $CD4^+$ and $CD8^+$ T cells in TILs expressed CD44 (Fig. 1c). Furthermore, approximately 40% and 80% of $CD44^+PD\text{-}1^+$ proportions were observed among $CD4^+$ and $CD8^+$ T cells in TILs, respectively (Fig. 1d). In TILs, 20% of $CD4^+$ and 40% of $CD8^+$ T cells were positive for both PD-1 and CD69, another early activation marker (data not shown). We speculated that those $CD44^+$ and $CD69^+$ T cells might be activated by tumor antigens. These findings suggested that tumor-infiltrating T cells are displaying their impaired phenotype by upregulating the expression of PD-1, a marker that represents exhausted T cells.

3.2. Increased population of T_{reg} cells and upregulation of inhibitory receptors by T_{reg} cells in TILs from heterotopic tumor model

It has been reported that T_{reg} cells exist at a high rate and suppress immune responses in tumor microenvironment. In order to

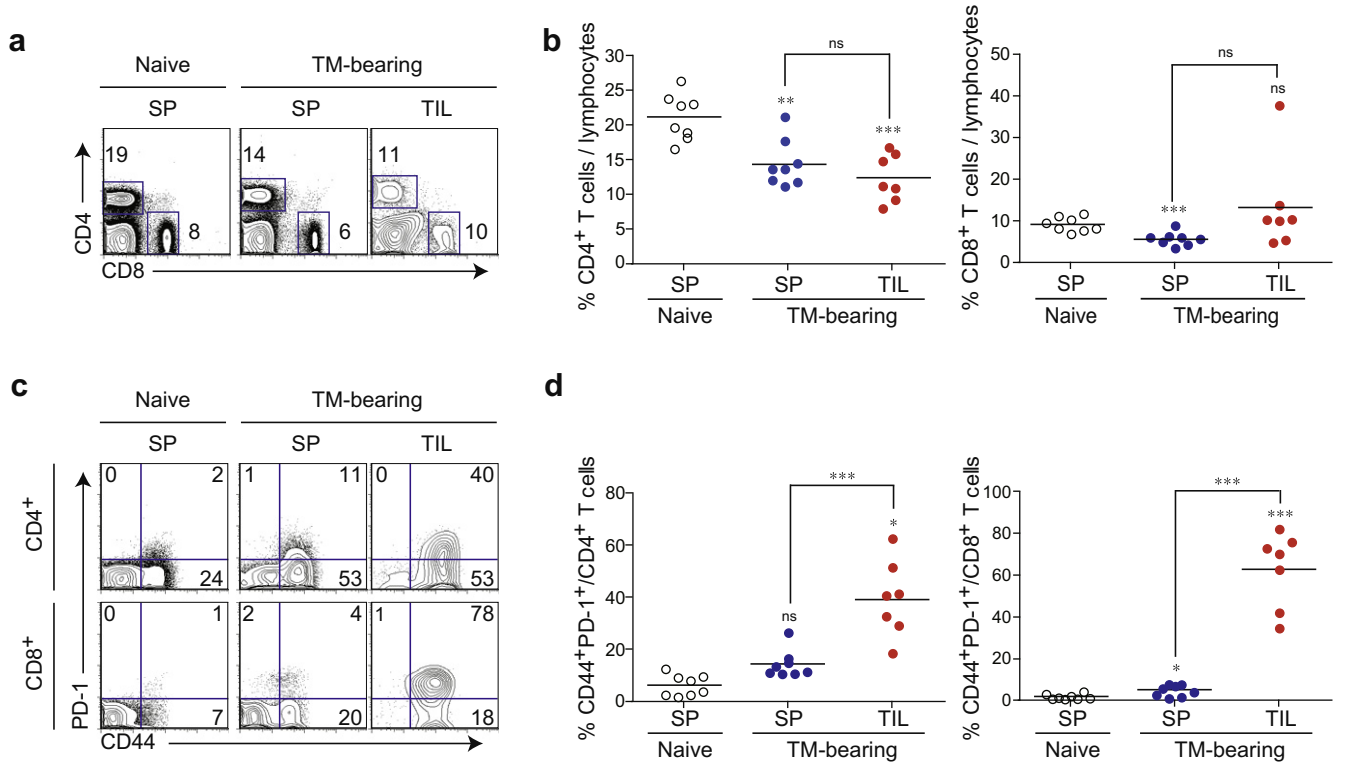

Fig. 1. High expression of PD-1 on the surface of T cells in TILs from heterotopic tumor model. Mice were implanted with MIH-2 and sacrificed at day 45 post tumor implantation. Lymphocytes isolated from either spleen or tumor burden were used for FACS analysis. (a) Frequency of $CD4^+$ and $CD8^+$ T cells in splenocytes (SP) and TILs (TIL) from naïve and tumor (TM)-bearing mice. The number in the plot indicates percentages of $CD4^+$ and $CD8^+$ T cells among lymphocytes. (b) Summary for the frequency of $CD4^+$ and $CD8^+$ T cells in different tissues from naïve and TM-bearing mice. The horizontal line is the mean value of individual result. (c) Expression of CD44 and PD-1 on the surface of $CD4^+$ and $CD8^+$ T cells in SP and TIL from naïve and TM-bearing mice. Upper and lower plots were gated on $CD4^+$ and $CD8^+$ T cells, respectively. The number in each quadrant indicates the percentage of the corresponding population. (d) Summary for the proportion of $CD44^+PD\text{-}1^+$ T cells among $CD4^+$ and $CD8^+$ T cells in SP and TIL. The horizontal line represents mean value of individual result. Data were pooled from two independent experiments. $n = 7$–8 mice per group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*t*-test). Symbol depicted above each column in the graph is *p*-value obtained when the corresponding SP isolated samples are compared to from naïve mice as control.

identify the sources of these suppressions, we decided to investigate the frequency and characteristics of T_{reg} cells in various organs using MIH-2 heterotopic tumor model. The frequency of T_{reg} cells was evaluated by measuring the expression level of an integral transcription factor of T_{reg} cells, Foxp3, in lymphocytes that were isolated from spleens and tumor burdens in a similar manner as in Fig. 1. Splenocytes of tumor-bearing mice showed much higher frequency of $Foxp3^+$ cell among $CD4^+$ T cells than naïve mice, despite the similar percentage of $CD25^+Foxp3^+$ cells among $CD4^+$ T cells between splenocytes of naïve and tumor-bearing mice. In contrast, $CD4^+$ T cells in tumor burden site exhibited fourfold higher frequency of $CD25^+Foxp3^+$ cells than those in splenocytes of naïve and tumor-bearing mice (Fig. 2a and b). To identify the phenotype of tumor-infiltrating T_{reg} cells, we examined the expression of PD-1 and CTLA-4 on T_{reg} cells. T_{reg} cells highly expressed PD-1 and CTLA-4 in tumor site as well as in spleen of tumor-bearing mice (Fig. 2c and d). Interestingly, while only some $CD4^+Foxp3^-$ conventional T (T_{conv}) cells upregulated the expression of PD-1 and CTLA-4, most $CD4^+Foxp3^+$ T_{reg} cells displayed high level of PD-1 and CTLA-4 (Fig. 2c). It was also observed that MFI values of PD-1 or CTLA-4 on T_{reg} cells and percentages of T_{reg} cells expressing PD-1 or CTLA-4 were significantly higher in TILs than in splenocytes from naïve mice (Fig. 2e and f). Meanwhile, we questioned whether tumor-infiltrating $CD4^+$ T cells also contain Th17 cells as well as T_{reg} cells. To address the question, we measured expression level of RORγt, a master transcription factor of Th17 cells, by tumor-infiltrating $CD4^+$ T cells in our heterotopic tumor model. RORγt-expressing $CD4^+$ T cells did not exist in TILs whereas Foxp3-expressing $CD4^+$ T cells were ~9% of total TILs (Supplemental Fig. 1a). When gated on $CD4^+$ T cells in TILs, it became more apparent that tumor-infiltrating $CD4^+$ T cells in our tumor model are composed of T_{reg} cells (~40%) and the other types of $CD4^+$ T cells, but not Th17 cells (Supplemental Fig. 1b). Taken together, these findings supported the idea that increased frequency of T_{reg} cells and upregulation of CD25, PD-1, and CTLA-4 indicate characteristics of tumor-infiltrating T_{reg} cells.

3.3. Highly exhausted phenotypes of T cells in TILs from orthotopic tumor model

We transplanted the other hepatocellular carcinoma cell line, Hca-I into the liver to get an orthotopic tumor mice model. To distinguish immune cells from peri-tumor site and tumor burden, we isolated the lymphocytes from the spleen, tumor-free region of the liver, and tumor burden. In tumor-bearing mice, the frequency of $CD4^+$ and $CD8^+$ T cells in TILs was very low compared to the lymphocytes from the spleen and tumor-free liver (Fig. 3a and b). In order to examine the phenotype of $CD4^+$ and $CD8^+$ T cells in TILs more extensively, we stained the cells in various tissues with CD44 and PD-1 antibodies. The percentage of $CD44^+PD\text{-}1^+$ population among $CD4^+$ and $CD8^+$ T cells was relatively higher in the liver, a peri-tumor site of tumor-bearing mice than in that of naïve mice. Notably, we discovered that approximately 55% of $CD4^+$ and 80% of $CD8^+$ T cells in tumor burden sites co-expressed CD44 and PD-1 (Fig. 3c and d). Because a high level of PD-1 expression was observed on tumor-infiltrating T cells, but not on the T cells present at distant site of tumor, in both heterotopic and orthotopic tumor

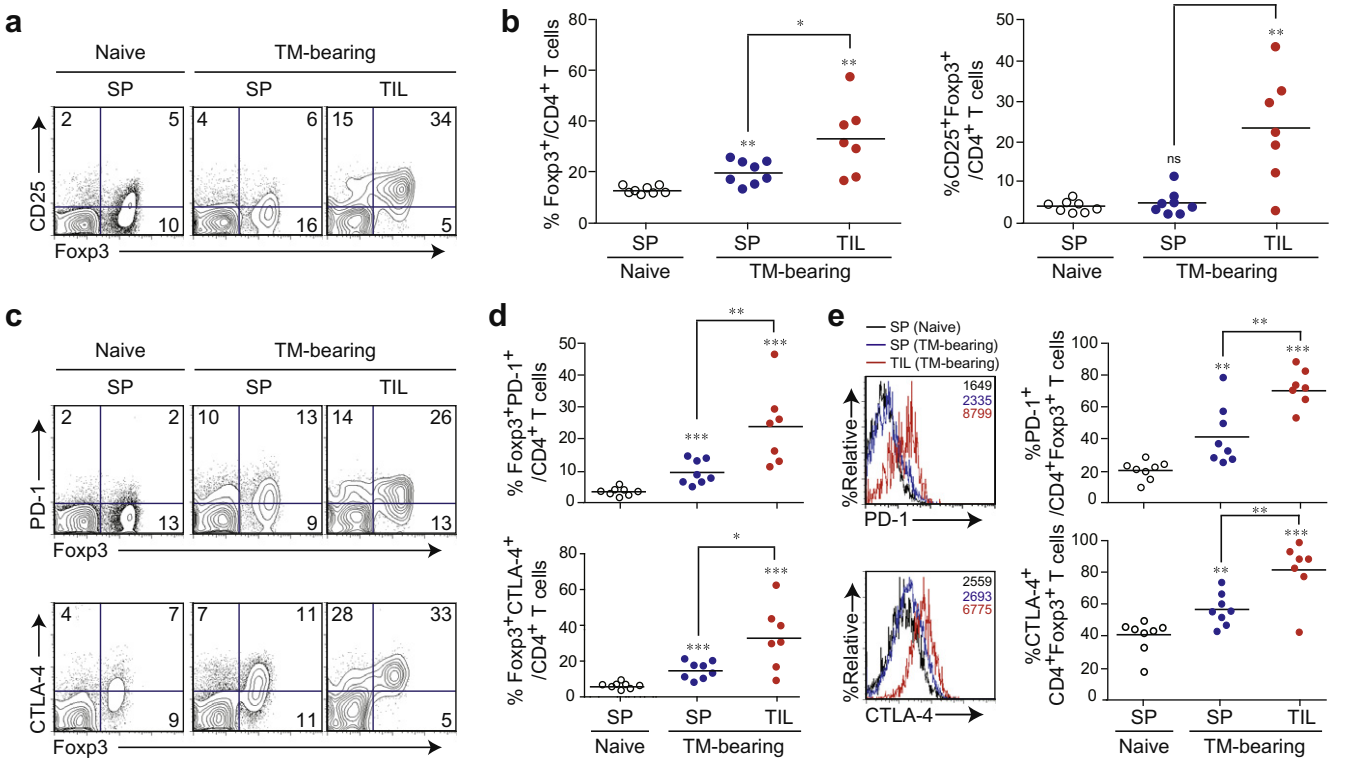

Fig. 2. High frequency of T_{reg} cells in TILs from heterotopic tumor model and upregulation of PD-1 and CTLA-4 in T_{reg} cells. Mice were implanted with MIH-2 and sacrificed at day 45 post tumor implantation. Lymphocytes isolated from either spleen or tumor burden were stained with Foxp3 antibody to detect T_{reg} cell population. (a) Expression of CD25 and Foxp3 on $CD4^+$ T cells in splenocytes (SP) and TILs (TIL) from naïve mice and tumor (TM)-bearing mice. The number in each quadrant indicates the percentage of the corresponding population. (b) Summary for the percentage of $Foxp3^+$ T_{reg} cells (left) and $CD25^+Foxp3^+$ T_{reg} cells among $CD4^+$ T cells (right). (c) Expression of PD-1 and CTLA-4 on T_{reg} cells in SP and TIL from naïve and TM-bearing mice. The number in each quadrant indicates the percentage of the corresponding population. (d) Summary result showing co-expression of PD-1 (upper) and CTLA-4 (lower) with Foxp3. (e) Comparison of mean fluorescence intensity (MFI) of PD-1 (upper) and CTLA-4 (lower) protein expression among $Foxp3^+$ T_{reg} cells in different tissues. (f) The percentage of PD-1^+ (upper) and CTLA-4^+ (lower) cells on total $Foxp3^+$ T_{reg} cells. Data were pooled from two independent experiments. $n = 7–8$ mice per group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (t-test). Symbol depicted above each column in the graph is p-value obtained when the corresponding SP isolated samples are compared to from naïve mice as control.

model (Fig. 3c and d; Fig. 4c and d), the phenotype is thought to be specific to tumor-infiltrating T cells.

3.4. Distinct profile of tumor-infiltrating T_{reg} cells from peripheral T_{reg} cells in orthotopic tumor model

We analyzed the distribution and characterization of T_{reg} cells in peri-tumor site and tumor burden using the same mice model. The result in Fig. 3a suggested low frequency of $CD4^+$ T cells in TILs when compared to liver lymphocytes of naïve mice. However, $CD4^+$ T cells in TILs or tumor-free liver lymphocytes consisted of higher numbers of $Foxp3^+$ T_{reg} cells than lymphocytes from liver of naïve mice (Fig. 4a and b). MFI value for CD25 on T_{reg} cells in TILs was prominently higher compared to the lymphocytes in other tissues (data not shown). Furthermore, most of $CD4^+Foxp3^+$ T_{reg} cells in TILs expressed PD-1 but only half of $CD4^+Foxp3^-$ T_{conv} cells expressed PD-1 (Fig. 4a). Compared with T_{reg} cells in peri-tumor site and T_{reg} cells isolated from naïve mice, tumor-infiltrating T_{reg} cells expressed remarkably high levels of various inhibitory receptors such as TIM-3, CTLA-4, GITR, and LAG-3 as well as PD-1 (Fig. 4c and d). It was also interesting that Granzyme B (GzmB), a molecule responsible for one of the direct cell killing mechanisms by T_{reg} cells, was also relatively upregulated in tumor-infiltrating T_{reg} cells compared to T_{reg} cells in peri-tumor site and T_{reg} cells isolated from naïve mice (Fig. 4c and d). The co-expression frequency of PD-1 and TIM-3, CTLA-4 and GITR, and LAG-3 and GzmB was more evident than each of their sole expression frequency (Fig. 4c and d). Collectively, these results demonstrate that increased expression of inhibitory receptors on T_{reg} cells is feature of tumor-infiltrating T_{reg} cells suggest that and their elevated expression or co-expression is involved in the enhancement of suppression function of TIL T_{reg} cells.

3.5. Increased population of MDSCs in TILs from heterotopic and orthotopic tumor model

We probed the distribution of MDSCs which is known as alternative immune response regulators in tumor microenvironment by utilizing heterotopic and orthotopic tumor mice model. The spleen and tumor burden of each tumor-bearing mice utilized in the previous experiments were stained with CD11b and Gr-1 antibodies. Splenocytes and TILs of mice that were heterotopically implanted with MIH-2 contained 11% and 24% of $CD11b^+Gr\text{-}1^+$ MDSCs (Fig. 5a and b). In orthotopic Hca-I liver tumor model, 14% and 45% of $CD11b^+Gr\text{-}1^+$ MDSCs were recorded on the splenocytes and TILs, respectively (Fig. 5c and d). In TILs, the frequency of MDSCs was significantly higher when compared to the splenocytes from naïve or the two different tumor-bearing mice model (Fig. 5b and d). These results imply that a high proportion of MDSCs can be a characteristic of TILs.

4. Discussion

In this study, we investigated the phenotype of T_{reg} cells as well as $CD4^+$ and $CD8^+$ T_{conv} cells which co-exist in tumor site by utiliz-

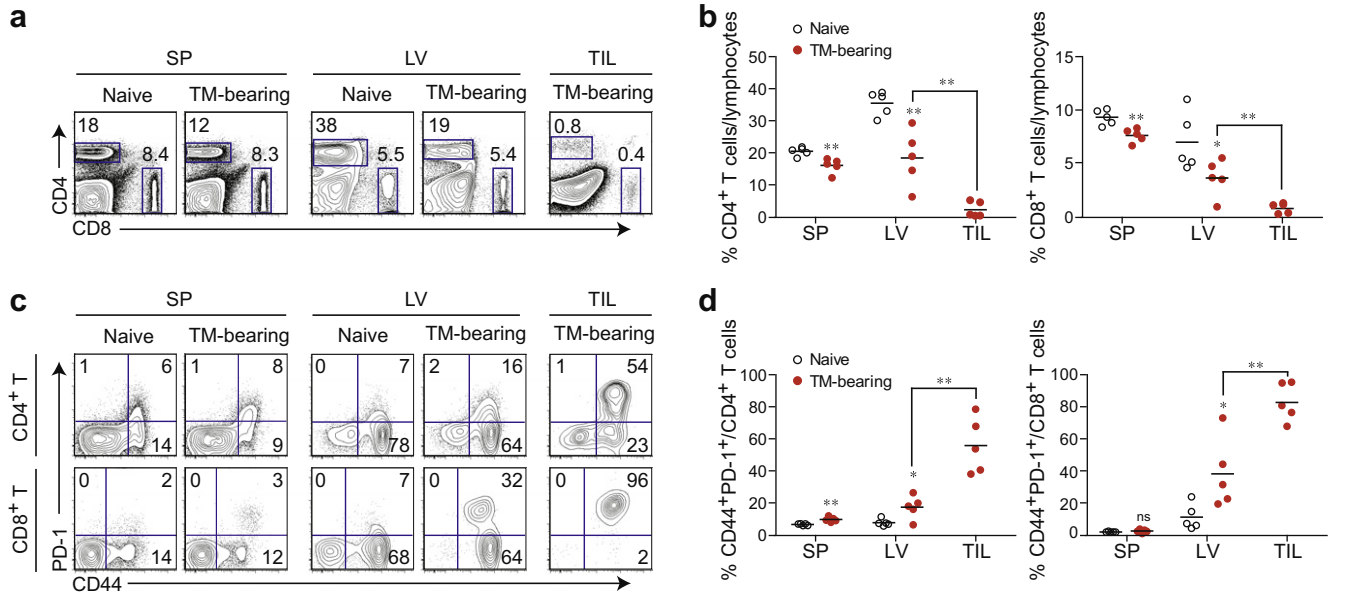

Fig. 3. Increased expression of PD-1 on the surface of T cells in TILs from orthotopic tumor model. Mice were implanted with Hca-I and sacrificed at day 18 after tumor implantation. Lymphocytes were isolated from the spleen, liver, and tumor burden. (a) Expression of CD4 and CD8 T cells in splenocytes (SP), liver lymphocytes (LV), and TILs (TIL) from naïve and tumor (TM)-bearing mice. The number in the plot indicates the frequency of $CD4^+$ and $CD8^+$ T cells. (b) Summary for the frequency of $CD4^+$ and $CD8^+$ T cells in different tissues from naïve and TM-bearing mice. The horizontal line is the mean value of individual result. (c) Expression of CD44 and PD-1 on $CD4^+$ and $CD8^+$ T cells in different tissues. The number of quadrant indicates the percentage of the corresponding population. (d) Summary for the proportion of $CD44^+PD\text{-}1^+$ T cells among $CD4^+$ and $CD8^+$ T cells in SP, LV, and TIL. The horizontal line represents mean value. All data are representative of two independent experiments. $n = 5$ mice per group in each experiment. ns, not significant; *, $P < 0.05$; **, $P < 0.01$ (t-test). Symbol depicted above each column in the graph is p-value obtained when the corresponding SP or LV isolated samples are compared to from naïve mice as control.

ing both heterotopic and orthotopic tumor models. As expected, most of $CD8^+$ T cells and less than half of $CD4^+$ T cells in TILs expressed PD-1, which indicates a functional exhaustion of PD-1-expressing T_{conv} cells. Surprisingly, most of T_{reg} cells among tumor-infiltrating $CD4^+$ T cells expressed high level of PD-1 and other inhibitory receptors, which has not been observed in naturally occurring T_{reg} (nT_{reg}) cells. Our study evidently demonstrated that tumor-infiltrating T_{reg} cells have a very distinct phenotype from nT_{reg} cells.

In the tumor microenvironment, T_{reg} cells have long been suspected to be the culprit behind local immunosuppressive environment. Studies suggested that T_{reg} cells hamper cytokine production and effector function as well as deteriorate anti-tumor immunity response by infiltrating into the tumor site [18–22]. Other experiments also found that the frequency of T_{reg} cells within tumor burden was significantly higher than that in peripheral tissues [20,23,24]. These results supported the results from our investigation that there was accumulation of $Foxp3^+$ T_{reg} cell population into the tumor.

The high frequency of T_{reg} cell population inside tumor microenvironment is prompting another question about their origin. A study reported that TGF-β which tumor cells secreted was triggering the migration of T_{reg} cells to tumor burden tissue and induced T_{reg} cells into $CD103^+$ state [24]. Another investigation argued that T_{reg} cells were accumulated at the tumor site through interactions of chemokine with chemokine receptors. Chemokines such as CCL5, CXCL12, CCL20, and CCL22 by tumor or TAMs can bind to T_{reg} cells on their recognition receptors such as CCR4, CCR5, or CCR6 [25–28]. It is also possible that tumor microenvironment is supporting the conversion of precursor CD4 T cells into $Foxp3^+$ T_{reg} cells lineage with enhanced TGF-β [29,30]. The origin or the migration pattern of $Foxp3^+$ T_{reg} cells into tumor microenvironment specifically will be an interesting topic for our future study.

Although tumor-infiltrating T_{reg} cells have been known to show much stronger suppressive function [18,31,32], the underlying mechanism is still unresolved. The first step to investigate the mechanism for the superior suppressive function of tumor-infiltrating T_{reg} cells would be the phenotypic characterization of the corresponding cells compared with nT_{reg} cells or T_{conv} cells. Tumor-infiltrating T_{conv} cells have been known to upregulate inhibitory receptors such as PD-1, TIM-3, and CTLA-4 [4–6,9]. Interestingly, it has been reported that T_{reg} cells also express limited levels of inhibitory receptors [32–36]. However, none of the studies compared directly the phenotypes of T_{reg} cells isolated from tumor burden, peri-tumor site, and tumor-free tissue of tumor-bearing mice. Here, we have shown that expression level of inhibitory receptors such as PD-1, TIM-3, CTLA-4, GITR, and LAG-3 was eminent in the tumor-infiltrating T_{reg} cells, compared to nT_{reg} cells of naïve mice and T_{reg} cells in peri-tumor site of tumor-bearing mice. In addition, the expression levels of PD-1 and TIM-3 on T_{reg} cells from peri-tumor site seemed to be in between those from distant site and tumor burden (Fig. 4c and d). Given that the potential correlated relationship between inhibitory receptor expression level and suppressive function by T_{reg} cells, it is possible to speculate that the suppressive ability of T_{reg} cells from peri-tumor site is better and worse than that from distant site and that from tumor burden, respectively. It is worth noting that expression levels of inhibitory receptors on T_{reg} cells correlated with the prevalence of tumor antigens. This observation also indicates that upregulation of inhibitory receptors on T_{reg} cells might be regulated by TCR stimulation or immune suppressive cytokine signaling achieved in tumor microenvironment.

Even though we have clearly showed the upregulation of inhibitory receptors on tumor-infiltrating T_{reg} cells, we did not yet evaluated the role of each inhibitory receptor in T_{reg} cell's suppressive function. There was reported that CTLA-4, GITR, or LAG-3 expressed by T_{reg} cells contributed to the inhibition of T_{conv} cell proliferation or function, and as a consequence of this proliferative breakage, it decreased antitumor immunity [20,33,36–39]. For example, T_{reg} cell-specific CTLA-4 deficiency impairs *in vivo* and *in vitro* suppres-

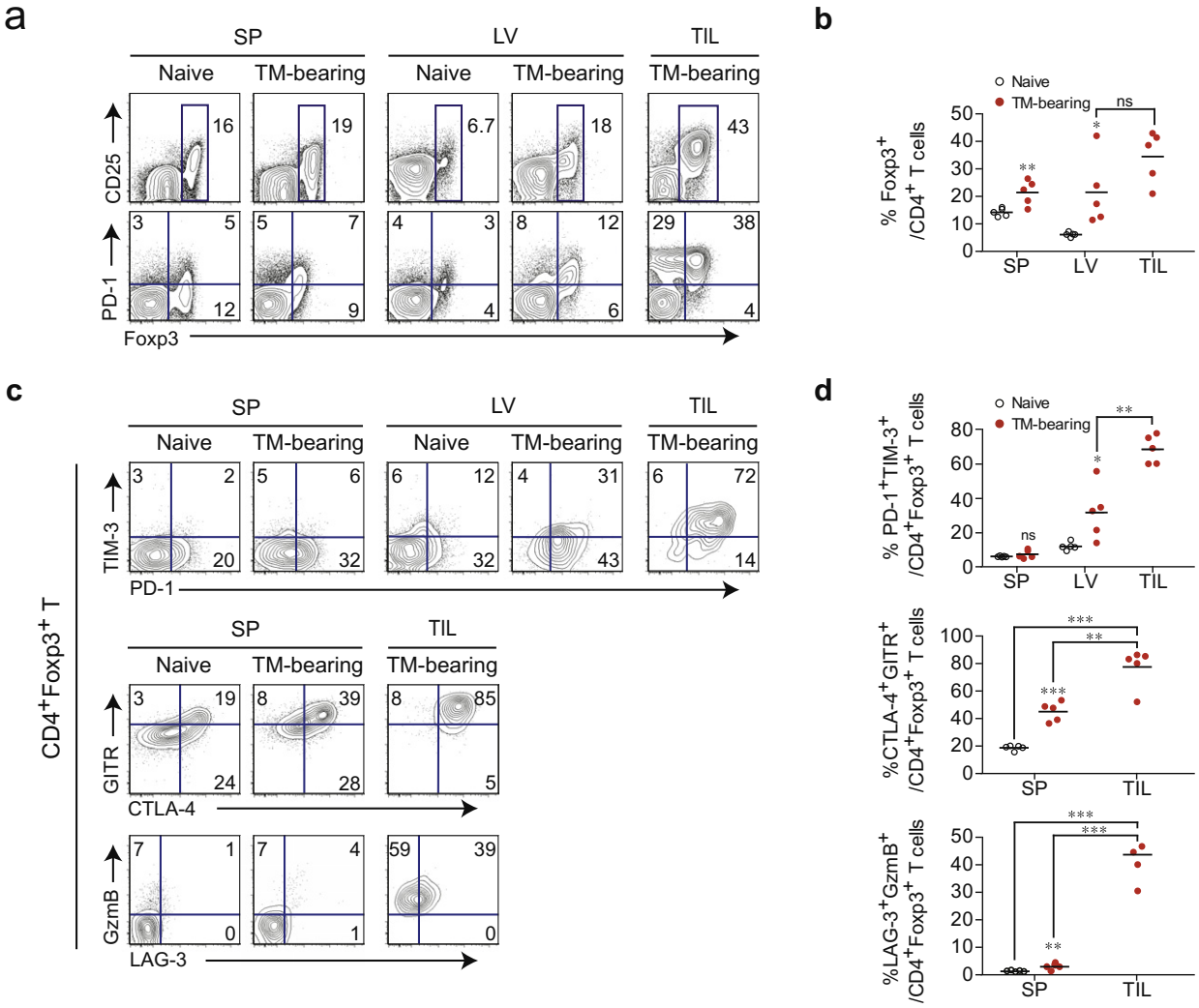

Fig. 4. Significantly increased inhibitory receptors on T_{reg} cells in TILs from orthotopic tumor model. Mice were implanted with Hca-I and sacrificed at day 18 after tumor implantation. Lymphocytes isolated from the spleen, liver, and tumor burden were stained with Foxp3 antibody to detect T_{reg} cell population. (a) Co-expression of Foxp3 with CD25 or PD-1 on $CD4^+$ T cells in splenocytes (SP), liver lymphocytes (LV), and TILs (TIL) from naïve and tumor (TM)-bearing mice. The number in each plot (upper row) indicates the frequency of $Foxp3^+$ cells among $CD4^+$ T cells. The number in each quadrant of the plots (lower row) indicates the percentage of the corresponding population. (b) Percentage comparison of $Foxp3^+$ T_{reg} cells among $CD4^+$ T cells in different tissues. The five individual data of each tissue were dotted in the plots with mean value (black line). (c) Expression of PD-1, TIM-3, CTLA-4, GITR, LAG-3, and GzmB on T_{reg} cells. Each plot was gated on $CD4^+Foxp3^+$ T_{reg} cells. The number in each quadrant of the plots indicates the percentage of the corresponding population. (d) Percentage of co-expression of various proteins in (c) among $CD4^+Foxp3^+$ T_{reg} cells in different tissues. The five individual data of each tissue were dotted in the plots with mean value (black line). Data are representative of two independent experiments. $n = 4–5$ mice per group in each experiment. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (t-test). Symbol depicted above each column in the graph is p-value obtained when the corresponding SP or LV isolated samples are compared to from naïve mice as control.

sive function of T_{reg} cells [39]. Because many studies demonstrated that tumor-infiltrating T_{reg} cells have been known to carry more potent suppressive ability than nT_{reg} cells [18,31] and our current study shows that tumor-infiltrating T_{reg} cells express higher level of PD-1 than nT_{reg} cells, it could be speculated that substantial expression of PD-1 by tumor-infiltrating T_{reg} cells is related to their enhanced suppressive function. Therefore, PD-1/PD-L1 blockade in T_{reg} cells might lead to the enhancement of anti-tumor immune responses by inhibiting their suppressive function. However, we still have to investigate whether inhibitory receptors including PD-1 highly upregulated by tumor-infiltrating T_{reg} cells actually can reinforce a basal suppressive function of T_{reg} cells. One piece of evidence was that tumor-infiltrating T_{reg} cells expressing inhibitory receptors express relatively higher level of GzmB, a molecule responsible for direct cell killing mechanism by T_{reg} cells, than nT_{reg} cells of naïve mice or T_{reg} cells at peri-tumor site of tumor-bearing mice. Since GzmB was reported to be important for T_{reg} cell-mediated suppression of tumor-specific T cells [40], it would be very interesting whether the correlation between inhibitory receptor signaling and GzmB expression. Furthermore, linking between distinct phenotype and enhanced suppressive function of tumor-infiltrating T_{reg} cells would be the next step to completely understand the suppressive mechanism.

Another anti-tumor immunity regulator, MDSCs, which are defined as double positive population of CD11b and Gr-1 were detected in elevated level in splenocytes and TILs from tumor-bearing mice [41–44]. Consistent with the finding of another report which argued that MDSCs alleviate the expansion of T_{reg} cells in the tumor microenvironment, we observed more MDSCs population in splenocytes and TILs from our two tumor models, compared to the splenocytes from naïve mice. Collectively, this discovery supports our current hypothesis that MDSCs, together with T_{reg} cells play important roles as immunosuppressive regulators in tumor microenvironment.

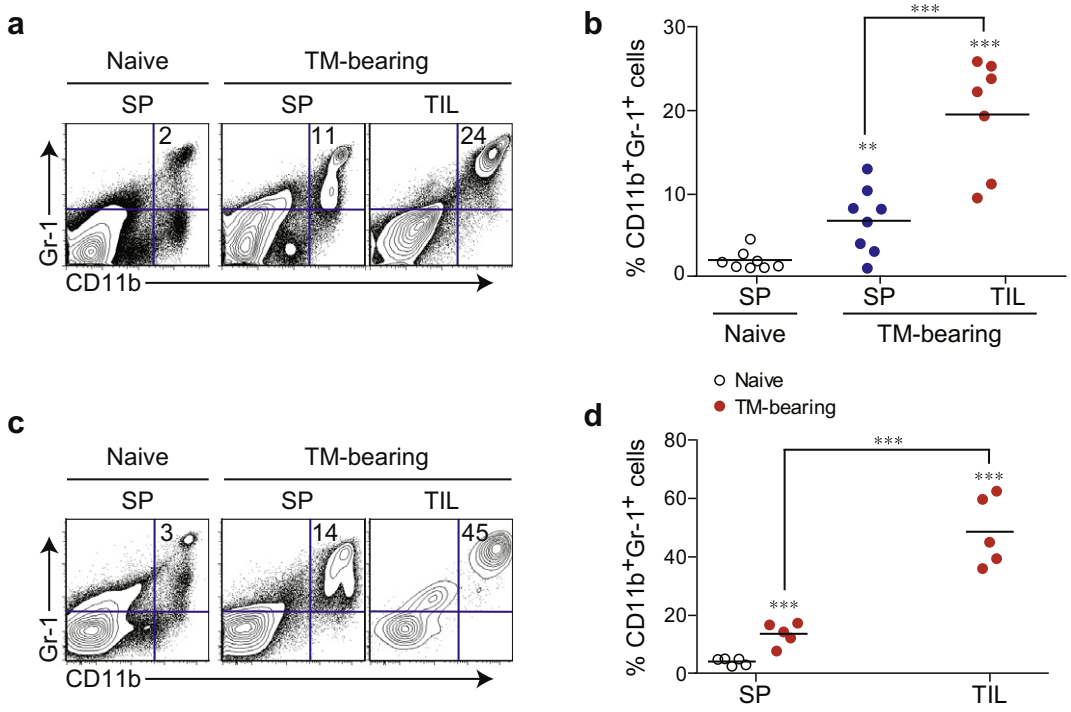

Fig. 5. Increased frequency of MDSCs in local tumor environment of heterotopic and orthotopic tumor models. Lymphocytes were extracted from spleen, liver and tumor burden of naïve, heterotopic, and orthotopic tumor (TM)-bearing mice. Each population from splenocytes (SP), liver lymphocytes (LV), and TILs (TIL) were stained with anti-CD11b and anti-Gr-1 Abs. Heterotopic TM-bearing mice were sacrificed at day 45 post MIH-2 tumor implantation. Orthotopic TM-bearing mice were sacrificed at day 18 post Hca-I tumor implantation. (a, c) Flow cytometry analysis of CD11b and Gr-1 protein in different tissues from heterotopic (a) and orthotopic (c) TM-bearing mice. The number in the plots indicates the percentage of $CD11b^+Gr\text{-}1^+$ cells. (b, d) Cumulative result of five individual data is presented in the form of $CD11b^+Gr\text{-}1^+$ cells percentage from heterotopic (b) and orthotopic (d) TM-bearing mice. The horizontal line indicates the mean value. The individual data of each tissue were dotted in the plots with mean value (black line). Data are representative of two independent experiments. $n = 7$–8 (b), $n = 5$ (d) mice per group. **, $P < 0.01$; ***, $P < 0.001$ (t-test). Symbol depicted above each column in the graph is p-value obtained when the corresponding SP isolated samples are compared to from naïve mice as control.

Lastly, we demonstrated that the immune cells accumulated in peri-tumor site and TILs were phenotypically different from each other. In conclusion, we found that tumor-infiltrating T_{reg} cells can be characterized by the elevation of frequency of T_{reg} cells and co-expression upregulation of inhibitory receptors such as PD-1, TIM-3, CTLA-4, GITR, LAG-3, and GzmB.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2012.07.001.

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