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ARTICLE

Frequency of CD45RO⁺ Subset in CD4⁺CD25^{high} Regulatory T Cells Associated with Progression of Hepatocellular Carcinoma

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Abstract

The purpose of this study was to assess the properties of CD4⁺CD25^{high/low/negative} T cell subsets and analyze their relation with dendritic cells (DCs) in patients with hepatocellular carcinoma (HCC). In HCC patients, the prevalence of CD45RO⁺ cells in CD4⁺CD25^{high} T cells was increased and associated with higher frequencies of plasmacytoid DCs. Larger proportions of this T cell subset were detected in the patients with larger tumor burdens. These results suggest that increased frequencies of the CD45RO⁺ subset in CD4⁺CD25^{high} Tregs in HCC patients may establish the immunosuppressive environment cooperatively with tolerogenic plasmacytoid DCs to promote disease progression of liver cancer.

Keywords: Regulatory T cell, Dendritic cell, Hepatocellular Carcinoma, CD45RO, Intracellular Cytokine

1. Introduction

Hepatocellular carcinoma (HCC) occurs primarily in individuals with cirrhosis related to hepatitis C virus (HCV) or hepatitis B virus (HBV) infections, and alcohol abuse. HCC is the fifth most common cancer, with increasing incidence worldwide. It is characterized by high mortality, frequent postsurgical recurrence and extremely poor prognosis [1-3].

CD4⁺CD25^{high}Foxp3⁺ regulatory T cells (Tregs) have been shown to suppress immune responses by direct interaction with other immune cell types or through immune suppressive cytokines and appear crucial in maintaining immune homeostasis, mediating peripheral tolerance and preventing autoimmunity [4-6]. Increased frequencies of Tregs have been documented in the peripheral blood and in some cases the tumor microenvironment in patients with several different tumor types [3-12]. It has been reported that, in HCC patients, increased Tregs are correlated with CD8⁺ T-cell impairment [11] and are related to poor prognosis [1].

Tregs are known to consist of heterogeneous subsets and to express various surface markers detectable by flow cytometry, including CD45RO, CTLA-4 (cytotoxic T lymphocyte associated antigen-4), GITR (glucocorticoid-induced TNF receptor-related protein), CD62L, HLA-DR, and CCR7 [8, 13-15]. The role of these markers in suppressor functions mediated by human Tregs is currently under discussion [8]. It has been suggested that GITR is associated with T cell activation[16, 17] and Treg subset expressing GITR are associated with disease activity in patients with Wegener's granulomatosis [17]. As for HCC, Ormandy et al. demonstrated that Tregs in HCC patients expressed high levels of HLA-DR and GITR [3]. However, there is a paucity of studies presenting the association of Treg subsets with disease progression.

In addition to Tregs, dendritic cells (DCs), a type of professional antigen-presenting cells (APCs), may be implicated in the regulation of immune

responses. The role of human DCs in modulating Tregs is not clear [18]. It has been suggested that immature and mature myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) may promote Treg cell differentiation, homeostasis and function [19]. It has been shown that lung cancer cells can convert mature DCs into TGF- β 1 producing cells, which demonstrate an increased ability to generate Tregs [20]. Conversely, Tregs can induce the generation of semimature DCs by which they can down-regulate immune responses [21]. These data suggest that there may be a mutual interaction between Tregs and DCs for the maintenance of immunosuppression.

In the present study, we evaluated the frequency and properties of CD4⁺CD25^{high} Foxp3⁺ T cells in HCC patients. Increased numbers of these cells produced more Th2 cytokine than CD4⁺CD25^{low/negative} cells. Furthermore, the proportion of CD45RO⁺ subset was increased in HCC patients. We also analyzed how the subset is related to DC frequencies, and found that some subsets were relevant to disease progression.

2. Materials and methods

2.1. Patients and Healthy Controls

Sixty-two HCC patients attending Kanazawa University Hospital (Ishikawa, Japan) between September 2006 and July 2008 were enrolled in this study with their informed consent. HCC was radiologically diagnosed by computed tomography(CT), magnetic resonance imaging(MRI), and CT angiography. Blood samples were taken from these HCC patients, as well as from 41 healthy controls, 17 patients with chronic hepatitis (CH) B and C and 16 patients with liver cirrhosis (LC) without a tumor. None of the patients received anticancer nor antiviral therapy at time of blood sample. Patients characteristics and disease classification are shown in Table 1.

2.2. Isolation of PBMC and CD4⁺ T Cells

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained blood by Ficoll-Hypaque (Sigma-Aldrich, St.Louis, MO). Total cell numbers were counted in the presence of a trypan blue dye to evaluate viability and immediately used for experiments. CD4⁺ T cells were isolated from freshly isolated PBMC by negative magnetic selection using the CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and QuadroMACS Separation Unit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated CD4⁺ T cells were purified by >90% as measured by flow cytometric analysis using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

2.3. Antibodies

The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD4-PerCP, anti-CD25-APC (BD Biosciences, San Jose, CA), anti-CD45RO-FITC (PROIMMUNE, Oxford, UK), anti-CTLA-4-PE, anti-CCR7-PE, anti-GITR (glucocorticoid-induced TNF receptor-related protein)-PE (R&D Systems, Minneapolis, MN), anti-CD62L-FITC, anti-HLA-DR-FITC, anti-CD45RA-PE (Exalpha

Biologicals, Watertown, MA), IOTest Conjugated Antibodies – (CD14+CD16)-FITC / CD85k(ILT3)-PE / CD123-PC5 Dendritic Cells “Plasmacytoid Subset” and IOTest Conjugated Antibodies – (CD14+CD16)-FITC / CD85k(ILT3)-PE / CD33-PC5 Dendritic Cells “Myeloid Subset” (Beckman Coulter, Miami, FL). Before use, all mAbs were titrated using normal PBMC to establish optimal staining dilutions.

2.4. Surface and Intracellular Staining

To determine the frequency of CD4⁺CD25^{high} T cells and the surface marker profile, CD4⁺ T cells (at least 2×10^5 cells/tube) were stained with mAbs in the above described panel for 30 minutes on ice. Appropriate isotype antibody controls were used for each sample. Cells were washed and examined by four-color flow cytometry.

For intracellular Foxp3 and cytokine staining, 2×10^5 CD4⁺ T cells/well in a 96-plate were stimulated with Leucocyte Activation Cocktail containing PMA, ionomycin, and brefeldin A, and then cultured at 37°C in a humidified CO₂ incubator for 4 hours. The activated cells were first incubated with anti-CD4-PerCP for 15 minutes on ice, followed by fixation and permeabilization of the activated cells for 20 minutes at room temperature with BD Cytofix /Cytoperm Buffer (BD Biosciences, San Diego, CA). Samples were then stained with anti-CD25-APC, anti-Foxp3-FITC (eBioscience) and PE-labeled anti-cytokine (IL-4, IL-10) antibodies (BD Biosciences) for 15 minutes at room temperature. Appropriate isotype controls were included for each sample.

2.5. Flow Cytometric Analysis

The samples were acquired on a FACSCalibur for four-color flow cytometry. Data analysis was performed using the CellQuest software (Becton Dickinson, CA, USA).

2.6 Statistical Analysis

Data are indicated as means \pm SD unless otherwise stated. The statistical significance of difference between the two groups was determined by applying the Mann–Whitney nonparametric U test. $P < 0.05$ was considered significant.

3. Results

3.1 Frequencies of CD4⁺CD25^{high} T Cells

To evaluate the frequencies of CD4⁺CD25^{high} T cell subsets that contain Tregs, MACS-sorted CD4⁺ T cell subsets obtained from the patients with CH, LC and HCC and healthy controls were analyzed by flow cytometry following the staining with anti-CD4 and anti-CD25 monoclonal antibodies (Fig. 1A and 1B). Although the frequencies of CD4⁺CD25^{high} T cells were not changed in patients with CH, they were increased in patients with LC compared to the controls ($P < 0.05$). As reported, it is remarkably elevated in patients with HCC ($P < 0.0001$). The results indicated that CD4⁺CD25^{high} T cell subset containing Tregs are increased in patients complicated with liver malignancies.

3.2. Intracellular Foxp3 and Cytokine Production of the CD4⁺CD25^{high} T Cell Subset in HCC Patients

The transcription factor Foxp3 is considered to be a specific marker for Tregs [22-24]. Intracellular Foxp3 levels were detected by using the specific mAb after the cell membrane permeabilization procedures (Fig. 2A). The percent of Foxp3⁺ cells in the CD4⁺CD25^{high} T cell subset in HCC patients was larger than that of CD4⁺CD25^{low/negative} subset, and it was also significantly larger than that of CD4⁺CD25^{high} T cells in healthy controls and CH patients. (Fig. 2B). Thus, not only is the number of CD4⁺CD25^{high} T cells in HCC patients larger, but also the frequency of Foxp3⁺ cells in HCC patients is higher than CH patient and healthy controls. This is consistent with previous reports of Tregs in patients with other malignancies.

Intracellular production of cytokines IL-4 and IL-10 of CD4⁺CD25^{high}Foxp3⁺ T cell subset was quantitated following the stimulation with PMA/ionomycin using the

specific mAbs by flow cytometry (Fig. 2C).

The levels of Th2 cytokines IL-4 and IL-10 were high in the CD4⁺CD25^{high} subsets. In addition, the levels of IL-4 and IL-10 were high in the CD4⁺CD25^{high}Foxp3⁺ T cell subset in HCC patient ($P < 0.005$) (Fig. 2D). These results suggest that the CD4⁺CD25^{high}Foxp3⁺ Treg subset in HCC patients may have a high potential to produce immunosuppressive cytokines.

3.4. Phenotypes of the CD4⁺CD25^{high} T Cell Subset in HCC Patients

To determine the phenotypical properties of CD4⁺CD25^{high} T cell subset increased in patients with HCC, the expression levels of the seven reported surface molecules, CD45RA, CD45RO, CD62L, CCR7, CTLA-4, HLA-DR and GITR were quantitated by flow cytometry. Among the seven molecules, the proportions of CD45RO⁺, HLA-DR⁺ and GITR⁺ cells were higher in the CD4⁺CD25^{high} T cell subset in all patient groups compared to the CD4⁺CD25^{low/negative} T cell subsets, except for GITR⁺ cells in CH patients ($P < 0.05$) (Fig. 3A and 3B). The percentage of CD45RO⁺ cells in HCC patients were elevated compared to the patients with advanced liver diseases and healthy controls ($P < 0.01$). These data demonstrate that the CD4⁺CD25^{high} T cell subset highly expresses the surface molecule CD45RO in HCC patients, which may reflect the memory properties of T cells.

3.5. CD4⁺CD25^{high} T Cell Subset and Dendritic Cells of HCC Patients

Several reports have suggested that the CD4⁺CD25^{high} T cell subset may interact with dendritic cells. To evaluate the frequencies of DCs in PBMC of HCC patients, whole blood cells were analyzed by flow cytometry following the staining with IOTest Conjugated Antibodies – (CD14+CD16)-FITC / CD85k(ILT3)-PE / CD123-PC5 Dendritic Cells “ Plasmacytoid Subset ” and IOTest Conjugated Antibodies – (CD14+CD16)-FITC / CD85k(ILT3)-PE / CD33-PC5 Dendritic Cells “Myeloid Subset ”. HCC patients were divided into two groups according to the frequencies of

CD45RO^{positive} cells in CD4⁺CD25^{high} T cell subsets (CD45RO⁺ vs. CD45RO⁺⁺). Patients with CD45RO⁺⁺ contained >83.8% positive cells in CD4⁺CD25^{high} T cells. The frequencies of CD123⁺ plasmacytoid DCs were significantly higher in CD45RO⁺⁺ group ($P < 0.05$) (Fig. 5A and 5B), although those of CD33⁺ myeloid DCs were not correlated with the subsets in CD4⁺CD25^{high} cells. These results showed that there are more tolerogenic plasmacytoid DCs in the PBMCs of HCC patients with higher frequencies of a memory subset of CD4⁺CD25^{high} T cells.

3.6. CD4⁺CD25^{high} T Cell Subset and Tumor Progression

To evaluate the association between CD4⁺CD25^{high} T cell phenotype and tumor progression, we compared the maximum tumor diameters, the number of tumors, tumor markers AFP (alpha-fetoprotein) and DCP (des-gamma-carboxyl prothrombin), TNM stages, Child-Pugh scores and fibrosis stages between two groups as described above. The levels of serum AFP and DCP and the maximum tumor diameters in CD45RO⁺⁺ group were larger than those in CD45RO⁺ group (Fig. 6). Others were not significantly different between two groups. These results imply that a subset of Tregs may contribute to the progression of liver tumors.

4. Discussion

CD4⁺CD25^{high} Foxp3⁺ regulatory T cells have been shown to increase in patients with malignancies to suppress the immune responses. In this study, we provide evidence that patients with HCC have increased frequencies of CD4⁺CD25^{high} T cells in their peripheral blood compared to healthy controls and chronic hepatitis patients. A large proportion of CD4⁺CD25^{high} T cells expressed Foxp3 and produced Th2 cytokines. We also showed that CD4⁺CD25^{high} T cells expressed high levels of CD45RO, HLA-DR and GITR, and, interestingly, the T cell frequencies expressing these surface molecules were associated with plasmacytoid DC numbers and maximum tumor diameters in HCC patients.

There are several reports of elevated numbers of Treg cells in the peripheral blood and tumor tissues of patients with different types of cancer [3-12]. The study of Unitt et al. provided the first report of increased CD4⁺CD25⁺ T cell frequency within tumor tissue compared to non-tumor tissue in HCC patients [13]. Ormandy et al. showed that the frequency of CD4⁺CD25^{high} T cells in peripheral blood of patients with HCC was significantly higher (3.92±3.3%) than in healthy donors (1.17±0.87%) and liver cirrhosis patients (0.78±0.43%) [3]. Our data revealed that a minimal increase in CD4⁺CD25^{high} T cells was detected in LC patients and more pronounced changes were found in HCC patients.

We showed that higher percentages of CD4⁺CD25^{high} T cells produced Th2 cytokines IL-4 and IL-10 in HCC patients. Tregs were recently observed to produce IL-10 [25-27], which can be a major mediator of immune suppression [28-30]. Voo et al. reported that Tregs in the peripheral blood of healthy donors secreted IL-10 but not IL-2, IFN- γ , or IL-4 [31]. Schmitz-Winnenthal et al. demonstrated the presence of Treg secreting IL-10 but not IL-4 or IFN- γ upon antigen recognition in chronic pancreatitis patients [32]. The present data demonstrated that larger numbers of Tregs produced not

only IL-10 but also IL-4 in HCC patients, which may contribute to the strong immunosuppressive properties of the T cells in liver malignancies.

It appears that Tregs consists of heterogenous populations within CD4⁺ T cells, and that a subset of CD4⁺CD25^{high} T cells could be subdivided into different functional subsets based on the expression of various surface molecules [6]. The proportions of Tregs expressing these molecules are reported to be different in the various forms of cancer. The prevalence of CD45RO⁺ and GITR⁺ Treg cells is higher in CD4⁺CD25^{high} T cells than in CD4⁺CD25^{low/negative} T cells in renal cell carcinoma [4]. In head and neck squamous cell carcinoma, however, CD4⁺CD25^{high} T cells express CTLA-4, Foxp3, and CD62L but little GITR, and CD25^{low/negative} T cells express intermediate to high levels of GITR and HLA-DR [8]. Our study showed that Tregs in HCC patients expressed significantly higher levels of CD45RO, HLA-DR and GITR compared to CD4⁺CD25^{low/negative} cells, suggesting that the activated populations of Tregs may contribute to the establishment of immunosuppressive microenvironments.

Little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated numbers of Treg cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells [20,33]. In peripheral blood, there are two distinct populations of DCs which can be distinguished based on phenotypical and morphological characteristics; myeloid DCs (mDCs) and plasmacytoid DCs (pDCs)[18,34]. Our data demonstrated that higher frequencies of CD45RO⁺CD4⁺CD25^{high} T cells were associated with higher frequencies of pDCs in the peripheral blood of HCC patients. When the tumor antigens are assumed by pDCs through Toll-like receptor 9 (TLR9) via receptor-mediated endocytosis, secretions of pro-inflammatory cytokines, such as type I interferons (IFNs), would be caused. On the contrary, pDC may regulate anti-tumor immunity and support immune evasion and tumor escape. They exhibit reduced IFN- α production upon TLR9 stimulation and can induce IL-10 producing CD4⁺ and CD8⁺ Treg [35,36]. This suggests that anti-tumor immune responses can be regulated through both modulation of pDC function by the

tumor and by limiting anti-tumor cytolytic activity through induction of CD8⁺ Treg.

Concerning the association of Tregs and prognosis, it has been reported that an increased number of circulating Tregs predicts poor survival of patients with renal cell carcinoma [4], gastric and esophageal cancers [7], myelodysplastic syndrome [37] and HCC [11]. In addition, tumor-infiltrating Tregs were associated with reduced survival in ovarian cancer [12] and HCC patients [1]. In addition, we found that CD45RO⁺CD4⁺CD25^{high} T cell subset was associated with larger tumor burdens, implying that a subset of Tregs may contribute to the promotion of tumor cell growth in the liver. However, it is also well possible that this just reflects stronger activation caused by a larger amount of antigen.

We performed the functional evaluation of Tregs derived from HCC patients by incubating with responder CD4⁺CD25⁻ T cells (Tresp). We observed that CD45RO⁺CD4⁺CD25^{high} T cells of HCC patients did not suppress the proliferation of responder T cells when co-cultured at Treg/Tresp ratios of 1:2 and 1:8 (data not shown). In contrast, Hoffmann et al confirmed that the CD45RA⁺CD4⁺CD25^{high} T cells of healthy volunteers give rise to a homogeneous and highly suppressive Treg cell population, whereas CD45RA⁻CD4⁺CD25^{high} T cells generate cell lines with mixed phenotype and function [38]. Although the reasons of these conflicting data were not clarified in the current study, cell viability, apoptosis susceptibility, involvement of Th1 cytokines, and interaction to helper T cell subsets of Tregs obtained from HCC patients need to be evaluated in the future experiments.

This study may be helpful for a better characterization of Treg subsets in the peripheral circulation of patients with HCC, which may establish the immunosuppressive environment to promote tumor progression. Furthermore, to gain insights into changes in the Treg subsets during the therapeutic option may lead to more effective immunotherapies against cancer and may improve prognosis.

Conflict of interest

None declared.

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FIGURE LEGENDS

Fig. 1. Frequencies of CD4⁺CD25^{high} T cells in peripheral blood of HCC patients and controls. (A) Representative flow cytometric analysis of PBMCs (peripheral blood mononuclear cells) of an HCC patient. Freshly isolated PBMCs were labeled with anti-CD4 and anti-CD25 antibodies as described in the Materials and Methods. (B) Percentages of CD4⁺CD25^{high} T cells in the peripheral blood of HCC (n=62), LC (n=16), CH (n=17) patient, and healthy controls (n=41). Percentages for individual patient analyzed are shown. The percentages represent the proportions of CD4⁺CD25^{high} T cells in total CD4⁺ cells. The prevalence of CD4⁺CD25^{high} T cells in HCC patients was significantly higher than in healthy controls or CH patients. CH, chronic hepatitis; HCC, hepatocellular carcinoma; LC, liver cirrhosis. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

Fig. 2. Analysis of intracellular Foxp3 expression and cytokine production in CD4⁺CD25^{high/low/negative} T cell subsets in HCC patients. (A) Representative expression of Foxp3 in CD4⁺ T cells from an individual subject. Intracellular Foxp3 was stained following membrane permeabilization. Intracellular Foxp3 was detected by the specific mAb. (B) Statistical analysis in the left side panel shows that the percent of Foxp3⁺ cells in the CD4⁺CD25^{high} T cell subset in HCC patients was significantly larger than that of CD4⁺CD25^{low/negative} T cell subsets, and in the right side panel shows that that of CD4⁺CD25^{high} T cell subset in HCC patients was significantly larger than that of CD4⁺CD25^{high} T cells in healthy controls and CH patients. (C) Statistical analysis shows that the levels of Th2 cytokines IL-4 and IL-10 were remarkably high in the CD4⁺CD25^{high} T cell subset. (D) Comparison of intracellular cytokine production in CD4⁺CD25^{high} T cell subsets between patients with and without HCC. Healthy controls, patients with chronic hepatitis and liver cirrhosis were included in the HCC(-) column. IL-4 and IL-10 levels were higher in the CD4⁺CD25^{high} T cell subset in HCC patients.

* indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

Fig. 3. Phenotypic analysis of CD4⁺CD25^{high/low/negative} T cell subsets in HCC patients. Freshly isolated CD4⁺ T cells (at least 2×10^5 cells/tube) from HCC patients were labeled with anti-CD4, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD62L, anti-CCR7, anti-CTLA-4, anti-HLA-DR and anti-GITR mAbs. (A) Representative CD45RO, HLA-DR, and GITR expression profiles in CD4⁺ T cell subsets that differ in CD25 expression. (B) Statistical analysis shows that the proportions of CD45RO⁺, HLA-DR⁺ and GITR⁺ were elevated in the CD4⁺CD25^{high} T cell subsets of all patient groups compared to the CD4⁺CD25^{low/negative} T cell subsets, except for GITR⁺ cells in CH patients ($P < 0.05$). The percentage of CD45RO⁺ cells in HCC patients was elevated compared to the patients with advanced liver diseases and healthy controls. * indicates $P < 0.05$, ** indicates $P < 0.01$ and *** indicates $P < 0.001$.

Fig. 4. Frequencies of plasmacytoid DCs in peripheral blood of HCC patients. Whole blood cells were analyzed by flow cytometry following staining with a combination of the mAbs. HCC patients were divided into two groups according to the frequencies of CD45RO^{positive} cells in CD4⁺CD25^{high} T cell subset (CD45RO⁺ vs. CD45RO⁺⁺). Patients with CD45RO⁺⁺ contained >83.8% positive cells in CD4⁺CD25^{high} T cells. (A) Representative dot plots of plasmacytoid DCs. Plasmacytoid DCs of CD45RO⁺ group are shown in the left panel and CD45RO⁺⁺ group in the right panel. (B) Statistical analysis shows that the frequencies of plasmacytoid DCs were significantly higher in CD45RO⁺⁺ group. * indicates $P < 0.05$.

Fig. 5. Prevalence of CD4⁺CD25^{high/low/negative} T cell subsets and tumor progression. The levels of AFP and DCP and the maximum tumor diameters in CD45RO⁺⁺ group were larger than those in CD45RO⁺ groups. AFP, alpha-fetoprotein; DCP, des-gamma-carboxyl prothrombin. * indicates $P < 0.05$.

Table 1. Clinical Characteristics of Hepatocellular Carcinoma, Liver Cirrhosis, Chronic Hepatitis Patients and Healthy Controls

Hepatocellular carcinoma (n=62)	
Age (yrs)	68.9 ±9.5
Gender (M / F)	37 / 25
Etiology of Liver Disease	
HBV / HCV / HBV+HCV / NBNC	19 / 34 / 2 / 7
TNM stages I / II / III / IV-A / IV-B	18 / 12 / 20 / 6 / 6
Largest tumor (mm)	37.6±34.4
Child-Pugh A / B / C	41 / 8 / 3
AFP (ng/mL)	10-35093 (52)
DCP (mAU/mL)	10-32818(34)
Liver cirrhosis (n=16)	
Age (yrs)	58.3±10.3
Gender (M / F)	11 / 5
Etiology of Liver Disease	
HBV / HCV / NBNC	4 / 7 / 5
Chronic hepatitis (n=17)	
Age (yrs)	58.9 ±10.4
Gender (M / F)	8 / 9
Etiology of Liver Disease	
HBV / HCV / NBNC	0 / 17 / 0
Healthy controls (n=41)	
Age (yrs)	46.1 ± 19.1
Gender (M / F)	16 / 25

Note: Results except for AFP and DCP are expressed as means ±SD. AFP and DCP values are expressed as range (median).

The reference range of normal values for the laboratory values : AFP <10ng/mL , DCP <40mAU/mL

M, Male; F, Female; HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC, non B non C; TNM, tumor-node-metastasis; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin

Fig. 1A

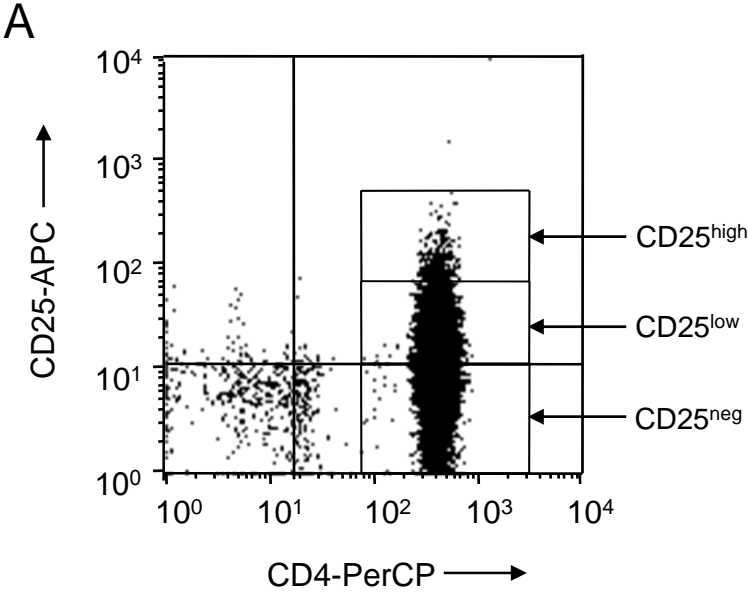


Fig. 1B

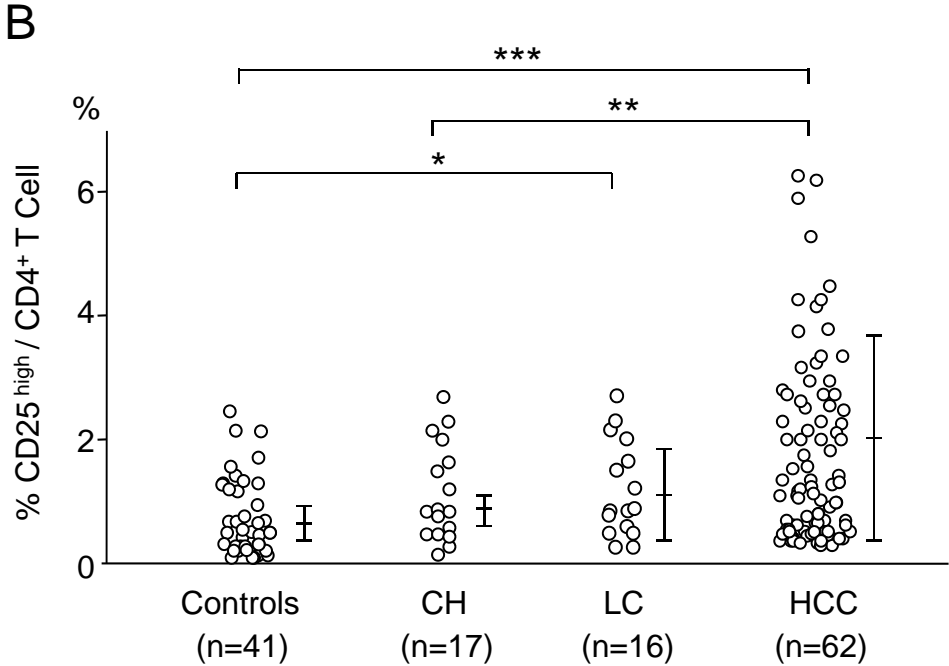


Fig. 2A

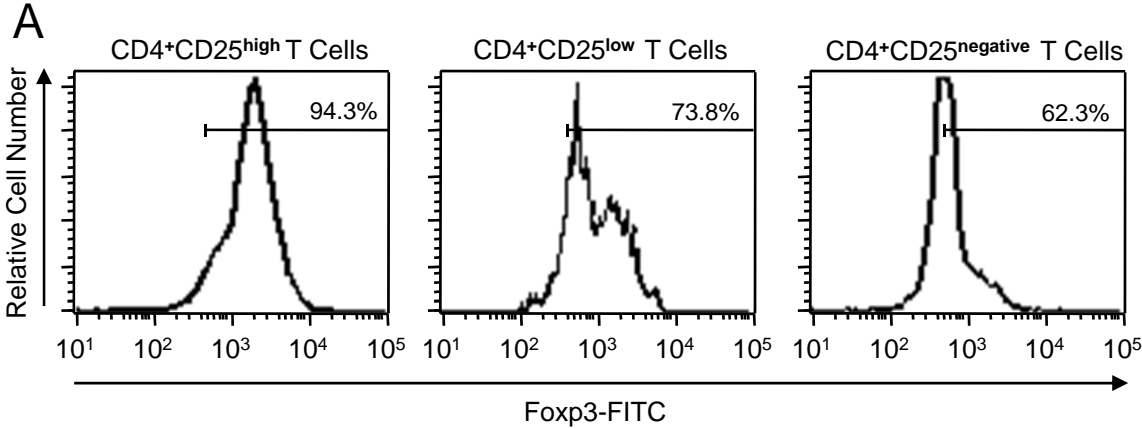


Fig. 2B

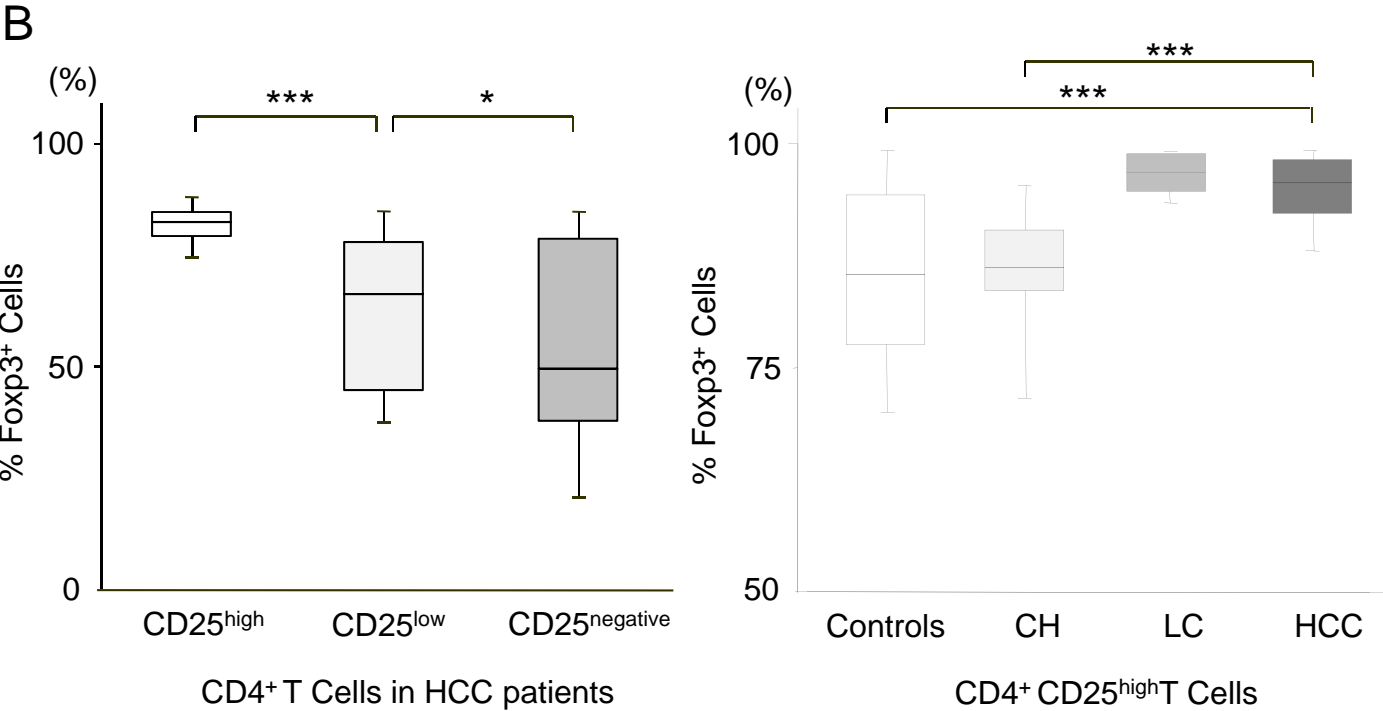


Fig. 2C

D

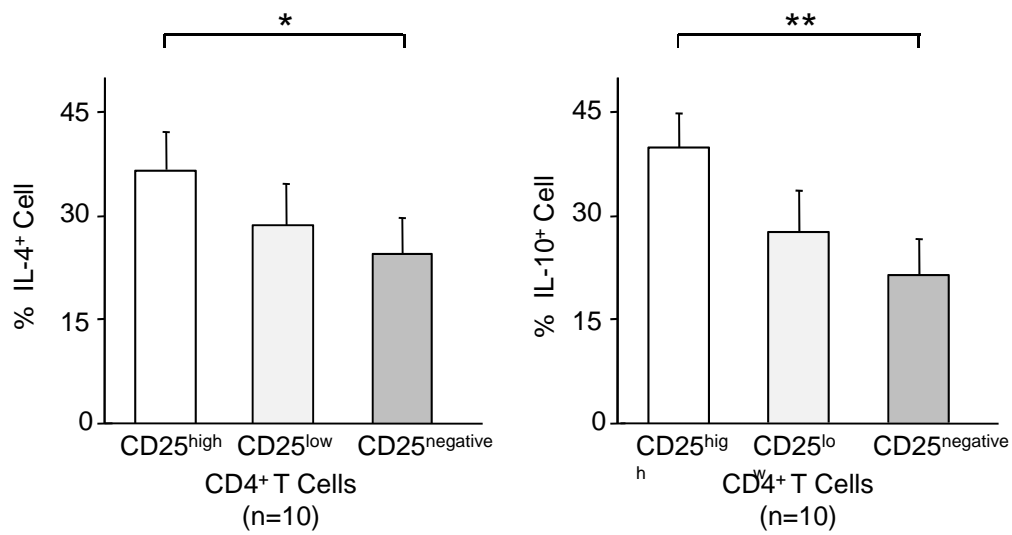


Fig. 2D

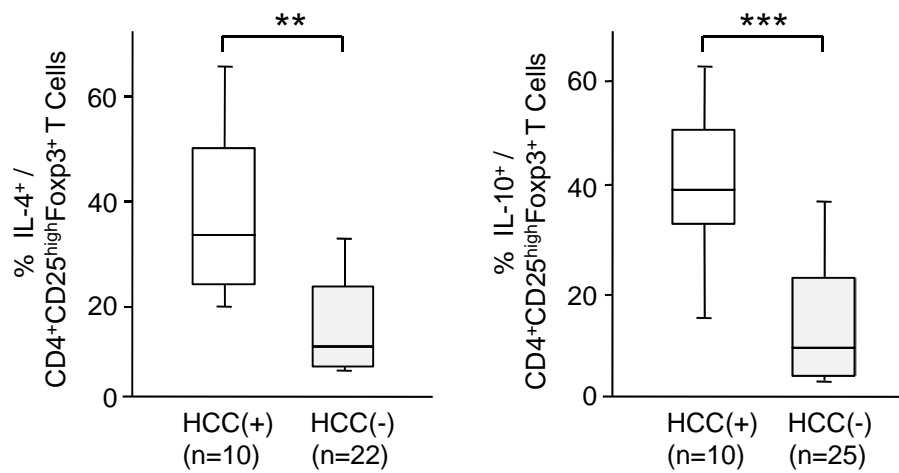


Fig. 3A

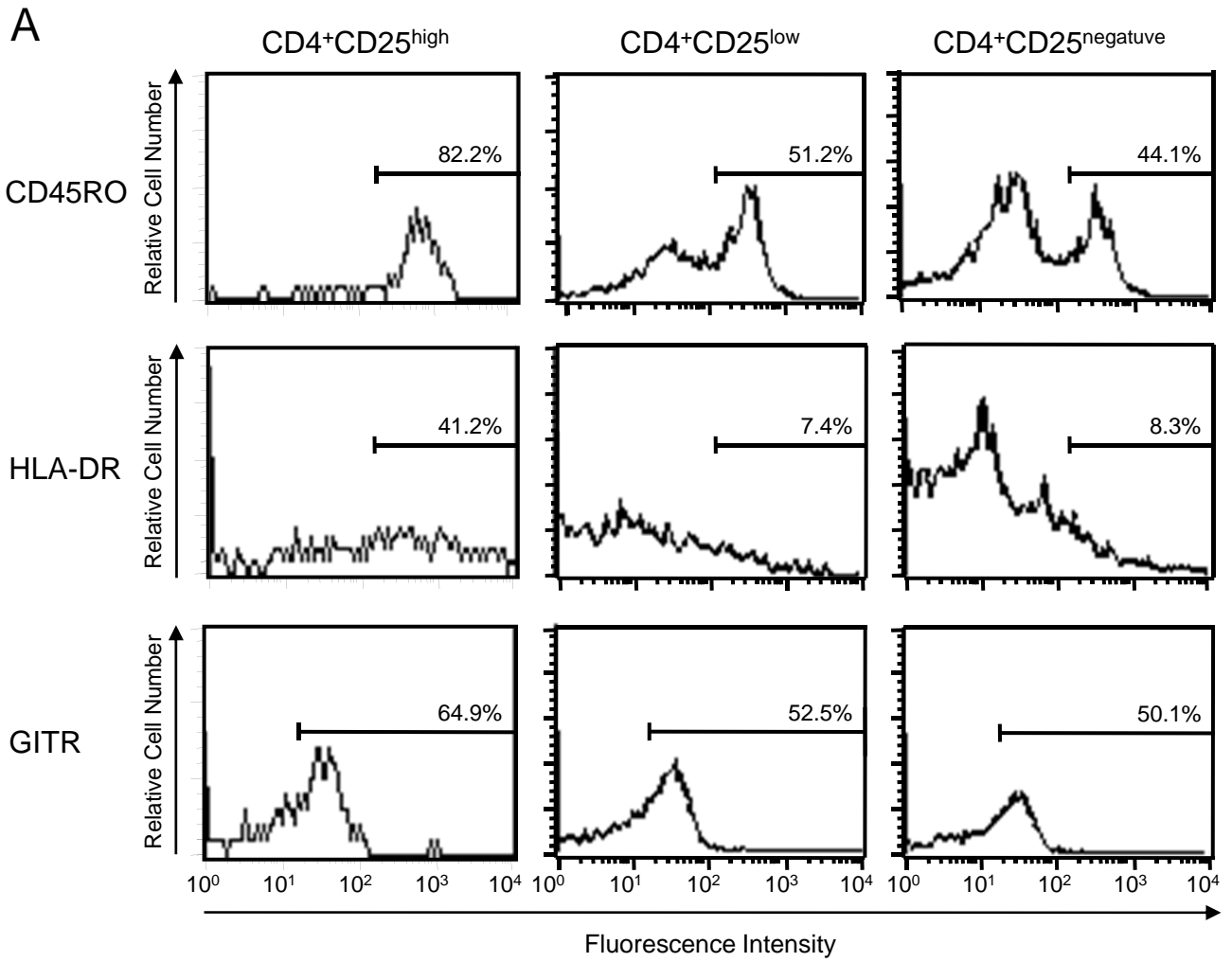


Fig. 3B

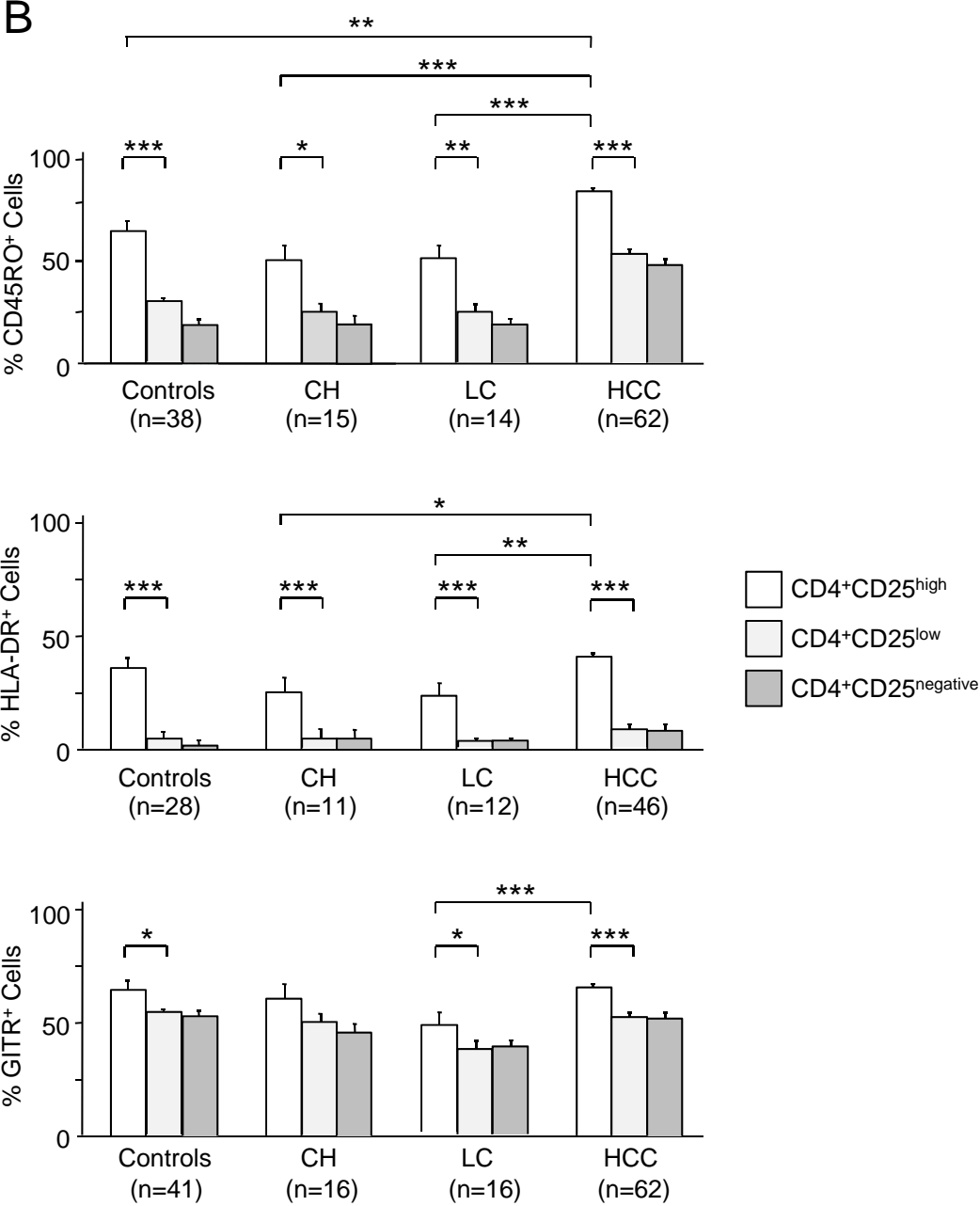


Fig. 4A

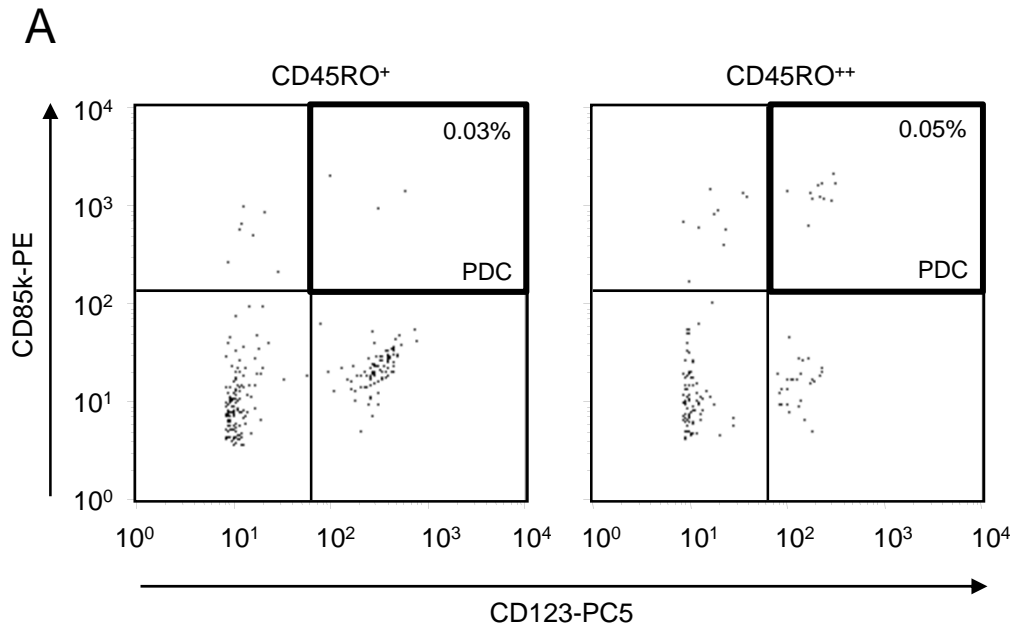


Fig.4B

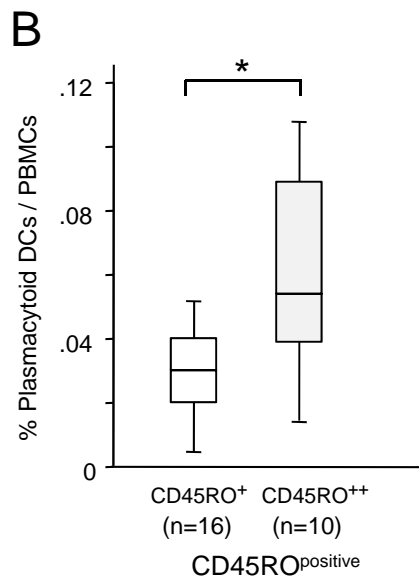


Fig. 5

