Frequency of CD45RO+ subset in CD4+CD25high regulatory T cells associated with progression of hepatocellular carcinoma

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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 hepatocelluar 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-β1 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 fleshly 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. Apropriate 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℃ in a humidified CO_2 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. Apropriate 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 ± 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 $CD45RO^+$, $HLA\text{-}DR^+$ 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 expess 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. Fleshly 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 $CD45RO^+$, $HLA\text{-}DR^+$ 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 $CD45RO^{positivee}$ 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 cacinoma (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

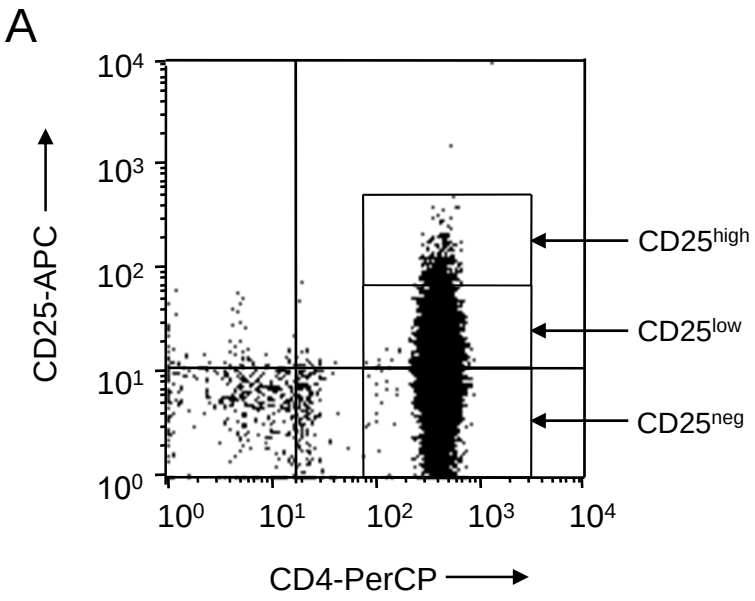

A
CD25-APC
CD4-PerCP
10^0
10^1
10^2
10^3
10^4
CD25high
CD25low
CD25neg

Fig. 1B

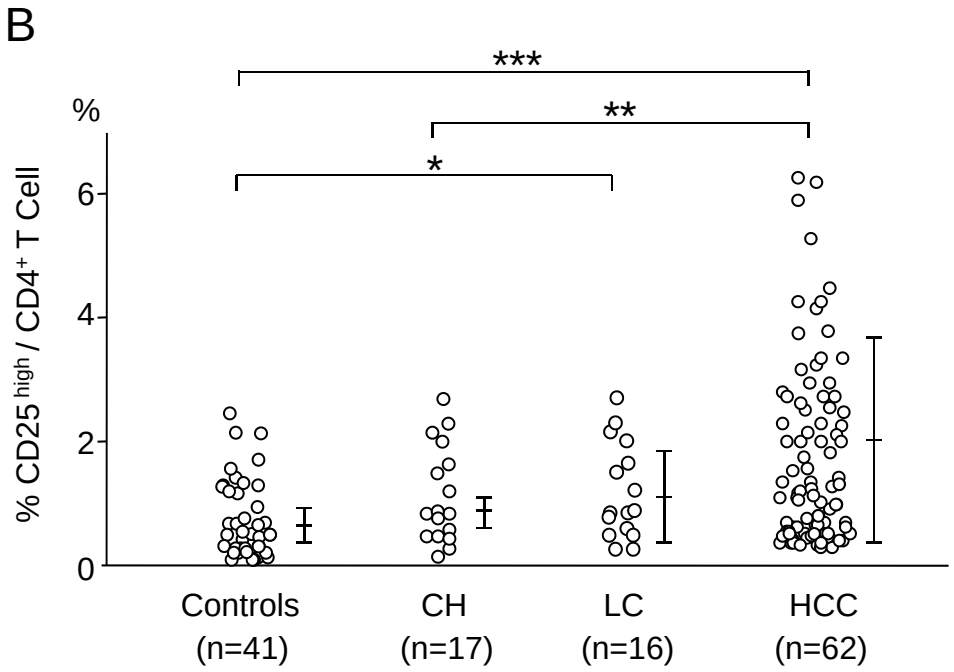


Fig. 2A

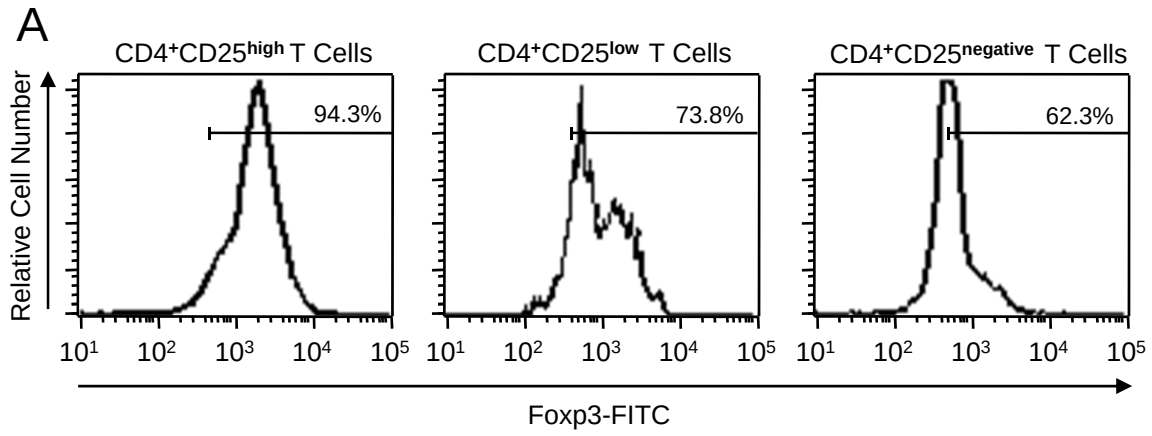

A
CD4+CD25high T Cells
CD4+CD25low T Cells
CD4+CD25negative T Cells
94.3%
73.8%
62.3%
Relative Cell Number
10¹ 10² 10³ 10⁴ 10⁵
Foxp3-FITC

Fig. 2B

B

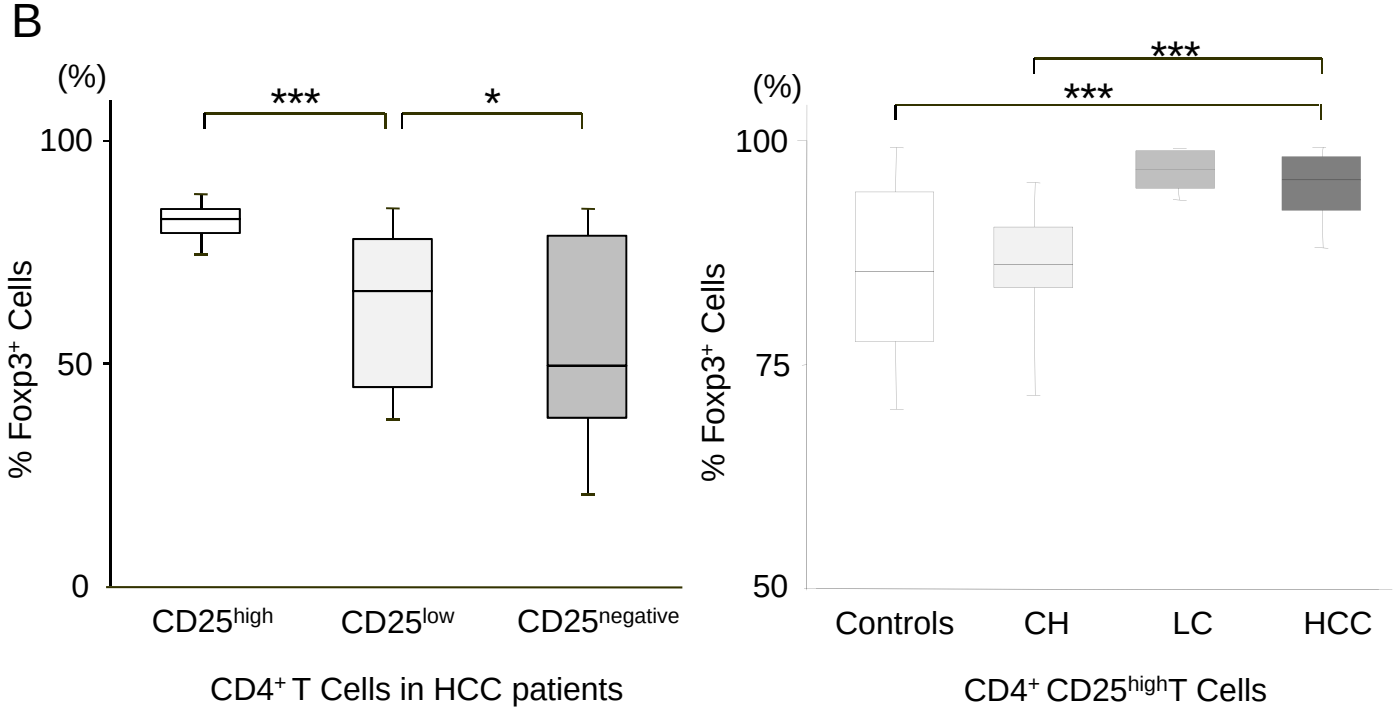

(%)
***
*
100
50
0
% Foxp3+ Cells
CD25high
CD25low
CD25negative
CD4+ T Cells in HCC patients
(%)
***
***
100
75
50
% Foxp3+ Cells
Controls
CH
LC
HCC
CD4+ CD25highT Cells

Fig. 2C

D

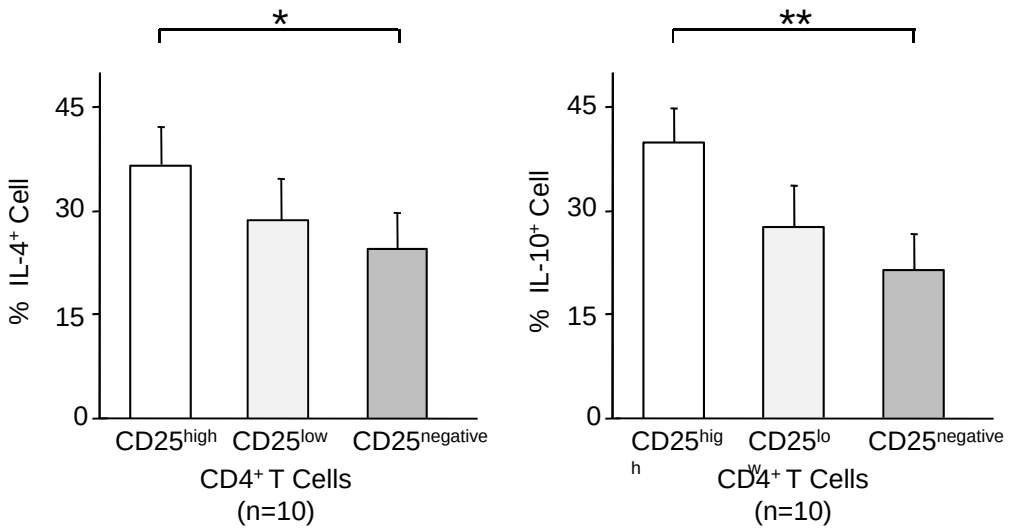

*
45
30
15
0
% IL-4+ Cell
CD25high
CD25low
CD25negative
CD4+ T Cells
(n=10)
**
45
30
15
0
% IL-10+ Cell
CD25high
CD25low
CD25negative
CD4+ T Cells
(n=10)

Fig. 2D

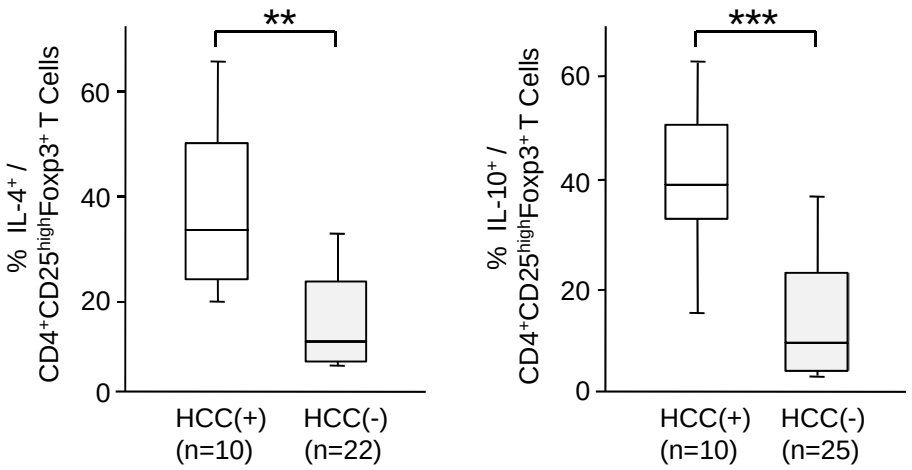

**
% IL-4+ / CD4+CD25highFoxp3+ T Cells
60
40
20
0
HCC(+) (n=10)
HCC(-) (n=22)
***
% IL-10+ / CD4+CD25highFoxp3+ T Cells
60
40
20
0
HCC(+) (n=10)
HCC(-) (n=25)

Fig. 3A

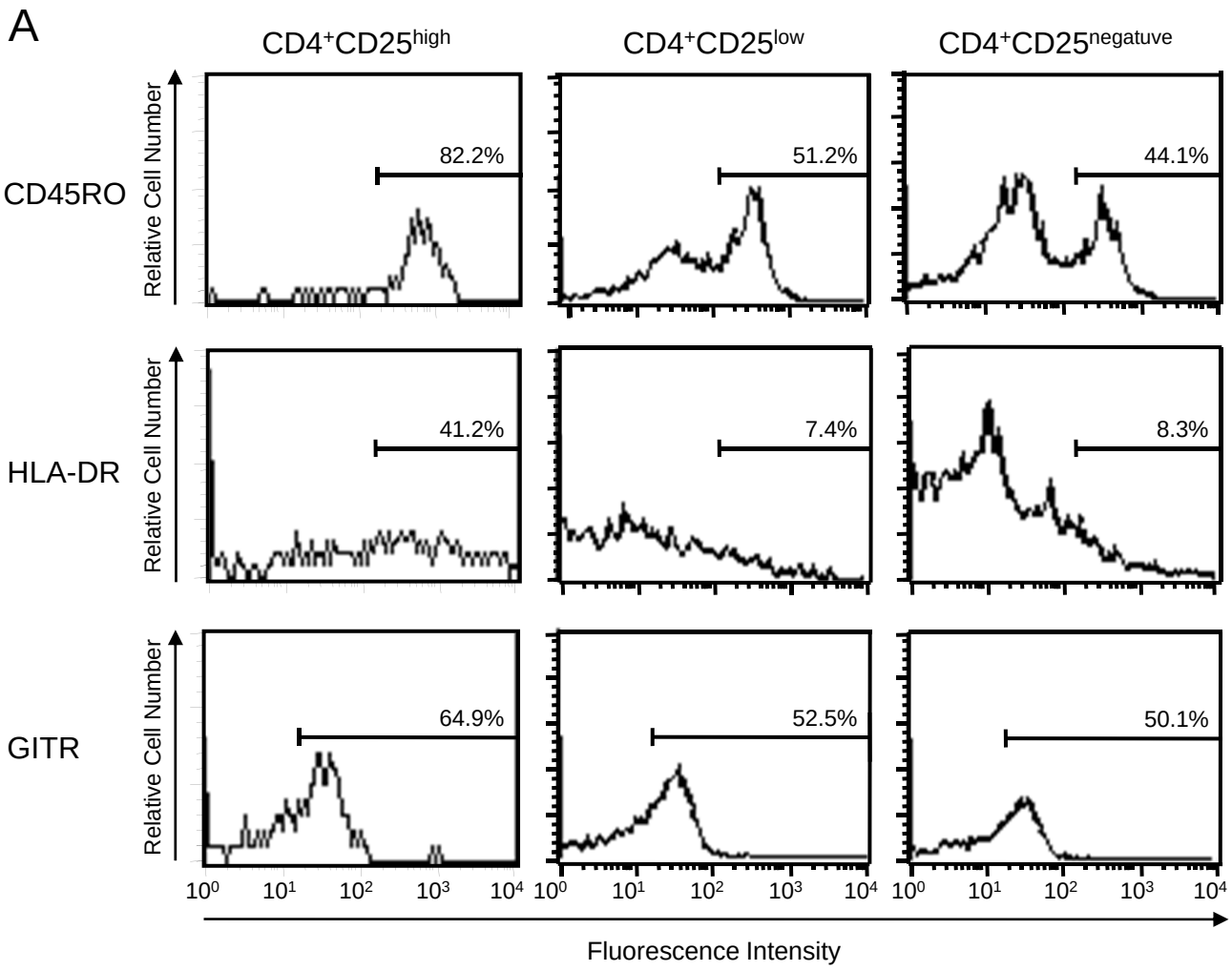

A
CD4+CD25high
CD4+CD25low
CD4+CD25negatuve
CD45RO
HLA-DR
GITR
Relative Cell Number
82.2%
51.2%
44.1%
41.2%
7.4%
8.3%
64.9%
52.5%
50.1%
10^0 10^1 10^2 10^3 10^4
Fluorescence Intensity

Fig. 3B

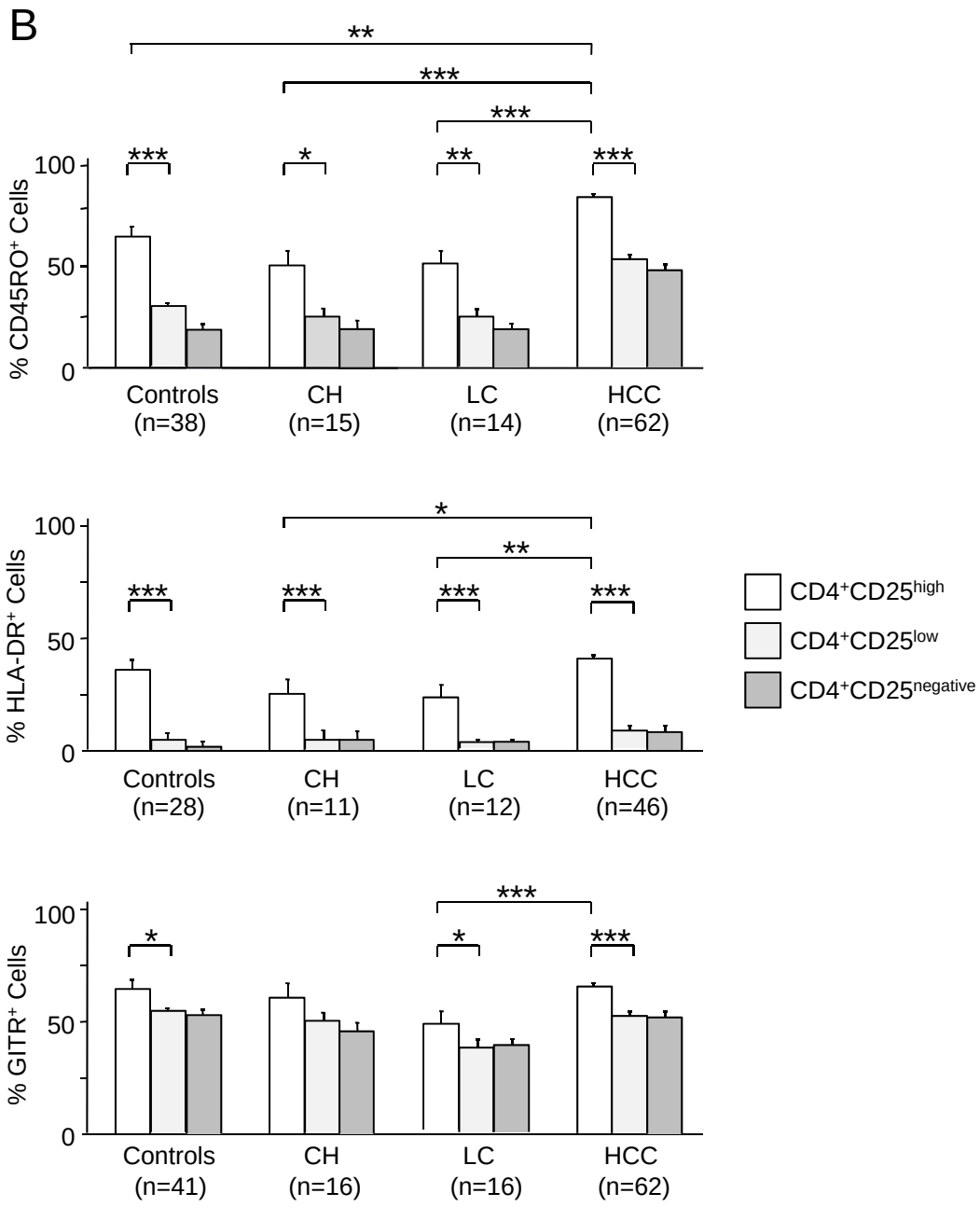

B
**
***
***
***
*
**
***
100
50
0
% CD45RO+ Cells
Controls (n=38)
CH (n=15)
LC (n=14)
HCC (n=62)
*
**
***
***
***
***
% HLA-DR+ Cells
Controls (n=28)
CH (n=11)
LC (n=12)
HCC (n=46)
***
*
*
***
% GITR+ Cells
Controls (n=41)
CH (n=16)
LC (n=16)
HCC (n=62)
CD4+CD25high
CD4+CD25low
CD4+CD25negative

Fig. 4A

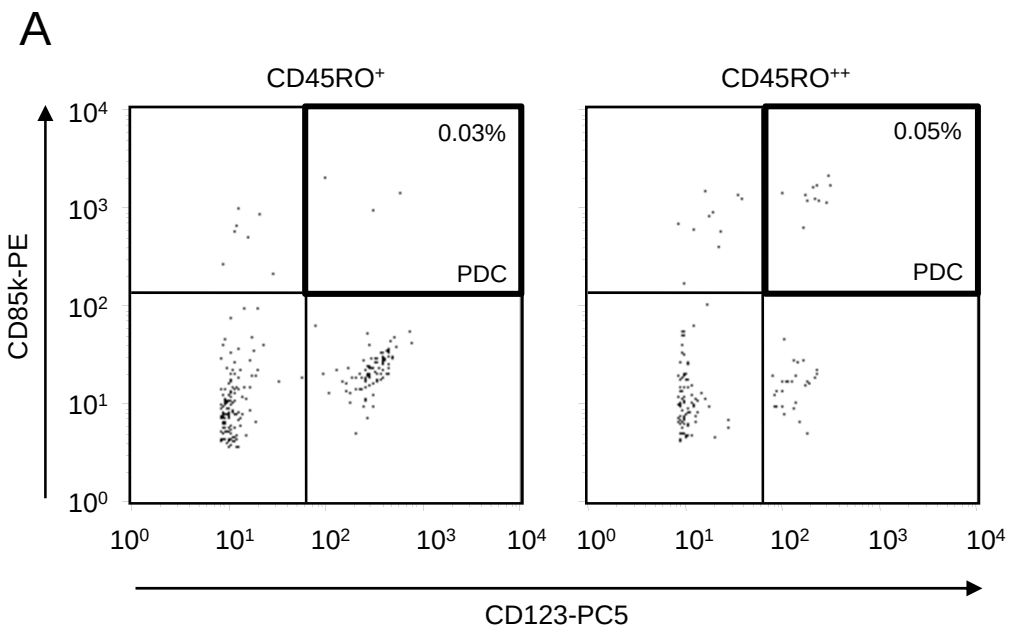

A
CD45RO+
CD45RO++
0.03%
PDC
0.05%
PDC
CD85k-PE
CD123-PC5
10^0
10^1
10^2
10^3
10^4

Fig.4B

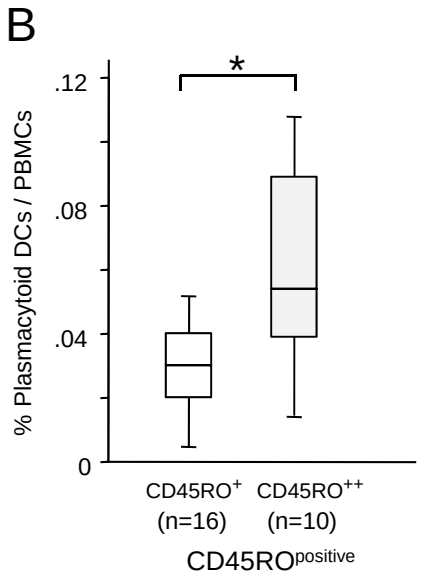
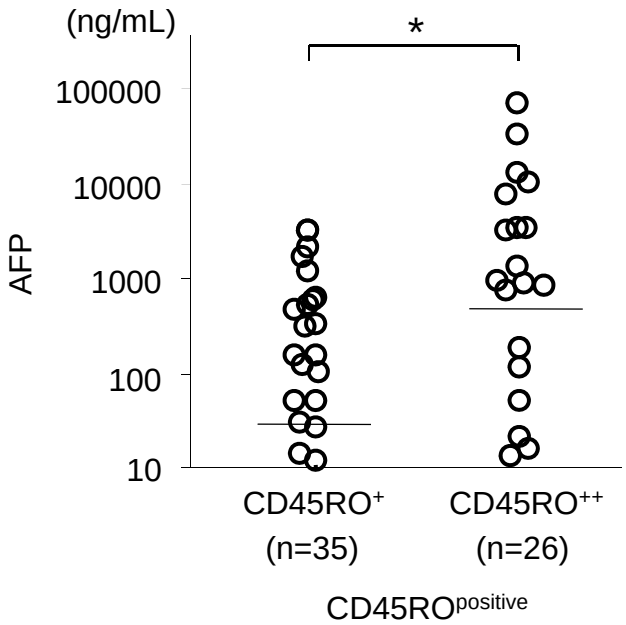
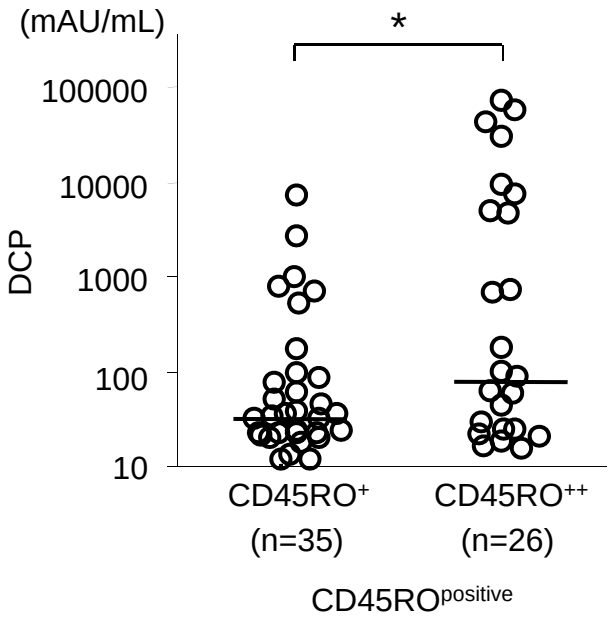
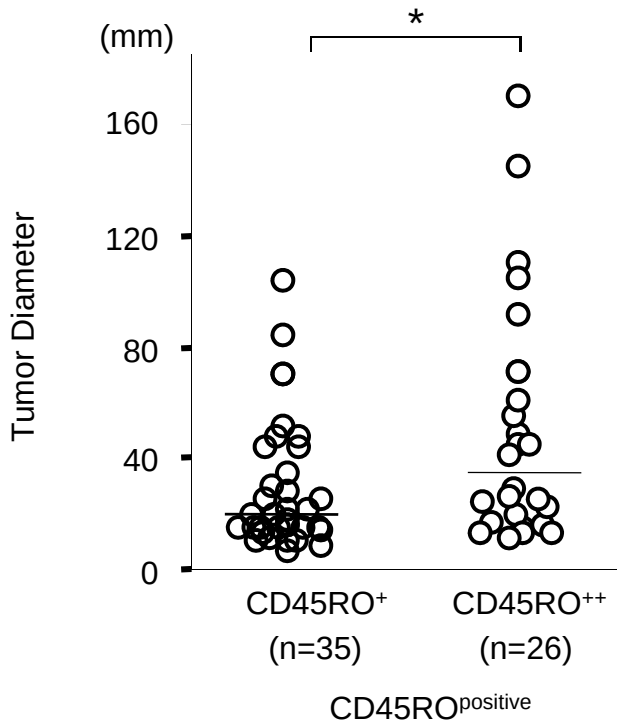

B
*
.12
.08
.04
0
% Plasmacytoid DCs / PBMCs
CD45RO+
(n=16)
CD45RO++
(n=10)
CD45ROpositive

Fig. 5


(ng/mL)
*
100000
10000
1000
100
10
AFP
CD45RO+
(n=35)
CD45RO++
(n=26)
CD45ROpositive


(mAU/mL)
*
100000
10000
1000
100
10
DCP
CD45RO+
(n=35)
CD45RO++
(n=26)
CD45ROpositive


(mm)
*
160
120
80
40
0
Tumor Diameter
CD45RO+
(n=35)
CD45RO++
(n=26)
CD45ROpositive