Circulating T Cell Profiling of Lymphedema Reveals Upregulated Immune Checkpoint Molecules and Preserved Stem Cell-Like Memory T Cells

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Abstract

Lymphedema is a common complication seen in patients with cancer who undergo lymph node dissection. Lymphedema is a debilitating progressive condition that severely restricts the quality of life owing to the accompanying cellulitis and angiosarcoma, which suggest that immune dysfunction is associated with lymphedema. However, the immune status of peripheral T cells during lymphedema remains poorly understood. Using the peripheral blood T cells of patients with lymphedema and age-matched healthy controls (HC), we compared the profile of various functional T cell subsets in this study. Expression of PD-1, Tim-3, and Lag-3 increased in peripheral CD4 + but not CD8 + T cell populations, and the proportion of regulatory T cells was higher in lymphedema patients than in HC. The proportions of naïve CD4 + and CD8 + T cells were lower in lymphedema patients than in HC, although proportions of stem cell-like memory T cell subsets were constant. These observations suggest the distinct immunosuppressive status of patients with lymphedema, which might be related to the development of the accompanying cellulitis and angiosarcoma. This study might provide new insights into the late complication after undergoing lymph node dissection as a cancer treatment.

Introduction

Lymphedema results in swelling of the limbs because of lymph retention following resection, radiotherapy, and lymph node dissection as part of cancer therapy. It is estimated that 20–40% of patients who undergo treatment for solid malignancies such as breast cancer, melanoma, gynecological or urologic tumors, or sarcomas go on to develop lymphedema [1, 2]. Patients with lymphedema develop progressive fibroadipose deposition in the affected limb and have an increased risk of developing infections and secondary malignancies, suggesting the coexistence of immune dysfunction [3]. Tissue fibrosis in lymphedema occurs due to a lymphatic leak with impaired lymphatic pumping and decreased formation of collateral lymphatics [3, 4]. Lymphatic flow disorder is associated with tissue/organ fibrosis. In particular, Th2-biased inflammatory responses promote tissue fibrosis by increasing collagen deposition and decreasing collagen breakdown [5–7]. A mouse model of axillary lymph node dissection demonstrated increasing infiltration of CD4 + T cells and regulatory T cells (Tregs) in lymphedematous tissues [8–13]. These studies showed that depletion of CD4 + T cells reduces chronic inflammation associated with lymphedema, while depletion of Treg aggravates inflammation and exacerbates lymphedema development. Depletion of other inflammatory cell types such as cytotoxic T cells, B cells, and macrophages either has no significant effect or worsens the severity of lymphedema. Thus, it is strongly suggested that there is some correlation between T cells and fibroadipose deposition in lymphedema.

Cellulitis is one of the most devastating complications for patients with lymphedema, and it has a high recurrence rate [14]. Moreover, at prolonged durations, lymphedema may develop into an angiosarcoma. The life expectancy with this condition is usually in the limited range of a few months to 2 years [15]. Increased risk of cellulitis and angiosarcoma has been considered a manifestation of immune dysfunction related to lymphedema. Previous studies have suggested that T cells may play a key role in progressive fibroadipose deposition in lymphedema; however, the immunoprofiling of T cell populations in lymphedema has not been thoroughly investigated. Most studies have been performed in animal models, while a few studies have defined T cell modulation in human lymphedema. To improve our understanding of the immune status in patients with lymphedema, more detailed investigation of peripheral T cells is warranted.

Regulatory pathways that limit the immune response of T cells are becoming increasingly well-characterized. Some of the well-studied immune regulatory molecules are programmed cell death protein 1 (PD-1), T-cell immunoglobulin

domain and mucin-3 (Tim-3), and lymphocyte activation gene-3 (Lag-3), which are collectively called immune checkpoint molecules or exhaustion markers [16, 17]. In chronic viral infection, PD-1, Tim-3, and Lag-3 expressions on T cells remain high, and these T cells become “exhausted”, with progressive loss of effector function and proliferative capacity taking place [18]. Further, conventional Tregs explicitly regulate the activation, proliferation, and effector functions of activated effector T cells to determine the outcomes of several immunological events, ranging from infectious diseases to immunopathology and autoimmunity [19]. Furthermore, naïve phenotype T cells are important to initiate an effective reaction to a target antigen. When naïve T cells react with cognate antigens during the immune response, a small proportion of the responding cells survives to form antigen-specific memory T cells [20]. The naïve T cell number represents the adaptive immune system’s potential to sense and respond to foreign pathogens and to the mutant proteins expressed by malignant cells [21]. Thus, T cells are assumed to play a crucial role in lymphedema pathophysiology. However, at least to our knowledge, no prior studies have focused on T cell subpopulations in the peripheral blood of patients with lymphedema.

The investigation of T cells in lymphedema is essential for a better understanding of immune alteration in patients undergoing lymph node dissection as a cancer treatment. The principal aim of this study was to elucidate the characteristics of peripheral CD4 + and CD8 + T cell subpopulations in lymphedema in comparison with those of healthy controls (HC) using T cell surface and intracellular markers.

Results

PD-1, Tim-3, and Lag3 expression on peripheral CD4 + and CD8 + T cells in patients with lymphedema and healthy controls

To understand the role of the exhaustion marker on CD4 + T-cells in lymphedema, we examined the expression patterns of PD-1, Tim-3, and Lag3 on peripheral CD4 + T cells in patients with lymphedema and HCs. Results of assays of PD-1, Tim-3, and Lag3 expression on CD4 + T cells showed a significantly higher population in the patients with lymphedema than in the HC, with the mean ± SEM at 32.1 ± 11.1 % vs 22.2 ± 6.8 % (p = 0.03) (Fig. 4A), 2.69 ± 1.61 % vs 1.44 ± 0.56 % (p = 0.02) (Fig. 4B), and 0.72 ± 0.31 % vs 0.43 ± 0.18 % (p = 0.02) (Fig. 4C), respectively (Table 2).

Similar to the investigation of exhaustion markers on CD4 + T cells, we next examined the expression patterns of PD-1, Tim-3, and Lag3 on peripheral CD8 + T cells in lymphedema and HC samples. In contrast to the results seen in the CD4 + populations, PD-1, Tim-3, and Lag3 expression on circulating CD8 + T cells did not exhibit significant differences between patients with lymphedema and HC, with the mean ± SEM at 20.6 ± 9.1 % vs 17.1 ± 6.2 % (p = 0.45) (Fig. 5A), 6.98 ± 4.03 % vs 4.40 ± 2.51 % (p = 0.08) (Fig. 5B), and 1.47 ± 0.86 % vs 0.98 ± 0.56 % (p = 0.14) (Fig. 5C), respectively (Table 2).

Treg population in patients with lymphedema and healthy controls

We compared the Treg population with three distinct subpopulations (Treg□, Treg□, and Treg□) between lymphedema patients and HC to understand the relationship between Treg and lymphedema (Fig. 6A). The total proportion of Foxp3-positive Treg (Treg□ + Treg□ + Treg □) in CD3 + CD4 + T cells was significantly higher in patients with lymphedema than in HC, with mean ± SEM at 5.49 ± 2.39 % vs 3.45 ± 1.96 % (p = 0.04), respectively (Fig. 6E). Although the proportion of Treg□ in CD3 + CD4 + T cells was similar between patients with lymphedema and HCs, with mean ± SEM at 0.65 ± 0.33 % vs 0.74 ± 0.48 % (p = 0.95), respectively (Fig. 6B) (Table 3), it was notable that Treg□ and Treg□ proportions in CD3 + CD4 + T cells were significantly higher in patients with lymphedema than in HC,

with mean ± SEM at 1.35 ± 0.91 % vs 0.55 ± 0.35 % ($p < 0.01$) (Fig. 6C) and 3.49 ± 1.59 % vs 2.15 ± 1.34 % ($p = 0.03$) (Fig. 6D), respectively.

Comparison of naïve and memory phenotype CD4 + T cells between patients with lymphedema and healthy controls

To understand the features of the functional subsets in peripheral CD4 + T cells in lymphedema, we compared the proportions of naïve and memory phenotypes among whole CD4 + T cells (Table 4). FACS representative data are described in Fig. 7A. Although no statistical differences were found in CM, EM and TE subpopulations between patients with lymphedema and HC, the proportions of naïve phenotype and naïve-like phenotype CD4 + T cells in patients with lymphedema were significantly lower than those in HC, with mean ± SEM at 40.7 ± 17.5 % vs 55.2 ± 10.6 % ($p = 0.04$) and 30.2 ± 15.5 % vs 46.0 ± 14.0 % ($p = 0.01$) (Fig. 7B), respectively. Naïve-like phenotype comprises three subpopulations: naïve, Tscm and Tmnp. We further analyzed ratios of those subsets among CD4 + naïve-like phenotype cells in patients with lymphedema and in HC (Fig. 7C). The proportion of naïve phenotype CD4 + T cells was significantly lower in patients with lymphedema patients than in HC, with mean ± SEM at 73.8 ± 19.6 % vs 83.2 ± 17.7 % ($p = 0.03$), respectively. In contrast, the proportion of Tscm in naïve-like CD4 + T cells was significantly higher in patients with lymphedema than in HCs, with mean ± SEM at 10.5 ± 6.0 % vs 5.4 ± 2.2 % ($p = 0.01$), respectively. The proportions of Tmnp in naïve-like CD4 + T cells were similar between the two groups.

Comparison of naïve and memory phenotype CD8 + T cells between patients with lymphedema and healthy controls

We also compared the naïve and memory subpopulations among CD8 + T cells (Table 5). FACS representative data are described in Fig. 8A. The proportion of naïve and naïve-like CD8 + T cells in patients with lymphedema was significantly lower than that in HC (mean ± SEM, 7.5 ± 7.7 % vs 13.4 ± 10.0 % for naïve cells, $p = 0.04$, respectively; 26.3 ± 17.8 % vs 42.2 ± 11.6 % for naïve-like cells, $p = 0.02$, respectively), while CM, EM and TE phenotype subsets did not show statistically significant differences (Fig. 8B). Similar to the analysis of CD4 + cells, we analyzed naïve, Tscm, and Tmnp ratios among CD8 + T cells with naïve-like phenotype in patients with lymphedema and in HC (Fig. 8C). Although no significant differences were found in the naïve and Tmnp subsets, the proportion of Tscm in naïve-like CD8 + T cells was significantly higher in the lymphedema group than in the HC, with mean ± SEM at 21.7 ± 13.3 % vs 10.0 ± 5.2 % ($p < 0.01$), respectively.

Discussion

In this study, we analyzed fresh peripheral blood samples and characterized the expression pattern of T cell markers in patients with lymphedema as a consequence of surgical cancer treatment. The expression of PD-1, Tim-3, and Lag3 and the frequency of Treg on CD4 + T cells were significantly upregulated in patients with lymphedema, compared with those in HC. On the other hand, we could not find any relationship between exhaustion markers on CD8 + T cells and lymphedema. Notably, for both CD4 + and CD8 + T cells, the proportions of the naïve phenotype subsets were decreased in patients with lymphedema patients, while the fraction of Tscm in the naïve cell-like phenotype was increased, suggesting that the Tscm compartment was retained under the concurrent shrinkage of naïve-like cell pools. To our knowledge, this is the first report of detailed observations of peripheral T cells in patients with lymphedema. Investigation of functional T cell landscapes in lymphedema is crucial for a deeper understanding of the immune status underlying this unique medical complication. Moreover, these findings might influence on prudent decision of cancer treatment, particularly in the case of the lymph node dissection.

The PD-1 regulatory pathway plays indispensable roles in downregulating the immune response, and in promoting tolerance to self-antigens by suppressing T cell activation through B7-CD28 co-stimulatory molecules that deliver

critical inhibitory signals [22]. In the context of autoimmune diseases, PD-1 is known as a protective molecule that acts by inducing apoptosis in activated effector T cells and reducing the apoptosis of regulatory T cells [23]. Tim-3, a member of the T cell Ig and mucin domain-containing molecule superfamily, is a key regulatory molecule for Th1 response. Initial studies on Tim-3 demonstrated its upregulation in activated Th1 cells [24–26]. The expression of Tim-3 in peripheral T cells is associated with the regulation of autoimmune encephalomyelitis (EAE) and rheumatoid arthritis [27, 28]. Lag-3 exhibits high affinity to major histocompatibility complex class II (MHC II) and regulates the proliferation, activation, and function of T cells [29, 30]. In this study, the expression of PD-1, Tim-3, and Lag3 on CD4 + T cells was significantly upregulated in patients with lymphedema, compared with that in HC. Importantly, these molecules are also known as markers of Treg subsets that possess enhanced immunosuppressive function [31, 32]. Previous studies on the immunopathology of lymphedema have shown that CD4 + T cells play a role in aggravating tissue fibrosis and lymphatic dysfunction [8–11]. Upregulated exhaustion markers on CD4 + T cell populations in lymphedema might reflect not only chronic consumption of effector CD4 + T cells, but also the counterbalancing enhancement of Treg function to inhibit the progress of tissue inflammation and fibrosis. This hypothesis is supported by our observations in the present study that the proportions of Treg II and Treg III, but not those of Treg I, were more significantly elevated in patients in lymphedema patients than in HC.

Treg cells compete for the T cell growth factor IL-2 via expression of high-affinity IL-2 receptor complexes and exert direct suppressive activity by secreting immunosuppressive cytokines such as TGF-β and IL-10 [33]. Treg □ cells are able to proliferate themselves upon T cell receptor stimulation and can convert to Treg □ cells [26]. The Treg □ subset is functionally important in regard to its potent suppressive function, which is caused due to its high expression of CTLA-4 and CD25 and its higher sensitivity to IL-2 than that of other Treg subpopulations [26]. Treg □ secretes a high amount of effector cytokines (IL-2, IL-17, and IFN-γ) without suppressive activity [19, 34]. Treg □ cells may be a heterogeneous subset between Treg cells and effector T cells. After dissection of the lymph node in an animal model, Treg infiltration is increased in lymphedematous tissues [12]. The accumulation of Treg in the skin subsequently impairs bacterial phagocytosis because of the development of a Treg-mediated immunosuppressive environment [12]. This observation suggests that, in the presence of chronic lymphedema, circulating Treg I may be prone to conversion into Treg □, which can swiftly downregulate local inflammation.

Naïve T cells are subjected to an abnormal drive for differentiation under the pressure of IL-6 and TNF [35]. Given that age strongly affects the number of circulating naïve T cells in healthy individuals, the proportions of naïve fraction observed in patients need to be age-matched [36, 37]. Irrespective of age, the reduction of naïve T cells was reported in patients with active rheumatoid arthritis [36] and autoimmune colitis [38], implying that such abnormalities may be common to immune-mediated inflammatory diseases. In this study, the number of naïve T cells was more significantly decreased in patients with lymphedema than in age-matched HC. The progressive depletion of the naïve pool induces homeostatic proliferation, where the naïve T cells turn over and differentiate into memory T cells [39]. Naïve T cell numbers and clonal diversity represent the potential of the adaptive immune system to sense and respond to foreign pathogens and to the mutant proteins expressed by malignant cells [21]. Loss of naïve T cells could leave many individuals vulnerable to infection.

Tscm cells exhibit enhanced proliferative capacity compared with that of CM phenotype T cells, the potential to differentiate into all other classically defined T cell memory subsets, and the ability to retain their phenotype following proliferation *in vitro* and *in vivo* model [40, 41]. Tscm cells are increased in chronic disease states. Specifically, Tscm cells are enhanced in individuals with type 1 diabetes [42], uveitis [43], systemic lupus erythematosus [44], cardiovascular disease [45], and rheumatoid arthritis [46]. Tscm frequency might correlate with disease activity and might indicate immune therapies [43]. In the present study, the proportion of the Tscm

population was retained even though the number of naïve phenotype cells was significantly decreased in CD4 + and CD8 + T cells. Such a Tscm pool might function as a reservoir of effector T cells relevant to chronic inflammation induced by environmental factors in lymphedema.

All patients in this study had history of surgical resection of cancer without relapse. A dramatic reduction in PD-1 expression and peripheral frequencies of Treg to the normal level was observed within weeks after surgical resection of the primary cancer [47, 48]. It is plausible that the expression patterns of T cells in the present study are contributed to by lymphedema, and not by the history of primary cancer.

Circulating T cell proportion in patients with lymphedema was investigated using several expression markers in the present study. It is well known that patients with lymphedema often experience cellulitis without definite skin injury. The pathogenesis and progression of the symptoms are very rapid, and redness and local fever quickly spread throughout the whole extremity within an hour, which completely differs from usual cellulitis without lymphedema [49]. Stewart and Treves reported that angiosarcomas associated with lymphedema develop after 9 years (range 8–24) from the onset of lymphedema [50]. The median survival has been quoted as 24 months, with an overall 5-year survival rate of approximately 10% [15]. Only early radical surgical removal, including amputation or disarticulation of the affected limb, or wide excision at an early stage offers the greatest chance of long-term survival. Frequent incidences of cellulitis and angiosarcoma cause heavy deterioration in the quality of life of patients with lymphedema. Because we believed that these pathological changes were associated with T cell modulation, we investigated circulating T cells using several expression markers in patients with lymphedema. We found that the exhaustion markers of CD4 + T cells and Treg were upregulated, and the naïve phenotype on CD4 + and CD8 + T cells was decreased in patients with lymphedema patients. These findings may be the result of the immune dysfunction that accompanies lymphedema.

Our study has some limitations. First, a small number of patients is always a cause of bias in this type of study. Second, we lack data on the similar profiling of T cells in age-matched patients with cancer but without lymphedema; therefore, we could not exclude the effects of pre-existing cancer from those of the lymphedema itself. In addition, the relationships between clinical conditions and therapeutic strategies need to be elucidated. A large-scale study in the future is warranted to assess the function of T cells in lymphedema more clearly.

In conclusion, the expression of PD-1, Tim-3, and Lag-3 in peripheral CD4 + T cells, and the relative proportions of Foxp3-positive Treg cells were upregulated in patients with lymphedema, compared with those in HC. The skewed number of naïve phenotype T cells and the increased frequency of Tscm both in naïve-like CD4 + and CD8 + T cells subsets were characteristic of the patients with lymphedema in our study. These distorted profiles of circulating T cells may be related to the frequent cellulitis and rare cases of angiosarcoma seen in patients with lymphedema. Our results may assist in designing new approaches for cancer treatment with the help of translational immunology.

Methods

Patients and healthy controls

This prospective study included patients with a diagnosis of lymphedema and age- and sex-matched healthy volunteers. Written informed consent was obtained according to the protocol approved by the institutional review board of Hiroshima University (number: E-1413). Lymphedema was diagnosed by histological examination and indocyanine green (ICG) lymphography [51] (Fig. 1). We excluded patients who underwent lymphatic venous anastomosis for the treatment of lymphedema [52] and those with comorbid medical disorders (heart failure, renal

failure, hepatic failure, endocrine abnormality, immunological disease). Data on the type of cancer that caused lymphedema, the radiation therapy used for cancer, duration of edema, frequency of cellulitis, and the Campisi clinical staging of lymphedema [53] were obtained for each patient (Table 1). Peripheral blood samples were collected from 13 patients with lymphedema and 11 HC. All patients underwent surgical resection of cancer with dissection of lymph nodes and were free from relapse. The median age of the patients was 54 years (range 39–70) and that of the HC was 52.5 years (range 39–66) ($p = 0.90$). The median duration of lymphedema was 42 months (range 1–120). Five patients had a history of cellulitis, of whom two experienced cellulitis more than 10 times. The characteristics of the patients are described in Table 1.

Preparation of peripheral blood mononuclear cells (PBMCs)

A 10 mL aliquot of fresh peripheral blood was collected from each patient and HC. Blood samples were also collected from the lymphedema-affected limb of the patients. Peripheral blood mononuclear cells (PBMCs) isolated with Lymphoprep gradient (Axis-Shiel PoC AS, Oslo, Norway) were used for each experiment after confirming viability > 95%, as determined by trypan blue staining.

Flow cytometry analysis

Cell surface marker staining of freshly isolated PBMCs was performed using appropriate combinations of mouse fluorescein-conjugated anti-human antibodies against the following molecules: CD3-fluorescein isothiocyanate (FITC), CD4-Brilliant Violet 510 (BV510), CD8-PerCP, PD-1-allophycocyanin (APC), Tim3-BV421, Lag3-phycoerythrin (PE), CD4-APC/Cyanine7 (APC/Cy7), CD8-PE/Cy7, CCR7-BV510, CD45RA-BV421, CD45RO-FITC, CD95-APC, and CXCR3-BV421 (all from Biolegend, San Diego, CA, USA,) and CD49d-PE (eBioscience, San Diego, CA, USA). Briefly, cell suspensions (1×10^6 to 2×10^6) were incubated with a cocktail of these antibodies in the dark for 30 min at 4°C. Intracellular staining of Foxp3 was performed with the FOXP3 Fix/Perm Buffer Set (Biolegend) according to the manufacturer's instructions. After surface labelling, PBMCs were permeabilized in 1 mL of fixation/permeabilization buffer at 20°C for 45 min in the dark. Next, the samples were stained with the anti-human FoxP3 monoclonal antibody and incubated in the dark for 30 min at 20°C. Isotype-matched control antibodies were used to establish background levels of staining. 7-Aminoactinomycin D and Zombie-NIR (Biolegend) were used to identify and exclude dead T cells. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACS Canto Ⅱ flow cytometer (Becton Dickinson and Company (BD), San Jose, CA, USA). Data were analyzed using the DIVA software (BD) and FlowJo (Flowjo LLC, San Jose, CA, USA).

Gating strategy and expression profiles of PD-1, Tim-3, and LAG-3 in peripheral CD4 + and CD8 + T cells are described in Fig. 2A. It is now accepted that Treg cells are heterogeneous in phenotype and function, with three distinct subpopulations identified in human peripheral blood [54, 55]: Treg I (CD45RA + FOXP3lo), Treg II (CD45RA-FOXP3hi), and Treg III (CD45RA- FOXP3lo) cells (Fig. 2B). Surface expression of T-cell memory markers, including CCR7, CD45RO, CD95, CD49d, and CXCR3, was used to judge the differentiation status of cells. We examined the compartmentalization of naïve cells (CCR7 + CD45RO-CD95-CXCR3-), memory T cells with a naïve phenotype (Tmnp; CCR7 + CD45RO-CD95-CD49d + CXCR3+), stem-cell like memory T cells (Tscm; CCR7 + CD45RO-CD95+), naïve-like T cells (CCR7 + CD45RO-), central memory (CM) cells (CCR7 + CD45RO+), effector memory (EM) cells (CCR7-CD45RO+), and terminal effectors (TE) (CCR7-CD45RO-). The gating strategy for differentiated memory T cells is described in Fig. 3.

Statistical analysis

The JMP statistical software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. Mann–Whitney U tests were used to compare the cell proportions of interest between the two groups. Two-sided p values less than

0.05 were considered statistically significant.

Declarations

Data Availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

Hirofumi Imai and Takakazu Kawase contributed to the design of this study, executed the experiments and wrote the manuscript.

Tatsuo Ichinohe and Isao Koshima contributed by inspecting the study and by proofreading the manuscript.

All other authors contributed to this study by collecting the information about patients.

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Tables

Table I. Clinical characteristics of patients

F: Female

Table II. Comparison of PD-1, Tim-3, and Lag-3 expression (%) on CD4+ and CD8+ T cells between lymphedema and healthy controls (HC)

	CD4+ T cells			CD8+ T cells		
	PD-1	Tim-3	Lag-3	PD-1	Tim-3	Lag-3
HC	22.2 (range, 9.1–34)	1.43 (0.55–2.41)	0.43 (0.14–0.69)	17.1 (9.3–23.7)	4.40 (1.53–9.54)	0.98 (0.32–2.03)
Lymphedema	32.1 (19.8–51.1)	2.69 (0.89–6.69)	0.72 (0.25–1.40)	20.6 (11.1–40.8)	6.95 (1.68–11.8)	1.47 (0.33–3.55)
P value	0.03	0.02	0.02	0.45	0.08	0.14

Table III. Comparison of percentage of regulatory T cell (Treg) subsets among CD3+CD4+ T cells (%) between lymphedema and healthy controls (HC).

Case	Age (years)	Sex	Lymphedema site	Cause of lymphedema	Radiotherapy for cancer	Duration of lymphedema (months)	Cellulitis	Campisi clinical staging
1	47	F	Right lower limb	Ovarian cancer	(-)	48	10 times	2b
2	57	F	Right lower limb	Uterine cancer	(-)	4	None	1
3	54	F	Left lower limb	Uterine cancer	(+)	96	30 times	2b
4	39	F	Left lower limb	Uterine cancer	(-)	24	6 times	2a
5	42	F	Left lower limb	Uterine cancer	(+)	1	None	1
6	58	F	Bilateral lower limb	Uterine cancer	(-)	120	Once	2a
7	70	F	Left lower limb	Bladder cancer	(-)	36	None	2b
8	49	F	Right upper limb	Pharyngeal cancer	(+)	72	None	2b
9	63	F	Left upper limb	Breast cancer	(-)	108	None	2b
10	46	F	Left lower limb	Adrenal cancer	(-)	60	Twice	2b
11	49	F	Right upper limb	Breast cancer	(+)	4	None	2b
12	56	F	Right upper limb	Uterine cancer	(-)	12	None	2a
13	66	F	Right lower limb	Breast cancer	(-)	5	None	2a

	Treg□	Treg□	Treg□	Foxp3+
HC	0.75 (range, 0.26–1.56)	0.55 (0.01–0.97)	2.15 (0.25–4.43)	3.45 (0.61–6.56)
Lymphedema	0.65 (0.23–1.41)	1.35 (0.23–3.41)	3.49 (1.53–6.61)	5.50 (2.10–8.95)
P value	0.95	0.005	0.03	0.04

Table IV. Comparison of naïve and memory phenotype frequencies in CD4+ T cells between lymphedema and healthy controls (HC).

	% of CD4+ T cells						
	Naive	Tscm	Tmnp	Naïve like	CM	EM	TE
HC	46.0 (range, 22.1–62.0)	2.93 (1.40–6.39)	6.21 (0.65–31.2)	55.2 (34.8–61.9)	23.0 (5.32–40.9)	20.7 (11.8–32.5)	1.18 (0.40–2.5)
Lymphedema	30.2 (11.2–51.7)	4.09 (1.73–14.1)	6.44 (1.41 –13.8)	40.7 (18.0–72.5)	29.3 (15.2–44.7)	28.4 (7.80–44.7)	1.72 (0.40–4.5)
P value	0.01	0.31	0.87	0.04	0.05	0.13	0.95
	% of CD4+ naive T cells						
HC	83.2 (3.71–93.2)	5.41 (3.38–10.7)	11.3 (1.87–52.2)				
Lymphedema	73.8 (26.2–91.0)	10.5 (4.20–22.9)	15.7 (5.12–68.1)				
P value	0.03	0.01	0.07				

Tscm: stem-cell like memory T cell

Tmnp: memory T cells with naïve phenotype

CM: central memory

EM: effector memory

TE: terminal effector

Table V. Comparison of naïve and memory phenotype frequencies in CD8+ T cells between lymphedema and healthy controls (HC).

	% of CD8+ T cells						
	Naive	Tscm	Tmnp	Naïve like	CM	EM	TE
HC	13.4 (range, 1.00–34.2)	4.35 (1.32–9.11)	24.4 (8.08–32.8)	42.2 (19.1–59.5)	4.95 (3.00–10.4)	29.8 (9.30–45.3)	23.0 (11.8–34.5)
Lymphedema	7.5 (0.32–22.5)	4.82 (0.80–9.29)	13.2 (4.57–46.0)	26.3 (6.9–64.2)	5.92 (2.40–11.2)	33.9 (12.3–73.2)	33.9 (10.2–71.8)
P value	0.04	0.43	0.03	0.02	0.58	0.62	0.28
	% of CD8+ naïve like T cells						
HC	30.6 (4.70–74.2)	9.97 (5.03–21.5)	59.4 (17.5–88.9)				
Lymphedema	25.8 (4.60–62.1)	21.7 (11.1–48.5)	52.6 (21.5–83.8)				
P value	0.41	0.003	0.49				

Tscm: stem-cell like memory T cell

Tmnp: memory T cells with naïve phenotype

CM: central memory

EM: effector memory

TE: terminal effector

Figures

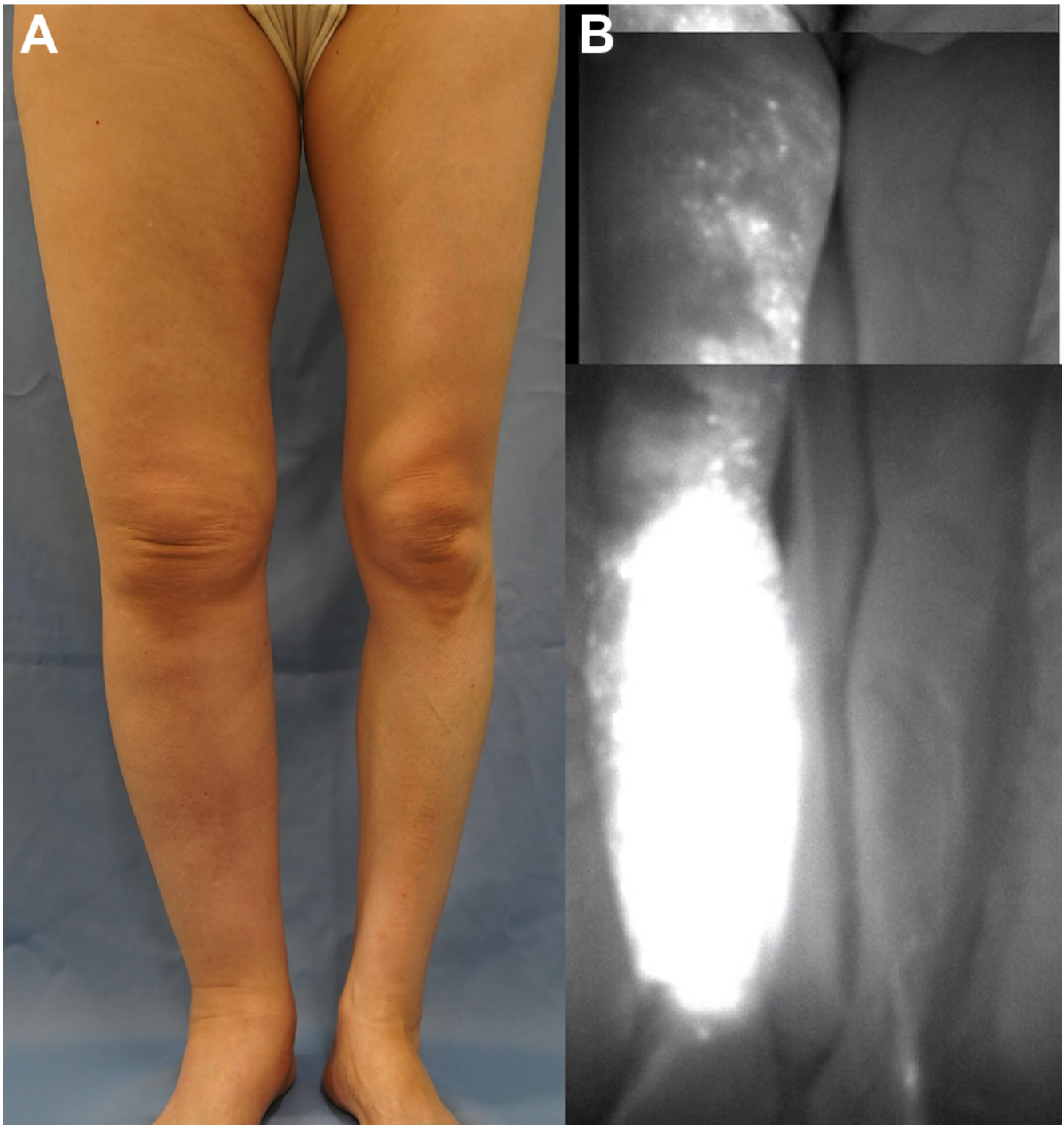


Figure 1

Edematous condition of patients with right lower limb lymphedema. A. Compared with those on the left side, the circumferences are higher on the right side. B. The indocyanine green lymphology shows marked dermal backflow on the right side, while a linear pattern can be noted on the left side

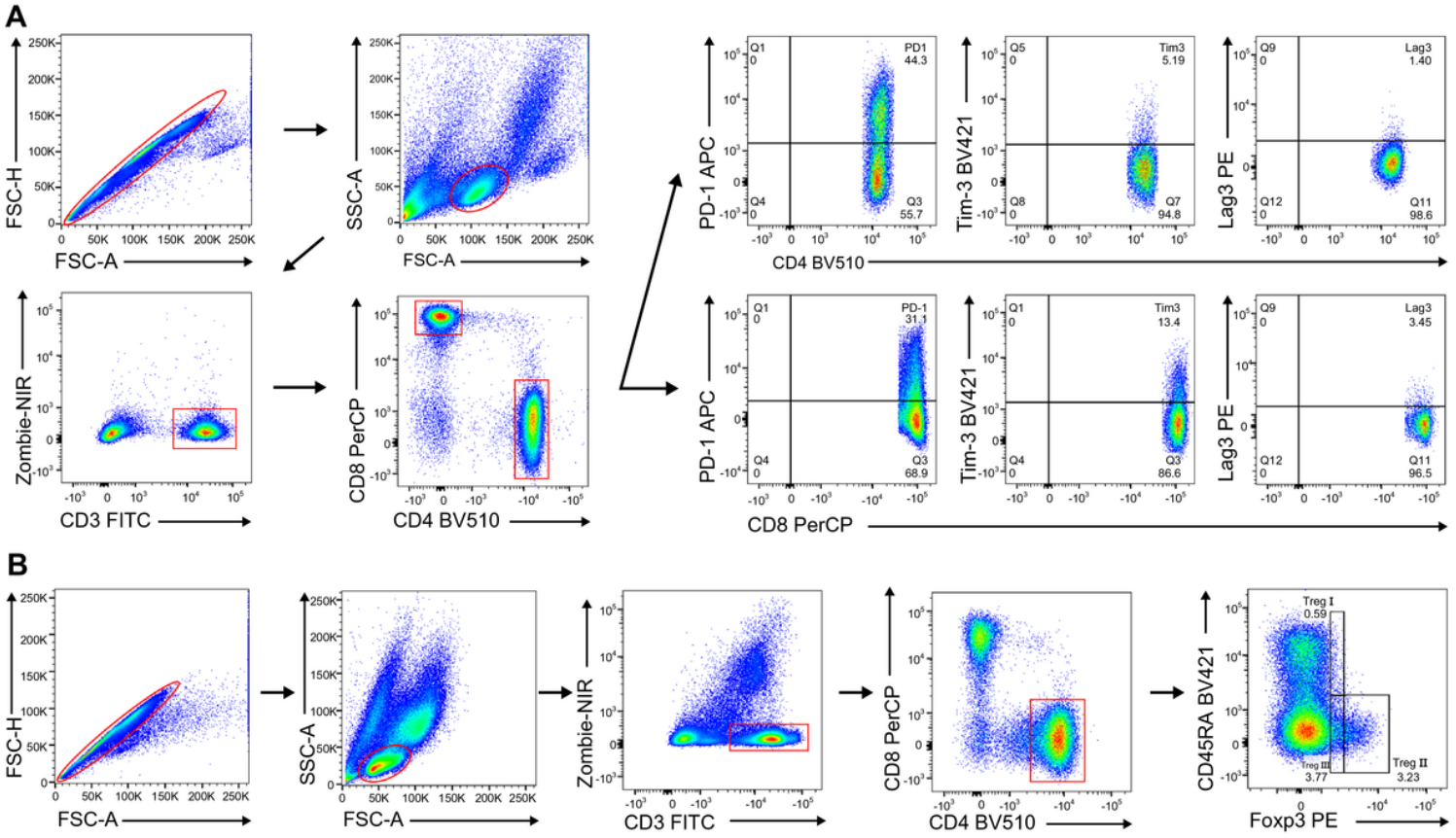
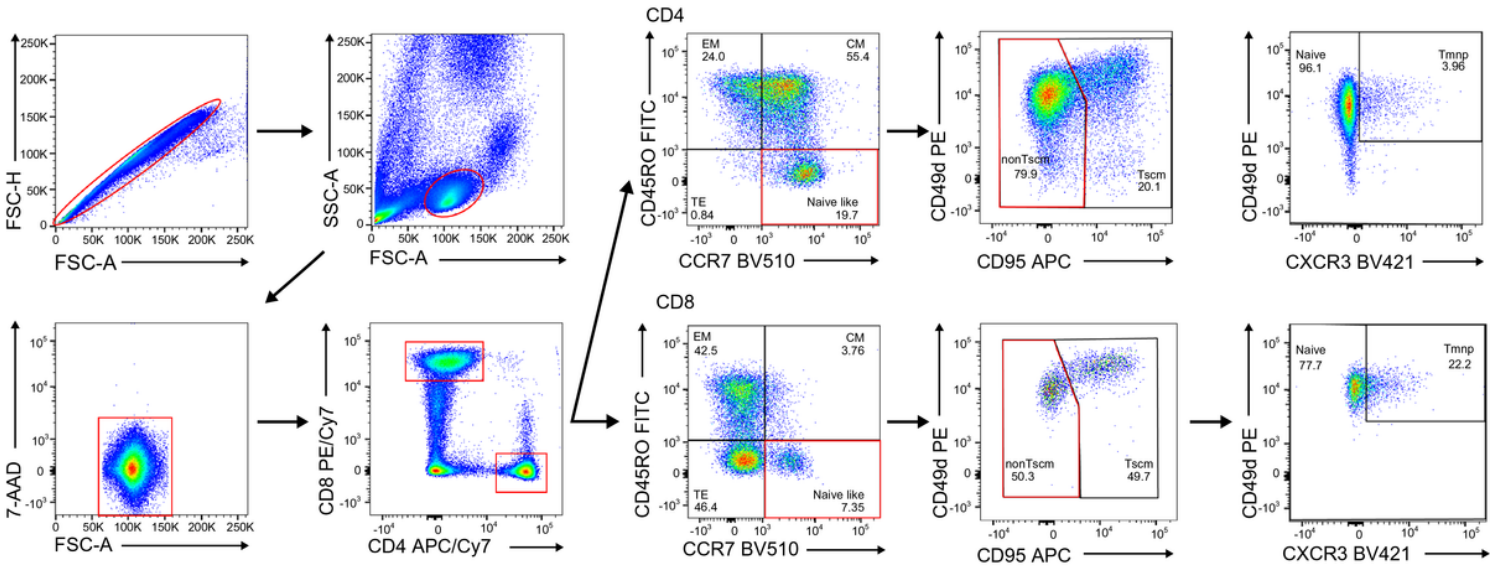


Figure 2

Gating strategy and expression profiles of PD-1, Tim-3, and LAG-3 in peripheral CD4+ and CD8+ T cells and regulatory T cells (Treg) of lymphedema. A. PD-1, Tim-3, and LAG-3 in peripheral CD4+ and CD8+ T cells. B. Treg in peripheral CD4+ T cells.



Naïve cell	CCR7+CD45RO-CD95-CXCR3
Stem-cell like memory T cell (Tscm)	CCR7+CD45RO-CD95+
Memory T cells with a naïve phenotype (Tmnp)	CCR7+CD45RO-CD95-CD49d+CXCR3+
Naïve like T cell	CCR7+CD45RO-
Central memory (CM)	CCR7+CD45RO+
Effector memory (EM)	CCR7-CD45RO+
Terminal effector (TE)	CCR7-CD45RO-

Figure 3

Gating strategy and expression profiles of naïve and memory phenotype on CD4+ T cells in lymphedema.

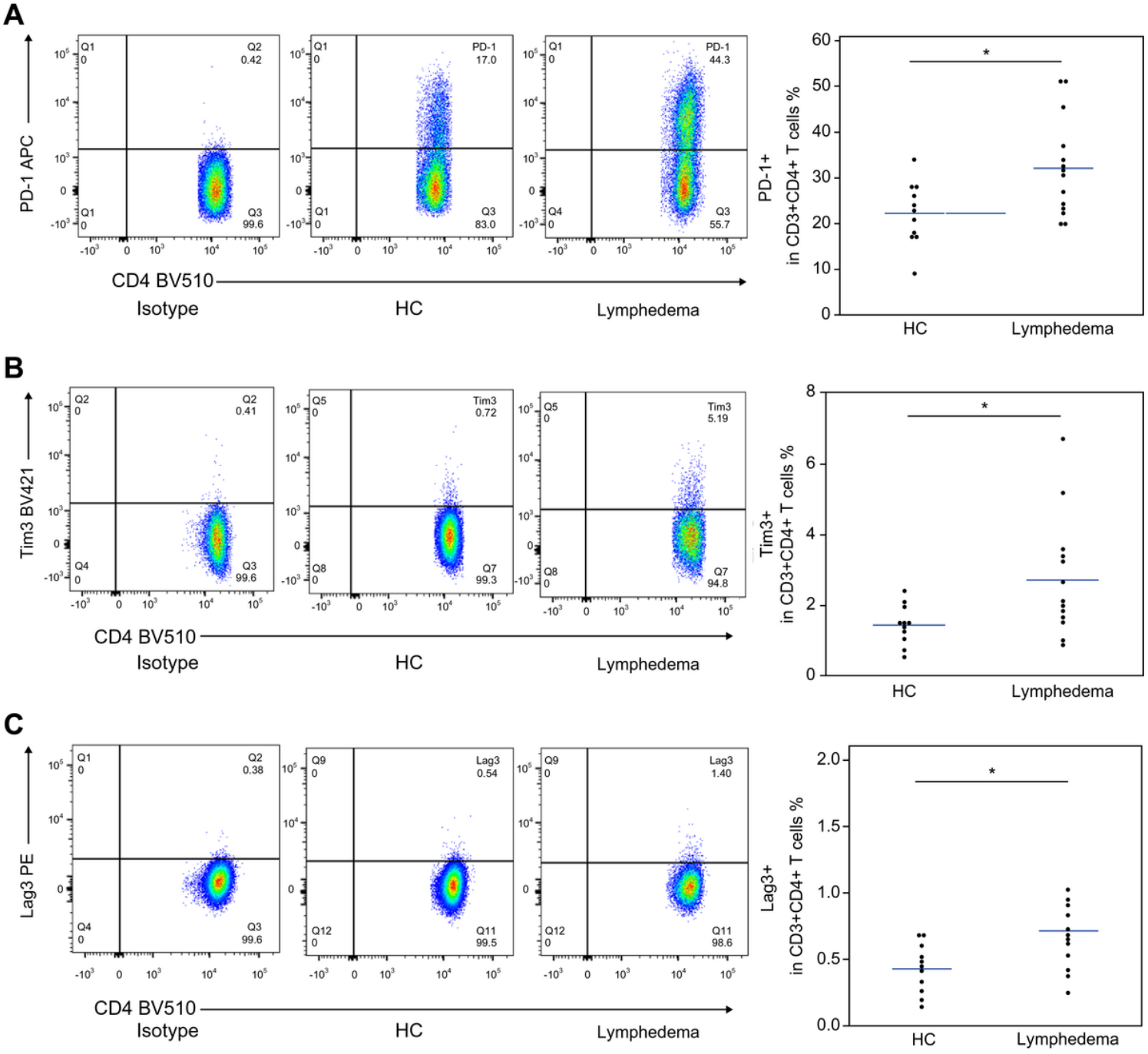


Figure 4

The comparison of PD-1, Tim-3, and Lag-3 expression in CD4+ T cells between patients with lymphedema and healthy controls (HC). A. Representative fluorescence-activated cell sorting data and flow cytometry percentage of PD-1 expression on CD4+ T cells from patients and HCs. B. percentage of Tim-3 expression on CD4+ T cells C. percentage of Lag-3 expression on CD4+ T cells. *, Results were considered statistically significant at $p < 0.05$.

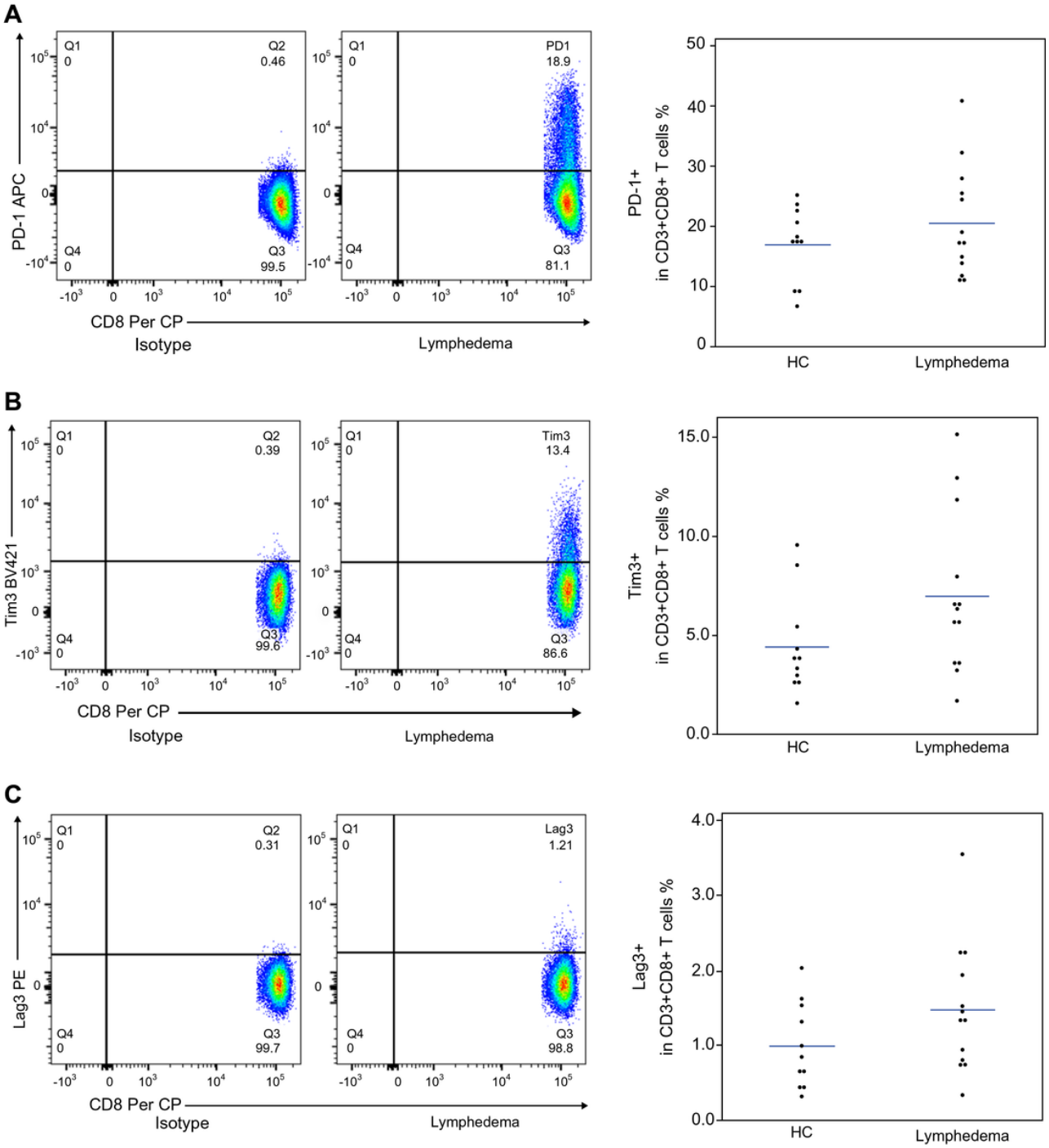


Figure 5

The comparison of PD-1, Tim-3, and Lag-3 on CD8+ T cell between patients with lymphedema and healthy controls (HC). A. Representative fluorescence-activated cell sorting data and flow cytometry percentage of PD-1 expression on CD8+ T cells from patients and HC. B. percentage of Tim-3 expression on CD8+ T cells C. percentage of Lag-3 expression on CD8+ T cells.

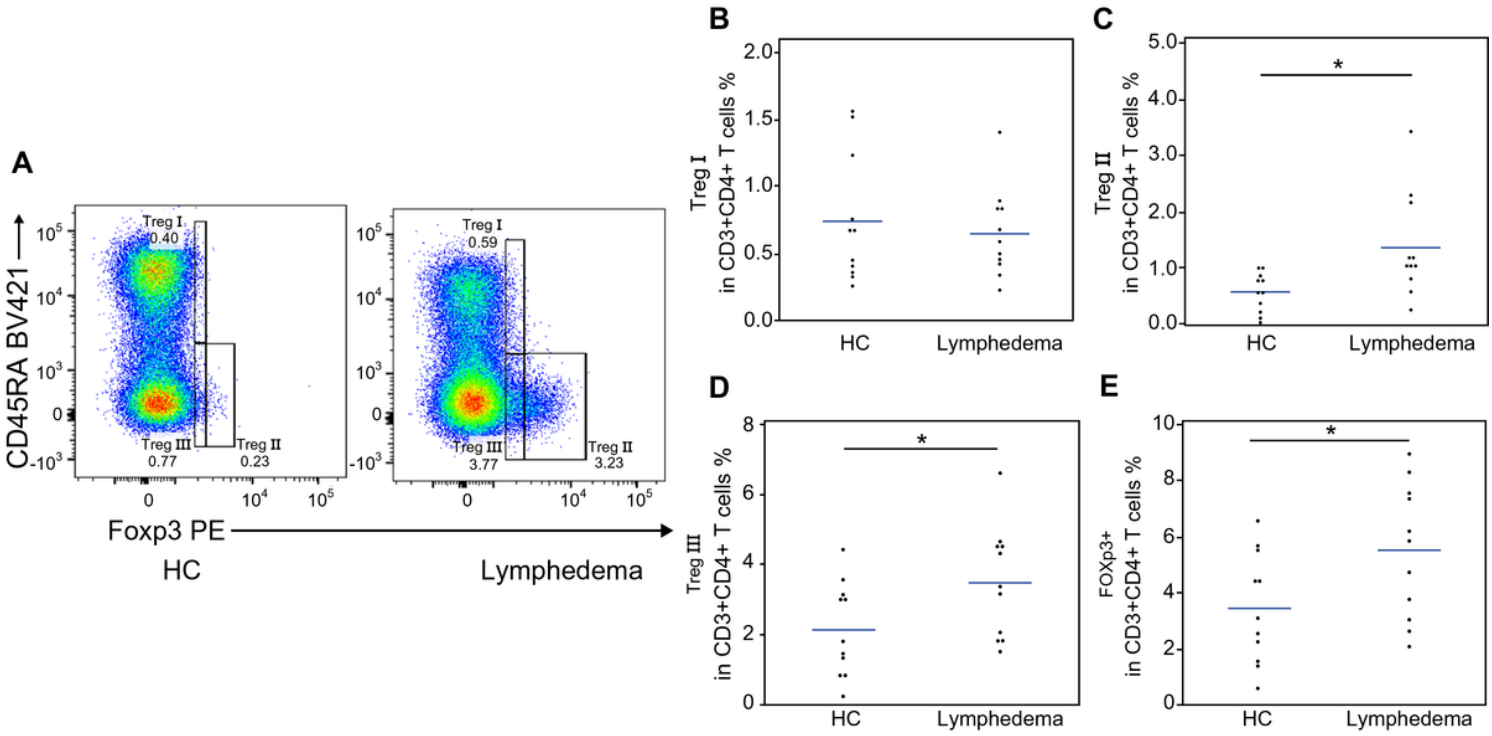


Figure 6

Percentage of Treg subsets in patients with lymphedema and healthy controls (HC). A. Representative fluorescence-activated cell sorting data of patients with lymphedema and HC. B. Treg Ⅰ in CD3+CD4+ T cells was similar in patients and HC. In contrast, Treg Ⅱ (C), Treg Ⅲ (D) and Foxp3+ (E) in CD3+CD4+ T cells were significantly higher in patients than in HC. ※, Results were considered statistically significant at $p < 0.05$.

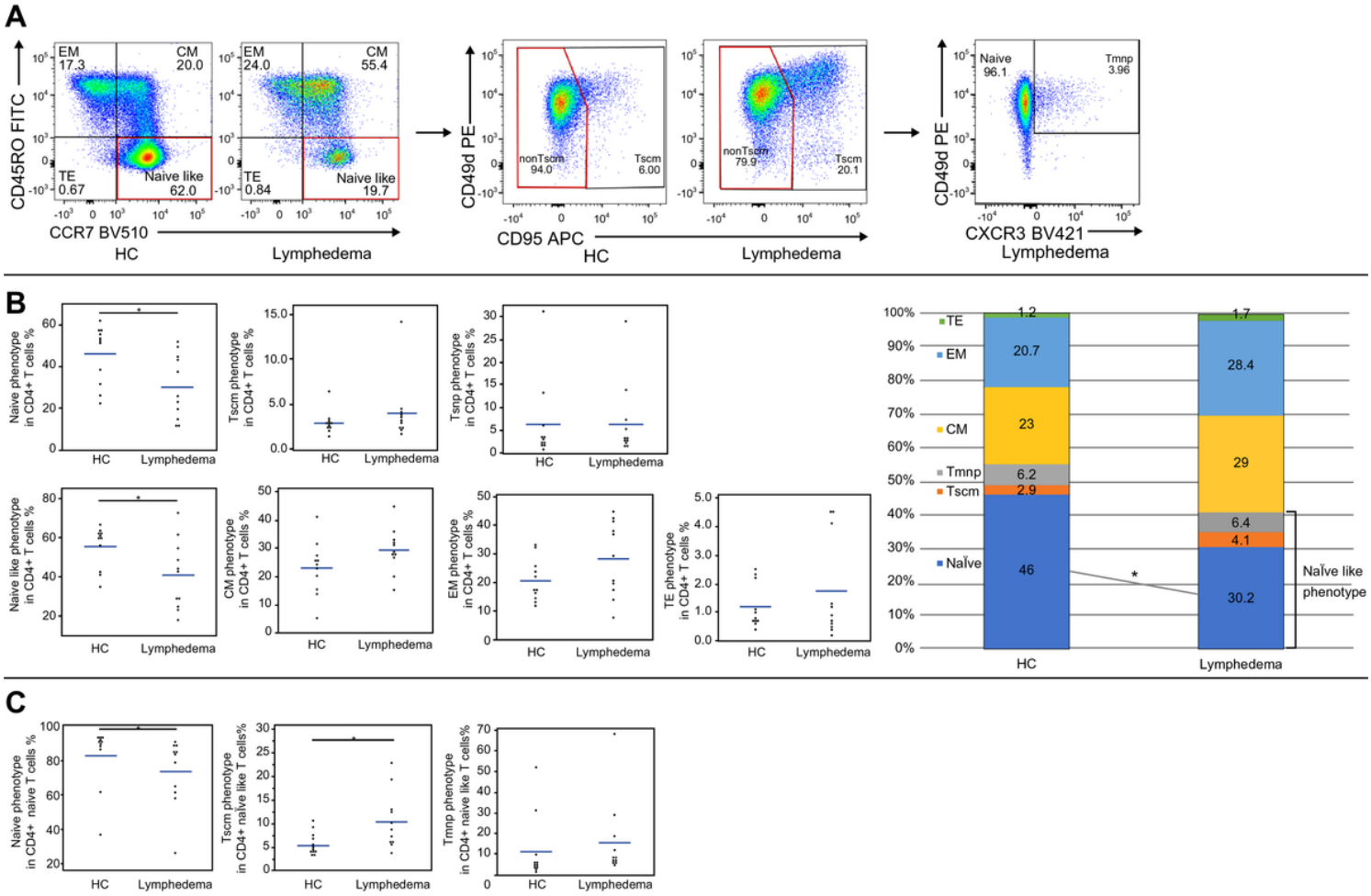


Figure 7

Naïve and memory phenotype frequencies on CD4+ T cells in patients with lymphedema and healthy controls (HC). A. Representative fluorescence-activated cell sorting data of patients with lymphedema and HC. B. naïve, stem-cell like memory T cell (Tscm), memory T cells with a naïve phenotype (Tmnp), naïve like, central memory (CM), effector memory (EM), and terminal effector (TE) frequencies in CD4+ T cells. The number of naïve and naïve like phenotypes on CD4+ T cells in patients with lymphedema had significantly reduced, compared with that in HC. C. Naïve, Tscm and Tmnp frequencies in CD4+ naïve like T cells. The frequency of naïve phenotype in CD4+ naïve like T cells was significantly reduced in patients with lymphedema compared with that in HC and the frequency of Tscm in CD4+ naïve like T cells was significantly higher in patients with lymphedema than in HC.

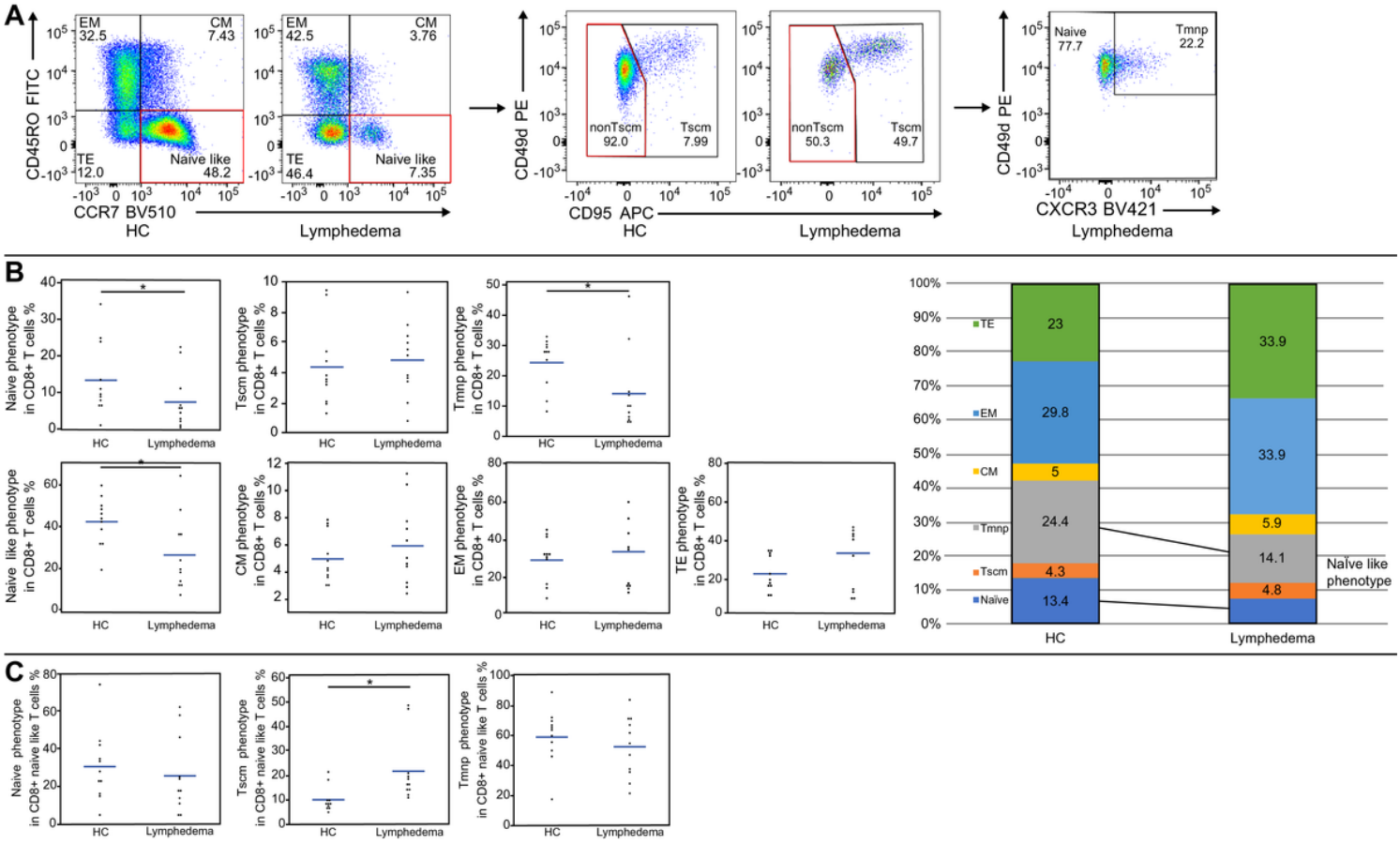


Figure 8

Naïve and memory T cell-like phenotype frequencies in CD8+ T cells in patients with lymphedema and healthy controls (HC). A. Representative fluorescence-activated cell sorting data of patients with lymphedema and HC. B. naïve, stem-cell like memory T cell (Tscm), memory T cells with a naïve phenotype (Tmnp), naïve like, central memory (CM), effector memory (EM), and terminal effector (TE) frequencies in CD8+ T cells. The frequencies of naïve, naïve like phenotype, and Tmnp on CD8+ T cells in patients with lymphedema were significantly reduced, compared with those in HC. C. Naïve, Tscm, and Tmnp frequencies in CD8+ naïve like T cells. The frequency of Tscm in CD8+ naïve like T cells was significantly higher in patients than in HC.