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ORIGINAL PAPER

Expression and anatomical distribution of TIM-containing molecules in Langerhans cell sarcoma

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Abstract Signals from the T cell immunoglobulin and mucin-domain (TIM)-containing molecules have been demonstrated to be involved in regulating the progress of carcinoma. However, the expression and anatomical distribution of TIMs in Langerhans cell sarcoma (LCS), which is a rare malignancy derived from dendritic cells of the epidermis, has yet to be determined. In this study, the expression of TIM-1, TIM-3 and TIM-4 in LCS samples were detected by immunohistochemistry. Our results showed that these three molecules were found in LCS sections. At the cellular level, these molecules were found on the cell membrane and in the cytoplasm. Immunofluorescence double-staining demonstrated that these TIMs were co-expressed with Langerin, a potential biomarker for detecting LCS. In addition, TIM-1 was also expressed on $CD68^+$ macrophages and CK-18^+ epithelial cells, while TIM-3 and TIM-4 were expressed on all cell types investigated, including $CD3^+$T cells, $CD68^+$ macrophages, $CD11c^+$ dendritic cells, $CD16^+$ NK Cells, $CD31^+$ endothelial cells and CK-18^+ epithelial cells. Interestingly, TIMs were also co-expressed with some members of the B7 superfamily, including B7-H1, B7-H3 and B7-H4 on sarcoma cells. Our results clearly showed the characteristic expression and anatomical distribution of TIMs in LCS, and a clear understanding of their functional roles may further elucidate the pathogenesis of this carcinoma and potentially contribute to the development of novel immunotherapeutic strategies.

Keywords Langerhans cell sarcoma · T cell immunoglobulin and mucin-domain containing molecules · Immunohistochemistry · Langerin · B7 superfamily

Introduction

Langerhans cell sarcoma (LCS) is a rare malignancy which has an aggressive clinical behavior. A combination of radiotherapy with chemotherapy is the best treatment for this disease. Nevertheless, the results are disappointing (Uchida et al. 2008). Currently, the diagnostic specificity for LCS has not been well determined, although Langerin (CD207) has been suggested to be a potential biomarker (Nakayama et al. 2010). It is critical to identify more specific markers for disease diagnosis.

The T cell immunoglobulin domain and mucin-domain (TIM) family is a newly described group of molecules, which have important immunological functions (Freeman et al. 2010). Three members of the TIM-containing molecules (TIMs), including TIM-1, TIM-3, and TIM-4, have been identified in humans. All these TIMs share a similar structure as type I membrane pro-teins, consisting of an N-terminal immunoglobulin variable (IgV)-like domain, a

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mucin-like domain, a transmembrane region and an intracellular tail (Rodriguez-Manzanet et al. 2009). TIM-1, an important susceptibility gene for asthma and allergy, is preferentially expressed on Th2 cells and function as a potent costimulatory molecule for T cell activation (Rennert 2011; Sonar et al. 2010). TIM-3 is preferentially expressed on T-helper 1 (Th1) cells and mediates an inhibitory signal (Sánchez-Fueyo et al. 2003; Monney et al. 2002). Furthermore, dendritic cell (DCs)-associated TIM-3 mediates phagocytosis of apoptotic cells and cross-presentation of antigen (Nakayama et al. 2009). In contrast, TIM-4 is exclusively expressed on antigen-presenting cells (APCs), and it mediates phagocytosis of apoptotic cells (Rodriguez-Manzanet et al. 2010; Miyanishi et al. 2007). In addition to playing an essential role in regulating immune responses, TIMs have been found to actively participate in the pathogenesis of several human diseases, including tumor development. For example, non-small cell lung cancer (NSCLC) patients whose tumor tissues were positive for TIM-3 had a significantly shorter survival time than those with TIM-3-negative tissues (Zhuang et al. 2012). In patients with hepatitis B virus (HBV)-associated hepatocellular carcinoma (HBV-HCC), the numbers of Tim-3^+ tumor infiltrating cells were negatively associated with patient survival (Li et al. 2012b). Moreover, the level of TIM-3 in B cell lymphoma endothelium closely correlated with both dissemination and poor prognosis (Huang et al. 2010).

Our previous work has shown that cancer-associated B7 molecules (B7s) may serve as potent prognostic biomarkers to identify LCS (Li et al. 2012a). However, uncertainty remains regarding the expression and anatomical distribution of TIMs in these patients. In this study, we analyzed the expression of both B7 molecules and TIMs in LCS samples by immunohistochemistry and further analyzed their phenotypes by immunofluorescence double-staining.

Materials and methods

Patients

Three cases of LCS samples and two cases of normal epidermis were collected at the Department of Pathology, 150th Hospital (Luoyang, Henan Province, China). The other two LCS samples were collected at the Department of Pathology, Xinqiao Hospital, Third Military Medical University (Chongqing, China). The samples in 3 cases were from skin and one sample was from the lymph nodes. The tissues were fixed in 10 % neutral buffered formalin and paraffin embedded (Li et al. 2012a, b). This study protocol was approved by the Ethics Committee of the Third Military Medical University review board.

Immunohistochemistry

The protocol used for immunohistochemistry was performed as published previously with slight modifications (Li et al. 2012a). Briefly, paraffin-embedded tissue blocks were cut into 2–3 μm sections and mounted on poly-L-lysine-charged glass slides. After the sections were dewaxed and rehydrated, antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH 6.0). The sections were cooled to room temperature (RT), and endogenous peroxidase activity was blocked by incubation with a solution of 0.5 % hydrogen peroxidase (H_2O_2) in 50 % methanol for 1 h. The sections were then incubated in 3 % BSA plus 0.1 % Nonidet P-40 in PBS for 1 h at RT to block nonspecific binding. Then, the sections wereincubated overnight at 4 °C with primary anti-TIM-1 (1:100, goat IgG, R&D Systems, San Diego, CA, USA), anti-TIM-3 (1:100, goat IgG, R&D Systems) or anti-TIM-4 (1:100, goat IgG, R&D Systems) antibodies that had been diluted in 1 % BSA. After washing, the sections were incubated with the corresponding secondary antibodies for 1 h at RT. The Vecta-stain ABC kit (Vector Laboratories, San Diego, CA, USA) was used for the avidin–biotin complex method according the manufacturer's instructions. Sections incubated with isotype-matched, concentration-matched immunoglobulin without primary antibodies were used as isotype controls. Peroxidase activity was visualized with the DAB Elite kit (K3465, DAKO, Copenhagen, Denmark), and brown coloration of tissues represented positive staining. The sections were lightly counterstained with hematoxylin, dehydrated through an ethanol series to xylene and mounted. Finally, sample sections were viewed using a light microscope (Zeiss Axioplan 2, Berlin, Germany).

Immunofluorescence double-staining

For immunofluorescence double-staining, the sections were incubated with primary anti-BTLA and anti-HVEM antibodies at 4 °C overnight. After washing with PBS (3 washes, 5 min per wash), the sections were incubated with Alexa Fluor® 555-conjugated goat anti-mouse/rabbit IgG antibodies (Invitrogen, San Diego, CA, USA) for 1 h. The sections were further incubated with anti-CD3 (1:50, clone:F7.2.38, Dako), anti-CD8 (1:50, clone: C8/144B, Dako), anti-CD56 (1:50, Santa Cruz, San Diego, CA, USA), anti-B7-H1, (1:100, clone, 29E.2A3, kindly provided by Dr. Gordon J. Freeman, Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, USA), anti-B7-DC (1:50, Polyclonal Goat IgG, R&D system; or 1:100, clone: 24F.7G12, kindly provided by Dr. Gordon J Freeman), anti-B7-H3 (2 μg/ml, R&D system), anti-B7-H4 (1:100, Santa Cruz), anti-CD68

(1:100, clone: 3F103, Santa Cruz), anti-CD31(1: 50, Santa Cruz) or anti-CK-18 (1:200, Santa Cruz) antibodies at 4 °C overnight and incubated with Alexa Fluor® 488-conjugated goat anti-mouse/rabbit IgG1 antibodies (Invitrogen) for an additional 1 h. Finally, the sections were incubated with 1 μg/ml DAPI (Sigma, St. Louis, MO, USA) for 10 min to stain the nuclei. Sections incubated with the appropriate isotype control primary antibodies and fluorescently labeled secondary antibodies were used as negative controls. The results were analyzed using fluorescence microscopy (Zeiss Axioplan 2).

Results

The expression and anatomical distribution of TIMs in sections from LCS

The specimens from all five cases of LCS were highly cellular tumors consisting of enlarged round cells. The neoplastic cells showed the presence of cytologic atypia with multiple hyperchromatic and prominent nucleoli, multinucleated giant cells and infiltrated lymphocytes (Fig. 1a). Immunohistochemistry showed that TIM-1, TIM-3, and TIM-4 positive cells were observed in all these cases. These molecules were found on cell membranes and in the cytoplasm. Positive cells were distributed throughout the tissue sections. The morphological characteristics indicated that the positive cells were sarcoma cells as well as infiltrating inflammatory cells (Fig. 1). Similar results were found by immunofluorescence staining (Fig. 2). Nevertheless, the expression of TIM-3 rather than TIM-1 and TIM-4 was found on normal epidermis and the expression of TIM-3 was seen on some blood endothelial cells (Fig. 1). Here, sections incubated with secondary antibodies only (goat-IgG1) were used as negative controls.

The Phenotypes of TIMs in sections from LCS

The phenotypes of these TIM-positive cells were further examined by immunofluorescence double-staining. Interestingly, all of the TIMs were expressed on Langerin$^+$ tumor cells (Fig. 3). Moreover, TIM-1 was expressed on CD68$^+$ macrophages and CK-18$^+$ epithelial cells, while it was

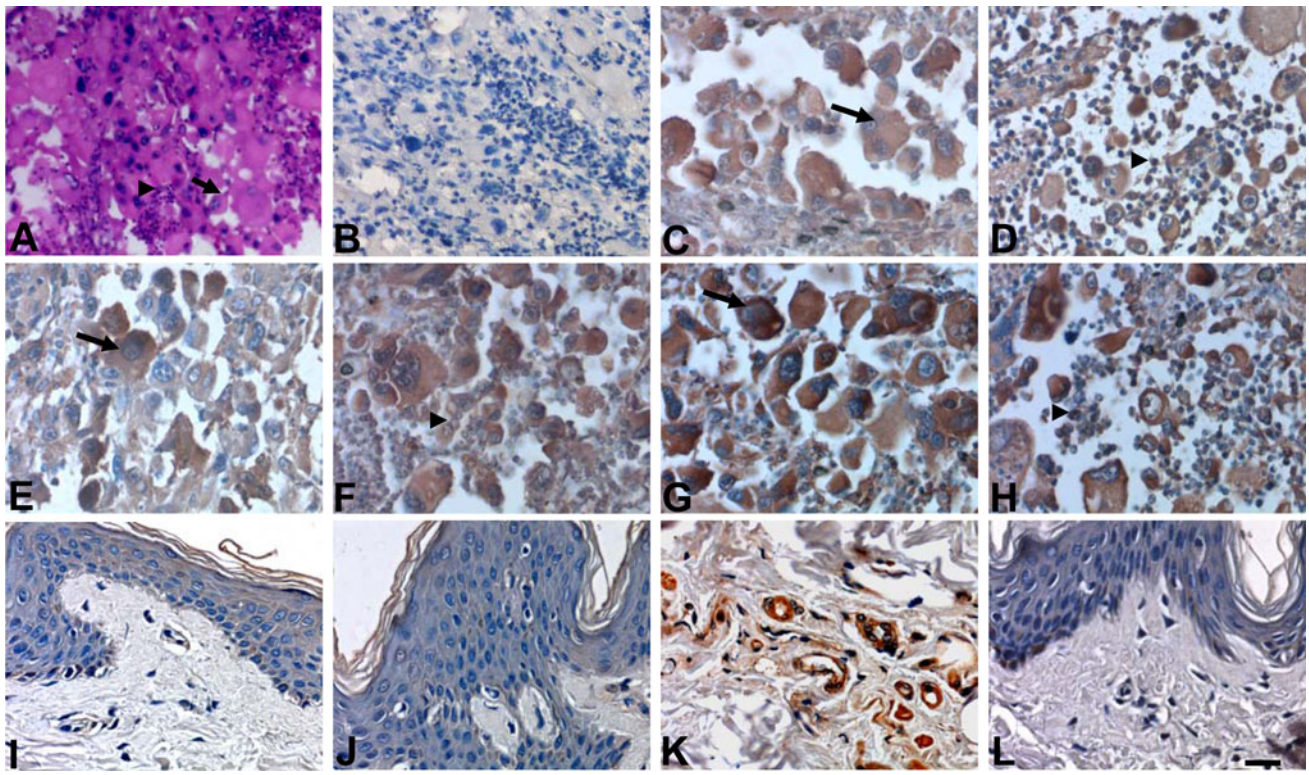
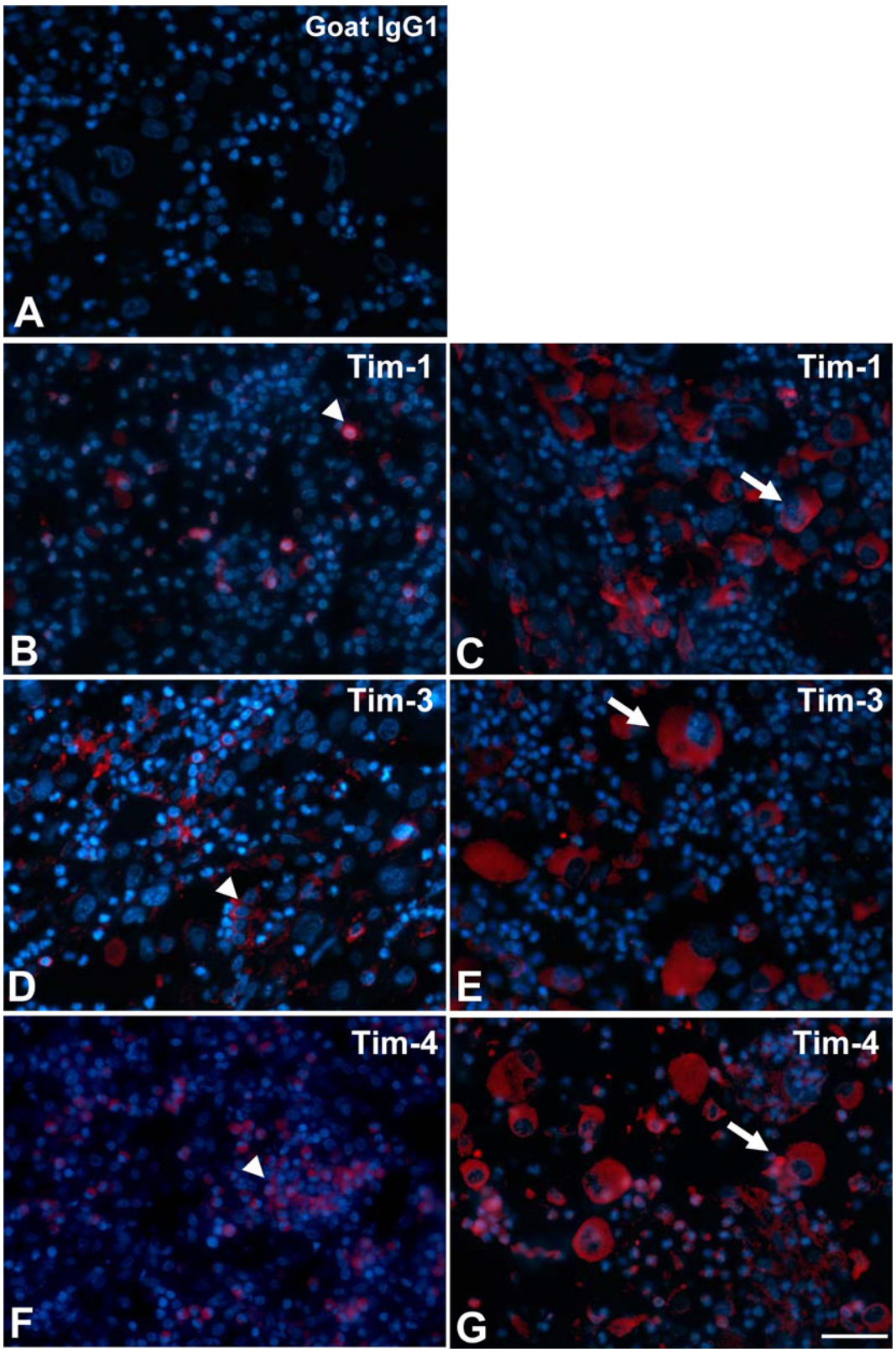


Fig. 1 The expression of TIM-1, TIM-3 and TIM-4 in LCS sample sections detected by immunohistochemistry. **a** H&E staining showed invasion of sarcoma cells into the epidermis in these LCS samples; **b** Goat IgG isotype control antibodies showed no positive staining; **c** TIM-1 was expressed on sarcoma cell aggregates; **d** TIM-1 was expressed on infiltrating lymphocytes; **e** the expression of TIM-3 was seen on sarcoma cell aggregates; **f** TIM-3 was expressed on infiltrating lymphocytes; **g** TIM-4 was expressed on sarcoma cell aggregates; **h** TIM-4 was expressed on infiltrating lymphocytes; **i** The expression of TIM-1 was absence in epithelial cells from normal epidermis; **j** The expression of TIM-3 was absence in epithelial cells from normal epidermis **k** the expression of TIM-3 was seen on some blood endothelial cells of normal epidermis tissues; **l** The expression of TIM-4 was absence in normal epidermis. *Arrows* indicate carcinoma cells and *arrowheads* indicate infiltrating cells. *Scale bar* = 20 μm



absent on $CD3^+$ T cells, $CD11c^+$ DC, $CD16^+$ NK cells and $CD31^+$ endothelial cells (Supplemental Fig. 1). However, TIM-3 and TIM-4 were expressed on all cell types examined, including $CD3^+$ T cells, $CD68^+$ macrophages, $CD11c^+$ DC, $CD16^+$ NK cells, $CD31^+$ endothelial cells and CK-18^+ epithelial cells (Supplemental Fig. 2 and Fig. 3).

◀ **Fig. 2** The expression of TIM-1, TIM-3 and TIM-4 in LCS sample sections detected by immunofluorescence staining. **a** Goat IgG isotype control antibodies showed no positive staining; **b** TIM-1 expression was found on infiltrating lymphocytes; **c** TIM-1 expression was found on sarcoma aggregates; **d** TIM-3 was expressed on infiltrating lymphocytes; **e** TIM-3 was expressed on sarcoma aggregates; **f** TIM-4 was expressed on infiltrating lymphocytes; **g** TIM-4 was expressed on sarcoma aggregates. *Arrows* indicate carcinoma cells, and *arrowheads* indicate infiltrating cells. *Scale bar* = 20 μm

The relationships between TIMs and B7s in sections from LCS

Our previous work demonstrated that the expression of B7 superfamily members, including B7-H1, B7-H3 and B7-H4, was also detected in sections from LCS patients, suggesting that these molecules may be potential biomarkers to identify LCS (Li et al. 2012a). We also detected the relationships between TIMs and these B7 family members. Our results showed TIM-1, TIM-3 and TIM-4 were co-expressed with B7-H1, B7-H3 and B7-H4 in the same sarcoma cells but were not co-expressed with B7-DC (Fig. 4). The characteristic expression of TIMs was summarized in Table 1.

Discussion

LCS is a rare malignancy derived from dendritic cells of the epidermis characterized by cytological atypia, frequent mitoses, and aggressive clinical behavior (Uchida et al. 2008; Nakayama et al. 2010). Most studies have shown that LCS has an immunophenotype characterized by expression of CD1a, S-100, CD68 and Langerin (Lau et al. 2008). However, the pathological diagnosis of LCS is limited, and it is essential to identify more specific markers to identify and to diagnosis this disease.

The TIM-containing molecules are newly discovered proteins that are actively involved in the development of inflammatory conditions, including rheumatoid arthritis, asthma, systemic lupus erythematosus, multiple sclerosis, diabetes, and tumor development. These molecules are worthy of further investigation (Freeman et al. 2010; Rodriguez-Manzanet et al. 2009). There are three members of TIM that have been identified in humans, including TIM-1, TIM-3 and TIM-4. TIM-1 is expressed on activated $CD4^+$ T cells and cross-linking of TIM-1 with its agonist monoclonal antibody (clone: 3B3) or its potential "receptor" PtdSer, provides a potent costimulatory signal to

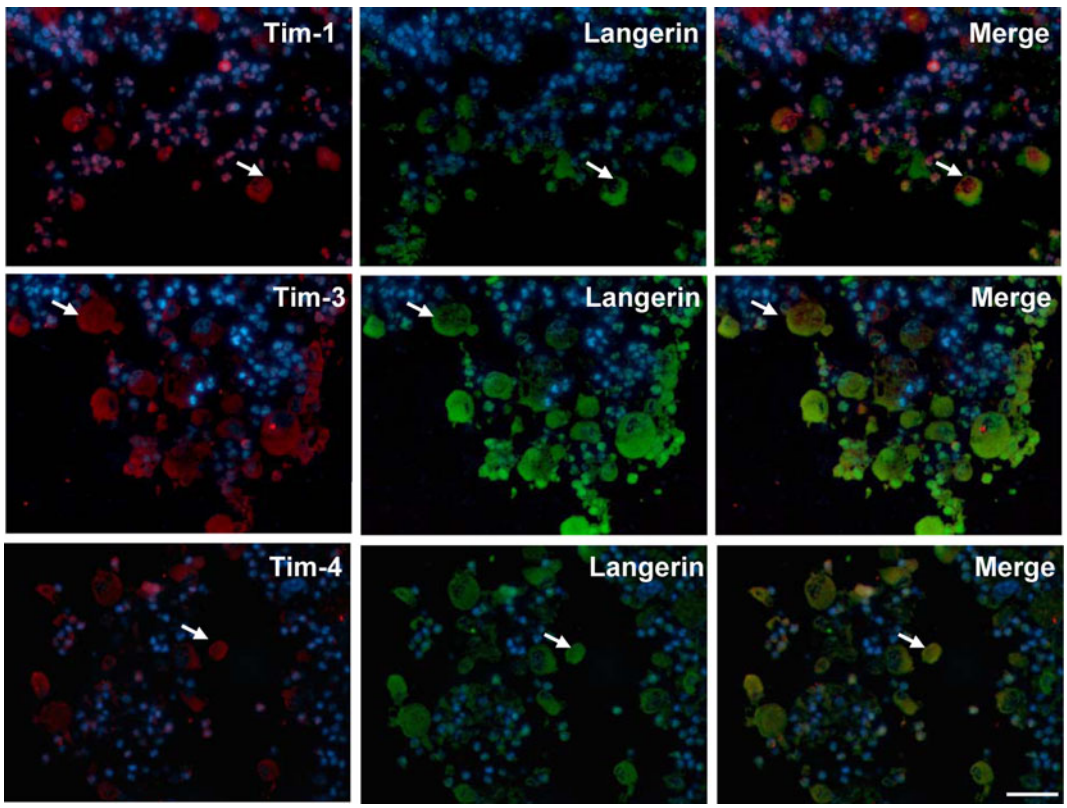


Fig. 3 The relationship between the expression of TIMs and Langerin in LCS sample sections was detected by immunofluorescence double-staining. The results showed that TIM-1, TIM-3 and TIM-4 were found on $Langerin^+$ tumor cells. *Arrow* indicates positive cells. Nuclei were stained with DAPI. *Scale bar* = 20 μm

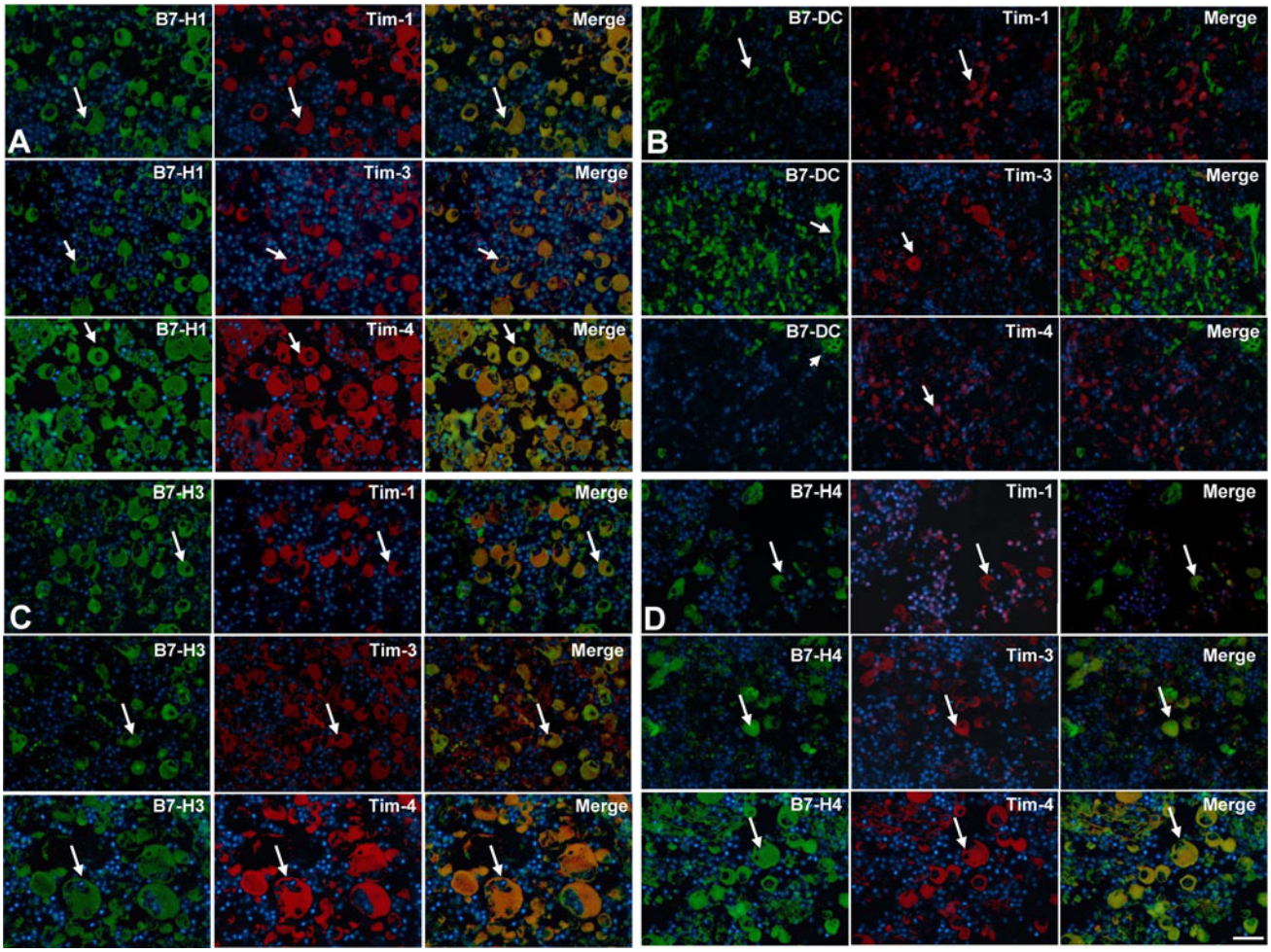


Fig. 4 The relationship between the expression of TIMs and some members of the B7 superfamily in LCS sample sections detected by immunofluorescence double-staining. Immunofluorescence double-staining showed that the expression of some members of the B7 superfamily, including B7-H1, B7-H3 and B7-H4, but not B7-DC,were co-expressed with TIMs on the same carcinoma cells. Nuclei were stained with DAPI. *Scale bar* = 20 μm

Table 1 The characteristic expression of TIMs in LCS samples

Cell types	TIMs		
	TIM-1	TIM-3	TIM-4
Langerin$^+$ tumor cells	+	+	+
CD68$^+$ macrophages	+	+	+
CK-18$^+$ epithelial cells	+	+	+
CD31$^+$ endothelial cells	−	+	+
CD3$^+$ T cells	−	+	+
CD11c$^+$ DC	−	+	+
CD16$^+$ NK cells	−	+	+
B7-H1$^+$ tumor cells	+	+	+
B7-DC$^+$ tumor cells	−	−	−
B7-H3$^+$ tumor cells	+	+	+
B7-H4$^+$ tumor cells	+	+	+

CD4$^+$ T cells (Rodriguez-Manzanet et al. 2009). In addition, the expression of TIM-1 was also observed on mast cells, invariant natural killer cells (iNK) and tubular epithelial cells (TEC) following kidney injury (Rodriguez-Manzanet et al. 2009; Nozaki et al. 2012). Nevertheless, whether TIM-1 was also expressed on carcinoma tissues has not been investigated. Our results showed that TIM-1 was expressed in sections from LCS patients and morphological analysis indicated that TIM-1 positive cells were sarcoma cells as well as infiltrating inflammatory cells. Further investigation showed that TIM-1 was also expressed on CD68$^+$ macrophages and CK-18$^+$ epithelial cells.

The expression of TIM-3 was reported chiefly on Th1 cells, mast cells, activated macrophages, DC, NK and NKT cells (Sánchez-Fueyo et al. 2003; Monney et al. 2002).

Galectin-9 and PtdSer have been identified as the ligands for TIM-3, and engagement of TIM-3 by galectin-9 leads to Th1 cell death as well as a consequent decline in IFN-γ production. This decline results in attenuated EAE severity, suppressed collagen-induced arthritis (Seki et al. 2008) and prolonged survival of fully mismatched cardiac allografts (He et al. 2010). The expression of TIM-3 was also found on tumor tissues including NSCLC, HBV-HCC, renal cell carcinoma and patients with follicular B cell non-Hodgkin's lymphoma (Anderson 2012). The presence of TIM-3 modulates tumor development and carcinoma traits (Chiba et al. 2012). Here, TIM-3 was also detected on sarcoma as well as some infiltrating inflammatory cells in sections from LCS patients and morphologic analysis demonstrated that TIM-3 was also present on $CD3^+$ T cells, $CD68^+$ macrophages, $CD11c^+$ dendritic cells, $CD16^+$ monocytes, $CD31^+$ endothelial cells and CK-18^+ epithelial cells.

TIM-4 is a recently identified member of the TIM superfamily and is expressed exclusively on APC, including some subsets of macrophages and $CD11c^+$ DCs (Wong et al. 2010). Several studies suggest that TIM-4 may play a role in maintaining oral tolerance and preventing food allergy (Yang et al. 2007). Here, the ectopic expression of TIM-4 was seen on sarcoma cells as well as infiltrated inflammatory cells of LCS sections. More interestingly, the phenotypic analysis indicated that TIM-4 was found on $CD3^+$T cells, $CD68^+$ macrophages, $CD11c^+$ dendritic cells, $CD16^+$ monocytes, $CD31^+$ endothelial cells and CK-18^+ epithelial cells.

Our previous work demonstrated that the expression of some members of the B7 superfamily, including B7-H1, B7-DC, B7-H3 and B7-H4, was found on LCS samples (Nozaki et al. 2012). B7-H1 and B7-DC are two immunoregulatory molecules that bind to the PD-1 receptor to reduce T cell activation (Saresella et al. 2012). B7-H3 is a type I transmembrane protein that promotes T cell proliferation, cytotoxicity and IFN-γ production through cross-linking its hypothetical receptor, triggering receptor mediated expression of myeloid cell-like transcript 2 (TLT-2) on activated T cells (Hashiguchi et al. 2008). B7-H4 is a GPI-linked protein that inhibits T cell responses through an identified receptor (Sica et al. 2003). We investigated the relationships between TIMs and these members of the B7 superfamily in sections from LCS patients, and the results showed that TIMs were co-expressed with B7-H1, B7-H3 and B7-H4.

In summary, this report investigated the expression of TIMs in sections from LCS patients, and an understanding of the functional roles of these molecules could aid in the development of novel strategies for disease diagnosis or immunotherapy.

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Conflicts of interest None of the authors have any conflicts of interest related to this manuscript.

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