

An IL-27/Stat3 axis induces expression of programmed cell death 1 ligands (PD-L1/2) on infiltrating macrophages in lymphoma

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Immune escape and tolerance in the tumor microenvironment are closely involved in tumor progression, and are caused by T-cell exhaustion and mediated by the inhibitory signaling of immune checkpoint molecules including programmed death-1 (PD-1), cytotoxic T-lymphocyte associated protein 4, and T-cell immunoglobulin and mucin domain-containing molecule-3. In the present study, we investigated the expression of the PD-1 ligand 1 (PD-L1) in a lymphoma microenvironment using paraffin-embedded tissue samples, and subsequently studied the detailed mechanism of upregulation of PD-L1 on macrophages using cultured human macrophages and lymphoma cell lines. We found that macrophages in lymphoma tissues of almost all cases of adult T-cell leukemia/lymphoma (ATLL), follicular lymphoma and diffuse large B-cell lymphoma expressed PD-L1. Cell culture studies showed that the conditioned medium of ATL-T and SLVL cell lines induced increased expression of PD-L1/2 on macrophages, and that this PD-L1/2 overexpression was dependent on activation of signal transducer and activator of transcription 3 (Stat3). *In vitro* studies including cytokine array analysis showed that IL-27 (heterodimer of p28 and EBI3) induced overexpression of PD-L1/2 on macrophages via Stat3 activation. Because lymphoma cell lines produced IL-27B (EBI3) but not IL-27p28, it was proposed that the IL-27p28 derived from macrophages and the IL-27B (EBI3) derived from lymphoma cells formed an IL-27 (heterodimer) that induced PD-L1/2 overexpression. Although the significance of PD-L1/2 expressions on macrophages in lymphoma progression has never been clarified, an IL-27-Stat3 axis might be a target for immunotherapy for lymphoma patients.

Recent studies have shown the significant involvement of macrophages in tumor progression in several kinds of human malignant tumors.^(1–4) In addition, there are numerous research articles that describe the protumor functions of macrophages that have infiltrated into the microenvironment of hematological malignancies such as leukemia/lymphoma.⁽⁵⁾ Macrophages infiltrating into the tumor microenvironment are referred to as tumor-associated macrophages (TAM). TAM are considered to be related to tumor progression through their association with tumor cell survival, angiogenesis, metastasis, invasion and immune suppression.⁽⁶⁾

Immune escape and tolerance in the tumor microenvironment are closely involved in tumor progression, and are caused by T-cell exhaustion and mediated by inhibitory signals due to the activation of immune checkpoint molecules, including programmed death-1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and T cell immunoglobulin and mucin domain-containing-3 (TIM3).^(7,8) The PD-1 ligand 1 (PD-L1, also known as B7-H1) is known to be widely expressed by leukocytes and tumor cells, whereas PD-L2 (also known as B7-DC) is expressed mainly by dendritic cells and

macrophages.⁽⁹⁾ Anti-tumor responses after blocking monoclonal antibodies against PD-1 signals are currently being reported in some kinds of malignant tumors, including lymphoma, and, therefore, blocking of immune checkpoint molecules is considered to be a new anti-tumor therapy.^(10–12) A number of research studies have focused on PD-L1 expression in stromal cells in the tumor microenvironment,^(13,14) however, only a few studies have investigated PD-L1/2 expression on myeloid cells in human malignant tumors.^(15,16)

In the present study, we demonstrated PD-L1/2 expressions on TAM in almost all malignant lymphoma cases that are frequently seen in Japan, including adult T cell leukemia/lymphoma (ATLL), follicular lymphoma (FL) and diffuse large B-cell lymphoma (DLBCL). We then investigated the detailed mechanisms of PD-L1/2 overexpression on TAM using human monocyte-derived macrophages and some lymphoma cell lines.

Materials and Methods

Tissue samples. Paraffin-embedded tumor samples from lymph node biopsies diagnosed as ATLL (16 cases), FL

(25 cases) and DLBCL (56 cases) during 2005–2014 were examined. Written informed consent was obtained from all patients in accordance with protocols approved by the Kumamoto University Review Board.

Immunohistochemistry. Rabbit monoclonal antibody against PD-L1 (clone E1L3N; Cell Signaling Technology, Danvers, MA, USA) or PD-L2 (clone D7U8C; Cell Signaling Technology), mouse monoclonal antibody against CD163 (clone 10D6; Leica Biosystems, Wetzlar, Germany), CD19 (Clone LE-CD19, DAKO) or anti-IL-27B (Epstein–Barr virus induced 3, EBI3; clone 15k8D10, Novus Biologicals, Minneapolis, MN, USA), and rabbit polyclonal antibody against IL-27p28 (Abcam, Cambridge, UK) were used as primary antibodies for immunostaining. Briefly, after samples were reacted with the primary antibodies, they were incubated with HRP-labeled goat anti-rabbit secondary antibodies (Nichirei, Tokyo, Japan). Can Get Signal Solution (TOYOCO, Tokyo, Japan) was used to dilute the antibodies to enhance the immunoreaction of PD-L1 and PD-L2. Reactions were visualized using the diaminobenzidine substrate system (Nichirei). For double immunostaining, goat anti-mouse antibody labeled with Alexa 488 and goat anti-rabbit antibody labeled with Alexa 546 (ThermoFisher, Waltham, MA, USA) were used as secondary antibody. Two pathologists (HH and YK), who were blinded to information about the samples, evaluated the immunostaining of PD-L1/2 and CD163.

Cell lines. The human ATLL cell lines (ATN-1, ATL-T, MT-1), T-cell lymphoma cell lines without HTLV-1 infection (SKW-3, MOLT-4), EBV-infected B-cell lines (103-LCL, 141-LCL) and B-cell lymphoma cell lines (DAUDI, TL-1, NALM, Raji, SLVL, BALL1) were maintained in RPMI supplemented with 10% FBS. ATL-T and MT-1 were previously established by Taylor and Matsuoka⁽¹⁷⁾ and other cell lines were purchased from RIKEN Cell Bank (Wako, Tokyo, Japan). The mycoplasma test was performed using a PCR Detection Kit (Takara Bio, Otsu, Japan).

Macrophage culture. Peripheral blood mononuclear cells were obtained from healthy volunteer donors, who had all provided written informed consent for the use of their cells in accordance with the study protocols approved by the Kumamoto University Hospital Review Board (No. 1169). CD14⁺ monocytes were isolated using CD14-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). These monocytes were plated in 12-well culture plates (2×10^5 cells/well, UpCELL; CellSeed, Tokyo, Japan) and were cultured in 2% human serum, 1 ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF; Wako), and 50 ng/mL macrophage-colony stimulating factor (M-CSF; Wako) for 7 days to induce differentiated macrophages. In some experiments, the Stat3 inhibitor WP1066 dissolved in DMSO was added at a final concentration of 20 μ M. IL-27 (heterodimer of p28 subunit and EBI3) was obtained from BioLegend (San Diego, CA, USA).

Flow cytometry. Cells were detached using cell dissociation buffer (Thermo Fisher Scientific, Waltham, MA, USA), and stained using PE-labeled anti-PD-L1 antibody and APC-labeled anti-PD-L2 antibody (BioLegend) with Fc receptor blocking solution (BioLegend). Isotype-matched control antibodies were also obtained from BioLegend. The stained cell samples were analyzed on a FACSverse (Becton Dickinson, Franklin Lake, NJ, USA) flow cytometer with FACSuite (Becton Dickinson) software.

Real-time quantitative PCR. Total RNA was isolated using RNeasy Plus (Takara Bio). RNA was reverse-transcribed by means of a PrimeScript RT Reagent Kit (Takara Bio Inc.). Quantitative real-time PCR was performed using TaqMan

polymerase with SYBR Green Fluorescence (Takara Bio) and an ABI PRISM 7300 Sequence Detector (Applied Biosystems, Foster City, CA, USA). The primers used were as follows: for PD-L1, sense 5'-GAC.CAG.CAC.ACT.GAG.AAT.CAA.CAC-3', antisense 5'-TTG.GAG.GAT.GTG.CCA.GAG.GTA.G'-3'; for PD-L2, sense 5'-GAA.ACT.TCA.GCT.GTG.TGT.TCT.GG-3', antisense 5'-GCA.GAA.GGG.GAT.GAA.AAT.GTG-3'; for IL-27B (EBI3), sense 5'-CTC.CCT.ACG.TGC.TCA.ATG.TCA.C-3', antisense 5'-GGC.TTG.ATG.ATG.TGC.TCT.GTT.ATG-3'; for IL-27p28, sense 5'-TCC.CTG.ATG.TTT.CCC.TGA.CC-3', antisense 5'-TGA.AGC.GTG.GTG.GAG.ATG.AAG-3'; for CCL20, sense 5'-GTG.CGC.AAA.TCC.AAA.ACA.GAC-3', antisense 5'-AAA.CCT.CCA.ACC.CCA.GCA.AG-3'. for β -actin, sense 5'-ATT.CCT.ATG.TGG.GCG.ACG.AG-3', antisense 5'-AAG.GTG.TGG.TGC.CAG.ATT.TTC-3'.

Western blot analysis. Cells were lysed in ice-cold lysis buffer (50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% NP-40) with a phosphatase inhibitor cocktail (R&D, Minneapolis, MN, USA) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). PVDF membrane was reacted with antibodies against I κ -B α , pStat3, Stat3 and PD-L1 (all antibodies were from Cell Signaling) or with anti- β -actin antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). HRP-Goat anti-mouse or rabbit IgG (Invitrogen, Camarillo, CA, USA) was used as the second antibody. Immunoreactive bands were visualized using the Pierce Western Blotting Substrate Plus Kit (Thermo Scientific, Rockford, IL, USA) and Image-Quant LAS-4000 mini (Fuji Film, Tokyo, Japan).

Cytokine array of culture supernatant. The cytokine array kit was purchased from R&D Systems (Human XL Cytokine Array Kit #ARY022, Minneapolis, MN, USA). The signal densities of spots were evaluated using Image J software (<https://imagej.nih.gov/ij/>).

Statistics. Statistical analysis of *in vitro* and *in vivo* data was carried out using JMP10 (SAS Institute, Chicago, IL, USA) and StatMate III (ATOMS, Tokyo, Japan). A *P*-value < 0.05 was considered to be statistically significant. The χ^2 -test, Mann–Whitney *U*-test, and paired *t*-test were used for statistical analysis. All data used in the cell-culture study were representative of at least three independent experiments.

Results

PD-L1/2 expressions were detected on tumor-associated macrophages in almost all cases. Immunostaining for PD-L1 was performed on paraffin sections of the tumors of 25, 56 and 15 cases of FL, DLBCL and ATLL, respectively. Characteristics of the patients are shown in Table 1. The lymphoma cells of 1, 2 and 1 cases of FL, DLBCL and ATLL, respectively, stained positive for PD-L1 (Table 2); however, we found that in almost all cases in which the lymphoma cells were negative for PD-L1 the TAM stained positive for PD-L1 (Fig. 1a).

Table 1. Characteristics of patients

	FL	DLBCL	ATLL
Age (mean)	36–81 (56)	35–87 (61)	31–86 (67)
M/F	14/11	30/26	8/8
Grade 1/2/3	9/12/4	—	—
GBC/Non-GBC	—	19/37	—

ATLL, adult T cell leukemia/lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma.

Table 2. PD-L1/2 expression on lymphoma cells

	Lymphoma cells	
	PD-L1 (negative/positive)	PD-L2 (negative/positive)
FL		
Grade 1	9/0	9/0
Grade 2	12/0	12/0
Grade 3	3/1	4/0
DLBCL		
GCB	19/0	19/0
nonGCB	35/2	35/2
ATLL	15/1	16/0

ATLL, adult T cell leukemia/lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; GCB, germinal center B-cell.

PD-L1 expression in TAM (Fig. 1b) and in lymphoma cells (Fig. 1c,d) was confirmed by double-fluorescent immunostaining. Next, the same lymphoma case set was immunostained for PD-L2. PD-L2 expression on lymphoma cells was seen in 0, 2

and 0 cases in FL, DLBCL and ATLL, respectively (Fig. 1e, Table 2). However, the staining intensity of PD-L2 on TAM was too weak to evaluate adequately and we could, therefore, not investigate PD-L2 expression on TAM in tissue sections (Fig. 1e). Cases in which PD-L1 was positive for lymphoma cells were different from cases in which PD-L2 was positive for lymphoma cells. Four DLBCL cases in which lymphoma cells were positive for either PD-L1 or PD-L2 belonged to the non-GCB type (Table 2), and three of these patients died within 2 years from diagnosis (data not shown).

PD-L1 and PD-L2 expression on macrophages was increased by the conditioned medium of SLVL, MT-1 and ATL-T cell lines. Both PD-L1 and PD-L2 are known to be expressed on macrophages. We then tested if PD-L1 and PD-L2 expression on macrophages is changed by secreted factors from lymphoma cells using human monocyte-derived macrophages. Culture supernatants of B- and T-cell lymphoma cell lines were collected and used as conditioned medium (CM). Macrophages were stimulated with CM for 24 h following which PD-L1 and PD-L2 expression on macrophages was evaluated using flow

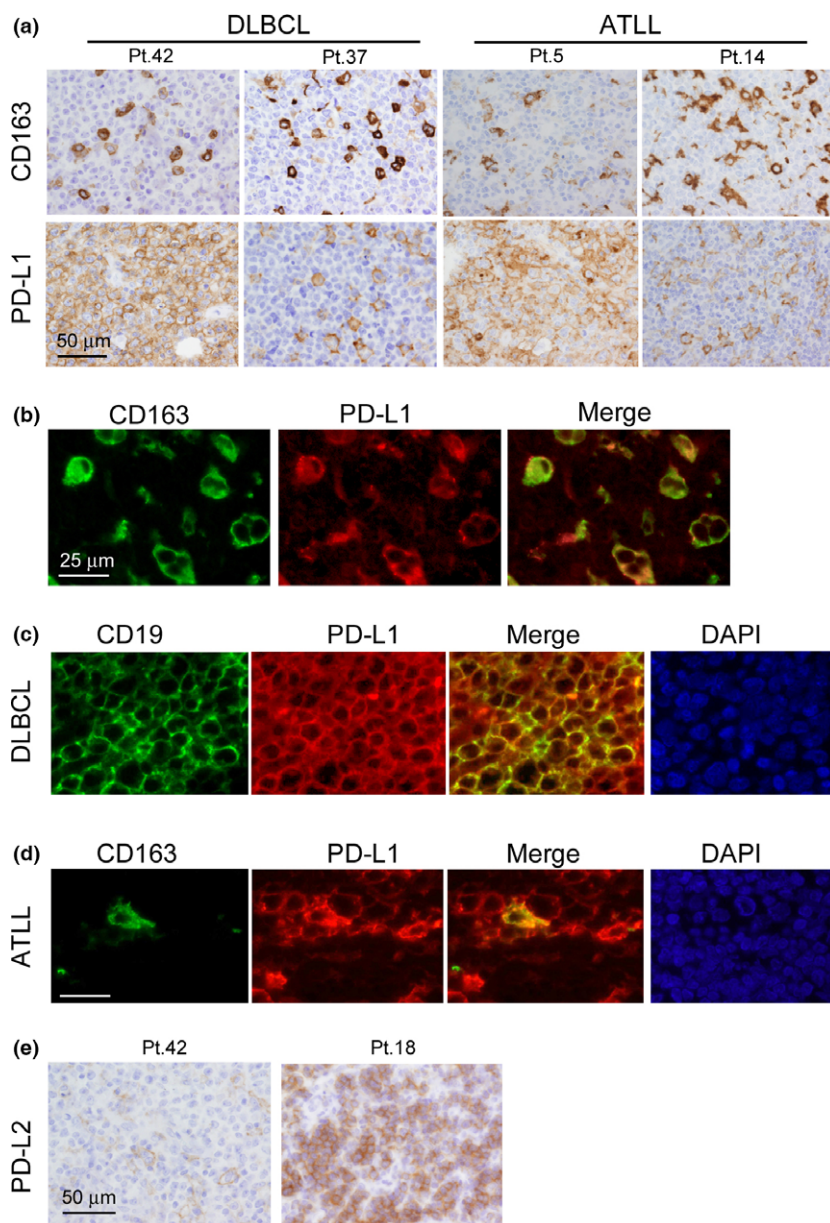


Fig. 1. PD-L1 expression in lymphoma tissues. (a) Representative immunostaining of PD-L1 and CD163 in DLBCL and ATLL is shown. Lymphoma cells in patient (Pt.5 and Pt.42) stained positive for PD-L1, whereas tumor-associated macrophages (TAM) in Pt.14 and Pt.37 stained positive for PD-L1. (b) Double-fluorescent immunostaining of PD-L1 and CD163 in Pt.37 confirmed that TAM express PD-L1. Scale bar: 25 μ m. (c) Double-fluorescent immunostaining of PD-L1/CD19 and PD-L1/CD163 is shown. In DLBCL (Pt.42), CD19-positive lymphoma cells stained positive for PD-L1. (d) In ATLL (Pt.5), CD163-positive TAM and CD163-negative lymphoma cells stained positive for PD-L1. Scale bar: 25 μ m. (e) PD-L2 positive signals were detected on TAM and lymphoma cells in Pt.42 and Pt.18, respectively.

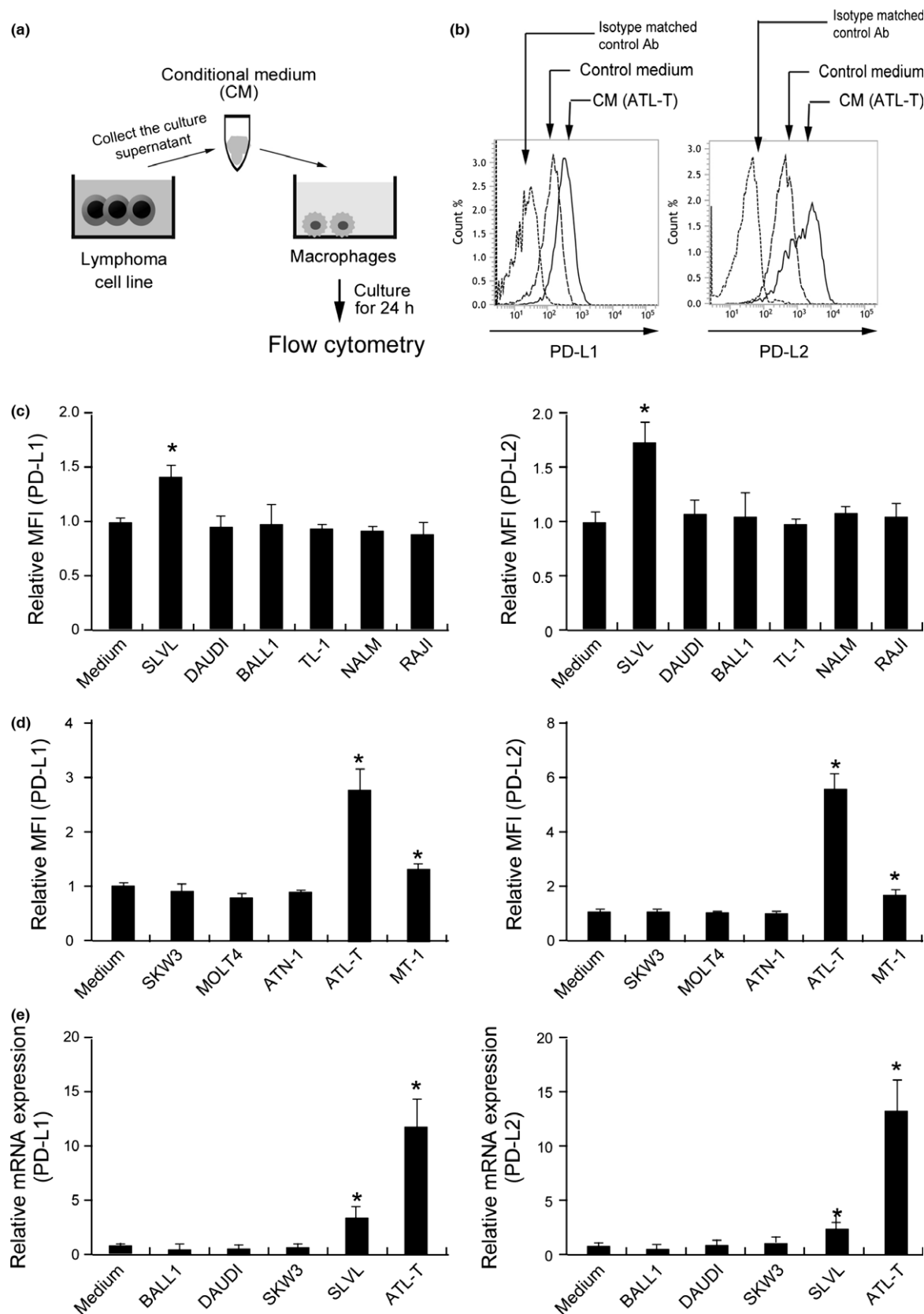


Fig. 2. PD-L1/2 expression on cultured macrophages. (a) Scheme for testing the effect of the conditioned media (CM) from lymphoma cell lines on PD-L1/2 expressions in macrophages. (b) Flow cytometric analysis of the effect of the CM of ATL-T on PD-L1 and PD-L2 expression. (c, d) Histogram data of flow cytometric analysis of PD-L1 and PD-L2 expression on macrophages with or without stimulation with the CM of B-cell lymphoma cell lines (c) or T-cell lymphoma cell lines (d) (MFI, mean fluorescent intensity, $n = 3$ for each group). (e) The mRNA expression of PD-L1/2 in cells stimulated with the CM of the indicated cell lines was evaluated using RT-qPCR. * P -value < 0.05.

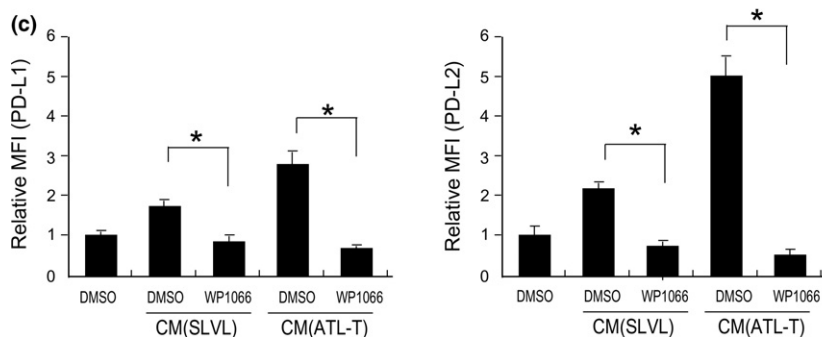
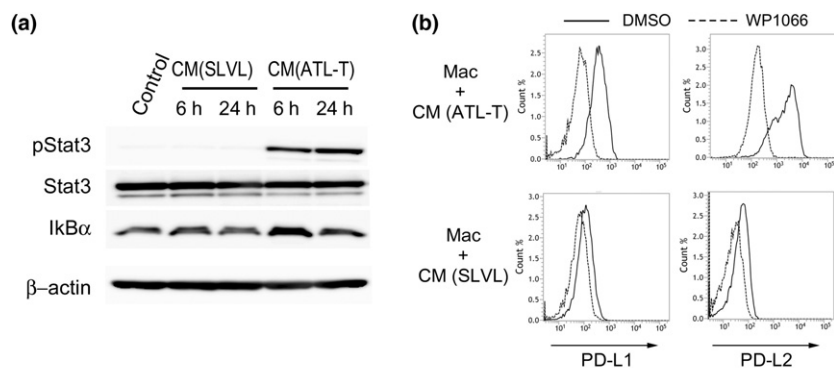


Fig. 3. Stat3 activation and PD-L1/2 upregulation on cultured macrophages. (a) Western blot analysis of pStat3, Stat3 and IκBα expression in cell lysates of cultured macrophages simulated with the conditioned media (CM) of SLVL and ATL-T cells for the indicated times. β-actin was analyzed as a loading control. (b, c) The effect of the Stat3 inhibitor WP1066 on CM-induced overexpression of PD-L1/2 on macrophages (Mac) was tested using flow cytometry (MFI, mean fluorescent intensity; $n = 3$ for each group). * P -value < 0.05.

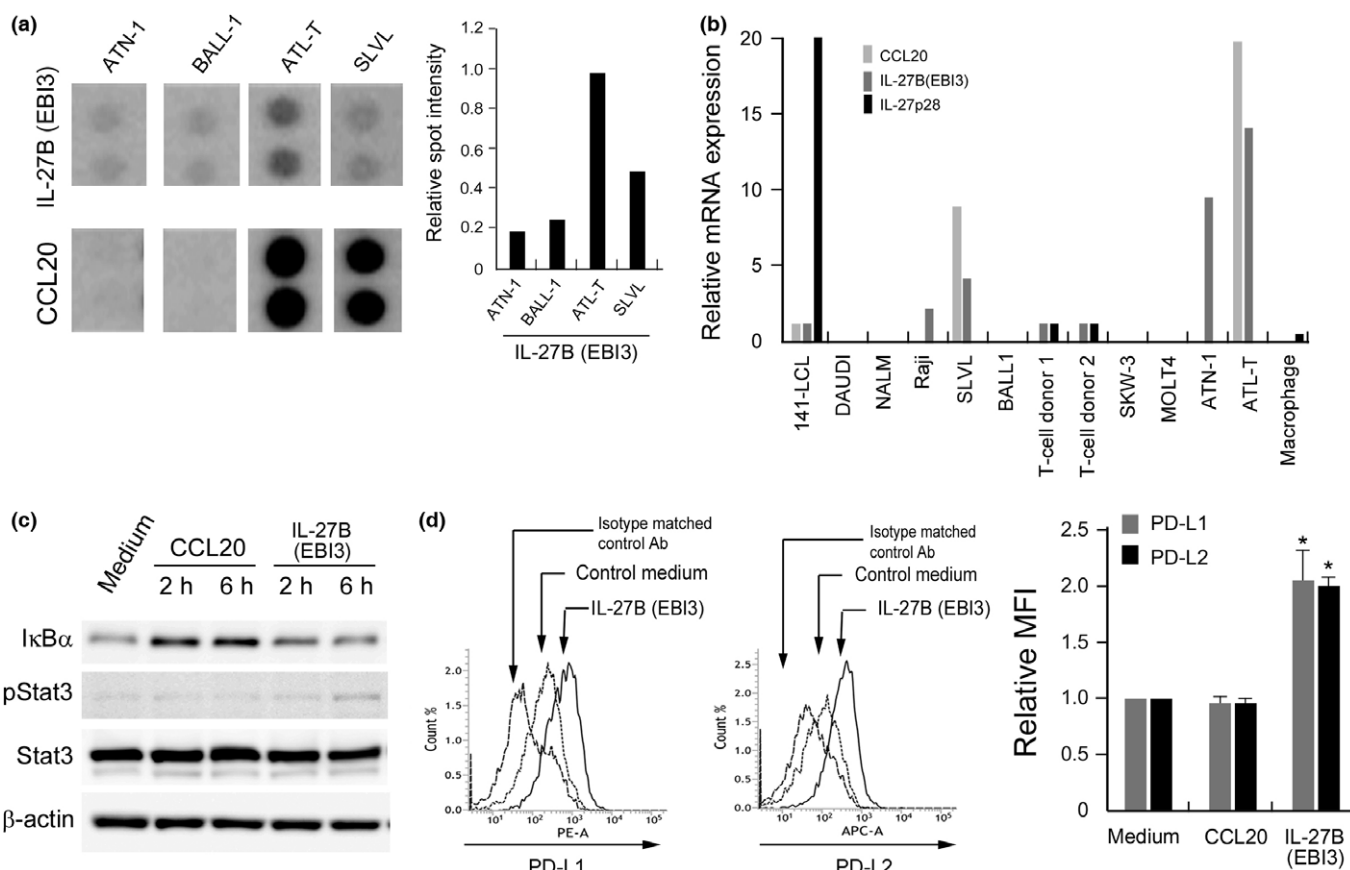


Fig. 4. IL-27B (EBI3) and PD-L1/2 expressions on macrophages. (a) Cytokine array analysis showing the spots of CCL20 and IL-27B (EBI3) derived from the conditioned media (CM) of the indicated cell lines. Spot intensity was evaluated using ImageJ software. (b) The mRNA expressions of CCL20, IL-27 (EBI3), and IL-27p28 in cell lines and activated T cells were evaluated using RT-qPCR. (c) Western blot analysis of pStat3, Stat3, IκBα and β-actin (control) expression in the cell lysates of cultured macrophages simulated with IL-27B (EBI3) or CCL20 protein for the indicated times. (d) Macrophages were stimulated with the recombinant IL-27B (EBI3) or CCL20 protein for 24 h, following which PD-L1/2 expressions on macrophages was tested using flow cytometry (MFI, mean fluorescent intensity; $n = 3$ for each group). * P -value < 0.05.

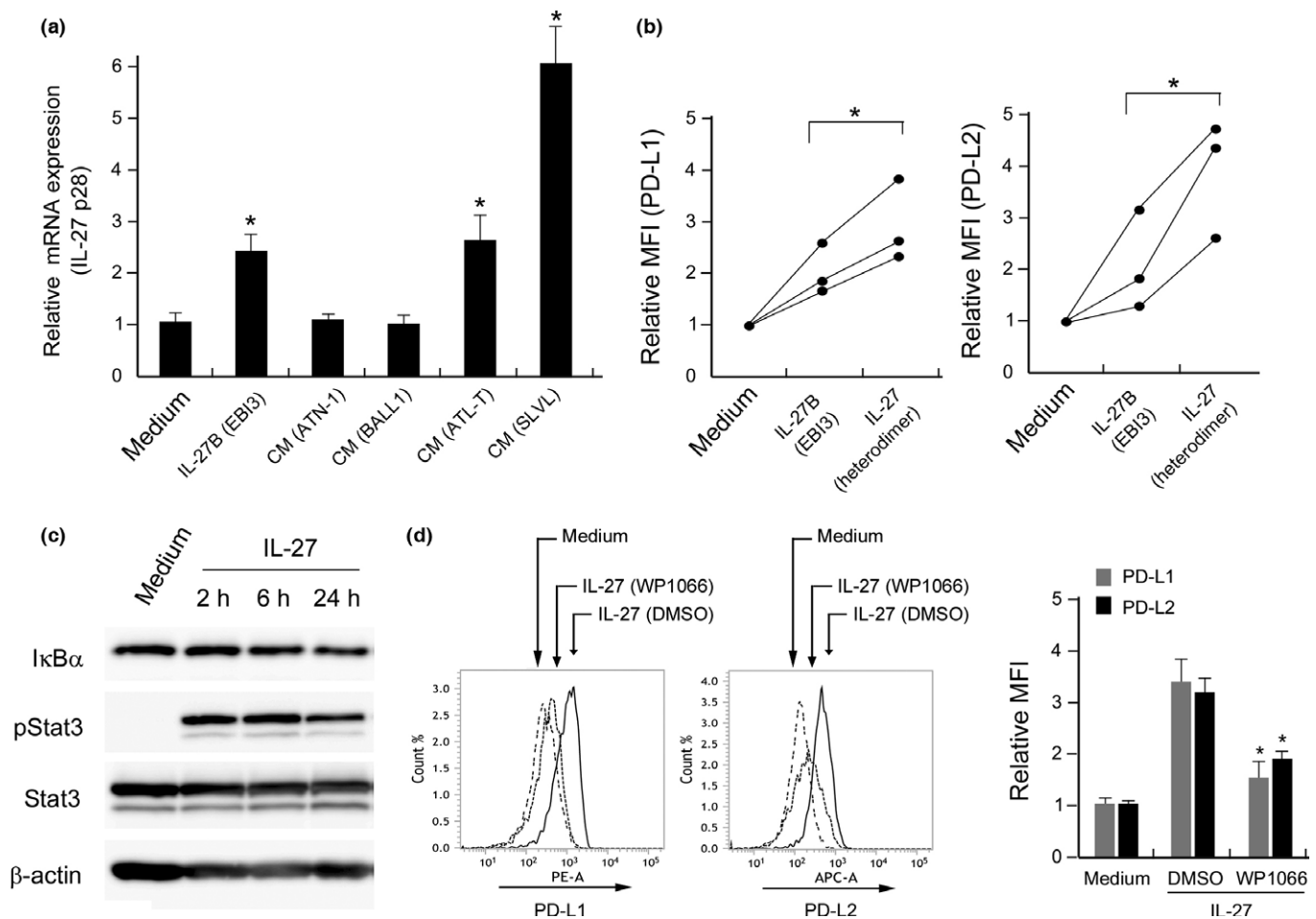


Fig. 5. The IL-27 (heterodimer) induced greater overexpression of PD-L1/2 on macrophages than IL-27B (EBI3). (a) Macrophages were stimulated with the same concentration (20 ng/mL) of recombinant IL-27B (EBI3) or IL-27 (heterodimer) for 24 h, following which PD-L1/2 expression on the macrophages was tested using flow cytometry (MFI, mean fluorescent intensity). The data of three donors are shown. The results were statistically analyzed using a paired t-test. (b) Macrophages were stimulated with IL-27B (EBI3) or the conditioned medium (CM) of the indicated cell lines for 24 h, following which the mRNA expression of IL-27p28 was analyzed using RT-qPCR. (c) Western blot analysis of pStat3, Stat3, IκBα and β-actin (control) expression in cell lysates of cultured macrophages stimulated with IL-27 (heterodimer) for the indicated times. (d) The effect of the Stat3 inhibitor WP1066 on IL-27-induced overexpression of PD-L1/2 on macrophages was analyzed using flow cytometry (MFI, mean fluorescent intensity, $n = 3$ for each group). * P -value < 0.05 .

cytometry (Fig. 2a). The results showed that the CM of SLVL, MT-1 and ATL-T cell lines induced significant upregulation of PD-L1 and PD-L2 in macrophages (Fig. 2b–d). No significant changes in PD-L1 or PD-L2 levels were induced by the CM of other cell lines. Increased mRNA expression of PD-L1 and PD-L2 in macrophages was also observed by stimulation with the CM of SLVL and ATL-T cell lines (Fig. 2e).

Upregulation of PD-L1 and PD-L2 expression on macrophages was mediated by Stat3 activation. NF-κB and Stat3 signals are known to be related to PD-L1 and PD-L2 expression. We therefore next tested the signal molecules activated in macrophages by the CM of the SLVL and ATL-T cell lines. Although degradation of IκBα was not observed following stimulation with CM, significant activation of Stat3 was induced by the CM of ATL-T cells (Fig. 3a). The Stat3 inhibitor WP1066 significantly abrogated the PD-L1 and PD-L2 overexpression induced by the CM of ATL-T cells (Fig. 3b,c). Although Stat3 activation by the CM of SLVL cells was not obvious, the Stat3 inhibitor WP1066 also significantly abrogated the PD-L1 and PD-L2 overexpression that was induced

in macrophages stimulated with the CM of SLVL cells (Fig. 3b,c).

IL-27B (EBI3) derived from lymphoma cells was involved in PD-L1 and PD-L2 expression on macrophages. We next investigated what kinds of soluble molecules in CM were associated with PD-L1 and PD-L2 overexpression on macrophages by using a cytokine array kit containing a total of 204 spots (102 soluble human proteins). Because PD-L1 and PD-L2 overexpression was induced by the CM of SLVL and ATL-T cell lines but was not induced by the CM of ATN-1 and BALL1 cell lines, proteins that were detected at a high level in the CM of SLVL and ATL-T cells and at a low level in the CM of ATN-1 and BALL1 cell lines were selected for further analysis. As shown in Figure 4(a) and Figure S1, CCL17, CCL20, IL-27B (EBI3), sICAM-1, GM-CSF and G-CSF were included in this list. Because CCL20 and IL-27B/EBI3 are known to link to NF-κB and Stat3 signals, CCL20 and IL-27B (EBI3) were focused on in further studies. The mRNA expression of CCL20 was significantly higher in SLVL and ATL-T cells, whereas the high mRNA expression of IL-27B (EBI3) was seen in SLVL, ATN-

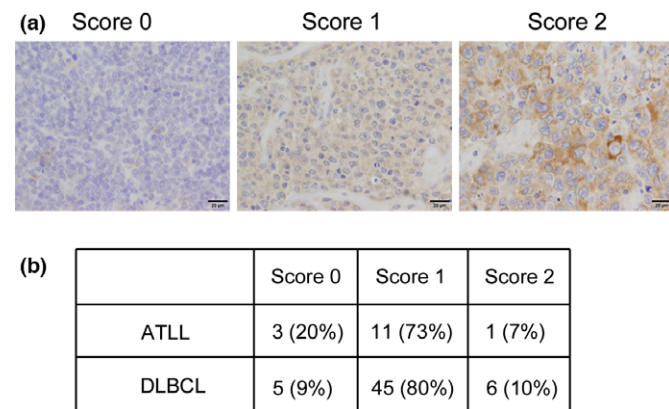


Fig. 6. IL-27B (EBI3) expression on lymphoma cells. (a, b) The staining intensity of IL-27B (EBI3) was classified into three groups (negative, score 0; weakly positive, score 1; strongly positive, score 2). Scale bar: 20 μ m.

1 and ATL-T cells (Fig. 4b). The degradation of I κ B α , which reflects NF- κ B activation, was not influenced by either recombinant CCL20 or IL-27B (EBI3), whereas slight activation of Stat3 was induced by IL-27B (EBI3) (Fig. 4c). PD-L1 and PD-L2 overexpression on macrophages was significantly upregulated by IL-27B (EBI3); however, PD-L1 and PD-L2 expression was not changed by CCL20 (Fig. 4d).

IL-27 (heterodimer of EBI3 and p28) induced greater upregulation of PD-L1 and PD-L2 on macrophages than IL-27B (EBI3). It is well known that IL-27, which is a heterodimer of IL-27B (EBI3) and IL-27p28, is more highly related to Stat3 activation than IL-27B (EBI3), and, moreover, that IL-27p28 is preferentially produced by myeloid cells, including macrophages.^(18,19) No mRNA expression of IL27p28 was observed in all lymphoma cell lines, as shown in Figure 4(b), whereas 141-LCL, activated T cell and macrophages express mRNA of IL27p28. We therefore considered that lymphoma cell-derived IL-27B (EBI3) and macrophage-derived IL-27p28 might be coupled in the lymphoma microenvironment, and that the subsequent IL-27 heterodimer might then induce PD-L1 and PD-L2 overexpression on TAM. Indeed, IL-27B (EBI3) and the CM of ATL-T cells both induced increased IL-27p28 mRNA expression in macrophages compared to medium alone (Fig. 5a). As shown in Figure 5(b), the IL-27 heterodimer did induce higher PD-L1 and PD-L2 overexpression on macrophages than IL-27B (EBI3) at the same concentration, and also induced

strong activation of Stat3 on macrophages (Fig. 5c). WP1066 significantly abrogated the PD-L1 and PD-L2 overexpression on macrophages that was stimulated by the IL-27 heterodimer (Fig. 5d).

IL-27B (EBI3) is expressed on lymphoma cells. We next examined the distribution of IL27B (EBI3) in tumor tissue by immunostaining. The distribution of IL-27p28 could not be examined because the anti-IL-27p28 antibody was not suitable for immunostaining of paraffin sections. The staining intensity was categorized into three groups (negative, score 0; weakly positive, score 1; strongly positive, score 2) as shown in Figure 6(a). IL27B (EBI3) expression was observed on lymphoma cells of 80% of ATLL and 91% of DLBCL cases. The numbers of weakly positive (score 1) cases were significantly higher than the numbers of strongly positive (score 2) cases (Fig. 6b).

IL-27 induced PD-L1 and PD-L2 expression on lymphoma cells. As the final step, we evaluated PD-L1 protein expression in lymphoma cell lines by means of western blot analysis and PD-L1/2 mRNA expression using real-time quantitative PCR (RT-qPCR). Western blot analysis of PD-L1 expression showed strong expression of PD-L1 in ATL-T and SLVL cells and weakly positive signals in 103-LCL, 141-LCL and ATN-1 cells (Fig. 7a). RT-qPCR analysis indicated a similar expression pattern of PD-L1/2 mRNA (Fig. 7b).

Discussion

In the present study, we demonstrated that TAM in malignant lymphoma expressed PD-L1 and potentially express PD-L2. The double immunofluorescent staining data indicated that almost all of the PD-L1-positive stromal cells are CD163-positive TAM, and that no PD-L1 expression was seen on other stromal cells such as endothelial cells and lymphocytes. Similar observations of the expression of PD-L1 by TAM were reported in glioma and malignant lymphoma.^(16,20) In the study of glioma patients, circulating monocytes isolated from glioma patients expressed higher levels of PD-L1 as compared with monocytes in healthy donors, and IL-10 was suggested to be involved in PD-L1 overexpression in monocytes and TAM.⁽²⁰⁾ With regard to PD-L2, the staining intensity of PD-L2 was too weak to evaluate the positive cells adequately in the present study; however, the staining pattern of PD-L2 appeared to be similar to that of PD-L1 and CD163. Based on the *in vitro* study of the present article, TAM are considered to express PD-L2 as well as PD-L1. The observed difference in the

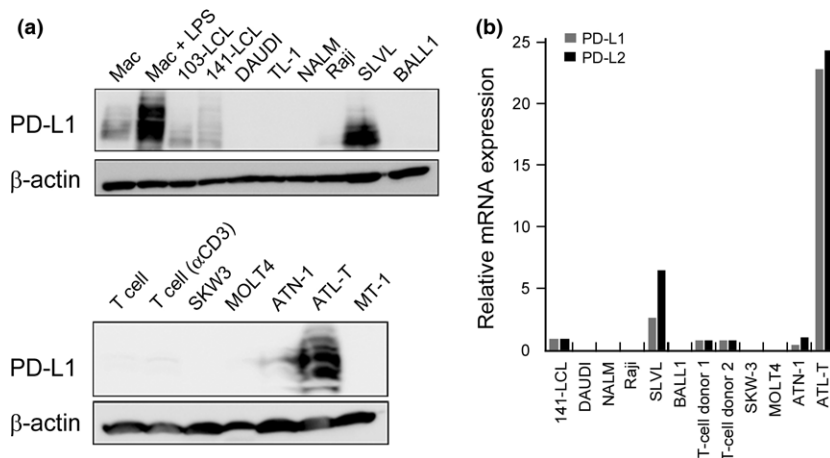


Fig. 7. PD-L1/2 expressions on lymphoma cell lines. (a) The protein expression of PD-L1 in lymphoma cell lines was analyzed using western blot analysis. β -actin was analyzed as a loading control. (b) The mRNA expression of PD-L1/2 in lymphoma cell lines was analyzed using real-time quantitative PCR.

immunostaining of PD-L1 and PD-L2 is considered to be due to the lower specificity of the anti-PD-L2 antibody that was used in this study as compared with that of the anti-PD-L1 antibody. In support of this finding, as shown in Figure 7, the anti-PD-L1 antibody was suitable for western blot analysis; however, the anti-PD-L2 antibody was not useful for western blot analysis.

Analysis of the effect of the CM from several lymphoma cell lines on macrophages indicated that the CM from SLVL and ATL-T cells strongly induced overexpression of PD-L1/2 on macrophages via Stat3 activation. PD-L1/2 expressions on macrophages are known to be mediated by NF- κ B and Stat3 activation;^(21,22) however, NF- κ B in macrophages was not influenced by the CM of the lymphoma cells in the present study. NF- κ B activation in macrophages was also not induced by CCL20, IL27B (EBI3) or the IL27 heterodimer. These findings indicate the significance of the Stat3 pathway in PD-L1/2 overexpression on macrophages. Stat1 was also activated by the CM from SLVL and ATL-T cells (unpublished data), and PD-L1 expression has been shown to be regulated by Stat1 as well as by Stat3 in some cancer cells.^(23,24) IL-27 is also known to activate the Stat1 pathway in addition to the Stat3 pathway.⁽¹⁸⁾ Carbotti *et al.*⁽²⁵⁾ demonstrated that IL-27-induced activation of Stat1 and Stat3 was involved in PD-L1 expression in cancer cells; however, they did not investigate which signal pathway is more important for PD-L1 expression. Because a specific chemical inhibitor against Stat1 is not commercially available, we could not evaluate whether Stat1 or Stat3 is more closely related to PD-L1/2 expression on macrophages in the present study. Some previous studies have shown Stat3-dependent PD-L1 expression in myeloid cells and cancer cells,^(26–28) whereas Stat1-dependent PD-L1 expression was reported in macrophages.⁽²⁹⁾ We are currently planning further studies to address this problem, and, therefore, we did not present the data regarding Stat1 in this article.

Important questions that remain to be answered are the significance of PD-L1 expression in stromal cells and its role in therapeutic responses as an immune checkpoint inhibitor. High expression of PD-L1 in infiltrating macrophages and dendritic cells correlated well with better clinical response of advanced cancer patients to treatment with an anti-PD-L1 antibody.⁽³⁰⁾ High PD-L1 expression in infiltrated immune cells also predicted better clinical response of advanced bladder cancer patients to treatment with an anti-PD-L1 antibody, although it was unclear if the PD-L1-positive cells were myeloid cells.⁽³¹⁾ An increased number of TAM expressing PD-L1 is associated with an elevated number of infiltrating regulatory T cells in ovarian cancer.⁽³²⁾ The function of PD-L1 on TAM has not yet been elucidated. However, these findings indicate that TAM are involved in immune suppression in the tumor microenvironment via a PD-L1-PD-1 axis.

In the present study, lymphoma cell-derived soluble factors were also found to be involved in PD-L1/2 overexpression in TAM via Stat3 activation, and IL-27B (EBI3) derived from lymphoma cells was suggested to be involved in PD-L1/2 overexpression on TAM. However, Stat3 activation due to IL-27B (EBI3) was significantly weaker than that due to the CM of ATL-T, and, therefore, we considered that TAM-derived IL-27p28 and lymphoma-derived IL-27 (EBI3) would form IL-27 (heterodimer), which is known to be related to PD-L1 expression, as summarized in Figure S2. As expected, IL-27p28 expression on macrophages was increased by stimulation with IL-27B (EBI3) or the CM of ATL-T cells, and IL-27 (heterodimer) induced strong activation of Stat3 on macrophages as compared with that induced by IL-27 (EBI3).

In Figure 3, the expressions of PD-L1/2 were decreased by WP1066, although Stat3 activation in macrophages stimulated with CM of SLVL cells was not activated. We evaluated the phosphorylation of Tyr 705 of Stat3 in the present study. Other phosphorylation sites such as Ser727 are also known as a target for Stat3 inhibitor,⁽³³⁾ and this indicated that CM of SLVL cells induced phosphorylation of other sites of Stat3.

In Figure 6, we found that IL-27B (EBI3) was expressed on lymphoma cells in many cases. Strong IL-27B (EBI3) expression was observed in 7% and 10% of ATL and DLBCL cases, respectively; however, these cases are negative for PD-L1/2. Cases with PD-L1/2-positive lymphoma cells showed weak expression of IL-27B (EBI3), whereas the *in vitro* study using lymphoma cell lines showed that IL-27B (EBI3) and PD-L1/2 expressions were well correlated. Epigenetic changes or differences of genome between primary cells and cell lines might provide an explanation for this discrepancy.

In conclusion, PD-L1, and also probably PD-L2, were highly expressed on TAM in almost all cases of lymphoma studied. *In vitro* study suggested that Stat3 activation was closely involved in PD-L1/2 overexpression on TAM. IL-27 was suggested to be involved in Stat3 activation and PD-L1/2 overexpression. Although the significance of PD-L1/2 expressions on macrophages in lymphoma progression and response to anti-lymphoma therapy remains to be clarified, the IL-27-Stat3 axis might be a target for immunotherapy for lymphoma patients.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Summary of cytokine array analysis of the conditioned medium (CM) of the indicated cell lines.

Fig. S2. Hypothesis of induction of PD-L1/2 expressions on tumor-associated macrophages (TAM).