Impairment of Plasmacytoid Dendritic Cells for IFN Production by the Ligand for Immunoglobulin-Like Transcript 7 Expressed on Human Cancer Cells

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Abstract

Purpose: Plasmacytoid dendritic cells (pDC) are specialized cells to produce type I IFN. Infiltration of pDCs in cancer tissues that have impaired ability to produce IFN-α has been suggested to play immunosuppressive roles in tumor immunity. To identify potential mechanisms causing pDC impairment in the cancer microenvironment, expression of immunoglobulin-like transcript 7 ligands (ILT7L), which inhibits pDC production of type I IFNs on the surface of various human cancer and noncancer cells, was examined.

Experimental Design: To detect unidentified ILT7L, reporter cells, which express green fluorescent protein on interaction with ILT7L, were constructed. ILT7L expression on various human cancer cell lines as well as various noncancerous stromal cells and immune cells was examined. Cytokines and signals involved in the ILT7L expression were also investigated.

Results: ILT7L was detected on all of the various types of human cancer cell lines tested. IFN-α, IFN-β, IFN-γ, tumor necrosis factor-α, interleukin-1β, transforming growth factor-β, lipopolysaccharide, and imiquimod induced ILT7L expression on cancer and noncancer cells. High ILT7L-expressing cancer cells inhibited production of IFN-α and tumor necrosis factor-α by pDC stimulated with CpG. ILT7L does not appear to be a member of classic or nonclassic HLAs. Additionally, NF-κB and mammalian target of rapamycin are involved in regulating ILT7L expression.

Conclusions: ILT7L expression on cancer cells may be one of the mechanisms for impairment of pDCs in the cancer microenvironment. ILT7L/ILT7L signaling may normally enable a negative immune response feedback following viral infection. Intervention of the ILT7L/ILT7 system may be useful for enhancing antitumor immunity as well as antiviral immunity. (Clin Cancer Res 2009;15(18):OF1–11)

Recent clinical immunotherapy trials have indicated that immunosuppression by existing cancer cells is a major problem leading to relatively weak antitumor effects in cancer patients. In the clinical trials, although adoptive transfer of activated T cells reactive to the MART-1 melanoma antigen following lymphodepletive treatment, which reduces various immunosuppressive factors including immunosuppressive regulatory T cells, has shown to be very effective (1), active immunization with MART-1 only produces a weak antitumor effect (2). Thus, understanding the mechanisms involved in inducing immunosuppression in cancer patients is important for developing effective immunotherapy (3). Numerous immunosuppression mechanisms in cancer patients have been reported including cancer cell production of a variety of immunosuppressive molecules for T cells and dendritic cells as well as activate various immunosuppressive cells, including regulatory T cells, myeloid-derived suppressor cells, and regulatory dendritic cells (4, 5).

Dendritic cells are professional antigen-presenting cells capable of activating naive T cells (6, 7) and are one of the major targets for immunosuppressive molecules produced by cancer
cells. Dendritic cells are composed of two major subsets: myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). pDCs produce high amounts of type I IFNs (α, β, and ω) following viral infection, which link innate and adaptive immune responses together by promoting activation and differentiation of natural killer (NK) cells, T cells, B cells, and mDCs (8). In addition, murine pDCs can augment immune responses through a type I IFN–independent manner. CD40 ligand expressed by pDCs activates mDCs to secrete interleukin (IL)-12 through CD40-CD40 ligand interactions (9). On the other hand, several studies showed that pDCs could also show immunosuppressive activities. CpG-oligodeoxynucleotides (CpG-ODN)–activated pDCs induce the generation of CD4+CD25+ regulatory T cells, which produce immunosuppressive IL-10 and transforming growth factor-β (TGF-β; ref. 10). Therefore, pDCs exhibit both immunoaugmenting and immunosuppressive activities. CpG-ODN–activated pDCs are immunosuppressive through CD4+CD25+ regulatory T cells, which produce immunosuppressive IL-10 and transforming growth factor-β (TGF-β; ref. 10). Therefore, pDCs exhibit both immunoaugmenting and immunosuppressive functions depending on the environmental condition.

Emerging evidence indicates that pDCs have a significant clinical importance for the treatment of cancer patients. In melanoma patients, pDCs are consistently found in the sentinel lymph node. Sentinel lymph node–infiltrated pDC in patients with metastasis was significantly higher than in those without metastasis and show an impaired ability to produce characteristic IFN-α (11). Also, infiltration of pDCs in primary breast cancers was significantly correlated with poor prognosis (12). Most infiltrating pDCs in human non–small cell lung cancers were immature and only secreted low amounts of IFN-α after Toll-like receptor (TLR) 9 stimulation (13). pDCs isolated from cancer ascites from ovarian cancer patients induced CD8+ regulatory T cells, which suppress tumor antigen-specific T cells through production of IL-10 (14). These observations suggest that impaired pDCs with low IFN-α production have an immunosuppressive role in cancer microenvironments. However, treatment of basal cell carcinoma with imiquimod, a synthetic protein specifically expressed on the cell surface of human pDCs (18). ILTs are a family of inhibitory and activating immune receptors. Inhibitory ILTs have immunoreceptor tyrosine-based inhibition motifs in cytoplasmic domains, whereas activating ILTs lacking cytoplasmic signaling domains associate with an adaptor protein such as FcRγ to mediate signals through the immunoreceptor tyrosine-based activation motifs of adaptor protein. ILT7 has been classified as an activating ILT, which associates with FcεRIγ to form a receptor complex and transduces signals that negatively modulate TLR7- or TLR9-mediated pDC type I IFN production. Although a specific ligand for ILT7 (ILT7L) has not been identified, some ILTs are known to recognize classic or nonclassic MHC class I molecules. Therefore, in this study, using the reporter cells that can detect expression of ILT7L, we evaluated expression of ILT7L by 48 human cancer cell lines and found that a variety of human cancer cell lines have constitutive or cytokine-induced expression of ILT7L and inhibit IFN-α production by pDC. The mechanisms for the constitutive expression of ILT7L appear to be heterogeneous, but a NF-κB inhibitor, DHMEQ, inhibits ILT7L expression in some of the renal cell carcinoma (RCC). Our results suggest human cancer cells may impair pDC functions through expression of ILT7L and subsequent ILT7L/ILT7L interactions, leading to immunosuppression in cancer patients.
Fig. 1. Constitutive or IFN-γ–induced expression of ILT7L in cancer cell lines. A, 2B4-NFAT-GFP reporter cells expressing both ILT7 and FcεRIγ and control reporter cells expressing one of the two molecules were cultured for 36 h in the 48-well plate precoated with α-ILT7 or murine IgG1 at the concentration of 10 μg/mL. GFP expression of these cells was measured by flow cytometry and shown by histograms (left) and dot plots (right). Left, thin and thick lines, mIgG1 and α-ILT7, respectively; shaded histograms, GFP from each reporter cell cultured without any antibody. B and C, cancer cell lines (LU99 and ACHN) cultured with or without IFN-γ (800 units/mL) for 48 h were cocultured with reporter cells, and GFP expression from reporter cells was measured. Thick line, GFP expression from reporter cells expressing both ILT7L and FcεRIγ cocultured with cancer cells; thin and dashed lines, GFP from reporter cells expressing ILT7 alone and FcεRIγ alone cocultured with cancer cells; shaded histograms, GFP from reporter cells expressing both ILT7L and FcεRIγ cultured alone.
conjugated anti-mouse CD3e (BD Bioscience), anti-human IRF-1 (C-20; Santa Cruz Biotechnology), anti-HLA-E (4D12; MBL), phycoerythrin-conjugated anti-HLA-G (87G; EXBIO), anti-β2-microglobulin (p2M; Beckman Coulter), anti-HLA class I (W6/32; Abcam), FITC-conjugated anti-human CD123 (Miltenyi Biotec), phycoerythrin-conjugated anti-BDCA-2 (Miltenyi Biotec), anti-phospho-human H6K (The; Cell Signaling Technology), anti-human H6K (Cell Signaling Technology), anti-lamin B1 (Abcam), anti-human glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology), and mouse IgG1 (Ancell). Anti-human CD3 (OKT-3) antibody was purified from supernatant of the hybridoma (American Type Culture Collection).

Cloning and construction of expression plasmids. Human ILT7 and FcRγ were amplified by reverse transcription-PCR from human tonsil total RNA (Clontech). Human β2M, HLA-E, HLA-F (isoform 1, NM_001098479), HLA-G (isoform 1, NM_002127), and IRF-1 were cloned from human mature dendritic cells, APC-1, L199, placenta total RNA (BD Bioscience), and HCT116, respectively. Expression vectors for transient transfection were constructed using SRs promoter-driven pME18S plasmid. Retroviral vectors were constructed using pMX-s-puro and pMXs-neo (both kindly provided by Dr. T. Kitamura; ref. 20). For transient transfection, 5 × 10⁵ cells in 60 mm dish were transfected with 3 μg DNA using FuGENE6 (Roche) following the manufacturer’s instructions. Expression of HLA-F and glyceraldehyde-3-phosphate dehydrogenase were confirmed by reverse transcription-PCR with HLA-F specific primers (5′-AGTATTGGGAGTGGACCACAGG-3′ and 5′-CGTCTCTCTCTCATTCACGG-3′) and glyceraldehyde-3-phosphate dehydrogenase specific primers (5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCACACACTGTTCGCTGA-3′).

Reporter cell construction. Human ILT7 and FcRγ were introduced by retroviral vectors into the 2B4 cell line expressing NFAT-green fluorescent protein (GFP) reporter construct (kindly provided by Dr. H. Arase; ref. 21). Retroviral vectors pMXs-neo-ILT7 and pMXs-neo-FcRγ were transduced into PLAT-E packaging cells (gift from Dr. T. Kitamura) and produced virus supernatant was used to infect the 2B4-NFAT-GFP cells. Infected cells were selected by G418 and/or puromycin. As controls, 2B4-NFAT-GFP reporter cells expressing ILT7 alone or FcRγ alone were also established.

Coculture of reporter cell with cancer or normal cells. Cancer or normal cell lines treated with various cytokines/reagents were collected by using PBS supplemented with EDTA and washed repeatedly to prevent carryover of the reagents. To avoid competing ILT7L expression for different number of cells, these were γ-irradiated (100 Gy for cancer cells and 50 Gy for normal cells and immune cells) to stop their proliferation. Then, 2 × 10⁵ of cells to be examined for ILT7L expression were cocultured with 1 × 10⁵ of reporter cells in a 48-well plate for 36 h. Whole cells were collected and stained with anti-mouse CD3ε-allophycocyanin. GFP expression of reporter cells was measured from CD3ε-positive populations using FACS Calibur (BD Bioscience).

Cytokines/TLR ligands/inhibitors treatment of cancer or normal cell lines. Cancer or normal cell lines were treated with following cytokines, TLR ligands, or inhibitors to see the results. Intensities of bands corresponding to NFAT and 389 to 414 of the human β2M coding sequence, respectively. The sense strand sequences are 5′-AAACCCUGAUAUCCGAGAUGCGCUG-3′ for the β2M si#124 and 5′-UCCUGGACUCUCCUAAUGUGCG-3′ for the β2M si#389. These small interfering RNAs were transfected into LU99 cells using siLentFect (BIO-RAD) at the concentration of 40 nmol/L according to the manufacturer’s protocol. Decrease of β2M and HLA class I were confirmed by flow cytometry using anti-β2M antibody and anti-HLA class I antibody.

Electrophoretic mobility shift assay. Nuclear extracts were prepared according to the method described by Andrews and Faller (22). Nuclear extracts (12 μg) and poly(1C) (1 μg; Sigma) were incubated with 32P-labeled DNA probe and then separated on a 4% native polyacrylamide gel. The DNA probe used for NF-κB binding was the double-stranded oligonucleotide composed of two oligonucleotides (5′-AGTCTACAGGGGACCTTCCGAG-3′ and 5′-TGACACCTCGGAAAGTCTTGTCA-3′). BAS-5000 (Fujifilm) was used to visualize the results. Intensities of bands corresponding to NF-κB−bound probes were estimated using the Image Gauge software (Fujifilm). Equal loading of nuclear extracts were confirmed by Western blotting using anti-lamin B1 antibody. Band intensities were estimated with ImageJ program (NIH).

Results

Constitutive or cytokine-induced expression of ILT7L in various human cancer cell lines. To detect ILT7L expression on human cancer cells, human ILT7 and FcRγ were stably introduced into a mouse T hybridoma 2B4 NFAT-GFP reporter cell line (21). In this reporter cell line, ILT7-FcRγ complex recognizes an ILT7-specific ligand, which leads to NFAT activation and,
subsequently, induction of GFP expression. As control reporter cells, 2B4-NFAT-GFP reporter cells expressing ILT7 alone or FcεRIγ alone were also established to clarify the ILT7/ILT7L specific interaction. Cross-linking of ILT7 with an ILT7-specific antibody induced strong GFP expression by the reporter cells expressing both ILT7 and FcεRIγ but did not express GFP in control reporter cells that express ILT7 or FcεRIγ alone (Fig. 1A). Isotype-matched control antibody did not induce GFP expression in all three reporter cells (Fig. 1A). Thus, the reporter cells express GFP when ILT7 on the reporter cells specifically recognize ILT7L on cancer cells.

Surface expression of ILT7L on the various human cancer cell lines was evaluated by induction of GFP expression in the reporter cells when cocultured with cancer cell lines. Because HLA-G, a known ligand for ILT2 and ILT4, was shown to be inducible on human cancer cells after IFN-γ treatment (23), we have tested possible ILT7L expression with or without pre-treatment with IFN-γ. As shown in the representative results of Fig. 1B and C, some cancer cells including LU99 lung cancer cell line constitutively express high levels of ILT7L even without IFN-γ treatment, whereas other cancer cell lines including ACHN RCC line only express high ILT7L levels after treatment
Fig. 3. Induction of ILT7L by various cytokines and TLR ligands. A, cancer and noncancer cell lines were treated with various cytokines [IFN-γ (800 units/mL), IFN-α (1,000 units/mL), TNF-α (100 ng/mL), IL-1β (800 units/mL), TGF-β (10 ng/mL), vascular endothelial growth factor (25 ng/mL), or IL-6 (100 ng/mL)] or LPS (100 ng/mL) for 48 h, and ILT7L expression was estimated by coculturing with reporter cells. B, C32 and T98G cells transfected with IRF-1 or mock were examined for ILT7L expression. Thin, thick, and dashed lines, mock transfectant, IRF-1 transfectant, and parental cancer cell line. Protein expression of IRF-1 and glyceraldehyde-3-phosphate dehydrogenase of parental lines and transfectants are also shown. C, human immune cells derived from PBMCs were treated with cytokines and TLR ligands in the same condition as in A and examined for their ILT7L expression by reporter cells. Dotted line, GFP expression from reporter cells without coculturing. Representative of more than two independent experiments.
with IFN-γ. No GFP emission was detected in control reporter cells, indicating that these observed results indicate specific interactions between ILT7-FcεRIγ-expressing reporter cells and a ILT7-specific ligand on the surface of cancer cells.

Expression of a ILT7L was evaluated for a total of 48 human cancer cell lines, with 8 cell lines for each cancer type, including melanoma, colon cancer, pancreatic cancer, RCC, lung cancer, and glioma (Supplementary Fig. S1). Figure 2 shows three representatives for each cancer type. Some of the cell lines such as RCC7, 769-P, and U1199 highly express ILT7L, without any cytokine stimulation. IFN-γ treatment induced only slight increase of ILT7L, whereas some cell lines such as C32, 1363mel, or T98G showed profound up-regulation of ILT7L following IFN-γ treatment. GFP emission was not detected in these cancer cell lines when cocultured with either ILT7- or FcεRIγ-transfected reporter cells (data not shown). The constitutive ILT7L expression and IFN-γ-induced ILT7L expression are observed in 6 of 8 (75%) and 8 of 8 (100%) cell lines analyzed in melanoma, 3 of 8 (37.5%) and 6 of 8 (75%) in colon cancer, 4 of 8 (50%) and 7 of 8 (87.5%) in pancreatic cancer, 5 of 8 (62.5%) and 8 of 8 (100%) in RCC, 6 of 8 (75%) and 6 of 8 (75%) in lung cancer, and 3 of 8 (37.5%) and 5 of 8 (62.5%) for glioma, respectively (Supplementary Table S1). Constitutive ILT7L expression was frequently observed in melanoma, RCC, and lung cancers. Lung cancer cell lines tested include large cell carcinoma (LU99 and H661), adenocarcinoma (RERF-LC-MS, RERF-LC-OK, and PC-9), squamous cell carcinoma (LK-2 and LC-1sq), and small cell carcinoma (SBC-2). Intensity of ILT7L expression in IFN-γ-treated cancer cell lines was profoundly increased (Fig. 2; Supplementary Fig. S1). Our results showed that various human cancer cells express ILT7L constitutively or up-regulated by IFN-γ stimulation.

Induction of ILT7L on human cancer cells and various noncancer cells by various cytokines and TLR ligands. To investigate other stimuli capable of inducing ILT7L expression, we next examined expression levels of ILT7L on human cancer and noncancer cells after stimulation with various cytokines and a TLR4 ligand, including IFN-α, IFN-β, TNF-α, IL-1β, LPS, TGF-β, vascular endothelial growth factor, IL-6, and LPS (Fig. 3A). Cancer cell lines (SF126, WM115, and ACHN) and cultured noncancer cells (fibroblasts and human vascular and umbilical vein endothelial cells) were stimulated for 48 h and then cocultured with the reporter cells. IFN-α and IFN-β treatments induced greater expression of ILT7L on all three cancer cell lines similar to IFN-γ treatment, but the effects of TNF-α and IL-1β treatments differed among cancer cells. TNF-α up-regulated ILT7L on SF126 and WM115 but not on ACHN and IL-1β induced ILT7L only on SF126. TGF-β induced ILT7L on ACHN; however, LPS, vascular endothelial growth factor, and IL-6 had no effect on these cancer cells. Hence, signals mediated by type I and II IFNs, TNF-α, IL-1β, and TGF-β have the ability to induce ILT7L expression on various human cancer cells. Because IFN treatments greatly increased ILT7L expression, we then evaluated whether IFN-associated signaling pathways potentially up-regulate surface ILT7L on the cells. Introduction of IRF-1, which is a IFN and TNF-α–induced transcription factor that participates in many IFN-specific signaling pathways (24), by transfection into C32 and T98G cancer cell lines resulted in increased ILT7L (Fig. 3B). This indicates that ILT7L is a IFN-1-inducible gene and possibly contains IFN-stimulated response element motif in its promoter.

Fig. 4. Expression of ILT7L correlated with inhibition of IFN-α and TNF-α from pDCs. 2 × 10^4 of pDCs isolated from PBMCs were cocultured for 24 h with 4 × 10^5 of 1363mel, AaPC-1, T98G, or SBC-2 treated with or without IFN-γ (800 units/mL, 48 h) after removing IFN-γ by repeated washing. CpG-ODN type A was then added at the concentration of 5 µmol/L. Supernatants of the culture were collected after 18 h from the CpG-ODN stimulation. IFN-α and TNF-α in the supernatant were measured by ELISA. Inset, purity of isolated pDCs. Bars, SD (n = 3).
Among the noncancer cells, constitutive ILT7L expression was not detected in fibroblasts; however, all the IFNs, TNF-α, IL-1β, and LPS induced ILT7L on fibroblasts (Fig. 3A). Human umbilical vein endothelial cells expressed constitutively ILT7L at low level, and all the IFNs, TNF-α, and IL-1β increased ILT7L expression in human umbilical vein endothelial cells. The expression of ILT7L on various immune cells from healthy donors was also examined (Fig. 3C). ILT7L was not detected on CD14+ and CD14- populations in PBMCs isolated from healthy volunteers. In the CD14+ population, we confirmed that T and NK cells did not express ILT7L even after inducing activation with anti-CD3 antibody and IL-2 or IL-2, respectively. Stimulation with type I or type II IFNs could not induce ILT7L on T and NK cells (data not shown). From the CD14+ population, mDCs and macrophages were generated and examined for their ILT7L expression. Immature and matured mDCs express ILT7L at very low levels and were not increased by IFN treatment. Macrophages had no constitutive expression of ILT7L. Treating macrophages with type I and II IFNs and TLR4-stimulating LPS did not induce ILT7L expression (data not shown). However, interestingly, TLR3-stimulating poly(I:C) weakly increased ILT7L and TLR7-stimulating R837 (imiquimod) induced greater expression of ILT7L than poly(I:C) stimulation. Our data indicate that various noncancer cells also express ILT7L. IFN-α produced by pDC may induce ILT7L on these stromal cells in the disease sites and subsequently inhibit pDC for IFN-α production as a feedback mechanism when viral infection occurs. Additionally, influenza virus containing TLR7-stimulating single-stranded RNA may induce ILT7L on macrophages and may be involved in the negative regulation of pDC IFN-α production in the virus-infected tissues.

Inhibition of pDC IFN-α and TNF-α production through ILT7L expressed on human cancer cells. ILT7-mediated signaling has been shown to inhibit production of type I IFN and TNF-α by TLR-stimulated pDCs when stimulated with anti-ILT7 antibody (18). We evaluated type I IFN and TNF-α production by human peripheral blood pDCs when cocultured with human cancer cell lines that highly express ILT7L after pretreatment with IFN-γ and cancer cell lines that do not up-regulated ILT7L, 1363mel, AsPC-1, T98G, and SBC-2 were pretreated with or without IFN-γ and cocultured for 24 h with pDCs isolated from human PBMCs. Then, CpG-ODN was added into the cultures to activate the pDCs and supernatants were collected for ELISA analysis. Both type I IFN and TNF-α were not detected in the supernatants of these cancer cell lines without pDCs (data not shown). Both IFN-α and TNF-α produced by pDC were inhibited in the presence of non-IFN-γ-treated cancer cells, strongly by AsPC-1 and T98G, which constitutively expressed ILT7L without IFN-γ stimulation, and only weakly by 1363mel and SBC-2 which did not express ILT7L without IFN-γ stimulation (Fig. 4). Furthermore, IFN-α and TNF-α production by pDC cocultured with IFN-γ-pretreated 1363mel, AsPC-1, and T98G, which expressed high ILT7L, were significantly inhibited compared with those by pDC cocultured with non-IFN-γ-treated cancer cells. IFN-α and TNF-α from pDC cocultured with SBC-2, which did not express ILT7L even after IFN-γ stimulation, were not significantly inhibited. The correlation of the inhibition of IFN-α and TNF-α by pDC with ILT7L expression on cancer cells along with the previous results of the inhibition of IFN-α production by pDC treated with anti-ILT7 antibody (18) indicated that ILT7L on cancer cells inhibits IFN-α and TNF-α production by pDC, although ILT7L-dependent inhibition should be further confirmed by specific blockade of ILT7/ILT7L interaction, which is currently not available.

ILT7L is neither a classic nor a nonclassic HLA molecule. Other ILT family molecules including ILT2 and ILT4 are known to bind to nonclassic MHC class I molecules, HLA-E, HLA-F, and HLA-G (25), which are expressed in some of human cancers (23, 26–29). HLA-G is induced by IFN in an IRF-1–dependent manner (30). Thus, we tested the possibility that the detected ILT7L could be a classic or nonclassic MHC molecule. The three nonclassic HLA class I heavy chains cDNAs were transfected into COS-7 cells along with human β2M cDNA and then cocultured with the reporter cells. Despite the expression of the HLA class I molecules

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**Figure 5.** ILT7L is neither a nonclassic HLA (HLA-E, HLA-F, and HLA-G) nor a classic HLA. A, COS-7 cells transfected with HLA-E, HLA-F, or HLA-G together with human β2M were examined for ILT7L expression (left). Thin and thick lines, mock transfectant and HLA transfectant, respectively. Expression of HLA-E and HLA-G for mock and HLA transfectants were examined by antibody for each HLA molecule and HLA-F mRNA expression was examined by reverse transcription-PCR (right). B, LU99 cells transfected with human β2M-specific small interfering RNAs were examined for ILT7L expression at 72 h after the transfection (right). Knockdown of β2M and down-regulation of HLA class I were confirmed by flow cytometry at 72 h after the transfection (left). Lines in the histograms on the left correspond to the right. A and B, shaded histograms, GFP expression from reporter cells cultured alone.
Fig. 6. Heterogenous mechanisms for the constitutive expression of ILT7L in human cancer cells. A, cancer cells (LU99, RCC7, and 769-P), which constitutively express ILT7L, and Caki-1, which does not express ILT7L, were treated with DHMEQ (10 μg/mL) for 12 h. NF-κB activities of these cells were evaluated by electrophoretic mobility shift assay using NF-κB specific probes. n.s., nonspecific band. Expression of lamin B1 was examined for the same amount of nuclear extracts used for electrophoretic mobility shift assay (middle). Relative NF-κB band intensities compensated by lamin B1 expression are shown in a histogram (bottom). B, above four cells were treated with rapamycin (100 ng/mL) for 12 h. S6K activities of these cells were evaluated by Western blotting using anti-phospho-S6K (Thr389) antibody. DM and rap, DMSO and rapamycin, respectively. C, RCC7, 769-P, and LU99 were treated with indicated inhibitors for 48 h with the same concentration above. ILT7L expression of these cells was estimated by coculturing with reporter cells. Shaded histograms, GFP expression from reporter cells cultured alone. Representative of more than two independent experiments. D, cancer cell lines treated with indicated inhibitors with the same concentration above for 48 h were γ-irradiated and 2 × 10^5 cells were cultured in a 48-well plate for 48 h before counting the cell number in each well. DH, DHMEQ. Bars, SD (n = 3).
(Fig. 5A, right), no GFP expression by the reporter cells was observed for all the three cases, indicating that the detected ILT7L is not HLA-E, HLA-F, or HLA-G (Fig. 5A, left). To test if ILT7L is a classic MHC class I, effects of down-regulation of all HLA class I on cancer cells by β2M-specific small interfering RNA were evaluated. Knockdown of β2M in LU99 cells, which constitutively express ILT7L at high level, did not affect ILT7L expression level (Fig. 5B, right), although both β2M and HLA class I expression were significantly decreased by β2M-specific small interfering RNA (Fig. 5B, left), indicating that ILT7L is not a classic HLA class I either. These results suggest that ILT7L is neither a classic nor a nonclassic HLA.

**Heterogeneous mechanisms for the constitutive expression of ILT7L in human cancer cells.** To elucidate the mechanisms for the constitutive expression of ILT7L on some of the human cancer cells, including RCC7, 769-P, and LU99, the effects of several inhibitors for signaling pathways frequently activated by genetic alterations in cancer cells were evaluated for the ILT7L expression. Inhibitors U0126, SP600125, SB203580, DHMEQ, LY294002, rapamycin, and Y-27632 were used to inhibit MEK1/2, c-Jun NH2-terminal kinase, p38 mitogen-activated protein kinase, NF-κB, phosphoinositide 3-kinase, mammalian target of rapamycin (mTOR), and ROCK, respectively. Among these inhibitors, NF-κB inhibitor DHMEQ and mTOR inhibitor rapamycin showed some effects on ILT7L expression. NF-κB and phosphoinositide 3-kinase/AKT/mTOR signals are frequently activated in various cancers. Basal activities of the target molecules and their inhibition by their specific inhibitors were evaluated (Fig. 6A and B). Band intensities corresponding to NF-κB activity were quantified and normalized by lamin B1 band intensities (Fig. 6A, histogram). NF-κB was highly activated in both RCC7 and 769-P and slightly activated in LU99 but not in Caki-1 (Fig. 6A). DHMEQ effectively inhibited these NF-κB activities. mTOR appears to be activated in all these cells as detected by phosphorylation of downstream molecule p70 S6K, which was inhibited by rapamycin (Fig. 6B). ILT7L expression was then measured by the reporter cell assay after 48 h pretreatment with or without inhibitors (Fig. 6C). DHMEQ partially decreased the ILT7L expression in RCC7 and 769-P, whereas rapamycin had no effect. Interestingly, DHMEQ slightly and rapamycin drastically increased the ILT7L expression in LU99. Observed differences in reporter cell GFP expression by the inhibitors are not due to the effects on cancer cell proliferation, because cell numbers after treatment with these inhibitors were not significantly affected (Fig. 6D). Similar increase of ILT7L by the rapamycin treatment was also observed in H661 large cell lung carcinoma, SF126 glioma, and A498 RCC line (data not shown). These results indicate that the mechanisms for constitutive expression of ILT7L differ among cancer cells. At least in the RCC, activated NF-κB appears to be one of the mechanisms involved in constitutive ILT7L expression.

**Discussion**

Immunosuppression induced by cancer cells, often targeting dendritic cells, is one of the major problems in the development of effective immunotherapy. pDCs are specialized cells, which produce large amounts of type I IFN in response to viral infections (31). Type I IFNs produced by pDCs promote the functions of other immune cells and link innate and adaptive immune responses including antitumor T-cell responses (8). Recent reports indicate that functionally impaired pDCs can infiltrate into tumor sites or sentinel lymph nodes in several types of cancers (11–13). One mechanism to modulate pDC type I IFN production is mediated by ILT7 specifically expressed on pDCs. ILT7 makes stable complex with FcγRIy and ILT7 ligation inhibits the production of type I IFNs and other cytokines from pDCs stimulated by CpG or influenza virus via ILT7/FcγRIy signaling (18).

In this study, using reporter cells capable of detecting ILT7L on target cells, we have evaluated expression of ILT7L on various human cancer cell lines and identified that ILT7L is expressed on all the cancer cell types evaluated constitutively and inducibly by several cytokines (Figs. 2 and 3; Supplementary Fig. S1). Cancer cell lines with high ILT7L expression induced by IFN-γ inhibited IFN-α production from human pDC (Fig. 4), indicating that the ILT7L/ILT7 interaction between the cancer cells and pDC may cause impairment of pDCs in the tumor microenvironment possibly leading to immunosuppression and poor prognosis of cancer patients as observed in the previous clinical studies (11, 12). Some cancer cells constitutively express ILT7L, and others express it by stimulation with cytokines, which are likely produced by surrounding stromal cells and immune cells.

Some of the cancer cell lines, particularly melanoma, RCC, and lung cancer, constitutively express ILT7L frequently. The elucidation of the mechanism for the constitutive ILT7L expression on cancer cells is important to understand the cancer cell triggered immunosuppression and to develop its overcoming methods. Inhibitors for MEK1/2, c-Jun NH2-terminal kinase, p38 mitogen-activated protein kinase, NF-κB, phosphoinositide 3-kinase, mTOR, and ROCK were used to evaluate the mechanisms associated with the constitutive ILT7L expression. Melanoma cells often have activation of mitogen-activated protein kinase signaling via BRAF mutation and Wnt/β-catenin signaling; however, treatment of MEK inhibitor or transfection of active forms of β-catenin (S37F mutant) did not affect ILT7L expression (data not shown). For lung cancer cell lines, none of the inhibitors decreased ILT7L expression. Rapamycin and DHMEQ rather increased ILT7L expression. Up-regulation of ILT7L by rapamycin in two large cell lung carcinoma lines suggests that a common mechanism may exist for large cell lung carcinomas. Additionally, RCC cells frequently have somatic mutations in the von Hippel-Lindau tumor suppressor gene (32, 33). 769-P, A498, 786-O, and SW839 have von Hippel-Lindau mutations, whereas RCC7, ACHN, KU19-20, and Caki-1 have wild-type von Hippel-Lindau (33). Thus, the status of von Hippel-Lindau mutation is not correlated with ILT7L expression. RCC frequently display activation of mitogen-activated protein kinase, Akt, mTOR, and NF-κB (34–38), but treatment with inhibitors for the most of the evaluated signaling pathways did not affect ILT7L expression on RCC. However, treatment with NF-κB inhibitor DHMEQ partially down-regulated ILT7L on RCC7 and 769-P, indicating that constitutively active NF-κB may be involved in the constitutive expression of ILT7L on these RCC lines. Therefore, constitutive ILT7L expression on some of RCC is caused by constitutively active NF-κB frequently observed in RCC. However, the mechanisms for the constitutive ILT7L expression are heterogeneous among cancers, and it remains to be elucidated for various types of human cancers.

A variety of noncancer cells, including stromal cells such as fibroblasts and endothelial cells as well as immune cells such...
as macrophages and dendritic cells, do not constitutively express ILT7L or only express low levels, but some normal cells can express it significantly when stimulated with various cytokines or TLR ligands (Fig. 3). ILT7L on these stromal cells induced by IFN-α from activated pDC may work as a negative feedback system to maintain immune homeostasis after viral infection. In addition, ILT7L on macrophages induced by influenza virus single-stranded RNA may also be involved in the negative regulation of IFN-α production by pDC in the viral infected tissues.

We tested whether ILT7L may be a nonclassic or classic HLA class I molecule because other ILTs such as ILT2 and ILT4 are known to bind them, and ILT7L was induced by IFN through IRF-1, which is similar to nonclassic or classic HLAs. However, nonclassic HLAs (HLA-E, HLA-F, and HLA-G) and classic MHC class I do not appear to be ILT7L (Fig. 5). Further study is required for the identification of ILT7L.

In summary, we have shown that ligand for ILT7 is expressed on various human cancer cells constitutively and inducibly by several cytokines though different signaling pathways among cancer, and it may be one of the mechanisms for generation of immunosuppressive tumor microenvironment. It was also suggested that ILT7/ILT7L may work for a negative feedback system during viral infection. Therefore, intervention on the ILT7/ILT7L system, including targeting signaling molecules in cancer cells, may restore immunocompetence in cancer patients and augment antitumor as well as antiviral immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

Impairment of Plasmacytoid Dendritic Cells for IFN Production by the Ligand for Immunoglobulin-Like Transcript 7 Expressed on Human Cancer Cells

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