

The Killer Cell Ig-like Receptor 2DL4 Expression in Human Mast Cells and Its Potential Role in Breast Cancer Invasion

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Abstract

The killer-cell Ig-like receptor (KIR) 2DL4 (CD158d) acts as a receptor for human leukocyte antigen (HLA)-G and is expressed on almost all human natural killer (NK) cells. The expression and function of KIR2DL4 in other hematopoietic cells is poorly understood. Here, we focused on human mast cells, which exhibit cytotoxic activity similar to that of NK cells. KIR2DL4 was detected in all examined human cultured mast cells established from peripheral blood derived from healthy volunteers (PB-mast), the human mast cell line LAD2, and human nonneoplastic mast cells, including those on pathologic specimens. An agonistic antibody against KIR2DL4 decreased KIT-mediated and IgE-triggered responses, and enhanced the granzyme B production by PB-mast and LAD2 cells, by activating Src homology 2-containing protein

tyrosine phosphatase (SHP-2). Next, we performed a coculture assay between LAD2 cells and the HLA-G⁺ cancer cells, MCF-7 and JEG-3, and showed that KIR2DL4 on LAD2 cells enhanced MMP-9 production and the invasive activity of both cell lines via HLA-G. Immunohistochemical analysis revealed that the direct interaction between HLA-G⁺ breast cancer cells and KIR2DL4⁺ tissue mast cells (observed in 12 of 36 cases; 33.3%) was statistically correlated with the presence of lymph node metastasis or lymphovascular invasion (observed in 11 of 12 cases; 91.7%; $\chi^2 = 7.439$; $P < 0.01$; degrees of freedom, 1) in the clinical samples. These findings suggest that the KIR2DL4 on human mast cells facilitates HLA-G-expressing cancer invasion and the subsequent metastasis. *Cancer Immunol Res*; 3(8): 871–80. ©2015 AACR.

Introduction

KIR2DL4 (2DL4/CD158d) is a killer-cell Ig-like receptor (KIR; refs. 1, 2). KIRs are transmembrane proteins that possess two-domain (2D) or three-domain (3D) Ig-like extracellular domains. They are expressed on human natural killer (NK) cells and recognize major histocompatibility complex (MHC) class I molecules (2). In addition, KIRs are categorized according to the presence or absence of immunoreceptor tyrosine-based inhibitory motifs (ITIM), in other words, into KIR with long (L) or short (S) cytoplasmic domains. KIR2DL4 has two Ig-like domains and a long cytoplasmic domain; that is, it contains ITIM motifs, which allow it to transduce inhibitory signals to NK cells (3). The inhibitory signals transduced by KIR2DL4 are mediated by Src homology 2-containing protein tyrosine phosphatase (SHP)-1 and SHP-2 in NK cells (3). Several reports have shown that KIR2DL4 stimulates NK activity and IFN γ secretion (4–7).

Human leukocyte antigen (HLA)-G, which is expressed in fetal tissues during pregnancy and cancer cells, has been identified as the ligand of KIR2DL4 (1, 2). Therefore, it has been suggested that NK cells expressing KIR2DL4 might be involved in the maintenance of pregnancy and in cancer progression (8, 9). Such hypotheses were derived from analyses of NK cells, and, to the best of our knowledge, neither the role(s) nor expression of KIR2DL4 has been examined in human mast cells.

Mast cells are derived from hematopoietic stem cells and are immunocompetent (10). They are activated via KIT and immunoglobulin E (IgE) receptors and secrete Th2 cytokines. In fact, mast cell-derived Th2 cytokines are considered to cause allergic reactions. Furthermore, mast cells have been shown to be involved in both Th17 (11–14) and Th1 responses (11, 15–18). Similar to NK cells, mast cells also secrete the Th1 cytokine IFN γ (17, 19) and exert NK activity via granzyme B (GrB; refs. 20–24).

In this study, we hypothesized that KIR2DL4 plays a functional role in human mast cells, as is the case for NK cells, and so we analyzed KIR2DL4 expression on mast cells.

Materials and Methods

Cells and tissue culture

We prepared human CD34⁺ peripheral blood cell-derived mast cells (PB-mast) and two human mast cell lines (LAD2 and HMC1.2 cells). The PB-mast cells were obtained by culturing CD34⁺ cells in StemPro-34 (Invitrogen) containing recombinant human stem cell factor (rhSCF; Peprotech) and rhIL6 (Peprotech), as described by Kirshenbaum and colleagues (25). The LAD2 cells were cultured in StemPro-34 containing rhSCF (26), and the

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HMC1.2 cells were cultured in Iscove modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; ref. 27).

NK-92, HL60, MCF-7, and JEG-3 cells were purchased from the ATCC. The NK-92 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and rhIL2, and the HL60, MCF-7, and JEG-3 cells were cultured in IMDM supplemented with 10% FBS. All cell lines used were confirmed to be *Mycoplasma* free. No other authentication assay was performed.

Antibodies, cytokines, and reagents

One agonistic Ab against KIR2DL4 (mouse monoclonal IgG, clone 181703) was purchased from R&D Systems, and the other agonistic anti-KIR2DL4 Ab (mouse monoclonal IgG, clone 33) was from BioLegend. The anti-KIR2DL4 Ab (rabbit IgG, polyclonal, ab154386) used for the immunohistochemical (IHC) examinations and the anti-mast cell tryptase (MCT) Ab (mouse monoclonal IgG, clone AA1) were obtained from Abcam. The anti-HLA-G Ab for IHC examinations (mouse monoclonal IgG, clone 87G) was obtained from eBioscience, and the Fab fragment of the anti-HLA-G Ab (mouse monoclonal IgG, clone 87G) was from Exbio Praha. The isotype control Ab was obtained from BD Biosciences. Fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG Ab (for flow cytometry) was purchased from BD Biosciences, and the secondary Ab (for Western blotting) was peroxidase-labeled anti-rabbit or anti-mouse IgG Ab (Santa Cruz Biotechnology).

KIR2DL4-targeting shRNA lentiviral particles and the associated control particles were purchased from Santa Cruz Biotechnology. The infection and subsequent selection of these viral particles were performed according to the manufacturer's instructions.

The specific SHP-2 inhibitor PTP Inhibitor V PHPS1 (28) was also purchased from Santa Cruz Biotechnology. We used PHPS1 at a concentration of 10 $\mu\text{mol/L}$ in each experiment (28).

RT-PCR

Mast cells (the PB-mast and LAD2 cells after overnight cytokine depletion, and the HMC1.2 cells; density: 5×10^6 cells in all cases) were added with 600 μL of TRIzol (Invitrogen Life Technologies), lysed by pipetting up and down, and incubated at -20°C . The samples were added with 120 μL of chloroform, vortexed, and centrifuged at $12,000 \times g$ for 15 minutes. The upper phase of each mixture was placed into a new RNeasy column (Qiagen). Total mRNA was then obtained according to the manufacturer's instructions. Next, 500 ng of each mRNA was reverse transcribed (SuperScript III One-Step RT-PCR System; Invitrogen Life Technologies), and the resultant RT products were used in the KIR typing kit assay (Milenyi Biotec Inc.) or PCR. The PCR was performed using the following primers, 5'-CTGTCCCTGAGCTCTACAA-3' and 5'-CACTGAGTACCTAATCACAG-3', for 35 (Fig. 2A) or 20 cycles (Fig. 6B) of 30 seconds at 94°C (denaturing), 1 minute at 60°C (annealing), and 1 minute at 72°C (extension), followed by a final extension step of 10 minutes at 72°C (29).

Immunoblotting

Cell lysates were prepared, and the proteins were separated by electrophoresis, and the resultant gels were probed for immunoreactive proteins as described previously (30).

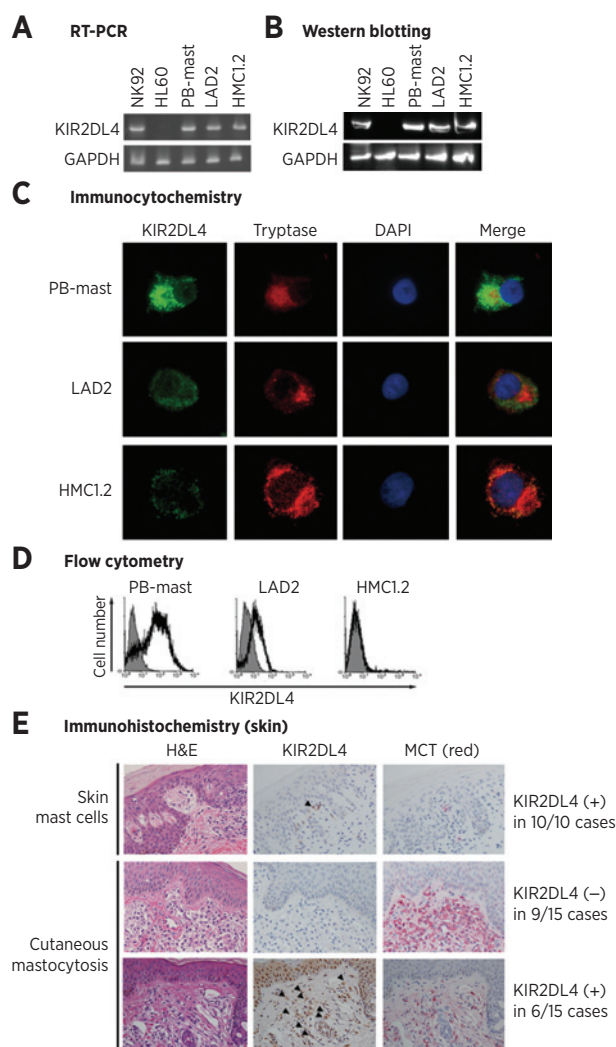


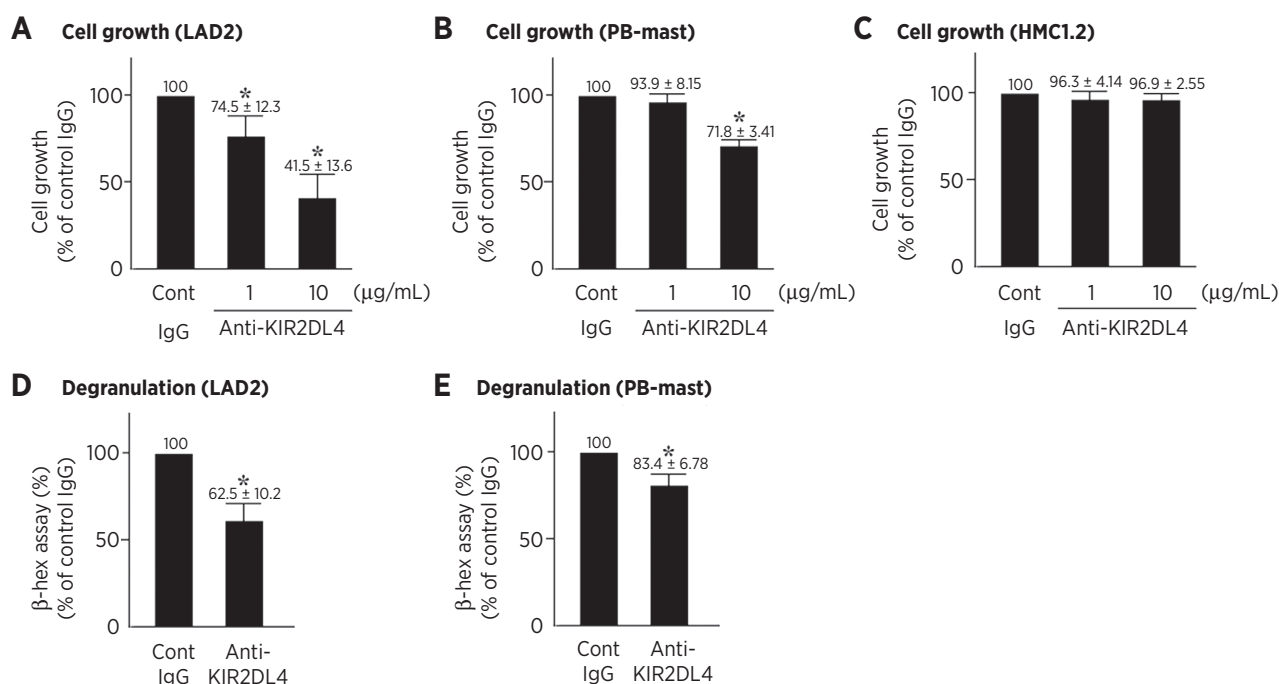
Figure 1. KIR2DL4 is expressed in human mast cells *in vitro* and *in vivo*. RT-PCR (A), immunoblot (B), immunocytochemistry (C), flow cytometry (D), and the IHC examination (E) of skin specimens were performed as described in Materials and Methods. NK92 cells were used as a positive control, and HL60 cells as a negative control in A and B.

Flow cytometry

PB-mast or LAD2 cells after overnight cytokine depletion, or HMC1.2 cells, were washed in phosphate-buffered saline (PBS), fixed in freshly prepared 4% paraformaldehyde (PFA) in 0.1 mol/L of phosphate buffer (pH 7.4), rewashed in PBS, and stained with the anti-KIR2DL4 or control IgG Ab overnight at 4°C , before incubation with anti-mouse IgG-FITC Ab for 3 hours at 4°C . The stained cells were then analyzed using a FACScan flow cytometer.

Immunocytochemistry

PB-mast, LAD2, or HMC1.2 cells were fixed with 100% ethanol for 1 hour at 4°C . After washing with PBS three times, the cells were incubated with anti-KIR2DL4 Ab (rabbit IgG) and anti-MCT Ab (mouse IgG) for 1 hour at 4°C . Alexa Fluor 488-conjugated

**Figure 2.**

Anti-KIR2DL4 Abs downregulate KIT-mediated growth and IgE-triggered degranulation of human mast cells. The effects of anti-KIR2DL4 Abs on the proliferation of LAD2 (A), PB-mast (B), and HMC1.2 cells (C). Cells were incubated for 24 hours with control IgG or anti-KIR2DL4 Ab (clone 181703; 10 µg/mL) in the presence or absence of SCF (10 ng/mL; $n = 3$ in all experiments). Growth was assessed as described in Materials and Methods. Relative values are based on a control IgG value of 100. *, $P < 0.05$, compared with the control IgG value. Effects of anti-KIR2DL4 Ab treatment on degranulation from LAD2 (D) and PB-mast cells (E). Degranulation was evaluated by measuring β-hex release, as described in Materials and Methods. LAD2 or PB-mast cells were sensitized overnight in cytokine-free medium containing biotinylated-human myeloma IgE (100 ng/mL) in the presence of control IgG or anti-KIR2DL4 Ab (clone 181703; 10 µg/mL) and then stimulated with streptavidin (100 ng/mL) without SCF for 30 minutes ($n = 3$ in all experiments). Relative values are based on a control IgG value of 100. *, $P < 0.05$, compared with the control IgG value.

anti-mouse IgG Ab (diluted at 1:200; Abcam) and Alexa Fluor 594-conjugated anti-rabbit IgG Ab (diluted at 1:200; Abcam) were added to the cells as secondary antibodies for 1 hour prior to mounting with fluoroshield mounting medium with DAPI (Abcam) after washing three times with PBS. The stained sections were imaged with a BX63 microscope (Olympus) equipped with an ORCA Flash 2.8 digital camera (Hamamatsu Photonics) and with fluorescent filters for Alexa Fluor 488, Alexa Fluor 594, and DAPI. The images were captured and composited using CellSens Dimension imaging software (Olympus); Alexa Fluor 488 signals were red, Alexa Fluor 594 signals were green, and DAPI signals were blue.

Immunohistochemistry

IHC was performed essentially as described previously (31). We obtained human tissue samples from 15 cases of cutaneous mastocytosis, 10 cases that were compatible with atopic dermatitis, and 36 cases of breast cancer via mastectomy resections in 2011–2013. All human tissue samples were collected at Kyoto University Hospital (Kyoto, Japan) and were approved for use in this study by the ethical committee of our hospital. Tissue sections were deparaffinized with xylene, rehydrated, and pretreated with 0.3% hydrogen peroxide for 5 minutes, and then steam-heated for 40 minutes. Anti-KIR2DL4 Abs (polyclonal rabbit IgG) were added as primary Ab, and the sections were incubated overnight at 4°C. Staining was performed using the ENVISION kit [(horse-radish peroxidase (HRP); Dako Cytomation) according to the manufacturer's instructions. To detect nonneoplastic or neoplas-

tic mast cells, serial sections were treated with anti-MCT Ab and then stained with the ENVISION kit (AEC). In the examinations of breast cancer, serial tissue sections were treated with anti-HLA-G Ab or anti-MCT Ab, and then each section was stained using the ENVISION kit (HRP). In other examinations of breast cancer, a tissue section was stained with anti-HLA-G Ab (mouse IgG, visualized with HRP) followed by staining with anti-MCT Ab (mouse IgG, visualized with AEC). Then, the section was imaged by a BX45 microscope (Olympus) equipped with a DP26 digital camera (Olympus). Then, the same section was stained with anti-KIR2DL4 Ab (rabbit IgG) followed by staining with biotin-conjugated anti-rabbit IgG Ab (Nichirei). FITC-conjugated avidin (Pierce Life Technologies) was added to the section followed by mounting with fluoroshield mounting medium with DAPI. The mounted section was imaged with a BX63 microscope (Olympus) equipped with an ORCA Flash 2.8 digital camera (Hamamatsu Photonics). To assess mast cell–breast cancer cell interaction, we evaluated maximum section of the resected specimens. Direct interactions between mast cells and cancer cells were scored when we observed more than 10 MCT⁺ cells localized adjacent to cancer cells per 10 high-power field.

Cell-proliferation assay

The Cell Counting Kit-8 (CCK-8; Dojindo) was used to evaluate the proliferation of PB-mast, LAD2, and HMC1.2 cells. PB-mast or LAD2 cells were cultured overnight in cytokine-free IMDM supplemented with 10% FBS. Then, PB-mast, LAD2, and HMC1.2

cells were recultured for 22 hours at a density of $1\text{--}3 \times 10^4$ cells/100 μL of RPMI-1640 medium containing 10% FCS, together with the control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 $\mu\text{g/mL}$, each) in the presence or absence of SCF (10 ng/mL). In some experiments, DMSO or PHPS1 was added with the Abs. We added 10 μL of CCK-8 solution for the last 2 hours and estimated the absorbance of the cultures at 450 nm, according to the manufacturer's instructions.

Degranulation assay

β -Hexosaminidase (β -hex) release was used as a measure of IgE-triggered degranulation activity (30). LAD2 or PB-mast cells were incubated overnight in cytokine-free StemPro cell culture medium together with biotinylated human myeloma IgE (100 ng/mL; Calbiochem, EMD Biosciences). After washing with HEPES buffer containing 0.04% bovine serum albumin (BSA), cells were reincubated in the same buffer supplemented with streptavidin (100 ng/mL; Sigma-Aldrich) and the control IgG or anti-KIR2DL4 Ab (clone 181703; 10 $\mu\text{g/mL}$ in both cases) for 30 minutes. The resultant supernatants were aliquoted into 96-well plates for the β -hex assay. Degranulation activity was calculated as follows: degranulation activity (%) = β -hex value for cells cultured with IgG or anti-KIR2DL4 Ab – background β -hex secretion (value for cultured cells alone)/maximum β -hex secretion (value for cells treated with Triton X-100) – background β -hex secretion.

ELISA for GrB

To examine GrB production/secretion, LAD2 or PB-mast cells were cultured overnight in cytokine-free IMDM supplemented with 10% FBS and then resuspended in cytokine-free StemPro-34 or StemPro-34 containing rhSCF. Next, 1×10^5 LAD2 cells (in 100 μL medium per well) were cultured for 6 hours with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 $\mu\text{g/mL}$, each). In some experiments, DMSO or PHPS1 was added with the Abs. Fifty-microliter samples of the resultant supernatants were collected and used in an ELISA for GrB (Human Granzyme B Platinum ELISA; eBioscience), according to the manufacturer's protocol.

Assessment of phospho-mitogen-activated protein kinases

We assessed the amounts of phospho-extracellular signal-regulated kinases (ERK), phosphor-c-Jun N-terminal kinases (JNK), and phospho-p38 using the mitogen-activated protein kinase (MAPK) Family (ERK, p38, JNK) Activation InstantOne ELISA Kit (eBioscience). LAD2 cells were cultured overnight in cytokine-free IMDM supplemented with 10% FBS and then resuspended in cytokine-free StemPro-34. Next, 1×10^5 LAD2 cells (in 1.5-mL tubes) were cultured for 30 minutes with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 $\mu\text{g/mL}$, each) in the presence or absence of rhSCF (10 ng/mL). After the cells had been centrifuged, the resultant cell pellets were processed and used for ELISA, which was performed according to the manufacturer's protocol.

Assessment of the invasive activity of MCF-7 and JEG-3 cells

The invasion of MCF-7 and JEG-3 cells into a gel-based matrix was assessed using Matrigel-coated Transwell polycarbonate membranes (8- μm pores; BD Biosciences). MCF-7 or JEG-3 cells (1×10^4 ; 100 μL) were added to cytokine-free medium in the upper chamber of the Transwell. Then, 400 μL of StemPro-34 alone, StemPro-34 containing 1×10^3 LAD2 cells that had been

transfected with the control vector, or StemPro-34 containing 1×10^3 KIR2DL4-knockdown LAD2 cells were added in the presence or absence of the Fab fragment of anti-HLA-G (clone 87G). After 6 hours of incubation at 37°C, cells that had migrated into the lower chamber were fixed in 4% PFA, stained with Diff-Quick stain (Sysmex), and counted using a microscope.

Assessment of the MMP-9 secretion by MCF-7 and JEG-3 cells

To determine MMP-9 levels, MCF-7 or JEG-3 cells were cultured in a 24-well dish until they were almost confluent. After the culture medium had been removed, 500 μL of StemPro-34 alone, StemPro-34 containing 5×10^3 LAD2 cells that had been transfected with the control vector, or StemPro-34 containing 5×10^3 KIR2DL4-knockdown LAD2 cells were added to each well containing JEG3 cells. After 6 hours of incubation at 37°C, culture supernatant aliquots (100 μL each) were collected and used in an ELISA for MMP-9 (human ELISA kit; Invitrogen), which was performed according to the manufacturer's protocol.

Statistical analysis

Data in Figs. 2–5 are expressed as the mean $\pm$ SE. Differences between groups were examined for statistical significance using the Student *t* test (Excel; Microsoft). *P* values of less than 0.05 were considered to indicate statistical significance. The χ^2 test was evaluated using Excel (Microsoft).

Results

KIR2DL4 was expressed by human mast cells *in vitro* and *in vivo*

We screened human CD34⁺ PB-mast cells (25) and human mast cell lines [LAD2 (26) and HMC1.2 cells (27)] for KIR2DL4 mRNA expression by PCR. The NK92 cells were used as a positive control, and the HL60 cells were used as a negative control (32). As a result, KIR2DL4 mRNA and protein were detected in all three

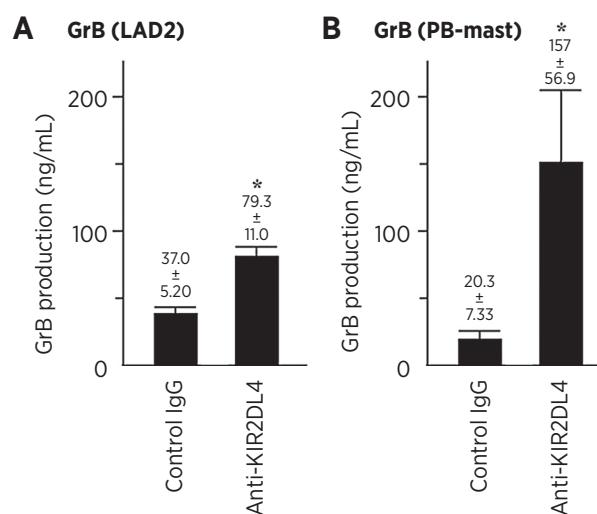
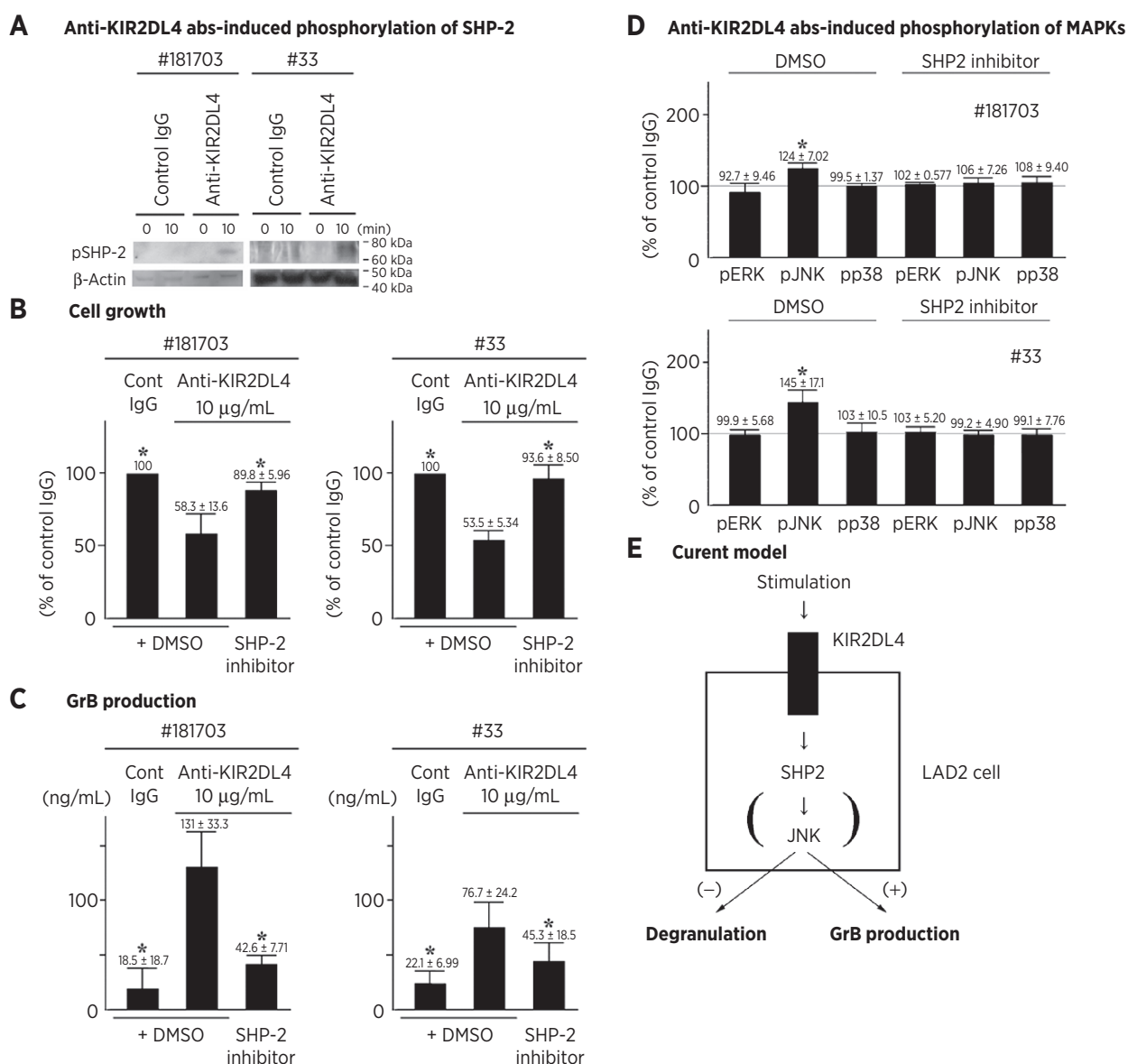


Figure 3.

Anti-KIR2DL4 Abs upregulate GrB production by human mast cells. The effects of anti-KIR2DL4 Ab treatment on GrB production by LAD2 (A), and PB-mast cells (B). A total of 1×10^6 LAD2 or PB-mast cells were cultured for 24 hours with control IgG or anti-KIR2DL4 Ab (Clone 181703; 10 $\mu\text{g/mL}$), and then their culture supernatants were used in the GrB ELISA.

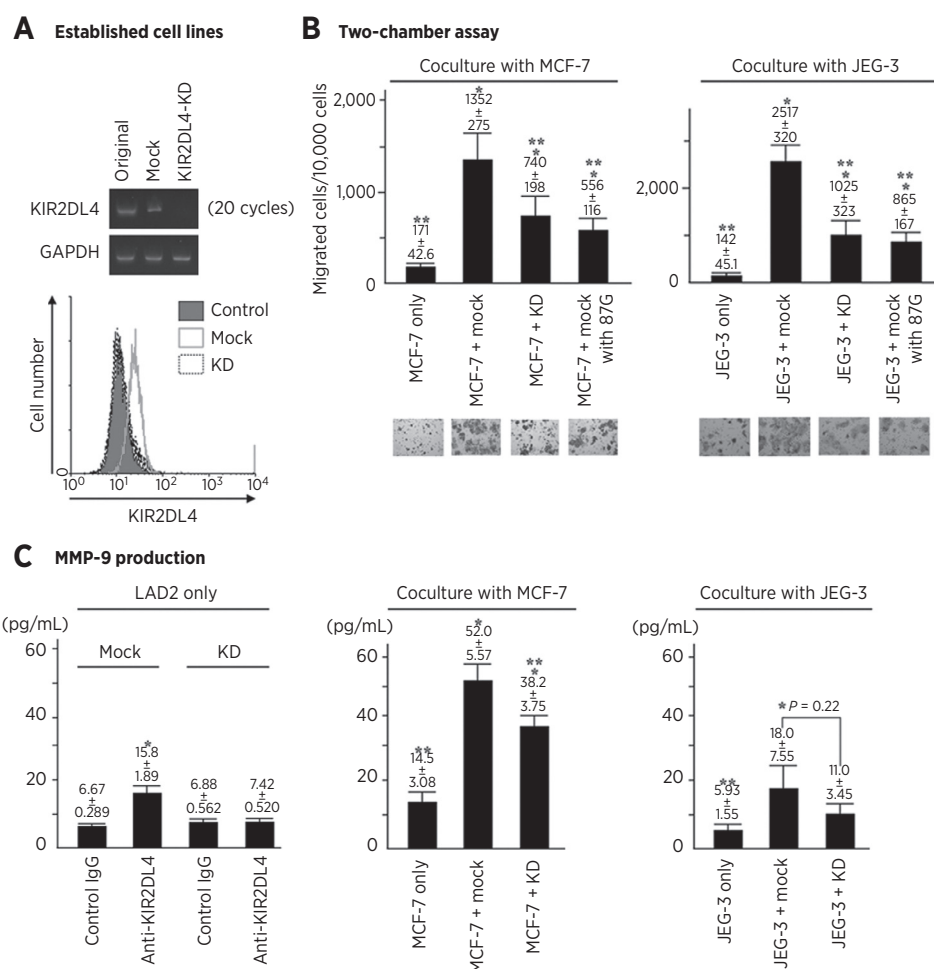
*, *P* < 0.05, compared with the control IgG value.

**Figure 4.**

Anti-KIR2DL4 Ab induces SHP-2 phosphorylation and SHP-2 inhibitor abrogates this effect. A, anti-KIR2DL4 Abs activated SHP-2 in the absence of SCF. LAD2 cells were incubated for 0 or 10 minutes with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 μg/mL, each) in the absence of SCF. Cell pellets were used for Western blot analysis. B, administration of an SHP-2 inhibitor abrogated the negative effect of anti-KIR2DL4 Abs on the growth of LAD2 cells. LAD2 cells were incubated for 24 hours with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 μg/mL, each) in the presence of SCF (10 ng/mL) together with dimethyl sulfoxide (DMSO) or SHP-2 inhibitor PHPS1 (10 μmol/L) dissolved in DMSO ($n = 3$ in all experiments). Growth was assessed as described in Materials and Methods. *, $P < 0.05$, compared with that of the anti-KIR2DL4 Ab. C, treatment with an SHP-2 inhibitor abrogated the positive effect of anti-KIR2DL4 Abs on GrB production by LAD2 cells. LAD2 cells were incubated for 24 hours with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 μg/mL, each) in the absence of SCF ($n = 3$ in all experiments) together with DMSO or SHP-2 inhibitor PHPS1 (10 μmol/L) dissolved in DMSO. The resultant culture supernatants were used for human GrB ELISA. *, $P < 0.05$, compared with the anti-KIR2DL4 Ab value. D, anti-KIR2DL4 Abs altered the phosphorylation of MAPK in LAD2 cells in an SHP-2-dependent manner. LAD2 cells were cultured for 30 minutes with control IgG or anti-KIR2DL4 Abs (clone 181703 or 33; 10 μg/mL, each) in the absence of SCF together with DMSO or SHP-2 inhibitor PHPS1 (10 μmol/L) dissolved in DMSO. After centrifugation, the resultant cell pellets were processed and used for MAPK Family (ERK, p38, JNK) Activation InstantOne ELISA (eBioscience), according to the manufacturer's protocol ($n = 3$ in all experiments). Relative values are based on a control IgG value of 100. *, $P < 0.05$, compared with the control IgG value with DMSO. E, current model.

cell lines, as in the positive control line NK92 (Fig. 1A and B). Next, we examined the localization of KIR2DL4 protein in these human mast cells. In IHC analysis, KIR2DL4 was localized mainly on the surface and in the cytoplasm of PB-mast and LAD2 cells, and mainly in the cytoplasm of HMC1.2 cells (Fig. 1C). In flow

cytometry, the PB-mast and LAD2 cells were found to express KIR2DL4 protein on their surfaces, when cells were fixed by 4% PFA (Fig. 1D). Conversely, we did not detect KIR2DL4 protein on the surface of HMC1.2 cells, when cells were fixed by 4% PFA (Fig. 1D).

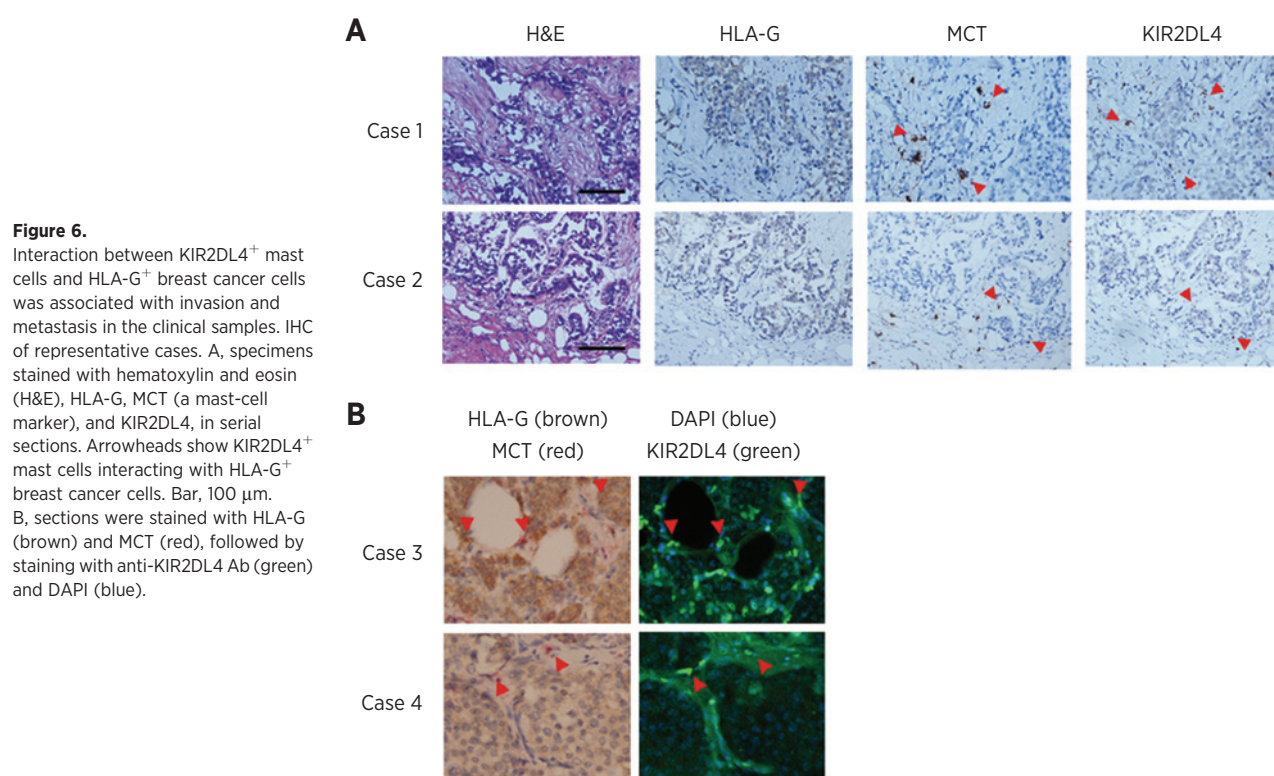
**Figure 5.**

KIR2DL4 on human mast cells upregulates the invasive activity of HLA-G⁺ cancer cell lines. A, KIR2DL4-knockdown LAD2 cells were prepared using KIR2DL4-targeting shRNA lentiviral particles (Santa Cruz Biotechnology) according to the manufacturer's instructions. After selection of the KIR2DL4-knockdown LAD2 cells, we collected mRNA from each cell line and performed RT-PCR and flow cytometry as described in Materials and Methods. B, invasion of HLA-G⁺ cancer cell lines, MCF-7 and JEG-3, that were cocultured with LAD2 cells through Matrigel. A two-chamber invasion assay was performed using Matrigel-coated Transwell polycarbonate membranes (8- μ m pores; BD Biosciences). We added to the upper chamber either 5×10^3 MCF-7 or JEG-3 cells alone, or 5×10^3 MCF-7 or JEG-3 cells together with 5×10^3 LAD2 cells that had been transfected with the control vector, or 5×10^3 MCF-7 or JEG-3 cells together with 5×10^3 KIR2DL4-knockdown LAD2 cells. In some experiments, the Fab fragment of anti-HLA-G (Clone 87G) was added. After 6 hours, cells that had migrated to the lower chamber were fixed by 4% PFA, stained with Diff-Quick stain (Sysmex), and counted using a microscope. Three to six independent experiments were performed. *, $P < 0.05$, compared with the value of MCF-7 or JEG3 cells alone. **, $P < 0.05$, compared with the value of MCF-7 or JEG3 cells together with mock LAD2 cells. C, MMP-9 production by LAD2 cells only (left), by HLA-G⁺ cancer cells, MCF-7 (middle), and JEG-3 (right), that were cocultured with LAD2 cells. 5×10^3 mock or KIR2DL4-knockdown LAD2 cells with or without anti-KIR2DL4 Abs (Clone 181703), MCF-7 or JEG-3 cells (grown to confluence) alone, MCF-7 or JEG-3 cells together with 5×10^3 LAD2 cells that had been transfected with the control vector, or MCF-7 or JEG-3 cells together with 5×10^3 KIR2DL4-knockdown LAD2 cells were cultured in a 24-well dish for 6 hours. The resultant culture supernatants were collected and used for the MMP-9 ELISA according to the manufacturer's protocol. *, $P < 0.05$, compared with the value of MCF-7 or JEG3 cells alone. **, $P < 0.05$, compared with the value of MCF-7 or JEG3 cells together with mock LAD2 cells.

IHC analysis detected KIR2DL4 in all of the examined nonneoplastic mast cell lines, including skin samples from patients who had been clinically diagnosed with atopic dermatitis (10 of 10 cases; Fig. 1E). In contrast, the neoplastic mast cells in 9 of the 15 skin samples from patients who had been diagnosed with cutaneous mastocytosis lacked KIR2DL4 expression. We did not detect any differences in clinical parameters (age, gender, site, or prognosis) between the KIR2DL4-positive and KIR2DL4-negative mastocytosis cases (data not shown).

Anti-KIR2DL4 Ab downregulated the KIT-mediated and IgE-triggered responses of human mast cells

Administration of anti-KIR2DL4 Ab (clone 181703) decreased the SCF-induced proliferation of LAD2 and PB-mast cells in a dose-dependent manner (Fig. 2A and B). However, when anti-KIR2DL4 Ab was added to HMC1.2 cells, it had no effect on their mutated KIT-associated proliferation (Fig. 2C). We also evaluated the effect of anti-KIR2DL4 Ab on the IgE-triggered degranulation activity of LAD2 and PB-mast cells in a β -hexosaminidase assay. When anti-KIR2DL4 Ab (clone 181703) was administered, the



degranulation activity of these cells was significantly reduced (Fig. 2D and E).

Anti-KIR2DL4 Ab upregulated the production of GrB from LAD2 cells

We evaluated the effect of anti-KIR2DL4 Ab on GrB secretion from LAD2 and PB-mast cells. GrB secretion from these cells was induced by anti-KIR2DL4 Ab (clone 181703) treatment (Fig. 3A and B).

SHP-2 is involved in both positive and negative effects of anti-KIR2DL4 Abs

Next, we explored the molecular mechanisms by which KIR2DL4 regulates human mast cell activity. Phosphorylation of SHP-2 was investigated by Western blot analysis. Both agonistic Abs against KIR2DL4 (clone 181703 or 33) activated SHP-2 even in the absence of SCF (Fig. 4A). However, we did not observe any marked status changes in SHP-1 (data not shown).

The addition of an SHP-2 inhibitor abrogated the KIR2DL4-induced suppression of KIT-mediated proliferation (Fig. 4B) as well as the KIR2DL4-induced enhancement of GrB secretion (Fig. 4C).

The effects of SHP-2 have been reported to be associated with MAPK activity (33, 34); therefore, we examined the activation of ERK, JNK, and p38. Even in the absence of SCF, these kinases were phosphorylated in LAD2 cells. Administration of anti-KIR2DL4 Abs (clone 181703 or 33) significantly increased the degree of JNK phosphorylation, but not those of ERK and p38, in LAD2 cells (Fig. 4D). However, this increase in JNK phosphorylation was abrogated by an SHP2 inhibitor (Fig. 4D). Taken together, stimulation with anti-KIR2DL4 Abs induced both positive and negative effects by

SHP-2 activation in human mast cells, possibly via the SHP-2-induced JNK phosphorylation (summarized in Fig. 4E).

Interaction between KIR2DL4 on human mast cells and HLA-G on cancer cells increased cancer-cell invasive activity

We prepared a coculture system involving the KIR2DL4⁺ human mast cell line LAD2 (Fig. 1B) and the HLA-G⁺ cancer cell lines MCF-7 and JEG-3 (35). To elucidate the role of KIR2DL4 on human mast cells, we prepared KIR2DL4-knockdown LAD2 cells (Fig. 5A). We confirmed that LAD2 cells express KIR2DL4 and no other HLA-G receptors, such as immunoglobulin-like transcript (ILT) 2 and ILT4 (data not shown). We checked the cytotoxic activity of LAD2 cells against MCF-7 or JEG-3 cells at the same cell number (E:T ratio = 1:1); LAD2 cells did not have a significant effect on the viability of these target cells at this E:T ratio (data not shown). Next, a two-chamber invasion assay was performed. Results from the assay revealed that coculture with LAD2 cells significantly enhanced the invasive activity of MCF-7 and JEG-3 cells (Fig. 5B). We checked the secretion of MMP-9, which is known to be produced by human mast cells and to play an important role in cancer invasion (36, 37). Stimulation of KIR2DL4 induced MMP-9 secretion from mock LAD2 cells, but not from KIR2DL4-knockdown LAD2 cells (Fig. 5C). Coculture of MCF-7 or JEG-3 cells with LAD2 cells in 24-well dish enhanced the magnitude of MMP-9 secretion (Fig. 5C). When MCF-7 or JEG-3 cells were cocultured with KIR2DL4-knockdown LAD2 cells, the number of invading cells and the magnitude of MMP-9 production was also decreased significantly (Fig. 5B and C). Treatment with Fab fragment of anti-HLA-G (clone 87G) also significantly decreased the number of invading cells and the magnitude of

Table 1. Clinical parameters of the examined breast cancer cases

	HLA-G ⁺ (27 cases)	HLA-G ⁻ (9 cases)	Total (36 cases)
Mast cell–cancer cell interaction (with ly ⁺ and/or pN ⁺)	12 (44.4%) 11/12	4 (44.4%) 1/4	16 (44.4%) 12/16
Gender			
Female	27 (100%)	9 (100%)	36 (100%)
Age, y	59.4 ± 2.60	59.3 ± 3.03	59.4 ± 2.07
Surgical procedure			
Mastectomy	27 (100%)	9 (100%)	36 (100%)
pT stage			
(y)pT1	13 (48.1%)	5 (55.6%)	18 (50%)
(y)pT2	12 (44.4%)	3 (33.3%)	15 (41.7%)
(y)pT3	2 (7.4%)	1 (11.1%)	3 (8.3%)
pN stage			
(y)pN0	9 (33.3%)	6 (66.7%)	15 (41.7%)
(y)pN1	13 (48.1%)	3 (33.3%)	16 (44.4%)
(y)pN2	1 (3.7%)	0 (0%)	1 (2.8%)
(y)pN3	4 (14.8%)	0 (0%)	4 (11.1%)
Lymphovascular invasion			
Positive	5 (18.5%)	0 (0%)	5 (13.9%)
Negative	22 (81.5%)	9 (100%)	31 (86.1%)
Preoperative chemotherapy			
Present	8 (29.6%)	2 (22.2%)	10 (27.8%)
Absent	19 (70.4%)	7 (77.8%)	26 (72.2%)
Histologic grading			
Grade 1	6 (22.2%)	2 (22.2%)	8 (22.2%)
Grade 2	12 (44.4%)	3 (33.3%)	15 (41.7%)
Grade 3	9 (33.3%)	4 (44.4%)	13 (36.1%)
Molecular subtype			
Luminal A	10 (37.0%)	5 (55.6%)	15 (41.7%)
Luminal B	9 (33.3%)	2 (22.2%)	11 (30.6%)
HER2	1 (3.7%)	0 (0%)	1 (2.8%)
Luminal-HER2	4 (14.8%)	0 (0%)	4 (11.1%)
Triple negative	3 (11.1%)	2 (22.2%)	5 (13.9%)

MMP-9 production to about equal levels as when cocultured with the KIR2DL4-knockdown LAD2 cells (Fig. 5B and C).

Interaction between KIR2DL4⁺ mast cells and HLA-G⁺ breast cancer cells is associated with invasion and metastasis in the clinical samples

Next, we evaluated the association of KIR2DL4 on human tissue mast cells with the invasive phenotypes of cancer cells using clinical samples. Thirty-six samples resected by mastectomy during the period of 2011 to 2013 at our hospital were prepared for this evaluation (summarized in Table 1). In all cases examined, KIR2DL4⁺ human tissue mast cells were detected. Breast cancer cells were positive for HLA-G in 27 cases (75%) by IHC. Direct interaction between KIR2DL4⁺ mast cells and HLA-G⁺ breast cancer cells was observed in 12 of 36 cases (33.3%); representative cases are shown in Fig. 6. Of the 12 cases with direct interaction between KIR2DL4⁺ mast cells and HLA-G⁺ breast cancer cells, 11 cases (91.7%) were accompanied with lymph node metastasis or with lymph-vessel invasion. The χ^2 test showed statistically significant correlation between the presence of KIR2DL4⁺ mast cells—HLA-G⁺ breast cancer cell interaction and the presence of lymph node metastasis or lymph-vessel invasion ($\chi^2 = 7.424$; $P < 0.01$; degrees of freedom, 1).

Discussion

In this study, we found that KIR2DL4 was expressed in all human nonneoplastic mast cells examined, and regulated human

cultured mast cells positively and negatively. In addition, the KIR2DL4 expressed on human mast cells enhanced the invasive activity of HLA-G⁺ cancer cells.

All nonneoplastic human mast cells (PB-mast and tissue mast cells included in the skin samples) examined expressed KIR2DL4 protein, as was found for NK cells (29). In addition, we observed KIR2DL4 expression in human cultured mast cells obtained by culturing CD4⁺/CD8⁺/CD11b⁺/CD14⁺/CD16⁺/CD19⁺ cells in methylcellulose medium supplemented with rhSCF and rhIL6, or human mast cells obtained by culturing CD34⁺ cells derived from peripheral blood cells in the same medium, or human mast cells collected and established from surgically resected lung tissue using anti-KIT Ab (data not shown; refs. 38, 39). Nevertheless, we also found a lack of KIR2DL4 protein expression on the human neoplastic mast cell line HMC1.2 and some neoplastic mast cell samples from patients with cutaneous mastocytosis. These observations suggest that a lack of KIR2DL4 protein expression could be a marker of neoplastic changes in mast cells. KIR2DL4 expression suppressed the proliferation of human nonneoplastic mast cells; therefore, KIR2DL4 deficiency might aid the uncontrolled proliferation of neoplastic mast cells. However, we could not detect any clinical differences between cases of cutaneous mastocytosis involving KIR2DL4⁺ and KIR2DL4⁻ mast cells. This might be because cutaneous mastocytosis is too indolent for significant differences in clinical parameters to be detected (40).

Structurally, KIR2DL4 is an inhibitory receptor (1, 2); therefore, our finding that KIR2DL4 suppresses KIT-mediated

and IgE-triggered responses of LAD2 and PB-mast cells makes sense. Mast cells express various inhibitory receptors that can suppress KIT-mediated and/or IgE-triggered responses (30, 31, 41–45). These receptors have inhibitory effects by activating nonreceptor-type phosphatases, such as SHP-1 and SHP-2. KIR2DL4 belongs to this group of inhibitory receptors and acts by activating SHP-2. Accordingly, anti-KIR2DL4 Abs are expected to be a useful tool for treating allergic conditions mediated by mast cells, as has been found for Abs for other receptors.

We observed that the invasive properties of HLA-G⁺ breast cancer cells were enhanced by mast cells. Some HLA-G⁺ neoplasms, including breast cancers, were associated with metastatic phenotypes, and this observation has been explained by immune escape from NK cells and by induction of immune-suppressive regulatory T cells (46, 47). Our current data provide another explanation for these reports; that is, mast cells are involved in the HLA-G–KIR2DL4 axis and are able to enhance the invasive property of cancer cells. Mast cell infiltration has been reported to be associated with invasive or metastatic phenotypes of various cancers (48). Therefore, the interaction of HLA-G on cancer cells and KIR2DL4 on human mast cells would be expected to exist in such invasive or metastatic cases as we have demonstrated in this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Rajagopalan S, Long EO. KIR2DL4 (CD158d): an activation receptor for HLA-G. *Front Immunol* 2012;3:258.
- Rajalingam R. Overview of the killer cell immunoglobulin-like receptor system. *Methods Mol Biol* 2012;882:391–414.
- Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol* 2002;168:6208–14.
- Miah SM, Hughes TL, Campbell KS. KIR2DL4 differentially signals downstream functions in human NK cells through distinct structural modules. *J Immunol* 2008;180:2922–32.
- Kikuchi-Maki A, Catina TL, Campbell KS. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor γ protein. *J Immunol* 2005;174:3859–63.
- Kikuchi-Maki A, Yusa S, Catina TL, Campbell KS. KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN- γ production. *J Immunol* 2003;171:3415–25.
- Rajagopalan S, Fu J, Long EO. Cutting edge: induction of IFN- γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J Immunol* 2001;167:1877–81.
- Goodridge JP, Lathbury LJ, John E, Charles AK, Christiansen FT, Witt CS. The genotype of the NK cell receptor, KIR2DL4, influences INF γ secretion by decidual natural killer cells. *Mol Hum Reprod* 2009;15:489–97.
- Yan WH, Lin A, Chen BC, Zhou MY, Dai MZ, Chen XJ, et al. Possible roles of KIR2DL4 expression on uNK cells in human pregnancy. *Am J Reprod Immunol* 2007;57:233–42.
- Okayama Y, Kawakami T. Development, migration, and survival of mast cells. *Immunol Res* 2006;34:97–115.
- Dudeck A, Suender CA, Kostka SL, von Stebut E, Maurer M. Mast cells promote Th1 and Th17 responses by modulating dendritic cell maturation and function. *Eur J Immunol* 2011;41:1883–93.
- Hueber AJ, Asquith DL, Miller AM, Reilly J, Kerr S, Leipe J, et al. Mast cells express IL-17A in rheumatoid arthritis synovium. *J Immunol* 2010;184:3336–40.
- Piconese S, Gri G, Tripodo C, Musio S, Gorzanelli A, Frossi B, et al. Mast cells counteract regulatory T-cell suppression through interleukin-6 and OX40/OX40L axis toward Th17-cell differentiation. *Blood* 2009;114:2639–48.
- Nakae S, Suto H, Berry GJ, Galli SJ. Mast cell-derived TNF can promote Th17 cell-dependent neutrophil recruitment in ovalbumin-challenged OTII mice. *Blood* 2007;109:3640–8.
- Yu M, Eckart MR, Morgan AA, Mukai K, Butte AJ, Tsai M, et al. Identification of an IFN- γ /mast cell axis in a mouse model of chronic asthma. *J Clin Invest* 2011;121:3133–43.
- Mazzoni A, Siraganian RP, Leifer CA, Segal DM. Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. *J Immunol* 2006;177:3577–81.
- Kataoka TR, Komazawa N, Morii E, Oboki K, Nakano T. Involvement of connective tissue-type mast cells in Th1 immune responses via Stat4 expression. *Blood* 2005;105:1016–20.
- Coulombe M, Battistini B, Stankova J, Pouliot P, Bissonnette EY. Endothelins regulate mediator production of rat tissue-cultured mucosal mast cells. Up-regulation of Th1 and inhibition of Th2 cytokines. *J Leukoc Biol* 2002;71:829–36.
- Gupta AA, Leal-Berumen I, Croitoru K, Marshall JS. Rat peritoneal mast cells produce IFN- γ following IL-12 treatment but not in response to IgE-mediated activation. *J Immunol* 1996;157:2123–8.
- Williams CM, Coleman JW. Induced expression of mRNA for IL-5, IL-6, TNF- α , MIP-2 and IFN- γ in immunologically activated rat peritoneal mast cells: inhibition by dexamethasone and cyclosporin A. *Immunology* 1995;86:244–9.
- Kataoka TR, Morii E, Oboki K, Kitamura Y. Strain-dependent inhibitory effect of mutant mi-MITF on cytotoxic activities of cultured mast cells and natural killer cells of mice. *Lab Invest* 2004;84:376–84.
- Ito A, Morii E, Kim DK, Kataoka TR, Jippo T, Maeyama K, et al. Inhibitory effect of the transcription factor encoded by the mi mutant allele in cultured mast cells of mice. *Blood* 1999;93:1189–96.
- Pardo J, Wallich R, Ebnet K, Iden S, Zentgraf H, Martin P, et al. Granzyme B is expressed in mouse mast cells *in vivo* and *in vitro* and causes delayed cell death independent of perforin. *Cell Death Differ* 2007;14:1768–79.

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24. Strik MC, de Koning PJ, Kleijmeer MJ, Bladergroen BA, Wolbink AM, Griffith JM, et al. Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. *Mol Immunol* 2007;44:3462–72.
25. Kirshenbaum AS, Goff JP, Semere T, Foster B, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood* 1999;94:2333–42.
26. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res* 2003;27:677–82.
27. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res* 1988;12:345–55.
28. Hellmuth K, Grosskopf S, Lum CT, Würtele M, Röder N, von Kries JP, et al. Specific inhibitors of the protein tyrosine phosphatase Shp2 identified by high-throughput docking. *Proc Natl Acad Sci U S A* 2008;105:7275–80.
29. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity* 1997;7:753–63.
30. Kataoka TR, Kumanogoh A, Bandara G, Metcalfe DD, Gilfillan AM. CD72 negatively regulates KIT-mediated responses in human mast cells. *J Immunol* 2010;184:2468–75.
31. Kataoka TR, Fujimoto M, Moriyoshi K, Koyanagi I, Ueshima C, Kono F, et al. PD-1 regulates the growth of human mastocytosis cells. *Allergol Int* 2013;62:99–104.
32. Rajagopalan S, Long EO. A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med* 1999;189:1093–100.
33. Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. *Immunol Rev* 2009;228:149–69.
34. Yu M, Luo J, Yang W, Wang Y, Mizuki M, Kanakura Y, et al. The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked mast cell proliferation by activating the Rac/JNK pathway. *J Biol Chem* 2006;281:28615–26.
35. Pangault C, Amiot L, Caulet-Maugendre S, Brasseur F, Burtin F, Guilloux V, et al. HLA-G protein expression is not induced during malignant transformation. *Tissue Antigens* 1999;53:335–46.
36. Baram D, Vaday GG, Salamon P, Drucker I, Hershkovich R, Mekori YA. Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrine regulation by TNF-α. *J Immunol* 2001;167:4008–16.
37. Duffy MJ. Proteases as prognostic markers in cancer. *Clin Cancer Res* 1996;2:613–8.
38. Saito H, Kato A, Matsumoto K, Okayama Y. Culture of human mast cells from peripheral blood progenitors. *Nat Protoc* 2006;1:2178–83.
39. Kashiwakura J, Yokoi H, Saito H, Okayama Y. T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. *J Immunol* 2004;173:5247–57.
40. Brockow K, Metcalfe DD. Mastocytosis. *Chem Immunol Allergy* 2010;95:110–24.
41. Izawa K, Kitaura J, Yamanishi Y, Matsuoka T, Kaitani A, Sugiyama M, et al. An activating and inhibitory signal from an inhibitory receptor LMIR3/CLM-1: LMIR3 augments lipopolysaccharide response through association with FcγR in mast cells. *J Immunol* 2009;183:925–36.
42. Migalovich-Sheikhet H, Friedman S, Mankuta D, Levi-Schaffer F. Novel identified receptors on mast cells. *Front Immunol* 2012;3:238.
43. Karra L, Berent-Maoz B, Ben-Zimra M, Levi-Schaffer F. Are we ready to downregulate mast cells? *Curr Opin Immunol* 2009;21:708–14.
44. Hitomi K, Tahara-Hanaoka S, Someya S, Fujiki A, Tada H, Sugiyama T, et al. An immunoglobulin-like receptor, Allergin-1, inhibits immunoglobulin E-mediated immediate hypersensitivity reactions. *Nat Immunol* 2010;11:601–7.
45. Mizrahi S, Gibbs BF, Karra L, Ben-Zimra M, Levi-Schaffer F. Siglec-7 is an inhibitory receptor on human mast cells and basophils. *J Allergy Clin Immunol* 2014;134:230–3.
46. Curigliano G, Criscitiello C, Gelao L, Goldhirsch A. Molecular pathways: human leukocyte antigen G (HLA-G). *Clin Cancer Res* 2013;19:5564–71.
47. Elliott RL, Jiang XP, Phillips JT, Barnett BG, Head JF. Human leukocyte antigen G expression in breast cancer: role in immunosuppression. *Cancer Biother Radiopharm* 2011;26:153–7.
48. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* 2010;29:1093–102.

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