KLRG1 binds cadherins and preferentially associates with SHIP-1

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Abstract

The killer cell lectin-like receptor G1 (KLRG1) is a unique inhibitory receptor expressed on a phenotypically mature subset of resting NK cells as well as subsets of T cells in naive mice. In vivo, pathogenic immune system activation induces dramatic changes in the expression patterns of KLRG1 among the different cell subsets. In order to enhance our understanding of KLRG1 signaling properties and to clarify the functions of KLRG1 on these cells, we identified the broadly expressed N-cadherin molecule as a ligand for KLRG1. We further demonstrate that a second member of this superfamily of adhesion molecules, E-cadherin, binds to KLRG1. Additionally, we show that upon phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) tyrosine, KLRG1 recruits both SHIP-1 and SHP-2 but not SHP-1. We also delineate the key KLRG1 ITIM amino acid residues required for optimal association with these phosphatases. Finally, we demonstrate that KLRG1 engagement can inhibit sub-optimal TCR signaling. Taken together, our results indicate that KLRG1 may differentially regulate NK cell and T cell functions through the association with different ligands as well as the recruitment of distinct phosphatases.

Introduction

The killer cell lectin-like receptor G1 (KLRG1) is a C-type lectin inhibitory receptor that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain. It is a well-conserved molecule found on subsets of NK cells and T cells in both mouse and human (1–4). However, the acquisition, regulation and role of the KLRG1 molecule with respect to these two lymphocytes seem to be distinct. Indeed, recent in vivo studies have shown that the KLRG1 molecule is expressed on the most mature as well as recently activated NK cells (5), whereas KLRG1 expression on T cells is believed to be a signature of senescence and/or lack of proliferative capacity (4, 6–8). In addition, the molecular mechanism leading to the acquisition of KLRG1 differs in these two subsets of cells (3). Finally, KLRG1 expression on NK cells in the early phase of viral infection inversely correlates with the ability of NK cells to produce IFN-γ (9), whereas KLRG1+ CD8+ T cells do not appear to be impaired in their ability to synthesize cytokines (6). Therefore, depending on the cell expressing KLRG1, different roles for KLRG1 have been observed.

In addition, it is unclear whether the developmental status of the KLRG1+ cells is a cause of KLRG1 expression or a consequence of the KLRG1 engagement by its ligand(s). Interestingly, the KLRG1 molecule originally identified as the mast cell function-associated antigen in rats has been shown to have unique signaling properties (10, 11). However, in contrast to rats, KLRG1 is not expressed on mast cells in mice or humans (12) and it is difficult to translate the data seen in rats to the other species. Taken together, these data led us to hypothesize that KLRG1 engagement may differentially regulate signaling, altering cell cycle or inhibiting cytokine synthesis, depending on the KLRG1 tissue distribution and/or the quality of the signal provoked by its engagement. To test this hypothesis, we undertook to identify the KLRG1 ligand and to examine the signaling properties of the murine KLRG1 molecule. We found that N-cadherin and E-cadherin are ligands for KLRG1 and demonstrated that upon phosphorylation of the ITIM tyrosine, KLRG1 recruits both SHIP-1 and SHP-2 but not SHP-1 at its cytoplasmic tail. We also defined the key ITIM amino acid residues for optimal association of these two phosphatases with the KLRG1 molecule.
Method

Cells and antibodies

Mouse, rat and human cell lines P815, RAW, DO11, A20, L929, AML12, RMA-S, NIH 3T3, 3T3 expressing N- and E-cadherin (3T3 FS), RNK16, HEK-293T and Jurkat were grown in either DMEM or RPMI (Invitrogen Life Technologies, Carlsbad, CA, USA) with 8% FCS added. BWZ.36, BW.36 H/S64, BWZ.36 H/S64 YF reporter cells were cultured in 8% RPMI with hygromycin-B (BD biosciences) at 200 μg ml⁻¹.

The retroviral packaging cell line, Plat-E, was kindly provided by T. Kitamura (University of Tokyo) and was used for retroviral transduction.

The following antibodies were used: anti-Myc tag (clone 4A6), and anti-phosphotyrosine 4G10 (Upstate Cell Signaling Solutions, Charlottesville, VA, USA), anti-SHIP-1 (sc-8425), anti-SHIP-1 (sc-287), and anti-SHIP-2 (sc-280) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), purified anti-KLRG1 and anti-KLRG1-APC streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), purified anti-SHIP-1 mAb for flow cytometry (ECCD-2; Zymed), rabbit polyclonal anti-cadherin (ECCD-2; Zymed), rabbit polyclonal anti-β-actin (Abcam inc., Cambridge, MA, USA), anti-pan cadherin (Sigma), anti-E-cadherin mAb for western blot, isotype controls, anti-IL-2 mAbs (purified JES6-N37), and anti-SHP-1 (sc-287), and anti-SHP-2 (sc-280) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

KLRG1 tetramer

A construct encoding a 6-histidine tag, a BirA recognition site, and a thrombin cleavage site was first cloned in the pET-23b vector (named His-BirA-Throm tag). Using the full length KLRG1 cDNA as a template, the KLRG1 extracellular domain, beginning at proline 73, was amplified by PCR using the 2F1, eBioscience, San Diego, CA, USA), rat anti-E-cadherin mAb for flow cytometry (ECCD-2; Zymed), rabbit polyclonal anti-β-actin (Abcam inc., Cambridge, MA, USA), anti-pan cadherin (Sigma), anti-E-cadherin mAb for western blot, isotype controls, anti-IL-2 mAbs (purified JES6-N37-1A12 and biotinylated JES6-SH4) and streptavidin PE were purchased from BD PharMingen.

Expression cloning

A retroviral cDNA library from B6 total fetal day 14 tissue was constructed as follows. The tissues were flash frozen and ground up after which TRizol (Invitrogen) was added. Total RNA was extracted according to manufacturer's instructions and mRNA was purified using the Poly(A) Quik mRNA Isolation Kit (Stratagene) following the manufacturer's instructions. Conversion of mRNA to cDNA was performed away from reactants by gel filtration as described above. KLRG1 molecule was tetramered by adding streptavidin labeled with PE (BD PharMingen).

Reporter cells

A chimeric receptor, which includes both the Ly49H cyttoplasmic and transmembrane domains and the KLRG1 extracellular domain (Ly49H serine 72 to KLRG1 serine 64) was designed. The primers used for amplification of the cytoplasmic and transmembrane domains of Ly49H were as follows: 5’ primer 5’-GCTGCTGCATATGTTCC and 3’ primer 5’-CTATGCGGCGCCGCGCTTCGCTGCTAGTTAGGAAGGCTCTGTCT. The Ly49H fragment was digested with the HincII restriction enzyme and ligated to amplified extracellular part of KLRG1. To amplify the correctly ligated fragments, an additional PCR reaction was performed using the Ly49H 5’ and the KLRG1 3’ primers, followed by BamHI and NotI digestion and ligation in the multi-cloning sites of the pMXs retroviral vector (kindly provided by Toshiro Kitamura). The BamHI and NotI restriction sites, used for cloning the fragment in the retroviral vector, are underlined. DAP12 was cloned downstream the IRES of the same vector. As a control, a similar construct was designed with a non-functional DAP12, DAP12 YF, in which two critical tyrosine residues were mutated in phenylalanine residues. Oligonucleotides used for cloning were as follows: 5’ primer 5’-ATTAAGGATCCACTGACGTGA and 3’ primer 5’-GACCTAGGATCCACTGACGATCCAAGGACTCTACATGTTCC and 3’ primer 5’-CATGCGGCGCCGCGCTTCGCTGCTAGTTAGGAAGGCTCTGTCT. The Ncol and Sfl restriction sites used for cloning are underlined. The Plat-E packaging cell line was transfected with the different plasmids, using Fugene6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. For transduction, BWZ.36 reporter cells (derived from BW5147 by transformation of a construction consisting of the NFAT enhancer element of the IL-2 promotor driving the expression of β-galactosidase) (13) were incubated in retroviral supernatant containing N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate liposomal transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. High expressors were sorted and functionality determined by cross-linking with specific anti-KLRG1 mAbs. After incubation, cells were washed twice with PBS and fixed for 10 min at 4°C using 10% buffered formalin phosphate (Fisher), and 25% glutaraldehyde (Sigma). Cells were washed twice with PBS and treated with X-Gal substrate (Invitrogen) and incubated at 37°C for 8 h. Cells were imaged at ×10 with an Olympus DP70.
tagged KLRG1 mutants were generated by oligonucleotide-directed mutagenesis using the ZAP-cDNA Synthesis Kit (Stratagene), which allows unidirectional cloning in an expression vector, in this case the retroviral pMXs-puro vector, digested with EcoRI and XhoI restriction digestion enzymes. The fetal day 14 cDNA was enriched for fragments >500 bp by size fractionation according to the manufacturer’s protocol, and ligated into the digested vector. Following transfection of L929 cells with the FD14 retroviral cDNA library, KLRG1 tetramer-positive cells were enriched using three rounds of sorting. Retroviral inserts were then amplified by PCR using the 5’ primer 5’-CCCAGTGTTGGTGATCGGG-3’ and 3’ primer 5’-GCCACCGGCGCTACAGTG-3’.

**KLRG1, N-cadherin and E-cadherin cDNA constructs**

Using the full length KLRG1 cDNA as a template, Myc tagged KLRG1 wild type (WT) was generated using 5’ primer 5’-CGAGATCTACATGGCTAGACCTCTATTC-3’ and 3’ primer 5’-CTCGAGCTACATGGCTAGACCTCTATTC-3’. The PCR product was purified by agarose gel electrophoresis and ligated into the TOPO cloning vector (Invitrogen). The construct was sequenced and inserted into the unique BglII sites of the retroviral vectors MSCV-IRES-GFP and MSCV-IRES-puromycin. Mouse E-cadherin-expressing vectors were kindly provided by Dr. Cossart (Pasteur Institute, France) and Dr. Sasaki (National Institutes of Health, USA). In order to sub-clone E-cadherin in MSCV-IRES-GFP, E-cadherin designed with BglII/HpaI sites was made by PCR using 5’ primer 5’-CGAGATCTGATGGCGGTCGTCCACGCCCGTGACATGTCC-3’ and 3’ primer 5’-CGTTAACGCTAGTCGTCCTCACCACCGCCGTACATGTCC-3’. The construct was sequenced and inserted into the unique BglII and HpaI sites of the retroviral vector MSCV-IRES-GFP. To measure the effect of cross-linking on IL-2 production, DO11 cells and DO11 transfectants were treated with pervanadate (0.4 mM Na3Vo4, 0.04% H2O2) or left untreated. After the indicated treatments, 25 × 10^6 cells were solubilized in lysis buffer (1 Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitors) at 4°C for 1 h. Lysates were centrifuged for 20 min at 4°C. For immunoprecipitation, pre-cleared lysates were first incubated for 2 h at 4°C with 1.5 μg specific antibodies or control antibodies. Lysates were then incubated overnight with Protein G Sepharose (Amersham). Beads were washed five times with ice-cold lysis buffer and boiled in 2× reducing sample buffer (5% SDS, 10% glycerol, 3% dithiothreitol, 0.15 M Tris/ HCl, pH 6.8, 0.0125% bromphenol blue). For western blotting, samples were separated on 4–15% gradient SDS–PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in PBS with 5% BSA or milk for 1 h at room temperature and developed using Super Signal West Pico (Pierce, Bonn, Germany).

**Immunoprecipitation and western blotting**

DO11 cells and DO11 transfectants were treated with pervanadate (0.4 mM Na3Vo4, 0.04% H2O2) or left untreated. After the indicated treatments, 25 × 10^6 cells were solubilized in lysis buffer (1 Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitors) at 4°C for 1 h. Lysates were centrifuged for 20 min at 4°C. For immunoprecipitation, pre-cleared lysates were first incubated for 2 h at 4°C with 1.5 μg specific antibodies or control antibodies. Lysates were then incubated overnight with Protein G Sepharose (Amersham). Beads were washed five times with ice-cold lysis buffer and boiled in 2× reducing sample buffer (5% SDS, 10% glycerol, 3% dithiothreitol, 0.15 M Tris/ HCl, pH 6.8, 0.0125% bromphenol blue). For western blotting, samples were separated on 4–15% gradient SDS–PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in PBS with 5% BSA or milk for 1 h at room temperature and incubated with the indicated antibody overnight at 4°C. After washing, the membrane was incubated with the respective HRP-conjugated secondary antibody for 1 h at room temperature and developed using Super Signal West Pico (Pierce, Bonn, Germany).

**Flow cytometry/antibodies and reagents**

For cell surface staining, cells were suspended in buffer comprised of PBS, pH 7.4, containing 2% BSA (w/v). After 10 min of preincubation with the Fc receptor blocking 2.4G2 mAb, when necessary, the cells were stained at 4°C with 1 or 0.1 μM of immobilized anti-TCRβ (or anti-CD3ε) in combination with various concentration of anti-KLRG1 mAbs (or anti-Myc). The plates were then incubated at 37°C for 6 h and IL-2 levels in cell culture supernatants were determined by ELISA. The cytokine levels in cell culture

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The packaging cell line, Plat-E (kindly provided by Kitamura) was transfected with the MSCV-IRES-GFP or MSCV-IRES-puromycin vectors containing one of the KLRG1 constructs. N-cadherin or E-cadherin was detected using Lipofectamine Plus reagent (Invitrogen). For transduction, target cells were re-suspended in retroviral supernatant containing polybrene (8 mg ml⁻¹; Sigma–Aldrich). The cells were sorted for expression of GFP, KLRG1, N-cadherin or E-cadherin. Before each experiment, cell surface expression of the transduced genes was measured and mean fluorescence intensity compared by flow cytometry. Transient transfection of KLRG1 in the HEK-293T cell line was performed using Lipofectamine Plus reagent.
supernatants were detected using standard sandwich ELISA with a coated capture mAb and a biotinylated detection mAb, according to the manufacturer protocol (BD). Cytokine levels are expressed as mean ± SD of culture triplicates.

Results
Identification of cells expressing potential candidates for KLRG1 ligands
In order to identify the KLRG1 ligands, we generated both a KLRG1 tetramer and a reporter cell line. Using these tools, we identified several cell lines of different species that could potentially express the KLRG1 ligand including both murine and human cell lines (Fig. 1 and Table 1). The specificity of tetramer binding was confirmed by blocking this interaction with anti-KLRG1 mAbs (not shown). Likewise, the specificity of the signal obtained with the reporter cell line was confirmed by mAb blocking as well as with the DAP12 mutant reporter cell line (not shown). In addition, we took advantage of the recently reported association of some inhibitory receptors with their ligands in cis (14) as a method to determine the specificity of the tetramer staining. To investigate this, the KLRG1 tetramer-positive Jurkat cell line was transduced with either the KLRG1 WT or KLRG1 tail deleted and subsequently stained with the KLRG1 tetramer. We found that KLRG1 expression at the cell surface correlated with a loss of binding to the KLRG1 tetramer (Fig. 2B and data not shown). Altogether, the data suggested that the KLRG1 ligand is expressed on the cell surface, is conserved among different species and can associate in cis with its ligand.

Features of the KLRG1 ligand
Interestingly, we found that treating KLRG1 tetramer-positive cells with trypsin completely abolished KLRG1 tetramer binding (Fig. 2A) suggesting that the KLRG1 ligand is a membrane protein. Furthermore, we also found that depending on the number of passages of a given cell line, expression of the KLRG1 ligand may vary. For instance, we found that a recently obtained NIH 3T3 from the American Type Tissue Collection does not express the KLRG1 ligand, whereas a 3T3-derived cell line (named 3T3 FS), kept in culture long term, binds strongly to the KLRG1 tetramer (Fig. 1A).

N-cadherin and E-cadherin are ligands for KLRG1
To identify the KLRG1 ligand, we used a fetal day 14 murine cDNA retroviral library to transduce KLRG1 tetramer-negative L929 cells. Following cell enrichment, we attained a population (L929/FD14) that was >97% positive for KLRG1 tetramer binding (Fig. 3A) and stimulated the reporter cell line (not shown). Sequencing of the retroviral insert yielded a match to N-cadherin. To confirm the validity of this result, full length N-cadherin was cloned in the MSCV-IRES-GFP retroviral vector and L929 (not shown) and DO11 cells were transduced. The transduced cells bound the KLRG1 tetramer (Fig. 3B) and stimulated the reporter cells (not shown). We confirmed this result by immunoblot using the anti-pan cadherin antibody that detected a protein at the expected molecular weight in the L929/FD14 but not in L929 parental cell line (Fig. 3C). Interestingly, we found that both the NIH 3T3 (KLRG1 tetramer negative) and 3T3 FS (KLRG1 tetramer high) express members of the cadherin family suggesting that some cadherins, such as N-cadherin, are ligands and others are not. Therefore, we tested these cell lines with a specific mAb for E-cadherin by immunoblot and found that 3T3 FS cells express E-cadherin suggesting that E-cadherin could also be a KLRG1 ligand (Fig. 3C). To confirm this possibility, E-cadherin was cloned into the MSCV-IRES-GFP retroviral vector and L929, DO11 and NIH 3T3 cells were transduced. We found that expression of E-cadherin (Fig. 4C) coincided with KLRG1 tetramer binding and activation of the reporter cells (Fig. 4A and B). Altogether, these data demonstrate that both E- and N- cadherins are ligands for KLRG1.

KLRG1 associates with SHIP-1 and SHP-2 but not SHP-1
In order to investigate the molecular mechanisms underlying KLRG1 functions, the T cell hybridoma DO11 was stably transfected with WT and mutated KLRG1 molecules. All the receptors were expressed on the cell surface at similar levels in retrovirally transduced DO11 cell lines (Fig. 5A and 6A). To identify the phosphatase that binds to the KLRG1 ITIM in mouse cells, KLRG1 and KLRG1 mutants were immunoprecipitated from pervanadate-treated DO11 cells and both their phosphorylation status and associated molecules were identified by western blot. This treatment stimulated robust and specific phosphorylation of the ITIM tyrosine (Fig. 5B). Immunoprecipitates of KLRG1 co-precipitated SHIP-1 and SHP-2 (Fig. 5B). In contrast, SHIP-1 and SHP-2 were not immunoprecipitated with the KLRG1 Y7F mutant indicating that the formation of KLRG1/SHIP-1 and KLRG1/SHIP-2 complexes were dependent upon the KLRG1 ITIM tyrosine (Fig. 5B). To confirm the specificity of these results, we showed that following immunoprecipitation of SHIP-1 (Fig. 5C) and SHP-2 (not shown), KLRG1 (detected with an anti-Myc mAb) was co-precipitated in a phosphorylated tyrosine-dependent manner (Fig. 5C). Interestingly, SHP-1 binding to KLRG1 was undetectable despite high amount of KLRG1 immunoprecipitated (Fig. 5B, Myc immunoblot). It should be noted that SHP-1 was detected by immunoblot on lysate controls (not shown). Although unusual among NK cell inhibitory receptors, the lack of recruitment of SHP-1 could be due to the fact that KLRG1 possesses only one ITIM within its cytoplasmic tail. This is in agreement with reports that demonstrated that SHP-1 recruiting receptors possess two or more ITIMs (15). In further support of the association of SHP-2 with KLRG1 molecules, several reports have also demonstrated that a single ITIM is sufficient to recruit SHP-2 (15, 16).

Point mutations of the KLRG1 ITIM can either enhance or antagonize association with SHIP-1 and SHP-2
To identify the KLRG1 ITIM amino acid residues required for binding SHIP-1 and SHP-2, we used site-directed mutagenesis to establish point mutants. DO11 cells were transduced with different KLRG1 ITIM mutants and stained by flow cytometry with anti-KLRG1 mAbs (Fig. 6A). Only DO11 cells expressing comparable levels of KLRG1 cell surface expression were used for these studies (Fig. 6A). The Y + 3 leucine...
residue was identified as a critical residue for optimal association of KLRG1 with SHIP-1. Indeed, substitution of the ITIM leucine residue for alanine completely abrogated SHIP-1 recruitment despite similar levels of immunoprecipitated KLRG1 (Fig. 6B, Myc immunoblot) and comparable levels of phosphorylation (not shown) to the WT KLRG1. Interestingly, the Y + 1 serine residue appears to antagonize KLRG1 association with SHP-2 as substitution of the serine 8 for alanine enhances KLRG1 association with SHP-2 (Fig. 6B). Other mutations weakened the recruitment of both SHIP-1 and SHP-2 (Fig. 6B).

KLRG1 ligation partially inhibits T cell activation
It has been reported that KLRG1 engagement can inhibit NK cell cytotoxicity (17), cytokine production (9, 18) as well as Ag induced T cell division (19). To explore the KLRG1 ITIM

Fig. 1. Cell surface expression of KLRG1 ligand. (A) Flow cytometry profiles of different cell lines. Each panel is an overlay of the streptavidin PE (open histogram) and the KLRG1 tetramer (solid histogram). (B) BWZ.36 cells transduced with KLRG1/Ly49H chimeric protein or the control reporter cell lines were co-cultured with tetramer-positive cell lines for 16 h. β-Galactosidase expression was determined using X-Gal as a substrate. One of three independent experiments is shown.
inhibitory properties, we examined the IL-2 production of the DO11 hybridomas. Cells were co-cross-linked with anti-KLRG1 or anti-Myc mAbs and anti-TCR-β or anti-CD3 mAbs. Production of IL-2 by DO11 cells expressing WT KLRG1 was inhibited by plate bound KLRG1 mAbs in a dose-dependent fashion only when sub-optimal TCR cross-linking was induced (Fig. 7A). Inhibition was also observed when plate bound anti-Myc mAbs were used (Fig. 7B). This inhibition was clearly dependent on the ITIM tyrosine as no inhibition was observed when DO11 cells expressing KLRG1 Y7F were used (Fig. 7A and B). From these experiments, we concluded that (i) partial inhibition of IL-2 production was observed only with KLRG1 high expressors and not with low expressors (not shown), (ii) the inhibition is dependent on the ITIM tyrosine and (iii) KLRG1 can inhibit only a sub-optimal positive TCR signal.

Discussion
Inhibitory receptors expressed on NK and T cells have the potential to prevent cellular activation upon binding to their ligands (20–22). This inhibition is mediated in part by an
intracellular ITIM (I/VxYxxL) (23, 24). Among these receptors, the well-conserved KLRG1 molecule has recently piqued interest. However, in the absence of a known ligand and a better understanding of its signaling properties, it has been difficult to elucidate the functions of KLRG1. The presence of this inhibitory receptor on different cell subsets in the naive state, while having a dramatic inducible expression during activation, presumes the potential for differential signaling from KLRG1 ligation in both homeostatic conditions versus pathogenic challenge. The ability of KLRG1 to bind different ligands and associate with different phosphatases may contribute to this molecule’s diversity of function. Here, we show that KLRG1 binds to N- and E-cadherin and we demonstrate that the inhibitory properties of KLRG1 can account for its ability to recruit both SHIP-1 and SHP-2.

The identification of the ubiquitously expressed cadherins as ligands for KLRG1 may broaden the role of KLRG1 to include involvement in homeostatic conditions, development, cancer surveillance, as well as pathogenic immunity. Cadherins are a large family of evolutionarily conserved glycoproteins comprised of an extracellular domain responsible for cell–cell interactions, a transmembrane domain, and a cytoplasmic domain that is linked to the cytoskeleton (25, 26). They facilitate calcium-mediated homophilic binding between cells, and show various pattern expression associated with morphogenetic processes (26). It is well documented that the loss of intercellular adhesion, as with altered expression or function of cadherins, can facilitate tumorigenesis. Specifically, the down-regulation of E-cadherin is associated...
with tumor development, while the expression or switch to N-cadherin is indicative of increased cell motility and invasiveness (27, 28). This leads to the possibility that the KLRG1+ cells may only have a limited time to eliminate a transformed cell. In addition, restoration of E-cadherins in cancer cells results in decreased invasiveness, growth suppression and terminal differentiation (29–31).

A role for KLRG1/cadherin interaction during viral infection is also likely. It has been recently shown that during human papillomavirus infection, cell surface E-cadherin expression is reduced on keratinocytes (32). In addition, it has also been shown that hepatitis B virus protein represses E-cadherin expression at the transcription level (33). So it is possible that in addition to other mechanisms [for review, see (34, 35)], lack of KLRG1 engagement contributes to the clearance of viral-infected cells. In contrast, collateral damage on healthy uninfected cells caused by the innate inflammatory response to pathogen would presumably dissociate connected cells, enabling contact of cadherins with KLRG1. This interaction may act as a signal to control for excessive damage to the host. As E-cadherin is clustered at cell–cell contacts lining cavities of the body, it is also possible that this interaction simply allows NK cells passage through these barriers.

Moreover, it is possible that KLRG1 engagement with the cadherins may have biological consequences on cadherin-positive cells. Many lines of research have demonstrated that cadherins can function as signal-transducing molecules, in addition to their more extensively researched functions in cell adhesion (36). Cadherins harbor a number of serines, threonines and tyrosines within the β-catenin-binding domain that are putative phosphorylation sites. Consequently, KLRG1 engagement may influence cadherin-positive cell functions. These intriguing perspectives warrant future investigation.

In this report, we also demonstrate that KLRG1 associates with both SHIP-1 and SHP-2 phosphatases. These phosphatases have very different functions and the consequences of their recruitment to KLRG1 cytoplasmic membranes are likely to differ. SHIP-1 phosphatase is regulated by membrane targeting via binding of its SH2 domain to phosphorylated ITIMs, the best characterized of which is present in the inhibitory low-affinity receptor for IgG antibodies, FcγRIIB, expressed in B cells, mast cells and macrophages (37–39). The phenotypes of SHIP-deficient mice are consistent with the role of SHIP phosphatases as important negative regulators. SHIP-1/−/− mice die at an early age due to myeloid cell infiltration into the lungs, which is caused by enhanced signaling through a number of receptors (40, 41). SHIP-1 presumably hydrolyzes phosphatidylinositol 3,4,5-triphosphate (PIP3) into PI-3,4P 2, which is itself the product of an activation. SHIP-1 also recruits RasGAP via the adaptor molecule Dok-1 and therefore can potentially down-regulate the activation of MAPKs and subsequent cytokine gene

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**Fig. 6.** KLRG1 association with SHIP-1 depends on both tyrosine and leucine residues of the ITIM. (A) Flow cytometry profiles of DO11, DO11 KLRG1 WT or DO11 KLRG1 mutants. Each panel is an overlay of the isotype control (open histogram) and the anti-KLRG1 mAb (solid histogram). (B) All cell samples were treated with pervanadate or left untreated for 5 min at 37°C, lysed and the KLRG1 was immunoprecipitated with an anti-KLRG1 mAb. Samples were resolved by SDS–PAGE and transferred to nitrocellulose membrane for western blot analysis with the indicated mAbs. One of three independent experiments is shown.
KLRG1 possesses a serine residue at Y+ shown to associate preferentially to SHIP-1 and similar to bears an ITIM (ITYSLL) in its cytoplasmic tail has been found that SHIP-1 recruitment to KLRG1 is phosphotyrosine dependent as well as dependent on the phosphatases and found that SHIP-1 recruitment to KLRG1 is essential for optimal association of these two proteins. The engagement remains to be determined.

Understanding of the role of SHP-2 in the context of KLRG1 is well as for CD94/NKG2A and gp49B (16, 48, 49). A better understanding of the role of SHP-2 in the context of KLRG1 is acute regulatory in some cases (43, 44) and to mediate inhibition in other cases (45–47). An inhibition induced by recruitment of SHP-2 by presumably dephosphorylation of a variety of substrates has been shown for KIR receptors as well as for CD94/NKG2A and gp49B (16, 48, 49). A better understanding of the role of SHP-2 in the context of KLRG1 engagement remains to be determined.

In this study, we also defined the key KLRG1 ITIM amino acid residues required for optimal association of these two substrates and found that SHIP-1 recruitment to KLRG1 is phosphotyrosine dependent as well as dependent on the Y+3 leucine residue. Interestingly, FcRRIIB Fc receptor that bears an ITIM (ITYSLL) in its cytoplasmic tail has been shown to associate preferentially to SHIP-1 and similar to KLRG1 possesses a serine residue at Y+1 and a leucine residue at Y+3 (39). In the case of SHP-2, we found that a serine to alanine substitution at position Y-2 decreases SHP-2 association with KLRG1. In contrast, serine to alanine substitution at position Y+1 strongly increases SHP-2 association with KLRG1. Interestingly, using a combinatorial library method for the rapid identification of high-affinity peptides, Sweeney et al. (50) determined the consensus sequences that preferentially interact with several phosphatase SH2 domains. In support of our findings, they found that the Y+1 position was mostly occupied by an alanine for optimal binding to SHP-2.

In conclusion, we identified N-cadherin and E-cadherin as ligands for KLRG1 and delineate early KLRG1 mediated signaling. As we were concluding our experiments, two reports were published that also identified cadherins as ligands for KLRG1 (17, 19). The discovery of classical cadherins as ligands for the immune inhibitory receptor KLRG1 and its peculiar signaling properties opens unanticipated avenues of research combining immunology with structural biology.

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Abbreviations
ITIM immunoreceptor tyrosine-based inhibitory motif
KLRG1 killer cell lectin-like receptor G1
WT wild type

References
8 Ibegbu, C. C., Xu, Y. X., Harris, W., Maggio, D., Miller, J. D. and Kourti, A. P. 2005. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active,
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29 Ono, M., Bolland, S., Tempst, P. and Ravetch, J. V. 1996. Role of the inositol phosphatase SHIP in negative regulation of the immune system by the receptor Fc( gamma)RIIB. Nature 383:263.


