The Journal of Experimental Medicine

Activation of Natural Killer Cells and Dendritic Cells upon Recognition of a Novel CD99-like Ligand by Paired Immunoglobulin-like Type 2 Receptor

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Abstract

Paired receptors that consist of highly related activating and inhibitory receptors are widely involved in the regulation of the immune system. Here, we report a mouse orthologue of the human activating paired immunoglobulin-like type 2 receptor (PILR) β, which was cloned from a cDNA library of natural killer (NK) cells based on its ability to associate with the DAP12 signaling adaptor protein. The activating PILRβ was expressed not only on NK cells but also on dendritic cells and macrophages. Furthermore, we have identified a novel CD99-like molecule as a ligand for the activating PILRβ and inhibitory PILRα receptors. Transcripts of PILR ligand are present in many tissues, including some T cell lines. Cells expressing the PILR ligand specifically activated NK cells and dendritic cells that express the activating PILRβ. Our findings reveal a new regulatory mechanism of innate immunity by PILR and its CD99-like ligand.

Key words: natural killer cell receptor • tumor antigen • innate immunity • immunoglobulin superfamily • DAP12

Introduction

Activation of NK cells is controlled by both inhibitory and activating receptors (1–4). Human and mouse NK cells express inhibitory receptors that recognize MHC class I to avoid attack against normal cells. Inhibitory receptors possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains and deliver inhibitory signals via tyrosine phosphatases, such as SHP-1 (5, 6). On the other hand, NK cells may also express activating receptors that are highly homologous in their extracellular domains to the inhibitory receptors. Generally, genes for these paired inhibitory and activating NK receptors are located very closely in the genome and likely evolved by gene duplication (7). The activating receptors do not contain ITIM sequences in their cytoplasmic domains, and they exhibit a positively charged residue in transmembrane region that permits association with immunoreceptor tyrosine-based activation motif (ITAM)–bearing adaptor molecules, such as DAP12 (1). Unlike inhibitory receptors that recognize MHC class I, activating receptors generally do not recognize MHC class I, and physiological roles of most activating receptors have remained unclear. Receptor pairs that consist of highly related inhibitory and activating receptors are not restricted to NK receptors. For example, families of receptors, such as leukocyte Ig-like receptor (also called CD85 or Ig-like transcript),

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Abbreviations used in this paper: BM-DC, BM-derived DC; GFP, green fluorescent protein; IRES, internal ribosome entry site; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell Ig-like receptor; MCMV, mouse cytomegalovirus; PILR, paired Ig-like type 2 receptor, PILR-L, PILR ligand.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/02/525/9 $8.00
Volume 199, Number 4, February 16, 2004 525–533
http://www.jem.org/cgi/doi/10.1084/jem.20031885

paired Ig-like receptor (PIR), and signal-regulatory protein, are expressed on macrophages and DCs and consist of inhibitory and activating receptors (8).

Recently, we have proposed that mouse cytomegalovirus (MCMV) has acquired the m157 gene to encode a ligand for the inhibitory NK receptors to escape attack by NK cells (9). On the other hand, some MCMV-resistant mouse strains express activating NK receptors for m157 (9, 10). These findings suggested that NK cells may have evolved activating receptors from inhibitory receptors to control virus infection (11). This may have been accomplished by gene conversion in a process that used exons encoding the extracellular domain of an inhibitory receptor and an exon encoding the transmembrane of another activating receptor that allows for association with the DAP12 signaling adaptor (12). In addition, some activating human NK receptors, such as killer cell Ig-like receptor (KIR), exhibit a potential ITIM sequence in the 3′ untranslated region (13). From these observations, we have proposed a new hypothesis that activating receptors have evolved from inhibitory receptors (13). This hypothesis may explain why activating receptors corresponding to inhibitory receptors exist in the immune system. ITAM-bearing adaptor proteins, such as DAP12, FcRγ, and CD3ζ, are used for signal transduction by these paired activating receptors. Therefore, identification of receptors that associate with these adaptor molecules may be important to elucidate how NK cells recognize specific target cells, such as virus-infected cells.

An interesting characteristic of these ITAM-bearing adaptor molecules is their inability to be expressed stably on the cell surface in the absence of their partner receptor (14). By taking advantage of this property of the adaptor molecules and using retrovirus cDNA libraries, we have developed a sensitive screening system for receptors that associate with them. Using this system in the present work, we have identified a mouse orthologue of the human activating paired Ig-like type 2 receptor (PILR) β (15). The PILR family consists of PILRα (also called FDF03) and PILRβ (15, 16). Human PILRα contains an ITIM in its cytoplasmic domain and delivers inhibitory signals (15, 16). On the other hand, human PILRβ does not contain an ITIM in its cytoplasmic domain but possesses positively charged amino acids in the transmembrane region (15). Therefore, PILRβ has been suggested to be an activating receptor that associates with certain ITAM-bearing adaptor molecules. However, the physiological role, as well as ligands of PILR, have not been identified previously. In this paper, we demonstrate that mouse PILR recognizes a unique CD99-like protein expressed on several cell types, including T cells. Because the PILR is expressed not only on NK cells but also on macrophages, DCs, and granulocytes, our findings provide a new mechanism for the regulation of innate immunity.

Materials and Methods

Mice. C57BL/6 mice were purchased from Japan SLC. DAP12-deficient mice backcrossed to C57BL/6 for nine generations were maintained in the University of California San Francisco animal facility (17).

Expression Cloning of DAP12-associated Receptors. A NH_2-terminal FLAG-tagged DAP12 was transfected into the 2B4 T cell hybridoma by using the pMx-neo retrovirus vector. Thereafter, we selected a single cell clone that did not express FLAG-DAP12 on the cell surface, but did express high levels of FLAG-DAP12 upon transfection with the DAP12-associated Ly49D receptor. 2×10^7 FLAG-DAP12 2B4 T hybridoma cells were infected with a retrovirus cDNA library generated from IL-2–expanded C57BL/6 NK cells (18) at a viral titer to achieve ~30% infection. FLAG-expressing cells were isolated by using a MACS purification system (Miltenyi Biotech). After two rounds of enrichment of FLAG-positive cells by MACS, almost all of cells were stained by the anti-FLAG mAb M2 (Sigma-Aldrich) and approximately half of them were stained by mixture of anti-Ly49D (4E5; BD Biosciences) and Ly49H mAbs (provided by W.M. Yokoyama, Washington University School of Medicine, St. Louis, MO). Cells that did not express Ly49D and Ly49H were purified by MACS. Single cell clones expressing FLAG-DAP12, but not Ly49D or Ly49H, were obtained by limiting dilution. The integrated cDNA was derived from the FLAG-positive cells by PCR using sense primer (5′-GGTGGACCATCCTCTAGACT-3′) and antisense primer (5′-TTTATTTTATCGTCGATCGACC-3′), and the amplified genes were directly sequenced.

Functional Analyses for Adaptor Molecules Associated PILRs. NH_2-terminal FLAG-tagged PILRα or PILRβ were transfected into MA5.8 T cells that lack CD3ζ, FcRγ, and DAP12. ITAM-bearing adapters, including CD3ζ, FcRγ, and DAP12, were cotransfected into the FLAG-tagged PILR transfectants by using the pMx–internal ribosome entry site (IRES)–green fluorescent protein (GFP) retrovirus vector. The GFP-positive cells were sorted by using a FACSVantage™ (Becton Dickinson). The transfectants were stimulated with immobilized anti-FLAG mAb or control mAb. Culture supernatants were collected after 24 h, and amounts of IL-2 were measured by ELISA.

Generation of PILRα-Ig and PILRβ-Ig and Staining with Ig Fusion Proteins. cDNA fragments corresponding to the extracellular domains of PILRα and PILRβ were amplified by PCR using sense primer (5′-AATCTCGAGGGAAATTCAGAAAGATCCAA-3′) and antisense primer (5′-AATCTCGAGTGCCAACCCAACTGTGGTTT-3′), and sense primer (5′-AATCTCGAGGGAAATTCAGAAAGATACAACA-3′) and antisense primer (5′-AATCTCGAGCAGGTTCATCAGTGAAGGAT-3′), respectively. The cDNA for mouse PILRα was inserted into the XhoI cloning site of a modified pME18S expression vector that contained a mouse CD150 leader segment at the 5′ terminus, and the XhoI cloning site and the Fc segment of human IgG1 at the 3′ terminus. The cDNA-encoding mouse PILRβ was inserted into the XhoI cloning site of this expression vector, except that an additional IgA tail piece sequence was inserted at the 3′ end to generate a dodecameric Ig fusion protein (19). COS7 cells were transiently transfected with the plasmids, and culture supernatants were collected using standard methods. For the staining of PILR ligand (PILR-L), cell lines were mixed with saturating concentrations of these fusion proteins for 30 min on ice, followed by PE-conjugated goat anti–human IgG (Jackson ImmunoResearch Laboratories) for 30 min. Stained cells were analyzed by using a FACSCalibur™ (Becton Dickinson).

Retrovirus cDNA Library Construction. A retrovirus cDNA library was constructed as reported previously (18). In brief, cDNA was generated from mRNA purified from the EL-4 cell line using the Superscript plasmid system (Invitrogen) and was cloned into SalI and NotI sites of the pMxs retrovirus vector (18). Li-

gated cDNA was transformed into ElectroMAX competent cells (Invitrogen), and plasmids were purified by using a MAXI prep plasmid purification kit (QIAGEN).

Cloning of PILR-L and Generation of PILR-L Transfectants. 2×10^7 Ba/F3 cells were infected with a retrovirus cDNA library from EL-4 at an efficiency of ~30%. 2 d after infection, cells were stained with PILRβ-Ig and PE-conjugated goat anti–human IgG, and stained cells were purified by using a FACSVantage™. After expansion of the purified cells, the cells were stained with PILRβ-Ig plus PE-conjugated anti–human IgG and FITC-conjugated anti-Fc receptor mAb (2.4G2; BD Biosciences), and cells stained with PILRβ-Ig, but not 2.4G2 mAb, were obtained. Single cell clones that were stained by PILRβ-Ig were isolated by using a Clonocyte (Becton Dickinson). Library-derived genes were amplified by using the PCR GC Advantage system (CLONTECH Laboratories, Inc.) using sense primer (5′-GGTGGACCATCCTCTAGACT-3′) and antisense primer (5′-TTTATTTTATCGTCGATCGACC-3′). Amplified cDNA was cloned into the pMxs retrovirus vector, and nucleic acid sequences were determined. PILR-L cDNA was subcloned into the pME18S expression vector and pMx-IRES-GFP retrovirus expression vector and transfected into 293T cells and Ba/F3 cells, respectively.

Surface Biotinylation and Immunoprecipitation. Biotinylated Ba/F3 cells transfected with PILR-L were lysed with a lysis buffer containing 1% digitonin, 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 10 mM iodoacetamide at a concentration of 10^7 cells/ml. Cell lysates were immunoprecipitated with protein A preincubated with PILRα-Ig, PILRβ-Ig, and control Ig fusion protein, separated on SDS-PAGE gels, and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The biotinylated proteins were detected by using streptavidin peroxidase (VECSTAIN Elite ABC kit; Vector Laboratories), the enhanced chemiluminescence detection system (Amersham Biosciences), and LAS1000 (Fujifilm).

Real-time Quantitative PCR. cDNA from various tissues and purified cell populations were prepared as described previously (20). In brief, splenic B and T cells were stained with FITC-conjugated anti-B220 mAb (RA3–6B2; eBioscience) and PE-conjugated anti-CD3 mAb (145–2C11; BD Biosciences), respectively, and the stained cells were purified by using a MACS. Peritoneal macrophages and granulocytes were elicited by the i.p. injection of 3% sodium casein, and peritoneal cells were harvested after 1 d. Peritoneal exudates were separated with 90% Percoll gradient centrifugation (60,000 *g*, 20 min) (Amersham Pharmacia). Mac-1^+Gr-1^- and Mac-1^-Gr-1^+ cells were purified by using a FACSVantage™ and were used as macrophages and granulocytes, respectively. BM cells were cultured in the presence of 20 ng/ml of mouse IL-3 for 4 wk and used as BM-derived mast cells. Splenic NK cells and BM-derived DCs (BM-DCs) were prepared as described in the next two paragraphs. Quantitative PCR was performed by using a SYBER green PCR kit (QIAGEN) and analyzed by an iCycler (Bio-Rad Laboratories). Primers used for amplification were as follows: β-actin, sense primer (5′-TCTACAATGAGCTGCGTGTG-3′) and antisense primer (5′-GGTACGACCAGAGGCATACA-3′); PILRα, sense primer (5′-TGGGGTTTTGGGATTGATAG-3′) and antisense primer (5′-GCTGTTCCTGGTGAGGTCGG-3′); PILRβ, sense primer (5′-CCCAGTACTTTAGTCGAGTTAA-3′) and antisense primer (5′-TGAAGGATTCCTCTGGTCG-3′); and PILR-L, sense primer (5′-GGAGACTCAGGCGGCATCT-3′) and antisense primer (5′-GATAGGCCACGAAGCTCGAC-3′). Transcription levels of PILRα, PILRβ, and PILR-L were normalized to the amount of β-actin. The relative amount of transcripts in various organs was compared with the spleen by dividing the transcription level of each tissue by that of spleen.

NK Cell Cytotoxicity Assays. NKL human NK cell lines were transfected with pMx-IRES-GFP and pMx-IRES-GFP containing mouse PILRβ, and GFP-positive cells were collected by using a FACSVantage™. Mouse NK cells were purified as described previously (21). In brief, sIg^-, $CD4^-$, and $CD8^-$ splenocytes from C57BL/6 mice were stained with PE-conjugated DX5 mAb (eBiosocience), and $DX5^+$ cells were obtained by using a MACS purification system. $DX5^+$ NK cells were cultured in the presence of 1,000 U/ml IL-2 for 7 d. Cytotoxicity of NK cells against PILR-L–transfected Ba/F3 cells and 293T cells were analyzed by ^{51}Cr release assays using standard techniques.

Measurement of TNFα and NO Production. BM-DCs were generated by culturing 5×10^6 BM cells with RPMI 1640 medium containing 10% FCS and 200 U/ml mouse GM-CSF for 7–8 d in six-well culture plates. Nonadherent cells were collected and cultured for 1 d. More than 95% of the cells expressed CD11b and CD11c. 5×10^5 BM-DCs were cocultured with various numbers of parental or PILR-L–transfected 293T cells in 48-well plates, as indicated in Fig. 8. After 24 h, culture supernatants were collected, and amounts of NO_2 or TNFα were measured by using the Griess reagent (Sigma-Aldrich) or a TNFα ELISA kit (Genzyme), respectively.

Results

Cloning of Mouse Activating PILRβ. Most activating NK receptors associate with ITAM-bearing signal transducing adaptor molecules such as DAP12, FcRγ, and CD3ζ (21–23). Cell surface expression of these adaptor molecules is very low in the absence of the receptors that associate with them. To identify activating NK receptors that associate with DAP12, we generated a T cell hybridoma expressing a NH_2-terminal FLAG-tagged DAP12 (FLAG-DAP12) and isolated a single cell clone that lacks cell surface expression of FLAG-DAP12. We infected these cells with a retrovirus cDNA library generated from mouse NK cells (18) and selected and cloned cells expressing FLAG-DAP12 on the cell surface. Using this screening system, we cloned a mouse orthologue of the human activating PILRβ (15), as well as Ly49D and Ly49H that were already known to associate with DAP12 (24). The amino acid homology between mouse PILRβ and human PILRβ was 38% (15). When mouse PILRβ was transfected into the T cell hybridoma expressing FLAG-DAP12, cell surface expression of FLAG-DAP12 was induced by PILRβ (unpublished data). This suggested that DAP12 associates with mouse PILRβ.

Human and mouse PILRα (also called FDF03; references 15, 16) have been identified as an ITIM-containing inhibitory receptor that is highly related to PILRβ. Fig. 1 demonstrates the amino acid comparison between mouse PILRα and mouse PILRβ. PILRβ, but not PILRα, contains a positively charged amino acid in the transmembrane region that is required for association with DAP12 (25). Amino acid homology of the extracellular domain between mouse PILRα and PILRβ is 83%, with the NH_2 terminus of the extracellular domain showing very high similarity. This suggested that PILRα and PILRβ might recognize a common ligand.

```
        1                                                          60
PILRα   MALLISLPGGTPAMAQILLLLSSACLHAGNSERSNRKNGFGVNQPESCSGVQGGSIDIPF
PILRβ   MALLISLPGGTPAMAQVLLLLSSGCLHAGNSERYNRKNGFGVNQPERCSGVQGGSIDIPF
        ********************************************************************
        61                                                         120
PILRα   SFYFPWKLAKDPQMSIAWRWKDFHGEFIYNSSLPFIHEHFKGRLILNWTQGQTSGVLRIL
PILRβ   SFYFPWKLAKDPQMSIAWKWKDFHGEVIYNSSLPFIHEHFKGRLILNWTQGQTSGVLRIL
        ********************************************************************
        121                                                        180
PILRα   NLKESDQTRYFGRVFLQTTEGIQFWQSIPGTQLNVTNATCTPTTLPSTTAATSAHTQNDI
PILRβ   NLKESDQAQYFSRVNLQSTEGMKLWQSIPGTQLNVTQALNTTMRSPFIVTSEFTTAGLEH
        *******  ** ** ** ***   ************ *  *    *
        181                                                        240
PILRα   TEVKSANIGGLDLQTTVGLATAAAVFLVGVLGLIVFLWWKRRRQGQKTKAEIPAREPLET
PILRβ   TSDQ-RNPSLMNLGAMVTMLLAKVLVIVLVYGWMIFLRWKQRPAH----------------
        *     *     *   *    *     * * *   ** ** *
        241                                                        300
PILRα   SEKHESVGHEGQCMDPKENPKDNNIVYASISLSSPTSPGTAPNLPVHGNPQEETVYSIVK
PILRβ   ------------------------------------------------------------

        301
PILRα   AK
PILRβ   --
```

Figure 1. Amino acid comparison between mouse PILRα and PILRβ. Amino acid alignment of mouse PILRα and PILRβ is shown. Signal sequence and transmembrane region are indicated by double and single underline, respectively. A charged residue (K, bold and italicized) in transmembrane domain of PILRβ and the ITIM sequence in the cytoplasmic domain of PILRα are indicated (bold letters). Identical amino acids between PILRβ and PILRα are indicated by asterisks. GenBank/EMBL/DDBJ accession nos. of mouse PILRα and PILRβ are NM_153510 and AB122024/NM_133209, respectively.

Up-regulation of Cell Surface Expression of PILRβ by DAP12. We analyzed the role of ITAM-bearing adaptor proteins, including CD3ζ, FcRγ, and DAP12, on the cell surface expression and signal transduction of mouse PILRβ. NH_2-terminal FLAG-tagged PILRα (FLAG-PILRα) or PILRβ (FLAG-PILRβ) was transfected into a T cell hybridoma, MA5.8, that lacks CD3ζ, FcRγ, and DAP12. As shown in Fig. 2 A, FLAG-PILRα and FLAG-PILRβ were expressed on the cell surface of the MA5.8 transfectants, as demonstrated by staining with an anti-FLAG mAb. Of note, FLAG-PILRα was expressed at a higher level on MA5.8 than PILRβ. Next, we cotransfected CD3ζ, FcRγ, or DAP12 into M5.8 cells expressing FLAG-PILRβ (Fig. 2 B). The expression level of FLAG-PILRβ was substantially increased when DAP12, but not CD3ζ or FcRγ, was cotransfected into the MA5.8 cells bearing FLAG-PILRβ. In contrast, DAP10 did not substantially up-regulate the expression of FLAG-PILRβ (unpublished data). This suggests that DAP12 associates with PILRβ and stabilizes the cell surface expression of PILRβ.

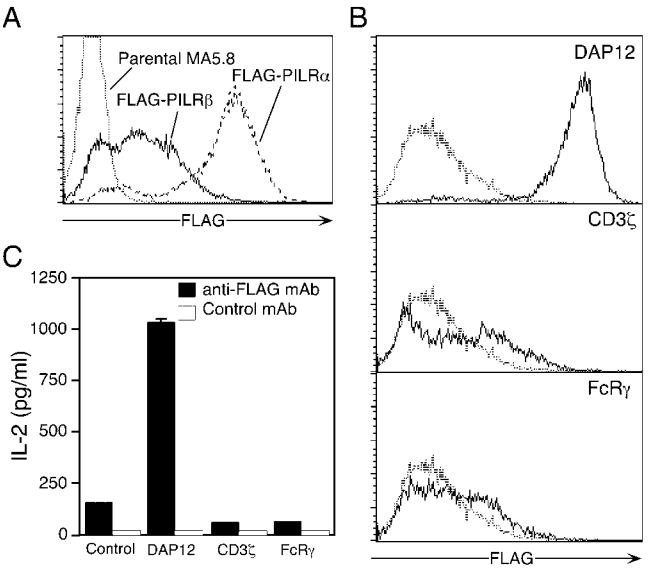

Figure 2. (A) Role of DAP12 in the cell surface expression and signal transduction of PILRβ, FLAG-PILRβ (solid line), and FLAG-PILRα (dashed line) was transfected into the MA5.8 cell line. Parental MA5.8 (dotted line) and the transfectants were stained with anti-FLAG mAb. Mean fluorescence intensities of anti-FLAG mAb staining for parental MA5.8, FLAG-PILRα, and FLAG-PILRβ–transfected cells were 2.93, 27.0, and 369.1, respectively. (B) DAP12, CD3ζ, or FcRγ were transfected into MA5.8-expressing FLAG-PILRβ using the pMx-IRES-GFP retrovirus vector. Transfectants were stained with anti-FLAG mAb and expression of FLAG on GFP-expressing cells is shown (solid line). FLAG expression on a mock transfectant was overlaid in this figure (dotted line). Mean fluorescence intensities of anti-FLAG mAb staining for FLAG-PILRβ–expressing cells transfected with DAP12, CD3ζ, and FcRγ were 1137.7, 56.4, and 33.9, respectively. (C) IL-2 production by MA5.8 cells and MA5.8 transfected with FLAG-PILRβ and DAP12, CD3ζ, or FcRγ and cultured for 1 d in the presence of immobilized anti-FLAG mAb (shaded bar) or control mAb (unshaded bar). IL-2 in the culture supernatants was measured by ELISA.

Next, we stimulated FLAG-PILRβ–transfected MA5.8 cells and FLAG-PILRβ and DAP12 cotransfected MA5.8 cells with immobilized anti-FLAG mAb (Fig. 2 C). MA5.8 cells expressing FLAG-PILRβ plus DAP12 were activated to produce IL-2 upon crosslinking of FLAG-PILRβ; however, IL-2 was not induced in cells transfected with FLAG-PILRβ alone, FLAG-PILRβ plus CD3ζ, or FLAG-PILRβ plus FcRγ. Together with the results that PILRβ was originally cloned as a molecule that induces cell surface expression of FLAG-DAP12, DAP12 associates with PILRβ and is required for high levels of cell surface expression and signal transduction of PILRβ.

Tissue Distribution of PILRα and PILRβ Transcripts. We analyzed the expression of PILRα and PILRβ transcripts by real-time quantitative PCR. Expressions of PILRα and PILRβ were relatively high in lung, liver, and spleen (Fig. 3 A). There was no significant difference in the tissue distribution between PILRα and PILRβ when analyzing their expression in total organs. We analyzed the expression of PILRβ in various purified populations (Fig. 3

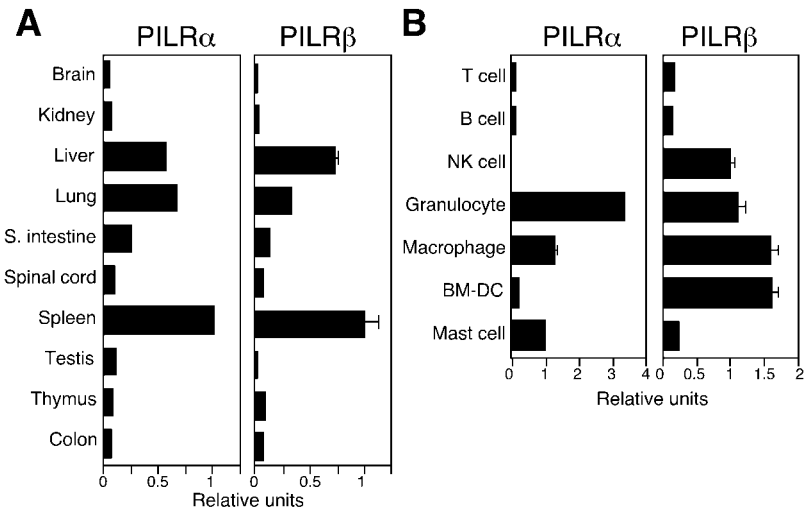

Figure 3. Transcription of PILRα and PILRβ in various tissues and purified populations. Transcription of PILRα and PILRβ in various tissues (A) and purified populations (B) was analyzed by real-time quantitative RT-PCR. Transcription of β-actin was used as a standard, and relative expression levels of PILRα and PILRβ compared with amounts in the spleen are shown. BM-DC, bone marrow–derived dendritic cells. S. intestine, small intestine.

B). Transcripts of PILRβ were detected not only in NK cells but also in BM-DCs, peritoneal macrophages, and granulocytes. Because DAP12 that is required for signaling of PILRβ is widely distributed not only in NK cells but also in cells of the myeloid lineage, including macrophages, granulocytes, and DCs, PILRβ may play a role in the activation of these populations. On the other hand, macrophages and granulocytes also expressed the inhibitory PILRα. In contrast, NK cells and BM-DCs expressed a very low amount of PILRα transcripts. Therefore, the expression pattern of PILRα and PILRβ seems to vary depending on cell types and their activation status.

Molecular Cloning of the Ligand for PILRβ. To identify the ligand for these receptors, we generated Ig fusion proteins containing the extracellular domains of PILRα and PILRβ. When various transformed cell lines were stained with the PILRα-Ig and PILRβ-Ig fusion proteins, we found that some T cell lines, such as EL-4 and 2B4, were stained with both PILRα-Ig and PILRβ-Ig (Fig. 4). These Ig fusion proteins did not stain other cell lines, such as B cell and macrophage lines. Because both EL-4 and 2B4 are IL-4–producing T cell lines, this suggested that expression of the ligand for PILRβ might correlate with Th2 differentiation.

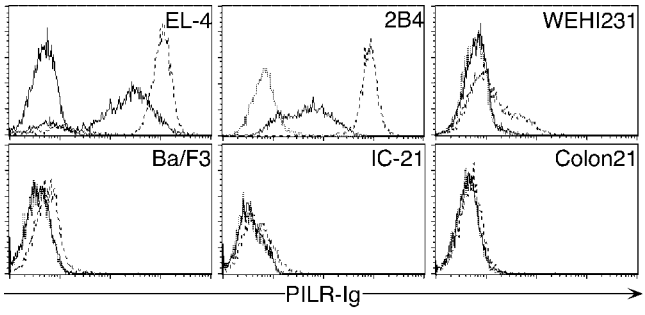

Figure 4. Expression of PILR-L. The cell lines indicated were stained with PILRβ-Ig (solid line), PILRα-Ig (dashed line), or control Ig (dotted line), followed by PE-conjugated goat anti–human IgG. Fluorescence intensities of PE-stained cells are shown.

To elucidate the function of PILRβ, we sought to identify the ligand by expression cloning. Because EL-4 was stained well by PILRβ-Ig (Fig. 4 A), we constructed a retrovirus cDNA library from EL-4 and undertook expression cloning using PILRβ-Ig. The EL-4 retrovirus cDNA library was transfected into Ba/F3 cells, which did not stain with the PILRα-Ig or PILRβ-Ig, and Ba/F3 cells expressing the ligand for PILRβ were purified by using a fluorescence-activated cell sorter. Single cell clones that were stained with PILRβ-Ig were obtained and genes derived from the cDNA library were amplified by PCR using primers corresponding to the flanking retrovirus long terminal repeat and sequenced. Fig. 5 A shows the nucleic acid and predicted amino acid sequence of a PILR-L. PILR-L is a type I membrane protein with a signal sequence, a small extracellular domain, a transmembrane region, and a short 17–amino acid cytoplasmic domain with no evident signaling motifs. The sequence of PILR-L was remarkable in two aspects. Surprisingly, the GC content of PILR-L is 75%; GC-rich transcripts are rare in mammalian cells. In addition, the short extracellular domain of PILR-L contained no sites for N- or O-linked glycosylation, again unusual for proteins expressed on the cell surface. PILR-L showed 45% similarity to human CD99, 36% to human CD99L2, and 32% to mouse CD99L2 (26). This indicates that PILR-L belongs to CD99 family.

When the PILR-L was transfected into Ba/F3 cells or 293T cells, the transfectants were stained well with PILRβ-Ig (Fig. 5 B). Furthermore, these PILR-L transfectants were also stained with PILRα-Ig. This indicated that PILR-L is a ligand for both PILRα and PILRβ. Indeed, when PILR-L was precipitated from cell lysates of PILR-L–transfected Ba/F3 cells using PILRα-Ig and PILRβ-Ig, PILR-L was clearly detected as a 17-kD molecule, although PILRβ-Ig precipitated much less PILR-L. A control Ig did not precipitate the 17-kD protein, and this protein was not immunoprecipitated from untransfected Ba/F3 cells (Fig. 5 C). The actual molecular weight was the same as the predicted molecular weight of PILR-L based

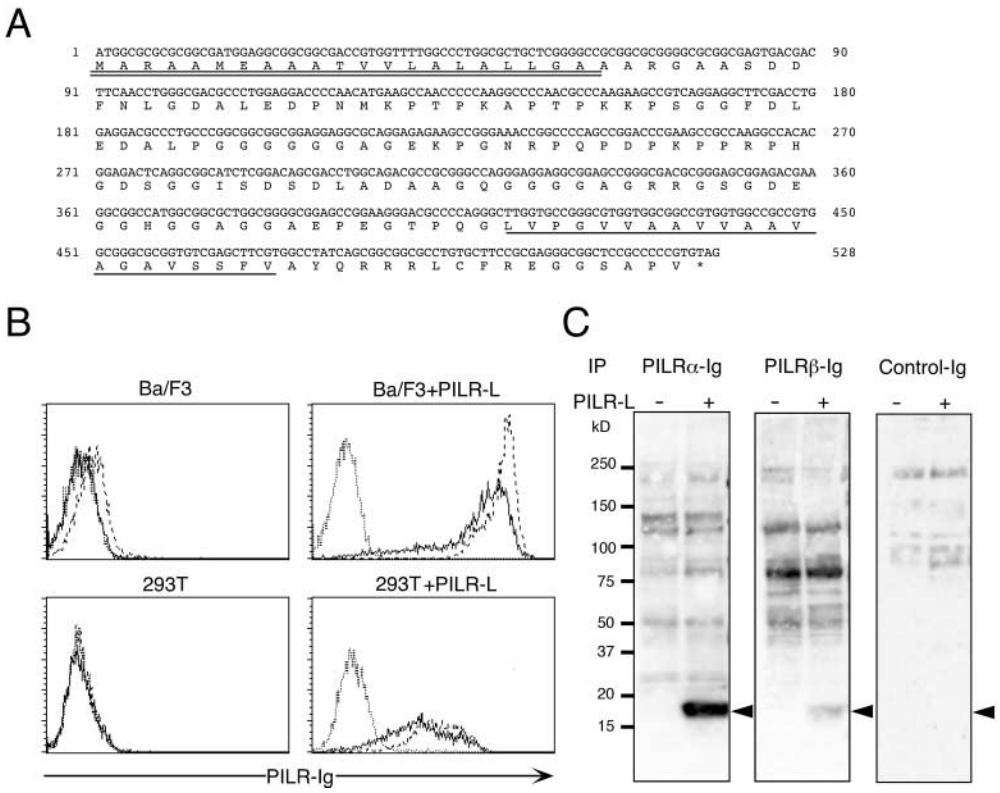

Figure 5. Molecular cloning of mouse PILR-L. (A) Nucleic and predicted amino acid sequences of mouse PILR-L are shown. The signal sequence and transmembrane domain are indicated by double and single underline, respectively. Sequence data of PILR-L are available from GenBank/EMBL/DDBJ under accession no. AB122023. (B) Ba/F3 and 293T cells were transfected with mouse PILR-L. Parental cells and the PILR-L transfectants were stained with PILRβ-Ig (solid line), PILRα-Ig (dashed line), or control Ig (dotted line), followed by PE-conjugated goat anti–human IgG. (C) SDS-PAGE analysis of PILR-L. PILR-L transfected (+) and parental (−) Ba/F3 cells were surface biotinylated, and cell lysates were precipitated with PILRα-Ig, PILRβ-Ig, or control Ig fusion protein as indicated. Precipitates were electrophoresed by SDS-PAGE and biotinylated proteins were detected by using horseradish peroxidase–conjugated streptavidin and the enhanced chemiluminescence detection reagent.

on the deduced amino acid sequence. We analyzed the expression of PILR-L transcripts by real-time quantitative PCR. PILR-L transcripts were present in most tissues, and expressions of PILR-L were relatively high in lung, spleen, thymus, liver, and spinal cord (Fig. 6).

Cytotoxicity by NK Cells against PILR-L–expressing Cells. To examine the function of mouse PILRβ and PILR-L, we transfected mouse PILRβ into NKL, a human NK cell line, and analyzed cytotoxicity of PILRβ-transfected NKL cells against mouse PILR-L–transfected 293T cells. As shown in Fig. 7 A, mouse PILRβ-transfected NKL cells showed cytotoxicity against PILR-L–transfected 293T, but not against parental 293T cells. Mock-transfected NKL did not show cytotoxicity against PILR-L–transfected or parental 293T cells, indicating that NKL cells do not express endogenous activating receptors that can recognize the mouse PILR-L. In addition, soluble recombinant human PILR-Ig did not bind to cells expressing mouse PILR-L (unpublished data). These data indicate that mouse PILRβ recognizes PILR-L–expressing cells and transduces an activating signal into NK cells. We observed that purified mouse PILRα-Ig and PILRβ-Ig did not block cytotoxicity mediated by mouse PILRβ-expressing NKL cells against PILR-L transfectants, probably due to insufficient affinity of these fusion proteins (unpublished data).

Next, we analyzed cytotoxicity of IL-2–expanded mouse NK cells against mouse PILR-L transfectants. Because mouse Ba/F3 pro–B cells are resistant to cytotoxicity mediated by mouse NK cells and do not express PILR-L, we transfected PILR-L into Ba/F3 and used these cells as targets. When cytotoxicity of C57BL/6 NK cells against the mouse PILR-L transfectants was analyzed, NK cells showed significant cytotoxicity against PILR-L transfectants compared with the parental Ba/F3 cells (Fig. 7 B). We analyzed NK cells from DAP12-deficient mice (17) because DAP12 is required for signal transduction by PILRβ (Fig. 2). As predicted, NK cells from DAP12-deficient mice showed almost the same cytotoxicity against PILR-L–transfected Ba/F3 cells and parental Ba/F3 cells. These data indicate that the DAP12-associated PILRβ is involved in target cell recognition by NK cells.

Activation of DCs by PILR-L–expressing Cells. As shown in Fig. 3, BM-DCs, as well as NK cells, express the activating PILRβ but express relatively very low amounts of inhibitory PILRα transcripts compared with macro-

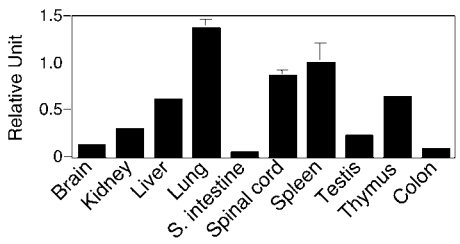

Figure 6. Transcription of PILR-L in various tissues. Transcription of PILRα and PILRβ in various tissues was analyzed by real-time quantitative RT-PCR. Transcription of β-actin was used as a standard and relative expression levels of PILR-L to the amounts present in spleen are shown. S. intestine, small intestine.

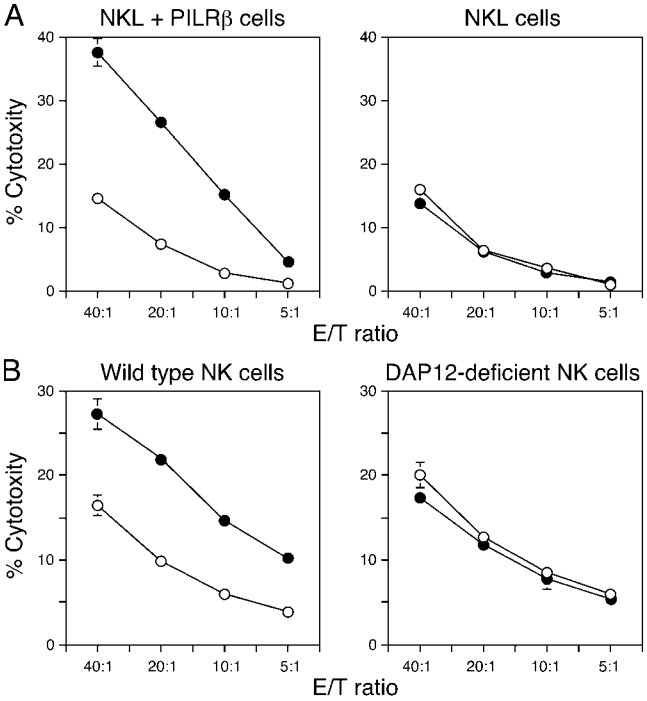

Figure 7. Cytotoxicity of NK cells against PILR-L–expressing cells. (A) Cytotoxicity of mouse PILRβ-transfected human NKL (left) or mock-transfected NKL cells (right) against mouse PILR-L–transfected 293T cells (closed circles) or parental 293T cells (open circles) is shown. (B) Cytotoxicity of IL-2 expanded NK cells from wild-type mice (left) or DAP12-deficient mice (right) against PILR-L–transfected Ba/F3 cells (closed circles) or parental Ba/F3 cells (open circles) is shown.

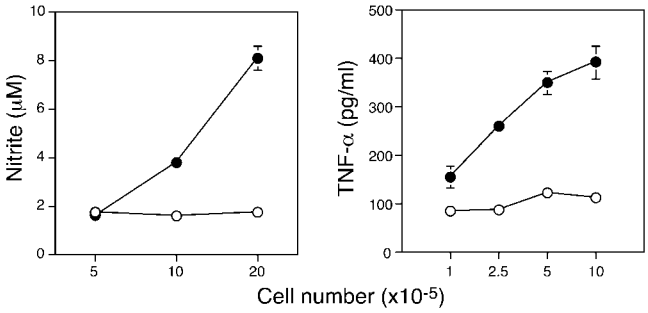

Figure 8. Activation of DCs by PILR-L–expressing cells. 5×10^5 BM-derived DCs were cocultured with PILR-L–transfected 293T cells (closed circles) or parental 293T cells (open circles) at the indicated cell number for 1 d. BM-derived DCs were also stimulated with 1 μg/ml LPS as a positive control. Nitric oxide and mouse TNF-α produced in the culture supernatants were measured. Amounts of nitric oxide and TNF-α produced by DCs cultured in medium only were 1.5 ± 0.1 μM and 48.5 ± 7.7 pg/ml, respectively, and those produced by DCs upon LPS stimulation were 59.1 ± 2.8 μM and 2048 ± 127.9 pg/ml, respectively.

phages and granulocytes. This suggested that PILR-L–expressing cells might activate not only NK cells but also DCs. Therefore, we analyzed the function of PILRβ on BM-DCs. Because DCs are known to produce nitric oxide and TNFα upon activation (27), we analyzed production of nitric oxide and TNFα production by DCs after coculture with PILR-L–expressing cells. When BM-DCs were stimulated with PILR-L transfectants, DCs produced significant amounts of nitric oxide and TNFα (Fig. 8). In contrast, DCs did not produce nitric oxide and TNF-α when they were cocultured with parental 293T cells lacking PILR-L. These data indicate that PILRβ is also involved in the activation of DCs.

Discussion

In the present work, we have described a mouse orthologue of human activating PILRβ. Furthermore, we identified its ligand by using PILRβ-Ig fusion protein. Expression of PILRβ was significantly up-regulated in the presence of DAP12, and PILRβ-expressing cells were activated only in the presence of DAP12. NK cells showed significant cytotoxicity against PILR-L–expressing cells, whereas NK cells from mice genetically lacking DAP12 did not. These results indicate that DAP12 mediates signal transduction for PILRβ.

Transcription of PILRβ was observed not only in NK cells but also in macrophages, DCs, and granulocytes. Because DAP12 is widely expressed in these populations, PILRβ may play an important role in the activation of various cell populations. DCs showed significant production of nitric oxide and TNF-α when they were cocultured with PILR-L–expressing cells, indicating that PILRβ directly recognizes PILR-L–expressing cells and delivers activation signals in various cell types. Of note, DAP12-deficient mice have been previously shown to have a defect in APC function (17, 28), manifested by the accumulation of DCs in the skin, an impaired delayed-type hypersensitivity response and resistance to the induction of myelin oligodendrocyte glycoprotein peptide–induced experimental autoimmune encephalomyelitis. Considering the finding that PILR-L is expressed on T cell lines, there is the possibility that interactions between PILRβ and PILR-L are involved in some of the disorders observed in the DAP12-deficient mice.

It is interesting that both the inhibitory PILRα and the activating PILRβ recognize the same ligand. Earlier analyses have indicated that human PILRα can inhibit ITAM-mediated activation signals (15, 16). However, activation of NK cells and DCs by coculture with PILR-L–bearing cells indicates either a subset of NK cells or DCs bear only the activating receptor or that the activating receptor is dominant in cells that coexpress both PILRα and PILRβ. Indeed, transcription levels of inhibitory PILRα in NK cells and DCs were very low compared with macrophages and granulocytes (Fig. 3 B). Specific mAbs that distinguish PILRα and PILRβ are needed to define the precise distribution of these receptors and to study their function in immune responses.

KIR, Ly49, NKG2/CD94, and NKR-P1 are paired receptors expressed by NK cells (8, 11). Similarly, myeloid and B cells have been shown to express the paired receptors, signal-regulatory protein, CD85 (also called Ig-like transcript and leukocyte Ig-like receptor), and paired Ig-like receptor (8, 11). *PILR*α and *PILR*β represent another

example of related genes, conserved in mice and humans, that encode activating and inhibitory receptors. Mouse PILRα and PILRβ are localized on chromosome 5, and human PILRα and β are localized on chromosome 7 (based on information derived from the National Center for Biotechnology Information Genome BLAST website). PILRα and PILRβ are located very closely on the genome, similar to other paired receptors. Although the amino acid homology between the extracellular domains of the paired activating and inhibitory receptors are often very high, often the activating receptor either fails to bind the ligand of the inhibitory receptor or do so with much reduced affinity. This is the case with KIR and Ly49 receptors for MHC class I (29, 30). Similarly, certain inhibitory CD85 receptors bind MHC class I, whereas the activating CD85 receptors do not (31, 32). Further studies are required to determine the precise affinity of mouse PILRα and PILRβ for PILR-L; however, we suspect that PILRβ will likely be lower than PILRα.

PILR-L exhibits 40% homology to human CD99. Although human CD99 is present on the pairing region of the human X and Y chromosomes, as yet the genomic localization of PILR-L has not been identified. Although it is premature to conclude that PILR-L is a mouse orthologue of CD99, there is a possibility that PILR-L is a mouse orthologue for human CD99. Human CD99 is widely expressed on most hematopoietic cells (34). Similarly, we detected mouse PILR-L transcripts in most tissues, suggesting a broad distribution (Fig. 6). In addition, human CD99 has been reported to be involved in apoptosis of T cells (33) and migration of monocytes through epithelial cells (34) by as yet undefined mechanisms. Expression of mouse PILR-L on the EL-4 and 2B4 T cell lines suggests a potential role in the regulation of T cell immune responses. In studies currently in progress, we have observed that naive $CD4^+$ T cells do not express PILR-L, but interestingly, when naive $CD4^+$ T cells were stimulated with Ag plus APC, PILR-L was expressed on activated $CD4^+$ T cells (unpublished data). This finding, together with expression of PILRβ on DCs (Fig. 3 B), suggests the possibility of a new pathway to regulate T cell–APC interactions and influence helper T cell differentiation.

What is the rationale for the evolution of paired immune receptors with activating and inhibitory function? We have proposed the hypothesis that pathogens may exploit inhibitory receptors by acquiring ligands that can dampen immune responses, but that the host may convert the inhibitory immune receptors to activating immune receptors by gene conversion due to selective pressure imposed by the pathogen. An example is provided by the paired inhibitory and activating Ly49 receptors on mouse NK cells that recognize a viral glycoprotein encoded by MCMV (9). According to this hypothesis, there is a possibility that the paired PILRs may recognize certain molecules derived from a pathogen. It is interesting to note that amino acid sequence of PILR-L demonstrates 20% homology with the PE–Pro-Glu polymorphic GC-rich repetitive sequence of *Mycobacterium tuberculosis* (35). Although further studies are necessary to reveal the biological role of PILR and PILR-L, our present findings provide evidence for a new receptor–ligand system involved in both the innate and adaptive immune responses.

We would like to thank A. Sakamoto, M. Unnno, and R. Shiina for technical assistance and Y. Kurihara and H. Yamaguchi for secretarial assistance.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (to T. Saito and H. Arase) and by the Uehara Memorial Foundation (to H. Arase). K. Ogasawara is supported by a Human Frontier Science Program Long-term fellowship. L.L. Lanier is an American Cancer Society Research Professor and is supported by National Institutes of Health grant CA89294 and the Sandler Family Supporting Fund.

Submitted: 3 November 2003
Accepted: 16 December 2003

References

1. Lanier, L.L. 2001. On guard—activating NK cell receptors. *Nat. Immunol.* 2:23–27.
2. Smith, H.R., A.H. Idris, and W.M. Yokoyama. 2001. Murine natural killer cell activation receptors. *Immunol. Rev.* 181:115–125.
3. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M.C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19:197–223.
4. Diefenbach, A., and D.H. Raulet. 2003. Innate immune recognition by stimulatory immunoreceptors. *Curr. Opin. Immunol.* 15:37–44.
5. Ravetch, J.V., and L.L. Lanier. 2000. Immune inhibitory receptors. *Science.* 290:84–89.
6. Daeron, M., and E. Vivier. 1999. Biology of immunoreceptor tyrosine-based inhibition motif-bearing molecules. *Curr. Top. Microbiol. Immunol.* 244:1–12.
7. Yawata, M., N. Yawata, L. Abi-Rached, and P. Parham. 2002. Variation within the human killer cell immunoglobulin-like receptor (KIR) gene family. *Crit. Rev. Immunol.* 22: 463–482.
8. Colonna, M., H. Nakajima, and M. Cella. 2000. A family of inhibitory and activating Ig-like receptors that modulate function of lymphoid and myeloid cells. *Semin. Immunol.* 12: 121–127.
9. Arase, H., E.S. Mocarski, A.E. Campbell, A.B. Hill, and L.L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science.* 296:1323–1326.
10. Smith, H.R., J.W. Heusel, I.K. Mehta, S. Kim, B.G. Dorner, O.V. Naidenko, K. Iizuka, H. Furukawa, D.L. Beckman, J.T. Pingel, et al. 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc. Natl. Acad. Sci. USA.* 99:8826–8831.
11. Arase, H., and L.L. Lanier. 2002. Virus-driven evolution of natural killer cell receptors. *Microbes Infect.* 4:1505–1512.
12. Mehta, I.K., H.R. Smith, J. Wang, D.H. Margulies, and W.M. Yokoyama. 2001. A "chimeric" c57l-derived ly49 inhibitory receptor resembling the ly49d activation receptor. *Cell. Immunol.* 209:29–41.
13. Arase, H., and L.L. Lanier. 2004. Specific recognition of virus-

infected cells by paired NK receptors. *Rev. Med. Virol.* In press.
14. Bakker, A.B., E. Baker, G.R. Sutherland, J.H. Phillips, and L.L. Lanier. 1999. Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. *Proc. Natl. Acad. Sci. USA.* 96:9792–9796.
15. Mousseau, D.D., D. Banville, D. L'Abbe, P. Bouchard, and S.-H. Shen. 2000. PILRα, a novel immunoreceptor tyrosine-based inhibitory motif-bearing protein, recruits SHP-1 upon tyrosine phosphorylation and is paired with the truncated counterpart PILRb. *J. Biol. Chem.* 275:4467–4474.
16. Fournier, N., L. Chalus, I. Durand, E. Garcia, J.J. Pin, T. Churakova, S. Patel, C. Zlot, D. Gorman, S. Zurawski, et al. 2000. FDF03, a novel inhibitory receptor of the immunoglobulin superfamily, is expressed by human dendritic and myeloid cells. *J. Immunol.* 165:1197–1209.
17. Bakker, A.B.H., R.M. Hoek, A. Cerwenka, B. Blom, L. Lucian, T. McNeil, R. Murray, J.H. Phillips, J.D. Sedgwick, and L.L. Lanier. 2000. DAP12-deficient mice fail to develop autoimmunity due to impaired antigen priming. *Immunity.* 13:345–353.
18. Arase, H., T. Saito, J.H. Phillips, and L.L. Lanier. 2001. The mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (α2 integrin, very late antigen-2). *J. Immunol.* 167:1141–1144.
19. Arthos, J., C. Cicala, T.D. Steenbeke, T.W. Chun, C. Dela Cruz, D.B. Hanback, P. Khazanie, D. Nam, P. Schuck, S.M. Selig, et al. 2002. Biochemical and biological characterization of a dodecameric CD4-Ig fusion protein: implications for therapeutic and vaccine strategies. *J. Biol. Chem.* 277:11456–11464.
20. Arase, H., T. Suenaga, N. Arase, Y. Kimura, K. Ito, R. Shiina, H. Ohno, and T. Saito. 2001. Negative regulation of expression and function of FcγRIII by CD3ζ in murine NK cells. *J. Immunol.* 166:21–25.
21. Arase, N., H. Arase, S.Y. Park, H. Ohno, C. Ra, and T. Saito. 1997. Association with FcRγ is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1$^+$ T cells. *J. Exp. Med.* 186:1957–1963.
22. Lanier, L.L., B.C. Corliss, J. Wu, C. Leong, and J.H. Phillips. 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature.* 391: 703–707.
23. Pessino, A., S. Sivori, C. Bottino, A. Malaspina, L. Morelli, L. Moretta, R. Biassoni, and A. Moretta. 1998. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J. Exp. Med.* 188:953–960.
24. Smith, K.M., J. Wu, A.B.H. Bakker, J.H. Phillips, and L.L. Lanier. 1998. Ly49D and Ly49H associate with mouse DAP12 and form activating receptors. *J. Immunol.* 161:7–10.
25. Lanier, L.L., B. Corliss, J. Wu, and J.H. Phillips. 1998. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity.* 8:693–701.
26. Suh, Y.H., Y.K. Shin, M.C. Kook, K.I. Oh, W.S. Park, S.H. Kim, I.S. Lee, H.J. Park, T.L. Huh, and S.H. Park. 2003. Cloning, genomic organization, alternative transcripts and expression analysis of CD99L2, a novel paralog of human CD99, and identification of evolutionary conserved motifs. *Gene.* 307:63–76.
27. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767–811.
28. Tomasello, E., P.-O. Desmoulins, K. Chemin, S. Guia, H. Cremer, J. Ortaldo, P. Love, D. Kaiserlian, and E. Vivier. 2000. Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. *Immunity.* 13:355–364.
29. Vales-Gomez, M., H.T. Reyburn, R.A. Erskine, and J. Strominger. 1998. Differential binding to HLA-C of p50-activating and p58-inhibitory natural killer cell receptors. *Proc. Natl. Acad. Sci. USA.* 95:14326–14331.
30. Nakamura, M.C., S. Hayashi, E.C. Niemi, J.C. Ryan, and W.E. Seaman. 2000. Activating Ly-49D and inhibitory Ly-49A natural killer cell receptors demonstrate distinct requirements for interaction with H2-D(d). *J. Exp. Med.* 192:447–454.
31. Cosman, D., N. Fanger, L. Borges, M. Kibin, W. Chin, L. Peterson, and M.-L. Hsu. 1997. A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity.* 7:273–282.
32. Colonna, M., J. Samaridis, M. Cella, L. Angman, R.L. Allen, C.A. O'Callaghan, R. Dunbar, G.S. Ogg, V. Cerundolo, and A. Rollick. 1998. Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* 160:3096–3100.
33. Pettersen, R.D., G. Bernard, M.K. Olafsen, M. Pourtein, and S.O. Lie. 2001. CD99 signals caspase-independent T cell death. *J. Immunol.* 166:4931–4942.
34. Schenkel, A.R., Z. Mamdouh, X. Chen, R.M. Liebman, and W.A. Muller. 2002. CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat. Immunol.* 3:143–150.
35. Brennan, M.J., and G. Delogu. 2002. The PE multigene family: a 'molecular mantra' for mycobacteria. *Trends Microbiol.* 10:246–249.