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Killer Cell Ig-Like Receptor and Leukocyte Ig-Like Receptor Transgenic Mice Exhibit Tissue- and Cell-Specific Transgene Expression

Danny Belkin,* Michaela Torkar,* Chiwen Chang,* Roland Barten,* Mauro Tolaini,† Anja Haude,* Rachel Allen,* Michael J. Wilson,* Dimitris Kioussis,† and John Trowsdale2*

To generate an experimental model for exploring the function, expression pattern, and developmental regulation of human Ig-like activating and inhibitory receptors, we have generated transgenic mice using two human genomic clones: 52N12 (a 150-Kb clone encompassing the leukocyte Ig-like receptor (LILR)B1 (ILT2), LILRB4 (ILT3), and LILRA1 (ILR6) genes) and 1060P11 (a 160-Kb clone that contains ten killer cell Ig-like receptor (KIR) genes). Both the KIR and LILR families are encoded within the leukocyte receptor complex, and are involved in immune modulation. We have also produced a novel mAb to LILRA1 to facilitate expression studies. The LILR transgenes were expressed in a similar, but not identical, pattern to that observed in humans: LILRB1 was expressed in B cells, most NK cells, and a small number of T cells; LILRB4 was expressed in a B cell subset; and LILRA1 was found on a ring of cells surrounding B cell areas on spleen sections, consistent with other data showing monocyte/macrophage expression. KIR transgenic mice showed KIR2DL2 expression on a subset of NK cells and T cells, similar to the pattern seen in humans, and expression of KIR2DL4, KIR3DS1, and KIR2DL5 by splenic NK cells. These observations indicate that linked regulatory elements within the genomic clones are sufficient to allow appropriate expression of KIRs in mice, and illustrate that the presence of the natural ligands for these receptors, in the form of human MHC class I proteins, is not necessary for the expression of the KIRs observed in these mice. The Journal of Immunology, 2003, 171: 3056–3063.

Natural killer cells are involved in immune responses to tumor cells and to viral, bacterial, and parasitic infections (1, 2). Attack by NK cells is regulated by a combination of signals from inhibitory and stimulatory receptors on the cell surface. MHC class I-specific inhibitory receptors are the best understood of these and include members of the human killer cell Ig-like receptor (KIR)5 family, their functional homologues the murine Ly49 family, and the NKG2/C9D94 receptors (present in both species) (3, 4). Additional immune receptors such as the leukocyte Ig-like receptors (LILR, also known as Ig-like transcripts (ILT), LIR, or CD85), and the murine paired Ig-like receptors are closely related to KIRs in structure and presumably also in function. Both KIRs and LILRs are encoded within the leukocyte receptor complex (LRC) on human chromosome 19q13.42, a region of the genome that exhibits marked inter- and intraspecies variation (5–7).

KIR and LILR receptors are members of the Ig superfamily and exist in both activating and inhibitory isoforms. The activating type, characterized by a short cytoplasmic tail, associate with adapter proteins via a positively charged arginine residue in their transmembrane domain. Associated transmembrane adapter proteins carry an immunotyrosine-based activation motif for signal transduction. Inhibitory type KIR and LILR, characterized by a long cytoplasmic tail-bearing immunotyrosine-based inhibitory motif, have been shown to inhibit cell activation of monocytes, B cells, NK cells, and T cells (8–11).

Different KIR gene products can be detected on overlapping sets of human NK cells (12, 13). Each KIR clone expresses at least one inhibitory receptor. Variegated patterns of expression suggest a stochastic mechanism of KIR expression (14). KIR expression is likely to be controlled by highly homologous upstream promoter sequences (with the exception of KIRD2DL4, which possesses a more divergent promoter and is constitutively expressed in all NK clones) (5). It has been demonstrated that the frequency with which cells express a combination of Ly49 receptors is a product of their individual frequencies, leading to the proposal of the “product rule” (15). Higher-order, chromatin-related regulation also seems to be involved, as there appears to be a correlation between methylation upstream of the transcriptional start site and reduced KIR expression (16). MHC class I molecules also exert an effect on the repertoire of NK receptor expression. An example of this was seen in MHC class I-deficient mice, which showed higher frequencies of NK cells coexpressing different inhibitory Ly49 receptors when compared with normal mice, indicating that the interaction of Ly49 receptors with their MHC class I ligands shape the receptor repertoire by limiting receptor coexpression (17, 18). This effect is consistent with a sequential regulated expression model, which proposes that the MHC reactivity of receptors expressed early in the sequence determines whether other receptors will be subsequently expressed. This happens to the extent that even MHC haplotypes, which did not detectably bind certain Ly49 receptors, impacted coexpression of these receptors with other Ly49 molecules (15, 17–20).
Many LILR-related sequences have been described to date. Most are transmembrane glycoproteins with an extracellular, ligand-binding region composed of either two or four Ig domains. LILR-mediated immune modulation may affect cells of both the innate and adaptive immune systems. LILRB1 (ILT2/LIR1) shows an exceptionally wide expression pattern and can be found on the majority of CD14+ myelomonocytic cells and B cells in addition to NK and T cell subsets (21, 22). LILRA2 (ILT1/LIR7) and LILRB3 (ILT5/LIR3) are expressed by myeloid cells, granulocytes, and subsets of NK or T cells (21, 23), while LILRB4 (ILT3/LIR5) is expressed only on myeloid cells (8). cDNA studies indicate that LILRB5 (LIR8) may be the only NK cell specific LILR (24). ILT7, ILT8 and LILRA1 (LIR6) are expressed in monocytes but not on B, T, or NK cells (24, 25). Little is known about the control of LILR gene expression, although there is preliminary evidence that some LILRs show similar clonotypic expression patterns to KIRs (21).

The ligands that have been identified for LILRB1, LILRB2 (ILT4/LIR2), and LILRA1 are all HLA class I molecules. LILRB1 and LILRB2 recognize classical, nonclassical, and viral orthologues of HLA class I (10, 21, 22, 26). LILRB2 can also recognize free H chain forms of HLA class I. LILRA1 has been shown to recognize HLA B27 but does not recognize the nonclassical HLA-F protein (27) (R. L. Allen and E. Lepin, unpublished observations), so it appears to have more restricted specificity.

Activation of monocytes through receptors such as FcγRIII and FcγRII can be inhibited by coligation of inhibitory LILR (8, 10). The inhibitory process is mediated by Src homology 2-containing phosphatases 1 and 2 (28). Activating LILRs use a similar signaling mechanism to the FcγR. LILR2A, an activating member of the LILR family, associates with the common Fcγ chain (25, 29). It is therefore likely that the signaling machinery in rodent and humans is conserved (24, 25), which could allow correct signaling through exogenous LILRs in transgenic mice.

A recently proposed mechanism governing the sequential expression of LRC-encoded receptors suggests that NK cell precursors in the bone marrow are initially unresponsive to self MHC, expressing the broadly self-specific LILRB1 and CD94/NKG2A receptors, and later begin expressing different KIRs, which stimulate the proliferation and survival of individual clones to form the repertoire of mature peripheral NK cells (30). Similar results have been shown for peripheral effecter CD8 T cells, which initially express LILRB1 and later show KIR expression in the same clones. These KIR+ cells survive activation-induced cell death and become long-term memory T cells (31).

Although KIRs have been better studied than LILRs with respect to ligands, immune modulation, and developmental regulation, these issues are not well understood in either receptor family. In vivo models would provide a system to allow such problems to be addressed. Previous work generated KIR2DL3 transgenic mice, and used them to investigate KIR function and the possible affects the ligand for this KIR, HLA-Cw3, exerts on KIR expression (32). This study showed that KIRs can be expressed without the need for the presence of their cognate human ligands, MHC class I. The use of the H-2kβ promoter in this transgene conferred an expression pattern that differed markedly from the one observed in humans.

In this study we describe the generation of two lines of LILR transgenic mice, and one line of KIR transgenic mice, using genomic constructs from the LRC. Official KIR and LILR nomenclature is described at http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml.

Materials and Methods

Transgene production

The P1 artificial chromosome (PAC) clones 1060P11 and 52N12 (constructed using the vector pCYPAC2 in the Escherichia coli host strain DH10B) were used for the creation of transgenic mice (Fig. 1) (33). For large scale, genomic DNA-free purification of PAC DNA, cesium chloride gradients and a Qiagen Large Construct kit (Valencia, CA) were used. The microinjection fragments were isolated from the vector by restriction with the restriction enzymes NgoMIV and NorI followed by centrifugation through a 10–25% sodium chloride gradient. Fractions were collected and analyzed by pulse field gel electrophoresis to assess for purity of insert from both vector and E. coli DNA.

Insert DNA was diluted, precipitated with ethanol, then resuspended twice in 0.25× Tris-taurin-EDTA. Before microinjection, DNA was passed through an Elutip minicolumn (Schleicher and Schuell, Keene, NH) following the manufacturer’s instructions. Eluted DNA was precipitated with ethanol then resuspended in 10 mM Tris-HCl, 2 mM EDTA, pH7.4 at a concentration of 5–10 μg/ml and then microinjected (43).

Monoclonal Abs

Anti-CD158a/β-FITC (HP-3E4), anti-CD158b-FITC (CH-1), anti-Ly49A/D-PE, anti-NK1.1-PE, anti-CD19-PE, anti-CD3-PE, DX5-PE, and DX5-Biotin, as well as Streptavidin-PerCP were purchased from BD Pharmingen (San Diego, CA). The anti-FcR mAb 2.4G2 (34) has been previously described. Anti-LILRB1 (HP-F1) was a gift by Dr. M. Colonna and anti-LILRB4 (ZM3.8) was a gift from Dr. M. Lopez-Botet.

Flow cytometry

PBLs, splenocytes, and thymocytes from transgenic and nontransgenic mice were preincubated with mouse serum and mAb to block Fc receptors. KIR/LILR expression on NK, T, and B cells was determined by staining with anti-LILRB1 and later show KIR expression in the same clones. These KIR+ cells survive activation-induced cell death and become long-term memory T cells (31).

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Flow cytometry

PBLs, splenocytes, and thymocytes from transgenic and nontransgenic mice were preincubated with mouse serum and mAb to block Fc receptors. KIR/LILR expression on NK, T, and B cells was determined by staining with anti-CD158a/β (which detects KIR2DL1/2DS1, but as only the latter is present on the PAC used to generate these mice only KIR2DS1 will be detected by this Ab), anti-CD158b (detecting KIR2DL2/2DS2), anti-LILRA1, anti-LILRB1, and anti-LILRB4 in conjunction with anti-NK1.1, anti-CD4, -CD8, -CD19, and DX5. Comparison with Ly49 expression was conducted using anti-Ly49A/D-PE.

Fluorescence in situ hybridization (FISH)

Chromosome spreads were obtained from mouse spleens after culturing in 20 mg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 40 h. The 1060P11 and

FIGURE 1. The LRC contains members of the Ig-SF receptor superfamily including the KIRs and LILRs. The genomic fragments contained in 1060P11 and 52N12, the PAC clones used to create the transgenics, are designated. (■), Pseudogenes.
52N12 transgenes were digoxigenin-labeled using a digoxigenin-nick translation mix (Roche Diagnostics, Mannheim, Germany) and mixed with salmon sperm and mouse cot-1 DNA (Life Technologies, Rockville, MD) before hybridization. Signal was detected using anti-digoxigenin Ab with Cy3-conjugated secondary reagent (Stratech Scientific, Cambridge, UK). Images were captured using a Photometrics CCD camera (Tucson, AZ) on a Zeiss Axioskop microscope (Don Mills, Ontario, Canada). Images were pseudocoloured and merged using SmartCapture software (Vyvis, Chicago, IL).

RT-PCR

Liver and spleen from transgenic and nontransgenic mice were used for RNA preparation using a Qiagen Rneasy kit. RT-PCR was performed on RNA isolated from tissue or sorted NK1.1+ cells using a Superscript One-step RT-PCR kit (Invitrogen, San Diego, CA) or an Omniscript RT kit (Qiagen), respectively. KIR-specific and mouse GAPDH primers were used.

Generation of LILRA1-specific mAb

Analysis of LILRA1 expression in this and in previous studies has been hampered by the lack of a specific Ab. Recombinant LILRA1-Fc fusion protein was produced using a rat myeloma-based expression system that had been established by Drs. Kathryn Armour and Mike Clark (Department of Pathology, University of Cambridge, Cambridge, U.K.), who kindly provided reagents and expertise. Protein (100 μg) was injected s.c. into rats and spleens removed for fusions. Hybridoma supernatants were screened with the LILRA1/Fc Ab. Recombinant LILRA1-Fc fusion protein was purified and used for fusions. Hybridoma supernatants were first screened with the LILRA1/IgG Ag by ELISA. To exclude Abs for the IgG part of the molecule, positive hybridoma supernatants were further tested using a LILRB4/Fc Ag. Finally, Ab specificity was tested on a panel of transfectants. mAb that bound LILRA1 specifically were selected for further studies. A single hybridoma-produced clone, which showed high affinity for LILRA1 and low cross-reactivity with other members of the LILR family, was grown and the purified mAb was used in subsequent experiments.

Results

Generation of KIR and LILR transgenic lines

Transgenic mice were generated using the recombinant constructs indicated in Fig. 1. The PAC 52N12 encodes three functional LILR genes: LILRB1, LILRB4, and LILRA1 in addition to two potential pseudogenes and partial KIR3DL3 and LILRA2 loci. The PAC 1060P11 contains a genomic fragment within which ten KIR genes are encoded, of which one (KIR3DL2) was cleaved during the DNA production process and one is a pseudogene (KIR3DP1/KIRX). Following egg injections with LILR DNA, nine mice were born one of which was transgene positive, whereas of 32 born after KIR DNA injection, nine mice were positive for the transgene and contained varying transgene copy numbers (data not shown). Founders from both sets of transgenics were crossed with CBA/Ca × C57BL/6 F1, nontransgenic mice.

The one LILR founder mouse was found by FISH to have three independent transgene integration sites. These sites were segregated in subsequent generations and led to the establishment of two LILR transgenic lines.

All nine KIR founders were bred and six transmitted the transgene (the rest either did not breed or did not transmit). Of those that transmitted the transgene, one (KIR5) showed KIR expression by flow cytometry (see below), and this line was chosen for further examination and subsequently bred to homozygosity. Two of the founders (KIR3 and KIR9) showed KIR2DL4 expression by RT-PCR (see below).

To determine whether the chromosomal integration site of the transgene was causing repression of transgene expression, KIR transgene integration in the founders that did not show KIR expression was examined by FISH. All examined KIR lines showed a single integration site, which varied in its location in different lines; both long-arm and peritelomeric locations were observed (data not shown).

Analysis of transgene expression

To detect transgene expression, we analyzed Ab binding to cells collected from transgenic animals.

LILR mice. mAbs were available for both LILRB1 and LILRB4 receptors. To examine the LILR expression pattern in the LILR transgenics, we stained spleen cells with LILRB1- or LILRB4-specific Abs, and this confirmed transgene expression in vivo. All mice carrying the transgene showed consistent cell surface expression of LILRB1 on B cells, as ascertained by FACS (Fig. 2A). To determine which cell types express the transgene, splenocytes were costained with Abs against mouse CD19 for B cells and NK1.1 as an NK cell marker. Furthermore, we compared LILRB1 and LILRB4 expression patterns in the transgenic mice to those seen for human PBLs, using Abs against human CD56 for NK cells and CD19 for B cells. These stainings show that almost all CD19+ B cells in transgenic mouse spleen expressed LILRB1 (Fig. 2B), similar to the expression pattern observed in human blood (Fig. 2C). Costaining with LILRB1 and NK1.1 indicated that the majority of NK cells also expressed the transgene (Fig. 2B), in contrast with human peripheral blood NK cells that showed no such expression (Fig. 2C). LILRB4 expression was detected on a high proportion of CD19+ transgenic spleen cells (Fig. 2B), whereas in human blood it is expressed only on a small percentage of B cells (Fig. 2C).

Thymocytes were also isolated from transgenic and nontransgenic littermates. Ab staining confirmed that thymic T cell populations in the transgenics displayed normal CD4:CD8 ratios (data not shown). Thymocytes were then analyzed for the presence of LILRB1 and LILRB4 using appropriate mAbs. LILRB1 was expressed at low levels in thymocytes whereas LILRB4 could not be detected on any thymocyte population (Fig. 2D).

KIR mice. To assess KIR expression in the KIR transgenic founder mice, PBLs were examined for CD158b (KIR2DL2/2DS2) expression, staining with anti-CD158b and DX5 (which reacts with NK and small T cell subpopulations) mAbs (Fig. 3A). Costaining with anti-CD158b and DX5, a CD158b/DX5 population was observed in three of eight founder mice examined (KIR5, -8, -9), suggesting that the transgenes are expressed by NK and T cell subsets in the transgenic mice. Subsequent RT-PCR experiments established that KIR2DL2, but not KIR2DS2, was being expressed in KIR5 mice (see below). Positive staining with the anti-CD158b Ab will therefore be referred to as detecting KIR2DL2.

The KIR5 line was studied in further detail (Fig. 3B): to establish the tissue specificity and type of cells that show KIR expression, PBLs and splenocytes from KIR5 offspring were double-stained with anti-CD158a/h or b in conjunction with anti-CD19 and anti-CD8. Both PBLs and splenocytes showed similar expression patterns and percentages of KIR2DL2 expressing cells, but no significant expression was observed on thymocytes (data not shown). KIR2DL2 expression in the transgenic mice was observed only on DX5+ cells. An apparent KIR2DS1+ population, observed in all mice, was the result of nonspecific binding to mouse B cells (data not shown). No KIR2DS1+ NK or T cell population was observed.

To examine how KIR expression correlates with the expression of the functional homologues of KIR in mice, namely the Ly49 receptors, dual color analysis using anti-CD158b and anti-Ly49A/D was conducted. This showed similar results in both PBLs and splenocytes, in which three distinct populations were seen: KIR2DL2-Ly49A/D, KIR2DL2-Ly49A/D+, and double positive KIR2DL2-Ly49A/D+.

To examine whether T cells constitute a subpopulation of the KIR-DX5+ population in KIR5 mice, costaining of splenocytes...
with anti-CD158b, anti-CD3, and DX5 was performed. This showed a small proportion of DX5<sup>+</sup> KIR2DL2<sup>+</sup> cells to be CD3<sup>+</sup>, indicating that a small percentage of KIR<sup>+</sup> cells are T cells (Fig. 3C).

RT-PCR was conducted on NK1.1<sup>+</sup> spleen cells from KIR5 mice to examine which additional KIRs were being expressed in the transgenic mice (Fig. 4A). Transcripts were detected for KIR2DL2, 2DL4, 3DS1, and 2DL5, but not for KIR3DL3, 2DS2, 2DS5, 2DS1, and 3DL2.

**KIR2DL4 expression variation**

KIR2DL4 is a KIR family member that shares structural features with both activating and inhibitory receptors. It bears a cytoplasmic immunotyrosine-based inhibitory motif, suggesting inhibitory function, and a positively charged amino acid in the transmembrane region, a feature typical of activating KIR (35). KIR2DL4 possesses a unique 14-Kb region upstream of its promoter, and unlike other clonally distributed KIRs, is transcribed by all KIR<sup>+</sup> NK and T cell clones. To assess KIR2DL4 expression in the different KIR founders, we performed RT-PCR on RNA isolated from the liver and spleen of a nontransgenic, KIR2DL2 expressing transgenic founder (KIR9), and a KIR2DL2 nonexpressing transgenic founder (KIR3), using KIR2DL4 RNA primers. Expression of KIR2DL4 was observed at the RNA level in both lines (Fig. 4B). Interestingly, KIR2DL4 RNA levels were much higher in the
mouse that did not express KIR2DL2. Only a low level was present in spleen and liver from the mouse that exhibited high levels of KIR2DL2 expression (KIR9).

In conclusion, KIR expression in KIR transgenic mice was restricted to those cells that normally express the genes in humans. Expression of both inhibitory and activating KIRs was observed, although not all KIRs contained in the 1060P11 PAC clone were expressed.

LILRA1 expression in vivo
Screening of human PBLs with the anti-LILRA1 Ab failed to identify any cell type expressing LILRA1. Similar results were previously observed for LILRA2 (36). Transgenic mice provide an opportunity to study receptor expression in tissue samples that are not readily available from humans. Various tissue sections were prepared from transgenic mice and stained for LILRA1 expression. Using the new Ab, LILRA1 protein was detected in spleen sections, in a ring of cells surrounding B cell areas, and in lymph nodes (Fig. 5). No LILRA1 expression was observed in thymus (data not shown).

Discussion
KIR and LILR transgene expression pattern
In the LILR mice we have examined so far, LILRB1 was expressed at high levels on almost all B and NK cells, while thymocytes expressed LILRB1 at much lower levels. When compared with LILRB1 expression in humans (8, 9, 22), the expression is
expression in a tissue- and cell type-specific pattern in mice. Interestingly, the KIR expression pattern in these transgenic mice appears consistent with the variegated expression pattern observed in humans.

The KIR expressing subset of both PBLs and splenocytes in the transgenic mice was independent of but overlapped with the fraction that expressed Ly49A/D receptors. The presence of a population expressing both human and murine receptors confirms that each receptor locus is expressed in an independent manner that leads to the observed variegated expression pattern. Future experiments examining transgenic KIR expression during mouse ontogeny will shed further light on the expression mechanisms as well as on the effects these two receptor types have upon each other.

Flow cytometry experiments showed that KIR2DL2 expression in KIR transgenic mice is limited to a proportion of NK cells and T cells, consistent with a variegated expression pattern. This expression pattern could stem from transgene position effect variegation (37), although this is unlikely because transgenic mice generated using large constructs (such as Y1 artificial chromosomes or PACs) are less likely to be influenced by transgene position effect variegation (38). The variegation observed in these mice may thus be due to an intrinsic stochastic mechanism that affects cis-regulatory elements and leads to a naturally variegated KIR expression pattern in NK cells.

This indicates that the sequences bringing about this expression pattern are included in the 160-Kb genomic transgene fragment. A similar conclusion can be reached regarding the LILR transgene, which brings about cell-specific expression in the LILR transgenics. These findings are even more significant when considering the fact that these transgenes originate from a different species, i.e., the KIRs and LILRs are expressed despite the fact that their natural ligand is lacking in mice—demonstrating that the presence of human MHC class I is not an essential requirement for KIR or LILR expression.

RT-PCR experiments detected transcripts for both inhibitory and a single activating KIR. A surprising observation was the lack of transcripts for additional activating KIRs, namely KIR2DS1, 2DS2 and 2DS5. It is possible that expression in the KIR gene complex favors those genes proximal to KIR2DL4 although this requires further investigation.

FISH experiments performed on splenocytes from LILR transgenic mice showed LILR transgene insertion sites on three different chromosomes, at different locations upon these chromosomes. All LILR transgenic mice that have been analyzed showed LILRB1 expression on all B cells. Thus, the transgene does not appear to be subject to repressive position effects. It is worth noting that the LILR and KIR gene clusters are naturally located near the telomere on human chromosome 19. Telomeres are heterochromatic and can induce variegated gene repression in yeast, Drosophila, and possibly mammals (39–41). This suggests that for appropriate regulation of LILR and KIR genes there may be a requirement for a powerful chromatin opening function to contend with this potentially repressive chromosomal environment.

The frequency of transgene expression in the KIR mice was lower than in humans, both in heterozygote and homozygote transgenic mice. This may be explained either by the lack of expression-driving enhancer regions or an LCR from the KIR transgene, by the transgene insertion site being close to a heterochromatin-rich region, by the lack of immune challenges during the development of the KIR transgenics, or by a combination of these factors. As large construct-transgenics seem to be less prone to positional effects, the second explanation seems less likely, and in addition to this the high percentages of expression observed from

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**FIGURE 4.** KIR expression in KIR transgenic mice. A, Expression in NK1.1+ cells from KIR5 transgenic mice spleen. RT-PCR was performed on (I) cDNA from transgenic mice (II) positive controls from PCR using cDNA from decidual human NK cells or cloned genes. Lane 1, KIR3DL3 (700 bp); lane 2, KIR2DS2 (290 bp): lane 3, KIR2DL2 (370 bp); lane 4, KIR2DL4 (1100 bp); lane 5, KIR3DS1 (900 bp); lane 6, KIR2DL5 (200 bp); lane 7, KIR2DS5 (330 bp); lane 8, KIR2DS1 (330 bp); lane 9, KIR3DL2 (800 bp). The control lane in this case was negative for KIR2DS5. B, KIR2DL4 expression in nontransgenic, KIR2DL2‘ transgenic founder (KIR3), and KIR2DL2‘ transgenic founder (KIR9) mice. RT-PCR was performed on RNA samples isolated from the liver and spleen of the three mice, using KIR2DL4 and GAPDH primers. KIR2DL4 expression can be seen in both types of transgenic mice.

observed in the expected cell types. LILRB4, which is expressed mainly on human myelomonocytic cells but is also found in B cells at very low levels, was expressed at a relatively low level on most B cells in the transgenic mice (8).

Using spleen section stainings, LILRA1 expression was observed in a ring of cells surrounding B cell areas. Little is known about the cellular expression of the LILRA1 receptor in humans. Additional experiments using our novel Ab should help clarify this and indicate whether these mice will prove an effective model for studying this activating receptor.

In the KIR transgenic mice, KIRs were expressed on NK and T cell subsets in spleen and PBLs, demonstrating that endogenous regulatory elements within the KIR region are sufficient to drive

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**A**

**B**

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**Liver**

- Non-Tg
- KIR3
- KIR9
- Human

**Spleen**

- KIR2DL4
- GAPDH
- No Template

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**KIR2DL4**

- 1Kb

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The KIR expressing subset of both PBLs and splenocytes in the transgenic mice was independent of but overlapped with the fraction that expressed Ly49A/D receptors. The presence of a population expressing both human and murine receptors confirms that each receptor locus is expressed in an independent manner that leads to the observed variegated expression pattern. Future experiments examining transgenic KIR expression during mouse ontogeny will shed further light on the expression mechanisms as well as on the effects these two receptor types have upon each other.

Flow cytometry experiments showed that KIR2DL2 expression in KIR transgenic mice is limited to a proportion of NK cells and T cells, consistent with a variegated expression pattern. This expression pattern could stem from transgene position effect variegation (37), although this is unlikely because transgenic mice generated using large constructs (such as Y1 artificial chromosomes or PACs) are less likely to be influenced by transgene position effect variegation (38). The variegation observed in these mice may thus be due to an intrinsic stochastic mechanism that affects cis-regulatory elements and leads to a naturally variegated KIR expression pattern in NK cells.

This indicates that the sequences bringing about this expression pattern are included in the 160-Kb genomic transgene fragment. A similar conclusion can be reached regarding the LILR transgene, which brings about cell-specific expression in the LILR transgenics. These findings are even more significant when considering the fact that these transgenes originate from a different species, i.e., the KIRs and LILRs are expressed despite the fact that their natural ligand is lacking in mice—demonstrating that the presence of human MHC class I is not an essential requirement for KIR or LILR expression.

RT-PCR experiments detected transcripts for both inhibitory and a single activating KIR. A surprising observation was the lack of transcripts for additional activating KIRs, namely KIR2DS1, 2DS2 and 2DS5. It is possible that expression in the KIR gene complex favors those genes proximal to KIR2DL4 although this requires further investigation.

FISH experiments performed on splenocytes from LILR transgenic mice showed LILR transgene insertion sites on three different chromosomes, at different locations upon these chromosomes (in the long arm, located close to the telomere, or close to the centromere). All LILR transgenic mice that have been analyzed showed LILRB1 expression on all B cells. Thus, the transgene does not appear to be subject to repressive position effects. It is worth noting that the LILR and KIR gene clusters are naturally located near the telomere on human chromosome 19. Telomeres are heterochromatic and can induce variegated gene repression in yeast, Drosophila, and possibly mammals (39–41). This suggests that for appropriate regulation of LILR and KIR genes there may be a requirement for a powerful chromatin opening function to contend with this potentially repressive chromosomal environment.

The frequency of transgene expression in the KIR mice was lower than in humans, both in heterozygote and homozygote transgenic mice. This may be explained either by the lack of expression-driving enhancer regions or an LCR from the KIR transgene, by the transgene insertion site being close to a heterochromatin-rich region, by the lack of immune challenges during the development of the KIR transgenics, or by a combination of these factors. As large construct-transgenics seem to be less prone to positional effects, the second explanation seems less likely, and in addition to this the high percentages of expression observed from...
all three transgene insertion sites in the LILR mice point to the first explanation. Thus, the existence of a putative LCR in the 52N12 construct region but not in the 1060P11 region may provide a simple explanation for the differences between the observed expression frequencies. In addition, the FISH experiments performed on the nonexpressing KIR transgenic founders showed varying single integration sites in these mice, of both heterochromatic and nonheterochromatic nature. This finding would indicate that the reduced expression percentage is less likely to be directly caused by a heterochromatinous integration site, and points toward the importance of an LCR in pushing expression to normal frequencies.

An alternative factor that could be relevant to the expression frequency observed is the lack of MHC class I, the natural ligand to the KIRs. Theoretically, this could cause a disturbance in the cellular education process of KIR-positive cells, by not allowing KIR\(^+\) cell proliferation, which may be brought about by the MHC class I-KIR interaction. Cambiaggi et al. (32) generated KIR2DL3 × HLA-Cw3 double transgenic mice, and their results showed no effect on KIR expression by the presence of its HLA ligand. As mentioned before, this model exhibited a significant overexpression of KIR on all lymphocytes. We intend to examine the possible effect of MHC class I on KIR expression in our transgenic model that more closely mimics the human situation by crossing the KIR transgenics with HLA transgenics.

Although KIR2DL2 expression was observed only in three of the nine transgenic founders that were created, RT-PCR results showed that KIR2DL4 is expressed both in one of the founder mice that does show KIR2DL2 expression, as well as on a mouse that does not express these KIRs. This suggests that flanking this gene are genetic elements that may allow KIR2DL4 to be expressed even if the entire transgene has integrated into a heterochromatic region that prevents expression of, at least, KIR2DL2. The region 14 Kb upstream of KIR2DL4 is unique, and the receptor has been shown to be expressed in all KIR\(^+\) human NK and T cell clones. This, and the fact that KIR2DL4 is one of the three "framework" KIR genes present in all haplotypes observed in humans and chimpanzees (42), points to an important conserved function.

In summary, the pattern of KIR and LILR expression we have observed in the transgenic mice indicates that the genomic constructs containing these gene families are sufficient to drive an expression pattern similar to the one seen in humans for at least

**FIGURE 5.** LILRA1 expression in LILR transgenic mice: tissue sections from LILR transgenics and nontransgenic littermate were stained with LILRA1 specific mAb 3/70.5. Spleen sections show a ring of positive stained cells in the transgenic animal (D) but not in the control animal (A) or with the isotype control (B and E). The stained cells form a ring or sheath apparently around B cell areas stained with CD19 (C and F), which may indicate that LILRA1 is expressed on tissue macrophages. A different LILRA1-specific mAb, 3/17.5, was tested to confirm these results (G). Some cells in lymph nodes do show LILRA1 expression (H), which is not seen using an isotype control (I).
part of the KIR genes contained on the 1060P11 PAC clone. The transgene expression pattern of those genes that were expressed seems to be encoded within the DNA sequences and is independent of the presence of the ligands for these receptor families. The KIR receptor family also seems to possess an inherent ability for variated expression.

The availability of these mics permits the developmental expression of LiLRs and KIRs to be studied, the ways in which their expression is regulated in response to infections, as well as their in vivo effects on disease susceptibility.

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References