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NK cell expression of the killer cell lectin-like receptor G1 (KLRG1), the mouse homolog of MAFA, is modulated by MHC class I molecules

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Using a new mAb, 2F1, we characterize a mouse natural killer (NK) cell antigen termed 'killer cell lectin-like receptor G1' (KLRG1; formerly mouse MAFA or 2F1-Ag). KLRG1 is expressed on 30–60 % of murine NK cells, and a small fraction of T cells, and is composed of a homodimer of glycosylated 30–38-kDa subunits. Strikingly, cell surface expression of KLRG1 by NK cells was substantially down-regulated in mice deficient for expression of class I molecules, in contrast to the Ly49 lectin-like NK receptors, which are up-regulated in class I-deficient mice. We could not demonstrate binding of KLRG1 to class I molecules in a cell-cell adhesion assay. Transgenic expression of KLRG1 under heterologous transcription elements was unaffected by class I deficiency, indicating that class I molecules do not affect the KLRG1 protein directly, and suggesting that regulation is at the level of expression of the endogenous KLRG1 gene. Evidence is presented that class I molecules regulate KLRG1 via interactions with class I-specific inhibitory Ly49 molecules and SHP-1 signaling. Thus, although KLRG1 and Ly49 molecules are both lectin-like inhibitory receptors that are regulated by class I expression, the effects of class I on the cell surface expression of the molecules are opposing, and the underlying regulatory mechanisms are distinct.

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1 Introduction

NK cell functions are regulated by the interplay of activating and inhibitory receptors [1]. The receptors defined to date are either members of the Ig superfamily, or are members of the lectin-like type 2 transmembrane receptor family, with inhibitory and activating receptors represented in each group. The inhibitory isoforms contain a characteristic immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains, which interacts with the SH2 domains of protein phosphatases such as SHP-1 [2, 3]. In contrast, the activating isoforms lack ITIM, and interact with signaling subunits such as KARAP/DAP12 [4, 5].

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Abbreviations: KLRG1: Killer cell lectin-like receptor G1 **MAFA:** Mast cell function-associated antigen **ITIM:** Immunoreceptor tyrosine-based inhibitory motif **A-LAK:** Adherent lymphokine-activated killer cells

Many if not all of the lectin-like receptors in the mouse are encoded in the NK gene complex, located on mouse chromosome 6 [6]. Among these are the NKR-P1 and Ly49 receptors, which exist as homodimers, and the CD94/NKG2 receptors, which are believed to function only as heterodimers [7]. The NKR-P1 receptors include at least three members, two of which are stimulatory, whose natural ligands are unknown. The Ly49 receptors interact with classical class I MHC molecules, and include at least ten members, eight of which contain ITIM, and two of which are thought to be stimulatory receptors [6, 8]. The CD94/NKG2A heterodimer is an inhibitory receptor that binds to a nonclassical class I molecule, Qa-1 in mice, presenting peptides derived from the signal peptides of many classical class I molecules [9]. The capacity of inhibitory isoforms of Ly49 and CD94/NKG2 receptors to respond to class I molecules is believed to account for the insensitivity of most normal class I-expressing cells to NK cells, and conversely, for the sensitivity of many class I-deficient target cells. The biological function of the activating isoforms of these receptors remains uncertain.

Other lectin-like receptors of unknown function have been described. A lectin receptor called mast cell

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function-associated antigen (MAFA) was identified on a rat basophilic leukemia cell line, RBL-2H3 [10]. Co-aggregation of the FcεR on RBL-2H3 cells with antibodies specific for rat MAFA was shown to inhibit granule exocytosis by the cells. The human homolog of MAFA is expressed, at least at the RNA level, by NK cells as well as by basophils, and its gene is located in the human NK cell complex on chromosome 12 [11]. Interestingly, the mouse homolog of rat MAFA is not detectably expressed by mast cells, suggesting that the MAFA nomenclature is misleading, at least in mice [12]. Recently, the Human Gene Nomenclature Database (http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/searchgenes.pl) has assigned the name 'killer cell lectin-like receptor G1' (KLRG1) to the gene. Here we adopt this new generic and standardized nomenclature.

In this report, we characterize KLRG1 expression using a monoclonal antibody (mAb) 2F1, specific for mouse KLRG1. Significantly, we demonstrate that KLRG1 expression is up-regulated by expression of MHC class I molecules. Evidence is presented that the effect of MHC class I is indirect, and can be mediated by interactions with class I-specific Ly49 inhibitory receptors. Furthermore, KLRG1 expression is diminished in mice with a loss of function mutation in SHP-1. The data suggest that KLRG1 expression is modulated by SHP-1 signaling downstream of class I specific Ly49 inhibitory receptors.

2 Results

2.1 mAb 2F1 recognizes a subset of NK cells

Popliteal lymph node cells from Syrian hamsters immunized with IL-2-activated NK cells (A-LAK cells) from B6 mice were fused with P3X63Ag8.653 myeloma cells. Hybridoma supernatants were screened for specific reactivity with A-LAK cells and freshly isolated NK1.1$^+$ cells from the spleen. One such hybridoma, designated 2F1, was characterized further. 2F1 mAb stained predominantly NK1.1$^+$ cells in the spleen, reacting with approximately 40 % of these cells in B6 mice (Fig. 1 A). Similarly, approximately 30–40 % of conventional NK cells from B6 mice, defined as NK1.1$^+$CD3$^-$Ig$^-$ cells, stained with 2F1 mAb (Fig. 1 B, F). A somewhat higher fraction of A-LAK cells stained with 2F1 mAb (Fig. 1 C). 2F1 mAb stained a subset of NK cells in all mouse strains tested, including B6, BALB/c, 129/J, C3H.SW, AKR/J and SJL (data not shown).

In further tests of cell specificity, we observed that 2F1 also reacted with a small fraction (approx. 1 %) of conventional T cells (Fig. 1 D) including CD4 as well as CD8

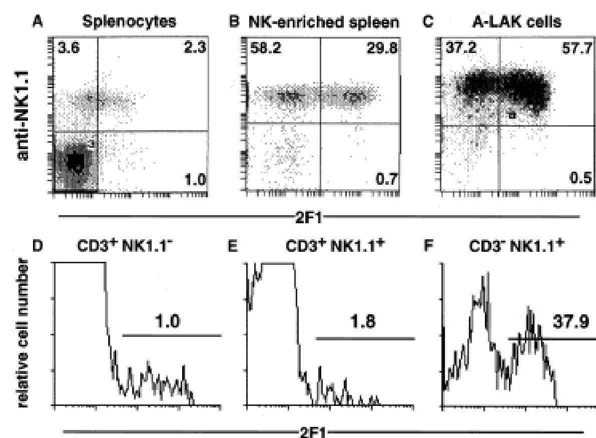

Fig. 1. Expression of KLRG1 on NK cells. Unseparated splenocytes (A), NK-enriched splenocytes (gated Ig$^-$ and TCR$^-$ cells) (B) and IL-2-activated NK cells (A-LAK cells) (C) were stained with 2F1 mAb. The unseparated spleen cells and A-LAK cells were stained in a two-step procedure and the NK-enriched spleen cells were stained with the three-color procedure A (see Sect. 4.5). (D–F) Nylon wool-nonadherent spleen cells were stained with 2F1 mAb, anti-CD3 and anti-NK1.1 according to procedure C (see Sect. 4.5). Numbers indicate representative percentages of KLRG1$^+$ cells among the indicated subpopulations based on comparison with an isotype-matched control mAb.

cells (data not shown). It also stained a small fraction (1–2 %, Fig. 1 E) of NK1.1$^+$ T cells weakly, but did not react with freshly isolated B cells (data not shown). The 2F1 mAb also failed to react with the large majority of Con A-activated T cells or LPS-activated B cells (data not shown). Furthermore, 2F1 mAb did not stain any of the cell lines tested, including EL-4, R8.15, C1498, S49.1, Yac-1, R1.1, R1E, P815, P388D1, or 7.17.A2 (data not shown). Included in this panel are T and B lymphomas, a dendritic epidermal $\gamma\delta$ T cell line, a macrophage cell line and a mastocytoma cell line. The failure of mast cells to react with the antibody was confirmed by analyses of *ex vivo* mast cell populations [12]. Thus, 2F1 mAb reacts with a substantial fraction of NK cells, and a small fraction of T cells, but does not react with the other lymphohematopoietic cells tested.

2.2 Subunit structure of the KLRG1

The antigen recognized by 2F1 mAb was immunoprecipitated from lysates prepared from A-LAK cells that had been subjected to surface ^{125}iodination, and analyzed by SDS-PAGE. Under nonreducing conditions, we observed a diffuse band of Mr 66–77 kDa compared to control immunoprecipitates with irrelevant hamster mAb

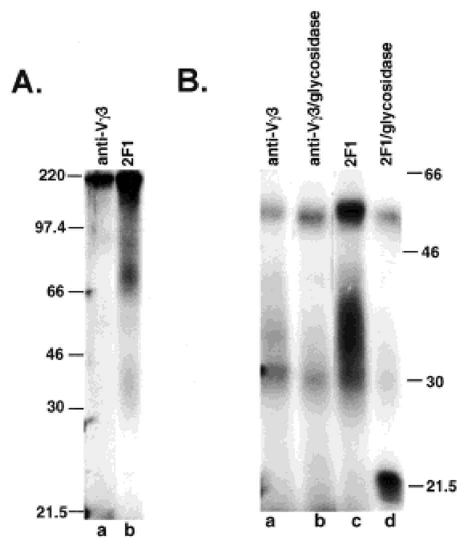

Fig. 2. Immunoprecipitation of KLRG1. Nonreducing (A) and reducing (B) SDS-PAGE of KLRG1 immunoprecipitated from ^{125}I-labeled A-LAK cells. The hamster mAb F536 (anti-Vγ3) was employed as a control. The immunoprecipitates were analyzed directly or after treatment with a mixture of endoF and N-glycosidase F, as indicated. The migration of molecular weight standards is indicated (in kDa).

as well as a fainter diffuse band of 30–40 kDa (Fig. 2 A). Under reducing conditions, a single specific diffuse band was evident, migrating with an M_r of 30 to 38 kDa (Fig. 2 B). The diffuseness of the band is probably due to heterogenous glycosylation of the protein since most of the reduced antigen migrated as a sharp 22-kDa band after removal of N-linked oligosaccharides with a mixture of N-glycosidase F and endoF (Fig. 2 B). The significant reduction in M_r as a result of glycosidase treatment suggests that the antigen recognized by 2F1 mAb is N-glycosylated at multiple sites. These observations suggest that the 2F1-antigen is expressed primarily as a disulfide-linked dimer composed of N-glycosylated subunits with a core molecular mass of 22 kDa.

As reported elsewhere, an expression cloning procedure was used to isolate a cDNA encoding the 2F1 antigen, now referred to as KLRG1 [12]. The nucelotide sequence predicts a protein with a core molecular mass of 21.3 kDa, with two N-glycosylation sites, consistent with the immunoprecipitation analysis of the 2F1 antigen from A-LAK cells. The protein exhibited 80 % identity to the rat MAFA at the amino acid level, including a conserved SIYSTL sequence in the putative cytoplasmic domain that may function as an ITIM. Importantly, a comparison of the immunoprecipitation results in Fig. 2 with those for rat MAFA [13] indicates that the two molecules have a very similar subunit structure. Collectively, these data indicate that the cloned cDNA encodes the mouse homolog of rat MAFA, and is the antigen recognized by the 2F1 mAb.

2.3 MHC class I expression impacts expression of KLRG1

The cell surface expression of class I-specific inhibitory Ly49 receptors on NK cell is partially down-regulated in mice that express the class I ligand for the receptor. As a consequence, Ly49 receptor levels are usually higher on NK cells from class I-deficient mice than on NK cells from class I$^+$ mice. Furthermore, the frequency of NK cells expressing a given Ly49 receptor is usually somewhat higher in class I-deficient mice than in class I$^+$ mice [14]. Since KLRG1 is a lectin-like receptor related to the Ly49 molecules, we examined the expression of KLRG1 in class I-deficient mice. We compared class I$^+$ B6, B10.D2 and B10.BR mice to corresponding mice that were class I-deficient because they harbored a homozygous mutation of the β2 microglobulin (β2m) gene. NK enriched spleen cells were prepared from nylon wool-nonadherent spleen cells by complement depletion of B cells and CD4$^+$ and CD8$^+$ T cells. The enriched cells were stained with 2F1 mAb, anti-CD3 and anti-mouse Ig antibodies. Gating on CD3$^-$Ig$^-$ cells yielded a population that was $>$90 % NK1.1$^+$ (data not shown). Surprisingly, KLRG1 expression was substantially down-regulated in class I deficient (β2m$^{-/-}$) mice, a pattern opposite to that seen with inhibitory Ly49 receptors. In B6-β2m$^{-/-}$ mice and B6-H-2^d-β2m$^{-/-}$ mice we observed an approximately threefold reduction in the frequency of KLRG1$^+$ NK cells compared to class I$^+$ control mice (Fig. 3 A), and a significant reduction in the mean fluorescence intensity (MFI) of the remaining positive cells (Fig. 3 B). In B10.BR-β2m$^{-/-}$ mice, we observed a more modest, though significant, reduction in the frequency of KLRG1$^+$ cells, and also a small reduction in the MFI of the positive cells. We also observed a reduction in the frequency of KLRG1-positive NK cells from mice with homozygous mutations in both the K^b and D^b genes, implicating classical class I molecules in regulating KLRG1 expression (Fig. 3 C). Mice deficient for the TAP-1 peptide transporter also exhibited reduced expression of KLRG1 (data not shown). There was no effect of class II MHC deficiency on expression of KLRG1 on NK cells (Fig. 3 C). These results suggests that deficiency in classical class I molecules results in a significant reduction in the number of KLRG1$^+$ NK cells and a lower level of cell surface expression of KLRG1.

To investigate the effects of allelic differences in MHC expression on KLRG1 expression, several MHC congenics on the B6/B10 genetic background were analyzed. NK-enriched spleen cells were prepared as before and

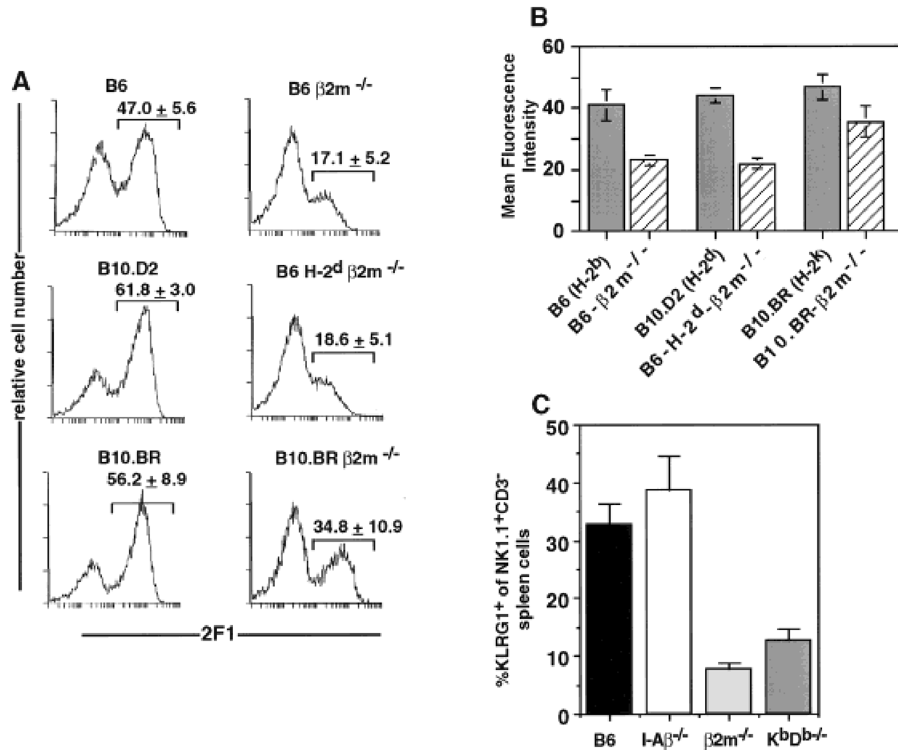

Fig. 3. Reduced expression of KLRG1 in class I-deficient mice. (A) The percentage of KLRG1⁺ NK cells was examined from mice of three MHC haplotypes (B6, B10.D2 and B10.BR in panels on left) and compared with $\beta2m^{-/-}$ mice with the same MHC genotypes (B6-$\beta2m^{-/-}$, B6-H-2^d-$\beta2m^{-/-}$ and B10.BR-$\beta2m^{-/-}$, in panels on right). The numbers represent the mean of four independent determinations ± SD. Splenocytes were enriched for NK cells by nylon wool passage and complement-mediated T cell depletion, and gated $CD3^-$ Ig^- populations were analyzed using the procedure B three-color analysis. Essentially identical results were obtained when gated $NK1.1^+$ cells were analyzed. (B) The mean fluorescence intensity ± SD of positive staining by mAb 2F1 on NK cells is depicted for the same mice as in panel A. (C) Nylon wool-nonadherent spleen cells from normal B6 mice or B6 mice harboring homozygous mutations in I-Aβ (I-$A\beta^{-/-}$), β2m ($\beta2m^{-/-}$) or K^b and D^b ($K^bD^{b-/-}$) genes were stained with 2F1 according to procedure C. Means ± SD of four animals are shown.

stained with 2F1 mAb, anti-NK1.1, anti-CD3 and anti-mouse Ig antibodies. Gating on $NK1.1^+CD3^-Ig^-$ cells, we observed that 35–55 % of NK cells expressed KLRG1 (Fig. 4). Some variation in the frequency of $KLRG1^+$ cells was observed, with a marginally higher frequency in H-2^d, H-2^k, H-2^f and H-2^r mice compared to H-2^b, H-2^q and H-2^s mice.

2.4 Class I deficiency does not affect transgenically expressed KLRG1

The higher expression of KLRG1 observed in the presence of MHC class I could be due to a direct stabilizing effect of class I molecules on KLRG1 proteins. Alternatively, class I might influence KLRG1 gene expression. To distinguish these possibilities, transgenic mice were generated that expressed the KLRG1 cDNA under the

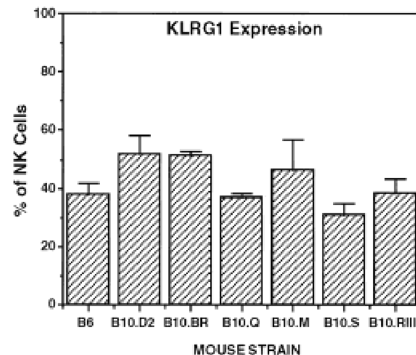

Fig. 4. Frequency of $KLRG1^+$ NK cells in MHC-congenic mice. Enriched NK cell populations, gated for $NK1.1^+$ TCR^- Ig^- cells (three-color procedure A), were analyzed for expression of KLRG1. The data represent the mean of three determinations ± SD.

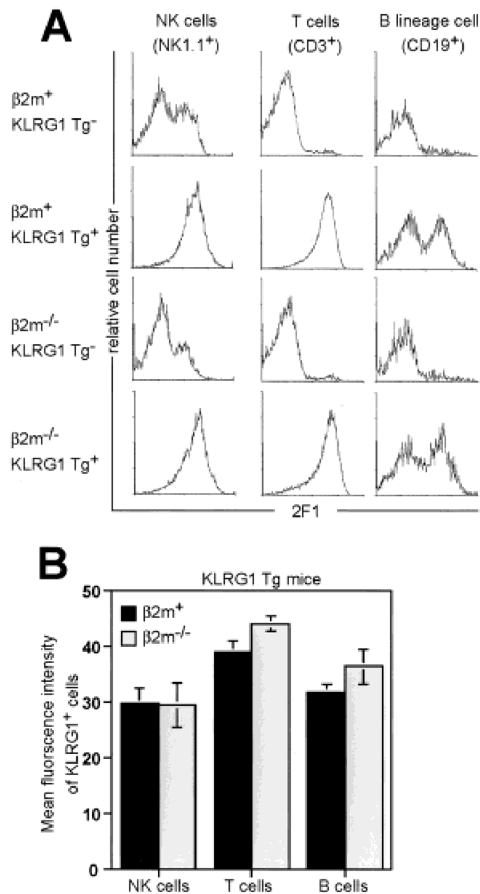

Fig. 5. The surface level of KLRG1 in KLRG1-transgenic mice is not altered by β2m deficiency. (A) Nylon wool-nonadherent splenocytes from 4-week-old mice of the indicated genotypes were stained for KLRG1 expression and representative mice are shown. 2F1-biotin was utilized in all cases, followed by streptavidin-PE (for expression on NK cells) or streptavidin-TRICOLOR (for expression on B and T cells). (B) The mean fluorescence intensity (MFI) of KLRG1 expression on KLRG1+ cells from KLRG1-transgenic mice is shown (mean ± SD, n = 3). Since a different secondary reagent was used for NK cells and B/T cells, MFI should not be compared across cell types.

control of the K^b promoter and the Ig enhancer [15]. These mice express KLRG1 on virtually all NK and T cells, and on a subset of B lineage cells (Fig. 5 A). The KLRG1 transgene was crossed onto a class I-deficient B6-$\beta 2m^{-/-}$ background, and the cell surface levels of KLRG1 in transgenic $\beta 2m^+$ and transgenic $\beta 2m^-$ mice were compared. In contrast to the results with endogenous KLRG1 in nontransgenic mice, we found that the cell surface levels of transgenically expressed KLRG1 were unaffected by class I deficiency (Fig. 5 A, B). This finding suggests that the expression of KLRG1 is not stabilized by a direct KLRG1-class I interaction, and that the effect of class I molecules is not at the level of KLRG1 protein. The data therefore support the alternative proposal that class I expression results in enhanced expression of the endogenous KLRG1 gene (see Discussion).

2.5 Failure to detect binding of KLRG1 to class I+ cells

As another means to address whether KLRG1 interacts directly with class I molecules, we utilized an adhesion assay in which class I+ cells were tested for adherence to COS cells that overexpress the receptor cDNA ([16, 17]). This assay readily detects binding of each inhibitory Ly49 receptor to its functionally relevant class I ligands [17], *e.g.* binding of Ly49C to $H\text{-}2^{b,d,k,f,q,r,s}$ molecules (Fig. 6). The mean fluorescence intensity of KLRG1 expression on the transfected COS cells was higher than that on NK cells, judging by staining with 2F1 mAb. We observed no binding of KLRG1-transfected COS cells to any cells tested, including lymphoblasts from seven different MHC congenic strains (Fig. 6) and several tumor cell lines including RMA, R8.15, MBL-2, C1498, Yac-1, R1.1 and P815 (data not shown). The absence of binding, while not definitive, argues that KLRG1 does not bind to class I molecules.

2.6 KLRG1 expression is influenced by inhibitory Ly49 receptors

The effects of MHC expression on the percentage of KLRG1+ NK cells could reflect an effect of signaling through class I-specific receptors. As one means to

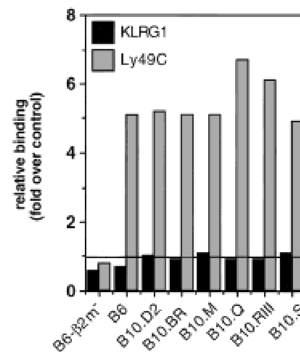

Fig. 6. Failure of KLRG1 to bind different MHC I molecules. ^{3}H-labeled Con A blasts from the indicated MHC-disparate and congenic strains were adhered to KLRG1-, Ly49C- or mock-transfected COS-7 cells. Relative binding over control was determined as "cpm KLRG1- or Ly49C-transfectants/ cpm mock transfectants". One representative of two independent experiments is shown.

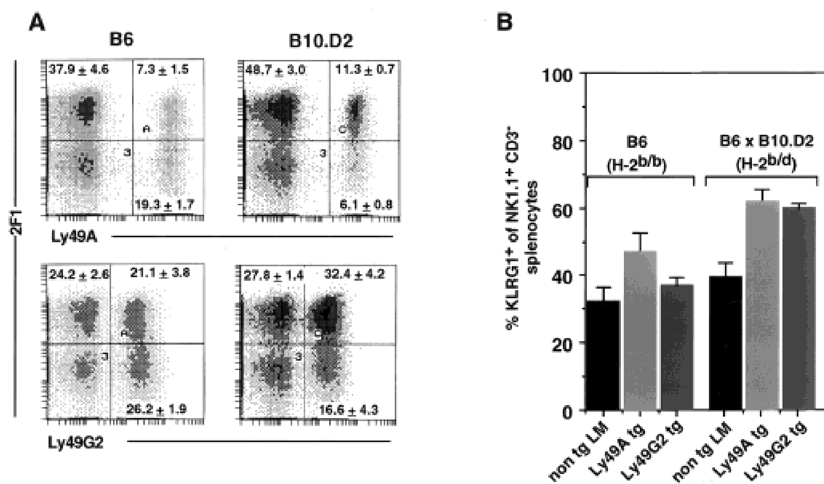

Fig. 7. Influence of Ly49 receptor expression on KLRG1 expression. (A) Enriched NK cell populations from B6 or B10.D2 mice were gated on $CD3^{-}Ig^{-}$ cells and analyzed for co-expression or KLRG1 and Ly49A or Ly49G2 as indicated (procedure B three color analysis). Greater than 90 % of the cells stained with anti-NK1.1 mAb in a parallel analysis (not shown). The numbers in the quadrants represent the means (± SD) of four determinations. (B) Increased expression of KLRG1 in mice constitutively engaging MHC with inhibitory Ly49 receptors. KLRG1 expression was determined by 2F1-FITC staining of gated $NK1.1^{+}$ $CD3^{-}$ cells from nylon wool-nonadherent spleen cells.

address the role of inhibitory class I-specific receptors, we determined whether KLRG1 expression correlated with expression of specific Ly49 inhibitory receptors. When co-staining with 2F1 mAb and antibodies against different Ly49 receptors, significant percentages of cells were present in all four quadrants of the staining analysis, indicating no exact correlation between the expression of the Ly49 receptor and expression of KLRG1 (Fig. 7 A, data not shown). However, it appeared that KLRG1 expression was higher as a percentage of $Ly49A^{+}$ or $Ly49G2^{+}$ NK cells in mice that expressed strong ligands for these receptors (B10.D2 mice) than in mice that did not (B6 mice). Thus, of $Ly49A^{+}$ NK cells, 65 % were $KLRG1^{+}$ in B10.D2 mice, but only 27 % were $KLRG1^{+}$ in B6 mice. Of $Ly49G2^{+}$ NK cells, 66 % were $KLRG1^{+}$ in B10.D2 mice, compared to 45 % in B6 mice. These differences raised the possibility that signaling by Ly49A and Ly49G2 augments KLRG1 expression in NK cells.

The effect of Ly49 expression on KLRG1 expression was confirmed by examination of two lines of Ly49-transgenic mice. In these mice, a cDNA encoding Ly49A [18, 19] or Ly49G2 [17] is expressed in all NK cells under heterologous control elements. Each of these transgenes was crossed into $H\text{-}2^{b/d}$ ((B10.D2 × B6)F1) mice to provide a strong class I ligand (D^{d}) for the receptors and into $H\text{-}2^{b/b}$ (B6) mice, which do not express a strong ligand for either receptor [6, 17]. In mice expressing a strong ligand for these receptors ($H\text{-}2^{b/d}$ mice) both transgenes resulted in a significant increase in the percentage of NK cells that expressed KLRG1, from approximately 40 % in nontransgenic mice, to approximately 60 % in each of the transgenic lines (Fig. 7 B). In $H\text{-}2^{b/b}$ mice, which lack a strong ligand for these receptors, the Ly49A transgene had a smaller effect and the Ly49G2 transgene had no significant effect on KLRG1 expression. These data confirm that expression of the Ly49A and Ly49G2 inhibitory receptors results in an elevated percentage of $KLRG1^{+}$ NK cells, especially in the presence of strong ligands for these receptors.

2.7 Down-regulation of KLRG1 in NK cells defective in class I receptor signaling

The above data raise the possibility that the effect of class I on KLRG1 expression is indirect. It appears that signals originating from the Ly49 class I specific receptors can influence KLRG1 expression. It was therefore of interest to examine KLRG1 expression in NK cells that harbor a mutation that prevents inhibitory signaling. SHP-1 is the protein tyrosine phosphatase that interacts with the ITIM in most, if not all, class I-specific inhibitory receptors, and is necessary for normal inhibitory signaling. The mutant mouse strain motheaten-viable (me^{v}/me^{v}) harbors a partial loss of function mutation in SHP-1, reducing SHP-1 activity by 80–90 % and resulting in a profoundly reduced capacity to mediate inhibitory signaling through class I-specific inhibitory receptors [3].

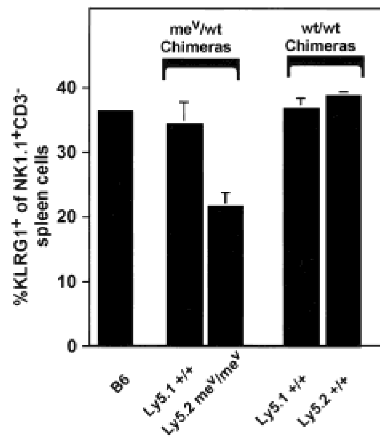

Fig. 8. Reduced percentage of KLRG1$^+$ cells among NK cells with a SHP-1 mutation (from me^v/me^v mice). A mixture of bone marrow cells from me^v/me^v (Ly5.2$^+$) mice and wild-type (Ly5.1$^+$) congenic mice was used to repopulate irradiated wild-type mice (me^v/wt chimeras). As a control, chimeras were prepared with a mixture of Ly5.1 and Ly5.2 bone marrow cells, both from wild-type mice (wt/wt chimeras). After 13 weeks, gated NK1.1$^+$CD3$^-$ cells were examined for expression of KLRG1 and Ly5.1 or Ly5.2. The bars indicate the average percentage ± SE of NK cells from each bone marrow source (Ly5.2 or Ly5.1) expressing KLRG1, from three chimeras of each type. Also shown are data from an age-matched unirradiated B6 mouse analyzed in parallel. Similar results were observed in an additional independent experiment.

Motheaten mice exhibit multiple hematopoietic disorders, including an aberrant activation of monocytes, which result in premature death at approximately 8–10 weeks of age.

To compare KLRG1 expression in me^v/me^v and wild-type NK cells maturing in a normal hematopoietic environment, we prepared irradiation chimeras in which a mixture of me^v/me^v and wild-type bone marrow cells were used to repopulate lethally irradiated normal mice. These mixed chimeras do not exhibit the characteristic motheaten syndrome [20]. The bone marrow cells differed in expression of Ly5 alleles in order to separately track cells of each type in the repopulated mice. After 13 weeks, we analyzed the NK cells by flow cytometry. Wild-type NK cells in the chimeras contained a normal percentage of KLRG1$^+$ cells, but me^v/me^v NK cells contained a significantly reduced percentage of KLRG1$^+$ cells (Fig. 8). These data indicate that high levels of KLRG1 expression are dependent on normal expression of the SHP-1 phosphatase by KLRG1$^+$ NK cells.

3 Discussion

Mouse KLRG1 is a dimeric receptor composed of two glycosylated subunits of approximately 30–38 kDa. The nucleotide sequence of the cDNA predicts a protein with a core molecular weight of 21.3 kDa, with two sites for N-glycosylation, in agreement with the M_r of the deglycosylated protein (22 kDa). KLRG1 exhibited a similar M_r to rat MAFA [13] before and after deglycosylation. The immunoprecipitation data are consistent with most KLRG1 being homodimeric, in line with the observation that COS7 cells require transfection only with the cloned KLRG1 cDNA to obtain ample cell surface expression.

As shown here, KLRG1 is expressed by a substantial subset of NK cells in mice, with few other hematopoietic cells expressing the antigen. Recent studies have shown that the human KLRG1/MAFA homolog is also expressed by NK cells [11]. Surprisingly, KLRG1 is not expressed by peritoneal or cultured bone marrow mast cells [12]. In contrast, evidence suggests that both rat and human KLRG1/MAFA are expressed by mast cells [11, 21]. The expression of KLRG1 on NK cells and some T cells but not mast cells suggests that a major function of the protein is in NK and/or T cells, at least in mice.

Interestingly, KLRG1 could be detected on only a very small fraction of NK1.1$^+$ T cells. Therefore, KLRG1 may mediate developmental processes and/or effector functions that are crucial for conventional NK cells, but not for NK1.1$^+$ T cells. The expression of KLRG1 on a small subpopulation of conventional T cells is reminiscent of the expression of other lectin-like NK cell receptors (*e.g.* Ly49 molecules) which can be expressed on a fraction of CD8 T cells [35]. It is also in line with recent findings that KLRG1 mRNA expression is induced in conventional CD8 T cells after LCMV infection *in vivo* [22].

In the rat basophilic leukemia cell line RBL-2H3, coligation of the FcεR with rat MAFA was shown to inhibit granule release compared to ligation of the FcεR by itself [10]. These data suggest that MAFA/KLRG1 functions as an inhibitory receptor. Thus far, we have been unable to discern effects of blocking KLRG1 with 2F1 mAb on NK cell functions *in vitro*. Lysis of both tumor cell targets and Con A blasts by NK cells was unaffected by the antibody (data not shown). Nor did we observe differences in the cytolytic capacity of sorted KLRG1$^+$ and KLRG1$^-$ NK cells. It is possible that KLRG1 is involved in other NK cell functions, or in interactions with molecules selectively expressed by certain target cells that were not tested in our assays. Alternatively, the antibody may fail to prevent ligand engagement by the receptor. Further studies will therefore be necessary to determine the biological role of the molecule.

It is striking that high cell surface expression of endogenous KLRG1 depends on class I expression. Ly49 receptor cell surface expression follows the opposite pattern, with higher levels in class I-deficient mice than in normal mice [14, 23, 24]. It was significant that mice deficient for expression of K^b and D^b also exhibited down-regulation of KLRG1, since it implicated the classical class I molecules in the process, as opposed to most nonclassical class I molecules. The only nonclassical class I molecule that should be affected in the K^b/D^b mutant mice is Qa-1^b, since it is known to present a D^b-derived leader peptide to its counter-receptor CD49/NKG2A [9, 25]. Despite the effects of class I expression on KLRG1 expression, the cell adhesion assay with Con A blasts revealed no binding of KLRG1 to classical class I molecules. No did we detected binging of KLRG1-transfected cells to several tumor cell lines we tested, including RMA, R8.15, MBL-2, C1498, Yac-1, R1.1, and P815. The cell-cell adhesion assay is sufficiently sensitive to detect binding of inhibitory Ly49 receptors to the functionally relevant class I ligand in almost every combination tested. Although we cannot exclude that KLRG1 binds class I molecules weakly, below the affinity threshold of the assay, it seems likely that KLRG1 is regulated by class I via an indirect mechanism.

The analysis of KLRG1 transgenic mice shed some light on the mechanism by which KLRG1 is regulated by MHC class I. In these mice, KLRG1 cDNA is under heterologous transcriptional control elements and is expressed on virtually all NK and T cells, and on some B cells. In contrast to endogenous KLRG1, we found that the absence of class I had no effect on the cell surface levels of transgenic KLRG1. By extension, this data suggests that regulation of endogenous KLRG1 by class I molecules is not at the level of the KLRG1 protein, and suggests instead that regulation occurs at the level of KLRG1 gene expression. A likely possibility is that class I expression indirectly enhances the transcription of KLRG1 in NK cells. An alternative possibility is that class I expression regulates the stability or translation of KLRG1 mRNA, via mediators that target regions of the transcript that are absent in the KLRG1 cDNA fragment incorporated in the transgene. The transgene incorporated 22 bp of 5' untranslated sequence and 272 bp of 3' untranslated sequence, but lacked an additional approximately 500 bp of untranslated sequences.

The results with the KLRG1 transgene were in marked contrast to previous results with Ly49A cDNA and Ly49G2 cDNA transgenic mice, which were made with the same transgene backbone and transcriptional control elements as were the KLRG1 transgenic mice, and therefore provide a good control for the present study. The cell surface levels of transgenically expressed Ly49A or Ly49G2, like those of the corresponding endogenously expressed receptors, were down-modulated by MHC class I ([19], data not shown). The results indicated that Ly49 receptors are down-regulated post-transcriptionally, probably as a consequence of ligand-induced receptor internalization. Thus, although KLRG1 and Ly49A are both lectin-like inhibitory receptors that are regulated by class I expression, the effects of class I are opposing, and the underlying regulatory mechanisms are distinct.

How do class I MHC molecules influence KLRG1 expression? We propose that signaling via Ly49 class I-specific inhibitory receptors results in an enhanced likelihood that an NK cell will express KLRG1. Part of the evidence for this proposition is that KLRG1 expression was up-regulated on NK cells that express Ly49A or Ly49G2, in mice that express ligands for these receptors. This was true whether the Ly49 receptors were expressed from the endogenous genes on an NK cell subset, or from transgenes on all NK cells. Up-regulation was clearly evident in mice expressing H-2^d, which provide a ligand (D^d) that is recognized by both Ly49A and Ly49G2. Some up-regulation of KLRG1 was also observed in Ly49A-transgenic H-2^b mice, which do not provide a strong ligand for Ly49A. Previous studies suggest, however, that the H-2^b haplotype provides a weak ligand for Ly49A, at least as tested in developmental assays [19]. Such as weak interaction may account for the effects observed here in Ly49A transgenic H-$2^{b/b}$ mice.

While the data show that inhibitory receptor engagement enhances the likelihood of KLRG1 expression, receptor engagement is apparently not sufficient to induce high level expression of KLRG1 in all NK cells. Indeed, not all Ly49A$^+$ NK cells in H-2^d mice expressed KLRG1 (Fig. 5). Nevertheless, expression of such as receptor may increase the probability that KLRG1 will be induced at any given point in time. The additional factors that influence KLRG1 expression remain to be determined.

The analysis of NK cells derived from me^v/me^v bone marrow stem cells extended these results by demonstrating that the high expression of KLRG1 depends on SHP-1 expression. Normal NK cells co-differentiating in the same bone marrow exhibited normal levels of KLRG1 expression. The SHP-1 phosphatase is the key mediator of signaling by class I-specific inhibitory receptors [26]. Thus we propose that the low expression of KLRG1 in class I-deficient mice is due to the absence of class I ligands for Ly49 receptors, whereas the low expression in me^v/me^v mice is due to impaired inhibitory signaling by these receptors.

An alternative explanation for the effect of SHP-1-deficiency on KLRG1 expression by NK cells is that

SHP-1 interacts with the cytoplasmic domain of KLRG1, and that high levels of KLRG1 expression on the cell surface depend on this interaction. We consider this less likely, because the cell surface levels of other receptors that interact with SHP-1, such as Ly49 receptors, were not down-regulated on the me^v/me^v NK cells (data not shown). It also remains possible that the interaction of SHP-1 with non-class I-specific receptors, such as c-Kit, is required for normal KLRG1 expression. However, this latter model does not explain the effect of class I deficiency on KLRG1 surface levels.

The dependence of KLRG1 on class I MHC expression is thus far unique among NK receptors, and opposite to the pattern exhibited by Ly49 receptors. We propose that KLRG1, and perhaps other NK-specific molecules, are up-regulated upon engagement of MHC class I-specific receptors and may serve as a marker for inhibitory receptor stimulation. In this sense, while class I expression has opposing effects on KLRG1 and Ly49 cell surface levels, it is possible that they function in the same inhibitory pathway. Whether KLRG1 plays a role in the process of inhibition or in other aspects of NK cell functionality will be addressed in future experiments with gene-targeted and transgenic mice.

4 Materials and methods

4.1 Animals

C57BL/6J (B6), B10.D2/nSnJ ($H\text{-}2^d$, B10.D2), B10.BR/SgSnJ ($H\text{-}2^k$, B10.BR), B10.M/Sn ($H\text{-}2^f$, B10.M), B10.Q/SgJ ($H\text{-}2^q$, B10.Q), B10.RIII (71NS)/Sn ($H\text{-}2^r$, B10.RIII) and B10.S/SgMcdJ ($H\text{-}2^s$, B10.S) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). B6-$\beta2m^{-/-}$ mice [27] had been backcrossed five times to B6 mice before intercrossing. B6-$\beta2m^{-/-}$ mice were crossed with B10.BR and B6-$H\text{-}2^d$ mice, and the F1 animals were intercrossed to generate B10.BR-$\beta2m^{-/-}$ and B6 $H\text{-}2^d$-$\beta2m^{-/-}$ mice, respectively. $K^bD^{b-/-}$ mice on the B6 background [28] were provided by F. Lemonnier, Pasteur Institut. B6 Ly5.1 congenic mice were purchased from NCI where they are listed as B6-Ly5.2/Cr, according to a previous nomenclature. C57BL/6J-*Hcph*$^{me\text{-}v/+}$ (motheaten viable) heterozygous mice were purchased from Jackson Labs and interbred to generate homozygotes. The generation of Ly49A and Ly49G2 transgenic mice have been described [17, 18]. Syrian hamsters were purchased from Simonsen, Gilroy, CA.

4.2 Antibodies

2F1 mAb was prepared by immunizing Syrian hamsters in the footpad with activated NK (A-LAK) cells from B6 ($H\text{-}2^b$) mice every week for 5 weeks. Three days after the final boost, the popliteal lymph node was removed and the cells were fused to the murine myeloma P3X63Ag.8.653. Hybridomas were screened for antibody specific for A-LAK cells, and the 2F1 hybridoma was recloned five times. 2F1 mAb was purified from hybridoma supernatants by affinity chromatography over protein G-agarose.

Other mAb included F536 (anti-Vγ3) [29], JR9–318 (anti-Ly49A) [30], 4D11 (anti-Ly49G2) [31], PK136 (anti-NK1.1) [32], RL-172 (anti-CD4), AD4(15) (anti-CD8), A20 (anti-Ly5.1), and 104 (anti-Ly5.2). 2F1 mAb and other antibodies were conjugated to biotin and fluorescein isothiocyanate (FITC) according to standard procedures.

For staining experiments we also employed goat $F(ab')_2$ anti-mouse IgG(H+L)-TRICOLOR, anti-CD3-TRICOLOR, H57-TRICOLOR (anti-TCRβ), and goat anti-hamster Ig-FITC, all from Caltag (Burlingame, CA), PK136-PE (Pharmingen, San Diego, CA), and streptavidin-PE (Southern Biotechnology Associates, Inc., Birmingham, AL).

4.3 Preparation of NK-cell enriched spleen cells and A-LAK cells

A-LAK cells cultured for a total of 9 days were prepared essentially as described [33], with modifications [34]. For enrichment of NK cells from splenocytes, nylon wool non-adherent cells were treated with anti-CD4 and anti-CD8 mAb plus complement. Viable cells were purified over Ficoll/Isopaque columns.

4.4 Immunoprecipitations

A-LAK cells were iodinated using the lactoperoxidase method [31], and lysed in NP40 lysis buffer. The lysates were pre-cleared three times with irrelevant F536 (anti-Vγ3) mAb coated on protein G-agarose. Equal amounts of lysate were immunoprecipitated with 2F1 mAb or with the negative control F536 mAb. The immunoprecipitates were subjected to SDS-PAGE on a 10 % gel under nonreducing conditions or reducing conditions. The gels were exposed to film for 10–40 days. In some cases, immunoprecipitates were treated with a mixture of EndoF and N-glycosidase F (Boehringer Mannheim) according to the manufacturer's instructions.

4.5 Staining experiments

KLRG1 expression was examined by two- or three-color flow cytometric analysis. For two-color analysis, cells were incubated with 2F1 mAb, washed, and incubated with anti-NK1.1-PE and goat anti-hamster IgG-FITC. For three-color analysis, four procedures were employed. In procedure A, cells were incubated first with goat $F(ab')_2$ anti-mouse IgG

(H+L)-TRICOLOR, followed by mouse IgG to block the free binding sites. In the third and fourth steps, cells were incubated with biotinylated-2F1 mAb followed by a mixture of anti-CD3-TRICOLOR, H57-TRICOLOR, anti-NK1.1-FITC and streptavidin-PE. Procedure B was identical to procedure A, except that anti-Ly49-FITC reagents were employed instead of anti-NK1.1-FITC in the last step. In both procedures, gated $CD3^{-}TCR\alpha\beta^{-}$ Ig^{-} cells were analyzed. In procedure C, mAb 2F1 staining was developed with goat anti-hamster IgG-FITC, and after blocking with hamster serum, cells were counter-stained with anti-CD3-biotin/SA-TC and anti-NK1.1-PE. A Coulter EPICS-XL-MCL flow cytometer was employed for the analysis.

4.6 Adhesion assay

Semiquantitative adhesion assays were performed as described [17]. Briefly, COS-7 cells (CRL1651, purchased from ATCC, Rockville, MD) were transfected with 5 μg of pME-Ly49C [17], pME-KLRG1 [12] or $Ly49G2^{inverse}$ as a control using Lipofectamine® (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Con A blasts from MHC-different mice were pulsed with 2.5 μCi/ml [^{3}H] thymidine (Amersham, Arlington Heights, IL). COS-7 transfectants were overlaid with Con A blasts and incubated at 37 °C for 2 h, followed by extensive washing. The remaining conjugates were lifted from the plates, and the radioactivity was determined using a β-counter. The binding index was determined as cpm KLRG1- or Ly49C-cDNA transfected well/cpm $Ly49G2^{inverse}$-transfected well.

4.7 KLRG1 transgenic mice

A 861-bp EcoRI/Pst1 fragment of the KLRG1 cDNA obtained by expression cloning [12] including the coding region as well as 22 bp of the 5'UTR and 272 bp of the 3'UTR was subcloned into the pHSE3 expression cassette, which incorporates the K^{b} promoter and the Ig enhancer [15]. Linearized Xho-1 fragments were injected into fertilized (B6 × CBA/JF_2) eggs, and founders were screened by Southern blotting as described earlier [12], RT-PCR and staining with mAb 2F1. The line described here (line 8) contained approximately 12 transgene copies as determined by Southern blotting and was backcrossed at least three times to B6 mice before analysis or crossing to B6-$\beta2m^{-/-}$ mice [27].

4.8 Motheaten chimeras

In order to distinguish *cis* and *trans* effects of the motheaten mutation, and to permit genetically me^{v}/me^{v} NK cells to mature in a relatively normal hematopoietic environment, mixed bone marrow chimeras were constructed essentially as has been previously described [20]. Briefly, bone marrow cells from C57BL/6 me^{v}/me^{v} mice (Ly5.2) were mixed in a 1:4 ratio with bone marrow cells from age-matched wild-type C57BL/6 (Ly5.1) congenic mice. As a control, a similar mixture of C57BL/6 wild-type (Ly5.2) and congenic (Ly5.1) bone marrow was prepared. Approximately $5 \times 10^{6} - 10 \times 10^{6}$ bone marrow cells were injected intravenously (subocularly) into irradiated (980 rad) B6 Ly5.1 mice. Reconstitution was allowed to proceed for at least 12 weeks. None of the characteristic symptoms of the motheaten syndrome (*e.g.* patchy fur, scabs on paws, premature death) were observed in these chimeras.

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