Dedicated immunosensing of the mouse intestinal epithelium facilitated by a pair of genetically coupled lectin-like receptors

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The integrity of the intestinal epithelium is constantly surveyed by a peculiar subset of innate-like T lymphocytes embedded in the epithelial cell layer, hence called intestinal intraepithelial lymphocytes (IELs). IELs are thought to act as “first-line” sentinels sensing the state of adjacent epithelial cells via both T-cell receptors and auxiliary receptors. Auxiliary receptors modulating IEL activity include C-type lectin-like receptors encoded in the natural killer gene complex such as NKG2D. Here, we report that the CTLR Nkrp1g is expressed by a subpopulation of mouse CD103⁺ IELs allowing immunosensing of the intestinal epithelium through ligation of the genetically coupled CTLR Clr-f that is almost exclusively expressed on differentiated intestinal epithelial cells (IECs). Most of these Nkrp1g-expressing IELs exhibit a $\gamma\delta$ TCR^{bright}Nkg2a[−] phenotype and are intimately associated with the intestinal epithelium. As Clr-f expression strongly inhibits effector functions of Nkrp1g-expressing cells and is upregulated upon poly(I:C) challenge, Clr-f molecules may quench reactivity of these IELs towards the epithelial barrier that is constantly provoked by microbial and antigenic stimuli. Altogether, we here newly characterize a genetically linked C-type lectin-like receptor/ligand pair with a highly restricted tissue expression that apparently evolved to allow for a dedicated immunosurveillance of the mouse intestinal epithelium.

INTRODUCTION

Mammalian epithelia cover body surfaces directly exposed to the environment and are populated by a complex community of commensal microorganisms. In addition, these epithelia are constantly challenged by potential pathogens. Hence, epithelial cells not only constitute a physical barrier of skin, intestine, and other exposed organs, but also are actively involved in pathogen recognition and immune defense.^{1–3} In the intestine, a single thin layer of intestinal epithelial cells (IECs) protects a huge area (~200 m² in humans)⁴ against pathogenic microorganisms also comprising specialized types of cells that produce protective mucus or secrete anti-microbial peptides.^{2,5,6} Although most host–microbiota interactions are of symbiotic nature, the immense number and density of bacteria in the gut constitutes a particular challenge for the immune system. Therefore, pathogen recognition and subsequent initiation and modulation of immune responses are major tasks of IECs

administered in a complex network together with innate and adaptive immune cells. A peculiar subset of the latter resides in tight contact with IECs within the epithelial barrier and in close proximity to the intestinal lumen. These so-called intestinal intraepithelial lymphocytes (IELs) act as a first line of defense, mostly comprising antigen-experienced $\alpha\beta$ T cells or $\gamma\delta$ T cells. They commonly express activation markers, such as CD69, and the integrin CD103 that mediates gut homing.⁷ Apart from T-cell receptor (TCR) engagement, activity of these IELs is regulated by auxiliary receptors such as killer cell lectin-like receptors (KLRs) encoded in the natural killer gene complex (NKC).⁸ Prominent NKC-encoded KLRs are NK1.1, NKG2D, CD94/NKG2A, and various Ly49 receptors.^{7,9,10} Although NK1.1 (also known as Nkrp1c) is a member of the NKR1 family with a yet unknown ligand,¹¹ other members of the NKR1 subfamily have been shown to ligate likewise C-type lectin-like receptors (CTLRs) of the CLEC2 family, with genes

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of both NKR1 receptors and CLEC2 ligands intermingled in the NKC.^{12–15} Members of the CLEC2 family are CD69, the human CTLRs LLT1, AICL, KACL, as well as the mouse C-type lectin-related (Clr) molecules.^{8,11} Certain mouse Clr molecules have been shown to act as ligands of NKR1 receptors expressed by NK cells and T cells, whereas expression and function of most of these Clr molecules are poorly defined.^{11,12,14–17} Also, function and immunological relevance of these genetically linked receptor/ligand pairs *in vivo* remain unknown.

Hence, we set out to characterize the expression and function of mouse Clr molecules. In our initial studies, we unexpectedly observed a rather tissue-restricted RNA expression pattern of some, but not all, Clr family members, well in line with a more recent study by Makrigiannis and co-workers.¹⁸ In particular, Clr-f transcripts were almost exclusively found associated with the mouse intestine prompting further in-depth studies of cellular expression of Clr-f and its receptor Nkrp1g. Using novel antibodies, our subsequent studies revealed colocalized expression of Clr-f glycoproteins and inhibitory Nkrp1g receptors within the intestinal epithelium by IECs and adjacent IELs, respectively, suggesting that this receptor/ligand pair contributes to the immunosurveillance of mouse intestinal epithelium in a tissue-specific manner.

RESULTS

Predominant expression of the mouse CLEC2 family member Clr-f in gut epithelium

The mouse Clr multigene family clustered in the mouse NKC comprises at least seven members that are predicted to encode for functional proteins.^{11,13,15} As we had recently observed a highly tissue-specific expression of the human CLEC2 family

member *CLEC2A* encoding for the keratinocyte-associated CTLR KACL,^{19,20} we hypothesized that also certain mouse CLEC2 family proteins may be distinguished by a tissue-specific expression pattern. Hence, we set out to determine expression of mouse Clr family members in various tissues by quantitative PCR (qPCR). In line with previous reports,^{12,16,21,22} we found a rather broad and uniform expression of the gene *Clec2d* (encoding Clr-b) in most tissues except the brain (**Figure 1a** and **Supplementary Figure S1** online). In contrast, expression of *Clec2i* (Clr-g) apparently is biased towards hematopoietic organs. Strikingly, the expression pattern of *Clec2h* (Clr-f) turned out to be completely different from that of both Clr-b and Clr-g with a predominant expression in the gut, including both small and large intestine (**Figure 1a**). Significant Clr-f expression was also noted for kidneys, whereas in all the other organs Clr-f transcripts were sparse or not even detectable. This peculiar gut-restricted expression pattern of Clr-f was consistently observed for both male and female mice, as well as for C57BL/6 and BALB/c mice (**Figure 1a** and **b**). Sectional analyses of the gastrointestinal tract revealed a rather uniform Clr-f expression throughout all parts of the small intestine and colon, whereas no expression was found in the esophagus or stomach (**Figure 1c**). To define the type of intestinal cells specifically expressing Clr-f, small intestines of C57BL/6 mice were dissociated and populations of IECs and IELs purified by FACS sorting, respectively (**Supplementary Figure S2**). Subsequent qPCR analyses revealed a virtually exclusive expression of Clr-f by IECs (**Figure 1d**), which was unexpected given the common expression of human and mouse CLEC2 family members by hematopoietic cells.

Figure 1 Tissue-specific expression of Clr-f. (a) Relative abundance of Clr-b (left), Clr-g (middle), and Clr-f (right) transcripts, respectively, in various tissues of female C57BL/6 mice. (b and c) Relative abundance of Clr-f transcripts (b) in selected tissues of male C57BL/6 mice (left), female BALB/c mice (right), or (c) in various sections of the gastrointestinal tract of female C57BL/6 mice. (d) Levels of Clr-f transcripts in FACS sorted IEL (CD45.2⁺CD103⁺EpCAM⁺) and IEC (CD45.2⁺EpCAM⁺), respectively. Purity of IEL and IEC sorted from the crude preparations of intestinal cells was verified by analysis of transcripts of CD45 and Glut2, respectively. (a–d) Levels of transcripts were determined by qPCR, normalized with 18S rRNA, and arbitrarily set relative to transcript levels of respective tissues marked with an asterisk (ND, not detectable). Asc. colon, ascending colon; Clr, C-type lectin-related; desc. colon, descending colon; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; qPCR, quantitative polymerase chain reaction.

Figure 2 Clr-f is a disulfide-linked homodimeric CTLR. **(a)** Surface expression of Clr molecules on 293T (left) and CHO cells (right) transfected with FLAG-tagged Clr-f, Clr-b, or Clr-g cDNA detected by FLAG-tag mAb M2 (solid line; shaded: isotype staining). **(b)** Immunoblotting of lysates of 293T transfected with FLAG-tagged Clr-f, Clr-b, or Clr-g cDNA. Clr molecules were detected by FLAG-tag mAb M2 after reducing SDS-PAGE (SDS-polyacrylamide gel electrophoresis upper left), after reducing SDS-PAGE of PNGase-F-treated lysates (upper right) or after nonreducing SDS-PAGE (lower). Clr, C-type lectin-related; CTLR, C-type lectin-like receptor.

Clr-f is a bona fide homodimeric cell surface CTLR

To explore whether Clr-f represents a typical cell surface CTLR, the putative open reading frame cloned from cDNA of small intestines was ectopically expressed in 293T and CHO cells. Flow cytometric analyses of the resulting transfectants showed that Clr-f is readily expressed at the cell surface comparable to Clr-b and Clr-g (Figure 2a). Immunoblots of corresponding 293T lysates revealed glycoproteins with molecular masses of ~30 kDa (Clr-f) and ~35 kDa (Clr-b; Clr-g), respectively (Figure 2b). Deglycosylation of all proteins reduced the apparent molecular masses to ~25 kDa (Figure 2b) matching the predicted molecular masses and indicating the usage of one (Clr-f), three (Clr-b) or three to four (Clr-g) N-linked glycosylation sites, respectively. Similar results were also obtained when Clr-f was expressed in the human intestinal epithelial cell line HCT116 (Supplementary Figure S3). Under nonreducing conditions, these glycosylated CTLRs appeared with molecular masses of ~60 kDa (Clr-f) and ~70 kDa (Clr-b, Clr-g), respectively (Figure 2b), demonstrating occurrence as disulfide-linked homodimers.

Selective expression of Clr-f on differentiated intestinal epithelial cells

Detection of endogenous Clr-f expression was hampered by the lack of Clr-f-specific antibodies. Hence, we expressed the soluble C-type lectin-like domain (CTLD) of Clr-f (sClr-f) in 293 cells and immunized rats with purified sClr-f to generate Clr-f-specific mAb. Exclusive specificity of resulting mAbs 10A6 and 10A8 for Clr-f was shown by flow cytometry using CHO cells ectopically expressing Clr-f (CHO-Clr-f) as compared with other Clr molecules and by staining of sClr-f-coated microspheres (Figure 3a; Supplementary Figure S4). To assess endogenous expression of Clr-f by IECs, freshly isolated intestinal cells were stained with mAb 10A6 and

analyzed by flow cytometry. 10A6 markedly bound to IECs, but not to EpCAM-negative cells including IELs, and binding to IECs was specifically blocked by sClr-f (Figure 3b). Of note, IECs did not uniformly express Clr-f as there was a significant subset of IECs with an absent or low expression of Clr-f (Figure 3b). To allocate Clr-f-expressing cells within the intestinal epithelium, sections of the small intestine were stained with mAb 10A6. Notably, differentiated IECs lining the intestinal villi prominently and homogeneously expressed Clr-f, whereas crypt cells did not express Clr-f (Figure 3c and d; Supplementary Figure S5). Clr-f expression was also noted for the colon epithelium well in line with the presence of Clr-f transcripts (Figure 3e).

Nkrp1g is a receptor for Clr-f expressed by a subset of IELs

Distinct expression of Clr-f by IECs was intriguing and raised immediate questions regarding the immunological significance thereof. Recently, it has been reported that Clr-f is a ligand of Nkrp1g, a member of the mouse family of NKRP1 receptors.^{13,15} Of note, the genes of Clr-f (*Clec2h*) and Nkrp1g (*Klrb1g*) are located vis-à-vis in the NKC in a tail-to-tail orientation, following the principle of genetic linkage of NKRP1/CLEC2 receptor/ligand pairs¹¹ (Figure 4a). In contrast to other members of the mouse NKRP1 family, physiologic expression of Nkrp1g remained undefined, mainly due to the lack of Nkrp1g-specific mAb.²³ Hence, we generated Nkrp1g-specific mAb by immunizing rats with the soluble CTLD of Nkrp1g (sNkrp1g) expressed in 293T cells. The resulting mAb 8A10 and 4A2 specifically bound to NK-92MI cells ectopically expressing Nkrp1g, but did not cross-react with other NKRP1 receptors (Figure 4b and Supplementary Figure S6). We assayed an interaction of Nkrp1g expressed in the context of NK-92MI cells with Clr-f using BWZ.36 reporter cells²⁴ expressing Clr-f-CD3ζ hybrids. BWZ.36-Clr-f reporter cells

Figure 3 Clr-f expression on differentiated intestinal epithelial cells. (a) MAb 10A6 and 10A8 specifically bind to Clr-f on CHO-Clr-f cells (solid lines) comparable to FLAG-tag mAb M2 (dotted), but not when pretreated with sClr-f (dashed). (b) MAb 10A6 detects Clr-f on freshly isolated EpCAM⁺ IEC, but not when pretreated with sClr-f (dashed line) and not on CD45⁺ IEL (solid line). (a and b) Isotype stainings are shaded. (c and d) Frozen sections of mouse C57BL/6 small intestine stained with (c) mAb 10A6 (right) and Mayer's hematoxylin or (d) with phalloidin (white), anti-EpCAM (green) and mAb 10A6 (red), respectively. Merged pictures include DAPI stainings (blue). (e) Frozen sections of mouse C57BL/6 ascending colon stained with mAb 10A6 (right) and Mayer's hematoxylin. (c and e) IgG2a served as a control (left). Asc. colon, ascending colon; Clr, C-type lectin-related; DAPI, 4,6-diamidino-2-phenylindole; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte.

strongly responded to cocultured NK-92MI-Nkrp1g cells (Figure 4c). Responses of the reporters could largely be blocked by the addition of anti-Clr-f mAb 10A8 or anti-Nkrp1g mAb 8A10, respectively (Figure 4c). Further, there was also a pronounced binding of sClr-f tetramers to NK-92MI-Nkrp1g cells that was completely blocked by the addition of mAb 8A10 (Figure 4d) and that was irrespective of sClr-f glycosylation (Supplementary Figure S7). Collectively, these results not only corroborated Nkrp1g/Clr-f interaction previously shown by using Nkrp1g-CD3 ζ reporter cells,^{13,15} but also qualified mAb 10A8 and 8A10 as efficient blocking reagents of this interaction for further functional studies.

Subsets of intestinal epithelial $\gamma\delta$ T cells and $\alpha\beta$ T cells express Nkrp1g

To determine and localize the cells expressing Nkrp1g, various tissues of C57BL/6 mice were screened by qPCR. Nkrp1g transcripts were most abundant in the spleen and small intestine, and also present in colon and other hematopoietic organs (Figure 5a). Further dissection of intestinal Nkrp1g expression revealed a predominance of Nkrp1g transcripts in

IELs (~ 80 -fold of small intestine) whereas these were not detectable in IECs (Figure 5b), constituting an intestinal epithelial expression pattern obverse to that of Clr-f. Expression of Nkrp1g on IELs was further studied using mAb 8A10. Nkrp1g expression was detectable on a subset of CD3⁺ CD103⁺ IELs isolated from BALB/c mice and included both $\gamma\delta$ TCR⁺ and $\alpha\beta$ TCR⁺ IELs (Figure 5c). Specificity of staining was verified by blockade with sNkrp1g (Figure 5c). Comprehensive analyses of IELs from BALB/c and C57BL/6 mice revealed higher frequencies of Nkrp1g⁺ IELs in BALB/c mice as compared with C57BL/6 mice (Figure 5d). Furthermore, frequencies of Nkrp1g⁺ IELs were quite variable in individual mice ranging from 0.5 to 13% for $\gamma\delta$ T cells and up to 2% for $\alpha\beta$ T cells (Figure 5e). Frequencies of Nkrp1g⁺ $\gamma\delta$ IELs significantly increased in older mice, whereas the overall frequency of Nkrp1g⁺ IELs did not change (Figure 5f). The latter is likely due to an overall reduction of $\gamma\delta$ IELs (Supplementary Figure S8) caused by a constant expansion of the so-called "induced $\alpha\beta$ IELs".⁷ To allocate these Nkrp1g⁺ intestinal lymphocytes, sections of the small intestine were stained with anti-Nkrp1g mAb 4A2 or 8A10, respectively,

Figure 4 Clr-f ligates Nkrp1g. (a) Genes of Clr-f (*Clec2h*) and its receptor Nkrp1g (*Klrp1g*) are tightly linked in the NKC. Boxes indicate genes, arrows transcriptional orientation. (b) MAb 8A10 specifically binds to NK-92MI-Nkrp1g cells, but not to control transductants (solid line). Isotype staining shaded. (c) BWZ.36 reporter cells expressing FLAG-tagged Clr-f-CD3ζ chimera respond to cocultured NK-92MI-Nkrp1g strictly depending on Nkrp1g/Clr-f interaction. Either reporter cells were pretreated with anti-Clr-f (10A8) or isotype control (open bars) or NK-92MI cells with anti-Nkrp1g (8A10) antibodies or an isotype control (gray bars) before coculture. Addition of medium only or PMA/ionomycin to reporter cells served as negative and positive control, respectively. One-way analysis of variance Tukey's *post hoc* (***) $P < 0.0001$. (d) sClr-f tetramers (solid line), but not control reagent (shaded), bind Nkrp1g on NK-92MI-Nkrp1g cells unless Nkrp1g was blocked by preincubation with mAb 8A10 (dotted line). Clr, C-type lectin-related; NKC, natural killer gene complex; PMA, phorbol 12-myristate 13-acetate.

revealing Nkrp1g⁺ cells in intimate contact with intestinal epithelial cells (Figure 5g).

Nkrp1g⁺ IELs exhibit a $\gamma\delta$ TCR^{bright}Nkg2a⁻ phenotype

To further characterize Nkrp1g-expressing IELs, Nkrp1g⁺ and Nkrp1g⁻ $\gamma\delta$ T cells were FACSsorted using the Nkrp1g-specific mAb 8A10 (Supplementary Figure S9a). Faithful distinction of these subsets by mAb 8A10 was confirmed by qPCR analyses of the sorted cells (Figure 6a) that were also subjected to microarray-based differential transcriptional profiling (Supplementary Figure S9b). For gene products for which suitable antibodies were not available, results from microarray studies were validated by qPCR of independently sorted IELs pooled from various mice. Consistently, an about twofold increase of transcripts of the integrin subunit CD11d and the close Nkrp1g relative Nkrp1f was detected in Nkrp1g⁺ $\gamma\delta$ T cells, whereas the levels of the chemokine CCL3/MIP-1 α were twofold lower (Figure 6b). Nkrp1g⁺ $\gamma\delta$ T cells also expressed transcripts for TGF- β 1 and TGF- β 3, as well as for the keratinocyte growth factor (Supplementary Figure S9d).

Differential expression of Nkg2a could be confirmed by flow cytometry, with Nkg2a preferentially expressed on Nkrp1g⁻ $\gamma\delta$ IELs (Figure 6c). Notably, Nkrp1g⁺ $\gamma\delta$ T cells are characterized by a strong expression of the $\gamma\delta$ TCR complex (Figure 6d and e).

Potent inhibition of effector cell responses by Nkrp1g/Clr-f interaction

Considering the contiguity of Nkrp1g⁺ IELs and Clr-f⁺ IECs in the intestinal epithelium, we wondered about the functional implications of this tissue-specific Nkrp1g/Clr-f interaction. As functional consequences of Nkrp1g ligation have not yet been reported, we directly addressed Nkrp1g function in cocultures of NK-92MI-Nkrp1g cells with CHO cells. NK-92MI-Nkrp1g cells readily degranulated when cocultured with CHO cells, whereas no degranulation was detectable in the presence of CHO cells ectopically expressing Clr-f (Figure 7a and b). Degranulation towards CHO-Clr-f cells could be restored in the presence of anti-Clr-f mAb 10A8, while addition of the non-blocking Clr-f mAb 10A6 had no effect (Figure 7a and b). Accordingly, CHO-Clr-f cells were resistant to cytolysis by NK-92MI-Nkrp1g cells. However, resistance to cytolysis was abolished in the presence of mAb 10A8, resulting in a cytolysis comparable to control CHO cells (Figure 7c). Altogether, these data demonstrate that Nkrp1g acts as a potent inhibitory receptor when engaged by its cognate ligand Clr-f.

Enhanced Clr-f expression on IECs upon poly(I:C) treatment can be sensed via Nkrp1g

On the basis of the findings reported in this study, one may hypothesize that Clr-f expression on IECs dampens effector functions of Nkrp1g⁺ IELs and that alterations in Clr-f expression levels, e.g., in course of an infection or an inflammation, may serve to modulate the responsiveness of these IELs. To address a potential regulation of Clr-f expression during an inflammatory challenge, mice were intraperitoneally injected with poly(I:C) and subsequently analyzed for expression of Clr-f and Nkrp1g, respectively. Sixteen hours post injection, there was a significant increase in Clr-f expression by IECs detected in situ by immunostaining and *ex vivo* by flow cytometry (Figure 8a-c). Increased expression of Clr-f following poly(I:C) injection was paralleled by a lower frequency of Nkrp1g⁺ IELs and a decrease in the respective Nkrp1g expression levels, possibly indicating ligand-induced downregulation of Nkrp1g (Figure 8d and e). To establish functional recognition of Clr-f on IECs by Nkrp1g, we used reporter cells expressing an Nkrp1g-CD3ζ chimeric receptor.¹⁵ Human intestinal carcinoma cells ectopically expressing Clr-f (HCT116-Clr-f) were recognized by these reporter cells in a Clr-f-specific manner demonstrating that, in principle, Clr-f molecules on intestinal epithelial cells are amenable to Nkrp1g-mediated detection (Figure 8f and g). Finally, Nkrp1g⁺ reporter cells were cocultured with IECs freshly isolated from phosphate-buffered saline-treated or poly(I:C)-treated mice. These reporter cells responded to IECs isolated from both groups of mice, but significantly stronger to IECs from poly(I:C)-treated mice, whereas responses were fully inhibited

Figure 5 Nkrp1g is expressed on a subset of intraepithelial lymphocytes (**a** and **b**) Relative abundance of Nkrp1g transcripts (**a**) in various tissues of female C57BL/6 mice and (**b**) FACSorted IEL and IEC. Tissues and cells of female C57BL/6 mice were analyzed by qPCR, levels of Nkrp1g transcripts were normalized to 18S rRNA and arbitrarily set relative to tissues marked with an asterisk. (**c**) Intestinal IEL (gate: CD103⁺, CD3⁺) (upper) were assessed for expression of Nkrp1g (upper) or Nkrp1g co-expression with $\gamma\delta$ TCR (lower) using mAb 8A10. Specificity of staining is shown by blockade with sNkrp1g (right); biotinylated rat IgG1 served as a control (left). (**d** and **e**) Compilation of frequencies of (**d**) Nkrp1g⁺ CD3⁺ CD103⁺ cells and of (**e**) Nkrp1g⁺ $\gamma\delta$ T cells and Nkrp1g⁺ $\alpha\beta$ T cells among IEL from small intestines of BALB/c and C57BL/6 mice, respectively. (**f**) Frequencies of Nkrp1g⁺ $\gamma\delta$ T cells (left) and Nkrp1g⁺ CD3⁺ CD103⁺ cells (right) and among IEL from small intestines of differently aged BALB/c. Statistical differences in frequencies of Nkrp1g⁺ cells are indicated by asterisks. One-way analysis of variance Tukey's *post hoc* (NS, not significant; ***P* < 0.005). (**g**) Localization of Nkrp1g⁺ IEL in situ. Frozen sections of mouse BALB/c small intestine stained for Nkrp1g (green) with mAb 4A2 (left and second left) or mAb 8A10 (second right), EpCAM (red), and counterstained with DAPI (blue). A representative isotype control staining (for mAb 8A10 and 4A2) is also shown (right). Clr, C-type lectin-related; DAPI, 4,6-diamidino-2-phenylindole; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; ND, not detectable; qPCR, quantitative polymerase chain reaction.

in the presence of the anti-Clr-f mAb 10A8, demonstrating that they were Clr-f-specific (**Figure 8h**). Altogether, these data show that upregulation of the intestinal epithelial Clr-f glycoproteins following poly(I:C)-exposure can be specifically sensed by Nkrp1g-expressing cells.

DISCUSSION

We here report the tissue-specific expression of the C-type lectin-like molecule Clr-f on differentiated mouse intestinal epithelial cells which contrasts the broad tissue expression of its close relative Clr-b, the best-studied member of the Clr family of CTLR. Although the original description of mouse Clr genes already provided first preliminary hints that their tissue expression may be differentially regulated,¹⁶ this was not elaborated by more in-depth studies until very recently.^{18,25} The subsequent characterization of certain Clr molecules as ligands of the genetically linked NKR1 receptors fueled the interest in Clr family members, but for the most part, studies on

Clr expression remained limited to the levels of transcripts due to the lack of specific antibodies.^{18,25} Only for Clr-b, pronounced cell surface expression could be shown for various hematopoietic cells and for cell lines of various origins.¹² The broad tissue expression of Clr-b and its interaction with the inhibitory Nkrp1d receptor on NK cells led to the hypothesis that the Nkrp1d/Clr-b receptor/ligand pair may act as a second missing-self recognition system analogous to MHC class I.^{12,14,26,27} This idea was further supported by the observation that Clr-b expression is reduced on many tumor cell lines and downregulated upon viral infection or cellular stress.^{12,14,21,26,27} In line with these previous studies, our tissue screen revealed a very broad and fairly uniform tissue expression of Clr-b except for the brain, where the non-NKC-encoded CLEC2 family member BACL is selectively expressed.²⁸ Unlike Clr-b, Clr-g transcripts primarily locate to hematopoietic organs such as bone marrow and spleen, suggesting a preferential Clr-g expression by hematopoietic cells similar to the human CLEC2

Figure 6 Characteristics of Nkrp1g⁺ IEL. (a) Freshly isolated IEL from small intestines of BALB/c mice were FACSsorted for Nkrp1g⁺ or Nkrp1g⁻ subsets of αβ T cells or γδ T cells using mAb 8A10 and purity of sorted cells verified by qPCR assessment of Nkrp1g expression. (b) Relative abundance of CCL3, Nkrp1f, and CD11d transcripts in FACSsorted Nkrp1g⁺ and Nkrp1g⁻ γδ IEL. (a and b) Levels of transcripts were normalized to 18S rRNA and set relative to samples marked with an asterisk. (c–e) IEL from C57BL/6 (c) and BALB/c mice (d) were analyzed for co-expression of Nkrp1g and (c) Nkg2a, (d) CD3e or γδ TCR, respectively. (e) Median fluorescence intensity (MFI) of γδ TCR on Nkrp1g⁺ vs. Nkrp1g⁻ γδ T cells. Statistical differences in MFI of γδ TCR are indicated by asterisks. T-test, unpaired, two-tailed (****P* < 0.0005). IEL, intraepithelial lymphocyte; qPCR, quantitative polymerase chain reaction; small int., small intestine.

family members AICL and LLT1.^{11,17,29,30} The gut-associated expression pattern of Clr-f reported here was consistently observed independent of strain, sex, and age of mice and thus appears as a characteristic trait of the mouse intestinal epithelium. Apparently, Clr-f protein expression is abruptly acquired during IEC differentiation when leaving the crypts as only villous epithelial cells, but not crypt cells, express Clr-f molecules. The rather exclusive expression of Clr-f by intestinal epithelial cells is reminiscent of the gut-restricted expression of the MHC class I-like molecule TL, a ligand of CD8αα homodimers characteristic of the so-called “natural” IELs.^{31,32} Recent studies showed that engagement of TL on IECs by CD8αα on IELs contributes to the generation of a pool of high-affinity effector memory CD8αβ IELs.³³ Similarly, we show that IEC-specific expression of Clr-f is matched by expression of its receptor Nkrp1g on a subset of IELs. These are “natural” IELs as they typically lack expression of CD5 (Supplementary Figure S9c) and include both intestinal αβ and γδ T cells, with a significantly higher representation among γδ T cells. We consistently observed lower frequencies of Nkrp1g-expressing IELs in C57BL/6 mice as compared with BALB/c mice indicating strain-specific genetic determinants of Nkrp1g expression. Our current data also show increased frequencies of Nkrp1g⁺ γδ IEL among aged mice in line with an accumulation of senescent γδ T cells. It was previously shown, that colonic γδ T cells express proinflammatory cytokines and chemokines such as IL-1β, CXCL1, CXCL2, and CXCL9 upon mucosal injury³⁴ and that these are also produced by γδ IELs of the small intestine.³⁵ We found Nkrp1g⁺ γδ T cells to express transcripts for TGF-β1 and TGF-β3, as well as for the keratinocyte growth

factor and this may mediate both suppressive and regenerative effects on epithelial cells. Interestingly, expression of Nkg2a and Nkrp1g is discordant on IELs and may coincide with different effector functions. In humans, triggering of the inhibitory CTLR NKG2A on γδ T cells has been shown to suppress cytotoxic αβ T cells by the release of TGF-β1 in patients with celiac disease.⁹

Strangely enough, not only the genes of the receptor/ligand pair Nkrp1g/Clr-f are opposed in tight genetic linkage in the NKC, but also the corresponding gene products are expressed by intimate, yet unrelated neighbors in the mouse intestinal epithelium (Figure 5g). Attribution of Nkrp1g and Clr-f expression to the intestinal epithelial compartment raises questions about the functional relevance thereof. Although direct assessment of the functional implications of the Nkrp1g/Clr-f interaction in the gut epithelium must await the availability of genetically modified mice, our data clearly demonstrate that the Nkrp1g/Clr-f interaction provides a strong inhibitory signal in line with the presence of a putative immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic domain of Nkrp1g. As Clr-f molecules are expressed at the surface of intestinal epithelial cells and thus can be sensed by adjacent IELs via the inhibitory receptor Nkrp1g, it is conceivable that γδ T cells are capable to survey the “state” of intestinal epithelial cells by means of the Nkrp1g/Clr-f interaction. As previously reported for Clr-b,^{21,27} down-regulation of Clr-f may indicate cellular stress, pathogenic challenge, or mucosal injury, and unleash effector responses of Nkrp1g-expressing γδ T cells, such as the release of proinflammatory cytokines and chemokines. In light of the

Figure 7 Nkrp1g potentially inhibits effector functions upon Clr-f engagement. **(a and b)** Degranulation of NK-92MI-Nkrp1g in cocultures with mock- or Clr-f-transfected CHO cells, in the presence of Clr-f-specific mAb 10A8 (blocking Nkrp1g/Clr-f interaction) or 10A6 (non-blocking) or isotype controls, respectively. **(a)** Representative dot plots and **(b)** compilation of experimental data. **(c)** Cytolysis of mock- or Clr-f-transfected CHO cells by NK-92MI-Nkrp1g cells in the presence of mAb 10A8 or isotype control. **(b and c)** Errors bars of triplicates are shown and statistical significance of differences between respective data pairs is given (two-way analysis of variance Bonferroni post tests: *** $P < 0.001$; ** $P < 0.01$; NS, not significant). Clr, C-type lectin-related.

highly proliferative nature and demanding microbial exposure of the intestinal epithelium, such a dedicated immunosurveillance mechanism may aid in controlling the integrity of the epithelial barrier. Alternatively, Nkrp1g may constrain the reactivity of IELs by the continuous engagement of Clr-f, and this mechanism may contribute to the control of IEL effector responses in an environment rich in immunostimulatory compounds and microbiota. First support for this latter idea was gained from experiments demonstrating that expression of Clr-f on IEC is substantially increased upon treatment with poly(I:C) and that this increase can be sensed via Nkrp1g. Such a mechanism may provide increased protection from IEL-mediated damage.

Taken together, we find Clr-f almost exclusively expressed by differentiated intestinal epithelial cells matched by a rather specific expression of Nkrp1g on a subset of intestinal IELs. As

engagement of Clr-f by Nkrp1g strongly inhibits effector responses, we hypothesize that this cognate receptor/ligand pair may contribute to the dedicated immunosurveillance of the epithelial barrier of the mouse gut.

METHODS

Mice. C57BL/6 and BALB/c mice were purchased from Harlan Laboratories and housed at the local animal facility. 6- to 8-week-old BALB/c mice were injected intraperitoneally with 30 μ g poly(I:C) per gram body weight (high molecular weight poly(I:C) from Invivogen, Toulouse, France). After 16 h mice were killed for isolation of IEC and IEL. Animal experiments were approved by the local authorities (Regierungspräsidium, Darmstadt, Germany; permit numbers F146/04 and F146/Anz03) and performed in full compliance with the respective national guidelines.

Quantitative PCR. RNA was isolated from cells using peqGOLD TriFast (Peqlab, Erlangen, Germany) or RNAqueous-Micro Kit (Life Technologies, Darmstadt, Germany) and converted into cDNA using M-MLV RT (H-) (Promega, Mannheim, Germany) according to the manufacturer's protocol after treatment with DNase I for 30 min at 37 °C. cDNA was amplified on the real-time PCR system StepOnePlus (Applied Biosystems, Foster City, CA) using SYBR Green chemistry (Roche, Mannheim, Germany) for detection (primer pairs are shown in **Supplementary Table S1**). Copy numbers were normalized with copy numbers of 18S rRNA (Δ Ct method).

Transfectants. 293 and 293T cell lines were cultured in Dulbecco's Modified Eagle's medium. CHO, HCT116, and NK-92MI cell lines were cultured in Iscove's Modified Dulbecco's Medium. CHO, HCT116, and 293T cells were transfected using FuGENE HD (Roche) with cDNA of *Clec2h*, *Clec2d*, or *Clec2i* containing a carboxyterminal FLAG- and hexahistidine-tag in RSV.5neo expression vector. Stably transfected cells were selected in G418 containing medium (2 mg ml⁻¹). NK-92MI were transduced with retroviruses generated by transfection of phoenix-ampho cells (Nolan Laboratory, Stanford, CA) with the vector pMXsIP kindly provided by Toshio Kitamura (University of Tokyo)³⁶ containing the Nkrp1g cDNA with a carboxyterminal FLAG-tag. Transduced NK-92MI cells were selected in puromycin-containing medium (7 μ g ml⁻¹).

Chimeric reporter construct. For the generation of reporter constructs, the nucleotide sequence for the CTLD of Clr-f was amplified with the following pairs of oligonucleotides: 5'-CAGCAACATGCCGGCCATGGAAGACCGTGAA-3' and 5'-CTCGAGCACATGCCTTGGTACACTTC-3' from mouse small intestine cDNA. These oligonucleotides contained overlapping sequences for fusion with sequences encoding for the stalk region and transmembrane domain of Ly49a and the cytoplasmic domain of mouse CD3 ζ , amplified from an existing reporter construct (NKG2D-Ly49a-CD3 ζ). The resulting fusion products were equipped with SacII and XhoI restriction sites and cloned into an RSV.5neo expression vector containing C-terminal FLAG- and hexahistidine tags.

Reporter cell assay. BWZ.36 reporter cells, a kind gift of N. Shastri, University of California, Berkeley,²⁴ were cultured in RPMI 1640. Transfection of BWZ.36 cells was performed by electroporation using 20 μ g of plasmid DNA and a Gene Pulser Xcell (Bio-Rad, Munich, Germany) (250 V, 950 μ F) followed by selection with G418 containing medium (1.8 mg ml⁻¹). BWZ.36 reporter cells (1 $\times$ 10⁵ per well) were cocultured overnight with 1 $\times$ 10⁵ target cells in the presence of IgG, anti-Clr-f mAb 10A8, or anti-Nkrp1g mAb 8A10 (10 μ g ml⁻¹), respectively. Cells were lysed in the presence of chlorophenol red- β -D-galactopyranoside (final concentration: 150 μ M; Sigma, Steinheim, Germany) and β -galactosidase activity was determined by measuring absorbance at 571 nm. For controls, BWZ.36 reporter cells were cultured in medium or stimulated with 25 ng PMA (phorbol 12-

Figure 8 Upregulation of Ccr-f molecules on IEC subsequent to poly(I:C) treatment can be sensed by Nkrp1g. **(a)** Frozen sections of the small intestine of BALB/c mice treated with PBS (upper) or poly(I:C) (lower) stained with mAb 10A6 (yellow) and anti-EpCAM (red) and counterstained with DAPI (blue). **(b and c)** Flow cytometric analyses of IEC from PBS- or poly(I:C)-treated mice for Ccr-f surface expression (mAb 10A6). Representative dot plots **(b)** and compiled data from six mice per group **(c)**. **(d and e)** Flow cytometric analyses of IEL from PBS- or poly(I:C)-treated mice for Nkrp1g expression (mAb 8A10). Compiled data (six mice per group) for **(d)** frequencies of Nkrp1g⁺ cells or **(e)** MFI of Nkrp1g expression levels on Nkrp1g⁺ cells. **(f)** Nkrp1g-CD3ζ expression of BWN3G reporter cells assessed with mAb 8A10 (left panel). HCT116 cells stably transfected with FLAG-tagged Ccr-f or Ccr-b, or mock-transfected cells stained with FLAG-tag mAb M2 (solid lines). Isotype control stainings are shaded. **(g and h)** Functional recognition of Ccr-f **(g)** on HCT116-Clr-f cells or **(h)** on freshly isolated IEC from PBS- or poly(I:C)-treated BALB/c mice by cocultured BWN3G-Nkrp1g reporter cells. Specific recognition of Ccr-f was abrogated by addition of anti-Clr-f mAb 10A8 to the cocultures. Negative (medium) and positive controls (PMA/ionomycin) are also shown. One-way analysis of variance Tukey's *post hoc* ($***P < 0.0001$). Ccr, C-type lectin-related; DAPI, 4,6-diamidino-2-phenylindole; IEC, intestinal epithelial cell; IEL, intraepithelial lymphocyte; MFI, median fluorescence intensity; NS, not significant; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate.

myristate 13-acetate) and 1 μ M ionomycin. BWN3G reporter cells expressing chimeric Nkrp1g-CD3ζ molecules and assay conditions were described previously.¹⁵ A total of 5×10^4 BWN3G reporter cells were cocultured with 1×10^5 HCT116 or 1.25×10^5 IECs, respectively. Stimulator cells and reporter cells were incubated overnight in the presence of 10 μ g ml⁻¹ rat IgG1 or anti-Clr-f mAb 10A8. Reporter cells were analyzed for induction of EGFP expression by flow cytometry.

Isolation of IECs and IELs. IECs and IELs were purified with modifications as described previously.³⁷ Briefly, after removal of Peyer's patches, sections of the small intestines were washed with ice-

cold phosphate-buffered saline, incubated at 37°C in Hank's Balanced Salt Solution containing 1 mM EDTA and 1 mM DTT for 45 min and vortexed before enrichment of lymphocytes by Percoll gradient centrifugation (GE Healthcare, Munich, Germany). Small intestines of 6- to 12-week-old mice were used unless stated otherwise.

Recombinant proteins and antibodies. Soluble recombinant ectodomains of Nkrp1g (sNkrp1g: Pro66 through Val214) and Ccr-f (sCcr-f: Pro78 through Val218) were purified from supernatants of 293 cells stably transfected with the respective cDNA containing BirA-, c-myc-, and hexahistidine tags as previously reported for rNkp80.²⁹ For gel filtration analysis, sCTRL were loaded onto a HiLoad 16/60 Superdex

200 gel filtration column at a flow rate of 1 ml min⁻¹. Ectodomains were biotinylated using BirA ligase (Avidity, Aurora, CO) and, before use, either immobilized on streptavidin-coated microspheres (Bangs Laboratories, Fishers, IN) or tetramerized using fluorochrome-labeled streptavidin (Jackson ImmunoResearch, Newmarket, UK). Clr-f-specific mAb 10A6 and 10A8 were generated by immunizing LOU/C rats (Harlan, Boxmeer, The Netherlands) with sClr-f and resulting hybridoma screened for specific reactivity against CHO-Clr-f transfectants. Nkrp1g-specific mAb 8A10 and 4A2 were generated by immunizing CD rats (Charles River, Köln, Germany) with sNkrp1g and resulting hybridoma screened for specific reactivity against NK-92MI-Nkrp1g transductants.

Cytotoxicity, degranulation. CHO transfectants (targets, T) were labeled with ⁵¹Cr for 2 h at 37 °C and cocultured with NK-92MI transductants (effectors, E) for 4 h at 37 °C at different E:T ratios. Supernatant were mixed with OptiPhase Supermix scintillation cocktail (PerkinElmer, Waltham, MA) in an IsoPlate-96 and measured with a MicroBeta² plate counter (PerkinElmer). In blocking experiments, antibodies (anti-Clr-f mAb 10A8, isotype) were used at a final concentration of 10 µg ml⁻¹. Percent-specific lysis was calculated as follows: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Degranulation of NK-92MI transductants was quantified by analysis of surface CD107a after 1 h coculture with CHO transfectants in the presence of mAb 10A8 or an isotype control (10 µg ml⁻¹).

Immunohistology. Immunohistochemistry was performed on cryosections of small intestine samples from C57BL/6 mice. Sections were fixed in cold acetone for 10 min and subsequently dried at room temperature. Specimens were stained with the MaxHetero Rat on Mouse Polymer HRP Detection Kit (MaxVision Bioscience, Bothell, WA) following manufacturers' protocol with anti-Clr-f mAb 10A6 or respective isotype control as primary mAbs. Finally, cryosections were developed with DAB (3,3'-diaminobenzidine) and counterstained with Mayer's hematoxylin. For immunostaining, cryosections of mouse intestinal samples were fixed with acetone. Slides were air dried and rehydrated before blocking in 10% donkey or goat serum. Sections were incubated in the presence of the anti-Clr-f antibody (mAb 10A6) or hybridoma supernatant containing anti-Nkrp1g mAb 8A10 or 4A2 for 1 h at room temperature or the respective isotype control. Secondary antibody staining was performed using a Cy3-conjugated donkey anti-rat antibody (Jackson ImmunoResearch) or Cy3-conjugated goat anti-rat antibody (BioLegend, London, UK) for 0.5–1 h at room temperature. After washing, sections were stained with an APC-conjugated EpCAM antibody (eBioscience, Frankfurt, Germany) and fluoroscein isothiocyanate-conjugated phalloidin (Sigma) or with an Alexa647-conjugated EpCAM antibody (BioLegend). The specificity of the primary antibody was confirmed by preincubation for 1 h at room temperature in the presence or absence of 10 µg ml⁻¹ of sClr-f. Slides were mounted in 4,6-diamidino-2-phenylindole-containing Vectashield (Biozol, Eching, Germany). Tissue sections were visualized using an ApoTome-equipped AxioPlan 2 microscope connected to an AxioCam Mr digital Camera (Carl Zeiss MicroImaging, Jena, Germany) or a DMI 6000B microscope connected to a DFC3000 camera (Leica Microsystems, Heerbrugg, Switzerland).

Immunoblotting. For immunoblot analysis, transiently transfected 293T cells were lysed using lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40) containing the complete protease inhibitor cocktail (Roche). For deglycosylation, samples were treated with PNGase-F (New England Biolabs, Frankfurt, Germany) according to manufacturer's instructions for 1 h at 37 °C. Thirty micrograms of total lysate were separated by SDS-polyacrylamide gel electrophoresis in a Mini-PROTEAN Tetra Cell (Bio-Rad) and transferred to nitrocellulose membranes (GE Healthcare) by semi-dry blotting. Membranes were stained with FLAG-tag mAb M2 (Sigma) in a final

concentration of 1 µg ml⁻¹ followed by detection using a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch).

Flow cytometry and cell sorting. For analysis of cell surface expression, cells were washed with ice-cold FACS (fluorescence-activated cell sorting) buffer (phosphate-buffered saline, 2% fetal calf serum, 2 mM EDTA, 0.01% sodium azide) and Fc receptors were blocked with 30 µg ml⁻¹ mouse IgG (Sigma) for 20 min at 4 °C. Cells were then stained with relevant antibodies for 20 min at 4 °C and washed again after each staining step with FACS buffer. Soluble Clr-f-coated microspheres were also stained for 20 min at 4 °C. Flow cytometry analysis was performed with a FACS Canto II (BD Biosciences, Heidelberg, Germany) and data analyzed using FlowJo (Tree Star, Ashland, OH). Antibodies used: anti-CD3ε-PerCP, anti-CD103-PE, anti-γδTCR-PE (all from BioLegend), anti-human CD107a-APC, anti-human CD56-PE (all from BD Biosciences), FLAG-tag mAb M2 (Sigma), anti-Nkrp1g mAb 8A10, and anti-Clr-f mAb 10A6. Unconjugated antibodies were stained with Alexa647-conjugated F(ab)₂-fragments of mouse anti-rat IgG (Jackson ImmunoResearch). Single-cell suspensions of intraepithelial lymphocytes were sorted (purity > 98%) using a BD FACSaria III cell sorter (BD Biosciences).

Statistical analysis. Statistical analyses as detailed in the figure legends were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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