A Murine Intestinal Intraepithelial NKp46-Negative Innate Lymphoid Cell Population Characterized by Group 1 Properties

Graphical Abstract

Highlights
- Identification of an intraepithelial NKp46−Ly49E+ innate lymphoid population
- These NKp46−ILCs are dependent on T-bet expression and IL-15 signaling
- Their gene expression profiles differ from NK cells, iCD8α cells, and known ILC1s
- These NKp46−ILCs produce IFN-γ, suggesting a contribution to Th1-mediated immunity

Authors
Aline Van Acker, Konrad Gronke, Aindrila Biswas, ..., Ildiko Rita Dunay, Andreas Diefenbach, Georges Leclercq

Correspondence
georges.leclercq@ugent.be

In Brief
Van Acker et al. define an intestinal intraepithelial innate lymphoid cell population that is dependent on T-bet and IL-15 and displays a group 1 ILC gene profile uniquely different from NK cells, iCD8α cells, and previously described ILC1 cells. Upon stimulation, these cells produce the Th1-related cytokine IFN-γ.

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A A Murine Intestinal Intraepithelial NKp46-Negative Innate Lymphoid Cell Population Characterized by Group 1 Properties

Aline Van Acker,1,2 Konrad Gronke,3,4 Aindrila Biswas,5 Liesbet Martens,6 Yvan Saeyes,6 Jessica Filtjens,1 Sylvie Taveirme,1 Els Van Ammel,1 Tessa Kerre,1 Patrick Matthys,7 Tom Taghon,1 Bart Vandekerckhove,1 Jean Plum,1 Ildiko Rita Dunay,5 Andreas Diefenbach,5,6 and Georges Leclercq1,9,*

1Laboratory of Experimental Immunology, Ghent University, 9000 Ghent, Belgium
2Center for Infectious Medicine, Department of Medicine Huddinge, Karolinska Institutet, 171 77 Stockholm, Sweden
3Institute of Medical Microbiology and Hygiene, University Medical Center of the Johannes Gutenberg University Mainz, 55131 Mainz, Germany
4Max-Planck-Institute of Immunobiology and Epigenetics, 79108 Freiburg, Germany
5Institute of Inflammation and Neurodegeneration, Otto-von-Guericke University Magdeburg, 39120 Magdeburg, Germany
6VIB Inflammation Research Centre, 9000 Ghent, Belgium
7Laboratory of Immunobiology, Rega Institute for Medical Research, Department of Microbiology and Immunology, KU Leuven - University of Leuven, 3000 Leuven, Belgium
8Department of Microbiology, Charité - University Medical Centre Berlin, 12203 Berlin, Germany
9Lead Contact
*Correspondence: georges.leclercq@ugent.be
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SUMMARY

The Ly49E receptor is preferentially expressed on murine innate-like lymphocytes, such as epidermal Vγ3 T cells, intestinal intraepithelial CD8αα+ T lymphocytes, and CD49αα* liver natural killer (NK) cells. As the latter have recently been shown to be distinct from conventional NK cells and have innate lymphoid cell type 1 (ILC1) properties, we investigated Ly49E expression on intestinal ILC populations. Here, we show that Ly49E expression is very low on known ILC populations, but it can be used to define a previously unrecognized intraepithelial innate lymphoid population. This Ly49E-positive population is negative for NKp46 and CD8αα, expresses CD49α and CD103, and requires T-bet expression and IL-15 signaling for differentiation and/or survival. Transcriptome analysis reveals a group 1 ILC gene profile, different from NK cells, iCD8α cells, and intraepithelial ILC1. Importantly, NKp46 CD8αα Ly49Eαα* cells produce interferon (IFN)-γ, suggesting that this previously unrecognized population may contribute to Th1-mediated immunity.

INTRODUCTION

In recent years, several innate lymphoid cell (ILC) populations have been described. Characterized by the absence of rearranged antigen-specific receptors, these cells have been classified into group 1, 2, and 3 ILCs, with each group mirroring a respective T helper cell population (Fuchs et al., 2013; Klose et al., 2014; Robinette et al., 2015). Group 1 ILCs comprise natural killer (NK) cells and ILC1 that are defined by a dependence on the Eomes or T-bet transcription factors, respectively. These cells have the capacity to respond to interleukin (IL)-12, IL-15, and IL-18 to produce interferon (IFN)-γ (Fuchs et al., 2013; Klose et al., 2014). Group 2 ILCs are defined by a dependence on the transcription factors RORγt and RORα and the capacity to respond to IL-1β and IL-23 by the production of IL-22, IL-17A, and/or IL-17F (Fuchs et al., 2013; Klose et al., 2014; Robinette et al., 2015; Tait Wojno and Artis, 2012).

Abundant at mucosal barrier surfaces (Klose et al., 2014; Robinette et al., 2015), ILCs have been assigned important roles in a number of immunological processes. Whereas ILC1s have been implicated in the control of intracellular parasite infections (Klose et al., 2014; Schulthess et al., 2012) and gut inflammation (Bernink et al., 2013b; Fuchs et al., 2013), ILC2s are important in the clearance of helminth (Neill et al., 2010) and viral infections (Monticelli et al., 2011) and in the progression of asthma and lung allergies (Chang et al., 2011; Mjöberg et al., 2011; Nussbaum et al., 2013; Spits and Cupedo, 2012; Klein Wolterink et al., 2012). ILC3s, in turn, contribute to tertiary lymphoid organogenesis (van de Pavert and Mebius, 2010), the containment of commensal bacteria (Sonnenberg and Artis, 2012), and clearance of bacterial infections in the gut (Cella et al., 2009; Diefenbach, 2013; Klose et al., 2013; Satoh-Takayama et al., 2008; Sonnenberg et al., 2011), and they have been implicated in the pathology of inflammatory bowel disease (Bernink et al., 2013b; Buonocore et al., 2010; Geremia et al., 2011). It should be noted that some of the described functions are attributable to ex-RORγt ILC3s (Bernink et al., 2013b; Vonarbourg et al., 2014).
where bidirectional functional plasticity between ILC1 and ILC3 classes in response to distinct environmental stimuli was recently demonstrated (Bemink et al., 2015). Whereas group 2 ILCs appear to be the most homogeneous cells, considerable heterogeneity exists within group 1 and 3 ILCs. In the gut, a distinction can be made within group 1 ILCs among conventional natural killer (cNK) cells, lamina propria ILC1 (Klose et al., 2014) and intraepithelial ILC1s (Fuchs et al., 2013), and ex-RORγt ILC3s (Bemink et al., 2013b; Vonarbourg et al., 2010). The cNK cells are considered to be CD27+/CD127+/IL-7Rα- NKp46− NK1.1+ cells that express both Eomes and T-bet. Whereas cNK cells are dramatically reduced in Eomes−/− × Vav-Cre mice (Gordon et al., 2012), they are only slightly reduced in Tbx21−/− mice (Klose et al., 2014; Townsend et al., 2004). NK cells are cytotoxic in nature, and they may also be stimulated to produce the signature cytokine IFN-γ. Intraepithelial ILC1 (Fuchs et al., 2013) and lamina propria ILC1 (Klose et al., 2014) have a CD27+/CD127+/NKp46+/NK1.1+ phenotype. Intraepithelial ILC1s, similar to NK cells, express both Eomes and T-bet. However, in contrast to cNK cells, intraepithelial ILC1s are largely independent of IL-15 signaling. Importantly, in vitro stimulation with IL-12, IL-15, and IL-18, or in vivo infection, induces IFN-γ expression by these cells, confirming the ILC1-like nature and the capacity to contribute to Th1-mediated immune responses.

RESULTS

Ly49E is Primarily Expressed on NKp46-Negative Intestinal IEL

We have previously shown that the Ly49E receptor is expressed on several innate-like cell populations in adult C57BL/6 mice (Filtjens et al., 2013; Taveirne et al., 2011; Van Acker et al., 2014; Van Beneden et al., 2002). Recent years have seen the discovery of a number of innate lymphoid populations, i.e., the ILCs, with particular diversity of these cells in the small intestine (Robinet et al., 2015). Klose et al. (2014) demonstrated that intestinal ILC populations may be successfully segregated on the basis of CD27 and CD127 expression. As such, we set out to investigate whether Ly49E is expressed on small intestinal ILC subsets. Our results showed that Ly49E was expressed only by a small subpopulation of intraepithelial NKp46+/NK1.1+ ILCs: 2%–5% of CD27+CD127− cNKs expressed Ly49E. Ly49E expression was even lower on CD27+CD127+ ILC1s, and it was almost absent on CD27−CD127+ ILC3s. A small number of CD27−CD127− cells could also be detected within the NK1.1+NKp46−gated lymphocytes. We hypothesized that these cells represent terminally differentiated NK cells, as loss of CD27 expression on such cells in the periphery has previously been reported (Hayakawa and Smyth, 2006). Comparable to CD27− NK cells, a mean of 2.5% of CD27−CD127− NK cells expressed Ly49E (data not shown). Similarly, Ly49E expression was low/absent on lamina propria NKp46− NK1.1+ ILC populations (Figure 1A). Importantly, Ly49E expression was most abundant on the NKp46− subpopulation of intraepithelial CD3− CD45− cells. Whereas the percentage of Ly49E-expressing cells in the total CD3− CD45− NKp46− population was relatively low (Figure 1A), the absolute number of these cells was 7-fold higher as compared to the Ly49E-expressing CD3− CD45− NKp46− intestinal IEL fraction (Figure 1B). Even higher absolute numbers of CD3− CD45−Ly49E+ IELs were present in the small intestine of Rag−/− and NOD-SCID mice (Figures 1C and 1D), confirming their innate nature. Therefore, for practical purposes, all future experiments were performed with small intestinal IELs of Rag−/− or NOD-SCID mice.

NKp46-Negative Ly49E+ IELs Have an ILC-like/Non-NK Surface Phenotype and a Granular Lymphoid Morphology

Based on CD4, CD8β, and CD8x expression, two main subpopulations of CD3− CD45− NKp46− Ly49E+ cells could be distinguished: that were CD8β− and CD8β+, respectively (Figure 2A). We further performed a detailed phenotypic characterization of these two populations and of spleen NK cells for comparison. Both CD8β− and CD8β+ Ly49E-expressing populations were CD49a−CD103− (Figure 2B), suggestive of a tissue-resident intraepithelial ILC phenotype (Fuchs et al., 2013; Robinette et al., 2015). A fraction of cells from both populations expressed CD49b but at lower levels compared to NK cells. Furthermore, both populations were CD127− CD27− CD11bint CD11cint.
Figure 1. Ly49E Expression in NKp46⁺ and NKp46⁻ Innate Lymphocytes in the Intestinal Epithelium and Lamina Propria

(A) Flow cytometric analysis of small intestinal innate IELs and LPLs from WT mice. cNK-, ILC1-, ILC3-, and NKp46-negative cells were gated as indicated and analyzed for Ly49E expression.

(B) Absolute numbers (mean ± SEM; n = 5) of small intestinal intraepithelial NKp46⁺ cNK cells, ILC1s, and ILC3s and NKp46⁻ cells in WT mice and the absolute numbers of Ly49E-expressing cells therein.

(C) Flow cytometric analysis of small intestinal IEL from WT, Rag⁻/⁻, and NOD-SCID mice. NKp46⁻ cells were first gated for Ly49E expression and analyzed for CD3 and CD45 expression.

(D) Absolute numbers (mean ± SEM; n = 5) of CD3⁺CD45⁺NKp46⁺Ly49E⁺ cells in the small intestinal epithelium of C57BL/6, Rag⁻/⁻, and NOD-SCID mice. Statistical analysis was performed using the two-tailed Mann-Whitney U test (**p < 0.001). (A and C) Numbers in the dot plots represent the percentage of cells in the indicated gate or quadrant. Data are representative of five independent experiments.
Figure 2. Surface Marker Expression of Intestinal NKp46-Negative CD8αα+ Ly49E+ and CD8αα-C0 Ly49E+ IELs

(A) CD8αα-C0 and CD8αα+ subpopulations were identified in intestinal CD45+NKp46-C0 Ly49E+ IELs from Rag−/− mice, as indicated.

(B) Surface expression profile of CD8αα+ Ly49E+ (red line) and CD8αα-C0 Ly49E+ (blue line) cells, gated as in (A) and stained with the indicated antibodies. The black line represents spleen NK cell staining. Gray-shaded histograms represent Fluorescence Minus One controls. Data are representative of three independent experiments.
CD8α+ Ly49E+ LECEs were sorted to high purity (>88%) from small intestinal IELs of NOD-SCID mice. Intestinal intraepithelial CD3+CD45+ T lymphocytes were sorted from WT mice. Cells were fixed and stained with Giemsa.

![Figure 3. Intestinal CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs Have a Lymphoid Morphology](image)

**Intestinal NKp46-Negative CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs Have a Distinct Transcription Factor Expression Profile and Dependency**

ILCs may be classified into group 1, 2, or 3 ILCs on the basis of transcription factor expression and dependency. Intracellular staining of intestinal CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs revealed that both populations lacked Eomes expression, in contrast to NK cells, but expressed T-bet. Whereas a very small proportion of CD8α+ Ly49E+ cells was RORγt positive, CD8α+ Ly49E+ cells did not express RORγt (Figure 4A). When CD8α+ Ly49E+ and CD8α+ Ly49E+ cells were isolated from RORγt-fate map (fm) mice, a minor fraction of cells from each subpopulation was RORγt+ (Figure 4B), suggesting that only a few cells are ex-RORγt+ ILC3s that have lost RORγt expression (Bernink et al., 2013b). Alternatively, the minor fraction of RORγt or RORγt+ cells in the CD8α+ Ly49E+ IELs could be contaminating LTi cells from the lamina propria. Complete separation of epithelium and lamina propria gut fractions is technically challenging with the currently available methods, and as such a small number of LTi RORγt+ cells may account for the RORγt signal in a minor fraction of CD8α+ Ly49E+ IELs.

To analyze the transcriptional programs required for differentiation or maintenance of Ly49E+ ILCs, the cell numbers of intestinal CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs, and as controls the cNK, ILC1, and NKp46+ ILC3 populations, were determined in Eomes−/−, Tbx21−/−, and Rorc(γt)−/−. As compared to wild-type (WT) mice, even greater numbers of CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs were present in Eomes−/− mice. It has previously been observed that in mice genetically lacking one ILC population, other ILC populations are increased (Sanos et al., 2009). At current, the mechanistic basis for this finding is unknown. We hypothesize that this may be due to competition of ILC subsets for space or higher availability of ILC-sustaining cytokines, such as IL-15. Additionally, the lack of competition with NK cells for IL-15 in Eomes−/− mice could support not only expansion but also survival of these IELs. Tbx21−/− mice had strongly reduced numbers of CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs. Numbers of CD8α+ Ly49E+ and CD8α+ Ly49E+ cells were unaffected in Rorc(γt)−/− mice. Control populations showed the expected transcription factor dependency, as NK cells were virtually absent in Eomes−/− mice, whereas ILC1s and NKp46+ ILC3s were strongly reduced or absent, respectively, in Tbx21−/− mice, and ILC3s were strongly reduced in Rorc(γt)−/− mice (Figure 4C).

**NKp46-Negative CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs Are Dependent on IL-15, but Not IL-7, Signaling**

Previous studies have shown that ILCs require signaling through the common gamma chain (γc) for their survival (Bernink et al., 2013a, 2013b; Satoh-Takayama et al., 2010). To investigate the cytokine dependency of intestinal CD8α+ Ly49E+ and CD8α+ Ly49E+ IELs, we determined their cell numbers in Il12g (γc)−/−, Il17a−/−, and Il15ra−/− mice. As shown in Figure 4C, in CD8α+ Ly49E+ IELs were increased in number in Il12g−/− mice and absent in Il17a−/− and Il15ra−/− mice. This is in agreement with the absence of CD127 on these cells but intermediate levels of CD122 expression (Figure 2B). Increased ILC numbers have previously been reported in the gut of Il7r−/− mice (Klose et al., 2014; Vonarnbourg et al., 2010). CD8α+ Ly49E+ IELs were similarly independent of IL-7 and dependent on IL-15 signaling (Figure 4F), as previously reported for iCD8α cells (Van Kaer et al., 2014).
lamina propria-localized ILC1s and NK cells and partially separated from iCD8α and from IEL, liver, or spleen ILC1s. CD8αα-Ly49E+ IELs did not cluster with ILC2 and ILC3 populations. CD8αα+Ly49E+ IELs clustered with iCD8α cells (Figure 6A).

More detailed gene expression analysis showed that CD8αα-Ly49E+ IELs, in contrast to CD8αα+Ly49E+ IELs, showed increased transcript levels of Rorc, Rora, and Eomes. In accordance with the flow cytometric data, Tbx21 (T-bet) expression levels were high in CD8αα-Ly49E+ IELs, and they were comparable to those of NK and intestinal intraepithelial and lamina propria ILC1 populations. Both CD8αα-Ly49E+ and CD8αα+Ly49E+ IEL subsets expressed high levels of Id2, which is required for the development of all ILC subsets (Spits and Cupedo, 2012) (Figure 6B). Cytokine and cytokine receptor expression profiles indicated that CD8αα-Ly49E+ IELs showed increased expression of the chemokine receptor CCR9. Both CD8αα-Ly49E+ and CD8αα+Ly49E+ IELs expressed high levels of the chemokines CCL3 (MIP1α) and CCL5 (RANTES) (Figure 6C). CD8αα-Ly49E+ IELs showed increased expression of Ifng and Tnf as compared to CD8αα+Ly49E+ IELs. Additionally, CD8αα-Ly49E+ IELs differed significantly from CD8αα+Ly49E+ IELs in the expression of cytokine receptors, where CD8αα-Ly49E+ IELs highly expressed Il2ra, Il17r, and Il18r1, while only low expression of these genes could be detected in CD8αα+Ly49E+ IELs. Il2rb and Il12rb were expressed at high and intermediate levels, respectively, in both populations. CD8αα-Ly49E+ IELs expressed Prf1, Grzma, and Grzmb, but levels were lower than in CD8αα+Ly49E+ IELs (Figure 6D). Finally, comparative analysis of the expression levels of genes of the recently described NK cell-distinct ILC core signature (Robinette et al., 2015) showed that these genes were expressed.

Figure 4. Transcription Factor Expression and Dependency of Intestinal CD8αα-Ly49E+ and CD8αα+Ly49E+ IELs

(A) Expression of Eomes, T-bet, and RORγt by intestinal CD8αα-Ly49E+ (red line) and CD8αα+Ly49E+ IELs (blue line) and spleen NK cells (black line) of NOD-SCID mice. The light shaded histogram represents non-stained cells.

(B) Analysis of small intestinal CD8αα-Ly49E+ (red line) and CD8αα+Ly49E+ IELs (blue line) and spleen NK cells (black line) from RORγt-fate map (Rorcγt-CreTg3 × Rosa26Rfluorescent reporter mice). The expression of RORγt-fate map (fm) is shown in the indicated populations. (A and B) Data are representative of three independent experiments.

(C) Absolute numbers (mean ± SEM; n = 2–3) of the indicated IEL populations in WT, EomesΔΔ, Tbx21ΔΔ, and RorcγtΔΔ mice. Statistical analysis was performed using the two-tailed Mann-Whitney U test (**p < 0.01 and ***p < 0.001).
CD8α− Ly49E+ Cells Do Not Kill YAC-1 Cells but Produce IFN-γ

Both intestinal CD8α− Ly49E+ and CD8α+ Ly49E− IEL populations contain cytoplasmic granules (Figure 3) and express Prf1, GzmA, and GzmB mRNA (Figure 6D). Intracellular flow cytometric analysis confirmed perforin, granzyme A, and granzyme B expression at the protein level (Figure 7A), indicating that these cell populations may have cytotoxic potential. We therefore sorted these cells to high purity and tested their capacity to kill NK-sensitive YAC-1 cells. Neither CD8α− Ly49E+ nor CD8α+ Ly49E− IELs killed YAC-1 cells, whereas the tested control populations, i.e., intestinal intraepithelial and splenic NK cells, displayed efficient cytotoxicity (Figure 7B). Collectively, this indicates that the CD8α− Ly49E+ and CD8α+ Ly49E− IELs are potentially cytotoxic but that they do not have the capacity to kill YAC-1 cells.

As CD8α− Ly49E+ IELs displayed intermediate expression of IL7ra and strong expression of Il15ra and Il18ra, we stimulated intestinal IELs in vitro with IL-12, IL-15, and/or IL-18 and determined IFN-γ expression. Whereas CD8α+ Ly49E− IELs expressed moderate levels of IFN-γ following stimulation with IL-12 + IL-15 + IL-18, CD8α− Ly49E+ IELs were avid producers of IFN-γ (Figure 7C). With respect to the function of the Ly49E receptor in the immune system, our group has conclusively demonstrated that Ly49E is an inhibitory receptor in NK cells, restricting NK cell cytotoxicity and cytokine production upon interaction with its ligand uPA (Van Den Broeck et al., 2008). We therefore also stimulated the cells with IL-12 + IL-15 + IL-18 in the presence of uPA or BSA as a negative control. The presence of uPA did not alter IFN-γ production of cytokine-stimulated CD8α+ Ly49E− or CD8α+ Ly49E+ IELs (Figure 7D).

These results suggest that CD8α− Ly49E+ IELs, similarly to NK cells and ILC1s, may be involved in mediating Th1-type immunity. To test this, we assessed a putative function for these cells in mediating T. gondii immunity. Our results indicate that CD8α− Ly49E+, but not CD8α+ Ly49E−, IELs contribute in immunity to T. gondii infection through production of IFN-γ (Figure 7E). As a control, we confirmed that IFN-γ was produced by lamina propria ILC1s upon T. gondii infection (data not shown), as previously described by Klose et al. (2014).

DISCUSSION

The intestinal epithelium harbors a number of lymphocyte populations, including innate-like and adaptive T cells, NKp46-expressing NK cells and ILC1s, and the recently described iCD8α cells (Fuchs et al., 2013; Van Kaer et al., 2014). In this paper, we show that the intestinal epithelium is also home to a previously unrecognized CD3− innate population that can be minimally characterized as NKp46 CD8α+ Ly49E+ . Further study of these cells revealed a lymphoid morphology and a...
Figure 6. The Transcriptome of Intestinal CD8αα/Ly49E+ IEL Clusters with that of Lamina Propria-Localized ILC1s and NK Cells

Intestinal CD8αα/Ly49E+, CD8αα/Ly49E+, and CD8αα/Ly49E− IELs, additionally gated as CD3− CD45+, were sorted to high purity (>98%) from NOD-SCID mice in three different experiments. Transcriptome analysis was performed, and it was compared to publicly available gene expression data of intestinal IELs and/or lamina propria NK, ILC1, ILC2, NKP46+ ILC3, and CD4+ or CD4− ILC3 lymphoid tissue inducer (LTi) cells (see the Experimental Procedures).

(A) Non-supervised hierarchical clustering of the indicated populations based on all expressed genes is shown.
(B–E) Comparative analysis of individual gene transcript expression between the indicated cell subpopulations for the indicated gene groups.
CD103^+CD127^− surface phenotype. CD103 is expressed by intestinal epithelium-homing leukocytes, including T cells and iCD8α cells. As such, CD103 expression reflects the intraepithelial localization of CD8αα^−Ly49E^+ IELs. CD127 surface expression may be used to identify ILCs, except NK cells, in many mouse tissues (Robinette et al., 2015). CD8αα^−Ly49E^+ IELs did not express cell surface CD127. However, microarray analysis revealed large numbers of *Il7r* transcripts, suggesting that *Il7r* expression may not, or not sufficiently, be translated onto the protein level in these cells to allow flow cytometric detection. Alternatively, CD8αα^−Ly49E^+ IELs may reside in an IL-7φ environment and downregulate CD127. As intraepithelial ILC1 and iCD8α cells have also been shown to lack detectable surface expression of CD127 (Fuchs et al., 2013; Van Kaer et al., 2014), it is worthwhile to consider that absence of CD127 on the surface membrane of these three cell populations may be a feature of their intraepithelial localization, whereas ILCs located in the lamina propria are CD127^+ (Klose et al., 2014). This notion is further corroborated by data from human studies, which have also described the existence of intraepithelial...
CD103+ ILC1 cells that are CD127lo− (Hazenberg and Spits, 2014).

Specification of intestinal CD8αα'Ly49E+ IELs by hierarchical clustering indicated that these cells showed close similarities with intestinal lamina propria Nkp46+ NK cells and Nkp46+ ILC1s but differed from ILC2 and ILC3 subsets. Detailed cytokine and effector molecule gene expression analysis confirmed a group 1 ILC profile for CD8αα'Ly49E+ IELs, with the presence of large numbers of transcripts for Ccl3, Ccl5, Ifng, Tnf, Gzma, and Gzmb. We also demonstrated that CD8αα'Ly49E+ IELs were IL-15 dependent. As NK cells as well as lamina propria ILC1s, but not intraepithelial ILC1 cells, are dependent on IL-15 signaling (Fuchs et al., 2013; Klose et al., 2014; Van Kaer et al., 2014), these data further support the group 1 ILC-like nature of CD8αα'Ly49E+ IELs. However, intestinal CD8αα'Ly49E+ IELs are not NK cells. Comparing our data with the recently described NK cell-distinct core signature, i.e., a set of transcripts common to ILC1 from all tissues that are more highly expressed than in NK cell subsets and that also show high expression in ILC2 and ILC3 subsets (Robinette et al., 2015), CD8αα'Ly49E+ IELs showed strong expression of the NK cell-distinct ILC core signature genes, whereas CD8αα'Ly49E+ IELs displayed low expression of NK cell-specific signature genes. Other arguments are that, contrary to NK cells, CD8αα'Ly49E+ IELs have an Nkp46− NK1.1+ phenotype and express CD49a, a marker present on tissue-resident ILC1s and absent on NK cells (Klose et al., 2014; Robinette et al., 2015). In addition, these cells do not express the typical NK receptor NKG2D, and, most importantly, CD8αα'Ly49E+ IELs develop independently of Eomes and are dependent on T-bet. Finally, although CD8αα'Ly49E+ IELs contain cytoplasmic granules and express perforin, granzyme A, and granzyme B, they do not kill the NK-sensitive YAC-1 cells.

There are also important differences between the intestinal CD8αα'Ly49E+ IELs described in the present paper and the previously described intestinal intraepithelial and lamina propria ILC1s. In contrast to CD8αα'Ly49E+ IELs, murine intestinal intraepithelial ILC1s have an Nkp46NK1.1+ phenotype and are negative for CD103. In addition, intraepithelial ILC1s are largely independent of IL-15Rx (Fuchs et al., 2013), which is opposite to what we observed for CD8αα'Ly49E+ IELs. Alongside and unlike CD8αα'Ly49E+ IELs, lamina propria ILC1s have an Nkp46NK1.1+ phenotype and express CD127, CD90, and CD117 (Klose et al., 2014). Thus, although CD8αα'Ly49E+ IELs have group 1 ILC characteristics, including their overall transcriptional profile, these cells have several crucial differences when compared to NK cells and previously described intestinal ILC1s.

CD8αα'Ly49E+ IELs, in contrast to CD8αα'Ly49E+ IELs, showed increased transcript levels of the transcription factors RORγt, RORα, and Eomes. As these three transcription factors are associated with the ILC3, ILC2, and NK cell lineages, respectively, this could indicate that CD8αα'Ly49E+ IELs represent a developmental intermediate and/or an activation-induced fraction of some larger, previously described population. Therefore, we tested the in vivo stability of this population upon transfer to immunodeficient recipients. The results show that CD8αα'Ly49E+ IELs represent a stable population, with 80%–97% retention of Ly49E expression.

A role for both intraepithelial and lamina propria ILC1s in mediating Th1-type immune response has been demonstrated (Fuchs et al., 2013; Klose et al., 2014). Intestinal Nkp46-negative CD8αα'Ly49E+ IELs expressed Il12r, Il2rb, and, in comparison to Nkp46-expressing NK cells and Nkp46+ ILC1s, expressed even higher Il18r1 levels, which may reflect their ability to be readily stimulated by IL-18 in conjunction with IL-12 and/or IL-15 to express IFN-γ. We evaluated IFN-γ production by these cells following incubation in the presence of IL-12 alone, IL-12 + IL-15, or IL-12 + IL-15 + IL-18. Whereas IL-12 alone induced IFN-γ, there was a progressive increase when IL-15 and IL-15 + IL-18 were added. As we have previously shown that uPA triggers the Ly49E receptor (Van Den Broeck et al., 2008), we also stimulated the Ly49E+ IELs with IL-12 + IL-15 + IL-18 in the presence of uPA or BSA as a negative control. The presence of uPA did not alter IFN-γ production of cytokine-stimulated CD8αα'Ly49E+ or CD8αα'Ly49E+ IELs. Therefore, at present, we do not have evidence for a functional role of the Ly49E receptor on CD8αα'Ly49E+ IELs. However, this does not preclude a function for Ly49E. We have shown before that Ly49E expression on NK cells restrains IFN-γ production and CD107 expression in an Ly49E-dependent manner (Van Den Broeck et al., 2008). IFN-γ induction in these experiments required stimulation of cells through an activating NK receptor. This can be generalized to the function of the other inhibitory NK receptors, as their inhibitory function has always been demonstrated in combination with triggering of an activating NK receptor. To the best of our knowledge, there have been no reports of inhibitory NK receptor function in cytokine-mediated activation. Moreover, it has been shown by others that IL-12 as well as IL-18 override the inhibitory Ly49G2 receptor blockade for IFN-γ production in Ly49D-triggered or tumor cell-triggered NK cells, both in vitro and in vivo (Ortaldo and Young, 2003). Therefore, a possible Ly49E function on CD8αα'Ly49E+ IELs should not be discounted at this stage, but additional work is needed in this context.

To discern whether CD8αα'Ly49E+ IELs contribute to Th1-mediated immunity in vivo, we evaluated IFN-γ production by these cells 4 days following infection of NOD-SCID mice with the parasite T. gondii. We show that CD8αα'Ly49E+ IELs express IFN-γ in this model, more so than CD8αα'Ly49E+ IELs, corroborating our in vitro data.

In parallel, we showed that intestinal CD8αα'Ly49E+ IELs had several characteristics in common with iCD8x cells. CD8αα'Ly49E+ cells had a CD45+CD11b+CD11c+CD103+Grzma−/− NKp46−NK1.1− phenotype, as also described for iCD8x cells (Van Kaer et al., 2014). Furthermore, we showed that these cells express CD49a, suggesting a tissue-resident ILC1-like phenotype (Fuchs et al., 2013; Klose et al., 2014; Robinette et al., 2015). CD8αα'Ly49E+ IELs had a lymphoid morphology and were granular, in accordance with high expression of Gzma and Gzmb of iCD8x cells (Van Kaer et al., 2014). As well as this, CD8αα'Ly49E+ IELs were dependent on IL-15-mediated signaling, and total numbers were strongly reduced in γc−/− mice. CD8αα'Ly49E+ IELs expressed Id2, as do iCD8x cells. However, our microarray data indicated lower T-bet expression levels for CD8αα'Ly49E+ as compared to CD8αα'Ly49E+ IELs, lamina propria NK cells, and ILC1s, and CD8αα'Ly49E+ cells were T-bet dependent, whereas iCD8x are reportedly T-bet independent (Van Kaer et al., 2014).
In comparison with CD8α+Ly49E+ IELs, CD8α−Ly49E− IELs also had higher gene expression of Ccr9, Ifng, Tnf, Il2ra, Il7r, Il18r, Il1r1, Pod1fl, Tmem176a, and Tmem176b.

Conclusively, we have defined an intestinal intraepithelial ILC1-like population that is distinct from the previously described NKp46−/NK1.1+CD160+ intraepithelial ILC1 population (Fuchs et al., 2013), the NKp46+ NK1.1+CD127+ lamina propria ILC1 population (Kloese et al., 2014), and also distinct from NK cells. Of note, a recent paper also describes the existence of a population of salivary gland ILC1 groups 1 ILCs distinct from both NK and ILC1s, thus highlighting the heterogeneity of group 1 ILCs at multiple mucosal sites (Cortez et al., 2016).

Moreover, a contemporary paper describes the presence of non-circulating innate-like T cell receptor αβ and γδ lymphocytes, in addition to ILC1-like cells, in mammary tissue (Dadi et al., 2016). Both mammary innate-like T cells and ILC1-like cells express NK1.1, CD103, and CD49a; express high T-bet levels; are negative for CD127; and are IL-15 dependent. Thus, these cells show many similarities to the intestinal NKp46−/NK1.1−/CD160− IELs described in this paper. Most intriguingly, mammary-resident innate-like T cells and ILC1-like cells express Ly49E (Dadi et al., 2016). We therefore conclude that Ly49E receptor expression is characteristic for and restricted to specific tissue-resident innate-like lymphocyte populations, including intestinal CD8α− cells (Van Kaer et al., 2014), intestinal innate-like CD8α+ γδ T lymphocytes (Shires et al., 2001; Taveirne et al., 2011; Van Acker et al., 2014), liver CD49a+ NK cells (Filijens et al., 2013), skin epidermal γδ T lymphocytes (Van Beneden et al., 2002), mammary gland innate-like IELs and ILC1-like cells (Dadi et al., 2016), and intestinal NKp46−/CD8α−/Ly49E− IELs (this paper).

**EXPERIMENTAL PROCEDURES**

**Mice**

C57BL/6 WT, BALB/c NOD-SCID, and C57Bl/6 Rag−/− and Rag−/−γc−/− mice were purchased from Charles River Laboratories. Eomes−−/− (EomesCre/+ x T-Cre) (Arnold et al., 2008; Perantoni et al., 2005), Tbx21−/− (Szabo et al., 2002), Rorcγt−/−/C0 (Eberl et al., 2004), Il7r−/− (Peschon et al., 1994), Il15ra−/− (Kennedy et al., 2003), and RORγt−/−tm (Rorcγt−/−Cre+ x Rosa26Rcre−/−) mice (Eberl and Littman, 2004) were on a C57BL/6 background. Both females and males were used at the age of 2–6 months. All animal experimentation was performed after approval and according to the guidelines of the Ethical Committee for Experimental Animals at the Faculty of Medicine and Health Sciences of Ghent University, the Landesuntersuchungsamt Koblenz, and the Landesverwaltungsamt Sachsen-Anhalt.

**Intestinal IEL and Lamina Propria Lymphocyte Isolation**

IELs were isolated from the small intestine as described previously (Van Acker et al., 2014) (see the Supplemental Experimental Procedures). Briefly, small intestinal fragments were mechanically digested in a Ca/Mg-free buffer, and IELs were enriched by density gradient centrifugation. Thereafter, the small intestinal fragments were dispase/collagenase treated, and lamina propria lymphocytes (LPLs) were isolated by density gradient centrifugation (see the Supplemental Experimental Procedures).

**Immunofluorescence Staining and Antibodies**

Following isolation, cells were labeled with antibodies for flow cytometric analysis (see the Supplemental Experimental Procedures). Flow cytometry was performed using a BD LSRII flow cytometer, and samples were analyzed with FACSDivia Version 6.1.2 software (BD Biosciences) or FlowJo 8.7.1.

**Cell Sorting and Cytospin**

CD3−/CD45−/CD8α−/Ly49E− and CD3−/CD45−/CD8α−/Ly49E− IELs were sorted to high purity (>98%) from the small intestine of NOD-SCID mice using a BD FACSaria III cell sorter (BD Biosciences). For cytospins, cells were spun onto a glass slide, fixed, and stained with Giemsa.

**Microarray**

Small intestinal CD3−/CD45−/CD8α−/Ly49E−, CD3−/CD45−/CD8α−/Ly49E−, and CD3−/CD45−/CD8α−/Ly49E− IELs of NOD-SCID mice were sorted to high purity (>98%) in three independent experiments using a BD FACSaria III cell sorter (BD Biosciences). Transcriptome analysis was performed using the Affymetrix Mouse Gene 1.0ST Array (Affymetrix) (see the Supplemental Experimental Procedures).

**In Vitro Stimulation**

Intestinal IELs were isolated from NOD-SCID mice and cultured in complete RPMI medium in the presence of IL-12 (20 ng/mL; R&D Systems), and/o IL-15 (10 ng/mL; R&D Systems), and/o IL-18 (2.5 ng/mL; Medical and Biological Laboratories) for 15 hr. Alternatively, wells were precoated with uPA (Molecular Innovations) or BSA at 200 ng/mL for 6 hr, before the different cell populations were cultured in the presence of different cytokine combinations for an additional 15 hr. In both experimental conditions, Brefeldin A (Golgistop, BD Biosciences) (1 μg/mL) was added during the last 12 hr. Cells were harvested and stained as described.

**Cytotoxicity Assay**

Freshly isolated NOD-SCID intestinal intraepithelial NK (CD3−/CD45−/NKp46+), CD3−/CD45−/NKp46+ CD8α−/Ly49E−, and CD3−/CD45−/NKp46+ CD8α−/Ly49E− cell populations and spleen NK cells were sorted to high purity (>98%) and used as effectors. Killing of the NK-sensitive YAC-1 (European Collection of Cell Cultures) target cells was assessed in a standard 51Cr-release assay, as previously described (Filijens et al., 2013). Percentage specific lysis was calculated as 100 x ([experimental release – spontaneous release]/[total release – spontaneous release]).

**T. gondii Infection**

T. gondii cysts of type II strain ME 49 were harvested from the brains of female NMRI mice infected intraperitoneally with T. gondii cysts 8–10 months earlier. Brains obtained from infected mice were mechanically homogenized in 1 mL sterile PBS, and the cysts were counted using a light microscope. NOD-SCID mice were infected by oral gavage with ten T. gondii cysts.

**Statistical Analysis**

Statistical analysis was carried out using PASW Statistics 22 Software (SPSS) or GraphPad Prism5 Software. Data were analyzed using the non-parametric two-tailed Mann-Whitney U test. A p value ≤ 0.05 was considered statistically significant.

**ACCESSION NUMBERS**

The accession number for the array data reported in this paper is GEO: GSE83895.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and one figure and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.04.068.

**AUTHOR CONTRIBUTIONS**

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