Ly49E Expression on CD8αα-Expressing Intestinal Intraepithelial Lymphocytes Plays No Detectable Role in the Development and Progression of Experimentally Induced Inflammatory Bowel Diseases

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Abstract

The Ly49E NK receptor is a unique inhibitory receptor, presenting with a high degree of conservation among mouse strains and expression on both NK cells and intraepithelial-localised T cells. Amongst intraepithelial-localised T cells, the Ly49E receptor is abundantly expressed on CD8αα-expressing innate-like intestinal intraepithelial lymphocytes (iIELs), which contribute to front-line defense at the mucosal barrier. Inflammatory bowel diseases (IBDs), encompassing Crohn’s disease and ulcerative colitis, have previously been suggested to have an autoreactive origin and to evolve from a dysbalance between regulatory and effector functions in the intestinal immune system. Here, we made use of Ly49E-deficient mice to characterize the role of Ly49E receptor expression on CD8αα-expressing iIELs in the development and progression of IBD. For this purpose we used the dextran sodium sulphate (DSS)- and trinitrobenzenesulfonic-acid (TNBS)-induced colitis models, and the TNFalpha ileitis model. We show that Ly49E is expressed on a high proportion of CD8αα-positive iIELs, with higher expression in the colon as compared to the small intestine. However, Ly49E expression on small intestinal and colonic iIELs does not influence the development or progression of inflammatory bowel diseases.

Introduction

Inflammatory bowel diseases (IBDs), encompassing Crohn’s disease and ulcerative colitis, are chronic and relapsing disorders of the gastrointestinal tract [1]. Although the etiology of IBD is incompletely understood, inflammatory bowel disorders are believed to present in genetically predisposed individuals exposed to undefined microbial and environmental triggers. In this, IBD pathogenesis has been linked to deregulation of the fine homeostatic balance that exists between the mucosal immune system and commensal microbiota [2–5]. With the highest worldwide prevalence, European figures show an estimated 1 in 200 people affected by ulcerative colitis, and 1 in 300 affected by Crohn’s disease [6,7]. Current IBD treatment options include the administration of anti-inflammatory drugs, immunosuppressives and immunobiological agents [8,9]. For unresponsive patients, surgical intervention may provide a temporary relief from symptoms. However, specificity is lacking in these modes of treatment, and none are capable of inducing complete remission [8,10]. Therefore, additional research is required to further elucidate IBD mechanisms and facilitate the development of specific and effective new therapies.

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Intestinal intraepithelial lymphocytes (iIELs) are resident lymphocytes of the intestinal epithelium, and constitute one of the largest lymphocyte populations of the body [11]. In mice, five main subpopulations of iIELs have been identified: CD4, CD8β and CD8αα T cell receptor (TCRαβ iIELs, and CD4/CD8 double-negative (DN) and CD8αα TCRγδ iIELs. TCRαβ CD4 and TCRαβ CD8β iIELs have been described as being ‘induced’ iIELs, whereas TCRδβ CD8αα, TCRγδ DN and TCRγδ CD8αα iIELs are also referred to as ‘natural’ iIELs [12]. More recently, Muicida et al. [13] have also described the existence of a sixth iIEL subpopulation, the TCRδβ CD4 CD8αα double-positive (DP)
iIELs. Numerous reports indicate an important role for iIELs in maintaining mucosal homeostasis. In this context, iIELs have been implicated in the recognition of stress signals [14], repair of the intestinal epithelium [15–17], may function as memory cells [18], show cytotoxic activity [19,20], and have autoreactive properties [21,22]. In addition, iIELs are characterized by an ‘activated yet resting’ phenotype [20], suggesting the capacity to respond rapidly to in vivo stimuli and the need for tight regulation of iIEL effector function. However, exact mechanisms involved in iIEL regulatory and effector functions remain largely unknown.

Unique to iIELs is the large number of cells expressing the CD8αα homodimer [23]. Recently, our group showed that Ly49E, an inhibitory receptor, is abundantly expressed on CD8αα-expressing iIELs of the small intestine [24]. We demonstrated that iIELs expressing inhibitory Ly49 receptors, including Ly49E, are hypersensitive to TCR-mediated stimulation [24]. Importantly, in vitro TCR-triggering results in upregulation of Ly49E receptor expression on iIELs [25]. Furthermore, we were able to show that the Ly49E receptor can be triggered by the non-MHC-related protein urokinase plasminogen activator (uPA). Interestingly, several studies have indicated a role for uPA in IBD, with increased levels of tissue uPA present in Crohn’s disease and ulcerative colitis patients as compared to healthy controls [26,27]. Thus, we hypothesized that Ly49E expression on CD8αα-expressing iIELs constitutes a novel mechanism through which iIEL function can be regulated. To test our hypothesis in vivo, we have generated Ly49E knockout (KO) mice on a C57BL/6 background. Here, we investigated a role for Ly49E expression on iIELs in the context of IBD development and progression, making use of the dextran sodium sulphate (DSS)- and trinitrobenzene-sulfonic-acid (TNBS)-induced colitis models, and the TNFARE/WT–TNFWT/WT for a kind gift from Dr. G. Kollias (Institute for Immunology, Athens, Greece). Heterozygous TNFARE/WT (Ly49EWT/KO) mice were interbred to obtain homozygous Ly49EWT/WT and Ly49EKO/KO mice. TNFARE/WT mice were a kind gift from Dr. G. Kollias (Institute for Immunology, Biomedical Sciences Research Center “Alexander Fleming,” Attica, Greece). Heterozygous TNFARE/WT (Ly49EWT/KO) mice were interbred to obtain homozygous TNFWT/WT and Ly49EKO/KO mice in order to obtain TNFWT/WT Ly49EKO/KO iIELs. Male TNFARE/WT Ly49EWT/KO offsprings were bred to obtain homozygous TNFWT/WT and female TNFWT/WT Ly49EWT/KO offsprings were bred to obtain homozygous TNFWT/WT and TNFARE/WT Ly49EWT/KO mice. All mice were housed and bred in our animal facility, and all animal experimentation was performed according to the guidelines of the Ethical Committee for Experimental Animals at the Faculty of Medicine and Health Sciences of Ghent University (Ghent, Belgium).

Materials and Methods

Mice

The generation of Ly49E KO mice on a C57BL/6 background was outsourced to Ozgene (Bentley DC, WA, Australia), and the targeting strategy is explained elsewhere [28]. Heterozygous Ly49EWT/KO mice were interbred to obtain homozygous Ly49EWT/WT and Ly49EKO/KO mice. TNFARE/WT mice were a kind gift from Dr. G. Kollias (Institute for Immunology, Biomedical Sciences Research Center “Alexander Fleming,” Attica, Greece). Heterozygous TNFARE/WT (Ly49EWT/KO) mice were interbred to obtain homozygous Ly49EWT/WT and Ly49EKO/KO mice in order to obtain TNFWT/WT Ly49EKO/KO iIELs. Male TNFARE/WT Ly49EWT/KO offsprings were bred to obtain homozygous TNFWT/WT and female TNFWT/WT Ly49EWT/KO offsprings were bred to obtain homozygous TNFWT/WT and TNFARE/WT Ly49EWT/KO mice. All mice were housed and bred in our animal facility, and 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 (Ghent, Belgium).

Genotyping of mice

Genomic DNA was extracted from tail tissue samples, according to the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR amplification of genomic DNA for the TNF locus was carried out with the following primers: forward-5’-CTGTTTGGATCTGTTTTCTTCC-3’ and reverse-5’-AATTACGGTGATGGCTCCTGTTTCC-3’. Following agarose gel electrophoresis, TNFWT/WT and wild-type (WT) alleles yield bands of 626 and 560 bp, respectively. PCR amplification of genomic DNA for the Ly49E locus was carried out making use of the following primers: for WT allele, forward-5’-TCGCCTTTGAATGTCTGTTTCC-3’ and reverse-5’TCTCACCCTGACTGCAATCT-3’, for the KO allele, forward-5’GGAATATTGCTTTACATTAG in combination with reverse-5’TCTCACCCTGACTGCAATCT-3’. Ly49E WT and KO alleles yield bands of 1070 and 1200 bp, respectively.

DSS-induced colitis

Colitis was induced in 8-week old mice by administering 4% (w/v) DSS (molecular weight 36,000–50,000, MP Biomedicals, Cleveland, USA) in drinking water and allowing mice to drink ad libitum for 7 days. Following 7 days of DSS treatment, drinking water containing DSS was replaced by normal drinking water and mice were allowed to recover. Control mice received normal drinking water only.

Assessment of the disease activity index in DSS-induced colitis

The disease activity index (DAI) was scored as follows: weight loss, score 0–5: 0: <1% change in weight, 1: 1–5% change in weight, 2: 6–10% change in weight, 3: 11–15% change in weight, 4: 16–20% change in weight, 5: >20% change in weight), appearance, score 0–2: 0: healthy appearance, 1: unkempt fur coat, 2: arched back), fecal consistency, score 0–2: 0: normal, 1: soft pellets, 2: diarrhea) and fecal occult blood, score 0–5, using the ColoScreen Hemoccult test (Helena Laboratories, Texas, USA) 0: Hemoccult negative, 1: Hemoccult +, 2: Hemoccult ++, 3: Hemoccult ++++, 4: Hemoccult ++++ or marked bleeding, 5: visible indications of rectal bleeding and prolapse). An individual score was assigned to each mouse on a daily basis.

TNBS-induced colitis

Prior to administration of TNBS, mice were sedated through intraperitoneal injection with a mixture (2.5 ml/g body weight) of ketamine (50 mg/ml: 4 volumes) and xylazine (2%: 1 volume). For induction of TNBS-induced colitis, mice were rectally administered a solution (0.1 ml/mouse) of 2.5% (w/v) TNBS (Sigma-Aldrich) in 50% ethanol solution. Control mice received a solution (0.1 ml/mouse) of PBS in 50% ethanol solution. Mice were kept in a vertical position for 1 min to prevent rectal leakage.

iIEL isolation

iIELs from 8- to 14-week old mice were isolated as follows. Briefly, intestines were removed and cleaned of mesenteric fat. Peyer’s patches were excised, and intestines were rinsed with DPBS to clear intestines of fecal content. Subsequently, intestines were opened longitudinally, cut into 0.5-cm pieces, and transferred to a 50-ml conical tube. Intestines were incubated twice for 20 minutes at 37°C in Ca/Mg-free Hank’s Balanced Sodium Solution (HBSS; Invitrogen, Carlstad, CA, USA) containing 5% FCS, 1 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen) and 1 mM dithiotreitol (Sigma-Aldrich) at slow rotation. Cell suspensions were passed through a 40-μm cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) and pelleted by centrifugation at 480 g. Pellets were resuspended in 44% Percoll (GE Healthcare, Buckinghamshire, UK) on an underlay of 67% Percoll, and centrifuged for 20°C at 2000 g. pellet and 1900 g, respectively.
phosphate buffered saline (PBS) for 5 minutes at 840 g, and resuspended in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids (all Invitrogen) and 50 μM 2-mercaptoethanol (Sigma-Aldrich).

Antibodies

mAbs used for staining of iIELs were as follows: anti-TCRβ (APC/Cy7-conjugated, clone H57.597), anti-CD4 (peridinin chlorophyll protein/Cy5.5-conjugated, clone GK1.5), anti-CD69 (biotin- or FITC-conjugated, clone YTS156.7.7), all from BioLegend, San Diego, CA, USA. Anti-ly49E (PE- or FITC-conjugated, clone CM4; kindly provided by Dr C. G. Brooks (Paris, France)), anti-Ly49D/C (biotin- or FITC-conjugated, clone 4D12, made and labeled in-house) [29] and anti-Ly49IF/F (FTC-conjugated, clone CM4; kindly provided by Dr C. G. Brooks (Newcastle on Tyne, UK) [30]. mAb 4D12 (Ly49C/E) in combination with mAb CM4 (Ly49E/F) were used to identify Ly49E/C-expressing cells (CM4+/4D12−), Ly49E-expressing cells (CM4+/4D12+) and Ly49E-expressing cells (CM4+/4D12−). In B6 (H-2b) mice, mAb 5E6 stains Ly49I, whereas Ly49C is very hard to detect [31]. Anti-Ly49H (biotin-conjugated, clone 3D10; kindly provided by Dr. W. Yokoyama, St.Louis, MO, USA) and anti-NKG2A/C/E (FTC-conjugated, clone 389, generated and labeled in-house) [29].

Prior to staining, cells were blocked with anti-FcγRII/III (unconjugated, clone 2.4G2, kindly provided by Dr J. Unkeless, Mount Sinai School of Medicine, New York, USA). Propidium iodide was used to discriminate live and dead cells. Flow cytometry was performed using a BD LSRII flow cytometer, and samples were analysed with FACSDiva Version 6.1.2 software (BD Biosciences).

Histology

For histological analysis, tissue sections of the distal ileum and distal colon were fixed in 4% formaldehyde solution (VWR, Radnor, Pennsylvania, USA) and embedded in paraffin. 5 μm paraffin-embedded sections were stained with hematoxylin and eosin (Sigma-Aldrich). Intestinal inflammation was scored blindly by two independent observers using a validated scoring system, as previously described [32].

Statistics

Statistical analysis was carried out using PASW Statistics 22 Software (SPSS, Chicago, IL, USA). Data was analysed using the non-parametric two-tailed Mann-Whitney U-test or ANOVA, as indicated. A P value ≤0.05 was considered statistically significant.

Results

Ly49E KO mice have normal iIEL population frequencies and NK receptor expression

As the function of Ly49E expression on iIELs of the intestine is currently unknown, we initially sought to clarify whether Ly49E expression on iIELs affects differentiation of intestinal iIELs. In this respect, we found that total numbers of iIELs present in the small intestine of Ly49E WT and Ly49E KO mice (8.60 ± 10^5 vs. 6.60 ± 10^5) were similar. Furthermore, iIEL subpopulation frequencies, and the total number of cells in each iIEL subpopulation, did not differ significantly between Ly49E WT and Ly49E KO mice (Fig. 1A and 1B, and data not shown). We and others have previously shown that several NK receptors are expressed on the surface of small intestinal iIELs. In particular, Ly49E is expressed on a higher proportion of CD8^+ iIELs than other Ly49 receptors [24,33]. Surprisingly, the frequency of Ly49E-expressing colonic TCRγδ CD8^+ iIELs exceeds the frequency of Ly49E-expressing TCRγδ CD8^+ iIELs in the small intestine, whereas we found the proportion of Ly49E-expressing TCRγδ CD8^+ iIELs in the small intestine and colon to be comparable. As expected, Ly49E expression is absent on iIELs of Ly49E KO mice. Small intestinal and colonic iIEL NK receptor expression was unchanged between Ly49E WT and Ly49E KO mice (Fig. 2A and 2B). Furthermore, co-expression of Ly49 receptors on the iIEL surface was unaltered between Ly49E WT and Ly49E KO mice (Fig. 2C and 2D). Ly49E expression on CD8^+ iIELs was low or absent (data not shown).

Ly49E expression on CD8^+ iIELs in Inflammatory Bowel Diseases

To study a possible role for Ly49E expression on colonic CD8^+ iIELs in the context of colitis, we performed DSS-induced colitis with Ly49E WT and Ly49E KO mice. DSS was administered to mice in drinking water for a period of 7 days, after which DSS-containing water was replaced by normal drinking water. Mice were analysed on a daily basis for a total of 11 days. As shown in Fig. 3A, we observed no difference in relative weight loss between Ly49E WT and Ly49E KO mice. Additionally, we analysed the DAI on a daily basis. Here, we observed no significant difference between the DAI of Ly49E WT and Ly49E KO mice between days 0–11 (p=0.35; Mann-Whitney test) (Fig. 3B). This was corroborated by histological scores of distal colon formalin fixed paraffin-embedded (FFPE-) sections obtained from Ly49E WT and Ly49E KO mice at days 7, 9 and 11 following colitis induction, which indicate a similar degree of inflammation for Ly49E WT and Ly49E KO mice (Fig. 3C and 3D). Studying a role for Ly49E expression on colonic iIELs, we analysed iIEL subpopulation frequencies and iIEL NK receptor and activation marker expression at days 0, 7 and 11 of DSS-induced colitis. As illustrated, iIEL subpopulation frequencies and iIEL NK receptor and activation marker expression were unchanged on days 7 and 11 as compared to untreated mice on day 0, and were unchanged between Ly49E WT and Ly49E KO mice (Fig. 4A, 4B and 4C). Hall et al. [34] reported an increased frequency of NKG2D expression on NK cells in DSS-induced colitis. As iIELs have a number of innate-like properties and are crucial to front-line defense of the intestinal mucosal barrier [12,18–22], we examined whether iIELs upregulate NKG2D expression upon DSS-induced colitis. Using littermate DSS-treated and control mice, we observed a trend towards upregulation of NKG2D expression on TCRγδ CD8^+ iIELs and TCRγδ CD8^+ iIELs in DSS-induced colitis, but this was not significant (p=0.083; Mann-Whitney test). NKG2D expression between Ly49E WT and Ly49E KO mice was unchanged on days 0, 7 and 11 as compared to untreated mice on day 0, and were unchanged between Ly49E WT and Ly49E KO mice (Fig. 4A, 4B and 4C). Hall et al. [34] recently reported an increased frequency of NK cell frequencies upon NK cells in DSS-induced colitis. As iIELs have a number of innate-like properties and are crucial to front-line defense of the intestinal mucosal barrier [12,18–22], we examined whether iIELs upregulate NKG2D expression upon DSS-induced colitis. Using littermate DSS-treated and control mice, we observed a trend towards upregulation of NKG2D expression on TCRγδ CD8^+ iIELs and TCRγδ CD8^+ iIELs in DSS-induced colitis, but this was not significant (p=0.083; Mann-Whitney test). NKG2D expression between Ly49E WT and Ly49E KO mice was unchanged on days 0, 7 and 11 as compared to untreated mice on day 0, and were unchanged between Ly49E WT and Ly49E KO mice (Fig. 4A, 4B and 4C). Hall et al. [34] recently reported an increased frequency of NK cell frequencies upon NK cells in DSS-induced colitis. As iIELs have a number of innate-like properties and are crucial to front-line defense of the intestinal mucosal barrier [12,18–22], we examined whether iIELs upregulate NKG2D expression upon DSS-induced colitis. Using littermate DSS-treated and control mice, we observed a trend towards upregulation of NKG2D expression on TCRγδ CD8^+ iIELs and TCRγδ CD8^+ iIELs in DSS-induced colitis, but this was not significant (p=0.083; Mann-Whitney test). NKG2D expression between Ly49E WT and Ly49E KO mice was unchanged on days 0, 7 and 11 as compared to untreated mice on day 0, and were unchanged between Ly49E WT and Ly49E KO mice (Fig. 4A, 4B and 4C). Hall et al. [34] recently reported an increased frequency of NK cell frequencies upon NK cells in DSS-induced colitis. As iIELs have a number of innate-like properties and are crucial to front-line defense of the intestinal mucosal barrier [12,18–22], we examined whether iIELs upregulate NKG2D expression upon DSS-induced colitis. Using littermate DSS-treated and control mice, we observed a trend towards upregulation of NKG2D expression on TCRγδ CD8^+ iIELs and TCRγδ CD8^+ iIELs in DSS-induced colitis, but this was not significant (p=0.083; Mann-Whitney test).
DSS-treated mice was comparable (Fig. 5A and 5B). Thus, Ly49E expression on colonic CD8αα-expressing iIELs does not appear to influence DSS-induced colitis development or progression.

Ly49E expression on CD8ββ-expressing iIELs of the colon does not affect TNBS-induced colitis

A second frequently used model in the study of mucosal immunity is that of TNBS-induced colitis. Here, we show that colitis induction in both Ly49E WT and Ly49E KO occurred rapidly, with the first cases of mortality noted at 5 and 3 days post-treatment for Ly49E WT and Ly49E KO mice, respectively. Seven days following TNBS-colitis induction, 50% of mice had died in both Ly49E WT and Ly49E KO-treated groups, and the decision was made to terminate treatment and euthanize the remaining mice (Fig. 6A). Maximum weight loss for Ly49E WT and Ly49E KO mice occurred at day 5 and day 3, respectively, following the start of treatment, and weight loss differences between Ly49E WT and KO mice were not significant (Mann-Whitney test) on all days analysed (Fig. 6B). FFPE-sections of the distal colon of Ly49E WT and Ly49E KO mice were obtained 72 h following induction of colitis, and scored as described. As shown in Fig. 6C and 6D, colonic inflammation in the colon of both Ly49E WT and Ly49E KO-treated mice was comparable in severity. A role for Ly49E expression on colonic CD8ββ-expressing iIELs was investigated by comparing iIEL numbers, iIEL subpopulation frequencies, and iIEL NK receptor expression of Ly49E WT and Ly49E KO-treated mice 72 h after treatment start. Here, we found the total number of iIELs, iIEL subpopulation frequencies and iIEL NK receptor expression of Ly49E WT and Ly49E KO-treated mice 72 h after treatment initiation as compared to healthy control mice (data not shown). Therefore, we conclude that Ly49E expression on colonic CD8ββ-expressing iIELs does not alter TNBS-induced colitis development or progression.

**Figure 1. iIEL subpopulation frequencies in the small intestine and colon of Ly49E WT versus Ly49E KO mice.**

A) iIEL subpopulation frequencies in the small intestine of Ly49E WT versus Ly49E KO mice. TCRββ and TCRγδ iIEL population frequencies are shown as a percentage of the total iIELs present in the small intestine and colon of Ly49E WT versus Ly49E KO mice. TCRββ, CD4, TCRβδ, CD8ββ and TCRγδ CD8ββ iIEL subpopulation frequencies are shown as a percentage of the total TCRββ iIELs. TCRγδ DN and TCRγδ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRγδ iIELs. B) iIEL subpopulation frequencies in the colon of Ly49E WT versus Ly49E KO mice. Representation of iIEL subpopulation frequencies is the same as for Fig. 1A. iIEL subpopulation frequencies are shown as mean values (n = 5, small intestine; n = 5, colon, where each value is derived from a pool of 3 mice).

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TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice display similar ileitis development and progression

To study the role of Ly49E expression on small intestinal iIELs in the context of ileitis development and progression, we employed the TNF-ARE model of ileitis. Harbouring a deletion of the TNF 3’ AU-rich elements, TNF-ARE/WT mice develop spontaneous ileitis that highly resembles human Crohn’s disease [35,36]. Here, we bred Ly49E WT and Ly49E KO mice to heterozygous TNF-ARE/WT mice in two rounds, generating TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO offspring. Monitoring weight progression of TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice, and their littermate controls, we noted decreased weights for TNF-ARE/WT Ly49EWT/WT mice as compared to TNF-ARE/WT Ly49EKO/KO mice, as expected. Furthermore, we observed no significant difference in weight progression of TNF-ARE/WT Ly49EWT/WT versus TNF-ARE/WT Ly49EKO/KO mice between 5 and 14 weeks of age (Fig. 7A). Subsequently, we analysed iIEL numbers, subpopulation frequencies, NK receptor and activation marker expression in TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice and their littermate controls at the age of 10 weeks, by which time ileitis is fully developed. Total numbers of iIELs isolated from the small intestine of TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice were similar (8.57 × 10^6 ± 2.73 vs. 9.57 × 10^6 ± 3.39 iIELs, respectively). Studying iIEL subpopulation frequencies, we noted a significant increase in TCRββ CD4 iIELs (p < 0.0001; ANOVA), and a significant decrease in TCRγδ CD8αα iIELs (p < 0.0001; ANOVA), in the intestines of TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice as compared to their littermate controls. However, no differences were observed in iIEL subpopulation frequencies between TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice (Fig. 7B). Similarly, we noted no significant differences in the NK receptor expression of iIELs from TNF-ARE/WT Ly49EWT/WT and TNF-ARE/WT Ly49EKO/KO mice (data not shown). To confirm that iIELs were activated in this TNF-ARE/WT ileitis model, we stained iIELs for expression of the activation marker CD69. Small intestinal iIELs from TNF-ARE/
WT Ly49EWT/WT and TNF\textsubscript{ARE}/WT Ly49EKO/KO mice expressed significantly higher CD69 levels as compared to their littermate controls for both TCR\textsubscript{ab} CD8\textsubscript{aa} iIELs and TCR\textsubscript{cd} CD8\textsubscript{aa} iIELs in the small intestine of Ly49E WT versus Ly49E KO mice. Co-expression of Ly49 receptors on the iIEL surface of TCR\textsubscript{ab} CD8\textsubscript{aa} iIELs and TCR\textsubscript{cd} CD8\textsubscript{aa} iIELs in the small intestine of Ly49E WT versus Ly49E KO mice. Numbers indicate the percentage of cells expressing or co-expressing the indicated Ly49 receptors, and are representative of 3 mice. doi:10.1371/journal.pone.0110015.g002

**Discussion**

In this paper we show that the Ly49E NK receptor is abundantly expressed on CD8\textsubscript{aa}-expressing iIELs of the small intestine as well as the colon. Herein, the frequency of Ly49E-expressing colonic CD8\textsubscript{aa}-positive iIELs is greater than the frequency of Ly49E-expressing small intestinal CD8\textsubscript{aa}-positive iIELs. Because of the relatively high Ly49E expression on iIELs, we initially sought to investigate whether Ly49E expression influences iIEL differentiation or the iIEL NK receptor expression profile. In this regard, we show that Ly49E KO mice have unchanged iIEL subpopulation frequencies and a similar NK receptor expression profile to Ly49E WT. Thus, we show that Ly49E expression on iIELs does not affect the development or NK receptor expression profile of basal resting iIELs.

Upon iIEL activation, a number of reports have demonstrated a function for these cells in regulating the development and progression of ulcerative colitis. Recently, Meehan et al. [37] illustrated that interaction of CD100 on \gamma\delta-iIELs with plexin B2 on epithelial cells is required for keratinocyte-growth factor (KGF-1)-mediated epithelial repair following DSS-induced colitis. A role

Figure 2. iIEL NK receptor expression in the small intestine and colon of Ly49E WT versus Ly49E KO mice. A) NK receptor expression on TCR\textsubscript{ab} CD8\textsubscript{aa} iIELs and TCR\textsubscript{cd} CD8\textsubscript{aa} iIELs in the small intestine of Ly49E WT versus Ly49E KO mice. B) NK receptor expression on TCR\textsubscript{ab} CD8\textsubscript{aa} iIELs and TCR\textsubscript{cd} CD8\textsubscript{aa} iIELs in the colon of Ly49E WT versus Ly49E KO mice. C) Co-expression of Ly49 receptors on the iIEL surface of TCR\textsubscript{ab} CD8\textsubscript{aa} iIELs and TCR\textsubscript{cd} CD8\textsubscript{aa} iIELs in the small intestine of Ly49E WT versus Ly49E KO mice. Numbers indicate the percentage of cells expressing or co-expressing the specific Ly49 receptors, and are representative of 3 mice. iIEL NK receptor expression is presented as the mean \pm SD (n = 5, small intestine; n = 5, colon, where each value represents the mean of a pool of 3 mice).

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Figure 3. Clinical symptoms of DSS-induced colitis in Ly49E WT versus Ly49E KO mice. Colitis was induced in Ly49E WT and Ly49E KO mice by administration of DSS in drinking water for 7 days. Thereafter, mice received normal drinking water. Ly49E WT and Ly49E KO mice were scored and compared at the indicated days. A) Relative weight loss (mean \pm SD; n = 71). The reference weight was taken as the weight on day 0, at the start of the experiment. B) Disease activity index (mean \pm SD; n = 71). C) Colon histological score (mean \pm SD; n = 6 on day 7, n = 5 on day 9 and n = 10 on day 11). D) Representative hematoxylin/eosin-stained paraffin sections of the distal colon. doi:10.1371/journal.pone.0110015.g003
Figure 4. iIEL NK receptor expression upon DSS-induced colitis in Ly49E WT versus Ly49E KO mice. A) Colon iIEL subpopulation frequencies (mean ± SD; n = 5 on day 0, n = 3 on day 7 and n = 4 on day 11, where each value is derived from a pool of 3 mice). TCRαβ CD4, TCRγδ
for iIELs in TNBS-induced colitis has been shown by Inagaki-Ohara et al. [38], who demonstrated that CD4 T cell-deficient (Cd42/2) mice show high susceptibility to TNBS-induced colitis at young age, and that subsequent transfer of γδ iIELs to Cd42/2 mice ameliorates TNBS-induced colitis. Roselli et al. [39] corroborated this result by illustrating that a probiotic-induced increase in γδ-positive iIELs suppresses TNBS-induced colitis development in treated mice. Most recently, Jiang et al. [40] were able to show that adoptive transfer of iIELs to NOD2/−/− mice, harboring a strongly reduced number of iIELs, significantly reduced the susceptibility to TNBS-induced colitis. Together, these reports suggest an important role for iIELs in the regulation and prevention of ulcerative colitis.

Alongside, NK receptor expression has been implicated in colitis development. Hall et al. [34] showed that NKG2A expression on NK cells protected mice from DSS-induced colitis development, where NK cells downregulated reactive oxygen species and cytokine production by activated neutrophils through direct cell-to-cell contact involving the NK cell inhibitory receptor NKG2A. Additional data from human studies supports a possible role of NK receptor expression in ulcerative colitis development and progression. In this context, the frequency of KIR2DL1 and KIR2DL3 genotypes was shown to be lower in ulcerative colitis patients as compared to control individuals [41–43]. Moreover, it was shown that KIR2DL1/HLA-C2 interaction negatively correlates with IBD development [44]. Inversely, KIR2DL2 and KIR2DS2 expression was linked to an increased incidence of ulcerative colitis [45]. Interestingly, several studies have suggested KIR receptors to be the human equivalent of Ly49 receptors in mice [46–48]. Taken together, these studies illustrate that NK receptor expression on colonic NK cells and some T cell subsets may influence the course of colitis development. However, to our
knowledge, a role for NK receptor expression on iIELs, in the context of ulcerative colitis, had not previously been investigated.

Recently, our group demonstrated that expression of inhibitory Ly49 receptors on iIELs promotes hyporesponsiveness of these cells [24], and that in vitro TCR-triggering results in upregulation of Ly49E receptor expression on iIELs, indicating a negative feedback loop [25]. Thus, we hypothesized that Ly49E expression on colonic CD8αα-expressing iIELs might provide a new mechanism through which iIEL function can be regulated in colitis development and progression. Here, we show that Ly49E expression on colonic CD8αα-expressing iIELs does not influence the development or progression of DSS-induced colitis and TNBS-induced colitis. Relative weight progression, disease activity index scores, and histological scoring of FFPE-embedded colon sections showed no significant difference in DSS-induced colitis progression between Ly49E WT and Ly49E KO mice. Furthermore, iIEL numbers, iIEL subpopulation frequencies and iIEL phenotype were unaltered throughout DSS-induced colitis progression in Ly49E WT versus Ly49E KO mice. Similarly, survival rates, relative weight progression and iIEL kinetics were not statistically significant in TNBS-induced colitis between Ly49E WT and KO mice.

A role for iIELs in ileitis, as modeled by the mouse TNF+ARE model, has been reported by Apostolaki et al. [49], who showed that intestinal inflammation in TNF+ARE/WT mice is associated with a reduced presence of CD8αα-expressing iIELs. As in ulcerative colitis, human data studies show a negative influence of KIR2DL2/KIR2DL3 in Crohn’s disease development and a protective effect for the KIR2DL1/HLA-C2 interaction [41–44]. Here, we crossed Ly49E WT and Ly49E KO mice to heterozygous TNF+ARE/WT mice in two rounds, generating TNF+ARE/WT Ly49EWT/WT and TNF+ARE/WT Ly49EKO/KO offspring for the study of the role of Ly49E expression on iIELs in ileitis development and progression. Our results show that TNF+ARE/WT Ly49EWT/WT and TNF+ARE/WT Ly49EKO/KO mice display with similar ileitis disease kinetics. Concurrent with results from Apostolaki et al. [49], we observe a significant decrease of TCRβ CD8αα iIELs in the small intestine of TNF+ARE/WT mice compared to their littermate controls. Alongside, we note a significant increase of TCRβ CD4 iIELs in TNF+ARE/WT mice compared to their littermate controls. Furthermore, we show activation of TNF+ARE/WT small intestinal iIELs in ileitis, with significantly higher CD69 expression in TNF+ARE/WT mice as compared to littermate controls. However, we observe no significant differences in iIEL numbers, subpopulation frequencies, NK receptor expression or activation marker expression between TNF+ARE/WT Ly49EWT/WT and TNF+ARE/WT Ly49EKO/KO mice, illustrating that Ly49E expression on small intestinal CD8αα-expressing iIELs does not influence ileitis development or progression.

Conclusively, we report that Ly49E expression is abundant on iIELs of the small intestine and colon. In this, Ly49E is expressed on a high proportion of CD8αα-expressing iIELs. However,
Ly49E expression on CD8αα-expressing iIELs does not influence the development or progression of inflammatory bowel diseases.

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