Aline Van Acker

The role of Ly49E expression on intraepithelial T and ILC lymphocytes in mediating intestinal immune response

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Graphic depiction of an intestinal villus

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
αLP	Alpha lymphoid progenitor
AOM	Azoxymethane
Apc	Adenomatous polyposis coli
ARE	AU-rich elements
B_2m	B_2-microglobulin
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma extra large
Bim	Bcl-2-interacting mediator of cell death
BM	Bone marrow
CHILP	Common helper innate lymphoid progenitor
CLP	Common lymphoid progenitor
CPRG	Chlorophenol red-β-D-galactopyranoside
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DN	Double-negative
DP	Double-positive
DSS	Dextran sodium sulphate
(e)GFP	(Enhanced) green fluorescent protein
Egr2	Early growth response 2
FACS	Fluorescence activated cell sorting
FD	Fetal day
GALT	Gut-associated lymphoid tissue
γc-	Common gamma chain
GATA-3	GATA binding protein 3
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPR(18)	G-protein coupled receptor (18)
IBD	Inflammatory bowel disease
iCD8α	Innate CD8α
IEC	Intestinal epithelial cell
IEL	Intestinal intraepithelial T lymphocyte
IFN-γ	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
ILF	Isolated lymphoid follicle
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KGF-1	Keratinocyte growth factor-1

KIR	Killer cell immunoglobulin-like receptor
KLRG1	Killer-cell lectin like receptor G1
KO	Knock-out
LCMV	Lymphocytic choriomeningitis
Lineage	Lin
LT(α)β(2)	Lymphotoxin (α)β(2)
LTi	Lymphoid tissue inducer
Mcl-1	Myeloid cell leukemia 1
MHC	Major histocompatibility complex
MIC-A	MHC class I polypeptide-related sequence A
MLN	Mesenteric lymph node
Nfil3	Nuclear factor, interleukin-3 regulated
NKG2D	natural-killer group 2, member D
MyD88	myeloid differentiation primary response gene 88
NK	Natural killer
NOD2	Nucleotide-binding oligomerisation domain-containing protein 2
Nur77	Nerve growth factor IB
OPN	Osteopontin
PCA	Principal component analysis
PP	Peyer's patch
PZLF	Promyelocytic leukaemia zinc finger
RAG	Recombinase activating gene
RORα	RAR-related orphan receptor alpha
RORγt	RAR-related orphan receptor gamma t
Runx3	Runt-related transcription factor 3
SAGE	Serial analysis of gene expression
SCID	Severe combined immune deficiency
SP	Single-positive
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF-β	Transforming growth factor β
ThPOK	T-helper inducing POZ-Kruppel like factor
TL	Thymus leukemia antigen
TNBS	Trinitrobenzenesulphonic acid
TP	Triple-positive
TRIF	Toll/interleukin 1 receptor domain containing adaptor inducing IFN-β
TSLP	Thymic stromal lymphopoietin
uPA	Urokinase plasminogen activator
WT	Wild-type

SUMMARY

The single-cell intestinal epithelial layer is composed of cells specialized for the uptake and exchange of nutrients, and cells of the immune system. Together, these form a tightly controlled barrier, regulating the passage of components from the intestinal lumen to the rest of the body. Pathogens such as specific bacteria, viruses and parasites may breach this barrier. Homeostasis may also be challenged by stress-, inflammation- or substance-induced cell transformation. As such, there is a need for intestinal intraepithelial immune cells to respond to barrier invasion and transformation with a rapid and robust response.

Intestinal intraepithelial T lymphocytes (IEL) are resident immune cells of the gut epithelium, characterised by an 'activated yet resting' phenotype. Intrinsically this implies that IEL are quiescent upon exposure to a large number of antigens, promoting tolerance and homeostasis. However, specific antigen-mediated triggering of these cells results in rapid and efficient immune response. Type 1 innate lymphoid cells (ILC1s) are the putative innate counterpart of adaptive immune IEL cells. These cells lack re-arranged antigen receptors, but similarly have the capacity to respond rapidly to breaching of the intestinal epithelium. Together, IEL and ILCs provide crucial front-line defense at the intestinal mucosal surface.

The Ly49E receptor is a member of the Ly49 NK receptor family, exhibiting a unique expression profile and ligand binding. As such, previous work showed that Ly49E is expressed by a number of innate-like immune populations, including natural killer (NK) cells and CD8αα^{+} IEL in the small intestine. Moreover, Ly49E expressed on the cell surface can be triggered by urokinase plasminogen activator (uPA), where uPA has been implicated in numerous pathologies, including inflammatory bowel disease (IBD) and colorectal cancer.

Here we show that Ly49E is not only expressed by small intestinal IEL, but also shows relatively high expression on colonic IEL. Expression of Ly49E by small intestinal and colonic IEL does not influence the development or progression of IBD, nor that of intestinal cancer. Surprisingly, however, we identified a novel NKp46-negative ILC population that could be characterised by Ly49E expression and ILC1-like properties. This population was also resident to the intestinal epithelium, and preliminary data suggests that this population may be important in Th1-mediated immune responses.

SAMENVATTING

Het darmepitheel is een eencellige laag opgebouwd uit cellen gespecialiseerd voor de opname en het uitwisselen van nutriënten, en cellen van het immuun systeem. Tezamen vormen ze een sterk gereguleerde barrière die instaat voor de gecontroleerde passage van stoffen van de luminale zijde van de darm naar het inwendige lichaam. Pathogenen, waaronder bepaalde bacteriën, virussen en parasieten, zijn in staat om door de darmbarrière te dringen en intestinale homeostase te verstoren. Daarnaast leidt stress- of inflammatie-gemedieerde cel transformatie vaak ook tot het ontwrichten van de intestinale balans. Om darm homeostase te bewaren en het lichaam te beschermen tegen pathogenen, is er nood aan immuuncellen die op snelle en efficiënte wijze reageren op invasie en cel transformatie.

Intestinale intraepitheliale T lymfocyten (IEL) zijn residente immuuncellen van het darmepitheel, gekarakteriseerd door een 'geactiveerd maar rustend' fenotype. Dit impliceert dat IEL een interne rusttoestand behouden bij blootstelling aan een grote waaier van intestinale antigenen. Deze cellen worden echter snel geactiveerd door triggering van antigeen-specifieke receptoren. Omwille van deze eigenschap kunnen IEL zowel bijdragen tot het behoud van darm homeostase als darm bescherming. Type 1 innate/aangeboren lymfoïde cellen (ILC1) kan men beschouwen als de aangeboren equivalenten van adaptieve IEL immuuncellen. ILC1 cellen brengen geen herschikte antigeen receptoren tot expressie, doch zijn zij, net als IEL, in staat om snel en efficiënt te reageren op verstoring van de darmbarrière. Samen staan IEL en ILC1 cellen aan de frontlinie in bescherming van de darm mucosa.

De Ly49E receptor behoort tot de familie van Ly49 NK receptoren, en heeft een uniek expressie patroon en ligand. Zo hebben voorgaande studies aangetoond dat Ly49E uitgedrukt wordt op de celmembraan van verschillende aangeboren celpopulaties in de dunne darm, zoals natural killer (NK) cellen en CD8αα$^+$ IEL. Bovendien werd aangetoond dat urokinase plasminogeen activator (uPA) de Ly49E receptor triggert. uPA is een protease dat betrokken is bij de ziektes van Crohn en colitis (tesamen IBD genoemd), alsook bij tumor ontwikkeling.

In deze thesis wordt aangetoond dat Ly49E niet enkel uitgedrukt wordt door IEL in de dunne darm, maar ook door IEL afkomstig van het colon. Ly49E expressie door IEL heeft geen invloed op de ontwikkeling en progressie van IBD, of darmtumour vorming. Daarnaast beschrijven we een nieuwe NKp46-negatieve ILC populatie in het darmepitheel, welke gekarakteriseerd wordt door Ly49E expressie en ILC1-like eigenschappen. Preliminaire data suggereren dat deze populatie een belangrijke rol zou kunnen hebben in het Th1-gemedieerd immuun antwoord.

Chapter 1:

General introduction and research objectives

I. General introduction

As we live our daily lives, our bodies are under constant attack from a wide array of pathogens, including bacteria, viruses and parasites. To defend ourselves from this onslaught, we have evolved and developed a sophisticated immune system. In general terms, the mammalian immune system can be divided into two branches – the innate and the adaptive immune system. The innate immune system is comprised of innate lymphoid cells (ILCs), which include natural killer (NK), innate lymphoid (IL)C1, ILC2 and ILC3 cells, and myeloid cells, such as dendritic cells (DCs), macrophages and neutrophils/eosinophils/basophils. Innate immune cells are programmed to provide a rapid and robust response to pathogen invasion. In this context, DCs and macrophages are programmed to recognize and engulf pathogens. Synergistically, ILCs are programmed to evoke a rapid cytotoxic and/or cytokine-mediated response. As a result, the innate immune system is capable of eliminating many threats. However, more often than not, eradicating a pathogen requires collaboration between cells of the innate and adaptive immune system. DCs and macrophages from the innate immune system, and B cells from the adaptive immune system, are capable of presenting pathogen peptides in the groove of major histocompatibility complex (MHC) molecules to T cells of the adaptive immune system. T cells expressing rearranged antigen receptors specific for a pathogen peptide may then mount an antigen-specific immune response. B cells, in parallel, may be activated to produce antigen-specific antibodies. Although the adaptive immune response is slower than the innate immune response, it is often of vital importance for the total clearance of pathogens from the body, and will also contribute to the formation of memory cells. These are important in subsequent encounters of the immune system with re-occurring pathogens.

Alongside the many immune cells that can be found circulating our bodies, we have also evolved specific tissue-resident immune cells. These can be found in many organs, but are particularly present at mucosal surfaces, such as the skin and gut.

The work in this thesis has focused on elucidating new functions for tissue-resident intestinal intraepithelial T lymphocytes (IEL) and intestinal ILCs.

II. Structure of the small intestine and colon

Structurally, the intestine is a complex organ composed of multiple layers (Fig. 1). Going inwards towards the lumen of the gut are the muscularis externa, submucosa and mucosa. The mucosa is composed of the lamina propria and epithelium, and folds onto itself into villi and microvilli. The purpose of this folding is to create the maximal possible mucosal surface area for absorption of e.g. nutrients and minerals from the lumen. For the purpose of this work, the emphasis is placed on the lamina propria and epithelium, which contain the immune cells of interest.

The lamina propria is a loose connective tissue layer and contains immune cells of both the myeloid and lymphoid lineage, as well as fibroblasts. The epithelium, in turn, is a single cell layer composed of enterocytes, and contains specialized cells such as enteroendocrine cells, Paneth cells, goblet cells and microfold cells, but also cells of the lymphoid immune system. Together, immune cells from the lamina propria and epithelium mount an effective immune response at the intestinal barrier surface.

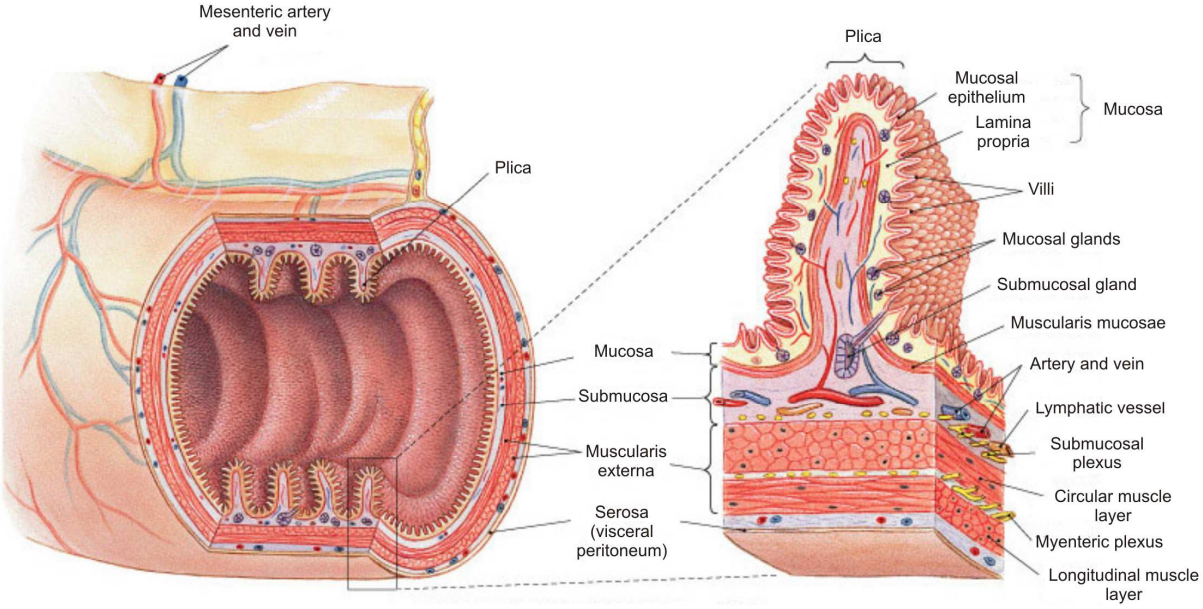


Fig. 1 Structure of the small intestine. *Illustration showing the structural layers that make up the intestinal wall. Going inward toward the lumen are the serosa, muscularis externa, submucosa and mucosa. The mucosa is comprised of the lamina propria and the mucous epithelium. Pearson Education, Inc. 2004.*

III. Immune subsets at the intestinal mucosal barrier

1. Intestinal intraepithelial T lymphocytes

i. Classification of intestinal intraepithelial T lymphocytes

T cells may be classified according to the composition of the T cell receptor (TCR) and the expression of co-receptors on the cell surface membrane. Conventional T cells are characterised by a TCR that is composed of an alpha(α)-chain and a beta(β)-chain. Together with a TCRαβ receptor, these cells often express the co-receptors CD4 or CD8αβ. Non-conventional T cells, on the other hand, often express a TCR that is composed of a gamma(γ)-chain and delta(δ)-chain, in combination with the co-receptors CD4, CD8αβ or CD8αα. Whereas conventional T cells are found circulating the periphery and are present in the majority of peripheral organs, non-conventional T cells are tissue resident and adapted to carry out tissue-specific functions.

Intestinal intraepithelial T cells (IELs) are evolutionary conserved cells and comprise a large number of the body's T cells, where >90% and 80-90% of intraepithelial lymphocytes in the small intestine and colon, respectively, are T cells [1-3]. On average, one in ten cells in the epithelium is thought to be an IEL [4]. IEL T cells are TCRαβ^{+} and TCRγδ^{+} cells, with further subset classification on the basis of CD4, CD8αβ and CD8αα co-receptor expression. This yields TCRαβ cells that express either CD4, CD8αβ or CD8αα (or double-positives that express both CD4 and CD8αα or CD8αβ), and TCRγδ cells that are either CD4 and CD8 double-negative (DN) or that express the CD8αα homodimer [1-3,5]. CD4 and CD8αβ TCRαβ cells are conventional T cells and are often termed 'type a/induced' IELs, whereas CD8αα TCRαβ and TCRγδ cells are non-conventional T cells and are often termed 'type b/natural' IELs. Classification is based on the mechanism by which 'type a' and 'type b' IELs become activated, and on the type of antigen recognized by each. Natural IELs become activated during development in the thymus upon exposure to self-antigens. Induced IELs are activated post-thymically following an encounter with non-self antigen presented by antigen-presenting cells, after which these cells are recruited to the intestinal epithelial surface [1-3,5]. Furthermore, type b/natural IELs may also be distinguished from type a/induced IELs by their oligoclonal TCR repertoire and lack of the classical T cell markers CD2, CD5, CD28 and CD90 (Thy-1) [3,6-10].

ii. Development of intestinal intraepithelial T lymphocytes

Intestinal IEL development has been a heavily debated topic for many years. Whereas there is a general consensus that conventional CD4 and CD8αβ TCRαβ cells develop in the thymus along the classically accepted paths of positive and negative selection, there is uncertainty as to the location and pathways by which non-conventional TCRαβ CD8αα and TCRγδ IELs develop. What follows is a short synopsis of different theories and evidence that has been put forward to elucidate non-conventional IEL development.

Early studies on TCRαβ CD8αα and TCRγδ IEL development found evidence to support an extrathymic origin for non-conventional IELs. In this respect, Guy-Grand *et al.* [11] showed that CD8αα-positive IELs are present in nude and thymectomised mice. Alongside, Saito *et al.* [12] demonstrated that intestinal cryptopatches contain Lineage (Lin)$^{-}$ CD117^{hi} CD127^{+} CD90$^{+/-}$ cells, where cryptopatches are small organized structures located in the basal lamina propria containing predominantly Lin (CD3, CD4, CD8, B220)$^{-}$ cells [13]. These cells, when adoptively transferred into severe combined immune deficiency (SCID) mice, give rise to mature TCRαβ- and TCRγδ-positive IELs [12]. To provide additional evidence for the existence of an IEL T cell progenitor residing in cryptopatches, athymic *CRγ$^{-/Y}$* mice were generated. These mice have a mutation in the common cytokine receptor gamma-chain (γc), and are devoid not only of a thymus, but also of Peyer's patches (PPs), cryptopatches and TCR^{+} cells [14]. Suzuki *et al.* [15] demonstrated that transplantation of wild-type (WT) T-cell depleted bone marrow (BM) cells into athymic *CRγ$^{-/Y}$* mice results in restoration of cryptopatch structures, but not PPs, and subsequent repopulation of the gut with TCR^{+} IELs, which are mainly TCRαβ CD8αα and TCRγδ cells. Taken together, this provides evidence for the existence of an extrathymic IEL progenitor that gives rise to TCRαβ CD8αα and TCRγδ IELs in the intestine.

However, a large array of studies have questioned athymic development of non-conventional IELs, and instead provide evidence for a thymic origin of TCRαβ CD8αα and TCRγδ IELs. A strong argument against the athymic development of IELs from a cryptopatch progenitor population is the appearance of TCRαβ CD8αα and TCRγδ IELs in the gut before the appearance of cryptopatches [16,17]. As well as this, cryptopatches have yet to be identified in humans, arguing against a cryptopatch origin for non-conventional IELs in humans [13,18].

Arguments to support a partial or complete thymic origin for non-conventional TCRαβ CD8αα and TCRγδ IELs are strong. Guy-Grande *et al.* [11] demonstrated that although CD8αα$^+$ IELs are present in the gut of athymic mice, numbers are strongly decreased as compared with WT mice, arguing against a solely extrathymic origin for development of these cells. A follow-up study by this group subsequently used reporter mice that express green fluorescent protein (GFP) under control of the promotor of recombinase activating gene (RAG)2, to demonstrate that RAG2 expression, and thus extrathymic T cell development, may occur in the mesenteric lymph nodes (MLN) and to some extent in the PPs, in nude mice. However, extrathymic development is blocked in healthy euthymic mice, and only observed in mice under conditions of severe lymphocytic depletion. Taken together, these data suggest that the thymus functions as the main lymphoid organ for IEL differentiation, but that extrathymic differentiation may occur under conditions of defective thymopoeiesis [19]. A thymic origin for non-conventional IEL development was further corroborated by a fate-mapping study performed by Eberl *et al.* [20]. Here, mice expressing enhanced GFP (eGFP) under control of the RAR-related orphan receptor gamma t (RORγt) promoter were analysed. The results showed that cryptopatch cells, double-positive (DP)-thymocytes and TCRαβ CD8αα IELs express RORγt, suggesting that TCRαβ CD8αα IELs go through a cryptopatch or DP progenitor stage. Survival of DP thymocytes and TCRαβ CD8αα IEL numbers are sharply decreased in *Rorc(γt)*$^{-/-}$ mice, but can be rescued by expression of a B-cell lymphoma extra large (Bcl-XL) transgene. Importantly, whilst cryptopatches are also absent in *Rorc(γt)*$^{-/-}$ mice, these structures cannot be rescued by Bcl-XL expression. Thus, this study elegantly demonstrated the ability of non-conventional IELs to develop in the absence of cryptopatches, and provided evidence for the existence of a DP thymic IEL progenitor. Further evidence to support TCRαβ CD8αα IEL differentiation from a thymocyte progenitor was provided by Gangadharan *et al.* [21] (Fig. 2). Studying heterogeneity of the DP thymocyte population, Gangadharan *et al.* showed that 6-9% of all CD4$^+$ CD8αβ$^+$ DP thymocytes express the CD8αα-homodimer, generating a CD4$^+$ CD8αβ$^+$ CD8αα$^+$ triple-positive (TP) thymocyte population. Interestingly, many TP cells survive and differentiate *in vitro* in response to agonist antigen and IL-15, with a majority of cells becoming mature TCRαβ CD8αα IELs. To confirm the differentiation of TP thymocytes into TCRαβ CD8αα IELs *in vivo*, TP (CD45.1$^+$) cells were sorted and adoptively transferred into (CD45.2$^+$) recipient B6 mice, where they were shown to differentiate into non-conventional TCRαβ CD8αα IELs with a CD5$^-$ Thy1$^-$ phenotype. Additional evidence to support agonist-based thymic selection for TCRαβ CD8αα IELs was also provided by Yamagata *et al.* [22] , who illustrated that some DP cells survive exposure to their cognate antigen and differentiate to single-positive (SP) TCRαβ CD8αα cells that express many transcripts in common with TCRαβ CD8αα IELs.

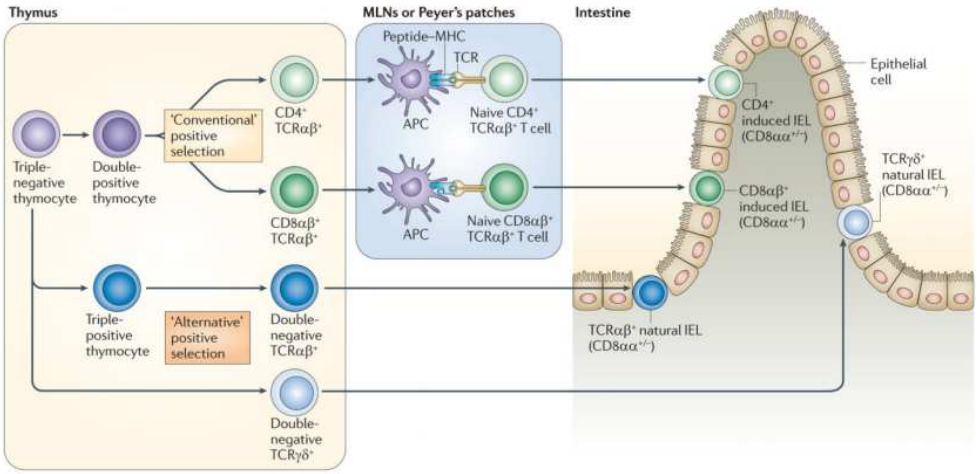


Fig. 2 A model for IEL development. *In the thymus, immature CD4+ CD8αβ+ CD8αα+ (triple-positive) thymocytes undergo agonist ('alternative') selection and differentiate into double-negative TCRαβ+ cells that are the precursors of natural TCRαβ+ CD8αα+ intestinal intraepithelial lymphocytes (IELs). CD4+ CD8αβ+ (double-positive) thymocytes undergo 'conventional' thymic selection and differentiate into CD4+ and CD8αβ+ TCRαβ+(single-positive) T cells that migrate to the periphery. These naive T cells may differentiate into effector T cells in response to peripheral antigens and subsequently migrate to the gut and become incorporated into the induced IEL compartment. APC: antigen-presenting cell; MLNs: mesenteric lymph nodes. Cheroutre et al. [1].*

Lambolez *et al.* [23] also suggest a thymic origin for non-conventional IELs, showing that thymic precursors at the DN2-DN3 developmental stage (prior to the DP stage) are capable of leaving the thymus and migrating to the gut. This was shown through transplantation of CD45.1+ neonatal thymi, or adoptive transfer of specific CD45.1+ thymic populations, into CD45.2+ *Rag2-/- Il2rg-/-* mice. Whereas a full neonatal thymus transplant, or transfer of T-cell committed DN2-DN3 precursors, resulted in reconstitution of CD8αα+ IELs in the gut, injection of more mature DP or SP thymocyte populations did not. Peaudecerf *et al.* [24] later showed the existence of a DN1-DN2 intermediate thymocyte population, characterised as Lin (CD3, CD4, CD8)- CD45+ CD117+ CD127int/- CD25int Sca-1int CD90int, which could egress from the thymus, colonize the gut, and differentiate into CD8αα IEL. Here, neonatal thymi from WT mice were transplanted into *Rag2-/- γc-/-* or *Rag2-/-* mice. In the first mouse strain, the cryptopatch niche is empty and immature local precursors are almost entirely absent. Thus, DN precursor cells from the transplanted neonatal thymus should be able to colonize the gut. In the latter mouse strain, the cryptopatch niche is full and this should hinder entry of new DN precursor cells from the neonatal transplanted thymus. Results showed that CD8αα+ IEL generation occurred in *Rag2-/- γc-/-* mice, but was virtually absent in *Rag2-/-* mice, suggesting that almost all CD8αα+ IELs derive from a DN progenitor. A breakthrough in elucidating the mechanism of TCRαβ CD8αα IEL development was achieved in 2012 by Podezinsky *et al.* [25], who showed that clonal deletion during negative selection in the thymus requires CD28-mediated co-stimulation of autoreactive thymocytes. Autoreactive thymocytes that did not express CD28 or that had sufficiently high *in vitro*-induced overexpression of the anti-apoptotic factors B-cell lymphoma 2 (Bcl-2) and myeloid cell leukemia 1 (Mcl-1) survived clonal deletion. Interestingly, these surviving thymocytes had a TCRαβ+ CD4- CD8- phenotype and were shown to preferentially home to the gut, where they subsequently re-expressed CD8α to become mature unconventional TCRαβ CD8αα IELs. Thus, TCRαβ

CD8αα IEL can develop from autoreactive thymocytes that escape clonal deletion and survive negative selection in the thymus. Finally, two recent papers by McDonald *et al.* [26] and Mayans *et al.* [27] illustrate that TCR specificity plays an important role in directing development of non-conventional TCRαβ CD8αα IELs. By cloning and expression of several endogenous TCRαβ CD8αα IEL TCRs in WT thymocytes, McDonald *et al.* [26] showed that unconventional IEL TCRs induced the generation of a DP (CD4, CD8)^{lo} population in the thymus. These cells developed into CD4^{-} CD8$^{-/lo}$ cells that had high expression of CD122, expressed markers such as 2B4, Ly49 and CD160, and had downregulated expression of CD90. Thus, these cells exhibited all characteristics of unconventional IELs and accordingly could be recovered from the intestinal epithelium. In contrast, TCRs cloned and expressed from conventional IELs were unable to generate DP^{lo} cells, but instead generated a population of DP^{hi} cells that could develop into conventional TCRαβ CD8αβ cells. Interestingly, DP^{lo} thymocytes also expressed high levels of CD69, early growth response 2 (Egr2), nerve growth factor IB (Nur77) and Bcl-2-interacting mediator of cell death (Bim), which have previously been associated with elevated TCR signalling and negative selection [28-30]. Indeed, a large proportion of these cells had increased caspase-3 activity and stained positive for annexin-V, suggestive of large-scale apoptosis following signalling through the TCR receptor. Investigating the underlying mechanism, the authors showed that thymocytes took on a DP^{lo} phenotype following exposure to their cognate antigen. However, some cells were able to survive thymic deletion (i.e. were agonistically selected) and migrated to the intestine where they developed into unconventional TCRαβ^{+} IEL. Hence, this paper showed conclusively that TCRαβ CD8αα IEL can develop from a DP^{lo} precursor population, and that the TCR specificity dictates thymocyte phenotype and development. In parallel, Mayans *et al.* [27] cloned TCRs from unconventional TCRαβ^{+} IEL and transfected these into BM cells, creating BM chimeras on an MHC-sufficient or MHC-restricted background. Results showed that all BM precursor cells developed into unconventional TCRαβ^{+} IELs, which were predominantly CD8αα^{+} CD69^{+} CD122^{+} and CD90^{lo}. Furthermore, the authors identified TCRαβ^{+} DN thymic precursors for these cells and demonstrated that the specificity of TCRs from unconventional TCRαβ^{+} IEL was not restricted to classical MHC-class I or II ligands, but also included specificity for β_2-microglobulin (β_2m)-dependent nonclassical MHC molecules and antigens presented in a transporter associated with antigen processing (TAP)-independent context. Once more, this study corroborates the importance of the TCR in lineage development of non-conventional TCRαβ^{+} cells, and provides evidence for the existence of an agonist-selected thymic precursor for these cells.

Very little is known with respect to the development of unconventional TCRαβ CD4^{+} CD8αα^{+} IELs [5,31]. However, a recent study by Van Kaer *et al.* [32] showed that CD4^{+} IEL can be differentiated *in vitro* to CD4^{+} CD8α^{+} IELs T cells in the presence of interleukin (IL)-7 and transforming growth factor (TGF)-β.

Several factors are known to influence development of IELs, including IL-7, T-bet, T-helper inducing POZ-Kruppel like factor (ThPOK), runt-related transcription factor 3 (Runx3) and G-protein coupled receptor (GPR)18. *Il7*$^{-/-}$ and *Il7rα*$^{-/-}$ mice lack γδ IEL, suggesting that IL-7 is crucial to γδ T cell development [33-35]. Indeed, Laky *et al.* [36,37] showed that IL-7 is required for both intrathymic and extrathymic TCRγδ IEL development. When *Il7*$^{-/-}$ thymi were grafted into TCR*β*$^{-/-}$ host mice, no graft-derived TCRγδ cells developed, indicating that intrathymic IL-7 is required for thymic IEL development. Alongside, it was

shown that TCRγδ cells can develop extrathymically in adult, thymectomized and irradiated mice reconstituted with *Il7*$^{-/-}$ BM, suggesting that peripheral IL-7 is sufficient to sustain extrathymic TCRγδ development [37]. Moreover, an elegant study performed by this same group illustrated that TCRγδ cells can develop in *Il7*$^{-/-}$ mice that overexpress IL-7 specifically in the intestinal epithelium [36]. More recently, Shitara *et al.* [38] have corroborated these results, showing the necessity of thymic IL-7 for TCRγδ IEL development by demonstrating that IL-7$^{flox/flox}$ FoxN1-Cre mice, in which thymic epithelial cells lack IL-7 expression, have significantly decreased numbers of TCRγδ cells in the gut epithelium. As well, Ye *et al.* [39] have demonstrated that IL-7 is required for initiating TCRγ gene rearrangements. Thus, IL-7 plays a crucial role in the development of TCRγδ–positive IELs. An interplay of transcription factors, most notably T-bet, Runx3 and ThPOK, has also been shown to influence IEL development and plasticity. All IELs express high levels of the transcription factor T-bet, with T-bet expression in these cells exceeding that of peripheral T cells. Consequently, *Tbx21*$^{-/-}$ mice, that are T-bet deficient, have severely reduced numbers of all CD8αα-expressing and TCRγδ IELs [40]. Included in this are induced CD4^{+} CD8αα^{+} IELs, which constitute a population of peripheral CD4^{+} T cells that have acquired CD8αα expression and an IEL phenotype in the gut [5,40]. Mucida *et al.* [5] demonstrated that CD4^{+} CD8αα^{+} IEL may evolve from CD4^{+} effector T cells that downregulate *ThPOK* expression and upregulate *Runx3* expression. Functionally, this results in reactivation of the cytotoxic T cell program and endows these cells with cytotoxic properties. Differentiation of CD4^{+} CD8αα^{+} cytotoxic T lymphocytes (CTLs) was shown to be driven by antigen and excessive cytokine stimulation *in vivo*. Interestingly, Reis *et al.* [40] show the ability of T-bet to bind transcriptional elements of *Runx3*, and in conjunction with Runx3 the ability to bind *ThPOK* regulatory elements, resulting in downmodulation of ThPOK. Indeed, *Tbx21*$^{-/-}$ ThPOK-GFP reporter mice have impaired CD8αα induction and display reduced ThPOK downregulation. Also, overexpression of *Tbx21* in CD4^{+} T cells resulted in enhanced CD8αα expression and ThPOK loss, where these effects were abrogated in the absence of Runx3 [40]. As such, changes in T-bet levels may regulate downstream *Runx3* and *ThPOK* expression, which in turn influence development and differentiation of specific IEL subpopulations. Factors mediating T-bet expression levels are diverse, and include IL-15, TGF-β, retinoic acid, interferon (IFN)-γ and IL-27 [21,25,40-43]. Lastly, a recent paper by Wang *et al.* [44] has identified GPR18 as important to the development of CD8αα-expressing IELs. *Gpr18*$^{-/-}$ mice have a significant reduction in the total numbers of IELs, with a marked deficiency in TCRγδ CD8αα and TCRαβ CD8αα IELs. Remaining *Gpr18*$^{-/-}$ TCRγδ cells are Thy1^{hi} , depleted for Vγ7, and show only low granzyme B expression, consistent with a less mature effector state [44]. The mechanism through which GPR18 regulates IEL differentiation is unknown.

iii. Survival of IELs

IELs readily undergo spontaneous apoptosis *ex vivo*, making it a challenge to study these cells [45,46]. Nonetheless, several factors have been shown to influence the survival of gut IELs, most notably IL-7 and IL-15 [45,47-49]. Yada *et al.* [45] showed that addition of IL-7 or IL-15 to IEL cultures decreased spontaneous apoptosis of IEL by 50-60%. Adding both IL-7 and IL-15 did not synergistically enhance the observed effect. Further study into the mechanisms underlying IL-7-mediated IEL survival showed that

addition of IL-7 reduced IEL caspase-3 activity. Additionally, IL-7 could prevent spontaneous apoptosis of IELs through inhibition of a Bcl-2 decrease, where Bcl-2 is an anti-apoptotic cell factor [45]. Similarly, IL-15 was shown to sustain IEL survival through maintenance of Bcl-2 and Bcl-XL [45,50,51]. More recently, Lai *et al.* [52] have shown that, in addition to maintaining Bcl-2 levels, IL-15 activation of the Jak3-Jak1-PI3K-Akt pathway also results in phosphorylation of Bim. In adoptive transfer studies, they subsequently showed that overexpression of Bcl-2 or Bim deficiency in CD8αα-positive IELs promoted their survival in *Il15*$^{-/-}$ mice. Moreover, a study by Jiang *et al.* [53] demonstrated that upstream signalling through the intracellular sensor nucleotide-binding oligomerisation domain-containing protein (NOD)2 is implicated in IL-15-mediated IEL survival. In this study, it was shown that *Nod2*$^{-/-}$ mice have severely reduced numbers of unconventional IELs. Loss of IELs in these mice is correlated to impaired expression of IL-15 by antigen presenting cells. Thus, under normal circumstances, microbial stimulation of antigen presenting cells, resulting in IL-15 expression, is important for IEL homeostasis and survival.

iv. Function of intestinal intraepithelial T lymphocytes

IELs in the small intestine are primarily 'natural' TCRγδ and TCRαβ CD8αα cells (~60%). In contrast, IELs of the large intestine are primarily 'induced' TCRαβ CD4 and TCRαβ CD8αβ T cells (~70%)[1,2]. The ratio of 'natural' to 'induced' IELs in the gut changes gradually with age, with accumulation of 'induced' IELs following exposure to exogenous antigen. As a result, the mucosal immune system develops an almost 'personal' immune repertoire, allowing for adaptation and an optimal response to challenges of the immune system [1].

An interesting property of IELs is their activation state, which has been described as 'activated but resting' [3]. This is due to high expression of effector molecules, such as granzymes A and B and FasL, as well as expression of the activation marker CD69, by IELs even when these cells are unchallenged. On the other hand, low expression levels of cytokine receptor chains CD25 (IL-2Rα) and CD215 (IL-15Rα), which are strongly induced upon activation, are reminiscent of a resting state. These results were confirmed by serial analysis of gene expression (SAGE), which showed that all IELs constitutively express genes associated with immediate response, whereas conventional cytokines and growth factors are underrepresented. Moreover, SAGE identified a high number of tags for the transcription factor JunB, where JunB is known to aid in maintaining highly differentiated cells in a resting state [3]. An 'activated yet resting' phenotype allows IELs to remain quiescent when exposed to numerous luminal antigens, yet simultaneously allows for a rapid response when challenged with an immunological attack [4].

IELs have been implicated in numerous immunological processes, both beneficial and pathological to the host. With respect to conventional TCRαβ CD8αβ IELs, these cells are known to have both protective and pathogenic effector functions [3]. Previous studies indicate that TCRαβ CD8αβ IELs have an antigen-specific cytotoxic potential that exceeds cytotoxicity of spleen CD8 T cells. Thus, these cells are very potent at fighting infection, and have been reported an important role in a number of infectious systems, including rotavirus [54-56], vaccinia virus [57], lymphocytic choriomeningitis virus (LCMV) [58,59] and

Toxoplasma gondii infections [60]. Alongside, numerous studies have shown that TCRαβ CD8αβ T cells are required for an effective anti-tumour response. In this setting, conventional TCRαβ CD8αβ IEL recognize tumour antigens expressed in an MHC class-I restricted manner. Upon recognition, activated TCRαβ CD8αβ IEL expand clonally and kill tumour cells through the release of lytic granules [61]. Cytotoxic CD8αβ IEL are crucial in the colorectal cancer anti-tumour response. Naito *et al.* [62] reported that infiltration of tumours by CD8 T cells expressing granzyme B is associated with better patient survival. Chiba *et al.* [63] confirmed the importance of intraepithelial CD8 T cells in immune response to colorectal cancer, showing that increased numbers of these cells are positively correlated with favourable tumour staging and patient survival. Moreover, Watson *et al.* [64] illustrated a role for TCRαβ CD8αβ IEL in colorectal cancer anti-tumour response, showing that tumours with low MHC class I expression conferred a significantly poorer survival prognosis. TCRαβ CD8αβ IEL have also been shown to exacerbate coeliac disease, where natural-killer group 2, member D (NKG2D) expressed on the surface of these cells interacts with MHC class I polypeptide-related sequence-A (MIC-A) on stressed intestinal epithelial cells (IECs), and elicits villous atrophy [65,66].

A role for TCRαβ CD4 IELs in mediating immune response to parasitic infection has been demonstrated by McDonald *et al.* [67]. In this study, infection of SCID mice with *Cryptosporidium muris* oocysts resulted in severe chronic infection, where co-transfer of IEL procured protection. Depletion of CD4 IEL abrogated the ability to provide immunity upon infection of SCID mice, thus showing the importance of TCRαβ CD4 IEL in this model [67]. TCRαβ CD4 IELs have also been attributed important regulatory functions, where a role for these cells has been demonstrated in the CD4 T cell transfer model of induced colitis. In this model, $CD4^+$ $CD45RB^{hi}$ splenic or lymph node cells are transferred to immune-deficient recipient mice, where transfer of these cells elicits severe intestinal inflammation [68,69]. In this model, co-transfer of $CD4^+$ $CD103^+$ [70,71] and $CD4^+$ $CD8\alpha\alpha^+$ [72] IELs has been shown to reduce intestinal pathology and inflammation. Nonetheless, aberrant $CD4^+$ are known to drive intestinal inflammation in both Crohn's disease and ulcerative colitis [73,74]. Conventional TCRαβ CD4 T cells may also be beneficial in cancer immunosurveillance, where through production of Th1-type cytokines, these cells aid in proliferation of cytotoxic TCRαβ CD8 T cells [61]. TCRαβ CD4 T cells may also function as a prognostic marker in colorectal cancer, where Matsuda *et al.* [75] described significant infiltration of CD4 T cells into moderately differentiated colon adenocarcinoma tissue, whereas infiltration was low in poorly-differentiated adenocarcinoma.

Testament to the plasticity of immune cells in the gut is the described presence of tumour-infiltrating TCRαβ CD4 $CD8\alpha\beta$ $CD103^+$ IEL in the context of colorectal cancer. Numbers of $CD4^+$ $CD8\alpha\beta^+$ cells were shown to be increased in metastatic colorectal cancer, and cells were shown to secrete high levels of IL-4 and IL-13, suggesting that these cells may promote immune tolerance and therein tumour growth and metastasis [76].

A role for TCRαβ CD4 $CD8\alpha\alpha$ IEL in intestinal immune response has been demonstrated by Das *et al.* [72], who showed that $CD4^+$ T cells stimulated with Th2 cytokines acquired $CD8\alpha\alpha$ expression when reaching

the gut upon adoptive transfer in *Rag1*$^{-/-}$ mice. Subsequent co-transfer of sorted CD4 CD8αα TCRαβ IEL with Th1-polarised cells in syngeneic *Rag1*$^{-/-}$ mice was shown to reduce inflammation, as compared with transfer of Th1-polarised cells alone, in these mice. Protection was shown to be IL-10-mediated, as transfer of CD4^{+} CD8αα^{+} IEL sorted from *Il10*$^{-/-}$ mice did not protect mice from development of intestinal inflammation in this model [72]. Thus, CD4^{+} CD8αα^{+} IEL may exert an important regulatory role in IBD. Contrary to these studies, and as previously mentioned, Mucida *et al.* [5] demonstrated that CD4^{+} CD8αα^{+} IEL can differentiate from CD4^{+} effector T cells that downregulate *ThPOK* and upregulate *Runx3* expression, respectively. Functionally, this results in reactivation of the cytotoxic T cell program, and endows these cells with cytotoxic properties. As such, it was shown that stimulation of diet-induced *ThPOK*$^{-}$ OT-II CD4^{+} CTLs with IL-15 results in marked upregulation of CD107a expression and a significant increase in IFN-γ and TNFα production by these cells [5]. These studies demonstrate and suggest that TCRαβ CD4 CD8αα IEL may differ in functionality and exert both protective and pathogenic roles in intestinal immunity dependent on the type of cytokine exposure during differentiation from CD4^{+} T cells.

Unconventional TCRγδ IELs have been designated an important role in a number of immune-mediated processes. TCRγδ cells have been studied extensively with regard to their role in infectious disease models, IBD, recognition of stress-induced ligands and repair of the intestinal epithelium. With respect to infectious disease models, an early study by Roberts *et al.* [77] demonstrated that infection of TCR*δ*$^{-/-}$ mice with *Eimeria vermiformis,* an intracellular protozoan parasite, results in a pro-inflammatory αβ T-cell mediated response. Interestingly, adoptive transfer of bulk IEL to these mice is able to significantly reduce inflammation, suggesting that TCRγδ cells exert a protective role in this model through regulation of the αβ T cell response. Subsequent studies have gone on to show an important role for γδ T cells in other infection models. Inagaki-Ohara *et al.* [78] showed that γδ T cells are important in resistance to infection with the nematode *Nippostrongylus brasiliensis*. In this model, TCR*δ*$^{-/-}$ mice showed more severe clinical disease and microscopic lesions, and succumbed more rapidly to infection as compared with WT controls. Moreover, nematode egg production and worm burden were significantly higher in TCR*δ*$^{-/-}$ as compared with WT infected mice. Here, intraepithelial γδ T cells were shown to produce IL-13, and administration of recombinant IL-13 to TCR*δ*$^{-/-}$ mice was able to partially ameliorate disease, reducing *N. brasiliensis* egg production. Furthermore, TCR*δ*$^{-/-}$ mice expressed higher levels of IFN-γ than WT mice, where αβ T cells were shown to be the main IFN-γ producers. This suggests that TCRγδ IEL may indirectly control *N. brasiliensis* infection through regulation of the intestinal αβ T cell compartment. Lastly, TCR*δ*$^{-/-}$ mice had a decreased crypt/villus ratio and fewer Ki67 (a proliferation marker)-positive epithelial cells, suggesting that γδ T cells are also involved in epithelial cell turnover, an important mechanism in nematode expulsion [78]. Protection against *T. gondii* infection is mediated in part by conventional TCRαβ CD8αβ cells. Interestingly, protection of *T. gondii*-infected mice by TCRαβ CD8αβ cells was shown to be partly dependent on the presence of TCRγδ IELs [79]. Here, primed CD8β^{+} T cells were isolated from WT mice at 6 days post-infection and adoptively transferred into naïve TCR*δ*$^{-/-}$ mice or WT control mice. Indeed, transfer of CD8β^{+} T cells into WT mice protected these mice from mortality upon challenge with *T. gondii*, whereas TCR*δ*$^{-/-}$ mice survival was reduced by 50%. Furthermore, TCR*δ*$^{-/-}$ mice had a significantly increased cyst burden as compared to infected WT mice [79]. TCRγδ IEL have also been implicated in

resistance to *Salmonella typhimurium* infection, where Zhiyuan *et al.* [80] showed that numbers of TCRγδ CD8αα are strongly increased following infection, and that IEL cytotoxicity towards infected intestinal epithelial cells is high. In both *T. gondii* and *S. typhimurium* infections, γδ T cells were additionally shown to exert a protective function through maintenance of the epithelial barrier, where epithelial transmigration of *T. gondii* and *S. typhimurium* is enhanced in TCR$\delta^{-/-}$ mice. Epithelial permeability in TCR$\delta^{-/-}$ mice is associated with a lack of occludin phosphorylation and claudin 3 as well as zona occludens-1 proteins in tight junction complexes, and reconstitution of these mice with TCRVγ7 IELs was shown to be sufficient for restoration of epithelial barrier function [81]. Beside the aforementioned infectious models, γδ T IELs have also been implicated in resistance to *Listeria monocytogenes* and *Encephalitozoon cuniculi* infections [82-84]. A function for TCRγδ IELs in IBD development has been extensively investigated through the use of both chemically-induced models of colitis, including dextran sodium-sulphate (DSS)- and trinitrobenzenesulfonic acid (TNBS)-induced colitis, and genetically induced models of IBD, including TNF$^{\Delta ARE/+}$ ileitis and *Il2*$^{-/-}$-mediated colitis. Kühl *et al.* [85] extensively examined the consequence of γδ T cell depletion or deficiency in DSS-induced colitis, *Il2*$^{-/-}$-mediated colitis and TNF$^{\Delta ARE/+}$ ileitis. Depletion of γδ T cells using a γδ T-cell depleting antibody aggravates DSS-induced colitis and TNF$^{\Delta ARE/+}$ ileitis. Similarly, TCR*γδ*$^{-/-}$ mice display exacerbated DSS-induced colitis. *Il2*$^{-/-}$ mice show increased mortality upon early (4 weeks of age), but not late (8 wks of age) γδ T cell depletion. Increased pathology in the gut of γδ T cell-depleted or deficient mice in all models was associated with an increase in lamina propria lymphocyte IFN-γ expression. As TCRγδ cells express very few IFN-γ transcripts, the authors hypothesize that TCRγδ cells regulate IFN-γ expression by αβ T cells through an as yet undefined mechanism. Complimentary studies by a number of groups have shown that TCRγδ cells may also exert a direct protective effect in IBD development and progression, through maintenance and repair of the intestinal epithelium. As such, Chen *et al.* [86] illustrated the importance of γδ T cell-produced keratinocyte growth factor-1 (KGF-1) in DSS-induced colitis, showing that both TCR*γδ*$^{-/-}$ mice and *Kgf*$^{-/-}$ mice have severe defects in repair of the intestinal epithelium following DSS treatment. More recently, Meehan *et al*. [87] showed that interaction of CD100 expressed by γδ-IELs, with plexin B2 on epithelial cells, is required for KGF-1 mediated epithelial repair following DSS-induced colitis. Inagaki-Ohara *et al*. [88] demonstrated that TCR$\delta^{-/-}$ mice are highly susceptible to TNBS-induced colitis, and that adoptive transfer of γδ IELs to TCR$\delta^{-/-}$ mice ameliorates TNBS-induced colitis. Roselli *et al.* [89] corroborated this result, showing that a probiotic-induced increase in γδ-positive IELs suppressed TNBS-induced colitis development in TNBS-treated mice. Taken together, this data illustrates a protective role for TCRγδ IELs in IBD development. A role for γδ T cells in cancer development and progression has also been illustrated. Matsuda *et al.* [90] showed that TCR$\delta^{-/-}$ mice are more susceptible to azoxymethane (AOM)-mediated colorectal cancer, and developed more adenocarcinomas as compared with TCR$\alpha^{-/-}$ and WT mice. However, a study in adenomatous polyposis coli (Apc)$^{Min/+}$ TCR$\delta^{-/-}$ mice showed that these mice develop significantly less adenomas than control Apc$^{Min/+}$ mice, suggesting that TCRγδ IEL may exert both beneficial and pathological roles in tumour development [91]. Additional data from human studies show a role for γδ T cells in tumour immunity, where γδ T cells are capable of recognizing stress- or transformation-induced cell ligands, such as tumour-expressed MIC-A and MIC-B proteins [92,93].

Unconventional TCRαβ CD8αα IEL are agonist-selected cells [26]. Despite their self-reactivity, these cells appear to have profound tolerogenic and regulatory functions [22,94]. As such, Saurer *et al.* [95] demonstrated that self-reactive TCRαβ CD8αα IEL isolated from mice expressing their cognate antigen had reduced expression of IL-2, IL-10 and IFN-γ when stimulated *in vitro* by plate-bound anti-CD3, as compared with TCRαβ CD8αα IEL isolated from mice lacking the cognate antigen. Alongside, *in vivo* LCMV-induced activation of TCRαβ CD8αα IEL did not result in upregulation of pro-inflammatory cytokines or cytotoxicity, confirming the tolerogenic nature of these cells [95]. TCRαβ CD8αα IEL have also been attributed a role in the immune response to bacterial sepsis, where a study by Tung *et al.* [96] illustrated that sepsis is accompanied by increased expression of IL-2Rβ, IL-15Rα, and IFN-γ mRNA by TCRαβ CD8αα IELs. With respect to IBD, a study by Poussier *et al.* [97] illustrated that TCRαβ CD8αα IEL are protective in the $CD4^+$ T cell transfer model of induced colitis. Here, co-transfer of TCRαβ CD8αα IEL and $CD4^+$ $CD45RB^{hi}$ splenic cells to SCID mice protected mice from colitis development, and this in contrast to co-transfer of $CD4^+$ $CD45RB^{hi}$ cells with TCRαβ CD8αβ IEL. Protection was IL-10-mediated, as TCRαβ CD8αα IEL isolated from $Il10^{-/-}$ mice were unable to prevent colitis development [97]. Unconventional IEL have been shown to recognize a number of non-MHC class I-restricted ligands. With respect to TCRαβ CD8αα IEL, an early study by Gapin *et al.* [98] demonstrated that numbers of these cells are reduced in $\beta_2m^{-/-}$ and $TAP^{-/-}$ mice as compared to WT mice, but a small population of cells remains. This suggests that at least some TCRαβ CD8αα IEL are not dependent upon MHC class I molecules. Indeed, Leishman *et al.* [99] showed that CD8αα can bind the non-classical MHC class-I molecule thymus leukemia antigen (TL). TL is abundantly expressed on IECs [100]. Interestingly, binding of CD8αα to TL modifies TCR-mediated cell responses upon antigen exposure, whereby stimulated-IEL proliferation decreased, and the cytotoxic killing capacity of TCRαβ CD8αα IEL was reduced [99]. Olivares-Villagomez *et al.* [101] corroborated these results in part, showing that colonic TCRαβ CD8αα IELs from $TL^{-/-}$ mice incorporated more BrdU, used as a proliferation marker, than TCRαβ CD8αα IELs from $TL^{+/+}$ mice. Thus, interaction of CD8αα with TL modifies the proliferation capacity of TCRαβ CD8αα IELs. Interestingly, TL absence does not influence DSS- or TNBS-induced colitis, wherein the epithelial barrier is breached. This suggests that TL expression on IECs may not be sufficient in these models to have a critical effect on colitis development [101]. Taken together, interaction of TCRαβ CD8αα IELs with TL appears to modulate IEL proliferation and function, aiding in maintenance of the 'activated yet resting' phenotype. Most recently, Huang *et al.* [102] have additionally shown that expression of TL by IECs is involved in the selection of mature TCRαβ CD8αβ memory cells that transiently express CD8αα. A function for TCRαβ CD8αα IELs in mediating intestinal tumour immune response has not been described to our knowledge. However, studies by Marsh *et al.* [91] and Matsuda *et al.* [75] demonstrate that numbers of CD8αα IELs are reduced in two models of colorectal cancer, namely $Apc^{Min/+}$ mediated and AOM-induced colorectal cancer.

v. Human intestinal intraepithelial T lymphocytes

IEL are abundantly present in both the murine and human intestine. Nonetheless, some differences between mouse and human IEL have been described. With respect to IEL subset composition it is notable that TCRγδ IEL represent only a minor fraction (10%) of all IEL in the human gut [103]. As well as this,

TCRαβ CD8αα IEL are present in great numbers in the human foetal intestine, but are rare in human adults [104]. As such, unconventional IEL are less prevalent in the human intestine as compared with the murine gut. Importantly, this does not reflect a diminished importance of these IEL subpopulations in contributing to intestinal mucosal immunity. Testament to this, for example, is the increase in TCRγδ IEL in coeliac disease [105]. In this disease setting, Bhagat *et al.* [106] demonstrated that individuals on a gluten-free diet have an increased frequency of NKG2A-expressing TCRγδ IELs as compared to patients with active coeliac disease. Triggering of the TCR or NKG2A receptor increases TGF-β expression by these cells. Also, co-culture of NKG2A$^+$ TCRγδ IELs with enterocytes and activated TCRαβ IELs decreases the percentage of cytotoxic CD8 TCRαβ IELs expressing IFN-γ, granzyme B and surface NKG2D. Thus, TCRγδ IEL exert a key suppressor/immunoregulatory function in human coeliac disease [105-107].

2. Innate lymphoid cells

i. Nomenclature of innate lymphoid cells

ILCs are a recently discovered aggregation of lymphoid cells whose precise classification, development and functions are still a matter of debate. Nonetheless, a consensus regarding the nomenclature for ILCs was reached in 2013 (Fig. 3). In this nomenclature, ILCs may be divided into three groups: group 1, group 2 and group 3 ILCs. Group 1 ILCs contain the well-known conventional NK (cNK) cells and ILC1 cells. Group 2 ILCs contain only ILC2 cells, which have previously been termed 'natural helper cells', 'nuocytes' and 'innate helper 2 cells' [108-110]. Group 3 ILCs are diverse and contain lymphoid tissue inducer (LTi) cells, as well as NKp46 negative (NCR$^-$) and NCR$^+$ ILC3 cells [111]. NCR$^+$ ILC3 were previously assigned different names, including NK22 [112], NCR22 [113], NKR-Lti [114] and ILC22 cells [115-117].

It is currently unknown whether additional ILC subsets exist. Also, due to the apparent functional plasticity of some ILC subsets, classification of ILC populations may differ depending on the circumstances under which these are studied.

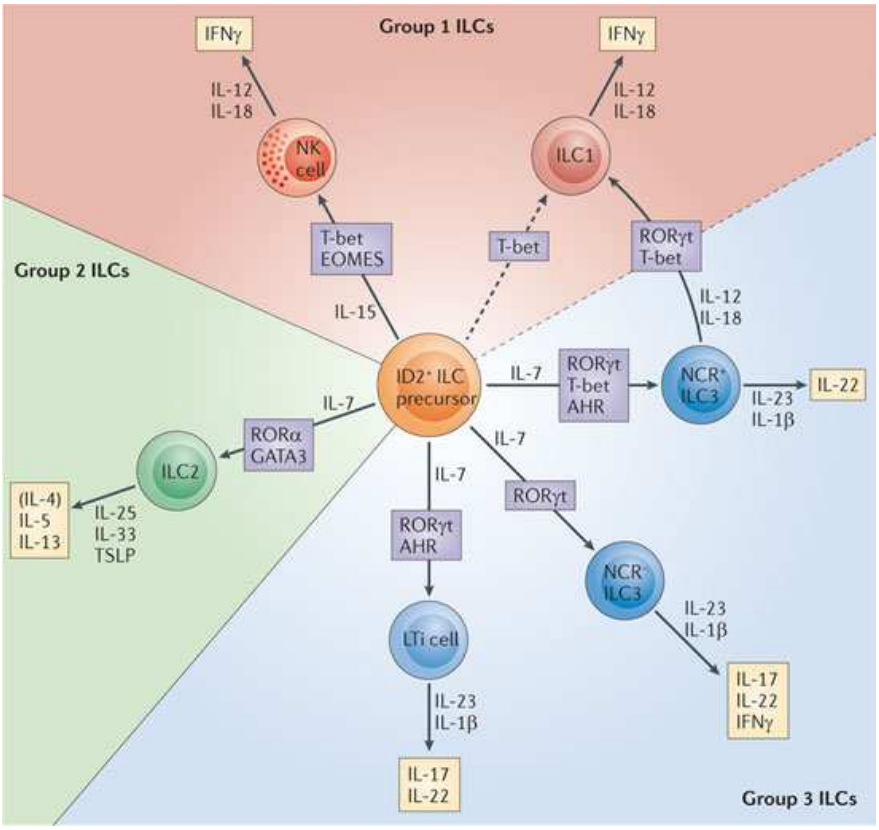


Fig. 3 Nomenclature of ILC subsets. *Group 1, 2 and 3 ILCs are defined on the basis of their functional characteristics. Spits et al. (2013) [111].*

ii. Development and classification of innate lymphoid cells

All lymphoid cells develop from a common lymphoid progenitor (CLP) [118] (Fig. 4). CLP are Lin$^-$ Id2$^-$ CD127$^+$ α4β7$^-$ Flt3$^+$ cells, and can give rise to all ILC subsets, including cNK cells, as well as B and T cells. Progenitor cells that can give rise to all ILCs, including cNK, but not to B/T cells have also been identified. These progenitor cells are highly similar to CLP, but express α4β7, and are therefore termed α-lymphoid progenitor (αLP) cells [118]. Several specific ILC precursors have been identified downstream of the αLP, all of which express the transcription factor Id2. Id2 is an antagonist of E proteins, which function as transcriptional activators and as such have the capacity to modulate gene transcription [119]. Testament to the importance of Id2 for the development of ILCs is the expression of Id2 by all known mature ILC populations [120,121]. Furthermore, deletion of Id2 abrogates the development of all ILCs, including cNK cells [108,113,122]. Two recently discovered Id2$^+$ ILC precursors will be discussed here. Klose *et al.* [123] described the existence of an Id2$^+$ common helper innate lymphoid progenitor (CHILP). The CHILP progenitor was first identified in the BM as being Lin$^-$ Id2$^+$ CD127$^+$ CD25$^-$ α4β7$^+$. Moreover, CHILP are CD93$^-$ Flt3$^-$ and CD122$^-$, distinguishing this progenitor from the CLP (CD93$^+$ Flt3$^+$) and committed NK cell progenitors (CD122$^+$). CHILP are negative for RORγt, T-bet and Eomes, and show only low expression of GATA binding protein 3 (GATA-3), thus asserting the immature phenotype of these cells. To assess the differentiation potential of the CHILP progenitor, cells were purified and adoptively transferred into

alymphoid mice. Here, CHILP differentiated into GATA-3$^+$ ILC2 cells, RORγt$^+$ ILC3 cells, and Eomes$^-$ ILC1 cells, but not into conventional Eomes$^+$ cNK cells. Thus, CHILP progenitors can differentiate to all 'helper' ILC populations, but not to cytotoxic cNK cells. Another ILC progenitor was identified on the basis of transcription factor promyelocytic leukaemia zinc finger protein (PZLF) expression. PLZF$^+$ Id2$^+$ CD3ε$^-$ CD127$^+$ CD117$^+$ CD90$^+$ α4β7$^+$ progenitor cells are found in fetal liver and adult BM, and have the capacity to differentiate to CD127$^+$ ILC1, ILC2 and ILC3 (except LTi) cells, but not LTi or cNK cells [124]. Thus, whereas CHILP give rise to all ILC3 (including LTi-ILC3) cells, PLZF$^+$ Id2$^+$ progenitors do not, demonstrating that PLZF$^+$ Id2$^+$ progenitors appear downstream of the CHILP, and highlighting a branching in the development of LTi-ILC3 and 'other' ILC3 cells. Interestingly, Klose *et al.* [123] were able to identify a subset of CHILP that expresses PLZF, suggesting that indeed PLZF$^+$ Id2$^+$ progenitors develop downstream of PLZF$^-$ Id2$^+$ progenitors and represent cells with more restricted developmental potential. A specific ILC2 progenitor (ILC2P) has also been described as being Lin$^-$ Id2$^+$ CD127$^+$ CD25$^+$ [120]. More committed ILC1 and ILC3 progenitor cells have yet to be discovered.

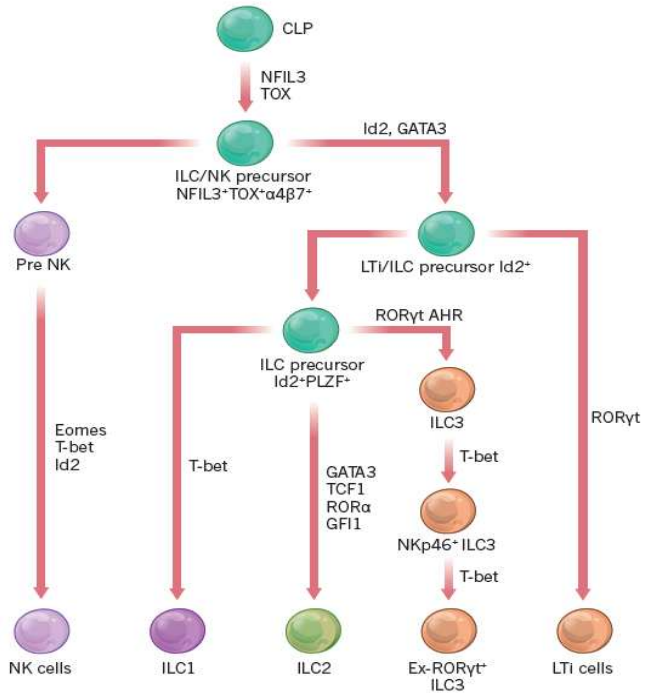


Fig. 4 A model for ILC development. *ILCs develop from a common lymphoid progenitor (CLP). The common ILC/NK cell precursor is contained within a population of cells that have the same phenotype as the CLP but also express the $\alpha_4\beta_7$ integrin. Downstream of the common ILC/NK cell precursor is an Id2-expressing precursor that can give rise to all ILCs, including lymphoid tissue inducer (LTi) cells and an Id2$^+$ PLZF$^+$ precursor that is restricted to ILCs but is unable to develop into LTi cells. Some ILC3s may downregulate RORγt$^+$ and upregulate T-bet expression to develop into IFNγ-producing cells. These IFN-γ-producing ILC1-like cells are called ex-RORγt$^+$ ILC3s. Artis and Spits (2015)[118] .*

ILC development takes place during fetal life and continues into adulthood [119]. Known sites for ILC development include the BM, fetal liver and fetal gut. With respect to the latter, a previous study by

Cherrier *et al.* [125] identified RORγt^{+} ILC3-restricted fetal liver precursors that migrate to the gut and subsequently differentiate to LTi cells.

Mature ILC subsets are characterised by a lymphoid morphology and lack expression of rearranged antigen-specific receptors [116]. Further classification of ILC populations is based on transcription factor dependency and functional properties. For the purpose of this thesis, only ILC populations present in the gut are discussed below.

Of all ILC populations, cNK cells are undoubtedly the best studied and most extensively characterised. Generally speaking, cNK cells may be defined by their developmental dependency on the transcription factors Eomes and nuclear factor interleukin-3 regulated (Nfil3). cNK cells are absent or severely reduced in *Eomes*$^{-/-}$ and *Nfil3*$^{-/-}$ mice, respectively [123]. cNK also strongly express T-bet, but *Tbx21*$^{-/-}$ mice show normal numbers of cNK, showing that development of cNK is not dependent on this transcription factor [123]. Furthermore, cNK cells require IL-15 for survival, and produce IFN-γ upon stimulation with the cytokines IL-12 and/or IL-18. NK cells are also highly granular, and contain lytic granules for exerting a cytotoxic function [116,126].

ILC1 cells, which together with NK cells constitute the group 1 ILCs, are perhaps the least well defined and studied of all ILC populations. In mice, three distinct gut ILC1 populations have been described in recent years [123,127,128]. The first of these is the intraepithelial ILC1 as defined by Fuchs *et al.* [127]. Here, ILC1 cells were isolated from the intestinal intraepithelial compartment of *Rag1*$^{-/-}$ mice. ILC1 cells were defined as CD3^{-} Nkp46^{+} NK1.1^{+} CD160^{+} cells. Importantly, intraepithelial ILC1 cells lack expression of CD127, the Il7rα chain, which is expressed on the surface of most ILC cells. Development of intraepithelial ILC1 cells was shown to be dependent on the transcription factors T-bet and Nfil3, as *Tbx21*$^{-/-}$ and *Nfil3*$^{-/-}$ mice have strongly reduced numbers of intraepithelial ILC1s. Conversely, development was independent of RORγt and aryl hydrocarbon receptor (AhR), two transcription factors required for development of ILC3 cells. Setting these cells apart from cNK cells is the lack of dependence on the cytokine IL-15 for survival, where intraepithelial ILC1 cells are only minimally reduced in *Il15rα*$^{-/-}$ mice. As is common to all group 1 ILCs, intraepithelial ILC1 cells were shown to produce IFN-γ upon stimulation with IL-12 and IL-15 [127]. A second ILC1 population was described by Klose *et al.* [123]. Here, ILC1 cells were identified in the lamina propria that are CD3^{-} Nkp46^{+} NK1.1^{+} CD127^{+} CD27^{+}. Furthermore, these cells have surface expression of the markers CD117 (c-kit) and CD90 (Thy-1), commonly expressed by ILCs. Lamina propria ILC1 cells are dependent on the transcription factor T-bet for their development, but independent of Eomes, thus corroborating that these cells are distinct from cNK cells. ILC1 cells are also RORγt-fate map (fm) negative, and thus are not "ex-ILC3s". Interestingly, lamina propria ILC1 development was also found to be partially dependent on the transcription factors Nfil3 and GATA-3, which are required for NK cell development and ILC2/ILC3 development, respectively, suggesting a previously unappreciated role for these transcription factors in ILC1 cell development. In contrast to the intraepithelial ILC1 cells described by Fuchs *et al.* [127], lamina propria ILC1 cells as defined by Klose *et al.* [123] were shown to require IL-15 for their maintenance and/or differentiation. Lamina propria ILC1 cells produce IFN-γ upon

stimulation with IL-12. Most recently, Van Kaer *et al.* [128] have described a new lymphoid ILC1-like population termed innate CD8α (iCD8α) cells. These cells, located within the intestinal epithelium, are characterised as CD3$^-$ CD8α$^+$ CD103$^+$ CD11b^{int} CD11c^{int} CD117$^-$ Nkp46$^-$ NK1.1$^-$. As for the intraepithelial ILC1 population described by Fuchs *et al.* [127], iCD8α cells do not express CD127, and *Il7rα*$^{-/-}$ mice contain equal numbers of iCD8α cells as WT mice. In contrast to previously described ILC1 populations, iCD8α cells are independent of T-bet and AhR, and express very low levels of RORγt. Thus, it remains to be determined which transcription factors are essential for the development of these cells. As is the case for lamina propria ILC1s [123], but unlike intraepithelial ILC1 cells [127], iCD8α cells are dependent on IL-15 for their survival and/or maintenance, and are avid producers of IFN-γ upon IL-12 stimulation [128].

As previously mentioned, all ILC populations are considered to derive from a common Id2$^+$ precursor. Using *Id2*$^{GFP/+}$ reporter mice, iCD8α cells were shown to express Id2. However, *Id2*$^{GFP/GFP}$ mice, which lack Id2 expression, contain normal numbers of iCD8α cells. Furthermore, transfer of fetal liver cells from *Id2*$^{GFP/GFP}$ mice to irradiated WT recipients results in full reconstitution of the iCD8α pool, demonstrating that iCD8α development is Id2-independent. As such, despite exhibiting many characteristics of ILC1 cells, the classification of iCD8α cells as ILC1 cells is still under dispute [128].

Group 1 ILCs are highly heterogeneous, and defining what distinguishes cNK from ILC1 cells has become more challenging with the identification of new ILC1 subpopulations. To date, the developmental dependency of cNK cells, but not ILC1 cells, on the transcription factor Eomes, appears to constitute a true distinction between these cell types. In turn, dependency on the transcription factor T-bet is common to all group 1 ILCs except cNK. Moreover, a recent large-scale transcriptome analysis of cNK and ILC subsets revealed that expression of *cd49a*, *Tmem176a* and *Tmem176b* by ILC1s, but not cNK cells, may also be used to efficiently distinguish between these group 1 ILC populations [129]. Furthermore, whereas ILC1 cells may develop from CHILP precursors, cNK cells do not [123].

Group 2 ILCs contain ILC2 cells, which may be characterised as Lin$^-$ ICOS$^+$ Sca-1$^+$ CD127$^+$ CD117$^+$ CD90$^+$ IL-25R$^+$ IL-33R (also known as ST2)$^+$ and thymic stromal lymphopoietin receptor (TSLPR)$^+$ cells [111]. Furthermore, killer-cell lectin like receptor G1 (KLRG1) may constitute a good marker for identification of ILC2 cells, as KLRG1 is expressed by virtually all ILC2 in adult mice [120]. ILC2 cells have been identified in the gut lamina propria [120,130] and depend on the transcription factors GATA-3 and RAR-related orphan receptor alpha (RORα) for their development [120,131-133]. Hoyler *et al.* [120] illustrated a role for GATA-3 in ILC2 development by showing that all ILC2 cells are characterised by continuously high GATA-3 expression. Subsequently, using conditional *Gata3*$^{\Delta ILC/T}$ mice, it was shown that KLRG1$^+$ GATA-3^{hi} cells and KLRG1$^+$ RORγt$^-$ cells (both representing ILC2 cells) are virtually absent in the lamina propria of GATA-3-deficient mice. In parallel, development of RORγt$^+$ ILC was shown to be unaffected in *Gata3*$^{\Delta ILC/T}$ mice. Yagi *et al.* [134] confirmed the importance of GATA-3 for maintenance of ILC2 *in vivo*, similarly showing that *Gata3*$^{flox/flox}$-*CreER*^{T2} mice have reduced numbers of KLRG1$^+$ ILC2s, but not RORγt$^+$ ILCs, in the small intestinal lamina propria following tamoxifen treatment. To investigate a role for RORα in ILC2 development, *Rorα*$^{sg/sg}$ mice have been used. These mice have a spontaneous deletion in the *Rora* gene that

results in RORα deficiency [133]. Halim *et al.* [132] demonstrated that *Rorα$^{sg/sg}$* mice, but not *Rorc(γt)$^{-/-}$*, lack ILC2 cells in all tissues studied, including lung, small and large intestine. Alongside, Wong *et al.* [133] generated BM chimeras by transfer of CD45.2 *Rorα$^{sg/sg}$* or WT BM cells into CD45.1 irradiated recipient mice. BM chimeras showed similar hematological repopulation, but transfer of *Rorα$^{sg/sg}$* BM failed to generate ILC2 cells, in contrast to WT BM. Moreover, IL-25 injection of *Rorα$^{sg/sg}$* mice did not result in expansion of ILC2 cells in these mice, as compared with ILC2 expansion in WT controls, confirming the importance of RORα for ILC2 development. Functionally, ILC2 cells are characterised by production of type 2 cytokines, most notably IL-5 and IL-13, but also IL-4, IL-6, IL-9 and granulocyte-macrophage stimulating colony factor (GM-CSF) upon stimulation of these cells by IL-25, IL-33 and/or TSLP [108-110,132,135,136].

Group 3 ILCs are heterogeneous, and a number of different ILC3 populations have been identified. LTi cells have been extensively studied, and may be characterised as Lin$^-$ CD127$^+$ CD4$^{+/-}$ CCR6$^+$ CD117$^+$ CD90$^+$ IL-1R$^+$ IL-23R$^+$ cells [111,137]. These cells are negative for NKp46, a marker that is used to discriminate between several murine ILC3 subpopulations. Development of LTi cells is dependent on the transcription factors RORγt and AhR. Eberl *et al.* [138] showed that in fetal *Rorc(γt)$^{GFP/+}$* mice, only LTi cells express RORγt. Furthermore, whereas *Rorc(γt)$^{GFP/+}$* mice have normal development of lymph nodes and PPs, *Rorc(γt)$^{GFP/GFP}$* mice lack these structures as well as LTi cells. Thus, demonstrating that RORγt is essential for the generation of LTi cells, and that LTi cells are necessary for the development of lymph nodes and PPs. Kiss *et al.* [139] showed that AhR is implicated in the postnatal expansion of LTi cells and in the formation of isolated lymphoid follicles (ILFs). Lee *et al.* [117] corroborated these results, demonstrating that *Ahr$^{-/-}$* mice have markedly less LTi cells than WT mice, and lack cryptopatches and ILFs. Lymph nodes and PPs are formed during embryonic life, whereas cryptopatches and ILFs function following birth. As such, RORγt and AhR may influence the development of LTi cells and the formation of gut-associated lymphoid tissue (GALT) both prior to and following birth [140,141]. LTi cells produce IL-17 and IL-22 upon stimulation with IL-1β and IL-23. Two additional subpopulations of ILC3 cells that can be distinguished are NCR$^-$ and NCR$^+$ ILC3s, lacking or expressing the NKp46 receptor, respectively [111,137]. NCR$^-$ ILC3 cells are Lin$^-$ CD127$^+$ CD4$^-$ CCR6$^-$ CD117$^-$ CD90$^+$ Sca-1$^+$ IL-1R$^+$ IL-23R$^+$ cells. These cells produce IL-17A, IL-22 and IFN-γ upon stimulation with IL-1β and IL-23. NCR$^+$ ILC3s are Lin$^-$ CD127$^+$ CD4$^-$ CCR6$^-$ CD117$^+$ CD90$^+$ IL-1R$^+$ IL-23R$^+$ cells [111,137]. These cells produce IL-22 upon stimulation with IL-1β and IL-23. Both NCR$^-$ and NCR$^+$ ILC3 depend on RORγt for development. The requirement of RORγt for NCR$^-$ ILC3 development was illustrated by Buonocore *et al.* [142]. NCR$^-$ ILC3 express high levels of RORγt, and *Rag$^{-/-}$ Rorc(γt)$^{-/-}$* mice show significantly reduced expression of IL-23R in the colon as compared to *Rag$^{-/-}$* mice, suggesting that RORγt may be important in the development of these cells. The requirement of RORγt for NCR$^+$ ILC3 development was shown by Klose *et al.* [123] who demonstrated that *Rorc(γt)$^{-/-}$* mice have significantly reduced numbers of NKp46$^+$ NK1.1$^+$ ILC3 as compared with WT mice. Additionally, NCR$^+$ ILC3s depend on AhR and T-bet for their development [111,118]. *Ahr$^{-/-}$* mice harbor a severely diminished population of CCR6$^-$ T-bet$^+$ RORγt$^+$ ILC3 cells [143]. *Tbx21$^{-/-}$* mice, in turn, have normal development of CCR6$^+$ LTi and CCR6$^-$ NKp46$^-$ RORγt$^+$ (NCR$^-$) ILC3 populations, but are devoid of CCR6$^-$ NKp46$^+$ RORγt$^+$ (NCR$^+$) ILC3s [143]. It has been suggested that NCR$^-$ ILC3 cells may differentiate into NCR$^+$

ILC3s. As such, a study was performed in which genetically labeled ILC3 subsets were transferred into alymphoid mice. Results showed that CCR6$^+$ LTi cells are a stable lineage. A subset of CCR6$^-$ NKp46$^-$ RORγt$^+$ (NCR$^-$) ILC3 cells, however, upregulated T-bet, lost RORγt and acquired NKp46 expression. Here, the commensal microbiota instructs T-bet expression, where germ-free and myeloid differentiation primary response gene 88 (*Myd88*)$^{-/-}$ toll/interleukin 1 receptor domain containing adaptor inducing IFN-β (*Trif*)$^{-/-}$ mice have strongly reduced numbers of NCR$^+$ ILC3 cells [143]. IL-12 and IL-15 have also been shown to accelerate RORγt loss in NCR$^+$ ILC3s, where RORγt$^+$ NCR$^+$ ILC3 cells produce IL-22, but *Rorc(γt)*$^{lo/-}$ NCR$^+$ ILC3 release IFN-γ [114]. Therefore, it appears that NCR$^-$ and NCR$^+$ ILC3 cells constitute flexible ILC3 populations that may alter in phenotype and function dependent on the gut environment. NCR$^+$ ILC3s have been shown to produce IL-22 and IFN-γ upon stimulation with IL-1β and IL-23, but they do not produce IL-17A [111,115,118].

iii. Function of innate lymphoid cells

ILC are distributed throughout the length of the intestine, with group 1 ILC located within the epithelium as well as the lamina propria compartment. Group 2 and group 3 ILCs are mainly located within the lamina propria. Due to their strategic localisation, ILCs are able to respond rapidly to breaches of the intestinal barrier and may mount an important primary immune response to viral or bacterial infection, as well as contributing to IBD and intestinal tumourigenesis.

Group 1 ILCs have been assigned a number of functions. NK cells, first described in 1975, are important players in frontline defense to infected and transformed cells [144,145]. In this, NK cells may function as either helper or cytotoxic cells, secreting IFN-γ and eliciting Th1-type immunity, or killing target cells. Killing is mediated through the secretion of perforins and granzymes, or through Fas/FasL-mediated caspase-dependent apoptosis [146]. NK cells have been implicated in protection against numerous infections. Ashkar *et al.* [147] showed that NK cells are involved in elimination of *S. typhimurium* infection, where *Il15*$^{-/-}$ mice have increased bacterial burdens, and depletion of NK cells in WT mice results in increased bacterial colonization. Schulthess *et al.* [148] demonstrated that lamina propria NK cells contribute to *T. gondii* infection, where IL-15 and IL-18 induction of NK cells results in CCL3 expression by these cells. NK CCL3 expression was indispensable for recruitment of inflammatory monocytes to the gut, where these cells aided in parasite control. As a last example, NK cells have also been shown to protect against *Yersinia enterocolitica* infection, where *Trif*$^{-/-}$ mice, which are defective in IFN responses and have impaired phagocytosis, present with greater *Y. enterocolitica* dissemination and mortality as compared to WT mice. Immunity to infection was mediated in part by IFN-γ$^+$ NK cells, where induction of IFN-γ increased macrophage bactericidal activity to sufficient levels for elimination of *Y. enterocolitica* pathogens [149]. A function for NK cells in IBD was recently demonstrated by Hall *et al*. [150], as NK cell depletion in the model of DSS-induced colitis impairs survival and increases colonic inflammation. Here, NK cells were shown to downregulate cytokine and reactive oxygen species production by activated neutrophils, which attenuates inflammation and tissue injury in treated mice. A limited role for NK cells in the context of colorectal cancer development has also been suggested, where several studies have shown

that infiltration of tumours by NK cells, in combination with $CD8^+$ T cells, improves survival rates [151-154].

ILC1 cells have been designated various functions, ranging from defense against bacterial infections to mediating IBD development [118,123,127,128]. Intraepithelial ILC1 cells were shown to contribute to inflammation in the model of anti-CD40-mediated colitis [127]. In this model, injecting anti-CD40 antibody into immunodeficient *Rag1*$^{-/-}$ mice induces rapid and robust colitis development [155]. Thirty-six hours following anti-CD40 injection, intraepithelial ILC1 cells from *Rag1*$^{-/-}$ mice were shown to produce copious amounts of IFN-γ, contributing to gut inflammation. Moreover, depletion of both cNK and intraepithelial ILC1 cells with an anti-NK1.1 antibody reduced colon inflammatory cell infiltration and epithelial damage as compared to control mice, suggesting that IFN-γ producing intraepithelial ILC1 cells may contribute to colitis in this particular IBD model [127]. Lamina propria ILC1 cells have in turn been attributed an important role in the protection against *T. gondii* parasite infection [123]. Four days following oral gavage of *Eomes*$^{GFP/+}$ *x Rorc(γt)-fm* mice with *T. gondii*, ILC1 cells were shown to be the main producers of both IFN-γ and TNF-α in response to infection. Moreover, *Tbx21*$^{-/-}$ mice, which lack ILC1 cells but have normal cNK cell numbers, show little IFN-γ production and are subsequently unable to control parasite load and infection [123]. In this, ILC1 were demonstrated to be important for the recruitment of inflammatory monocytes to the gut, which were previously shown to be crucial in control of *T. gondii* infection [156]. Thus, ILC1 are significant players in the response and control of *T. gondii* parasite infection [123]. iCD8α ILC1-like cells function in defense against bacterial infections, and have the capacity to engulf and kill bacteria, as well as to process and present antigen [128]. This was illustrated by Van Kaer *et al.* [128] in an elegant study whereby FITC-labeled *Citrobacter rodentium* or *Helicobacter pylori* bacteria were co-incubated with iCD8α cells. Fluorescence activated cell sorting (FACS) 4 h following incubation showed that iCD8α cells engulf bacteria, as measured by iCD8α-FITC expression. Addition of cytochalasin D, a phagocytosis inhibitor, abrogates the ability of iCD8α cells to engulf bacteria. Killing of bacteria by iCD8α cells was shown through incubation of these cells with *C. rodentium* bacteria for 4 or 24 h, where significantly fewer *C. rodentium* colonies were counted from plated cell lysates following 24 h as compared to 4 h of iCD8α-bacteria incubation [128]. Additionally, iCD8α cells had high expression of the cytokine osteopontin (OPN), which has been implicated in chemotaxis, wound healing and Th1-mediated immunity. Interestingly, Van Kaer *et al.* [128] showed that incubation of iCD8α with peptidoglycan or *L. monocytogenes* bacteria results in secretion of OPN by iCD8α cells, suggesting that iCD8α may also contribute to defense against bacterial infection through production of this Th1-type cytokine. Moreover, iCD8α cells were also shown to have the capacity for processing and presenting bacterial-derived antigen, suggesting that iCD8α cells may interact with cells of the adaptive immune system to modulate response to infection. Lastly, an *in vivo* role for iCD8α cells in control of bacterial infection was demonstrated in the *C. rodentium* infection model [128]. *H2-T3*$^{-/-}$ *Rag2*$^{-/-}$-infected mice, which have fewer iCD8α cells than infected *H2-T3*$^{+/+}$ *Rag2*$^{-/-}$ mice, showed significantly more weight loss and increased bacterial titers 6 days following infection. Alongside, the presence of iCD8α was shown to enhance IL-22 production by $NKp46^+$ ILC3s, whereby IL-22 is important in protection against *C. rodentium* infection [128]. Overall, iCD8α cells were illustrated to have a crucial role in immunity to bacterial infections.

Group 2 ILCs have been attributed numerous functions, ranging from aiding in immunity to extracellular helminth infection, mediating eosinophil homeostasis and function, and have been shown to interact with adaptive immune cells [108-110,135,157-159]. A role for ILC2 cells in contributing to defense against *N. brasiliensis* nematode infection has been illustrated by a number of research groups [108-110]. These showed that ILC2 cells are the primary non-T cell source of IL-13, which is crucial for expulsion of the gut nematode [108,110]. In addition, Neill *et al.* [109] showed that ILC2 cells fail to expand in the absence of IL-25 and IL-33 signalling, resulting in a defect in *N. brasiliensis* helminth expulsion. This defect could be restored through adoptive transfer of WT, but not IL-13 deficient ILC2 cells. Thus, ILC2 cells are important effectors in type 2-mediated immunity to helminth infection. Additionally, ILC2 cells are proposed to play a protective role in *Trichuris muris* helminth infection, where ILC2 have been shown to express amphiregulin, a growth factor important in immunity to this helminth [135,159]. Recently, ILC2 cells were shown to respond rapidly to changes in nutrient levels in the intestine [157]. Here, IL-13 expression by ILC2s was shown to be suppressed as a result of fasting in mice. Alongside, IL-5 expression by ILC2s was shown to increase in response to vasoactive intestinal peptide, a neuropeptide that is induced upon feeding. Interestingly, IL-5 plays an important role in the development and survival of eosinophils. Thus, this research demonstrated for the first time that tissue ILC2 cells regulate eosinophil homeostasis and accumulation through cytokine expression, levels of which may be regulated by nutrient intake levels. Lastly, work by Oliphant *et al.* [158] has illustrated that ILC2 cells interact with cells of the adaptive immune response to co-ordinate type 2 immunity. In this context, ILC2 cells were shown to express ligands in an MHC class-II-restricted context, activating T cells to produce IL-2 (albeit with a lower efficiency than dendritic cells). In turn, IL-2 has the capacity to stimulate ILC2 proliferation and production of Th2-associated cytokines, which contributes to protection against the parasite *N. brasiliensis* [158].

Group 3 ILCs are diverse, not only in classification but also in function. These cells are important mediators of immunity in bacterial infection, contribute to host-microbiota homeostasis, are involved in organogenesis, and have been implicated in IBD and tumour immune response [20,141-143,160-165].

LTi cells have been extensively studied and implicated in GALT formation before and after birth [138,141,166]. Briefly, PPs and MLN are believed to develop between embryonic days E13.5 to E15.5, whereas cryptopatches and ILFs develop following birth [137,140]. LTi cells aid in the formation of GALT through production of lymphotoxin (LT)α1β2 that binds to LTβ receptors expressed by stromal cells. Subsequently, stromal secretion of chemokines and upregulation of adhesion molecules results in attraction and binding of leukocytes, ultimately resulting in formation of secondary and tertiary lymphoid structures [167,168]. In addition to their important role in organogenesis, production of the cytokine IL-22 by LTi cells was shown to be crucial for epithelial repair following host-versus-graft disease [169]. Furthermore, a study by Goto *et al.* [170] showed that LTi-derived IL-22, in co-operation with lymphotoxin, leads to fucosylation of the intestinal epithelium. Interestingly, disruption of epithelial fucosylation results in increased susceptibility to *S. typhimurium* infection. Thus, LTi cells also contribute

significantly to maintaining protective epithelial barrier function and mediating immune response to bacterial infections.

NCR$^-$ and NCR$^+$ ILC3 cells have been implicated in numerous immune-mediated processes. An important function for ILC3 cells was shown in innate immunity to *C. rodentium* infection [162,163]. In an early study by Satoh-Takayama *et al.*, [163] NKp46$^+$ RORγt$^+$ ILC3 were shown to express IL-22. Resistance to *C. rodentium* infection is critically dependent on IL-22, where IL-22 promotes expression of antimicrobial peptides by intestinal epithelial cells [164,165]. As such, *Rag2*$^{-/-}$ *Il2rβ*$^{-/-}$ mice (lacking T/B and cNK cells, but retaining NCR$^+$ ILC3 cells), were shown to be more resistant to *C. rodentium* infection than *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice (additionally lacking NCR$^+$ ILC3 cells), which rapidly succumb to infection. Thus, in the absence of cNK cells, NCR$^+$ ILC3 cells provide sufficient IL-22 to protect mice from *C. rodentium* infection [163]. Later, Satoh-Takayama *et al.* [162] additionally demonstrated that *Ncr1*$^{-/-}$ *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice were equally resistant to *C. rodentium* infection, indicating that NKp46 is not required for the generation of IL-22$^+$ ILC3s that mediate the defense against this pathogen. Taken together, both NCR$^-$ and NCR$^+$ IL-22-producing ILC3 are likely to play an important role in mediating defense to *C. rodentium* infection. A specific function for NCR$^+$ ILC3 cells in mediating immunity to *S. typhimurium* has also been demonstrated by Klose *et al.* [143]. Here, NKp46$^+$ T-bet$^+$ RORγt-fm$^+$ RORγt$^{lo/-}$, but not NKp46$^-$ T-bet$^+$ RORγt$^+$ ILC3 cells were shown to be the main producers of IFN-γ in the control of *S. typhimurium* infection. Besides exerting an important role in innate immunity to bacterial invasion, both NCR$^-$ and NCR$^+$ ILC3 cells have been shown to contribute to colitis development [114,142,161]. Buonocore *et al.* [142] identified a function for NCR$^-$ ILC3 cells in colitis, first showing that *H. hepaticus*-induced colitis in *Rag*$^{-/-}$ mice is characterised by an increase in RORγt$^+$ ILC3 cells that express IL-22, IL-17A and IFN-γ. Subsequent depletion of these cells protects mice from colitis, illustrating that NCR$^-$ ILC3 cells are pathogenic in the context of *H. hepaticus*-mediated colitis. In a second model of anti-CD40-mediated colitis, Buonocore *et al.* [142] additionally showed that depletion of NCR$^-$ ILC3 cells protected *Rag*$^{-/-}$ mice from colitis development, where pathogenicity of NCR$^-$ ILC3 was IFN-γ- rather than IL-17-dependent. Powell *et al.* [161] illustrated a function for NCR$^-$ ILC3 in a third model of colitis, namely *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ ulcerative colitis (TRUC). TRUC development in mice is dependent on IL-23-mediated IL-17A induction in the gut, where depletion of IL-17A through administration of a neutralizing IL-17A or IL23p19 antibody significantly improves disease. Interestingly, TRUC mice were shown to harbor large numbers of IL-17A$^+$ NCR$^-$ ILC3 cells, and depletion of CD90$^+$ cells results in a sharp decrease in IL-17A-producing ILC3s, which protects TRUC mice from colitis development. Thus, IL-17A NCR$^-$ ILC3 cells have an important function in mediating TRUC [161]. A function for NCR$^+$ ILC3 cells in colitis was illustrated by Vonarbourg *et al.* [114], who demonstrated that colon RORγt$^-$ NCR$^+$ ILC3 may produce IFN-γ in response to IL-23. Here, in a model of anti-CD40-mediated colitis in *Il15*$^{-/-}$ mice, which lack cNK, IFN-γ production by NKp46$^+$ cells was shown to mediate colitis. Moreover, following transfer of highly purified colonic lamina propria cells from RORγt-fm mice to alymphoid anti-CD40-treated mice, results indicated that NCR$^+$ RORγt- (RORγt-fm$^+$) ILC3, but not cNK (RORγt-fm$^-$), are the main producers of IFN-γ. Thus, NCR$^+$ ILC3 cells are important mediators of colitis development [114]. Although little research has been conducted with respect to a possible role for ILCs in cancer development, some preliminary studies exist that indicate a role for ILC3 cells in colorectal cancer

development and colon epithelial repair [160,171]. Transgenic overexpression of IL-23 in *Rag1*$^{-/-}$ mice induces the formation of intestinal adenomatous polyps [172]. Using this model, Chan *et al.* [160] demonstrated that tumourigenesis is mediated in part by IL-17A-expressing Thy1^{+} IL-23R^{+} NCR^{-} ILC3 cells, as tumour development is inhibited in *Rag*$^{-/-}$ *Il17*$^{-/-}$ mice. Alongside, Kirschberger *et al.* [173] showed that colon cancer in genetically susceptible 129SvEv.*Rag*$^{-/-}$ mice infected with *Helicobacter hepaticus* and treated with the carcinogen AOM is accompanied by accumulation of IL-17^{+} IL-22^{+} NCR^{-} ILCs. Interestingly, depletion of these cells in mice with severe inflammation prevents the development of invasive colon cancer as compared to control mice [173]. Taken together, these studies highlight a previously unappreciated role for ILC3 cells in tumour development and cancer progression. Lastly, it is important to note that ILC3 cells have been shown to interact with the gut microbiota in regulating mucosal immunity [174,175]. For example, Sawa *et al.* [175] showed that germ-free or antibiotic-treated *Rorc(γt)*$^{GFP/+}$ mice harbored similar numbers of RORγt^{+} ILC3 as WT mice, however, IL-22 expression was greatly increased in these mice. Microbiota were subsequently shown to repress IL-22 expression by ILC3s indirectly through epithelial production of IL-25. Importantly, this mechanism was de-repressed by epithelial damage, allowing for epithelial repair upon damage to the intestinal barrier. Similarly, interaction between host microbiota and ILC3 cells may aid in creating a tolerogenic gut environment. As such, Mortha *et al.* [174] showed that intestinal microbiota elicit GM-CSF production by ILC3s. This, in turn, enhanced DC and regulatory T-cell function, promoting intestinal homeostasis. Thus, host microbiota-ILC3 interactions may be highly beneficial to promoting intestinal immune response.

Developmental and functional plasticity between ILC1 and ILC3 cells has been demonstrated, and is linked with varying expression levels of the transcription factors RORγt and T-bet [114,143]. As such, differentiation of NCR^{-} and NCR^{+} ILC3 cells to IFN-γ-producing CD127^{+} ILC1 cells may occur under influence of IL-2 and IL-12. In turn, the reverse differentiation of CD127^{+} ILC1 cells into IL-22-producing ILC3 cells may occur under the influence of IL-2, IL-23 and IL-1β. Functionally, this plasticity suggests that these cells may adapt their phenotype and function differently under specific disease or homeostatic conditions in the gut. As such, ILCs are optimally adapted to providing rapid and robust innate immune responses [114,143].

iv. Human innate lymphoid cells

All three groups of ILC cells are present in the human gut. Group 1 ILC cells, as is the case for mouse ILCs, are not yet well-defined. Although NK cells were identified many years ago, helper ILC1 cells have only recently been characterised. Human helper ILC1s include two subpopulations which can be distinguished on the basis of their localisation and cell surface marker expression profile. Intraepithelial ILC1s are characterised by a CD127^{low} profile, and are CD103^{+} CD160^{+} CD56^{+} CD94^{+} NKp44^{+} cells. In contrast, lamina propria ILC1 are CD127^{high}, and lack expression of CD56, CD94 and NKp44. Both intraepithelial and lamina propria ILC1 express high levels of T-bet and express IFN-γ when stimulated with IL-12 + IL-15/IL-18. Group 2 ILCs are innate CD127^{+} CRTH2^{+} cells. Like murine ILC2 cells, human ILC2 cells depend on GATA-3 for their development and can be stimulated by IL-25, IL-33 and TSLP to express IL-5, IL-13

and amphiregulin. Human group 3 ILCs contain LTi cells, NCR^+ (NKp44 in humans) and NCR^- ILC3s. NCR^+ and NCR^- ILC3 are $CD127^+$ $CD117^+$ $CCR6^+$ $IL1\beta R^+$ $IL23R^+$ cells. As is the case for mouse ILC3s, human ILC3 respond to IL-1β and IL-23 to secrete IL-22. Functional plasticity between human ILC1 and ILC3s has also been demonstrated [176-178].

IV. Ly49 receptors

NK cells express a wide array of cell surface receptors. Amongst these are several families of receptors, including the NKG2/CD94, CD69-related, NKRP1 and Ly49 receptor families [126]. Of importance to this thesis are the Ly49 receptor family members, and Ly49E in particular, which are discussed in more detail below.

i. Activating and inhibitory Ly49 receptors

The Ly49 NK cell receptor family has been most extensively characterised in the mouse C57BL/6 strain, with a total of 15 Ly49 receptor genes identified. Ly49 genes are located distally on mouse chromosome 6, in a 620-kb cluster [126]. Seven Ly49 genes encode an inhibitory receptor, (*ly49a, c, e, f, g, i and q*), two Ly49 genes encode for an activating receptor (*ly49d, h*) and the remaining genes are pseudogenes [179]. Fig. 5 provides an overview of Ly49 receptor expression and functions known to date. Inhibitory Ly49 receptors have been extensively implicated in the process of NK cell education. In NK cell education, NK cells become "licensed" and fully functional following the interaction of a self-specific inhibitory Ly49 receptor with self-MHC-I. In agreement with this notion, NK cells from Ly49-deficient mice have an impaired "missing-self" response against MHC-I-deficient target cells [180]. Similarly, specific interaction of Ly49A with the non-classical MHC-I molecule H2-M3 has been implicated in NK cell education, where $Ly49A^+$ NK cells from H2-$M3^{-/-}$ mice showed reduced expression of IFN-γ as compared to $Ly49A^+$ NK cells isolated from WT H2-$M3^{+/+}$ mice [181]. Ly49C/I have been implicated in leukemia, where use of a blocking Ly49C/I antibody was shown to be therapeutic in the treatment of disease [182]. With respect to Ly49Q, two independent studies showed that Ly49Q is expressed by plasmacytoid DCs. Subsequent analysis of Ly49Q-deficient mice demonstrated that plasmacytoid DCs isolated from these mice express decreased levels of TLR9-mediated IFN-α, and are more susceptible to MCMV-mediated infection as compared with wild-type littermates [183,184]. A possible explanation for this paradoxical stimulatory effect of Ly49Q on IFN-α expression levels was proposed in that Ly49Q/DAP12 may negatively regulate TLR7/9 signal transduction, resulting in decreased IFN-α responses in Ly49Q-deficient mice [184]. Ly49Q is also expressed by macrophages, where cross-linking of this receptor induces cytoskeletal re-arrangements and cell spreading [185]. A function for the Ly49F receptor has, to our knowledge, not been described to date. Looking at a function of the two activating Ly49 receptors, a study by Mason *et al.* [186] demonstrated that expression of Ly49D by NK cells augments IFN-γ expression by these cells upon interaction of the receptor with its ligand H-$2D^d$. Interestingly, NK cells co-expressing Ly49D and the inhibitory receptor Ly49G exhibited decreased levels of IFN-γ as compared to cells expressing Ly49D alone, additionally

demonstrating the dominant inhibitory nature of the Ly49G receptor [186]. With respect to the activating receptor Ly49H, Ly49H-deficient mice were illustrated to have an increased susceptibility to MCMV-infection. In accordance, Ly49H was shown to recognize specifically the MCMV viral protein m157, expressed on the surface of infected cells [187].

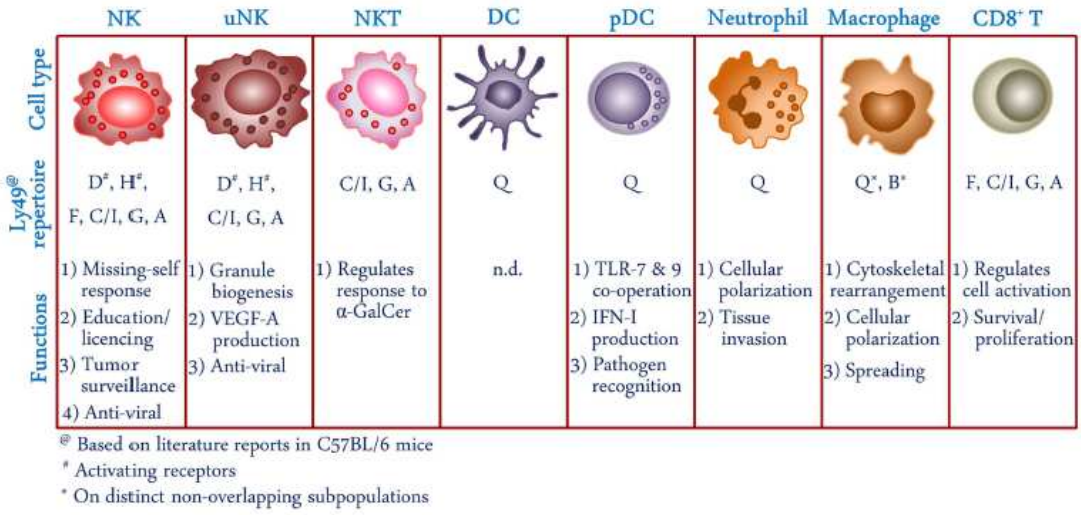


Fig. 5 Schematic depiction of cell types expressing Ly49 receptors. *The diversity of Ly49 receptors expressed by each cell type, and proposed functions for these receptors, are shown. (uNK, uterine NK cells; pDC, plasmacytoid. Rahim et al. (2014) [188].*

ii. Signalling mechanisms in Ly49 receptors

Structurally, Ly49 receptors are lectin-like receptors, which refers to the extracellular core c-type lectin-like domain (CTLD), common to all Ly49 receptors. Ly49 receptors are expressed as 92-kDa disulphide-linked homodimers [126].

Activating Ly49 receptors are characterised by the presence of positively charged transmembrane residues. The presence of a positively charged residue allows for the interaction of the receptor with adaptor signalling chains, such as DAP12, DAP10, FcεRIγ and CD3ζ, which facilitate the expression of the activating receptors. Furthermore, these signalling chains contain an immunoreceptor tyrosine-based activating motif (ITAM). Upon ligand binding, receptor cross-linking results in tyrosine phosphorylation at the ITAM. The phosphorylated ITAM tyrosine then recruits SYK or ZAP70 proteins for further downstream signalling, ultimately resulting in cell activation. In contrast, inhibitory Ly49 receptors are characterised by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain. Following ligand binding, the ITIM is tyrosine phosphorylated by a SRC-family tyrosine kinase. The phosphorylated ITIM tyrosine subsequently recruits SH2-domain-containing protein tyrosine phosphatase 1, which mediates further downstream signalling, ultimately resulting in inhibition of cell activation (Fig 6) [126,189].

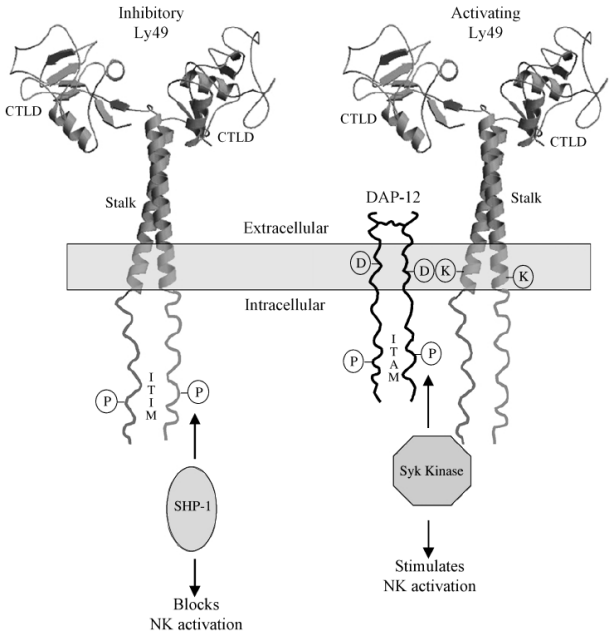


Fig. 6 Signalling of activating and inhibitory mouse NK Ly49 receptors. *Ly49 receptors are lectin-like glycoprotein receptors expressed as disulphide-linked homodimers on the cell surface. Upon triggering of a Ly49 inhibitory receptor, tyrosine phosphatase SHP-1 is recruited, where downstream signalling from SHP-1 results in inhibition of NK cell activation. Upon triggering of an activating Ly49 receptor, the adaptor signalling chain DAP-12 ITAM is phosphorylated, resulting in subsequent recruitment and activation of Syk kinase. Downstream signalling from the activated Syk kinase will initiate NK cell activation. Dimasi and Biassoni (2005) [189].*

iii. Ly49E

Ly49E is a unique member of the Ly49 NK receptor family, where the *Ly49e* gene is strongly conserved, in contrast to the allelic polymorphism observed for other Ly49 genes [190]. Furthermore, Ly49E is distinct from other members of this family on the basis of its structural properties, expression pattern and ligand recognition [126].

With respect to its structural properties, Ly49E is particular in displaying characteristics of both an activating and inhibitory NK receptor. In this sense, the intracellular domain of Ly49E contains an ITIM, characteristic of an inhibitory NK receptor. On the other hand, the Ly49E transmembrane domain also contains a positively charged residue, i.e. arginine. This would suggest that Ly49E can function as both an activating and/or inhibitory NK receptor. Ly49E, however, in contrast to other activating Ly49 receptors, has an arginine residue that is located at position 19 instead of position 10, as is the case for the activating receptors Ly49D/H. Previous studies have shown that the location of the charged residue within the transmembrane region is critical in the ability of the receptor to pair with the adaptor signalling chain [191]. Therefore, it is unlikely that Ly49E can function as an activating receptor.

In contrast to other Ly49 receptors, Ly49E is expressed on a range of innate-like cells. With regard to NK cells, expression of other (non-Ly49E) Ly49 receptors commences at 1 week following birth, and adult

Ly49 receptor expression levels are reached at 6-8 weeks of age [192]. In contrast, Ly49E is highly expressed on fetal and neonatal NK cells, and Ly49E expression decreases from 2 weeks following birth, to reach low levels on adult NK cells [94,192-194]. An exception to this are DX5-negative NK cells in the liver, which express relatively high levels of Ly49E, also in adult mice. With respect to this, it was shown that up to 50% of fetal day (FD)17 NK cells, the majority of which are DX5-negative, express Ly49E [195]. Ly49E is also expressed by T cells, where 25-35% Ly49E expression has been observed in FD17 Vγ3 and adult Vγ3 skin intraepithelial T cells [192]. Furthermore, Ly49E expression by CD8αα-expressing IELs of the small intestine has been demonstrated, where 20-30% of TCRαβ CD8αα IELs and 10-20% of TCRγδ IELs express Ly49E [196].

Most Ly49 receptors have MHC-class I ligands. Ly49E, in contrast, is unique in its ability to be triggered by a non-MHC-class I-related ligand, i.e. urokinase plasminogen activator (uPA) [197]. Briefly, triggering of Ly49E by uPA was shown *in vitro* through the use of a BWZ.36 reporter cell line. In this model, BWZ.36 cells express a hybrid Ly49 receptor, where the extracellular Ly49E domain is coupled to the transmembrane and intracellular domains of the activating Ly49H receptor. A DAP-12 signalling chain is also expressed (H/ED12 cells). As such, triggering of the chimeric Ly49E receptor will result in cell activation and signalling, and ultimately β-galactosidase production. Using this reporter model, uPA was selected from a cDNA library as the protein that triggers the Ly49E receptor, and this was confirmed by the demonstration that coated recombinant uPA strongly stimulates H/ED12 cells [197].

Interaction of uPA with Ly49E has been shown to inhibit NK cell function. In this context, IFN-γ production by NK cell lines that strongly express Ly49E, i.e. KY and NK03, is inhibited upon exposure to coated uPA. Importantly, addition of anti-Ly49E $(Fab')_2$ fragments restores IFN-γ production, showing the Ly49E specificity of uPA [197]. Additionally, coated uPA reduces the cytotoxicity of IL-2 cultured FD17 thymocytes upon NK1.1-mediated stimulation, as assessed by CD107-surface expression. Thus, triggering of Ly49E by uPA inhibits the cytotoxic potential of fetal NK cells. With respect to other innate-like cell populations, data from our group also went on to show that IEL upregulate Ly49E expression following TCR-mediated stimulation of these cells [198]. However, a function for Ly49E expression by IEL remains to be determined. Importantly, uPA is a serine protease that has been implicated in a number of physiological processes, including tissue remodeling, tumour invasion and tumour metastasis, as well as development and progression of IBD [197,199-201].

V. *In vivo* disease models for the study of Ly49E function on intestinal intraepithelial T lymphocytes and innate lymphoid cells

To study the functional role of Ly49E expression on IEL and ILC cells, a wide range of *in vivo* disease models can be used. Listed below are *in vivo* disease models relevant to this PhD thesis, with a brief description of each model used.

IBD encompasses Crohn's disease and ulcerative colitis. Whereas Crohn's disease is characterised by a predominant Th1-type immune response, ulcerative colitis is characterised primarily by a Th2-type immune response. However, it should be mentioned that the Th1/Th2 nature of these disease is not clear-cut, and an overlap in the pathogenesis of Crohn's disease and ulcerative colitis exists [73]. Furthermore, a Th17-type immune response is also prominent to both Crohn's disease and ulcerative colitis [202]. Three popular models for the study of Crohn's disease and ulcerative colitis in mice are $TNF^{\Delta ARE}$-mediated ileitis, DSS-induced colitis and TNBS-induced colitis.

$TNF^{\Delta ARE}$-mediated ileitis develops in mice that harbor a deletion of the AU-rich elements (ARE) of the *Tnfa* gene. Deletion of the ARE 3' UTR regulatory element results in loss of posttranscriptional regulation of TNF-α. As a result of deregulated expression, TNF-α levels are systemically increased, and $TNF^{\Delta ARE/+}$ mice develop spontaneous IBD as well as inflammatory polyarthritis [203,204]. Similar to Crohn's disease patients, intestinal inflammation in $TNF^{\Delta ARE/+}$ mice is transmural and primarily localized to the terminal ileum [205,206]. The first symptoms of ileitis are detectable at 3 weeks of age, and disease is fully developed at 8 weeks of age [203,204].

One of the most widely used mouse models of colitis, is that of DSS-induced colitis. DSS is a water soluble polysaccharide that is capable of establishing linkages with medium-chain-length fatty acids present in the colon lumen. Once complexed, DSS-fatty acid nanovesicles fuse with colonocyte membranes and are toxic to colon epithelial cells [207]. As a result, the intestinal barrier is breached and bacteria are capable of disseminating across the lumen into the gut. The spread of bacteria and bacterial products, in turn, triggers a response from both the innate and adaptive immune system [208,209]. DSS-treated mice develop inflammation that affects the mucosal layer of the intestinal wall (but is not transmural), where inflammation is primarily localized to the distal colon [210,211]. DSS-induced colitis is induced through the administration of DSS in drinking water to mice. Both acute and chronic intestinal inflammation can be modeled, depending on the concentration and frequency of DSS administration. Where acute DSS-induced colitis is characterised by a Th1 cytokine profile, with expression of cytokines such as TNF-α, IFN-γ, IL-1β and IL-12, chronic DSS-induced colitis exhibits a pre-dominant Th2 cytokine profile, with expression of IL-4, IL-6 and IL-10 [208]. DSS-induced colitis is also modulated by signalling through the IL-23/IL-17 axis. In this model, IL-23 and IL-17A have been shown to improve disease outcome, whereas IL-17F exacerbates colitis [212,213]. As is the case for a number of murine models, susceptibility to DSS-induced colitis is influenced by the genetic background of mice, where C3H/HeJ, NOD/Ltj, and NOD-SCID mouse strains are very susceptible to DSS-induced colitis, whereas C57BL/6 mice are more resistant [208,209].
A second model commonly used for the study of colitis is that of TNBS-induced colitis, where TNBS is a chemical that haptenises colonic autologous and microbial proteins, rendering these immunogenic to the host [214]. TNBS is administered intrarectally in ethanol, and colitis induction is rapid. Intestinal inflammation in TNBS-induced colitis is transmural and localized to the distal colon [209,211]. Development of colitis is associated with high expression levels of Th1-type cytokines, where IFN-γ is key. Here, signalling through the IL-23 receptor, and IL-17A, were shown to improve disease outcome. As is the case for DSS-induced colitis, disease severity is dose-dependent. Also, genetic background influences the

degree of TNBS-induced colitis development, where SJL/J and BALB/c for example are highly susceptible to colitis, whereas C57BL/6 mice are relatively resistant [215-217].

Importantly it should be noted that other models, such as the *Il10*$^{-/-}$ mouse colitis model, or the T cell transfer model of colitis, show a deficiency in IL-23 and IL-17 to result in less severe colitis development [202]. These findings are in contrast to those observed for the DSS-induced and TNBS-induced colitis models. This highlights a key aspect of IBD development, where IL-23/IL-17 mediated immune responses can be both beneficial and pathogenic to colitis development [202,214].

For the study of intestinal cancer, we utilized two specific murine models: Apc^{Min}-mediated intestinal cancer and AOM-induced colorectal cancer.

Apc$^{Min/+}$ mice are frequently used for the study of intestinal tumour development. These mice have a dominant mutation in the *Apc* gene, resulting in truncation of the gene product at amino acid 850, and the formation of non-functional Apc protein. Under normal circumstances, Apc is involved in the Wnt-signalling pathway, where Apc is complexed with GSK3β/Actin/CKIα and is crucial for phosphorylation and degradation of β-catenin. In the absence of functional Apc protein, β-catenin accumulates, translocates to the nucleus in increased amounts, and subsequently deregulates target gene transcription. In this process, transcriptional activation of numerous genes is altered, including for example the known oncogenes c-*Myc* and *Kras*. As a result, Apc$^{Min/+}$ mice develop many (up to 100) adenomatous polyps and adenomas throughout the length of the intestine, with most located in the small intestine. Tumour development can be noted as early on as 3-4 weeks of age, and progressively increases as mice age. Homozygous Apc^{Min} mice are embryonic lethal, and as such cannot be used for intestinal tumour research [218,219].

The AOM-induced colorectal cancer model is often used for the study of inflammation-driven colon cancer. Azoxymethane is a potent carcinogen, where a metabolite of AOM causes DNA mutations by altering nucleotides from G-C to A-T [220]. To induce carcinogenesis, mice are usually injected intraperitoneally with a single dose (10 mg/kg) of AOM. Subsequently, treatment is often supplemented with varying doses/cycles of DSS. In this context, inflammation-driven tumourigenesis takes place in the gut, with development of multiple adenomas and adenocarcinomas throughout the colon and rectum. Tumour development classically takes place over a period of 3-5 months, and serves as an excellent model for colorectal cancer development in humans [220,221].

VI. Research objectives

The intestinal epithelium, a polarised single-cell layer, is all that separates our 'clean' bodies from the 'dirty' environment, containing potentially harmful pathogens. Therefore, the intestinal epithelium must be optimally equipped to react quickly and correctly to breaching. To enable it to carry out this function, a multitude of immune cells are interspersed between the epithelial cells that constitute this barrier.

iELs have long been shown to reside in the intestinal epithelium, but a complete understanding of IEL function and regulation is lacking. ILCs are a novel set of immune cells, and a subgroup of ILC1 cells have also been shown to reside in the intestinal epithelium. The function of these cells remains elusive.

The Ly49E receptor has previously been shown to be expressed on innate-like cells, such as cNK cells, epidermal Vγ3 T cells, $DX5^-$ liver NK cells and $CD8\alpha\alpha^+$ small intestinal IEL. A first objective of this thesis is to investigate whether Ly49E is expressed not only by small intestinal but also by colonic IEL. Previous studies have demonstrated differences in IEL subset composition and function between the small intestine and colon [1,222]. As such it is important to examine Ly49E expression in the colon as well as the small intestine, and to evaluate how Ly49E expression on IEL of both gut compartments translates to IEL function of these cells. Subsequently, the question of whether Ly49E expression influences IEL differentiation will be addressed. Here, Ly49E KO mice will be used to examine differences in IEL subset composition and cell surface receptor expression as compared to Ly49E WT. IEL will be isolated from naive mice and examined in a basal resting state. If Ly49E affects IEL development and/or function in the basal resting state, this knowledge will be of importance when the role of these cells in intestinal disease is subsequently studied.

The second main aim of this thesis is to examine whether IEL Ly49E expression affects development and/or progression of inflammatory bowel diseases and intestinal tumour immune response. Here development refers to the establishment of disease, whereas progression refers to disease kinetics. With respect to inflammatory bowel diseases, disease progression may be measured through parameters such as the rate of weight loss, faecal consistency and occult blood, and the extent of mucosal inflammation. With respect to intestinal cancer development, disease progression may be evaluated through parameters such as the rate of tumour development and tumour size. A role for Ly49E expression by IEL in the development and/or progression of inflammatory diseases and intestinal tumour immune response will be examined for both IEL of the small intestine and colon. Where inflammatory bowel diseases are concerned, a role for IEL Ly49E expression in the small intestine will be examined through the use of the $TNF^{\Delta ARE/+}$ ileitis model, whereas a role for IEL Ly49E expression in the colon will be examined through the use of the DSS-induced and TNBS-induced colitis models. To study a possible role for Ly49E expression by IEL in the small intestinal tumour immune response, development of intestinal cancer in $Apc^{Min/+}$ mice will be evaluated. For the study of a possible role for Ly49E expression by IEL in colon intestinal tumour immune response, development of colorectal cancer in AOM/DSS-treated mice will examined.

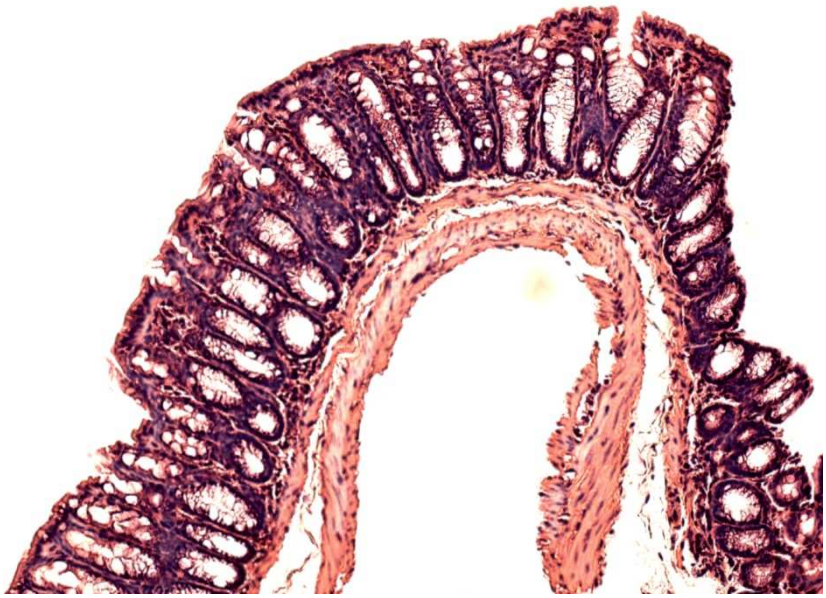
The third, and final, objective of this thesis is to investigate whether the Ly49E receptor is expressed by intestinal ILCs. To gain a full understanding of Ly49E function in intestinal mucosal immunity, it is important to have knowledge of all gut populations that express, and are possibly regulated by, this receptor. ILCs represent a novel innate immune population of which little is known to date, and as such it is of importance to examine whether Ly49E is also expressed by these cells.

Taken together, the overall objective of this PhD thesis is to gain valuable insights into the functional role of the Ly49E receptor in the immune response of murine intestinal intraepithelial T cells and ILCs. With respect to inflammatory bowel diseases and tumour immune response, identifying a function of Ly49E expression by IEL may constitute a first step towards combating these intestinal diseases. With respect to ILC cells, examining Ly49E expression on murine ILC subpopulations will contribute to ILC knowledge and may provide additional clues as to a role for Ly49E in regulation of mucosal immunity.

Chapter 2:

Results

Part I



Paraffin-embedded section of colonic crypts from an Ly49E WT mouse

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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 $TNF^{\Delta ARE}$ 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.

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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 relieve 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.

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, CD8αβ and CD8αα T cell receptor (TCR)αβ iIELs, and CD4/CD8 double-negative (DN) and CD8αα TCRγδ iIELs. TCRαβ CD4 and TCRαβ CD8αβ iIELs have been described as being 'induced' iIELs, whereas TCRαβ CD8αα, TCRγδ DN and TCRγδ CD8αα iIELs are also referred to as 'natural' iIELs [12]. More recently, Mucida *et al.* [13] have also described the existence of a sixth iIEL subpopulation, the TCRαβ 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 hyporesponsive 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 $TNF^{\Delta ARE}$ ileitis model. Our results indicate that Ly49E expression on both small intestinal and colonic CD8αα-expressing iIELs is abundant. However, abrogation of Ly49E expression on iIELs of the small intestine and colon does not influence development or progression of IBD.

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 $Ly49E^{WT/KO}$ mice were interbred to obtain homozygous $Ly49E^{WT/WT}$ and $Ly49E^{KO/KO}$ mice. $TNF^{\Delta ARE/WT}$ mice were a kind gift from Dr. G. Kollias (Institute for Immunology, Biomedical Sciences Research Center "Alexander Fleming," Attica, Greece). Heterozygous $TNF^{\Delta ARE/WT}$ ($Ly49E^{WT/WT}$) mice were bred to homozygous ($TNF^{WT/WT}$) $Ly49E^{KO/KO}$ mice in order to obtain $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/KO}$ offspring. Male $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/KO}$ and female $TNF^{WT/WT}$ $Ly49E^{WT/KO}$ offspring were bred to obtain homozygous $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ 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′CCTTCCTCACAGAGCCAGC-3′ and reverse-5′-AATTAC-GGTTAGGCTCCTGTTTCC-3′. Following agarose gel electrophoresis, $TNF^{\Delta ARE}$ 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 the WT allele, forward-5′-TCGCTTGGAATCTT-CTGTTTC-3′ and reverse-5′TCCTCACCTGGACTGCAAT-C-3′; for the KO allele, forward-5′GGAATAATTGCTGTTAC-CATTAG in combination with reverse-5′TCCTCACCTGGA-CTGCAATC-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 libidum* 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 µl/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 centrifugated for 20°C at 2000 g. iIELs were collected from the 44%/67% Percoll interface, washed twice with

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-CD8β (peridinin chlorophyll protein/Cy5.5-conjugated, clone YTS156.7.7), all from BioLegend, San Diego, CA, USA. Anti-TCRδ (PE- or FITC-conjugated, clone GL3), anti-Ly49D (FITC-conjugated, clone 4E5), anti-Ly49C/I (PE-conjugated, clone 5E6), anti-NKG2D (PE-conjugated, clone CX5), anti-CD69 (biotin-conjugated, clone H1.2F3), were obtained from Becton Dickinson, Franklin Lakes, NJ, USA. Anti-CD8α (PE/Cy7-conjugated, clone 53–6.7) from eBioscience, San Diego, CA, USA. Anti-Ly49G2-producing hybridoma (clone 4D11) was from ATCC and antibody was biotin- or FITC-conjugated in-house. Anti-Ly49A (biotin-conjugated, clone JR9-318; kindly provided by Dr J. Roland (Paris, France)), anti-Ly49E/C (biotin- or FITC-conjugated, clone 4D12, made and labeled in-house) [29] and anti-Ly49E/F (FITC-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 Ly49C-expressing cells ($CM4^-/4D12^+$), Ly49E-expressing cells ($CM4^+/4D12^+$) and Ly49F-expressing cells ($CM4^+/4D12^-$). In B6 ($H\text{-}2^b$) 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 (FITC-conjugated, clone 3S9, 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 ($8.68\times10^6\pm2.50$ vs. $6.60\times10^6\pm1.15$ iIELs, respectively) and colon of Ly49E WT and Ly49E KO mice ($0.32\times10^6\pm0.10$ vs. $0.46\times10^6\pm0.20$) 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 CD8αα-positive iIELs than other Ly49 receptors [24,33]. Here, we show that several NK receptors are also expressed on the surface of colonic CD8αα-positive iIELs. As for small intestinal iIEL expression, a high proportion of colonic iIELs express the Ly49E receptor. Surprisingly, the frequency of Ly49E-expressing colonic TCRγδ CD8αα-positive iIELs exceeds the frequency of Ly49E-expressing TCRγδ CD8αα-positive iIELs in the small intestine, whereas we found the proportion of Ly49E-expressing TCRαβ CD8αα-positive 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 CD8αα-negative iIELs was low or absent (data not shown).

Ly49E expression on CD8αα-expressing iIELs of the colon does not influence DSS-induced colitis

To study a possible role for Ly49E expression on colonic CD8αα-expressing 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 ($p\geq0.35$; 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] recently 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 observe a trend towards upregulation of NKG2D expression on TCRαβ CD8αα-positive iIELs and TCRγδ CD8αα-positive iIELs in DSS-induced colitis, but this was not significant ($p\geq0.083$; Mann-Whitney test). NKG2D expression between Ly49E WT and Ly49E KO

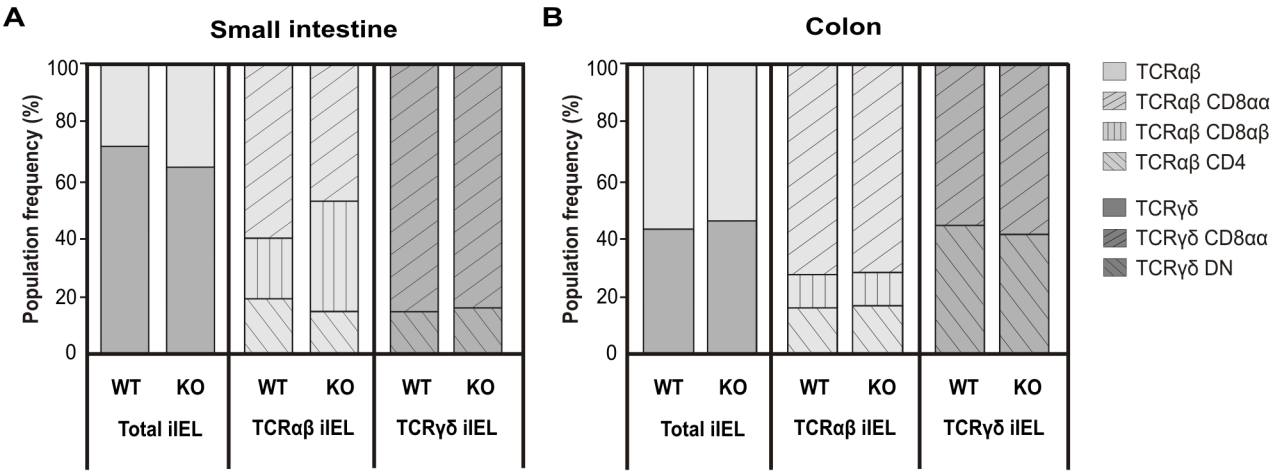

Figure 1. iIEL subpopulation frequencies in the small intestine and colon of Ly49E WT versus Ly49E KO mice. A) iIEL subpopulation frequencies in the small intestine of Ly49E WT versus Ly49E KO mice. TCRαβ and TCRγδ iIEL population frequencies are shown as a percentage of the total iIELs present in the small intestine and colon of Ly49E WT versus Ly49E KO mice. TCRαβ CD4, TCRαβ CD8αβ and TCRαβ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRαβ 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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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 CD8αα-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 CD8αα-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 to be unchanged between Ly49E WT and Ly49E KO mice 72 h after treatment initiation as compared to healthy control mice (data not shown). Therefore, we conclude that Ly49E expression on colonic CD8αα-expressing iIELs does not alter TNBS-induced colitis development or progression.

$TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ 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 $TNF^{\Delta ARE}$ model of ileitis. Harbouring a deletion of the TNF 3′ AU-rich elements, $TNF^{\Delta ARE/WT}$ mice develop spontaneous ileitis that highly resembles human Crohn's disease [35,36]. Here, we bred Ly49E WT and Ly49E KO mice to heterozygous $TNF^{\Delta ARE/WT}$ mice in two rounds, generating $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ offspring. Monitoring weight progression of $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice, and their littermate controls, we noted decreased weights for $TNF^{\Delta ARE/WT}$ mice as compared to $TNF^{WT/WT}$ mice, as expected. Furthermore, we observed no significant difference in weight progression of $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ versus $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice between 5 and 14 weeks of age (Fig. 7A). Subsequently, we analysed iIEL numbers, subpopulation frequencies, NK receptor and activation marker expression in $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ 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 $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice were similar ($8.57\times10^6\pm2.73$ vs. $9.57\times10^6\pm3.39$ iIELs, respectively). Studying iIEL subpopulation frequencies, we noted a significant increase in TCRαβ CD4 iIELs ($p\leq0.001$; ANOVA), and a significant decrease in TCRαβ CD8αα iIELs ($p\leq0.001$; ANOVA), in the intestines of $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice as compared to their littermate controls. However, no differences were observed in iIEL subpopulation frequencies between $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice (Fig. 7B). Similarly, we noted no significant differences in the NK receptor expression of iIELs from $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice (data not shown). To confirm that iIELs were activated in this $TNF^{\Delta ARE}$ ileitis model, we stained iIELs for expression of the activation marker CD69. Small intestinal iIELs from $TNF^{\Delta ARE/}$

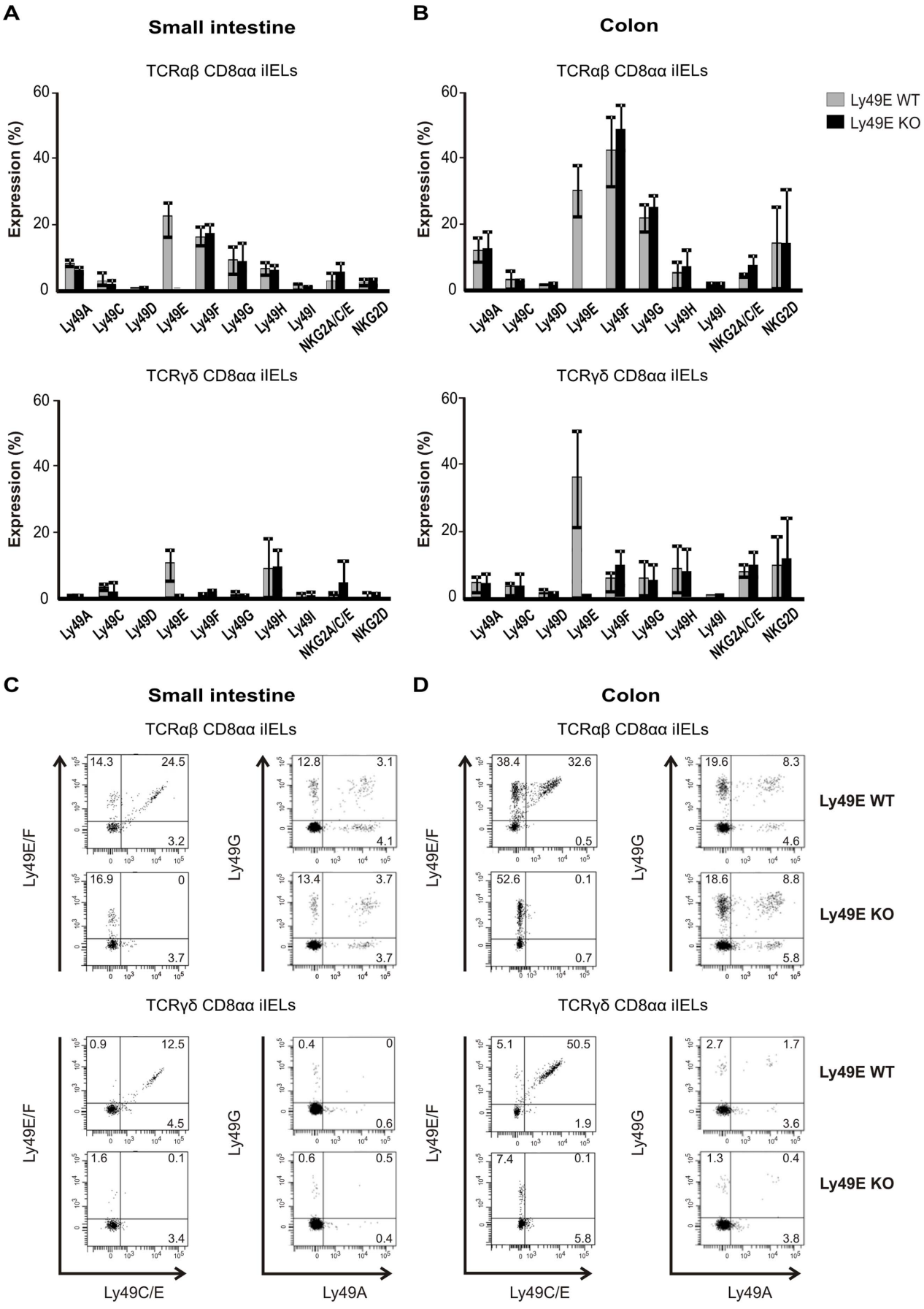

A
Small intestine
TCRαβ CD8αα iILEs
Expression (%)
60
40
20
0
Ly49A
Ly49C
Ly49D
Ly49E
Ly49F
Ly49G
Ly49H
Ly49I
NKG2A/C/E
NKG2D
TCRγδ CD8αα iILEs
B
Colon
TCRαβ CD8αα iILEs
TCRγδ CD8αα iILEs
Ly49E WT
Ly49E KO
C
Small intestine
TCRαβ CD8αα iILEs
14.3
24.5
3.2
16.9
0
3.7
12.8
3.1
4.1
13.4
3.7
3.7
TCRγδ CD8αα iILEs
0.9
12.5
4.5
1.6
0.1
3.4
0.4
0
0.6
0.6
0.5
0.4
Ly49E/F
Ly49G
Ly49C/E
Ly49A
D
Colon
TCRαβ CD8αα iILEs
38.4
32.6
0.5
52.6
0.1
0.7
19.6
8.3
4.6
18.6
8.8
5.8
TCRγδ CD8αα iILEs
5.1
50.5
1.9
7.4
0.1
5.8
2.7
1.7
3.6
1.3
0.4
3.8
Ly49E WT
Ly49E KO

Figure 2. iIEL NK receptor expression in the small intestine and colon of Ly49E WT versus Ly49E KO mice. A) NK receptor expression on TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs in the small intestine of Ly49E WT versus Ly49E KO mice. B) NK receptor expression on TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs in the colon of Ly49E WT versus Ly49E KO mice. C) Co-expression of Ly49 receptors on the iIEL surface of TCRαβ CD8αα iIELs and TCRγδ CD8αα 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. D) Co-expression of Ly49 receptors on the iIEL surface of TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs in the colon 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 ±SD (n = 5, small intestine; n = 5, colon, where each value represents the mean of a pool of 3 mice).
doi:10.1371/journal.pone.0110015.g002

^{WT} Ly49E$^{WT/WT}$ and TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$ mice expressed significantly higher CD69 levels as compared to their littermate controls for both TCRαβ CD8αα and TCRγδ CD8αα iIELs ($p \leq 0.001$; ANOVA). However, CD69 expression levels were comparable between TNF$^{\Delta ARE/WT}$ Ly49E$^{WT/WT}$ and TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$ mice, and between control Ly49E WT and Ly49E KO mice (Fig. 7C). All experiments were repeated with mice at 14 weeks of age, and similar results were obtained (data not shown). Together, this data suggests that Ly49E expression on small intestinal iIELs does not affect ileitis development or progression.

Discussion

In this paper we show that the Ly49E NK receptor is abundantly expressed on CD8αα-expressing iIELs of the small intestine as well as the colon. Herein, the frequency of Ly49E-expressing colonic CD8αα-positive iIELs is greater than the frequency of Ly49E-expressing small intestinal CD8αα-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 γδ-iIELs with plexin B2 on epithelial cells is required for keratinocyte-growth factor (KGF-1)-mediated epithelial repair following DSS-induced colitis. A role

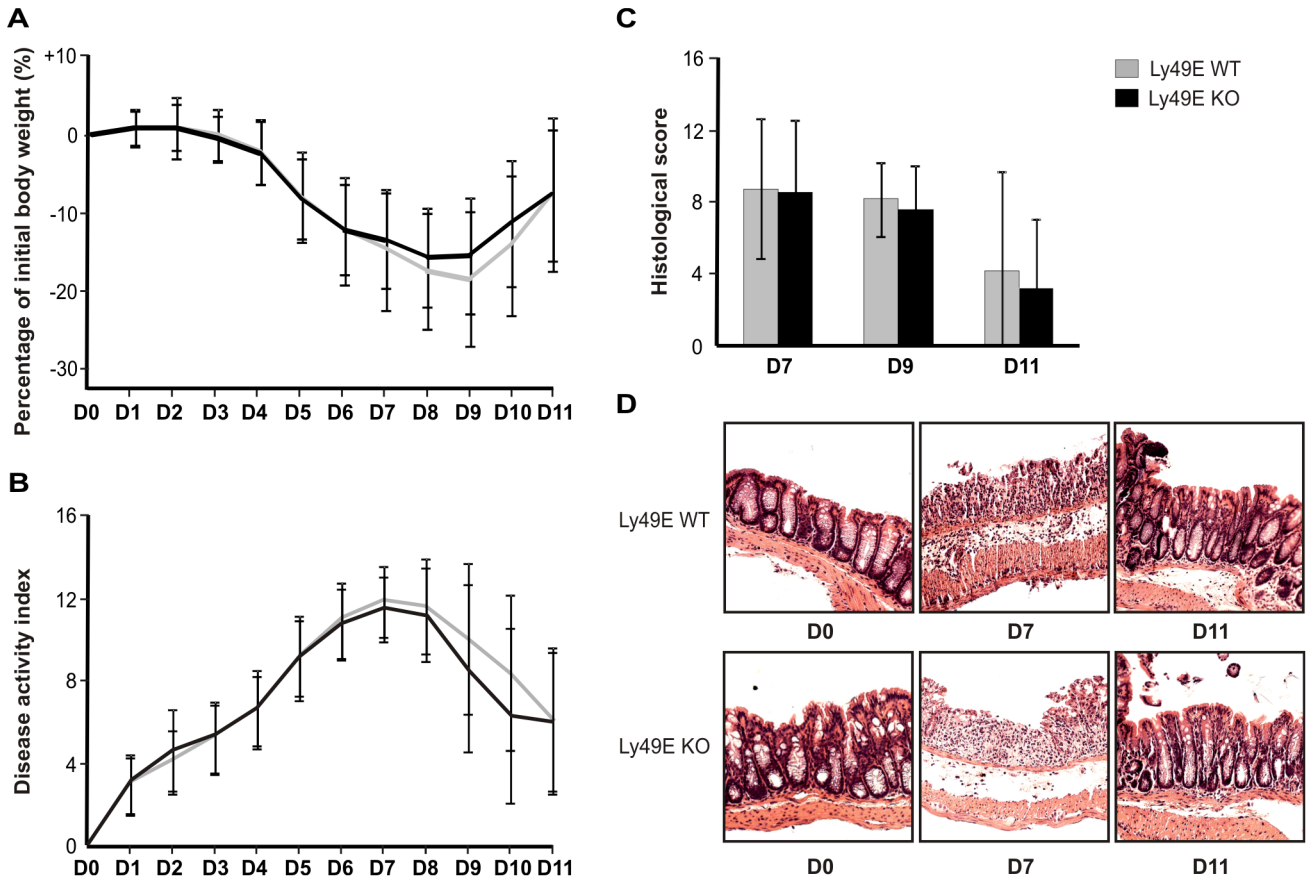
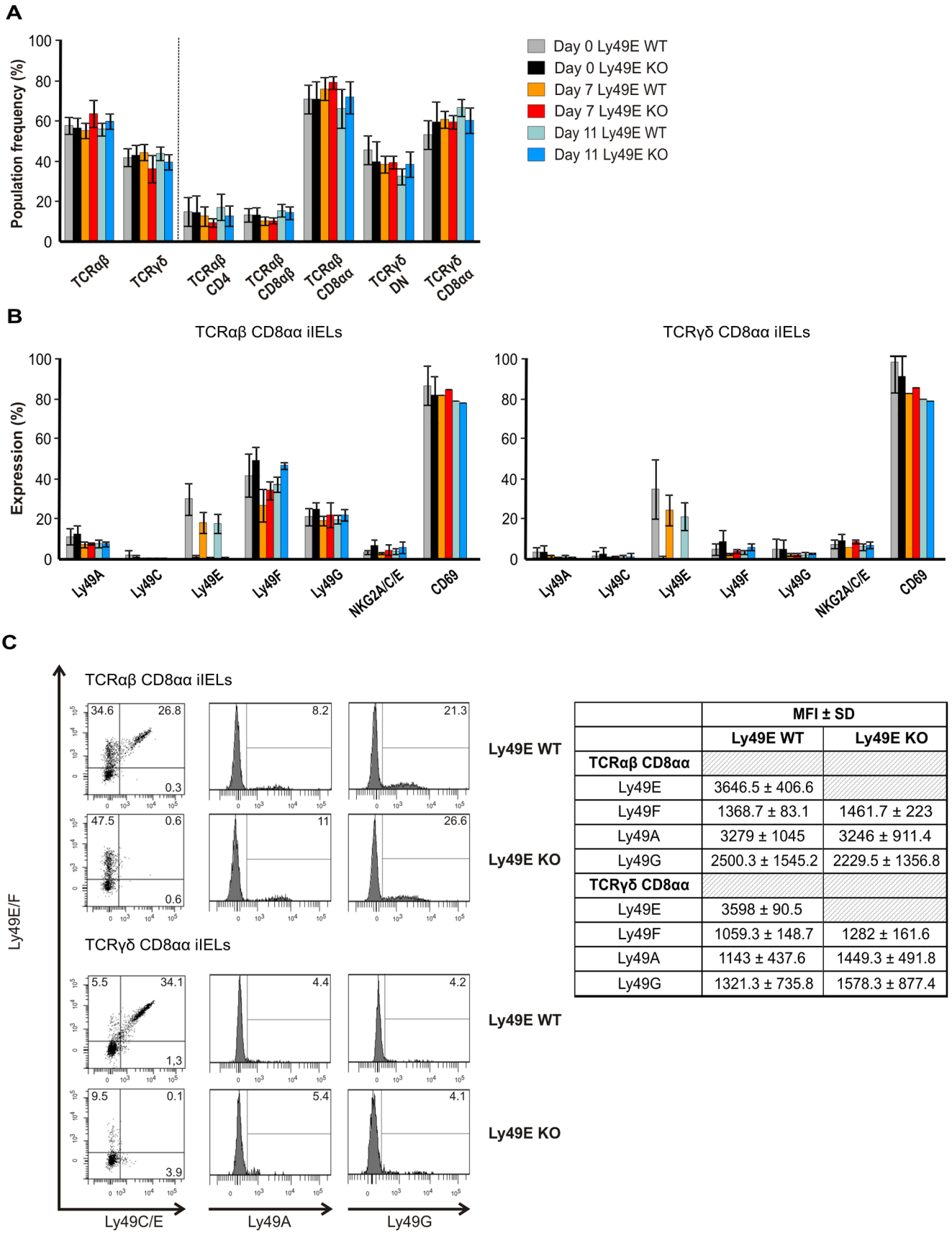


Figure 3. Clinical symptoms of DSS-induced colitis in Ly49E WT versus Ly49E KO mice. Colitis was induced in Ly49E WT and Ly49E KO mice by administration of DSS in drinking water for 7 days. Thereafter, mice received normal drinking water. Ly49E WT and Ly49E KO mice were scored and compared at the indicated days. A) Relative weight loss (mean ±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 ±SD; n = 71). C) Colon histological score (mean ±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



	MFI ± SD	
	Ly49E WT	Ly49E KO
TCRαβ CD8αα		
Ly49E	3646.5 ± 406.6	
Ly49F	1368.7 ± 83.1	1461.7 ± 223
Ly49A	3279 ± 1045	3246 ± 911.4
Ly49G	2500.3 ± 1545.2	2229.5 ± 1356.8
TCRγδ CD8αα		
Ly49E	3598 ± 90.5	
Ly49F	1059.3 ± 148.7	1282 ± 161.6
Ly49A	1143 ± 437.6	1449.3 ± 491.8
Ly49G	1321.3 ± 735.8	1578.3 ± 877.4

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, TCRαβ

CD8αβ and TCRαβ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRαβ iIELs. TCRγδ DN and TCRγδ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRγδ iIEL. B) Colon iIEL NK receptor and activation marker expression on TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs (mean ±SD; n = 5 on day 0, n = 3 on day 7 and n = 4 on day 11, where each value represents the mean of a pool of 3 mice). C) Colon iIEL NK receptor expression on TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs on day 7 following induction of DSS-induced colitis. Numbers indicate the percentage of cells expressing the indicated Ly49 receptors, and are each representative of 5 mice. Representative data are shown on the left. Mean fluorence intensity (MFI) values for each receptor are shown on the right.
doi:10.1371/journal.pone.0110015.g004

for iIELs in TNBS-induced colitis has been shown by Inagaki-Ohara *et al.* [38], who demonstrated that γδ T cell-deficient ($C\delta^{-/-}$) mice show high susceptibility to TNBS-induced colitis at young age, and that subsequent transfer of γδ IELs to $C\delta^{-/-}$ 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

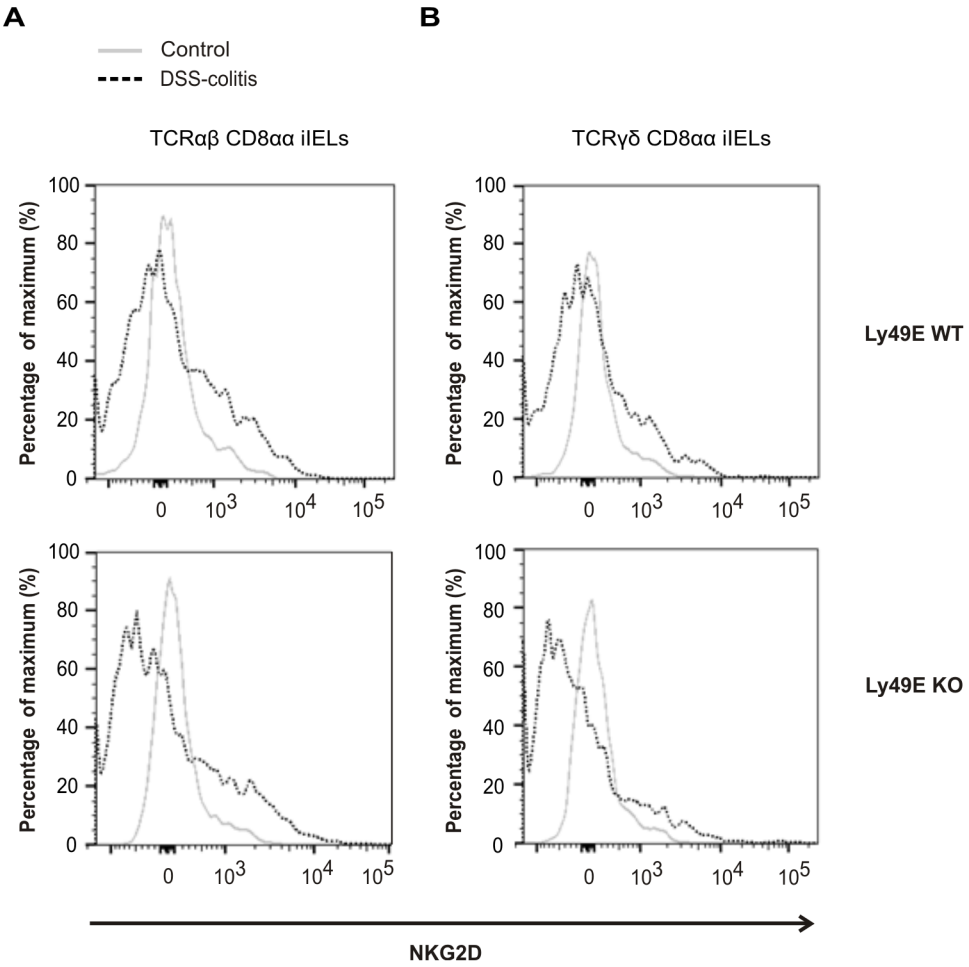


Figure 5. iIEL NKG2D expression upon DSS-induced colitis in Ly49E WT and Ly49E KO mice. Colitis was induced in Ly49E WT and Ly49E KO littermate mice by administration of DSS in drinking water for 7 days. A) NKG2D expression on TCRαβ CD8αα iIELs. B) NKG2D expression on TCRγδ CD8αα iIELs. N = 2 for control mice, n = 3 for DSS-treated mice.
doi:10.1371/journal.pone.0110015.g005

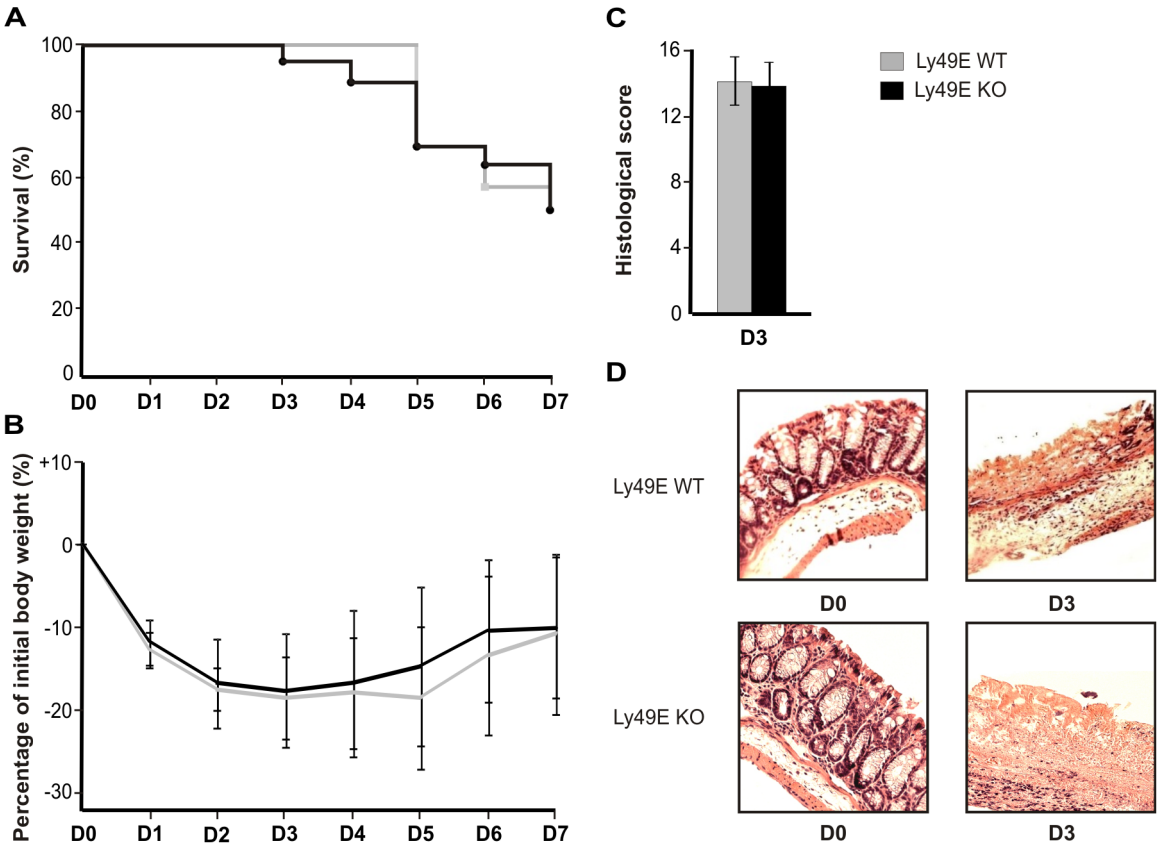


Figure 6. TNBS-induced colitis in Ly49E WT versus Ly49E KO mice. Colitis was induced in Ly49E WT and Ly49E KO mice by intra-rectal administration of TNBS. Mice were analysed at the indicated days. A) Survival (n = 16). B) Relative weight loss (mean ±SD; n = 16). The reference weight was taken as the weight on day 0, at the start of the experiment. C) Colon histological score (mean ±SD; n = 5). D) Representative hematoxylin/eosin- stained paraffin sections of the distal colon.
doi:10.1371/journal.pone.0110015.g006

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 $TNF^{\Delta ARE}$ model, has been reported by Apostolaki *et al.* [49], who showed that intestinal inflammation in $TNF^{\Delta ARE/WT}$ 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 $TNF^{\Delta ARE/WT}$ mice in two rounds, generating $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ offspring for the study of the role of Ly49E expression on iIELs in ileitis development and progression. Our results show that $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ mice display with similar ileitis disease kinetics. Concurrent with results from Apostolaki *et al.* [49], we observe a significant decrease of TCRαβ CD8αα iIELs in the small intestine of $TNF^{\Delta ARE/WT}$ mice compared to their littermate controls. Alongside, we note a significant increase of TCRαβ CD4 iIELs in $TNF^{\Delta ARE/WT}$ mice compared to their littermate controls. Furthermore, we show activation of $TNF^{\Delta ARE/WT}$ small intestinal iIELs in ileitis, with significantly higher CD69 expression in $TNF^{\Delta ARE/WT}$ 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 $TNF^{\Delta ARE/WT}$ $Ly49E^{WT/WT}$ and $TNF^{\Delta ARE/WT}$ $Ly49E^{KO/KO}$ 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,

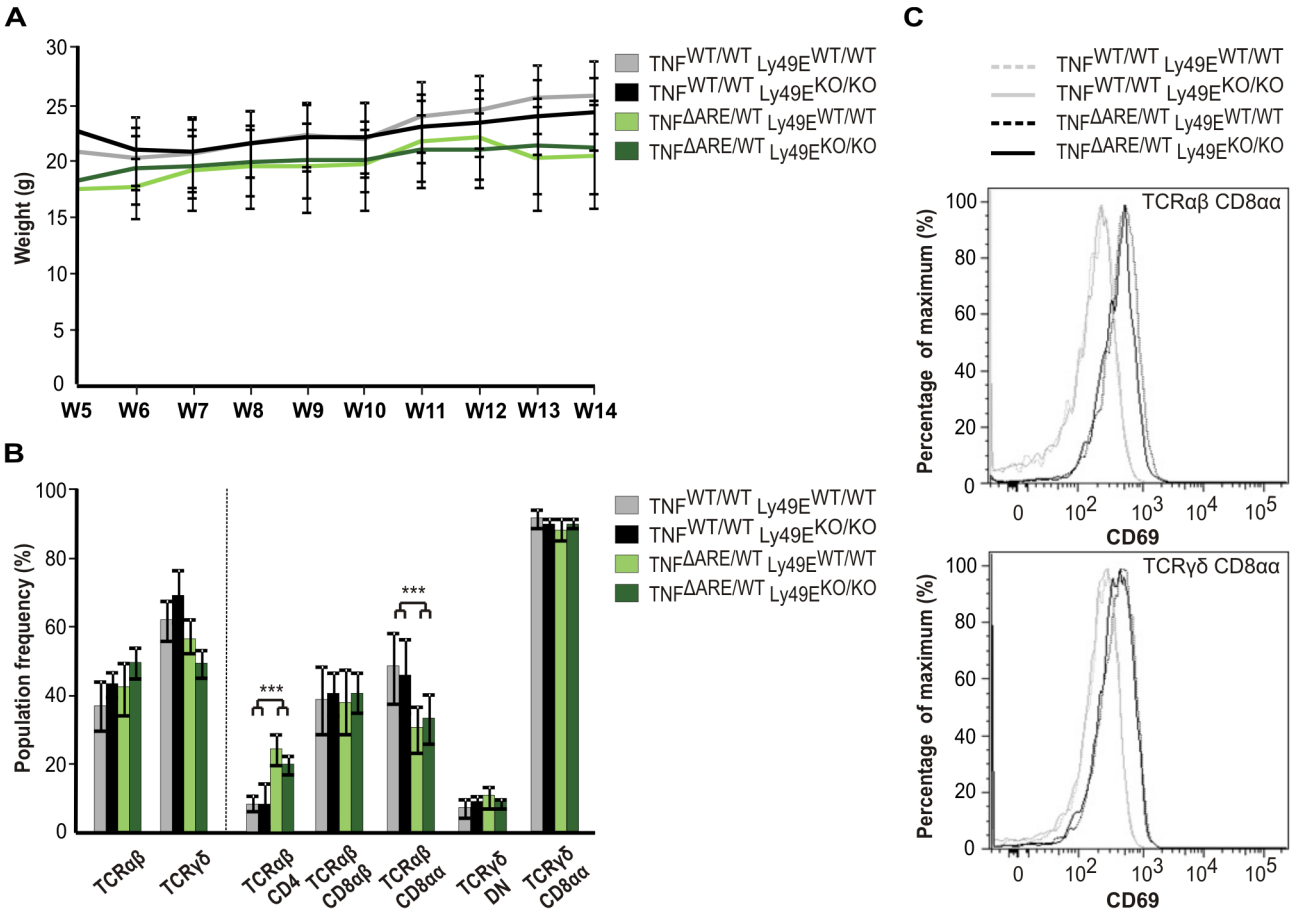


Figure 7. TNF$^{\Delta ARE}$-induced ileitis on an Ly49E WT versus Ly49E KO background. A) Weight progression of TNF$^{\Delta ARE/WT}$ Ly49E$^{WT/WT}$ and TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$ mice as compared to TNF$^{WT/WT}$ Ly49E WT and Ly49E KO mice (mean ±SD; n = 30 (TNF$^{\Delta ARE/WT}$ Ly49E$^{WT/WT}$), n = 22 (TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$), n = 31 (TNF$^{WT/WT}$ Ly49E WT) and n = 21 (TNF$^{WT/WT}$ Ly49E KO)). Weight was monitored weekly from the age of genotyping (5 weeks of age) up to 14 weeks of age. B) Small intestinal iIEL subpopulation frequencies in TNF$^{\Delta ARE/WT}$ Ly49E$^{WT/WT}$ and TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$ mice as compared to TNF$^{WT/WT}$ Ly49E WT and Ly49E KO mice (mean ±SD; n = 6). TCRαβ CD4, TCRαβ CD8αβ and TCRαβ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRαβ iIELs. TCRγδ DN and TCRγδ CD8αα iIEL subpopulation frequencies are shown as a percentage of the total TCRγδ iIEL. C) CD69 expression on small intestinal TCRαβ CD8αα iIELs and TCRγδ CD8αα iIELs of TNF$^{\Delta ARE/WT}$ Ly49E$^{WT/WT}$ and TNF$^{\Delta ARE/WT}$ Ly49E$^{KO/KO}$ mice as compared to TNF$^{WT/WT}$ Ly49E WT and Ly49E KO mice (mean ±SD; n = 6).
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Ly49E expression on CD8αα-expressing iIELs does not influence the development or progression of inflammatory bowel diseases.

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Author Contributions

Conceived and designed the experiments: AVA GL. Performed the experiments: AVA JF ST EVA MV. Analyzed the data: AVA JF SVW ST EVA MV LD. Contributed reagents/materials/analysis tools: TK TT BV JP. Wrote the paper: AVA GL.

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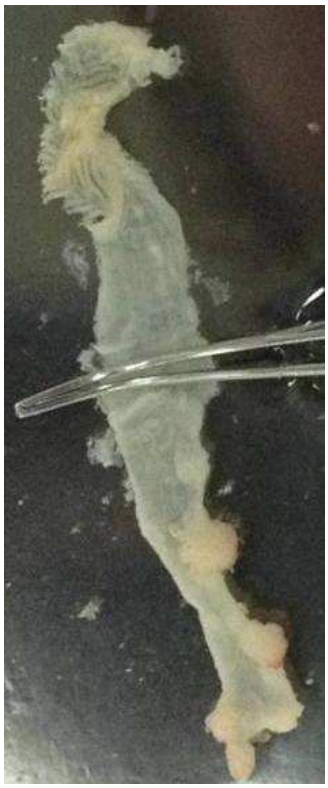
Part II



Tumour formation in the colon of an azoxymethane-treated Ly49E KO mouse.

The role of Ly49E receptor expression on intraepithelial lymphocytes in intestinal cancer development and progression

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Abstract

Ly49E is a member of the Ly49 family of natural killer (NK) receptors and is distinct from other members of this family on the basis of its structural properties, expression pattern and ligand recognition. Importantly, Ly49E receptor expression is high on small intestinal and colonic intraepithelial lymphocytes (IELs). Intestinal IELs are important regulators of the mucosal immune system and contribute to front-line defense at the mucosal barrier, including anti-tumour immune response. Whereas most Ly49 receptors have major histocompatibility complex (MHC) class-I ligands, we showed that Ly49E is instead triggered by urokinase plasminogen activator (uPA). uPA has been extensively implicated in tumour development, where increased uPA levels are linked to poor prognosis. As such, we investigated the role of Ly49E receptor expression on intestinal IELs in the anti-tumour immune response. For this purpose we compared Ly49E wild-type (WT) mice to Ly49E knockout (KO) mice in two established tumour models: $Apc^{Min/+}$-mediated and azoxymethane-induced intestinal cancer. Our results indicate that Ly49E expression on IELs does not influence the development or progression of intestinal cancer.

Introduction

Colorectal cancer represents the third most common type of cancer worldwide [1,2]. Of all cases, 10-30% are hereditary, such as familial adenomatous polyposis coli and hereditary non-polyposis colon cancer. The majority, however, can be attributed to non-familial sporadic mutations and colitis-associated cancer [3]. For patients, treatment options today are limited. Curative resection in combination with chemo- or radiotherapy is standard, but recurrence rates are high, and up to 50% [4]. Alongside, a variety of new immunotherapies have been developed, including peptide-, whole tumour lysate-, dendritic cell- and viral vector-based vaccines. Adoptive cell transfer, including chimeric antigen receptor therapy, and antibody-based therapies have also been introduced [5]. However, all currently available therapies struggle with low efficiency and limited success rates [5]. Therefore, sustained and additional research to elucidate the mechanisms that operate in intestinal cancer progression and development are crucial to improve outcome in therapies.

Intestinal intraepithelial lymphocytes (IELs) are key to maintaining intestinal homeostasis. Strategically positioned within the intestinal epithelium, these cells have been implicated in the recognition of stress signals [6] and repair of the intestinal epithelium [7]. Furthermore, IELs have been attributed a role in intestinal tumour immune response as demonstrated by a number of *in vitro* and *in vivo* studies. Cytotoxicity of intestinal IELs towards epithelial cell tumours was first reported in 1993 [8]. Later, Ebert *et al.* [9] showed that interaction of CD2 on the IEL surface with tumour-expressed CD58 induces expression of IL-8, TNF-α and IFN-γ by IELs, with a resulting enhanced anti-tumour response. Additionally, binding of IEL-expressed NKG2D to tumour MIC and ULBP ligands may trigger Fas-mediated lysis of tumour cells [10]. Moreover, Baker *et al.* [11] showed that colorectal cancers preferentially retain intestinal IEL rather than peripherally derived CD8 T cells. Together, these data illustrate that IELs, and NK receptors expressed on the IEL cell surface, play an important role in mediating intestinal tumour immune response.

Ly49E is an inhibitory member of the Ly49 natural killer (NK) receptor family [12], characterised by a unique expression profile. Ly49E expression is high on tissue-resident lymphocytes, including CD8αα-positive IEL [13,14]. IELs expressing inhibitory Ly49 receptors, including Ly49E, are hyporesponsive to TCR-mediated stimulation [13]. Importantly, *in vitro* TCR-triggering results in upregulation of Ly49E receptor expression on IELs [15]. Furthermore, whereas all other inhibitory Ly49 receptors have MHC class-I ligands, Ly49E is triggered by the non-MHC-related protein urokinase plasminogen activator (uPA) [16]. Importantly, uPA has been implicated in tumour development in general, and colorectal cancer in particular, as an increase in tumour uPA expression has been noted in the colorectal adenoma-carcinoma transition [17-22]. Also, in a clinical setting, increased uPA levels have been linked to poor patient prognosis [23,24]. Indeed, targeted uPA antibodies and inhibitors have been shown to reduce tumour growth, cancer cell spread and metastasis [25,26].

With the recent finding that uPA triggers the inhibitory Ly49E receptor, along with the observed constitutive expression of Ly49E on intestinal IELs and its additional upregulation upon TCR-mediated activation, we hypothesize that Ly49E receptor expression by IELs affects their *in vivo* immune response against intestinal tumours. If this hypothesis is correct, uPA produced by tumour cells would not only promote tumour invasion and metastasis, but would also be used as a novel tumour escape mechanism from host innate-like T cells. We tested this hypothesis by comparing Ly49E KO to Ly49E WT mice in two intestinal tumour models, $Apc^{Min/+}$-mediated and azoxymethane (AOM)-induced intestinal cancer.

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 [27]. Heterozygous $Apc^{Min/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). For the generation of $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice, male $Apc^{Min/+}$ $Ly49E^{WT/WT}$ mice were first bred to female $Apc^{+/+}$ $Ly49E^{KO/KO}$ mice, after which male heterozygous $Apc^{Min/+}$ $Ly49E^{WT/KO}$ offspring were bred to female heterozygous $Apc^{+/+}$ $Ly49E^{WT/KO}$ offspring to generate $Apc^{Min/+}$ $Ly49E^{WT/WT}$, $Apc^{Min/+}$ $Ly49E^{KO/KO}$, $Apc^{+/+}$ $Ly49E^{WT/WT}$ and $Apc^{+/+}$ $Ly49E^{KO/KO}$ mice at the expected ⅛ Mendelian ratio. All mice were housed and bred in our animal facility, and all animal experimentation was performed after approval by 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 using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA). PCR amplification of genomic DNA from the *Apc* locus was carried out with the following primers: forward-5'-TGAGAAAGACAGAAGTTA-3' and reverse-5'-TCCACTTTGGCATAAGGC-3'. Following agarose gel electrophoresis, the Apc^{Min} allele yields a band of 313 bp, whereas the Apc WT allele does not yield a band due to primer mismatch. PCR amplification of genomic DNA for the *Ly49E* locus was carried out making use of the following primers: for the WT allele, forward-5'-TCGCTTGGAATCTTCTGTTTC-3' and reverse-5'TCCTCACCTGGACTGCAATC-3'; for the KO allele, forward-5'GGAATAATTGCTGTTACCATTAG in combination with reverse-5'TCCTCACCTGGACTGCAATC-3'. Ly49E WT and KO alleles yield bands of 1070 and 1200 bp, respectively.

Azoxymethane-induced colorectal cancer

Azoxymethane-induced colorectal cancer was induced in 7 to 11-week old mice. At day 0, mice were intraperitoneally injected with 10 mg/kg azoxymethane (AOM) (Sigma-Aldrich, St. Louis, MO, USA). At day 7 of treatment, mice were administered 2.5% (w/v) dextran sodium sulphate (DSS) (molecular weight 36,000-50,000, MP Biomedicals, Cleveland, OH, USA) in drinking water and mice were allowed 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. At days 28 and 39, a second and third DSS-treatment cycle was initiated, and mice were administered 2% DSS in drinking water. Once more, mice were allowed to drink *ad libitum* for 7 days, following which drinking water containing DSS was replaced by normal drinking water and mice were allowed to recover. Control mice were not treated with azoxymethane, but were subjected to DSS treatment in an identical manner to azoxymethane-treated mice.

Assessment of mouse weight, intestinal length and tumour load/distribution

Mice were weighed weekly, and at each time point of analysis. After sacrifice by cervical dislocation, the small intestine or colon was removed and intestinal length was determined. Intestines were then cleaned of all mesentery and cut open longitudinally for microscopic analysis of tumour load, size and distribution. The maximal tumour diameter was used to determine tumour size. For tumour distribution, intestines were cut into three equal-sized segments and the number of tumours per segment was noted.

Isolation of tumour-infiltrating intestinal IELs

Tumour-infiltrating IELs were isolated as follows. Briefly, the small intestine or colon was removed and cleaned of mesenteric fat. Peyer's patches were excised and intestines were rinsed with DPBS to remove fecal content. Subsequently, intestines were opened longitudinally and screened for tumour load and distribution. Tumour-rich sections of the small intestine, or dissected colonic and colorectal tumours, were cut into 0.5-cm pieces, and 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 (DTT; Sigma-Aldrich) at slow rotation. Between the first and second incubation, intestinal pieces were collected and cut into 1 mm-fragments with surgical scalpel blades to ensure complete dissection of tumours. Following incubation, 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 min at 2000 g. IELs were collected from the 44%/67% Percoll interface, washed twice with 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). Small intestinal and colonic IELs were isolated from control mice according to the same protocol.

Antibodies

mAbs used for staining were as follows: anti-TCRβ (APC/Cy7-conjugated, clone H57.597), anti-CD4 (peridinin chlorophyll protein/Cy5.5-conjugated, clone GK1.5), anti-CD8β (peridinin chlorophyll protein/Cy5.5-conjugated, clone YTS156.7.7), all from BioLegend (San Diego, CA, USA). Anti-TCRδ (PE- or FITC-conjugated, clone GL3), obtained from Becton Dickinson, (Franklin Lakes, NJ, USA). Anti-CD8α (PE/Cy7-conjugated, clone 53-6.7) from eBioscience (San Diego, CA, USA). Anti-Ly49C/E (biotin- or FITC-conjugated, clone 4D12, made and labeled in-house) [28] and anti-Ly49E/F (FITC-conjugated, clone CM4; kindly provided by Dr C. G. Brooks (Newcastle on Tyne, UK)) [29]. mAb 4D12 (Ly49C/E) in combination with mAb CM4 (Ly49E/F) were used to identify Ly49E-expressing cells ($CM4^+/4D12^+$), Ly49C-expressing cells ($CM4^-/4D12^+$) and Ly49F-expressing cells ($CM4^+/4D12^-$). 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, NY, USA). Propidium iodide was used to discriminate live and dead cells. Flow cytometric analysis was performed using a BD LSRII flow cytometer, and samples were analysed with FACSDiva Version 6.1.2 software (BD Biosciences).

Immunohistochemistry

For histological analysis, tumour-rich sections of the distal ileum and distal colon were fixed in 4% formaldehyde solution (VWR, Radnor, PA, USA) and embedded in paraffin. 5 µm formalin-fixed paraffin-embedded sections were stained with hematoxylin and eosin (Sigma-Aldrich) for analysis of tumour morphology. Alternately, sections were immersed in target retrieval solution, pH6 (Dako, Glostrup, Denmark) for 20 min at 95°C for antigen retrieval. Subsequently, sections were stained for immunofluorescent analysis of tumour-infiltrating lymphocytes. Antibodies used for immunofluorescent staining were polyclonal rabbit anti-human/mouse CD3 (Dako) and Dylight649 donkey anti-rabbit (BioLegend). Cell nuclei were counterstained by mounting with ProLong Gold anti-fade reagent with DAPI (Invitrogen). Stained sections were analysed on a Leica TCS SPE (Leica Microsystems, Wetzlar, Germany) using the LAS4 and LASAF software.

uPA ELISA

Tumours were microscopically dissected and weighed. Subsequently, tumours were lysed in buffer composed of 1 part PBS/1 part caspase lysis buffer/2 parts blocking buffer (0.1 M Tris, 0.15 M NaCl, 3% BSA, pH 7.4), on a Precellys24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). The uPA content of the tumour lysates was determined using the Mouse uPA Total ELISA Kit (Innovative Research, Novi, MI, USA). Results are expressed as ng uPA per mg of tumour tissue.

Statistics

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

Results

Mouse weight, small intestinal length and tumour load is unaltered in $Apc^{Min/+}$ mice on an Ly49E KO background

To study the role of Ly49E in intestinal cancer development and progression in $Apc^{Min/+}$ mice, we generated $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates. When mice were weighed at 14 weeks of age, mouse weight did not differ significantly between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates, nor between $Apc^{Min/+}$ and control $Apc^{+/+}$ littermates (Fig 1A). A putative link between *Dvl2* gene expression and small intestinal length in Apc^{Min} mice has been reported [30]. To investigate whether *Ly49e* gene expression influences intestinal length in Apc^{Min} mice, we analysed small intestinal length in $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates at 14 weeks of age, when tumour load was highest. As illustrated in Fig 1B, small intestinal length was unchanged between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates, and between $Apc^{Min/+}$ and control $Apc^{+/+}$ mice. When analyzing the small intestine tumour load during aging, there was a clear increase in tumour load as mice aged from 8 to 14 weeks of age. However, tumour numbers were comparable between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates at all time-points analysed (Fig 1C). Tumour distribution, i.e. in the proximal, middle or distal parts of the small intestine, was also similar for $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates (data not shown). As expected, no intestinal tumours were detected in $Apc^{+/+}$ mice (data not shown).

Tumour-infiltrating T cells and tumour uPA expression levels are similar between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice

To examine possible differences in tumour-infiltrating T lymphocytes in $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates, we first stained tumour sections for $CD3^+$ infiltrating T cells. As illustrated in Fig 2A, tumour-infiltrating T cells were clearly present, but we did not observe discernible differences in the numbers of tumour-infiltrating T cells between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice. To investigate possible changes in subpopulations of tumour-infiltrating T lymphocytes between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates, tumour-infiltrating IEL were isolated from tumour-rich tissue and population frequencies were analysed, and compared to IEL subpopulations of $Apc^{+/+}$ littermate mice. At 14 weeks of age, we noted a slight increase in TCRαβ lymphocytes, and decrease in TCRγδ lymphocytes, for $Apc^{Min/+}$ mice compared to $Apc^{+/+}$ mice. TCRαβ and TCRγδ lymphocyte frequencies were not different in tumour-infiltrating T lymphocytes of $Apc^{Min/+}$ mice on an Ly49E WT versus Ly49E KO background. Within the TCRαβ lymphocyte fraction, we observed no differences between the CD4, CD8αβ and CD8αα subpopulations. Within the TCRγδ lymphocyte fraction, double-negative (DN: $CD4^-$, $CD8^-$) and CD8αα cell frequencies were unchanged (Fig 2B). The frequency of Ly49E-expressing TCRαβ and TCRγδ IELs did not differ significantly between $Apc^{Min/+}$ Ly49E WT and $Apc^{+/+}$ Ly49E WT littermates (data not shown). We have previously shown that uPA triggers Ly49E and that this results in inhibition of cytotoxicity as well as cytokine production of Ly49E-expressing lymphocytes [16]. Furthermore, extensive research has shown that uPA is critical in tumour development and metastasis [24,25,31]. To investigate a putative link between Ly49E and tumour uPA expression levels, we analysed uPA expression levels in lysates of intestinal tumours from $Apc^{Min/+}$ Ly49E WT versus $Apc^{Min/+}$ Ly49E KO mice. As illustrated in Fig

2C, tumour uPA expression levels were comparable between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice.

Mouse weight, colon length and colon tumour load are similar between azoxymethane (AOM)-treated Ly49E WT and Ly49E KO mice

To induce tumour formation in the colon, Ly49E WT and Ly49E KO mice were administered 10 mg/ kg AOM and subsequently subjected to 3 cycles of dextran sodium sulphate (DSS) treatment, eliciting colitis-associated colorectal cancer. Mice were analysed at 14, 16 and 22 weeks following the start of treatment. At 22 weeks, mouse weight was unchanged between AOM-treated and untreated control mice. Moreover, we did not observe weight differences between AOM-treated Ly49E WT and AOM-treated Ly49E KO mice (Fig 3A). As severe colitis is linked to colon shortening [1], we analysed colon length in untreated and AOM-treated mice. Colon length was not significantly different in AOM-treated Ly49E KO as compared to AOM-treated Ly49E WT mice, nor in AOM-treated versus untreated mice (Fig 3B). When analyzing colon tumour load, we observed that tumours of varying size developed. Overall, tumour load increased as treatment progressed. However, tumour load was comparable between AOM-treated Ly49E KO and AOM-treated Ly49E WT mice (Fig 3C). We did not note significant differences in tumour size (Fig 3D). Alongside, tumour distribution was similar for AOM-treated Ly49E KO as compared to Ly49E KO mice, with tumour development almost exclusively restricted to the middle and distal colon (data not shown), as has been reported previously [32].

Tumour-infiltrating T cell frequencies and tumour uPA expression levels are unchanged in AOM-treated Ly49E WT versus Ly49E KO mice

As illustrated in Fig 4A, immunofluorescent staining of tumour sections shows that there were no discernible differences in the frequencies of tumour $CD3^+$ infiltrating cells between AOM-treated Ly49E WT and AOM-treated Ly49E KO mice. Following dissection of colorectal tumours, the frequencies of tumour-infiltrating T lymphocyte subsets were analysed. We observed a strong and statistically significant increase in the frequency of TCRαβ and a decrease of TCRγδ tumour-infiltrating lymphocytes, as compared to IELs from untreated mice. Within the TCRαβ lymphocyte fraction of tumour-infiltrating lymphocytes there was an increase in conventional CD4 lymphocytes, and a corresponding decrease in the frequency of non-conventional CD8αα lymphocytes. Surprisingly also, within the TCRγδ lymphocyte fraction, we noted a sharp increase in the frequency of DN lymphocytes and a corresponding decrease in CD8αα lymphocytes (Fig 4B and 4C). However, we could not find differences in lymphocyte population frequencies in AOM-treated Ly49E WT versus AOM-treated Ly49E KO mice. Lastly, we analysed uPA expression by tumours of varying size, isolated from AOM-treated mice (Fig 4D). Tumour uPA expression increased steadily as tumours grew larger in size. In 3-4 mm and in ≥ 5 mm tumours, there was also a trend towards higher uPA expression in AOM-treated Ly49E WT versus Ly49E KO mice, but this was not significant.

Discussion

$Apc^{Min/+}$ mice were first described in 1990, and have been used extensively since for the study of intestinal cancer development [33]. In humans, 80% of sporadic colorectal cancers have a mutation in the *Apc* gene [34]. Moreover, mutations in *Apc* are present in familial adenomatous polyposis coli (FAP) [34]. Here, we studied a possible role for Ly49E in cancer development using $Apc^{Min/+}$ mice crossed onto an Ly49E KO background. To investigate if Ly49E affects mouse weight, we weighed $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice at 14 weeks of age, when tumour load reached a plateau, as similarly observed by Puppa *et al.* [35]. Our results show no differences in body weight, suggesting that $Apc^{Min/+}$ -mediated pathology in these mice is similar. This was confirmed by the absence of a difference in small intestinal length between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates. $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice developed numerous 1-2 mm adenomatous polyps along the duodenal to ileal axis, with a 100% penetrance. As has been previously reported, and in contrast to human colorectal cancers [33,36], tumours developed almost exclusively in the small intestine and were not detected in the colon. Tumour load increased with age and was not significantly different in $Apc^{Min/+}$ Ly49E WT as compared to $Apc^{Min/+}$ Ly49E KO mice, indicating that adenoma formation and growth is not affected by Ly49E expression.

Immunofluorescent staining showed that global T cell infiltration is comparable between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice. Marsh *et al.* [37] have previously shown that both $Apc^{Min/+}$ $TCR\beta^{-/-}$ and $Apc^{Min/+}$ $TCR\delta^{-/-}$ mice develop less tumours than $Apc^{Min/+}$ mice, implicating a role for both TCRαβ and TCRγδ cells in tumourigenesis. Examining tumour lymphocyte infiltration in $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO littermates and comparing these to IELs of $Apc^{+/+}$ littermate controls, we observed slightly increased frequencies of TCRαβ cells, and decreased frequencies of TCRγδ cells. The latter is in agreement with Marsh *et al.* [37] who similarly recovered fewer TCRγδ cells from $Apc^{Min/+}$ as compared to $Apc^{+/+}$ mice. Frequencies of the CD4, CD8αα and/or CD8αβ subpopulations of TCRαβ and TCRγδ cells were unaltered between $Apc^{Min/+}$ and $Apc^{+/+}$ mice. Moreover, we observed no differences in infiltrating lymphocyte composition between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice. Taken together, our data illustrate that Ly49E does not influence the number or frequencies of tumour-infiltrating lymphocytes in $Apc^{Min/+}$ mice.

Ploplis *et al.* [38] showed that $Apc^{Min/+}$ $Plau^{-/-}$ mice, which have an additional uPA deficiency, have a lower tumour load than $Apc^{Min/+}$ ($Plau^{+/+}$) mice. Our lab showed that uPA triggers the Ly49E receptor, thereby inhibiting cytokine production and cytotoxicity [39]. Furthermore, Ly49E expression on intestinal intraepithelial cells is high [13], where Ly49E is primarily expressed by CD8αα-expressing IELs, and expression is higher for the colon as compared to the small intestine [14]. As such, we wished to explore a possible link between Ly49E and tumour immunosurveillance. It has been shown that the immune response not only protects against tumour development but can also select for tumour cells of lower immunogenicity [40]. If this also applies to the $Apc^{Min/+}$ model, tumours in Ly49E WT mice would be enriched for uPA production, whereas this would not be the case for tumours in Ly49E KO mice. However, our results show no significant difference in tumour uPA expression levels between $Apc^{Min/+}$ Ly49E WT

and $Apc^{Min/+}$ Ly49E KO mice, indicating that interaction between Ly49E and uPA is not involved in small intestinal $Apc^{Min/+}$ tumour evasion of the immune response.

The AOM-induced colorectal cancer model accurately recapitulates the aberrant crypt foci-adenoma-carcinoma sequence and allows for the development of adenocarcinomas in a relatively short time span [32,41]. This in contrast to $Apc^{Min/+}$ mice, where adenomas rarely progress to invasive adenocarcinoma's [34]. Thus, whereas $Apc^{Min/+}$ mice are excellent for study of the mucosa-adenoma transformation, study of AOM-treated mice may provide additional information with regard to adenoma-carcinoma transformation. Furthermore, tumours develop almost exclusively in the middle to distal colon, mimicking human colorectal cancer [32]. To explore a possible role for Ly49E in later stages of colorectal cancer development, we treated Ly49E WT and Ly49E KO mice with AOM and monitored tumour development. Suzuki *et al.* [42] have previously shown that AOM does not influence body weight of mice, as compared to untreated mice. Similarly, we did not observe a difference in body weight between AOM-treated as compared to untreated mice, or between AOM-treated Ly49E KO and AOM-treated Ly49E WT mice. We observed no significant difference in colon length for AOM-treated Ly49E KO mice as compared to AOM-treated Ly49E WT mice. Following 14-22 weeks of AOM/DSS treatment, AOM-treated mice developed on average 5-10 tumours. Tumour distribution was similar for AOM-treated Ly49E KO mice as compared to AOM-treated Ly49E WT mice. Tumour load was comparable between AOM-treated Ly49E WT and Ly49E KO mice, and this for all time-points analysed. Also, we did not observe a difference in numbers of tumours of any particular size.

Immunofluorescent staining of tumour sections from AOM-treated Ly49E WT and Ly49E KO mice showed that total T cell infiltration was comparable. Matsuda *et al.* [43] previously showed that the frequency of tumour-infiltrated TCRγδ cells is lower in well-to-moderately differentiated colorectal adenocarcinoma as compared to healthy colon IELs. Moreover, tumour-infiltrating lymphocytes were found to be predominantly $CD4^+$ and $TCR\alpha\beta^+$ [43]. Analyzing the frequencies of tumour-infiltrating lymphocytes in AOM-treated and untreated control mice, we similarly noted a significant decrease in the frequency of TCRγδ lymphocytes in colorectal tumours of AOM-treated mice. Alongside, we noted an increase in the frequency of TCRαβ lymphocytes, with a significant increase in the frequency of conventional TCRαβ CD4 and CD8αβ cells. In contrast, the frequency of CD8αα lymphocytes was significantly decreased in both the TCRαβ and TCRγδ cell fractions. However, no differences in infiltrating lymphocyte populations were observed between AOM-treated Ly49E WT and Ly49E KO mice, suggesting that Ly49E expression does not influence the total number or frequency of subpopulations of tumour infiltrating lymphocytes.

uPA has been extensively implicated in the development of colorectal cancer development, where increased levels of both plasma and tissue uPA are present in patients with colorectal cancer [44]. Furthermore, increased uPA activity has been demonstrated for adenocarcinomas as compared with adenomatous polyps, showing that uPA expression levels correlate with colorectal cancer staging [45]. Examining uPA expression in tumours of varying size, we noted a gradual increase in relative tumour uPA expression as tumour size increased. There was a trend towards higher uPA expression in tumours from

AOM-treated Ly49E WT as compared to Ly49E KO mice, but this was not significantly different. Thus, we have no evidence that Ly49E expression on intestinal IELs results in immunoediting of AOM-induced colorectal cancer cells.

Overall, we have shown that intestinal cancer, as modeled in $Apc^{Min/+}$ mice or through AOM/DSS treatment, is accompanied by significant changes in the gut. Specifically, study of tumour-infiltrating lymphocytes shows that these cells are predominantly CD4 and CD8αβ TCRαβ cells. In contrast, the frequency of CD8αα-positive cells is reduced. Ly49E, expressed by IELs of the small intestine and colon, did not influence the frequencies of subpopulations of tumour-infiltrating lymphocytes in either model studied. Furthermore, Ly49E, triggered by uPA, did not influence relative tumour uPA expression levels. Thus, conclusively, Ly49E expression on intraepithelial T lymphocytes does not affect the development or progression of intestinal cancer.

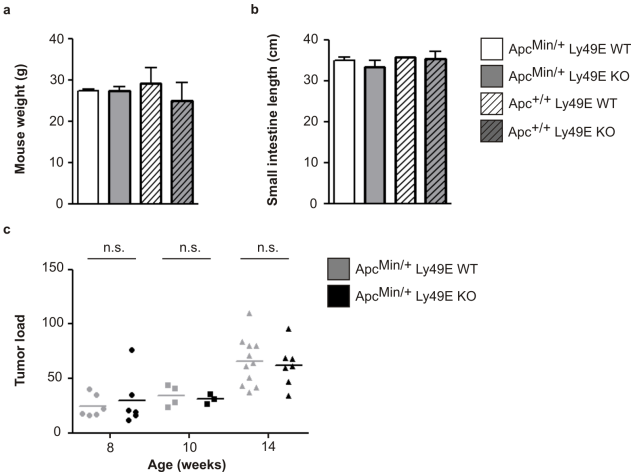


Fig. 1 Apc-mediated intestinal cancer on a Ly49E KO background (A) Body weight of $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice (mean ± SEM; n = 10 for $Apc^{Min/+}$ Ly49E WT, n = 5 for $Apc^{Min/+}$ Ly49E KO) and control Ly49E WT and Ly49E KO littermates (mean ± SEM; n = 6) at 14 weeks of age. (B) Length of the small intestine in $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice (mean ± SEM; n = 5 for $Apc^{Min/+}$ Ly49E WT, n = 3 for $Apc^{Min/+}$ Ly49E KO) and Ly49E WT and Ly49E KO littermates (mean ± SEM; n = 3) at 14 weeks of age. (C) Tumour load in the small intestine of $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice at 8, 10 and 14 weeks of age. n.s. = not significant.

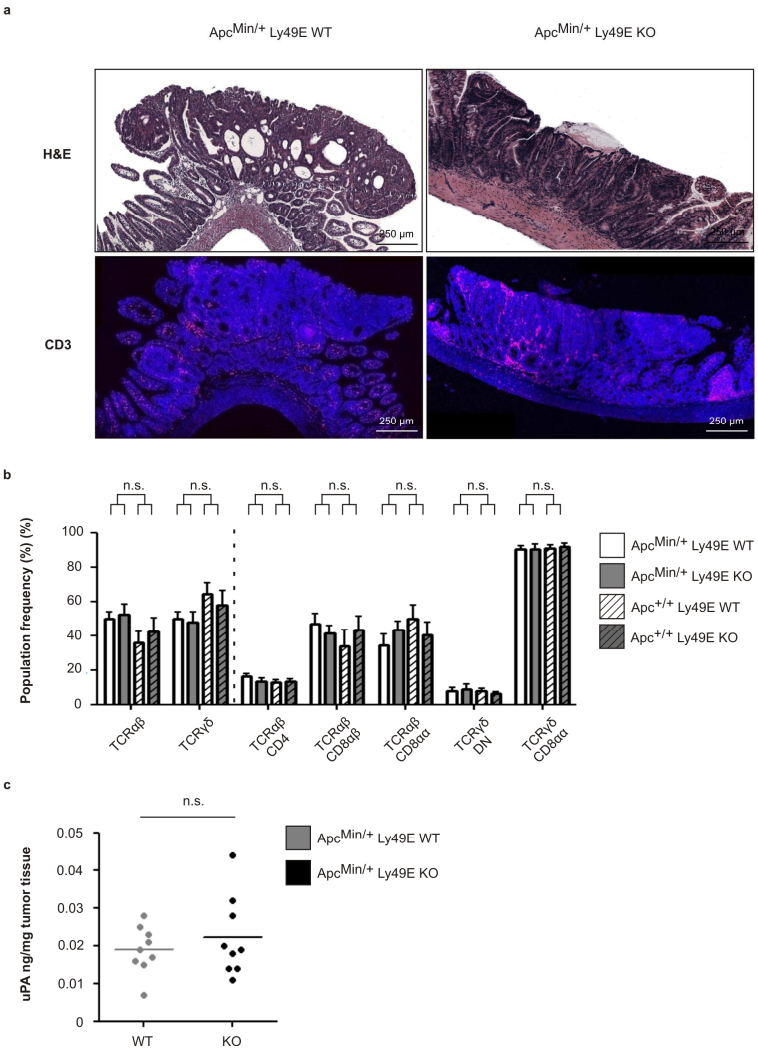
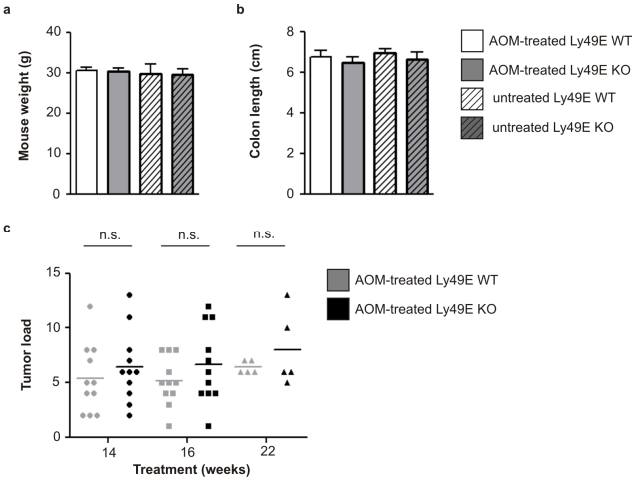


Fig. 2 Tumour-infiltrating T cells and tumour uPA expression in Apc$^{Min/+}$ Ly49E WT versus Apc$^{Min/+}$ Ly49E KO mice (A) Haematoxylin/eosin-(H&E) (upper), and CD3- (lower) stained paraffin tumour sections from Apc$^{Min/+}$ Ly49E WT and Apc$^{Min/+}$ Ly49E KO mice. Scale bar: 250 µm, x100 magnification. (B) Small intestinal tumour-infiltrating IEL subpopulation frequencies in Apc$^{Min/+}$ Ly49E WT and Apc$^{Min/+}$ Ly49E KO mice, and small intestinal IEL subpopulation frequencies from control littermates, at 14 weeks of age (mean ± SEM; n = 6). TCRαβ CD4, TCRαβ CD8αβ and TCRαβ CD8αα IEL subpopulation frequencies are shown as a percentage of the total TCRαβ IELs. TCRγδ DN and TCRγδ CD8αα IEL subpopulation frequencies are shown as a percentage of total TCRγδ IEL. (C) Tumour uPA expression in tumours from Apc$^{Min/+}$ Ly49E WT and Apc$^{Min/+}$ Ly49E KO mice at 14 weeks of age. n.s. = not significant.



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	Treatment (weeks)	Average number of tumors		
		≤ 2 mm	3-4 mm	≥ 5 mm
Ly49E WT	14	1.00 ± 1.00	2.33 ± 1.15	0.67 ± 0.58
Ly49E KO		4.67 ± 2.31	0.67 ± 1.15	0.33 ± 0.58
Ly49E WT	16	2.83 ± 1.72	1.50 ± 1.38	0.83 ± 0.75
Ly49E KO		4.00 ± 0.63	0.50 ± 0.84	1.17 ± 1.60
Ly49E WT	22	4.80 ± 1.79	1.20 ± 1.64	0.40 ± 0.55
Ly49E KO		4.80 ± 2.28	1.60 ± 1.14	1.60 ± 0.89

Fig. 3 Azoxymethane-induced colorectal cancer on a Ly49E KO background (A) Body weight of AOM-treated Ly49E WT and Ly49E KO mice (mean ± SEM; n = 8 for AOM-treated Ly49E WT, n = 7 for AOM-treated Ly49E KO) 22 weeks following the start of treatment, and of untreated mice at a similar age (mean ± SEM; n = 5). (B) Colon length of AOM-treated Ly49E WT and Ly49E KO mice (mean ± SEM; n = 8 for AOM-treated Ly49E WT, n = 7 for AOM-treated Ly49E KO) 22 weeks following the start of treatment, and of untreated mice at a similar age (mean ± SEM; n = 4). (C) Colon tumour load in AOM-treated Ly49E WT and Ly49E KO mice at 14, 16 and 22 weeks following the start of treatment. (D) Average number of tumours, when tumours are classified according to size, in AOM-treated Ly49E WT and Ly49E KO mice at 14, 16 and 22 weeks following the start of treatment (mean ± SEM; n = 3 at 14 weeks, n = 6 at 16 weeks and n = 5 at 22 weeks). n.s. = not significant.

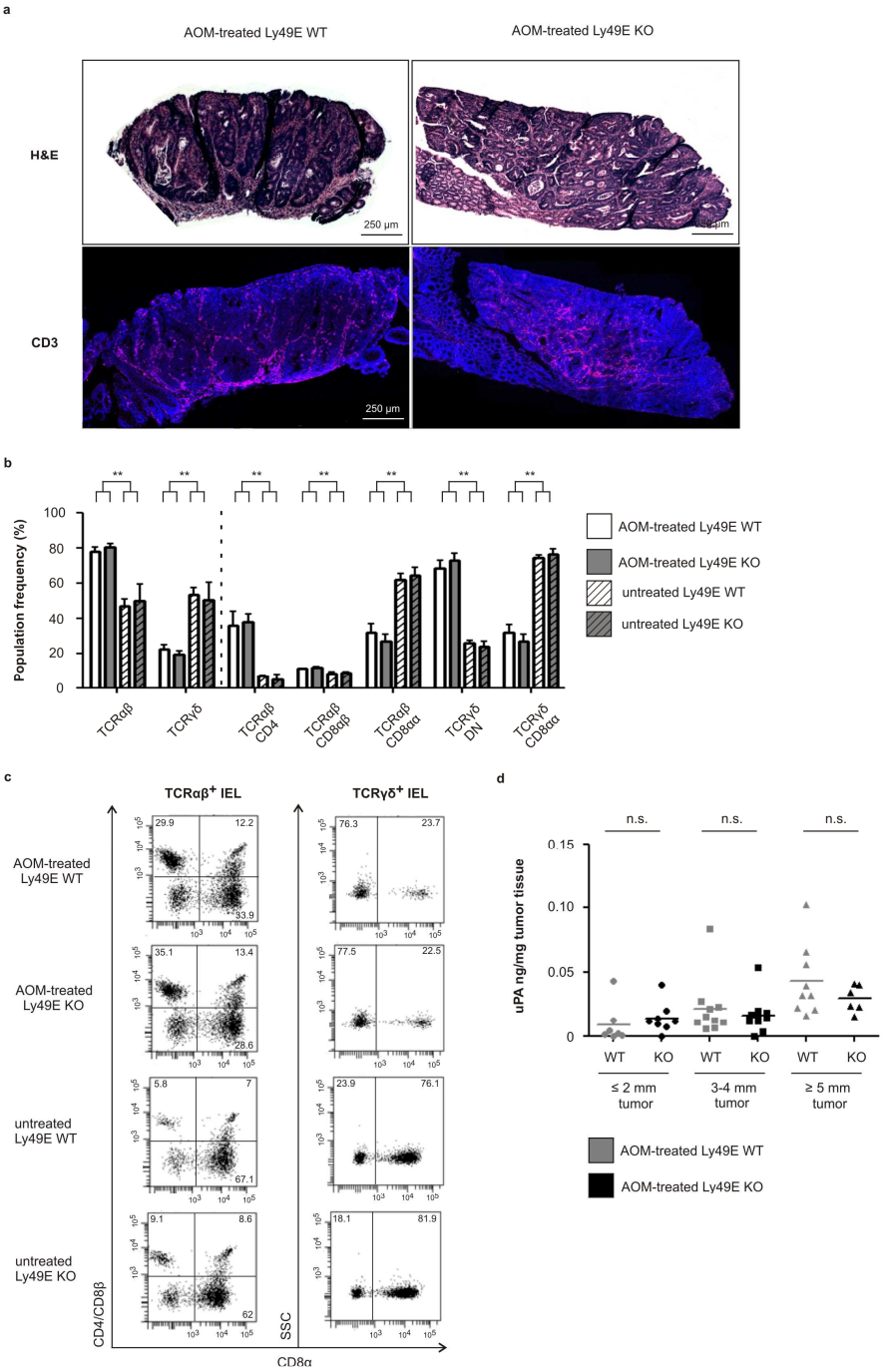

Fig. 4 Tumour-infiltrating T cells and tumour uPA expression in azoxymethane-treated Ly49E WT versus Ly49E KO mice (A) Haematoxylin/eosin-(H&E)- (upper), and CD3- (lower) stained paraffin tumour sections from AOM-treated Ly49E WT and Ly49E KO mice. Scale bar: 250 µm, x100 magnification. (B) Colon tumour-infiltrating IEL subpopulation frequencies in AOM-treated Ly49E WT and Ly49E KO mice 14-22 weeks following the start of treatment, and colon IEL subpopulation frequencies from untreated Ly49E WT and Ly49E KO mice (mean ± SEM; n = 5 for AOM-treated mice; n = 3 for untreated mice). TCRαβ CD4, TCRαβ CD8αβ and TCRαβ CD8αα IEL subpopulation frequencies are shown as a percentage of the total TCRαβ IELs. TCRγδ DN and TCRγδ CD8αα IEL subpopulation frequencies are shown as a percentage of the total TCRγδ IEL. (C) Dot plots are shown for CD4/CD8β versus CD8α

expression in colon tumour infiltrating IEL in AOM-treated Ly49E WT and Ly49E KO mice, and colon IEL from untreated Ly49E WT and Ly49E KO mice. Numbers indicate the percentage of cells in each quadrant. Dot plots are representative for n = 5 AOM-treated mice and n = 3 untreated mice. (D) Tumour uPA expression in tumours of varying size from AOM-treated Ly49E WT and Ly49E KO mice at 14-22 weeks following the start of treatment. n.s. = not significant.

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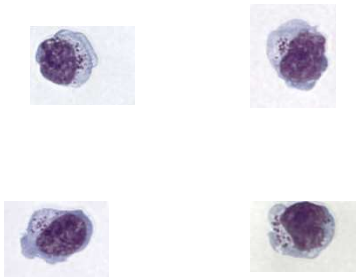
Part III



Innate lymphoid cells of the small intestine

A novel intestinal intraepithelial NKp46-negative innate lymphoid cell population characterised by Ly49E expression and group 1 ILC properties

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Abstract

Ly49E is an Ly49 receptor family member with unique characteristics, including its preferential expression on innate lymphocytes. On T cells, it is expressed by both innate-like epidermal Vγ3 T cells and intestinal intraepithelial CD8αα^{+} T lymphocytes. Regarding NK cells, Ly49E expression is low on conventional NK cells, but high on tissue-resident CD49a^{+} liver NK cells. As CD49a^{+} liver NK cells have recently been shown to have innate lymphoid cell type 1 (ILC1)-like properties, we investigated whether Ly49E is expressed on intestinal ILC populations. Here, we show that numbers of Ly49E-expressing conventional intestinal ILC1 and ILC3 are low. In contrast, Ly49E is expressed by a subpopulation of the recently characterised innate (iCD8α) lymphocytes, and defines a new and unique NKp46-negative innate lymphoid population that can be minimally characterised as NKp46^{-} CD8αα^{-} Ly49E^{+}. This population is positive for CD49a and CD103, expresses T-bet but not Eomes, and requires IL-15 signalling for survival. Further transcriptome analysis of these cells revealed a gene profile closely related to ILC1 and NK cells. However, NKp46^{-} CD8αα^{-} Ly49E^{+} cells are unique and distinct from NK cells and previously described intraepithelial and lamina propria ILC1 populations. Importantly, these cells are avid IFN-γ producers, suggesting that this novel population may contribute to Th-1 mediated immunity.

Introduction

In recent years, several new innate lymphoid cell (ILC) populations have been described [1]. Characterised by the absence of rearranged antigen-specific receptors, these cells have been classified into group 1, 2 and 3 ILCs, where each group appears to mirror a respective T helper cell population [2-4]. Group 1 ILC cells comprise NK cells and ILC1 cells that are defined by 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 [2,3]. ILC2 cells are defined by dependence on GATA-3 and the capacity to respond to IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) to produce type 2 cytokines, such as IL-5 and IL-13. Lastly, ILC3 cells are defined by dependence on RORγt and the capacity to respond to IL-1β and IL-23 by the production of IL-17 and/or IL-22 [2-5].

Abundant at mucosal barrier surfaces [3,4], ILCs have been attributed important roles in a number of immunological processes. Whereas ILC1 cells have been implicated in the control of intracellular parasite infections [3,6] and gut inflammation [2,7], ILC2 cells are important in the clearance of helminth [8,9] and viral infections [10], and in the progression of asthma and lung allergies [11-15]. ILC3 cells, in turn, contribute to tertiary lymphoid organogenesis [16], the containment of commensal bacteria [17] and clearance of bacterial infections in the gut [18-22], and have been implicated in the pathology of inflammatory bowel disease [7,23,24]. It should be noted that some of the described functions are attributable to "ex-RORγt" ILC3s [7,25], where bidirectional functional plasticity between ILC1 and ILC3 classes in response to distinct environmental stimuli was recently demonstrated by Bernink *et al.* [26].

Whereas group 2 ILCs appear to be the most homogeneous cells, considerable heterogeneity exists within group 1 and 3 ILCs [5,15]. In the gut, a distinction can be made within group 1 ILCs between conventional natural killer (cNK) cells, lamina propria [3] and intraepithelial ILC1s [2], and "ex-RORγt" ILC3s [7,25]. Within group 3 ILCs, four subsets can be distinguished: $CD4^-$ $NKp46^-$ and $CD4^+$ $NKp46^-$ lymphoid tissue inducer (LTi)-like ILC3s, and $NKp46^-$ $ROR\gamma t^+$ and $NKp46^+$ $ROR\gamma t^+$ ILC3s [4]. Recently, Van Kaer *et al.* also described the existence of innate CD8αα (iCD8α) cells, a new innate lymphoid population resident in the intestinal epithelium [27].

Ly49E is a unique member of the Ly49 NK receptor family, with preferential expression on innate lymphocytes. In this respect, the Ly49E receptor is expressed on innate-like epidermal Vγ3 T cells as well as intestinal $CD8\alpha\alpha^+$ intraepithelial T lymphocytes [28-30]. Alongside, Ly49E expression is low on conventional (c)NK cells from adult mice [31], but is highly expressed on $CD49a^+$ liver NK cells [32]. Interestingly, the latter have recently been shown to have ILC1-like properties [4,33]. As such, we investigated whether Ly49E is expressed on intestinal ILC populations. In this paper we show that, whereas Ly49E expression is low on conventional ILC1 and ILC3 populations, Ly49E is expressed by a subpopulation of the recently described innate CD8α (iCD8α) lymphocytes, and in addition defines a novel intraepithelial NKp46-negative lymphoid population. This novel intestinal intraepithelial NKp46-negative $Ly49E^+$ lymphoid population requires IL-15-, but not IL-7-mediated signalling, and has a gene expression

profile that is closely related to both lamina propria ILC1 and NK cells. Importantly, stimulation with the cytokines IL-12, IL-15 and IL-18 results in robust expression of IFN-γ, confirming that these cells are ILC1-like and may contribute to Th1-mediated immune responses.

Materials and Methods

Mice

C57BL/6 wild-type (WT), BALB/c NOD-SCID, and C57BL/6 $Rag^{-/-}$ and $Rag^{-/-}$ $\gamma c^{-/-}$ were purchased from Charles River (Wilmington, MA, United States). *Eomes*$^{-/-}$ (*Eomes*$^{fl/fl}$ *x T-Cre*) [34,35], *Tbx21*$^{-/-}$ [36], *Il7ra*$^{-/-}$ [37], *Il15ra*$^{-/-}$ [38] and RORγt fate map (fm) (*Rorc(γt)*-Cre^{Tg} x *Rosa26R*$^{Yfp/+}$) mice [39] were on a C57BL/6 background. 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) and the Landesuntersuchungsamt Koblenz (Mainz, Germany).

Intestinal intraepithelial and lamina propria lymphocyte isolation

For intestinal intraepithelial lymphocyte isolation, small intestines were removed and cleaned of mesenteric fat. Peyer's patches were excised, and intestines were rinsed with Dulbecco's phosphate-buffered saline (DPBS) to clear fecal content. Subsequently, intestines were opened longitudinally, cut into 0.5-cm pieces, and incubated twice for 20 minutes at 37°C in Ca/Mg-free Hank's Balanced Sodium Solution (HBSS; Invitrogen, Carlstad, CA, USA) containing 5% foetal calf serum (FCS), 1 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen) and 1 mM dithiotreitol (Sigma-Aldrich; Saint-Louis, MO, USA) at slow rotation. Cell suspensions were passed through a 40-µm cell strainer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and pelleted by centrifugation at 480 g. Cell pellets were resuspended in 44% Percoll (GE Healthcare, Buckinghamshire, UK) on an underlay of 67% Percoll, and centrifuged for 20 min at 2000 g. Intraepithelial lymphocytes were collected from the 44%/67% Percoll interface, washed twice with phosphate buffered saline (PBS) for 5 minutes at 480 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 β-mercaptoethanol (Sigma-Aldrich) (further indicated as 'complete RPMI medium'). For lamina propria lymphocyte isolation, small intestinal pieces were collected following two rounds of intraepithelial lymphocyte isolation and cut into fine pieces with a scalpel. Subsequently, the remaining tissue was incubated three times for 20 minutes at 37°C in Ca/Mg-free HBSS solution containing 2% FCS, collagenase D (0.5 mg/ml; Roche, Basel, Switzerland), DNase I (0.5 mg/ml; Roche) and dispase (50 U/ml; BD Biosciences). Cell suspensions were passed through a 40-µm cell strainer and pelleted at 480 g. Pellets were resuspended in 40% Percoll on an underlay of 80% Percoll, and centrifuged for 25 min at 1328 g. Lamina propria lymphocytes were collected from the 40%/80% interphase, washed twice with PBS and resuspended in complete RPMI medium.

Immunofluorescence staining and antibodies

Following isolation, cells were blocked with anti-FcγRII/III (unconjugated, clone 2.4G2, kindly provided by Dr J. Unkeless, Mount Sinai School of Medicine, New York, NY, USA). Cells were subsequently stained with the following antibodies: anti-CD45.2 (APC-Cy7; 104), anti-CD45 (PE-Cy7; 30-F11), anti-CD3 (PE-Cy7; 145-2C11), anti-CD5 (PE-Cy7; 53-7.3), anti-CD19 (PE-Cy7; 1D3), anti-Ly6G (PE-Cy7; RB6-85C), anti-CD127 (PerCP-Cy5.5; A7R34), anti-CD27 (FITC or PE; LG.7F9), anti-NKp46 (PE; 29A1.4), anti-NK1.1

(PerCP-Cy5.5; PK136), anti-CD160 (APC; CNX46-3), anti-CD8α (eFluor450; 53-6.7), anti-CD103 (PE; 2E7), anti-Sca-1 (PE; D7), anti-CD122 (PE; TM-β1), anti-Eomes (PE; Dan11mag), anti-T-bet (PE; 4B10), anti-RORγt (PE; AFKJS-9), all purchased from eBioscience (San Diego, CA, USA). Anti-CD3 (Pacific Blue; 500A2), anti-CD4 (PE; L3T4), anti-CD8β (PE; H35-17.2), anti-CD8α (APC; 53-6.7), anti-NK1.1 (APC; PK136), anti-NKp46 (AlexaFluor647; 29A1.4), anti-CD49a (PE; Ha31/8), anti-CD49b (PE; DX5), anti-CD11c (APC; HL3), anti-CD117 (PE; 2B8), anti-CD90.2 (PE; 53-2.1), anti-NK1.1 (PE; PK136), anti-NKG2D (PE; CX5), anti-CD127 (PE-CF594; SB/199) and anti-IFN-γ (PE; XMG1.2) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-CD4 (PerCP-Cy5.5; GK1.5) and anti-CD8β (PerCP-Cy5.5; YTS156.77) were bought from BioLegend (San Diego, CA, USA). Anti-CD11b (PE; M1/70.15.11.5) was obtained from Miltenyi (Bergisch Gladbach, Germany). Anti-Ly49A (biotin-conjugated; JR9-318; kindly provided by Dr J. Roland (Paris, France)), anti-Ly49E/C (biotin-conjugated; 4D12, made and labeled in-house) [31] and anti-Ly49E/F (biotin or FITC-conjugated; clone CM4; labeled in-house and kindly provided by Dr C. G. Brooks (Newcastle on Tyne, UK) [40]. mAb 4D12 (Ly49C/E) in combination with mAb CM4 (Ly49E/F) were used to identify Ly49E- ($CM4^+/4D12^+$), Ly49C- ($CM4^-/4D12^+$) and Ly49F-expressing cells ($CM4^+/4D12^-$). Anti-Ly49A/D (biotin-conjugated; clone 12A8; ascites kindly provided by Dr. J. Ortaldo (National Cancer Institute, Frederick, MD, USA), labeled in-house), anti-Ly49H (biotin-conjugated; clone 3D10; kindly provided by Dr. W. Yokoyama, (St.Louis, MO, USA)), anti-Ly49G (biotin-conjugated; 4D11; from ATCC and labeled in-house), and anti-NKG2A/C/E (biotin-conjugated; clone 3S9; generated and labeled in-house) [31]. Biotinylated antibodies were subsequently detected by streptavidin (APC-Cy7- or V500-conjugated, purchased from eBioscience or BD Biosciences, respectively).
For intracellular staining, cell membrane staining was performed first, following which cells were incubated in Cytofix/Cytoperm (BD Biosciences) and stained for cytokines. Intranuclear staining of transcription factors was performed using the Foxp3-staining kit (eBioscience). For surface stainings, propidium iodide was used to discriminate live and dead cells. For intracellular and intranuclear stainings, Live/Dead Fixable Aqua Staining (Molecular Probes, Eugene, OR, USA) was used. Flow cytometry was performed using a BD LSRII flow cytometer, and samples were analysed with FACSDiva Version 6.1.2 software (BD Biosciences) or FlowJo 8.7.1 (FlowJo, Ashland, OR, USA).

Cell sorting and cytospin

$CD3^-CD45^+NKp46^-CD8\alpha\alpha^-Ly49E^+$ and $CD3^-CD45^+NKp46^-CD8\alpha\alpha^+Ly49E^+$ cells were sorted to high purity (>98%) from the small intestinal intraepithelial lymphocyte fraction 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

$CD3^-CD45^+CD8\alpha\alpha^-Ly49E^+$, $CD3^-CD45^+CD8\alpha\alpha^+Ly49E^+$ and $CD3^-CD45^+CD8\alpha\alpha^+Ly49E^-$ cells were sorted to high purity (>98%) from the small intestinal intraepithelial lymphocyte fraction of NOD-SCID mice using a BD FACSAria III cell sorter (BD Biosciences). RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). Amplification and hybridization was carried out with the Ovation Pico WTA V2 kit (NuGen Technologies, San Carlos, CA, USA) and the Affymetrix Mouse Gene 1.0ST Array (Affymetrix, Santa

Clara, CA, USA). Samples were subsequently analysed using R/Bioconductor. All samples passed quality control, and the Robust Multi-array Average procedure was used to normalize data within arrays (probeset summarization, background correction and log2-transformation) and between arrays (quantile normalization). Only probesets that mapped uniquely to one gene were kept, and for each gene, the probeset with the highest expression level was retained. Correlation heatmaps were made by clustering the samples via the Ward method, and correlation was used to determine the distance between the samples. Microarray data and RNA Seq data were merged by first normalising and filtering the microarray data and the RNA Seq data separately. Quantile normalisation and ComBat were then used to correct for batch effects.

In vitro cell stimulation

Intestinal intraepithelial lymphocytes 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/or IL-15 (10 ng/ml; R&D), and/or IL-18 (2.5 ng/ml; Medical and Biological Laboratories, Nagoya, Japan) for 15 h. Brefeldin A (Golgistop, BD Biosciences) (1 μg/ml) was added during the last 12 h. Cells were harvested and stained as described.

Statistical analysis

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

Results

Ly49E is mainly expressed on NKp46-negative intestinal intraepithelial lymphocytes

We have previously shown that the Ly49E receptor is expressed on several innate-like cell populations in adult WT C57BL/6 mice, including intestinal intraepithelial CD8αα$^+$ T cells, epidermal Vγ3 T cells and tissue-resident liver CD49a$^+$ CD49b$^-$ NK cells [28-30,32]. Recent years have seen the discovery of a number of new innate lymphoid populations, i.e. the ILCs, with particular diversity of these cells in the small intestine [4]. As such, we set out to investigate whether Ly49E is expressed on small intestinal ILC subsets. We show that Ly49E was expressed only by a small subpopulation of intraepithelial NKp46$^+$ NK1.1$^+$ ILCs, where 2-5% of cNK expressed Ly49E, and where Ly49E expression was even lower on ILC1 and almost absent on ILC3 cells. Similarly, Ly49E expression was relatively low on lamina propria NKp46$^+$ NK1.1$^+$ ILCs, where expression was 3-4% on cNK, and also lower on ILC1 and ILC3 cells (Fig. 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 (Fig. 1A), the absolute number of these cells was seven-fold higher as compared to the Ly49E-expressing CD3$^-$ CD45$^+$ NKp46$^+$ intestinal intraepithelial fraction (Fig. 1B). Even higher absolute numbers of CD3$^-$ CD45$^+$ NKp46$^-$ Ly49E$^+$ intraepithelial cells were present in the small intestine of Rag$^{-/-}$ and NOD-SCID mice (Fig. 1C and 1D), confirming the innate nature of these cells. Therefore, for practical purposes, all future experiments were performed with cells isolated from the small intestinal intraepithelial lymphocyte fraction of Rag$^{-/-}$ and NOD-SCID mice.

NKp46-negative intraepithelial Ly49E$^+$ cells have an ILC-like/non-NK surface phenotype and a granular lymphoid morphology

Based on CD4, CD8β and CD8α expression, two main subpopulations of CD3$^-$ CD45$^+$ NKp46$^-$ Ly49E$^+$ cells could be distinguished, that were CD8αα$^+$ and CD8αα$^-$, respectively (Fig. 2A). We further performed a detailed phenotypic characterization of these two populations, and of spleen NK cells for comparison. Both CD8αα$^-$ and CD8αα$^+$ Ly49E-expressing populations were CD49a$^+$ CD103$^+$ (Fig. 2B), suggestive of a tissue-resident intraepithelial ILC phenotype [2,4]. Furthermore, both populations were CD127$^-$ CD11b^{int} CD11c^{int} CD117$^-$ CD90$^-$ Sca-1$^-$ CD122^{int}. Interestingly, whilst CD8αα$^+$ Ly49E$^+$ cells were negative for CD160, NK1.1 and NKG2A/C/E, the CD8αα$^-$ Ly49E$^+$ population showed intermediate expression of these NK cell markers. We could not detect expression of NKG2D or other (non-Ly49E) Ly49 receptors on either of these populations (Fig. 2B). In this, CD8αα$^+$ Ly49E$^+$ cells strongly resembled innate CD8α (iCD8α) cells, as recently described by Van Kaer *et al.* [27]. Thus, CD8αα$^+$ Ly49E$^+$ cells may define a subpopulation of iCD8α cells, whereas CD8αα$^-$ Ly49E$^+$ cells define a previously undescribed intraepithelial population with innate-type surface marker expression. Cytospins of highly purified CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ intraepithelial lymphocytes, and of CD3$^+$ Ly49E$^+$ intraepithelial T cells for comparison, showed that the Ly49E-expressing cells had a lymphoid morphology. Intriguingly, both CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ cells were highly granular (Fig. 3).

Intestinal intraepithelial NKp46-negative CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells 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 intraepithelial CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells revealed that both populations lacked Eomes expression, in contrast to NK cells, but expressed T-bet. Whereas a very small proportion of CD8αα- Ly49E+ cells was RORγt positive, CD8αα+ Ly49E+ cells did not express RORγt (Fig. 4A). When CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells were isolated from RORγt-fate map (fm) mice, the results indicated that a fraction of cells from each subpopulation was RORγt-fm positive (Fig. 4B). As intracellular RORγt staining of CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells from NOD-SCID mice yielded fewer, or no, RORγt-positive cells, respectively (Fig. 4A), this suggests that some of the observed RORγt-fm-positive cells are "ex-ILC3" cells that have lost RORγt expression [7,26]. To analyse transcription factor dependency, the cell numbers of intestinal intraepithelial CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells, and as control the cNK, ILC1 and ILC3 populations, were determined in *Eomes*$^{-/-}$ and *Tbx21*$^{-/-}$ versus WT mice. Normal numbers of CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells were present in *Eomes*$^{-/-}$mice. In turn, *Tbx21*$^{-/-}$ mice had strongly reduced numbers of CD8αα+ Ly49E+ cells, whereas the number of CD8αα- Ly49E+ cells was unaffected. The control populations showed the expected transcription factor dependency, as NK cells were virtually absent in *Eomes*$^{-/-}$ mice, whereas ILC1 and ILC3 populations were strongly reduced or absent, respectively, in *Tbx21*$^{-/-}$ mice (Fig. 4B).

NKp46-negative CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells are dependent on IL-15, but not IL-7 signalling

Previous studies have shown that ILCs require signalling through the common gamma chain (γc) for their survival [7,41,42]. To investigate the cytokine dependency of intestinal intraepithelial CD8αα- Ly49E+ and CD8αα+ Ly49E+ cells, we determined their cell numbers in γc$^{-/-}$, *Il7ra*$^{-/-}$ and *Il15ra*$^{-/-}$ mice. As shown in Fig. 5, CD8αα- Ly49E+ cells were increased in number in *Il7ra*$^{-/-}$ mice, and absent in *Il15ra*$^{-/-}$ and in *γc*$^{-/-}$ mice. This is in agreement with the absence of CD127 on the cell membrane of these cells, but intermediate levels of CD122 expression (Fig. 2B). Increased ILCs numbers have previously been reported in the gut of *Il7r*$^{-/-}$ mice [3,25]. CD8αα+ Ly49E+ cells were similarly independent of IL-7 and dependent on IL-15 signalling (Fig. 5), as previously reported for iCD8α cells [27].

NKp46-negative CD8αα- Ly49E+ cells have a group 1 ILC-like transcriptome

To gain a better understanding of the lineage relationship and potential function of NKp46-negative Ly49E+ cells, we carried out a global transcriptome analysis of sorted CD8αα- Ly49E+, CD8αα+ Ly49E+ and CD8αα+ Ly49E- intestinal intraepithelial NKp46- cell populations, and aligned our data with that of publicly available gene expression data of intestinal iCD8α cells and ILC subsets, including NK cells [4,27]. Hierarchical clustering revealed that CD8αα- Ly49E+ cells clustered with NK and ILC1 lamina propria subsets. CD8αα+ Ly49E+ cells, in turn, clustered with iCD8α cells, as expected, and thus represent a subpopulation of this innate lymphoid population (Fig. 6A). Principal component analysis (PCA) confirmed that CD8αα- Ly49E+ cells clustered with lamina propria NK and ILC1 cells (Fig. 6B). More detailed gene expression analysis of CD8αα- Ly49E+ and CD8αα+ Ly49E+ cell subsets showed that both populations express high levels of the transcription factor *Id2*, which is required for development of all ILC

subsets [15]. Furthermore, in accordance with our flow cytometric data, *Tbx21* (T-bet) expression levels were high and comparable to those of NK and intestinal intraepithelial and lamina propria ILC1 populations. In contrast to CD8αα$^+$ Ly49E$^+$ cells, CD8αα$^-$ Ly49E$^+$ cells also showed increased transcript levels of the transcription factors *Rorc, Nfil3, Ahr* and *Rora,* where transcript numbers were in the same range as those of lamina propria NK and/or ILC1 cells (Fig. 6C). Cytokine and cytokine receptor expression profiles indicate that both CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ cells express high levels of the chemokines *CCL3* (MIP1α) and *CCL5* (RANTES), whereas only CD8αα$^-$ Ly49E$^+$ cells showed increased expression of the chemokine receptor *CCR9* (Fig. 6D). CD8αα$^-$ Ly49E$^+$ cells also showed increased expression of *Ifng* and *Tnf* as compared to CD8αα$^+$ Ly49E$^+$ cells. Additionally, CD8αα$^-$ Ly49E$^+$ cells differed significantly from CD8αα$^+$ Ly49E$^+$ cells in the expression of cytokine receptors, where CD8αα$^-$ Ly49E$^+$ cells highly expressed the *Il18r1, Il2ra and Il7r* genes, whereas only low expression of these genes could be detected in CD8αα$^+$ Ly49E$^+$ cells. *Il2rb* and *Il12rb* were expressed at high and intermediate levels, respectively, in both populations. CD8αα$^-$ Ly49E$^+$ cells expressed *Grzma, Grzmb and Prf1*, but levels were lower than in CD8αα$^+$ Ly49E$^+$, NK and ILC1 cells (Fig. 6E). Finally, comparative analysis of the expression levels of genes of the recently described NK cell-distinct ILC core signature [4] showed that these genes were mainly expressed by CD8αα$^-$ Ly49E$^+$ cells (Fig. 6F upper panel), whereas the majority of NK-specific signature genes were only expressed at low levels (Fig. 6F lower panel). Transcriptome analysis of CD8αα$^+$ Ly49E$^-$ cells showed that the gene expression profile of these cells is indistinguishable from that of CD8αα$^+$ Ly49E$^+$ cells, and highly similar to that of iCD8α cells (Fig. 6A-F). In summary, we show that CD8αα$^+$ Ly49E$^+$ cells likely presents a subpopulation of iCD8α cells. In contrast, transcriptome analysis of CD8αα$^-$ Ly49E$^+$ cells reveals that these cells have a unique transcriptional profile that displays characteristics of core ILC subsets and a close correlation with both intestinal ILC1 and NK cell expression profiles.

CD8αα$^-$ Ly49E$^+$ cells are avid producers of IFN-γ

As CD8αα$^-$ Ly49E$^+$ cells displayed intermediate and strong expression of *Il12rb*, *Il2rb* and *Il18ra*, respectively, we stimulated intestinal intraepithelial lymphocytes *in vitro* with IL-12, IL-15 and/or IL-18 and determined IFN-γ expression. Whereas CD8αα$^+$ Ly49E$^+$ cells expressed moderate levels of IFN-γ following stimulation with IL-12 + IL-15 + IL-18, CD8αα$^-$ Ly49E$^+$ cells were avid producers of IFN-γ (Fig. 7A). These results suggest that CD8αα$^-$ Ly49E$^+$ cells, similarly to NK and ILC1 cells, may be involved in mediating Th1-type immunity.

Discussion

The intestinal epithelium harbors a number of lymphocyte populations, including innate-like and adaptive T cells, NKp46-expressing NK and ILC1 cells, and the recently described iCD8α cells [2,27]. In this paper we show that the intestinal epithelium is also home to a new and unique CD3$^-$ innate population that can be minimally characterised as NKp46$^-$ CD8αα$^-$ Ly49E$^+$ cells. Further study of these cells revealed a lymphoid morphology and a CD103$^+$ CD127$^-$ surface phenotype. CD103 is expressed by intestinal epithelium-homing leukocytes, including T cells and iCD8α cells [2,27,29]. As such, CD103 expression reflects the intraepithelial localization of innate lymphoid CD8αα$^-$ Ly49E$^+$ cells. CD127 surface expression may be used to identify ILCs, except NK cells, in many mouse tissues [4]. CD8αα$^-$ Ly49E$^+$ cells did not express cell surface CD127. However, microarray analysis revealed large numbers of *Il7r* transcripts in these cells, suggesting that *Il7r* expression may not, or not sufficiently, be translated onto the protein level in these cells to allow flow cytometric detection. As intraepithelial ILC1 and iCD8α cells have also been shown to lack detectable surface expression of CD127 [2,27], 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 do express cell surface CD127 [3]. This notion is further corroborated by data from human studies which have also described the existence of intraepithelial CD103$^+$ ILC1 cells that are CD127$^{lo/-}$ [43].

Specification of intestinal innate lymphoid CD8αα$^-$ Ly49E$^+$ cells by hierarchical clustering and PCA analysis indicated that CD8αα$^-$ Ly49E$^+$ cells had a unique transcriptional profile that showed some similarities to NKp46$^+$ NK 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$^+$ cells, with the presence of large numbers of transcripts for *Ccl5, Ifng, Tnf, Gzma* and *Gzmb*. We also demonstrated that CD8αα$^-$ Ly49E$^+$ cells were IL-15-dependent for their development and/or maintenance. As NK cells as well as lamina propria ILC1, but not intraepithelial ILC1 cells, are dependent on IL-15 signalling [2,3,27], these data further support the group 1 ILC-like nature of CD8αα$^-$ Ly49E$^+$ cells. However, intestinal CD8αα$^-$ Ly49E$^+$ intraepithelial lymphocytes are not NK cells. Robinette *et al*. [4] recently identified the NK cell-distinct ILC core signature, i.e. a set of transcripts common to ILC1 cells from all tissues that are more highly expressed than in NK cell subsets, and that also show high expression in ILC2 and ILC3 subsets. Analysis of CD8αα$^-$ Ly49E$^+$ cells showed strong expression of the NK cell-distinct ILC core signature genes whereas, vice versa, CD8αα$^-$ Ly49E$^+$ cells displayed low expression of NK cell-specific signature genes. There are several other indications that CD8αα$^-$ Ly49E$^+$ cells are not NK cells. Contrary to NK cells, these cells have an NKp46$^-$ NK1.1$^{-/low}$ phenotype and express CD49a, a marker present on tissue-resident ILC1 cells and absent on NK cells [3,4]. In addition, these cells do not express typical NK receptors, including NKG2D and NKG2A/C/E, and, most importantly, CD8αα$^-$ Ly49E$^+$ cells develop independently of Eomes, as testified by the presence of normal numbers of these cells in *Eomes*$^{-/-}$ mice. Furthermore, there are important differences between the intestinal intraepithelial CD8αα$^-$ Ly49E$^+$ cells described in the present paper and the previously described intestinal intraepithelial and lamina propria ILC1 cells. Fuchs *et al.* [2] identified murine intestinal intraepithelial ILC1 cells. In contrast to CD8αα$^-$ Ly49E$^+$ cells, intraepithelial

ILC1 cells have an NKp46^{+} NK1.1^{+} phenotype and are negative for CD103. In addition, intraepithelial ILC1 cells are dependent on T-bet and largely independent of IL-15Rα, which is opposite to what we observed for CD8αα^{-} Ly49E^{+} cells. Klose *et al.* [3] described lamina propria ILC1 cells. Moreover, and also in contrast to CD8αα^{-} Ly49E^{+} cells, lamina propria ILC1 cells have an NKp46^{+} NK1.1^{+} phenotype. In addition, these cells express CD127, CD90 and CD117, markers that are not expressed by CD8αα^{-} Ly49E^{+} cells. Finally, lamina propria ILC1 cells are also dependent on T-bet. Thus, although CD8αα^{-} Ly49E^{+} cells have group 1 ILC characteristics, including their overall transcriptional profile, these cells have several crucial differences as compared to NK cells and previously described intestinal ILC1 cells, and are therefore a new and unique intestinal intraepithelial ILC1-like population.

A role for both intraepithelial and lamina propria ILC1 cells in mediating Th1-type immune response has been demonstrated [2,3]. Intestinal intraepithelial NKp46-negative CD8αα^{-} Ly49E^{+} cells expressed *Il12r Il2rb* and, in comparison to NKp46-expressing NK and NKp46^{+} ILC1 cells, 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. Flow cytometric analysis of the transcription factor expression of CD8αα^{-} Ly49E^{+} cells revealed that these cells did not express Eomes protein, but did express high T-bet levels. Taken together with the IFN-γ production capacity, these data are in agreement with an ILC1-like phenotype of CD8αα^{-} Ly49E^{+} cells. Intriguingly, *Tbx21*$^{-/-}$ mice had normal numbers of CD8aa^{-} Ly49E^{+} cells in the gut, indicating that T-bet is not required for the development of these cells. Thus, it remains to be elucidated which transcription factor(s) are required for development of CD8αα^{-} Ly49E^{+} cells.

In parallel, we showed that intestinal intraepithelial CD8αα^{+} Ly49E^{+} cells represent a fraction of iCD8α cells. CD8αα^{+} Ly49E^{+} cells had a CD45^{+} CD11b^{int} CD11c^{int} CD103^{+} c-kit^{-} NKp46^{-} NK1.1^{-} phenotype, as also described for iCD8α cells by Van Kaer *et al.* [27]. Furthermore, we showed that these cells express CD49a, suggesting a tissue-resident ILC1-like phenotype [2-4]. CD8αα^{+} Ly49E^{+} cells had a lymphoid morphology and were granular, in accordance with high expression of *Grzma* and *Grzmb,* as reported by Van Kaer *et al.* [27]. As well as this, CD8αα^{+} Ly49E^{+} cells were dependent on IL-15-mediated signalling, and total numbers were strongly reduced in *γc*$^{-/-}$ mice. CD8αα^{+} Ly49E^{+} expressed Id2, as do iCD8α cells. However, although our microarray data indicated lower T-bet expression levels for CD8αα^{+} Ly49E^{+} as compared to CD8αα^{-} Ly49E^{+}, lamina propria NK and ILC1 cells, CD8αα^{+} Ly49E^{+} cells were T-bet dependent, whereas iCD8α are reportedly T-bet independent [27].

As such, we have defined a novel intestinal intraepithelial ILC1-like population, which is distinct from the previously described NKp46^{+} NK1.1^{+} CD160^{+} intraepithelial ILC1 population described by Fuchs *et al.* [2] and the NKp46^{+} NK1.1^{+} CD127^{+} lamina propria ILC1 population described by Klose *et al*. [3], and also distinct from NK cells. This new intestinal intraepithelial ILC1-like population, which may be minimally characterised as NKp46^{-} CD8αα^{-} Ly49E^{+}, requires IL-15 mediated signalling for development and/or

survival. Furthermore, we demonstrated that these cells display robust IFN-γ expression following *in vitro* activation. Lastly, we find that Ly49E is expressed on a subset of iCD8α cells. Thus, we show that Ly49E expression is not restricted to intestinal intraepithelial T lymphocytes, but may also be used to identify ILC1-like populations in the gut**.** Of interest, a recent paper by Dadi *et al.* [44] describes the presence of non-circulating innate-like T cell receptor αβ and γδ lymphocytes, in addition to ILC1-like cells, in mammary tissue. 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 novel intestinal NKp46^{-} CD8αα^{-} Ly49E^{+} population described in this paper. Most intriguingly, mammary-resident innate-like T cells and ILC1-like cells express Ly49E [44]. We can thus conclude that Ly49E receptor expression is characteristic for, and restricted to, specific tissue-resident innate(-like) lymphocyte populations, including intestinal intraepithelial NKp46^{-} CD8αα^{-} Ly49E^{+} cells, intestinal iCD8α cells [27], intestinal innate-like CD8αα^{+} T lymphocytes [28,29,45], liver CD49a^{+} NK cells [32] that were recently shown to be ILC1-like [4], skin epidermal Vγ3 T lymphocytes [30], and mammary gland innate-like T and ILC1-like cells [44].

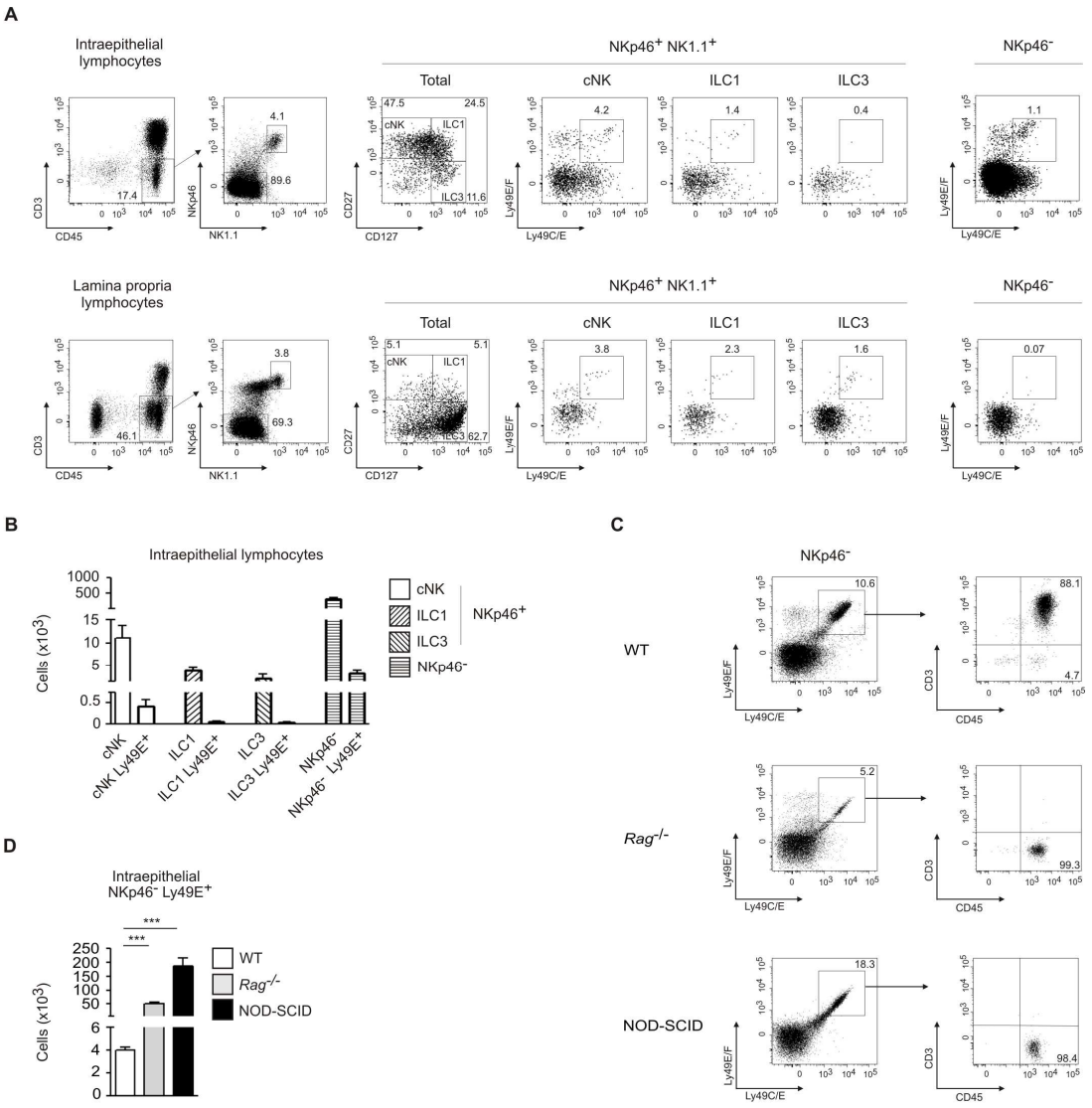

Fig. 1 NKp46⁺ and NKp46⁻ lymphocytes in the intestinal epithelium and lamina propria express Ly49E. (A) Flow cytometric analysis of small intestinal intraepithelial and lamina propria lymphocytes from wild-type (WT) mice. cNK, ILC1, ILC3 and NKp46-negative cells were gated as indicated, and analysed for Ly49E expression. (B) Absolute numbers (mean ± SEM; n = 5)) of small intestinal intraepithelial NKp46⁺ cNK, ILC1 and ILC3 cells, and NKp46⁻ cells in WT mice. The absolute number of Ly49E-expressing cells within these lymphocyte populations is also shown. (C) Flow cytometric analysis of small intestinal intraepithelial lymphocytes from WT, Rag$^{-/-}$ and NOD-SCID mice. NKp46⁻ cells were first gated for Ly49E expression, wherein CD3 and CD45 expression was further analysed. (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. *** indicates a significant difference with $p<0.001$. (A,C) Numbers in the dot plots represent the percentage of cells in the indicated gate or quadrant. Data are representative for 5 different experiments.

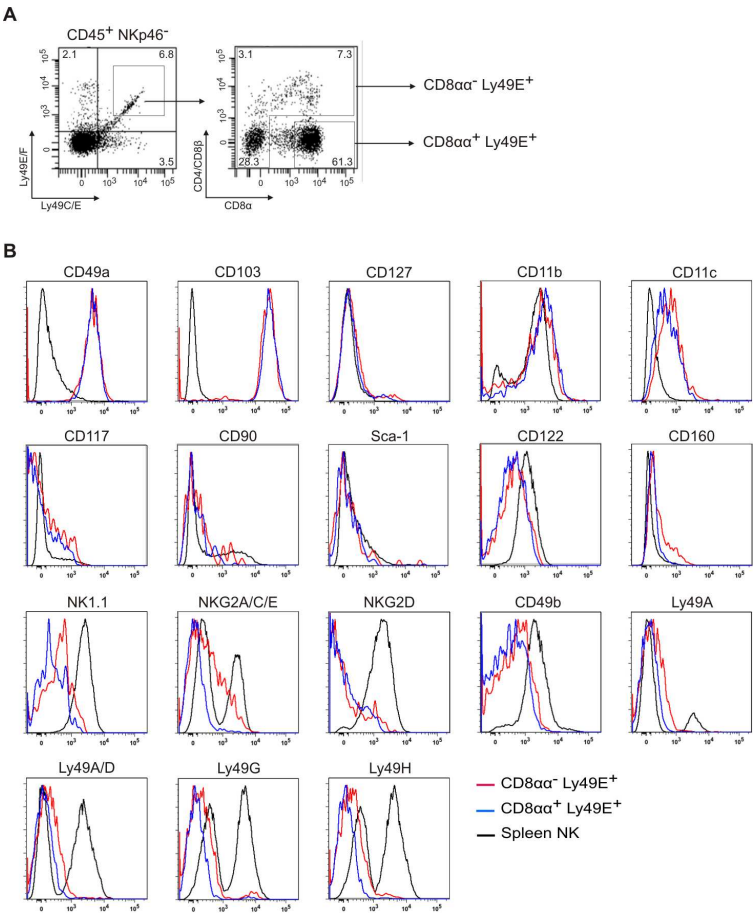

Fig. 2 Surface marker expression of NKp46-negative CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ lymphocytes in the intestinal epithelium. (A) CD8αα$^-$ and CD8αα$^+$ subpopulations were identified in CD45$^+$ NKp46$^-$ Ly49E-expressing intestinal intraepithelial lymphocytes from Rag$^{-/-}$ mice, as indicated. (B) Surface-expression profile of CD8αα$^-$ Ly49E$^+$ (red line) and CD8αα$^+$ Ly49E$^+$ (blue line) cells, gated as in (A), and stained with the indicated antibodies. The black line represents spleen NK cell staining. Data are representative of three independent experiments.

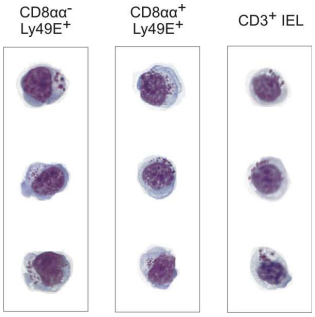

Fig. 3 CD8αα⁻ Ly49E⁺ and CD8αα⁺ Ly49E⁺ intestinal intraepithelial cells have a lymphoid morphology. CD8αα⁺ Ly49E⁺ and CD8αα⁻ Ly49E⁺ cells, additionally gated as CD3⁻ CD45⁺ NKp46⁻, were sorted to high purity (>98%) from the small intestinal intraepithelial lymphocyte fraction of NOD-SCID mice. Intestinal intraepithelial CD3⁺ CD45⁺ Ly49E⁺ T lymphocytes were sorted from WT mice. Cells were fixed and stained with Giemsa.

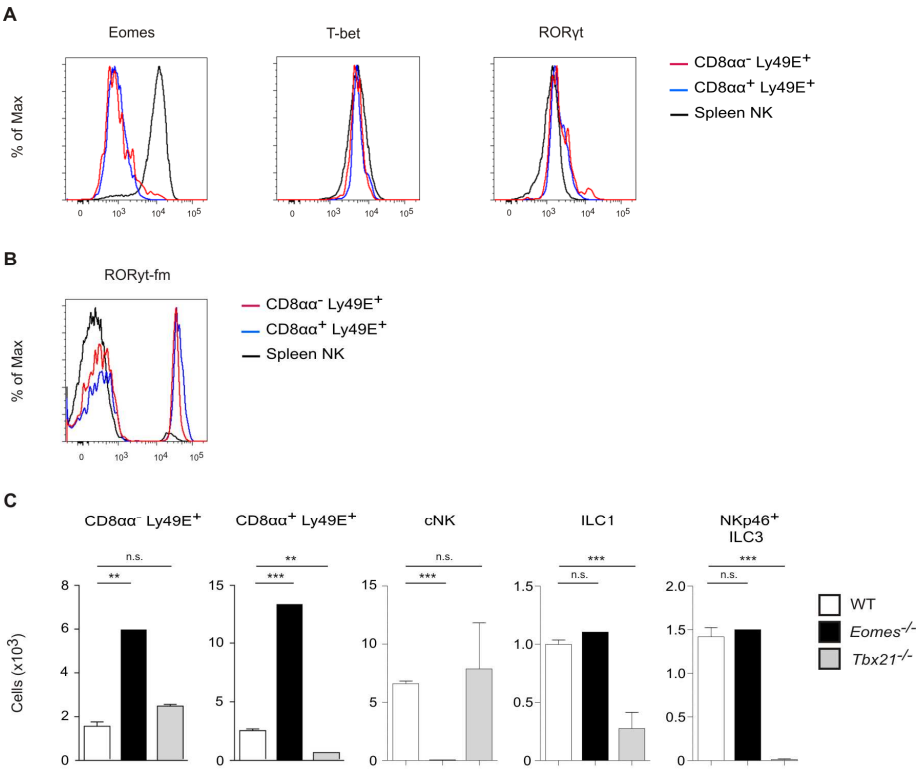

Fig. 4 Transcription factor expression and dependency of CD8αα⁻ Ly49E⁺ and CD8αα⁺ Ly49E⁺ lymphocytes in the intestinal epithelium. (A) Expression of the transcription factors Eomes, T-bet and RORγt by intestinal intraepithelial CD8αα⁻ Ly49E⁺ (red line) and CD8αα⁺ Ly49E⁺ lymphocytes (blue line), and spleen NK cells (black line), isolated from NOD-SCID mice. (B) Analysis of small intestinal intraepithelial CD8αα⁻ Ly49E⁺ (red line) and CD8αα⁺ Ly49E⁺ lymphocytes (blue line), and spleen NK cells (black line), from RORγt-fate map (*Rorc(γt)*-Cre^{Tg} x *Rosa26R*$^{Gfp/+}$) reporter mice. The expression of RORγt-fate map (fm) is shown in the indicated populations. (A,B) Data are representative of three independent experiments. (C) Absolute numbers (mean ± SEM; n = 2-3) of the indicated cell populations in the small intestinal epithelium of wild-type (WT), *Eomes*$^{-/-}$ and *Tbx21*$^{-/-}$ mice. Statistical analysis was performed using the two-tailed Mann-Whitney U-test. ** and *** indicate a significant difference with $p<0.01$ or $p<0.001$, respectively.

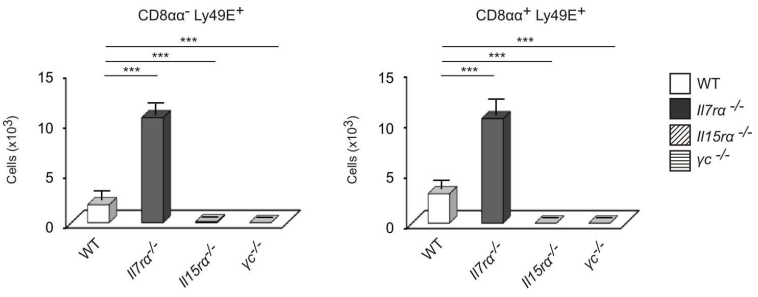

Fig. 5 Intestinal intraepithelial CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ cells are dependent on IL-15 signalling. (A) Absolute numbers (mean ± SEM; n = 3) of CD8αα$^-$ Ly49E$^+$ and CD8αα$^+$ Ly49E$^+$ lymphocytes isolated from the small intestinal epithelium of WT, *Il7ra*$^{-/-}$, *Il15ra*$^{-/-}$ and *yc*$^{-/-}$ mice, as indicated. Statistical analysis was performed using the two-tailed Mann-Whitney U-test. *** indicates a significant difference with $p<0.001$.

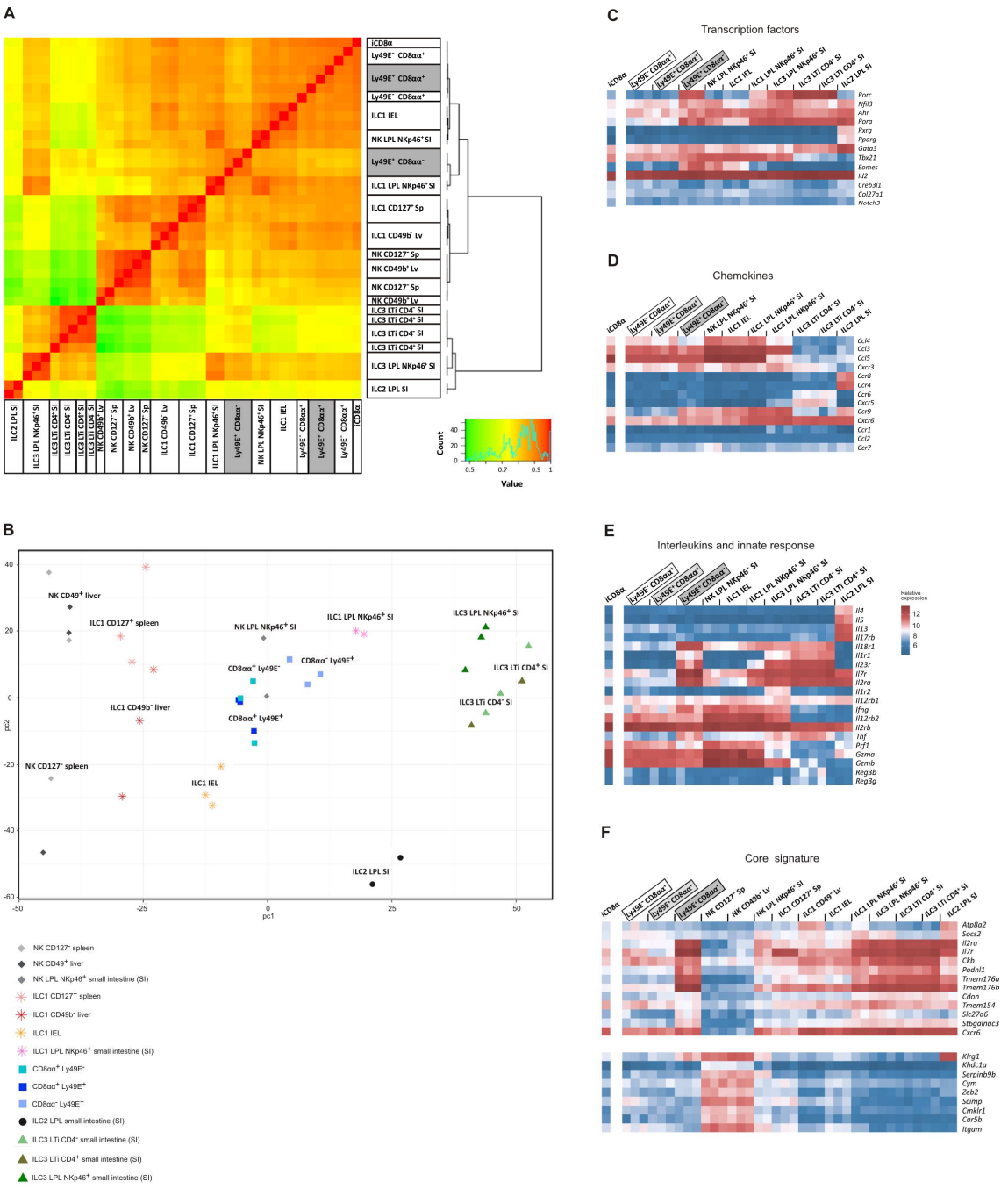

Fig. 6 Intestinal intraepithelial CD8αα⁻ Ly49E⁺ lymphocytes present with a group 1 ILC transcription profile. (A-F) Intestinal CD8αα⁻ Ly49E⁺, CD8αα⁺ Ly49E⁺ and CD8αα⁺ Ly49E⁻ innate lymphocytes, additionally gated as CD3⁻ CD45⁺, were sorted to high purity (>98%) from NOD-SCID mice in 3 different experiments. Transcriptome analysis was performed on these triplicates and was compared to publicly available gene expression data of spleen, liver, intraepithelial (IEL) and/or lamina propria (LPL) NK, ILC1, ILC2, NKp46⁺ ILC3, and CD4⁻ or CD4⁺ ILC3 lymphoid tissue inducer (LTi) cells (see materials and methods). (A) Hierarchical clustering of the indicated populations based on the 15 percent genes with the greatest variability. (B) Principal component analysis (PCA) of the selected populations. Numbers along axes indicate relative scaling of the principal variables. (C-F) Comparative analysis of gene expression by the selected cell populations.

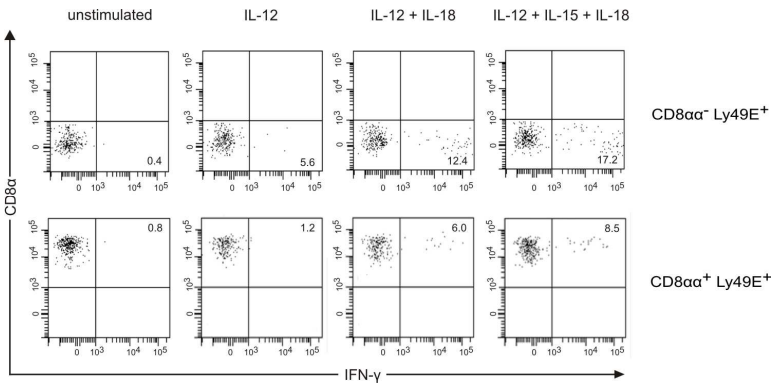

Fig. 7 CD8αα⁻ Ly49E⁺ innate lymphocytes express IFN-γ. (A) Intestinal intraepithelial lymphocytes were isolated from NOD-SCID mice and stimulated with the indicated cytokines, after which IFN-γ expression was determined. Numbers in the right quadrant represent the percentage of IFN-γ-expressing cells in the indicated population. Results are representative of 3 independent experiments.

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Chapter 3:

General discussion and future perspectives

Discussion and future perspectives

Part I

The Ly49E receptor is a unique receptor expressed on a number of innate-like lymphoid cell populations. Among these are NK and intestinal IEL cells, as well as our newly identified intestinal intraepithelial NKp46-negative ILC1-like population.

Previous and current studies in our lab have identified Ly49E receptor expression patterns, and also identified uPA as capable of triggering the Ly49E receptor. Our data indicate that Ly49E functions as an inhibitory NK receptor. As such, research at our lab showed that IFN-γ expression by several NK cell lines is reduced upon exposure to coated recombinant uPA [197]. Furthermore, NK1.1-mediated stimulation of FD17 NK cell cytotoxicity could similarly be reduced following incubation of these cells with uPA. Thus, triggering of Ly49E by uPA inhibits NK cell activation and function [197].

Interestingly, additional data shows that IELs upregulate Ly49E expression following stimulation. TCR-mediated stimulation increases Ly49E expression on TCRγδ CD8αα IEL from 12% to as much as 60%, indicating the existence of a negative feedback loop. Furthermore, our lab demonstrated that Ly49E expression is regulated by two different pathways wherein two different Ly49E promotors are active. More specifically, basal constitutive expression of Ly49E on resting cells is mainly driven by Pro2, whereas induced Ly49E expression on activated cells is driven by Pro3 [198].

Taken together, we hypothesized that basal Ly49E expression and TCR-induced upregulation of Ly49E on IELs might play an important role in tempering the mucosal immune response after initial activation, thus preventing intestinal autoimmunity and pathology.

To test our hypothesis, we examined two profound pathological systems in which uPA has previously been implicated, i.e. IBD and intestinal tumourigenesis. By comparing Ly49E WT to Ly49E knock-out (KO) mice in several established murine disease models, we examined whether Ly49E expression on IELs influences development and progression of these diseases. Here, development refers to the establishment of an intestinal disease, whereas progression refers to disease kinetics.

Taveirne *et al.* [196] previously showed that Ly49E is expressed on the surface of small intestinal IEL. However, at the start of this thesis, it was unknown whether Ly49E is also expressed by IELs of the colon, and whether IEL Ly49E expression affects differentiation of small intestinal and colonic IELs. We showed that Ly49E is indeed expressed by colonic IELs, and that Ly49E expression was almost exclusively restricted to CD8αα$^+$ IELs, as was the case for the small intestine. In this, we found that Ly49E expression by TCRαβ CD8αα IELs was comparable between the small intestine and the colon. Ly49E expression by TCRγδ CD8αα IEL, in turn, was higher in the colon compared to the small intestine. Additionally, we studied IEL subpopulation frequencies and NK receptor expression on IEL isolated from Ly49E WT and

Ly49E KO mice. Here we found that IEL subpopulation frequencies and expression of other NK receptors were similar between Ly49E WT and Ly49E KO mice, indicating that IEL differentiation and homeostasis is unaffected by Ly49E expression. Furthermore, we found that the cytokine expression profile of resting small intestinal IEL from Ly49E KO mice was similar to that of Ly49E WT mice (unpublished data). Together, this suggests that Ly49E expression does not affect IEL development or function when these cells are in a basal resting state.

A number of studies have shown a role for IEL in mediating immunity to ileitis and colitis. In this, both IEL numbers as well as IEL receptor-ligand interactions have been shown to contribute to protection from IBD development [53,87-89,150]. Alongside, an increase in uPA expression has been noted in IBD patients [223,224]. With the noted upregulation of Ly49E on CD8αα^{+} IELs upon TCR-mediated stimulation, we postulated that Ly49E expression by IELs may be involved in regulating the immune response in IBD. Studying a possible role for Ly49E expression by IEL in the development or progression of DSS-induced colitis, we noted that Ly49E KO mice developed colitis as did Ly49E WT mice. Examining disease progression through parameters such as the rate of weight loss, the disease activity index, and gut histology at defined time-points (prior to disease development, at the peak of colitis development and during disease recovery/remission) we observed no significant differences in colitis development or progression between Ly49E KO as compared to Ly49E WT mice. Furthermore, IEL subpopulation frequencies/receptor expression in DSS-treated Ly49E WT and Ly49E KO mice were similar. Thus, colitis development and progression was unchanged in Ly49E KO as compared to Ly49E WT mice in this murine IBD model. Similarly, we found comparable colitis development and progression in Ly49E WT and Ly49E KO mice using the TNBS-induced colitis model, where we monitored the mortality rate, weight loss, colon histology and IEL subpopulation frequencies/receptor expression. Thus, we concluded that Ly49E expression by colonic IEL does not influence colitis development or progression. Because we reasoned that IEL from the colon may carry out different functions as compared to small intestinal IEL, we also examined a possible role for Ly49E receptor expression on small intestinal IEL in the context of ileitis. By crossing Ly49E mice onto the TNF$^{\Delta ARE}$ background, we generated TNF$^{\Delta ARE/+}$ Ly49E WT and TNF$^{\Delta ARE/+}$ Ly49E KO mice. Although we observed severe ileitis development in these mice and noted IEL activation (as observed by an increase in CD69 expression), we found similar levels of intestinal inflammation and no differences in IEL subpopulation frequencies/receptor expression between TNF$^{\Delta ARE/+}$ Ly49E WT and TNF$^{\Delta ARE/+}$ Ly49E KO mice. Therefore, we conclude that Ly49E expression on small intestinal IEL does not influence ileitis development.

The IBD colitis models selected for the study of Ly49E function on IELs were chosen on the basis of previous studies showing an involvement of innate and γδ T cells, as well as NK cell receptors, in the pathogenesis of DSS-induced and TNBS-induced colitis [53,88,89,150]. As IEL are innate-like cells, and a significant proportion of IEL express the γδ T cell receptor, these models were deemed good candidates for study. Furthermore, DSS-induced and TNBS-induced colitis models are excellent models for the study of intestinal barrier function/mechanisms. The intraepithelial localisation of IELs [4], the previously shown role for IELs in mediating intestinal barrier repair [86,88], and the interaction of CD8αα expressed

on IEL with the epithelial cell ligand TL [99], all suggest that these cells have an important role in regulating epithelial barrier function. As such, again, the DSS-induced and TNBS-induced models provided the right environment in which to study the regulatory role of the Ly49E receptor in IEL function. However, it should be noted that studies of *Rag1*$^{-/-}$ mice in the context of DSS-induced [225] and TNBS-induced colitis [226] indicate a limited role for adaptive immunity in these colitis models, and as such alternative colitis models could also be considered for the study of the immunological response. Here, the *Il10*$^{-/-}$ model and the T cell transfer models of colitis have both been shown to involve T cell responses [214]. *Il10*$^{-/-}$ mice develop spontaneous inflammation of the colon characterised by an inflammatory infiltrate made up of lymphocytes, macrophages and neutrophils. Inflammation in this model is initially driven by a Th1/Th17-response but, for currently unknown reasons, is replaced by a more Th2-driven response over time [214]. Alongside macrophages, Foxp3^{+} T regulatory cells are the main drivers of disease in this model, where a more migratory and pro-inflammatory phenotype of intestinal macrophages arises due to the absence of IL-10 [227], and where deletion of IL-10 specifically from T regulatory cells has been shown to be sufficient for the induction of colitis [228]. In the T cell transfer model, transfer of CD4^{+} CD45RB^{hi} T cells to immunodeficient mice is sufficient for the induction of colitis in recipient mice [229]. Both Th1 and Th17-mediated immune responses have been implicated in this pathology [230]. Here too, T regulatory cells are crucial in preventing pathology, as transfer of CD4^{+} CD45RB^{lo} cells does not induce colitis in immunodeficient mice, where the mature CD4^{+} CD45RB^{hi} cell population was shown to harbour Foxp3^{+} T regulatory cells that suppress the development of inflammation in this model [229,231]. The T cell transfer model requires an immune-deficient background, where Ly49E KO mice were available on an immune competent C57BL/6 background. Furthermore, inflammation in the *Il10*$^{-/-}$ and T cell transfer models is mediated to a large extent by conventional T cells and T regulatory cells, that either do not express Ly49E or only display low Ly49E expression. Therefore, we considered these models less suitable for the study of IEL Ly49E function. However, it is not unforeseeable that IEL can interact with lamina propria lymphocytes to mediate colitis development, and as such these models could be considered for future study of IEL function in colitis. With respect to ileitis development, pathogenesis in TNF$^{\Delta ARE/+}$ mice is known to be mediated by both CD4 and CD8 T cells. Adoptive transfer of CD4 T cells from TNF$^{\Delta ARE/+}$ mice into *Rag1*$^{-/-}$ mice transfers disease to recipient mice [232]. A role for CD8 T cells was demonstrated in the expansion of pro-inflammatory CD8^{+} CD44^{hi} cells in TNF$^{\Delta ARE/+}$ mice. Interestingly, however, a subset of CD8^{+} CD103^{+} T cells was shown to attenuate ileitis in *Rag1*$^{-/-}$ mice co-transferred with pathogenic CD4 T cells, demonstrating that CD8 T cells have an important immunoregulatory function in disease development [232]. As IEL are CD103^{+} cells, and the majority of Ly49E^{+} IEL express the CD8αα homodimer [1], we selected TNF$^{\Delta ARE/+}$ -mediated ileitis as a suitable model for the study of IEL Ly49E function in the small intestine. However, an alternative is present in the SAMP1/Yit ileitis model. The SAMP1/Yit model was generated by twenty generations of brother-sister mating of a senescence-accelerated mouse line, and is characterised by the spontaneous development of inflammation in the ileum [233]. CD4 T cells are key to ileitis development, where transfer of activated CD4 T cells from ileitic mice into immunodeficient SCID mice transfers colitis. An increased frequency of CCR9^{+} CD8 T cells is also detected in early ileitis [234]. Together, this implicates both CD4 and CD8 T cell responses in mediating pathogenesis in this model. However, due to the difficulty of

breeding selective brother-sister pairs in combination with obtaining the correct Ly49E genotype, the $TNF^{\Delta ARE/+}$-mediated ileitis was chosen for evaluation of IEL Ly49E function.

Part II

uPA is an integral part of the plasminogen system, whereby plasminogen is converted to active plasmin. Plasmin, in turn, triggers a downstream cascade of extracellular matrix breakdown, ultimately resulting in tissue degradation. This process is beneficial in the context of tissue remodeling, however, may become detrimental in the process of tumour development and metastasis [223,235,236]. uPA has been linked to colorectal cancer development and progression [223,235-238]. As uPA was shown to trigger the Ly49E receptor [197], we wished to explore a possible role for Ly49E expression on IELs in mediating intestinal tumour immune response. Thus, we examined Ly49E WT and Ly49E KO mice in two models of intestinal cancer progression: $Apc^{Min/+}$-mediated intestinal cancer and AMO-induced colorectal cancer. We first examined tumour formation in mice through examination of the tumour load and tumour distribution in the small intestine and colon, respectively. Secondly, we studied tumour development at the cellular level in two ways: through immunofluorescent staining of tumour-infiltrating $CD3^+$ T cells, and through measurement of tumour uPA expression levels. Crossing Ly49E WT and Ly49E KO mice onto the $Apc^{Min/+}$ background, we found that these mice developed numerous spontaneous adenomas by 14 weeks of age. However, tumour load and distribution were similar for $Apc^{Min/+}$ Ly49E KO as compared to $Apc^{Min/+}$ Ly49E WT mice. At the cellular level, this was complemented with data showing no difference in T cell tumour infiltration or tumour uPA expression levels between $Apc^{Min/+}$ Ly49E WT and $Apc^{Min/+}$ Ly49E KO mice. Therefore, we concluded that IEL Ly49E expression does not affect adenoma formation in the small intestine of these mice.

The $Apc^{Min/+}$ model is limited, however, in that it does not allow for the study of adenocarcinoma formation [239]. Thus, it remained possible that Ly49E exerts a function during the adenoma-carcinoma transition, or at a later stage. AMO-treated Ly49E WT and Ly49E KO mice developed fully differentiated adenocarcinomas. However, here too, we did not observe differences in tumour load or distribution, T cell tumour infiltration or tumour uPA expression levels, and this for all tumour sizes analysed. Thus, we conclude that IEL Ly49E expression does not influence the development of adenomas nor adenocarcinomas in the gut.

An unexplored aspect of this research is the role of IEL Ly49E receptor expression in the process of intestinal metastasis. Patients with intestinal cancers may develop additional metastatic tumours at multiple sites, including the peritoneum, liver, lung, brain and bone. Of these, the liver and lungs are the most frequent sites of metastasis [240,241]. Moreover, uPA has been extensively implicated in metastasis [238], and as such it would be relevant to investigate whether putative Ly49E-uPA interactions on IELs influence the metastatic ability of intestinal tumour cells. $Apc^{Min/+}$ mice do not develop metastatic tumours, and metastasis in AMO-induced colorectal cancer has, to our knowledge, not been described [242]. Two currently available mouse models could be used to study the role of Ly49E in intestinal

metastasis. The first focuses on Smad3, where Smad3 is an intracellular protein active in, and required for, TGF-β signalling. In the intestine, TGF-β signals inhibit proliferation of the colonic epithelium, preventing aberrant proliferation that may result in tumour formation. Thus, *Smad3*$^{-/-}$ mice spontaneously develop 4-6 colonic tumours between 18 to 24 weeks of age, where tumours range from adenomatous polyps to invasive carcinomas. Metastasis to the thoracic and mesenteric lymph nodes is also observed in these mice, mimicking human colorectal cancer metastasis, which often initiates in the lymphatics and spreads to regional lymph nodes [243,244]. As such, crossing Ly49E KO mice onto a *Smad3*$^{-/-}$ background would allow for a comparative analysis of metastasis in these mice. Alternately, Hung *et al.* [245] developed the Apc$^{flox/flox}$/LSL-Kras^{G12D} model [245]. In this model, mice in which exon 14 of the *Apc* gene is homozygous floxed (Apc$^{flox/flox}$) are crossed to mice that are heterozygous for the Kras^{G12D} allele (Kras$^{G12D/+}$). As for the Apc$^{Min/+}$ model, an inactivating *Apc* mutation is sufficient for the induction of tumour formation in these mice. An additional activating mutation in the *Kras* proto-oncogene promotes progression from adenomas to carcinomas and ultimately metastasis. Apc$^{flox/flox}$/LSL-Kras^{G12D} mice are surgically incised and the distal colon is infected with adenovirus expressing the Cre enzyme. Following infection, these mice develop primary tumours in the distal colon only, where tumours differentiate as both adenomas and carcinomas 20 weeks following infection. Approximately 20% of mice also develop liver metastases starting 24 weeks following adeno-cre injection. Thus, this model accurately recapitulates human colorectal cancer development and progression on a number of levels: tumour development is localized to the colon, metastasis to the liver may occur, and tumourigenesis is the result of *Apc* and *Kras* gene mutations, where *Apc* and *Kras* genes are often mutated in human colorectal cancer cases [245]. A last additional advantage of this model is the low tumour load (+/- 3.6 tumours), which is coupled with a low lethality of mice and allows for the longitudinal study of metastases [245]. Thus, an excellent way to investigate a role for Ly49E in intestinal metastasis would be to cross Ly49E KO mice to Apc$^{flox/flox}$/LSL-Kras^{G12D} mice, generating Apc$^{flox/flox}$/LSL-Kras^{G12D} Ly49E WT and Apc$^{flox/flox}$/LSL-Kras^{G12D} Ly49E KO mice for a comparative study.

Another aspect that should be considered in studying a role for IEL Ly49E expression in intestinal disease development and progression through the use of full Ly49E KO mice, is the possibility that observed differences in disease development and/or progression may not be due to Ly49E expression by IELs, but may also be the result of Ly49E expression by other intestinal cell populations. In order to ensure that observed differences in phenotype between Ly49E KO and Ly49E WT mice are solely due to Ly49E expression/function on IEL, it would therefore be advisable to confirm any phenotype in a cell-specific Ly49E KO mouse. For this, one could breed mice carrying a floxed Ly49E exon to mice carrying the Cre-enzyme under control of an IEL-specific promotor. Our lab is already in possession of a mouse carrying a floxed Ly49E exon. A promotor that is specific to IEL is unknown to date as far as we are aware, however, use of the Lck (T-cell specific) promotor would allow for deletion of Ly49E specifically in all T cells. As lamina propria T cells express little or no Ly49E (unpublished data), differences between Ly49E WT and an Lck-Cre x Ly49E KO mouse are likely to be the result of a loss of Ly49E expression by IEL.

Part III

NK and LTi innate lymphoid cells were first described in the late 70's. Therefore, it is quite surprising that additional ILC subsets were only discovered more recently. Today, three groups of ILCs are defined: group 1, 2 and 3 ILCs. These ILCs are particularly abundant at mucosal surfaces, and the presence of all three ILC groups has been demonstrated in the gut [116,129,246]. As such, the intestine represents an ideal environment for the study of ILC diversity, function and plasticity.

With the recent identification of the ILC populations, and the knowledge that Ly49E is expressed on innate-like cell populations, we set out to explore whether the Ly49E receptor is expressed by intestinal ILCs ($CD3^-$) in addition to intestinal IELs ($CD3^+$). Ly49E staining of $CD3^-$ $CD19^-$ gated intraepithelial lymphocytes from WT C57BL/6 mice, and of total intraepithelial lymphocytes from T/B-deficient *Rag*$^{-/-}$ and NOD-SCID mice, revealed that Ly49E is indeed expressed by a non-T/B intestinal intraepithelial subset. Additional surface staining revealed that these intraepithelial non-T/B $Ly49E^+$ cells could be divided into two subpopulations, i.e. $CD8\alpha^+$ and $CD8\alpha^-$ populations. $CD8\alpha^+$ $Ly49E^+$ cells were shown to be a subset of the recently described iCD8α cells [128]. These innate lymphoid cells have an intraepithelial localisation and are characterised as $CD3^-$ $CD8\alpha^+$ $CD103^+$ $CD11b^{int}$ $CD11c^{int}$ $CD117^-$ $Nkp46^-$ $NK1.1^-$. iCD8α cells do not express CD127, and are reliant on IL-15-, rather than IL-7, -mediated signalling. Furthermore, these cells are independent of the transcription factors T-bet and AhR, and have low/absent levels of RORγt. iCD8α cells have a unique transcriptome and are avid producers of IFN-γ upon IL-12 stimulation [128].

In contrast, $CD8\alpha^-$ $Ly49E^+$ cells presented with a phenotype uniquely different from all previously reported ILC populations. Indeed, $CD8\alpha^-$ $Ly49E^+$ cells did not express CD127, CD90 and CD117, markers that are expressed by most other ILC populations. $CD8\alpha^-$ $Ly49E^+$ cells expressed CD49a and CD103, suggestive of an intraepithelial ILC1-like cell. Moreover, as $CD8\alpha\alpha^-$ $Ly49E^+$ cells have an $NKp46^-$ $NK1.1^{-/low}$ phenotype and do not express typical NK receptors, such as NKG2D, NKG2A/C/E and other (non-Ly49E) Ly49 receptors, they are not NK cells. To further explore the possibility that we had uncovered a novel ILC population present in the intestinal epithelium of mice, we performed a number of additional studies. First of all, cells were sorted to high purity to analyse their morphology. We found that the intestinal non-T/B $CD8\alpha^-$ $Ly49E^+$ intraepithelial cells have a lymphoid morphology and contain cytoplasmic granules. Secondly, we analysed the presence of $CD8\alpha^-$ $Ly49E^+$ cells in the intestinal epithelial fraction from a large array of mouse strains carrying specific deletions for transcription factors or cytokine receptors. Group 1 ILC NK cells and ILC1 cells are dependent on the transcription factor Eomes and T-bet respectively, group 3 ILCs are dependent on the transcription factor RORγt [111]. Our analysis revealed that $CD8\alpha^-$ $Ly49E^+$ cells are independent of Eomes, T-bet and RORγt for development. This suggests that $CD8\alpha^-$ $Ly49E^+$ cells cannot be classified on the basis of these common ILC transcription factors, and that $CD8\alpha^-$ $Ly49E^+$ cells perhaps constitute a separate ILC lineage. Nonetheless, intranuclear staining showed high expression of T-bet in $CD8\alpha^-$ $Ly49E^+$ cells, suggesting that these cells could have an ILC1-like phenotype. Additionally, the analysis showed that $CD8\alpha^-$ $Ly49E^+$ cells are dependent on signalling through the γc-chain for

development and/or survival. Specifically, we found CD8α$^-$ Ly49E$^+$ cells to be dependent on IL-15, but not IL-7 signalling. This is in accordance with intermediate expression levels of CD122, but absence of CD127, on the cell membrane of these cells. It is also in agreement with previous data showing dependency of most ILC populations on signalling through the γc-chain [116,129,246]. To gain a broader and more detailed insight into this novel subset of ILC cells, we performed a microarray analysis of sorted CD8α$^-$ Ly49E$^+$ cells, and compared our microarray data to that of established ILC populations. Indeed, global microarray analysis confirmed that CD8α$^-$ Ly49E$^+$ are a unique ILC population, with an expression profile intermediate to that of lamina propria NK and intestinal ILC1 cells. Finally, we wished to examine a putative function for this novel NKp46-negative CD8α$^-$ Ly49E$^+$ ILC1-like population. *In vitro* stimulation of CD8α$^-$ Ly49E$^+$ intraepithelial cells revealed that these cells can be stimulated by IL-12+IL-15+IL-18 to produce IFN-γ, as is the case for group 1 ILCs. Thus, our preliminary data suggests that CD8α$^-$ Ly49E$^+$ ILC may play an important role in Th1-mediated intestinal immunity.

Thus, conclusively, our research identified a novel NKp46-negative ILC1-like population, present in the intestinal epithelium. CD8αα$^-$ Ly49E$^+$ cells display characteristics of core ILC subsets, including NK cells and ILC1s. However, the lack of typical NK cell surface expression markers, in combination with their independence of Eomes, confirms that these cells are not NK cells. Furthermore, there are crucial differences between these CD8αα$^-$ Ly49E$^+$ cells and the previously described intestinal intraepithelial and lamina propria ILC1 cells. In contrast to intraepithelial ILC1 cells as described by Fuchs *et al.* [127], intraepithelial CD8αα$^-$ Ly49E$^+$ cells have an NKp46$^-$ NK1.1$^-$ phenotype, are positive for CD103 and negative for CD160. In addition, intraepithelial ILC1 are dependent on T-bet and largely independent of IL-15Rα [127], which is opposite to what we observed for CD8αα$^-$ Ly49E$^+$ ILCs. Lamina propria ILC1 have an NKp46$^+$ NK1.1$^+$ phenotype and express CD127, CD90 and CD117 [123], whereas these surface markers are not expressed by CD8αα$^-$ Ly49E$^+$ ILCs. Moreover, and again in contrast to CD8αα$^-$ Ly49E$^+$ ILCs, lamina propria ILC1 cells are dependent on T-bet. Thus, although CD8αα$^-$ Ly49E$^+$ ILCs have group 1 ILC characteristics, including their overall transcriptome and IFN-γ production capacity, they are uniquely different as compared to NK cells and to the previously described intestinal intraepithelial and lamina propria ILC1 cells. CD8αα$^-$ Ly49E$^+$ ILCs are therefore a novel and unique intestinal intraepithelial ILC1-like population.

Future research with respect to this novel ILC population should include an investigation of Id2 dependency. This could be investigated through the use of *Id2$^{GFP/GFP}$* mice, where absence of CD8αα$^-$ Ly49E$^+$ cells in these mice would be indicative of Id2-dependency. However, as *Id2$^{GFP/GFP}$* mice are growth retarded, findings should be complemented and confirmed through adoptive transfer experiments, wherein fetal liver hematopoietic stem cells are transferred from *Id2$^{GFP/GFP}$* mice to irradiated WT recipients. If fetal liver *Id2$^{GFP/GFP}$* cells are able to reconstitute CD8αα$^-$ Ly49E$^+$ ILC in the gut of recipient mice, this would indicate that Id2 is not required for development of CD8αα$^-$ Ly49E$^+$ ILC.

Our results suggest that CD8αα$^-$ Ly49E$^+$ ILCs have an ILC1-like phenotype and also have functional characteristics of ILC1 cells. We have demonstrated that CD8αα$^-$ Ly49E$^+$ ILC produce IFN-γ when

stimulated with specific cytokines *in vitro*. However, how these cells behave *in vivo*, and the full functional potential of these cells, is still unexplored. Therefore, the role of CD8αα^{-} Ly49E^{+} ILCs in specific models of intestinal disease should be investigated. A role for CD8αα^{-} Ly49E^{+} ILCs could be investigated by focusing on intestinal disease models that are reliant on Th1-mediated immunity such as anti-CD40-mediated colitis [127], *T.gondii* infection [123,156], *Clostridium difficile* infection [247] and *S. typhimurium* infection [143].

General discussion and future perspectives

Human studies have demonstrated a function for killer-cell immunoglobulin-like receptors (KIRs) in mediating susceptibility to IBD and colorectal cancer development. As such, a negative correlation between KIR2DL2 and KIR2DL3 [248], and a protective influence of KIRDL1, have been observed in development of Crohn's disease and ulcerative colitis [248,249]. Furthermore, a recent study shows that activating KIRs are associated with long-term disease free survival in colorectal cancer [250]. The human equivalent of the Ly49E receptor is currently unknown. Interestingly, a number of studies have suggested KIR receptors to be the human homologs of murine Ly49 receptors [126,251]. On the basis of currently available data, showing structural homology between Ly49E and human KIR2DL4, and the illustrated capacity of KIR2DL4 to function as an inhibitory receptor [126,252-254], we propose that KIR2DL4 could be the human equivalent of Ly49E. In order to extrapolate our data to human research, it would therefore be highly relevant to investigate whether this is indeed the case. As previously mentioned, we recently showed that uPA is capable of triggering the Ly49E receptor. Thus, to assert if KIR2DL4 is the human Ly49E homolog, one approach would be to establish whether human uPA is capable of triggering the KIR2DL4 receptor. To study the capacity of uPA to trigger the KIR2DL4 receptor, KIR2DL4 reporter cells could be developed, expressing the extracellular domain of the KIR2DL4 receptor fused to the transmembrane and intracellular domain of the activating KIR3DS1 receptor, as well as expressing the human DAP12 signalling chain. If KIR2DL4 is shown to be the human equivalent of murine Ly49E, advances can subsequently be made in studies of human disease. Although we have could not demonstrate a function for Ly49E expression on IEL in mediating IBD or intestinal cancer development and progression, one could continue work on the ILC aspect of this thesis, examining the human intestine for the presence of innate KIR2DL4-positive cells that have an ILC1-like phenotype and function.

Research conducted over the past few years highlights the heterogeneity and the functional diversity of lymphoid gut populations. Due to overlapping phenotypical and functional traits, classification of some populations remains unsure. However, it is becoming apparent that, as is the case for ILC1 and ILC3 cells, some subpopulations may transition from one cell type into another, underscoring the difficulty of assigning one particular phenotype or function to a specific population. Nonetheless, this heterogeneity and functional flexibility is an asset to the mucosal immune system, allowing it to optimally adapt to any immune challenge.

Where IELs ($CD3^+$) and ILCs ($CD3^-$) are concerned, one could argue that these are parallel and complimentary sides of the mucosal immune system, both exhibiting a phenotype that allows for a rapid and robust immune response. However, it remains possible that intermediate gut IEL-ILC-like populations exist that are as yet unidentified, and that carry out complimentary functions to individual IEL and ILC populations. An important and recent paper by Dadi *et al.* [255] indeed shows the existence of ILTC1 populations in mammary tissue, where ILTC1 cells exhibit phenotypical and functional characteristics of both IEL and ILC1 cells. ILTC1 cells were shown to be either TCRαβ- or TCRγδ-positive, and to express NK1.1, CD103 and high levels of CD49a, whereas these cells are negative for CD127. Principal component analysis (PCA) as well as gene expression analysis shows that these cells are more closely related to mammary ILC1-like (termed ILC1-like and not ILC1 due to their lack of CD127 expression and $Eomes^{lo}$ phenotype) and cNK cells (TCR^- $NK1.1^+$ $CD49a^-$) than to classical $TCR\gamma\delta^+$ $NK1.1^-$ and $TCR\alpha\beta^+$ $CD8\alpha^+$ $NK1.1^-$ populations. ILTC1 cells were also shown to express high T-bet levels, to have low or no detectable Eomes expression, and to be dependent on IL-15 for their development and/or survival. Thus, ILTC1 cells have a phenotype and expression profile that is intermediate to IEL and ILC1-like cells. Most interesting here, is the observation that both mammary ILC1-like cells and ILTC1 cells express intermediate cell membrane levels of Ly49E, with 10-15% and 20-30% Ly49E-positive cells in ILC1-like and ILTC1 populations, respectively. Additionally, ILTC1 cells express granzyme B and display cytotoxic activity towards mammary tumour cells [255].

We, and others, have previously shown that Ly49E expression is relatively high on innate-like unconventional IEL of the gut [94,196,256]. Furthermore, the research presented in this doctoral thesis describes the existence of a novel Ly49E-positive ILC population that is NKp46-negative and is characterised by ILC1-like properties. As this novel intestinal NKp46-negative $Ly49E^+$ ILC1-like population also has a $CD127^-$ $CD103^+$ $CD49a^{hi}$ tissue-resident phenotype, expresses high T-bet levels, is dependent on IL-15-mediated signalling for development and/or survival, and has an ILC1-like gene expression profile (including high granzyme B expression), this population is highly reminiscent of ILC1-like and ILTC1 cells in mammary tissue [255]. Thus, research conducted by us and Dadi *et al.* [255] demonstrates that Ly49E receptor expression is characteristic to innate-like intestinal IEL and tissue-resident $CD127^-$ ILC1-like cells, and mammary ILTC1 cell populations. Future research must elucidate whether ILTC1 cells also exist in the intestine, and whether these may similarly be characterised by Ly49E expression. Furthermore, we hypothesize that IEL, ILC and ILTC1 populations may not be restricted to the intestine or the mammary tissue, but may represent a common feature of all mucosal tissues. Again, future research should be conducted to examine this. And lastly, with the demonstration that mammary ILC1-like and ILTC1 cells are important regulators of tumour immune responses, it would be valid to investigate whether the novel intestinal intraepithelial NKp46-negative ILC1-like population also functions in mediating intestinal tumour immune response.

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Curriculum vitae

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Education

1991-1997	Berkenboom, Sint-Niklaas, Belgium
1997-1999	Prins-Willem Alexander School, Woking, U.K.
1999-2000	St.Maur's, Weybridge, U.K.
2001-2003	St.George's College, Addlestone, U.K.
2003-2006	IGCSE and Bilingual International Baccalaureate at the Antwerp International School, Ekeren, Belgium
2006-2009	Bachelor in Biology
	Free University of Brussels, Brussels, Belgium
	Graduated with honors
2009-2011	Master in Biology (Genetics, Cell- and Developmental- Biology)
	Free University of Brussels, Brussels, Belgium
	Graduated with great honors

Additional courses

2012	Laboratory Animal Science (FELASA Certificate)
2014	Biogazelle qPCR course - From qPCR experiment design to data analysis

Professional record

01/09/2011	PhD candidate Biomedical Sciences
-31/12/2015	Laboratory of Experimental Immunology
	Department of Clinical Biology, Microbiology and Immunology
	Ghent University, Ghent, Belgium
	Research was funded by a grant obtained from the IWT-Vlaanderen

01/01/2016 PhD candidate Biomedical Sciences

-present Laboratory of Experimental Immunology

Department of Clinical Biology, Microbiology and Immunology

Ghent University, Ghent, Belgium

Research was funded by a grant obtained from the Faculty of Health Sciences and Medicine, Ghent University

Publication record

- *A novel intestinal intraepithelial NKp46-negative innate lymphoid cell population characterised by Ly49E expression and group 1 ILC properties*
  Aline Van Acker, Konrad Gronke, Aindrila Biswas, Liesbet Martens, Yvan Saeys, Jessica Filtjens, Sylvie Taveirne, Els Van Ammel, Tessa Kerre, Tom Taghon, Bart Vandekerckhove, Ildiko Dunay, Andreas Diefenbach and Georges Leclercq.
  Manuscript submitted.

- *The role of Ly49E receptor expression on intraepithelial lymphocytes in intestinal cancer development and progression*
  Aline Van Acker, Els Louagie, Jessica Filtjens, Leen Pieters, Sylvie Taveirne, Els Van Ammel, Tessa Kerre, Tom Taghon, Bart Vandekerckhove and Georges Leclercq
  Manuscript submitted.

- *Ly49E expression on CD8αα-expressing intestinal intraepithelial lymphocytes plays no detectable role in the development and progression of experimentally induced inflammatory bowel diseases*
  Aline Van Acker, Jessica Filtjens, Sophie Van Welden, Sylvie Taveirne, Els Van Ammel, Mandy Vanhees, Lindsey Devisscher, Tessa Kerre, Tom Taghon, Bart Vandekerckhove, Jean Plum and Georges Leclercq
  PLoS ONE 2014: 9(10): e110015. doi:10.1371/journal.pone.0110015

- *Contribution of the Ly49E natural killer receptor in the immune response to Plasmodium berghei infection and control of hepatic parasite development*
  Jessica Filtjens, Lander Foquet, Sylvie Taveirne, Els Van Ammel, Mandy Vanhees, Aline Van Acker, Tessa Kerre, Tom Taghon, Bart Vandekerckhove, Philippe E. van den Steen and Georges Leclercq
  PLoS ONE 2014: 9(1):e87463. doi: 10.1371/journal.pone.0087463.

- *Abundant stage-dependent Ly49E expression by liver NK cells is not essential for their differentiation and function*
  Jessica Filtjens, Sylvie Taveirne, Aline Van Acker, Els Van Ammel, Mandy Vanhees, Tessa Kerre, Tom Taghon, Bart Vandekerckhove, Jean Plum and Georges Leclercq
  Journal of Leukocyte Biology 2013: 93(5):699-711

Congresses, symposia and meetings

- International Congress for Mucosal Immunology 2015 – Berlin, Germany. Oral presentation.
- Natural Killer Cell Symposium 2015 – Göttingen, Germany. Poster presentation.
- Natural Killer Cell Symposium 2014 – Hannover, Germany. Poster presentation.
- Science day 2014 – Ghent, Belgium. Oral presentation.
- International Congress for Mucosal Immunology 2013 – Vancouver, Canada.
- Natural Killer Cell Symposium 2013 – Heidelberg, Germany. Poster presentation.
- Gamma delta T cell Symposium 2012 – Freiburg, Germany.
- BIS Meeting 2011 – Hasselt, Belgium.