ORIGINAL ARTICLE

The composition and differentiation potential of the duodenal intraepithelial innate lymphocyte compartment is altered in coeliac disease

Frederike Schmitz, 1 Yvonne Kooy-Winkelaar, 1 Anna-Sophia Wiekmeijer, 1 Martijn H Brugman, M Luisa Mearin, 1,2 Chris Mulder, 3 Susana Chuva de Sousa Lopes, 4 Christine L Mummery, 4 Frank JT Staal, 1 Jeroen van Bergen, 1 Frits Koning 1

ABSTRACT

Objective Coeliac disease (CD), a gluten-induced enteropathy, alters the composition and function of duodenal intraepithelial T cells. The intestine also harbours four types of CD3-negative intraepithelial lymphocytes (IELs) with largely unknown function: CD56-CD127-CD56CD127-CD56CD127- and CD56CD127-. Here we aimed to gain insight into the potential function of these innate IELs in health and disease.

Design We determined the phenotypes, relative abundance and differentiation potential of these innate IEL subsets in duodenal biopsies from controls and patients with CD or patients with refractory CD type II (RCDII).

Results Hierarchical clustering analysis of the expression of 15 natural killer and T cell surface markers showed that innate IELs differed markedly from innate peripheral blood lymphocytes and divided innate IEL subsets into two main branches: a CD127- branch expressing high levels of interleukin (IL) 2/15Rβ but no IL-21R, and a CD127+ branch with the opposite phenotype. While CD was characterised by the contraction of all four innate IEL subsets, a selective expansion of CD56-CD127- and CD56CD127- innate IEL was detected in RCDII. In vitro, in the presence of IL-15, CD56-CD127- IEL from controls and patients with CD, but not from patients with RCDII, differentiated into functional natural killer and T cells, the latter largely dependent on notch-signalling. Furthermore, compared with non-coeliac controls, CD56-CD127- IEL from patients with CD expressed more intracellular CD3e and CD9y and gave more pronounced T cell differentiation.

Conclusions Thus, we demonstrate previously unappreciated diversity and plasticity of the innate IEL compartment and its loss of differentiation potential in patients with RCDII.

INTRODUCTION

In the intestine, a single-layer epithelium provides a barrier against potential pathogens and mediates the uptake of nutrients. Intraepithelial lymphocytes (IELs) scattered throughout the epithelium form one of the largest lymphocyte populations in the body and are thought to be essential for maintaining the integrity of this barrier. 1 IELs consist mainly of T cell receptor (TCR)αβ and TCRγδ T cells (T-IEL). In addition, an innate (CD3-negative) IEL compartment exists, 2-4 which harbours cells expressing a variety of natural killer (NK) cell receptors, 3-9 functional NK cells 8 and NK cell-like type 1 innate lymphoid cells (ILC1). 10 It has been reported that human innate IELs also contain T cell
Coeliac disease

Significance of this study

How might it impact on clinical practice in the foreseeable future?

► Our data show that RCDII can present with two subtypes of innate IEL, each with a distinct common γ-chain cytokine receptor profile, implicating the involvement of several common γ-chain cytokines, IL-21 in particular. This may lead to novel treatment strategies based on blockers of common γ-chain cytokine signalling.
► Furthermore, in contrast to innate IELs from controls, aberrant IELs failed to differentiate. This knowledge may help to understand how these cells transform and ultimately can give rise to enteropathy-associated T cell lymphoma.

precursors.11 12 We recently showed that innate IELs can be subdivided based on the presence or absence of the NK cell marker CD56, and based on the expression of CD127 (IL7Rα), a marker of ILCs.14 15 Thus, the human innate IEL compartment contains multiple subsets, but their phenotypes and functions are poorly understood.

Coeliac disease (CD) is associated with major changes in the duodenal IEL compartment. In active CD the number of IELs is greatly increased (>20 per 100 enterocytes). While TCRαβ and TCRγδ T cell numbers are increased, the innate IEL frequencies are reduced dramatically.7 8 9 16 17 Adherence to a gluten-free diet usually restores the TCRαβ T cell frequencies to normal, but the TCRγδ T cell frequencies remain increased.18 19 A small group of patients does not respond to the diet and therefore suffers from refractory CD (RCD). RCD type II (RCDII) is characterised by the presence of a large number of aberrant IELs, which in approximately 50% of patients derail into overt enteropathy-associated T cell lymphoma, a usually fatal complication of RCDII. Although these IELs express intracellular CD3 chains, TCR-CD3 complexes do not reach the cell surface due to the absence of functional TCR rearrangements.20 21 As these cells are phenotypically similar to innate IELs in non-coeliac controls, it has been suggested that aberrant IELs in RCDII derive from innate IELs rather than from T-IEL, most likely contained within the CD56−/CD127− innate IEL subset.13

Active CD is further characterised by an upregulation of the common γ-chain cytokines interleukin (IL) 15 and IL-21.22–26 IL-15 produced by epithelial cells23 25 converts TCRαβ+ IEL into NK-like cells that kill epithelial cells27 28 and IL-15 produced by lamina propria monocytes can enhance IL-21 production by local lymphocytes.29 Indeed, gluten-specific CD4+ T cells from the lamina propria of patients with CD secrete large amounts of IL-21.24 26 29 30 Although many functions of IL-21 have been described, including effects on B, NK and CD8+ T cells,31 the contribution of IL-21 to CD pathology is largely unclear.24 29 32 It is known that intestinal epithelial cells respond to IL-21 by producing the lymphocyte-attracting chemokine CCL20.33 In addition, mouse small intestinal ILC1 in the epithelium express IL21R transcripts,34 suggesting that IL-21 may also affect innate IELs.

To obtain insight into the dynamics of the innate IEL compartment during ageing and in disease, we conducted an extensive phenotypical analysis of innate IELs in biopsies from paediatric and adult non-coeliac controls, patients with CD and patients with RCDII, and tested the differentiation potential of lineage-negative innate IELs from such biopsies.

MATERIALS AND METHODS

Human specimens

Fetal intestines from elective abortions and duodenal biopsies were obtained as described previously.13 21 Informed consent was given for all human specimens.

Cell isolation

IELs were isolated from fetal intestinal material and duodenal biopsies as described.13 Briefly, the material was treated with 1 mM dithiothreitol (DTT) (Fluka, Buchs, Switzerland)/Hank’s Balanced Salt Solution (HBSS) (Life Technologies, Bleiswijk, the Netherlands) and subsequently 1 mM EDTA (Merck, Darmstadt, Germany)/HBSS. IELs from fetal intestine cells were further purified using a percoll (GE Healthcare, Hoevelaken, the Netherlands) gradient. Isolated cells were either kept on ice in phosphate buffered saline (PBS)/0.5% fetal calf serum (FCS), fixed for 10 min at 4°C with PBS containing 0.1% paraformaldehyde and 0.5% FCS, or viably frozen and stored at −80°C.

Flow cytometry

Fluorochrome-labelled antibodies directed against CD3ε, CD4, CD5, CD7, CD16, CD25, CD45, CD56, CD103, CD117, CD122, CD14, CD19, CD34, killer cell immunoglobulin-like receptor (KIR)3DL1 (DX9), IL-21R, IFNγ and TCRγδ were from BD Biosciences (San Jose, California, USA). KIR2DL2/DL3/D52 (GL183), KIR2DL1/2DS1 (EB6), CD244 and NKP46 were from Beckman Coulter (Fullerton, California, USA), and Nkp44, IL-15Rα, TCRβ, granzyme B and goat antihamster secondary antibody for HMT3-2, see below) were from Biolegend (San Diego, California, USA). Anti-CD127, CD45, ζ-chain and CD160 were from eBioscience (San Diego, California, USA) and CD8 from Invitrogen (Bleiswijk, the Netherlands). CD3γ (clone HMT3-2) was from Millipore (Billerica, Massachusetts, USA) and TCRδ (clone TCS-1) from ThermoScientific (Etten-Leur, the Netherlands).

Cells were incubated for 30 min with fluorochrome-conjugated antibodies for surface staining, and subsequent intracellular staining was done using the permilabelling kit from eBioscience. Cells were acquired on an LSRII or on a flow cytometry-activated cell sorter (FACS)AriaIII (BD Biosciences) for cell sorting. Results were analysed using FlowJo V10 software, and 4-GraphPad. Unsupervised hierarchical clustering of flow cytometry measurements was done using the complete clustering method with the Euclidean distance measurement. The result was plotted in a heat map using the heatmap.2 function from the R gplots package.

Differentiation assays and cell culture

OP9-DL1 cells35 were irradiated (1500 RAD) and plated at 5000 cells/well in 96-well plates. Lineage-negative (Lin−) cells were sorted from intestinal IEL as D45−/CD14−/CD19−/CD3−/CD7−/CD56−/CD127−/CD4+ and plated at 1 cell/well or 100 cells/well onto OP9-DL1. Analysis of cells directly after sorting (n=3, reanalysis of 500 sorted cells/well) showed that sort purity was on average 97.5% (SD 3%), with no contaminating T cells. These cocultures were maintained in Iscove’s modified Dulbecco’s medium (IMDM; LonzaVerviers, Verviers, Belgium) containing 10% pooled normal human serum (NHS), 5 ng/mL human interleukin-7 (Peprotech, Rocky Hill, New Jersey, USA), 10 ng/mL human stem cell factor (SCF), 10 ng/mL Flt3L as described in ref 39 with the addition of 10 ng/mL IL-15 (all from R&D systems Europe, Abingdon, UK) for 2 weeks.


http://gut.bmj.com/content/64/6/10.3326 Downloaded from on June 25, 2015.
Lin+IELs from duodenal biopsies were plated at 1 cell/well and feeder-cell mixture was added after 2 weeks of OP9-DL1 culture. This feeder-cell mixture contained 100 000 mixed irradiated allogeneic peripheral blood mononuclear cells, 1 μg/mL phytohaemagglutinin, 20 Cetus units/mL IL-2 (Proluekin, Chiron corporation, Emeryville, California, USA) and 10 ng/mL IL-15. Clones were maintained on IMDM/10% NHS containing 10 ng/mL IL-15 and 20 Cetus units/mL IL-2. In all cases, the phenotypes of the resulting cell populations were determined by flow cytometry.

Lin-CD127- and Lin-CD127+ IEL cell lines from patients with RCDII termed cell lines P1 and P4, respectively, were isolated from duodenal biopsies of patients with RCDII and maintained as described. Polyclonal NK cells were obtained from peripheral blood mononuclear cells by negative selection using magnetic-activated cell sorting (MACS) beads (Miltenyi, Bergisch Gladbach, Germany) and cultured for 2 weeks in AMV-I medium containing 10% NHS/IL-15. KS62 and Daudi cells were cultured in IMDM/8% FCS and split at regular intervals.

**NK and T cell assays**

For cytotoxicity assays, KS62 and Daudi cells were labelled with 100 μCi (111Cr) for 1 h at 37°C and washed. Target cells were incubated with a Lin+IEL-derived NK cell clone or blood-derived polyclonal NK cells at an effector:target (E:T) ratio of 25:1 for 4 h at 37°C. Spontaneous and maximum 111Cr-release was assessed by the addition of medium or 1% Triton X-100 (Pierce, Rockford, Illinois, USA), respectively. The percentage of specific lysis was determined by: (counts per minute (cpm) experimental release—cpm spontaneous release)/(cpm maximum release—cpm spontaneous release)×100%.

To determine granzyme B induction in primary cells, biopsy-derived IELs were cultured overnight in the absence or presence of IL-15 (10 ng/mL), after which the cells were stained for lineage-defining surface markers and intracellular granzyme B and analysed by flow cytometry. To investigate IFNγ or granzyme B induction in Lin-CD127-IEL-derived NK and T cells, non-tissue-culture treated plates (BD) were coated overnight with anti-CD3 and anti-CD28 (both at 2.5 μg/mL) or control antibodies (mouse IgG1 at 5 μg/mL), all from BioLegden, washed thrice with PBS and subsequently incubated for 1 h with 10% NHS/IMDM to block any remaining protein-binding sites. T or NK cells were activated with plate-bound anti-CD3/CD28, with plate-bound control antibodies or with a mix of phosphol-12-myristate-13-acetate (PMA) (20 ng/mL) and ionomycin (1 μg/mL, both from SigmaAldrich, St Louis Missouri, USA), IL-15 and IL-12 (both 10 ng/mL from R&D systems Europe, Abingdon, UK) for 4 h in the presence of brefeldin A (10 μg/mL) during the final 3 h of the incubation, after which cells were stained for intracellular IFNγ or granzyme B content and analysed by flow cytometry.

**RESULTS**

**Four phenotypically distinct populations of innate IEL**

In a previous study we identified four innate IEL subsets that could be distinguished on the basis of CD127 and CD56 expression. CD56+CD127+ IEL (Lin+CD127+), CD56+CD127+ IEL (Lin+CD127+), conventional CD56+CD127- NK cells (cNK) and CD56+CD127+ NK cells (CD127+ NK) (figure 1A). To determine the phenotypes of these innate IEL subsets, a large number (n=108) of duodenal samples was analysed by multicolour flow cytometry. Innate IELs were distinguished from other cells by selecting CD45+SSC<80CD14+CD19+CD3+CD77+ cells within a live lymphocyte gate (figure 1A). The expression of a panel of 15 lymphocyte markers was assessed on the innate subsets in the intestinal epithelium and peripheral blood lymphocyte (PBL): CD160, IL-21R, CD18, Nkp80, CD94, CD16, KIRs, CD122 (IL-15Rα), CD117 (c-kit), linker for activation of T cells (LAT), CD5, Nkp44, Nkp46, IL-15Rα and CD244 (see figure 1B, C and online supplementary figure S1).

To analyse this large data set in an unbiased fashion, unsupervised hierarchical clustering analysis of the entire set of flow cytometry data (see figure 1B and online supplementary figure S1) was performed (figure 1C). In this analysis, the innate IEL formed a separate cluster that was distinct from T-IEL as well as all peripheral blood subsets, including peripheral innate IELs (figure 1C). Compared with innate PBLs, innate IELs more frequently expressed Nkp44, Nkp46, CD244, IL-15Rα and CD160, while the opposite was true for KIRs, Nkp80, CD94 and CD18 (see online supplementary figure S1). The most striking difference between innate PBL and IEL was the expression of NK cell activation marker Nkp44, absent from innate PBL but expressed by most innate IELs (see online supplementary figure S1). Within the innate IELs, two main clusters could be distinguished: one cluster expressed no CD127 or IL-21R and high levels of IL-2/15Rβ (CD122), while the other cluster did express CD127 and IL-21R but had a lower surface density (mean fluorescence intensity) of IL-2/15Rβ (figure 1C). Strikingly, within these clusters the CD56− and CD56+ cells were relatively similar. Still, cNK could be distinguished from Lin+CD127+ cells by the presence of typical NK cell markers such as KIRs, CD94 and CD16, even though these were expressed at considerably lower frequencies than in peripheral blood cNK cells.

Taken together, these data show that innate IELs differ from T cells and from peripheral innate lymphocytes and consist of four phenotypically distinct subsets. Their cytokine receptor expression patterns indicate that while the Lin+CD127+ and CD127+ NK subsets respond to IL-7 and IL-21, the Lin+CD127− and cNK subsets would be more sensitive to IL-15.

**Age-associated and CD-associated changes in innate IEL subset composition**

As the size of the innate IEL population as a whole is decreased in patients with CD compared with non-coeliac controls, and as children show higher frequencies of Lin+CD127− IEL than adults, we considered the possibility that the composition of the innate IEL compartment is influenced by age and disease. We therefore determined the relative abundance of the IEL subpopulations in the fetal intestine, as well as duodenal biopsies from children and adults without CD, children and adults with CD, and adults with RCDII.

In the samples from individuals without CD, T cell frequencies were lowest in fetal IELs (on average ±40% of CD45−SSC<80 cells), intermediate (±65%) in children and highest in adults (±80%) (figure 2). Conversely, cNK and CD127+ NK populations were most abundant in fetal IELs (±20% and 15%, respectively) and significantly less prominent in IELs of children (±4%) and 2% adults (±1% and 1%). As reported earlier, Lin+CD127− IEL were most frequent in children (±7%), with lower percentages in fetal intestine (±3%) and adult biopsies (±2%). In conclusion, in non-CD controls the T cell population increases with ageing at the expense of the innate IEL population, but the composition of this intestinal innate IEL population changes as well, with CD56− subtypes being dominant in the fetal intestine, CD127− subtypes in the paediatric duodenum and Lin+CD127+ cells in the adult duodenum.

In agreement with previous reports, the percentage of T-IEL was increased in children and in adults with CD (figure 2A). In patients with CD, irrespective of age, each of the four innate
IEL subsets dropped to average frequencies below ±1% of CD45+ cells (figure 2). This drop was most noticeable in the dominant innate IEL subsets, that is, CD127− cells in children and Lin−CD127+ cells in adults (figure 2A). In striking contrast and in line with our previous report, in patients with RCDII the size of the Lin−CD127− (±40%) and the Lin−CD127+ (±10%) IEL subsets were significantly increased, accompanied by a decrease in the percentage of T-IEL (±40%) when compared with adults with CD (figure 2). The relative contributions of these subsets were patient dependent: in 7 out of 10 patients with RCDII the Lin−CD127− population was larger than the Lin−CD127+ population, but in the remaining 3 patients the Lin−CD127+ population was dominant (data not shown).

Taken together, these data show that the composition of the innate IEL compartment is dependent on age and disease status. In RCDII, Lin−CD127− and Lin−CD127+ innate IELs are expanded (figure 2), suggesting that a subset of aberrant IELs in these patients respond to IL-7 and IL-21 (see figure 1 and online supplementary figure S1) rather than IL-15. In line with this idea, Lin−CD127− RCDII line P4, but not Lin−CD127− RCDII line P1, proliferated in response to IL-7 (figure 2B).

Intracellular expression of CD3ε and CD3γ by lineage-negative innate IEL is increased in patients with CD Since Lin−CD127− IELs expanded in patients with RCDII express intracellular CD3ε (icCD3), we examined the
intracellular expression of the CD3γ, CD3ε and ζ-chain in the innate IEL populations in biopsies from non-RCDII patients, using PBLs as controls (figure 3). As expected, T cells in all samples expressed CD3γ, CD3ε (figure 3) and the ζ-chain, expressed by all peripheral blood NK cells. In individuals without CD, CD3γ and CD3ε were detected in a minority of Lin−CD127− and Lin−CD127+ innate IEL, while an even smaller fraction of the CD56+ innate IEL expressed these CD3 chains (figure 3). In marked contrast, in patients with CD the majority of Lin−CD127− and Lin−CD127+ IEL expressed icCD3γ and icCD3ε (figure 3). Furthermore, in these patients the majority of cNK and CD127+ NK cells expressed intracellular CD3γ chains, which has previously only been shown for fetal NK cells.19 As the ζ-chain was expressed by all innate IEL subsets (data not shown), these cells expressed CD3γ, CD3ε and ζ-chain. Due to the unavailability of suitable antibodies, we were unable to test the presence of CD3δ. In short, Lin−CD127− and Lin−CD127+ IELs expressed intracellular CD3 chains, and the expression of CD3ε and CD3γ in these cells was markedly increased in patients with CD.

Marked influence of disease status on the IL-15 dependent generation of NK cells and T cells from Lin−CD127−IEL

As both Lin− IEL subsets contained cells that expressed multiple CD3 chains intracellularly, we considered the possibility that these subsets might harbour the T cell precursors described previously by MacDonald et al.11,12 To test this, we decided to determine the in vitro differentiation potential of Lin− IEL isolated from biopsies obtained from non-coeliac controls, patients with CD and patients with RCDII. We focused our efforts on the Lin−CD127− IEL subset, because previous work had shown that these cells express a functional receptor for IL-15,13 a key cytokine in CD and RCDII pathogenesis. In all experiments, highly purified Lin−CD127− IELs were cultured in the OP9 stromal cell-based coculture system in the presence of IL-15, as initial experiments showed this cytokine to be essential for the survival and expansion of Lin−CD127− IEL in this system (figure 4A).

Notch receptor triggering has been shown to drive intracellular CD3 expression14 and T cell development in developing lymphocytes. As notch ligands are expressed in the intestinal...
Coeliac disease

Figure 3  Intracellular expression of CD3 chains by innate intraepithelial lymphocytes (IELs). (A) IELs from paediatric biopsies from patients with coeliac disease (CD) and patients without CD (no CD) were stained for surface CD45/CD14/CD19/CD3/CD7/CD56/CD127 and gated as shown in figure 1A. T cells (1), Lin<sup>−</sup>CD127<sup>−</sup> (2), Lin<sup>−</sup>CD127<sup>+</sup> (3) conventional natural killer (cNK) cells (4) and CD127<sup>−</sup>NK cells (5) were further stained for intracellular CD3ε (icCD3ε) and CD3γ (icCD3γ). Representative contour plots of the intracellular CD3ε and CD3γ staining of the indicated IEL subsets are shown. The numbers indicate the percentages of CD3ε<sup>+</sup> and CD3γ<sup>+</sup> cells for this representative experiment. (B) Intracellular CD3ε and CD3γ staining in IEL subsets from non-coeliac (n=3) and coeliac (n=3) biopsies and in peripheral blood lymphocytes (PBLs) (n=6). Bar graphs depict the percentages (mean±SD) of cells expressing intracellular icCD3ε and icCD3γ (icCD3γε<sup>+</sup>) cells within the indicated subsets.

In conclusion, Lin"CD127<sup>−</sup>" IEL cultured in a stromal cell-based differentiation system in the presence of IL-15 generated T and cNK cells. The NK/T cell ratio of the resulting cultures was markedly influenced by disease: Lin"CD127<sup>−</sup>" IELs from patients without CD yielded mostly cNK cells, while Lin"CD127<sup>−</sup>" IELs from patients with CD preferentially yielded T cells. In contrast, Lin"CD127<sup>−</sup>" IELs from patients with RCDII did not change their phenotype in these in vitro cultures.

**NK and T cells derived in vitro from Lin"CD127<sup>−</sup>" IEL are functional**

Lin"CD127<sup>−</sup>" IEL cultured in the OP9DL1-system with IL-15 yielded CD3<sup>−</sup>CD56<sup>−</sup>CD127<sup>−</sup> cells (figure 4), which are commonly classified as conventional NK (cNK) cells. However, human ‘helper’ ILCs can also express CD56, even in the absence of CD127. Therefore, expression of CD56 does not necessarily correlate with killing potential. To address this, we first determined the ability of IL-15, a cytokine crucial for NK cell differentiation, to induce granzyme B in primary IEL. Upon overnight culture, IL-15 induced accumulation of granzyme B in all innate IEL subsets, but not in TIEL (figure 5A). To investigate if Lin"CD127<sup>−</sup>" IEL-derived CD3<sup>−</sup>CD56<sup>−</sup> cells were indeed functional NK cells with the ability to kill target cells, a cNK cell clone and a control T cell clone from the OP9DL1 cultures were exposed to various stimuli. PMA/ionomycin stimulation induced IFNγ in the NK and the T cell clones, and only the
Figure 4  Lin^CD127^ IELs from non-CD controls and patients with coeliac disease (CD), but not from patients with refractory CD type II (RCDII), contain natural killer (NK) cell and notch-dependent T cell precursors. Lin^CD127^ IELs were isolated by FACS (gated as in figure 1A) and 100 cells/well were cultured with OP9DL1 or OP9 cells and Flt3L/IL-7/SCF for 14 days in the presence or absence of IL-15, after which the contents of each well were analysed separately by flow cytometry, using antibodies against CD45, CD14, CD19, CD3, CD7, CD56 and CD127. Each data point represents cells derived from 100 Lin^CD127^ IEL (1 well). *, statistically significant p<0.05, ns, non-significant p with Mann-Whitney test. (A) Lin^CD127^ IEL from adult non-CD biopsies were cultured with OP9 cells in the absence (-) or presence (+) of IL-15 and numbers (#) of CD45^+ cells within a live lymphocyte gate were counted. (B) Lin^CD127^ IEL from biopsies from patients with CD (n=4), non-coeliac biopsies (no CD, n=7), RCDII material (fresh intraepithelial lymphocytes (IELs) from one biopsy, short-term IL-15 cultures from two biopsies, RCDII lines from four biopsies) were cultured with OP9 or OP9DL1 cells in the presence of IL-15. The percentages of NK cells (CD3^-CD7^-CD56^+) and T cells (CD3^-CD7^+) within CD45^- cells in the resulting cultures are depicted. Note that Lin^CD127^ IEL from patients with RCDII retained their phenotype and yielded neither T cells nor NK cells. (C) Surface phenotypes of T cells (CD3^+CD7^+) and NK cells (CD3^-CD7^-) generated in panel B. DN: CD4^-CD8^-.

T cell clone was sensitive to TCR/CD3 cross-linking (figure 5B). Consistent with the induction of granzyme B by IL-15 in innate but not adaptive IELs (figure 5A), the NK cell clone constitutively expressed granzyme B (figure 5B) and was able to lyse the classical NK cell targets K562 and Daudi, albeit to a lesser extent than peripheral blood derived NK cells (figure 5C). Thus, the in vitro-generated CD3^-CD56^- T cells and CD3^-CD56^+ NK cells were functional NK cells with respect to IFNγ production and target cell lysis.

DISCUSSION

Our experiments show that (1) four subsets of innate IEL can be distinguished based on differences in their expression of CD127, CD56, IL-21R, CD3γ and CD3δ chains, that (2) with ageing and in CD, the relative sizes of these four subsets are altered, as is (3) the intracellular CD3 content and the differentiation potential of the Lin^CD127^- IEL subset in patients with CD and RCDII.

The main markers differentiating the innate IEL subsets are cytokine receptors. Whereas CD127^- innate IELs lack the IL-21R and express high levels of IL-2/15Rβ, CD127^+ innate IELs express the IL-21R and display significantly lower IL-2/15Rβ surface levels. In line with these findings, we previously demonstrated that Lin^CD127^- IELs, but not Lin^CD127^+ IELs, proliferate in response to IL-15, a cytokine that is upregulated in the duodenum of patients with CD. IL-21 is produced by gluten-specific CD4^+ T cells located in the lamina propria of patients with CD, and would be expected to activate the CD127^+ innate IEL subsets. It is not known whether the expression of IL-7 or thymic stromal lymphopoietin (TSLP), which bind receptors containing CD127, are altered in the duodenum of patients with CD. It will be important to further explore the effects of these and other cytokines on innate IEL function.

In recent years, ILCs have been the subject of intense investigation. In addition to cNK cells, which can be considered cytotoxic ILCs, a separate lineage of ‘helper’ ILCs has been defined. Three types of ‘helper’ ILCs have been identified thus far, each with distinct functional features and transcription factors matching those typifying Th1 (ILC1), Th2 (ILC2) and Th17 (ILC3) cells. ILCs in humans are generally defined as lineage-negative CD127^+ cells that can express CD56, suggesting that Lin^CD127^- and CD127^+ NK IELs belong to the ‘helper’ ILC lineage. In support of this idea, many Lin^CD127^- and CD127^+ NK IELs express Nkp44 and Nkp46, which are found on ILC1 and a subset of ILC3 cells. However, many Lin^CD127^- and CD127^+ NK IELs express CD16, which is absent from the ‘helper’ ILC lineages and is more typical of cNK cells. In line with the notion that the CD127^-CD56^- innate IELs are cNK cells, they express a functional IL-15 receptor and several NK cell receptors (Nkp46, KIRs, CD94, CD16). Thus far, only one single study has investigated the possibility that human IELs contain ILCs.

Our data indicate that innate IELs contain ILCs, an idea that is supported by recent experiments in mice.

Previous studies indicated that innate IELs contain T cell precursors, and our experiments suggest that these exist within...
the Lin−CD127− IEL subset. In line with the presence of multiple CD3 chains in Lin−IEL, coculture of Lin−CD127− IEL with OP9DL1 gives rise to T cells, and roughly equal numbers of cNK. Moreover, Lin−CD127− IELs from patients with CD, which are particularly rich in intracellular CD3 and the majority of which express CD3γ and CD3ε, yield considerably more T cells than cNK cells. The cNK cells generated from Lin−CD127− IEL express granzyme B, can produce IFNγ and lyse classical NK cell targets, indicating that these two innate IEL subsets are closely related. In humans, NK cell precursors have so far been described in secondary lymphoid tissues like lymph nodes, tonsil, liver, in the uterus, the thymus and as CD117+ cells in the lamina propria of the intestine, but not in the intestinal epithelium. Thus, our results indicate that Lin−CD127− IELs harbour precursors to NK and T cells, which may provide plasticity in response to environmental insults.

Although the outgrowth of contaminating NK and/or T cells could theoretically be confounding the results obtained in these in vitro differentiation experiments, several observations argue against this possibility. First, we used a strict gating strategy throughout our studies, which showed no T cell contamination. Second, culture of Lin−CD127− IEL from patients with RCDII did not lead to T cell outgrowth, even though T cells were present before the FACS sort. Third, bulk sorting of intestinal Lin−CD127− IEL followed by culture with ‘feeder mix’—optimal culture conditions for mature T cells and NK cells—did not yield any outgrowth of T cells or NK cells in four independent experiments (data not shown). Finally, the observation that notch-signalling augmented T cell differentiation points to the fact that T cells arising from Lin−CD127− IEL were derived from notch-dependent pre-T cells and thus not from contaminating mature T cells.

Inflammatory intestinal diseases are associated with major changes in local lymphocyte populations. CD, for example, is characterised by an expansion of intraepithelial T lymphocytes (T-IEL) and a reduction in CD3-negative or innate IELs. Our results suggest that local differentiation of T cells from Lin−IEL may contribute to this phenomenon. In contrast, RCDII is characterised by massive expansion and malignant transformation of aberrant IELs, and our previous and current results indicate that this is caused by proliferation of Lin−CD127− as well as Lin−CD127+ innate IELs. A striking difference between Lin−CD127− IEL from patients with RCDII and patients with CD is that the former have lost the ability to give rise to NK cells and T cells, most likely due to the chromosomal aberrations typically found in these premalignant cells (data not shown). In general, IL-15 has been held responsible for the expansion of aberrant IELs but the expression of CD127 (IL-7Rα) and the IL-21R by Lin−CD127+ IEL implies a potential involvement of IL-7 (or TSLP) and IL-21 as well. This implies that blocking of IL-15 alone may not be an effective treatment option for this often fatal condition.

In conclusion, our data provide evidence for substantial alterations in the composition, phenotype and differentiation potential of the innate IEL compartment in CD. The innate IEL subsets are equipped with distinct cytokine receptors which might allow responses to cytokines produced within the epithelium as well as in the lamina propria. This diversity and plasticity of the innate IEL compartment may allow for tailored responses to homeostatic and inflammatory stimuli. Finally, the expansion of Lin−CD127− and Lin−CD127+ innate IELs in...
RCID1 indicates that IL-15, and IL-7 and IL-21 contribute to the survival and expansion of these (pre-)malignant cells.

Acknowledgements The authors thank E de Haas and G de Roo for cell sorting, J Pahl, M Ostaïjen-ten Dam for K662/Daudi culture and the generation of blood-NK cell lines, J Hassing for processing material and K Lodder for material distribution.

Contributors FS designed the study, did experiments, analysed data and wrote the manuscript; AW-S did experiments; MHB analysed data; MLM and CJC provided patient tissue; SCCS provided fetal gut tissue; CLM facilitated provision of fetal gut tissue; FJS assisted in study design; JvB and FK designed the study, analysed the data and wrote the manuscript.

Funding Supported by the Celiac Disease Consortium, an Innovative Cluster approved by The Netherlands Genomics Initiative, and the Dutch government (grant BSIK03009). The funding body had no influence on the study design, the collection, analysis and interpretation of the data.

Competing interests None declared.

Ethics approval Medical ethical commission approval, and in accordance with the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES


**Coeliac disease**


The composition and differentiation potential of the duodenal intraepithelial innate lymphocyte compartment is altered in coeliac disease

Frederike Schmitz, Yvonne Kooy-Winkelaar, Anna-Sophia Wiekmeijer, Martijn H Brugman, M Luisa Mearin, Chris Mulder, Susana Chuva de Sousa Lopes, Christine L Mummery, Frank JT Staal, Jeroen van Bergen and Frits Koning

Gut published online May 12, 2015

Updated information and services can be found at: http://gut.bmj.com/content/early/2015/05/12/gutjnl-2014-308153

These include:

Supplementary Material
Supplementary material can be found at: http://gut.bmj.com/content/suppl/2015/05/12/gutjnl-2014-308153.DC1.html

References
This article cites 58 articles, 20 of which you can access for free at: http://gut.bmj.com/content/early/2015/05/12/gutjnl-2014-308153#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections

Coeliac disease (529)

Notes

To request permissions go to: http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to: http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to: http://group.bmj.com/subscribe/