Celiac disease : how complicated can it get?

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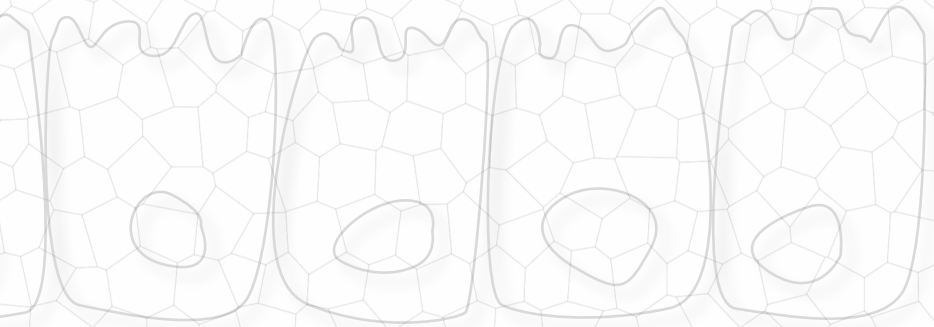

Chapter 3

DNAM-1 Mediates Epithelial Cell-specific Cytotoxicity of Aberrant Intraepithelial Lymphocyte Lines from Refractory Celiac Disease Type II Patients

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Abstract

In refractory celiac disease (RCD) intestinal epithelial damage persists despite a gluten-free diet. Characteristic for RCD type II (RCD II) is the presence of aberrant surface T cell receptor (TCR)-CD3$^-$ intraepithelial lymphocytes (IELs) that can progressively replace normal IELs and can eventually give rise to overt lymphoma. Therefore, RCD II is considered a malignant condition that forms an intermediate stage between celiac disease (CD) and overt lymphoma.

We now demonstrate that surface TCR-CD3$^-$ IEL lines isolated from three RCD II patients preferentially lyse epithelial cell lines. FACS-analysis revealed that DNAM-1 was strongly expressed on the three RCD cell lines, while other activating NK cell receptors were not expressed on all three RCD cell lines. Consistent with this finding, cytotoxicity of the RCD cell lines was mediated mainly by DNAM-1 with only a minor role for other activating NK cell receptors. Furthermore, enterocytes isolated from duodenal biopsies expressed DNAM-1 ligands and were lysed by the RCD cell lines ex vivo.

While DNAM-1 on CD8$^+$ T cells and NK cells is known to mediate lysis of tumor cells, this study provides the first evidence that (pre)malignant cells themselves can acquire the ability to lyse epithelial cells via DNAM-1. This study confirms previous work on epithelial lysis by RCD cell lines and identifies a novel mechanism that potentially contributes to the gluten-independent tissue damage in RCD II and RCD-associated lymphoma.

Introduction

A small proportion of adult-onset celiac disease (CD) patients develops a refractory state with persisting villous atrophy and an increase of intraepithelial lymphocytes (IELs) despite a gluten-free diet[1]. Refractory celiac disease (RCD) can be subdivided into RCD I and RCD II, distinguished by the respective absence or presence of an aberrant IEL population lacking surface expression of the T cell receptor-CD3 complex (sTCR-CD3)[2]. RCD II is now considered an intraepithelial lymphoma that can give rise to high-grade invasive lymphoma. Overt lymphoma likely derives from the aberrant IELs, as they share the sTCR-CD3$^-$ phenotype and display identical monoclonal TCR-γ gene rearrangements[3;4]. The cytokine interleukin-15 (IL-15) which is upregulated in the lamina propria and on epithelial cells of RCD patients[5;6] is thought to be crucial for the expansion and survival of aberrant IELs and lymphoma cells[7]. RCD II and RCD-associated lymphoma have poor 5-year survival rates of 44-58% and <20% respectively[1;8].

In active CD, many CD8$^+$TCR$^+$ IELs have acquired activating NK cell receptors such as NKG2C and NKG2D[9;10]. These receptors co-stimulate TCR-mediated lysis of epithelial cell lines *in vitro*[9;11]. Upon exposure to IL-15, CD8$^+$ IELs can display TCR-independent NKG2D-mediated cytotoxicity against epithelial cell lines[12]. Studies on the cytotoxic capacity of aberrant sTCR-CD3-negative IELs in RCD II indicated that such cells could lyse intestinal epithelial cell line HT29 in a granzyme/perforin dependent fashion[5;11]. Furthermore, lysis was induced by stimulation of NKG2D[11], boosted by IL-15 and could be partially inhibited by an antibody to CD103[5]. Blocking of NKG2D only partially inhibited the lysis of HT29, suggesting a role for additional activating NK cell receptors in the epithelial cytoxicity of aberrant IELs.

In the present study, we set out to identify these additional receptors and further defined the specificity of aberrant IEL cytotoxicity. To this end, we used cell lines isolated from small intestinal biopsies of three RCD II patients (hereafter called RCD cell lines) that display the typical aberrant sTCR-CD3$^-$ phenotype[13].

Materials and methods

Small intestinal biopsy specimens

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe, Tempe, Arizona) were taken from the second part of the duodenum[14]. Biopsy specimens were used for culture, direct flowcytometric analysis or cytotoxicity assays. All biopsy specimens were obtained after given informed consent in accordance with the local ethical guidelines of the VU Medical Center in Amsterdam and the Declaration of Helsinki.

Cell lines and cell culture

RCD cell lines P1, P2 and P3 were isolated from duodenal biopsies of three RCD II patients as previously described[13]. In short, after treatment with 1 mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylenediaminetetraacetic acid (Merck, Darmstadt Germany), biopsies were cultured in Iscove's modified Dulbecco's medium (IMDM, Lonza, Verviers, Belgium) with 10% normal human serum (NHS) containing 10 ng/ml IL-15 (R&D Systems Europe, Abingdon, United Kingdom). The predominant population of $CD3^-CD4^-CD8^-CD30^+$ cells was purified by FACS, thus giving rise to RCD cell lines P1, P2 and P3. RCD cell lines were propagated in IMDM with 10% NHS containing 10 ng/ml IL-15 and restimulated approximately every 4 to 5 weeks with 1 µg/ml phytohemagglutinin, 10 ng/ml IL-15 and 1 x 10^6/ml irradiated allogenous peripheral blood mononuclear cells as feeder cells. Three $CD8^+$ TCR^+ IEL lines were isolated from duodenal biopsies of three independent CD patients following the method described above and purified by FACS based on CD3-and CD8-positivity. These $CD8^+$ TCR^+ cells, that were homogeneously DNAM-1^+ $NKG2D^+$, were cultured in IMDM with 10% NHS containing 10 ng/ml IL-15 and restimulated every 2 weeks. 15 additional cell lines were isolated from biopsies of RCD II patients following the method for RCD cell lines P1, P2 and P3. These cell lines were not purified by FACS prior to FACS analysis.

Additional control cell lines for P1 and P3 were respectively a $CD8^+$ T cell line and a $CD4^+$ T cell clone isolated from a biopsy of the corresponding patient. Cells were restimulated every 2 weeks and maintained on IMDM with 10% NHS containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proleukin, Chiron corporation, Emeryville, CA). Cell lines HT29, Caco2, T84, A549, Daudi, K562, P815 and EBV-BC107 were cultured in IMDM with 10% fetal calf serum (FCS). EBV-BC107 is an EBV B-LCL cell line generated from a healthy blood bank donor[15].

Antibodies

Fluorochrome-conjugated anti-CD3, anti-CD226 (DNAM-1), anti-CD112, anti-CD30 anti-CD94 and anti-perforin were purchased from BD Biosciences (San Jose, CA). Anti-CD155, anti-NKG2D, anti-NKG2C were from R&D Systems Europe. Anti-NKp30, anti-NKp44 and anti-NKp46 were purchased from Beckman Coulter (Fullerton, CA). Anti-NKp80 was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany) and anti-epithelial specific antigen (ESA) from Biomeda (Foster city, CA). For blocking and redirected lysis experiments, anti-CD226 was purchased from BD Biosciences, anti CD30, anti NKG2D and isotype-matched control antibodies from R&D Systems Europe, anti-CD103 and anti-CD155 from Beckman Coulter and anti-CD112 from e-Bioscience (San Diego, CA).

Isolation of cells from duodenal biopsies

Enterocytes and lymphocytes were isolated from two or three duodenal biopsies treated with 200 µg/ml collagenase A (MP Biomedicals LLC, Eschwege, Germany) and 200 µg/ml DNAse II (Roche diagnostics, Almere, Netherlands). After rotating 1 hour at 37°C, cells were filtered through a 70 µm filter (BD Falcon, Erembodegem, Belgium) and washed twice with IMDM with 10% FCS. For subsequent flow cytometric analysis, cells were stained 30 minutes with fluorochrome-conjugated antibodies. For cytotoxicity assays, three duodenal biopsies were treated with 1 mM dithiothreitol (Fluka, Buchs, Switzerland) and 0.75 mM ethylene-diamine-tetra-acetic acid (Merck, Darmstadt Germany); the released cells were then used as target cells. Approximately 80% of the released cells were ESA^+ enterocytes.

Cytotoxicity assay

One million target cells were labeled with 100 µCi ^{51}Cr for 1 hour at 37°C. After extensive washing, labeled target cells were co-incubated with effector cells at effector-target ratios between 50:1 and 1.5:1 for 4 hours at 37°C. For blocking and redirected lysis experiments effector cells were pretreated for 20 minutes at room temperature with 20 µg/ml of the indicated antibodies. Masking ligands on target cells was achieved by the presence of 5 µg/ml monoclonal antibodies in the effector-target mixture during the 4 hour incubation period. Spontaneous chromium release and maximum chromium release by target cells was determined by addition of medium or 1% Triton X100 (Pierce, Rockford, Illinois) respectively. The percentage of specific cytotoxicity was as follows: ([cpm experimental release - cpm spontaneous release]/[cpm maximum release - cpm spontaneous release]) x 100 %.

BLT esterase assay

96-well non-tissue-culture-treated plates (BD Falcon) were coated overnight in duplicate with 5 µg/ml anti-NKG2D, anti-DNAM-1 or both. Subsequently, plates were blocked with 10% FCS/PBS and washed with PBS. 1 x 10^5 cells from cell lines P1, P2 or P3 were added. After 4 hours incubation at 37°C, supernatants were evaluated for esterase secretion using an N-benzyloxycarbonyl lysine thiobenzyl ester (Merck). OD values were obtained at an absorbance of 412 nm on a microplate reader (Bio-Rad Laboratories, Veenendaal, The Netherlands).

Immuno-electron microscopy

Cells were fixed in 2% paraformaldehyde with 0.1% glutaraldehyde and processed for immunogold staining as described elsewhere[16]. Ultrathin cryosections were incubated with granzyme B-specific monoclonal antibody GB11 (Sanquin, Amsterdam, The Netherlands), followed by rabbit-anti-mouse IgG (Dako, Heverlee, Belgium) and 15 nm Protein-A-gold particles. Sections were evaluated with a Philips 410 electron microscope.

Statistical analysis

For cytotoxicity assays and BLT esterase assays, each data point is the mean ± SEM of an experiment performed in duplicate. An unpaired two-tailed *t* test was performed to compare the effect of the antibodies used in this study with their respective controls. A P-value less than 0.05 was considered significant.

Results

RCD cell lines lyse intestinal epithelial cells

RCD cell lines P1, P2 and P3, isolated from three RCD II patients, have the characteristic phenotype of aberrant cells in RCD II: surface $TCR^-CD3^-CD4^-CD8^-CD103^+$, intracellular $CD3^+$ [13]. They grow in an IL-15 dependent fashion[13], which is of interest as IL-15 is known to be over-expressed in the intestine of patients with RCD and lymphoma[5]. Furthermore, they express granzyme B (Supplementary Figure S1) and perforin (not shown), both known to play a key role in lymphocyte-mediated cytotoxicity[17;18]. The RCD cell lines could be isolated repeatedly from successive duodenal biopsies of all three patients, indicating that the isolated cell lines represent a persisting population. Moreover, these cell lines have monoclonal TCR-γ gene rearrangements identical to the aberrant IELs in the patients and therefore closely resemble the aberrant IELs *in vivo*[13].

RCD cell lines P1, P2 and P3 were tested for their ability to lyse an array of target cells: intestinal epithelial cell lines (HT29, Caco2, T84), a non-intestinal epithelial cell line (A549), an erythroleukemia cell line (K562), a Burkitt lymphoma cell line (Daudi) and an EBV-lymphoblastoid cell line (EBV-BC107).

All three RCD cell lines lysed the intestinal epithelial cell lines HT29, Caco2 and T84, but not the non-epithelial cell lines Daudi and EBV-BC107 (Figure 1A-D). RCD cell line P3 was the only cell line that induced lysis of NK cell target K562 (Figure 1A-D), although lysis of K562 was much lower when compared to lysis of the epithelial cell lines (Figure 1D). The non-intestinal epithelial cell line A549 was lysed as well (Figure 1E), indicating that lysis is not confined to intestinal epithelium but can be directed against other types of epithelium. In contrast, three polyclonal $CD8^+TCR^+$ IEL lines derived from small intestinal biopsies of CD patients displayed far less cytotoxicity against HT29 (Figure 1F), but efficiently lysed non-epithelial cell line K562 (Figure 1F), showing that the weak lysis of HT29 was not due to an inherently low cytotoxic capacity. These results indicate that in comparison with $CD8^+$ TCR^+ IEL lines, RCD cell lines are more cytotoxic to epithelial cells. All three RCD cell lines expressed CD103 ($\alpha_E\beta_7$)[13], an integrin present on IELs to retain lymphocytes at the epithelial surface. The only known ligand of CD103 is E-cadherin, which is expressed selectively by epithelial cells[19]. Cytotoxicity of the RCD cell lines against the epithelial cell lines HT29, Caco2, T84 and A549 was indeed partially inhibited by a CD103-specific antibody (Figure 1G, 1H), consistent with a role for the integrin $\alpha_E\beta_7$

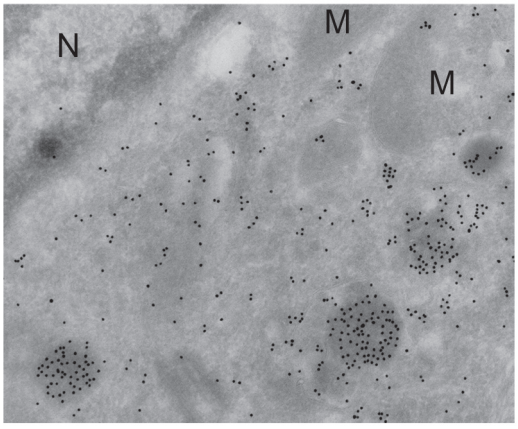

Figure S1. RCD cell lines express granzyme B. Granzyme B was detected using immunogold labeling and immuno-electron microscopy. The result for RCD cell line P1 is shown. "N" represents the nucleus; "M" represents a mitochondrium.

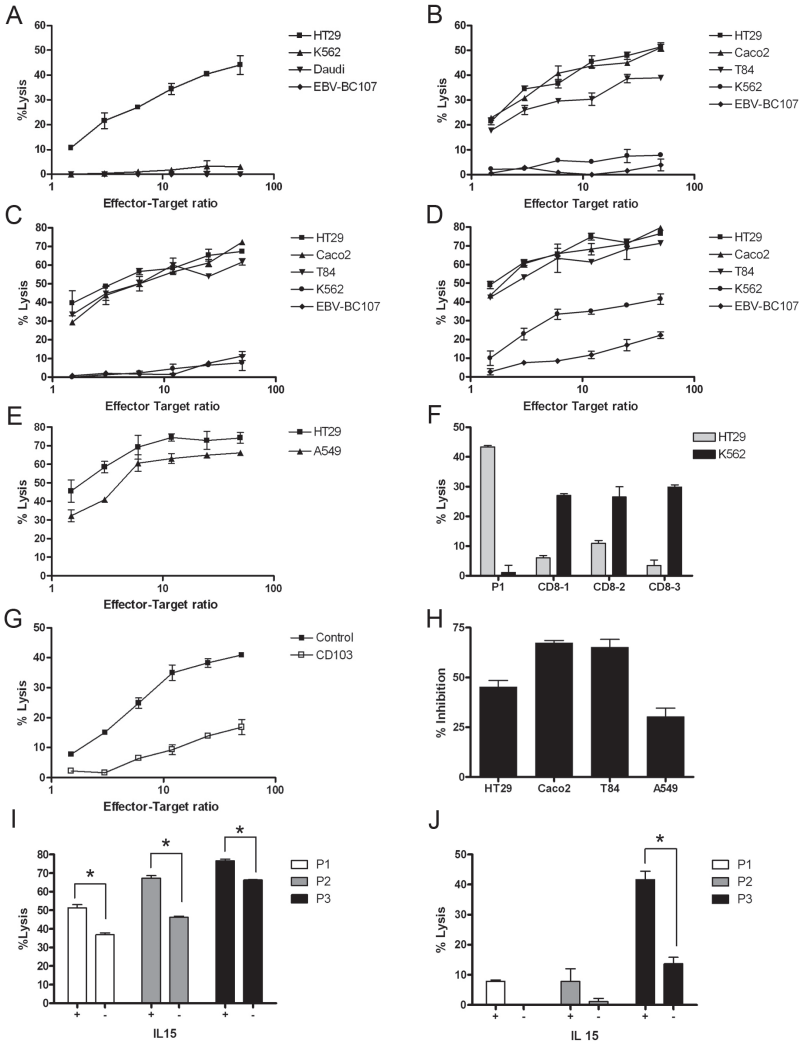

Figure 1. RCD cell lines preferentially lyse epithelial cells. (A) Lysis of HT29, K562, Daudi and EBV-BC107 by RCD cell line P1. Similar results were obtained with P2 and P3, except for lysis of K562 by RCD cell line P3. (B-D) Lysis of HT29, Caco2, T84, K562 and EBV-BC107 by: (B) RCD cell line P1. (C) RCD cell line P2. (D) RCD cell line P3. (E) Lysis of HT29 and A549 by RCD cell line P3. Similar results were obtained with P1 and P2. (F) Lysis of HT29 and K562 by RCD cell line P1 and three independent polyclonal $CD8^+NKG2D^+DNAM\text{-}1^+$ IEL lines in a 50:1 effector-target ratio. (G) Lysis of HT29 by RCD cell line P1 after incubation with an anti-CD103-antibody or with an isotype-matched control antibody. Similar results were obtained with P2 and P3. (H) Percentage of inhibition on the lysis of HT29, Caco2, T84 and A549 by RCD cell line P1 after incubation with an anti-CD103-antibody in a 50:1 effector-target ratio. Similar results were obtained with P2 and P3. (I+J) Cells from RCD cell lines P1, P2 and P3 were cultured for 72 hours in the presence of 10ng/ml IL-15 or in medium alone. After incubation cells were viable and were tested for cytotoxic activity against HT29, Caco2, T84 and K562. *$P < 0.05$. (I) Cytotoxic activity against HT29 in a 50:1 effector-target ratio. Similar results were obtained with the targets Caco2 and T84 (not shown). (J) Cytotoxic activity against K562 in a 50:1 effector-target ratio. All experiments shown are representative of at least three independent experiments. Each data point is the mean ± SEM of an experiment performed in duplicate.

in determining epithelial specificity.
Previous studies indicated that IL-15 potentiates effector functions of sTCR-CD3$^-$ cell lines[5;6]. In our hands, IL-15 indeed boosted the cytotoxicity of the RCD cell lines against intestinal epithelial cells (Figure 1I). However, cells deprived of IL-15 for three days still lysed epithelial target cells, suggesting that cytotoxicity against epithelial cells is an intrinsic property of these cell lines (Figure 1I). In contrast, three-day IL-15 deprivation abrogated lysis of K562 nearly completely (Figure 1J), suggesting that lysis of K562 is more dependent on IL-15 stimulation than the lysis of epithelial cell lines.
Together, these results indicate that RCD cell lines are cytotoxic and preferentially lyse epithelial cells. Furthermore, cytotoxicity against the epithelium was enhanced by IL-15, a cytokine highly upregulated in the epithelium and lamina propria of patients with RCD II and RCD-associated lymphoma[5].

RCD cell lines express DNAM-1 and other activating NK cell receptors

To determine which receptor(s) might mediate epithelial cell-specific cytotoxicity we analyzed the expression of activating receptors by the RCD cell lines. None of the cell lines expressed CD3 (Figure 2), consistent with the phenotype of aberrant IELs but not with that of T cells. In addition, RCD cell lines P1, P2 and >98% of RCD cell line P3 were negative for NK cell marker CD56, supporting the notion that the RCD cell lines were distinct from NK cells. NKG2C and NKG2D, NK cell receptors involved in epithelial cell lysis[9;11], were expressed on RCD cell line P1 and P3, but not P2 (Figure 2). The natural cytotoxicity receptors Nkp30, Nkp44 and NKp46 were only expressed on RCD cell line P3 and none of the RCD cell lines expressed NKp80 (Supplementary Figure S2). In contrast, only DNAM-1, an activating receptor mediating tumor cell lysis by CD8$^+$ T-cells and NK cells[20], was strongly expressed on the cell surface of all three RCD cell lines (Figure 2).

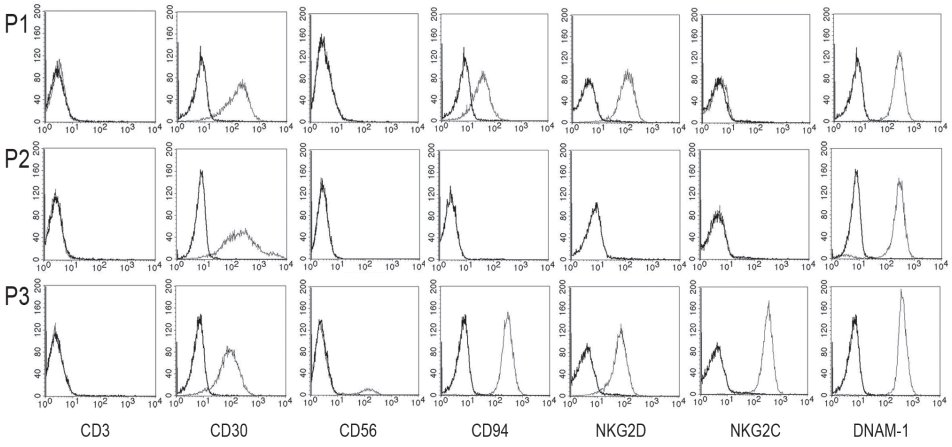

Figure 2. RCD cell lines express DNAM-1. FACS analysis of RCD cell lines P1, P2 and P3. The gray line in the histograms represents staining with the indicated antibody, the bold line the isotype-matched control. Analyses were performed within a live lymphocyte gate. The experiment shown is representative of three independent experiments.

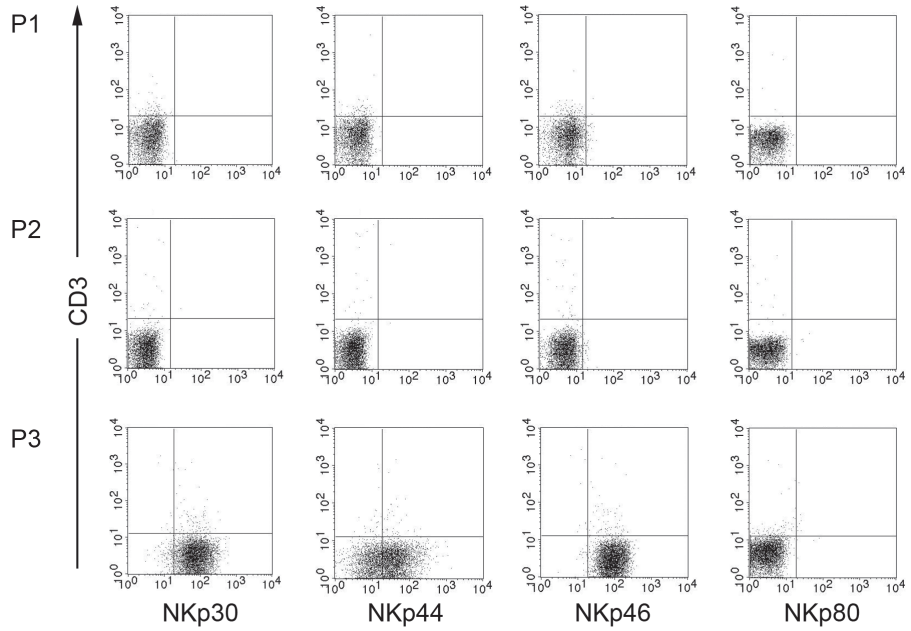

Figure S2. Expression of natural cytotoxicity receptors on RCD cell lines. FACS analysis of RCD cell lines P1, P2 and P3. Analyses were performed within a live lymphocyte gate and quadrants were based on staining with isotype-matched control antibodies (not shown). The experiment was performed twice and peripheral blood NK cells were used as positive control cells (not shown).

DNAM-1 is the main mediator of cytotoxicity of RCD cell lines against intestinal epithelial cells

The observation that all three RCD cell lines expressed DNAM-1 suggested that this receptor might be involved in the killing of intestinal epithelial cells. Indeed, a DNAM-1-specific antibody strongly inhibited lysis of intestinal epithelial cells by all three RCD cell lines (Figure 3A, 3B). In addition, while blocking of NKG2D inhibited lysis slightly in the case of the NKG2D$^+$ RCD cell lines P1 and P3, simultaneous blocking of DNAM-1 and NKG2D abrogated lysis nearly completely (Figure 3A, 3B). As expected, lysis by the NKG2D$^-$ cell line P2 could not be inhibited by an antibody to NKG2D (Figure 3B). In contrast, lysis of K562 by RCD cell line P3 was not inhibited by blocking of DNAM-1 (data not shown). In accordance with previous studies, a CD103-specific antibody was able to partially inhibit lysis of HT29 cells[5] (Figure 1G). When CD103 and DNAM-1 were blocked simultaneously, lysis was prevented completely (Fig. 3C), indicating synergy between DNAM-1 and CD103.

To test whether the DNAM-1 mediated cytotoxicity of RCD cell lines P1, P2 and P3 was independent of other receptor-ligand interactions, a redirected lysis experiment was performed. RCD cell lines P1, P2 and P3, pre-incubated with antibodies specific for DNAM-1, CD103, NKG2D or CD30 or with isotype-matched control antibodies, were co-cultured with Fc-receptor-bearing P815 cells. While neither the CD30-specific antibody nor the control antibody induced lysis of P815 cells, strong lysis was induced by the DNAM-1-specific antibody (Figure 3D). Much weaker effects were observed in the case

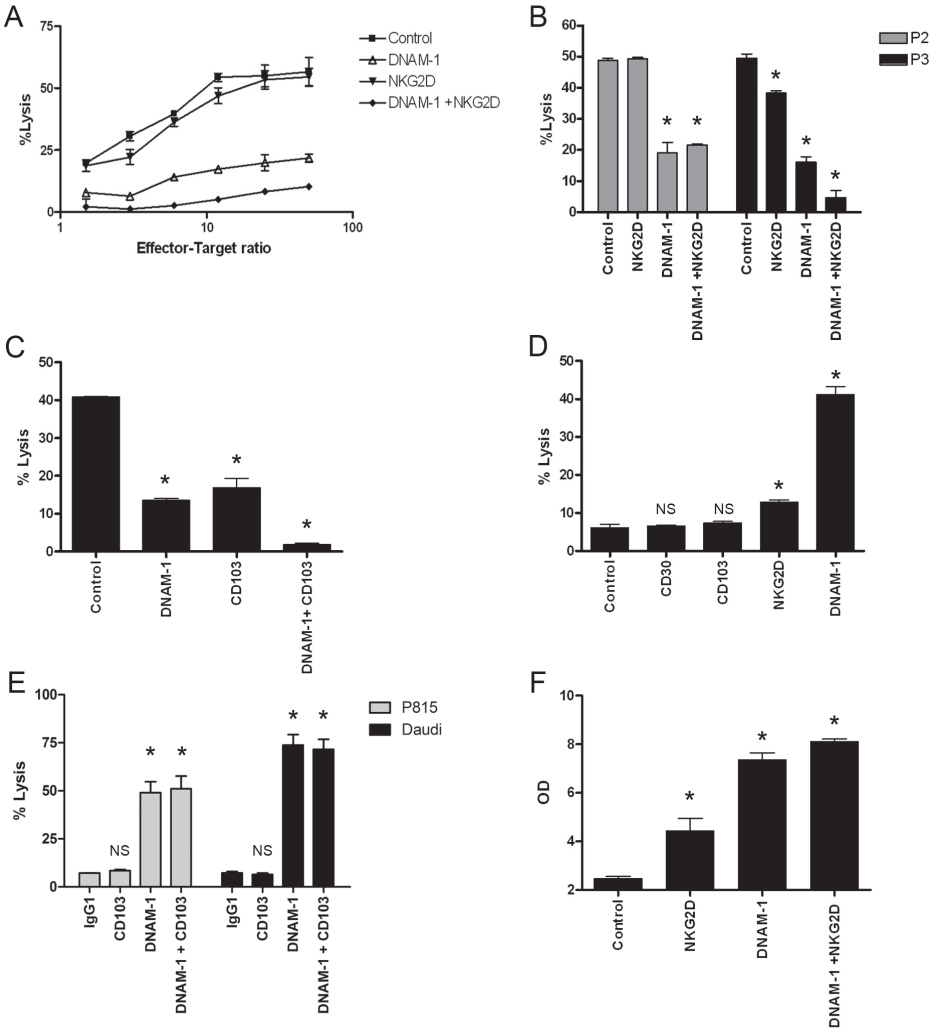

Figure 3. Lysis of epithelial cells by RCD cell lines is mediated mainly by DNAM-1. Cytotoxicity of RCD cell lines P1, P2 and P3 against HT29 in the presence of (combinations of) antibodies directed against DNAM-1, NKG2D, CD103, CD30 or the appropriate isotype control antibodies. Similar results were observed for targets Caco2 and T84. Each data point is the mean ± SEM of an experiment performed in duplicate. *P < 0.05, NS = not significant versus the respective control. (A) Lysis of HT29 by RCD cell line P1. (B) Lysis of HT29 by RCD cell line P2 and P3 in a 25:1 effector-target ratio. (C) Lysis of HT29 by RCD cell line P1 in a 50:1 effector-target ratio. Similar results were observed for RCD cell lines P2 and P3. (D) Redirected lysis of Fc-receptor+ cell line P815 by RCD cell line P3 in a 50:1 effector-target ratio. Similar results were observed for RCD cell line P1 and P2, except for the absence of NKG2D-induced lysis by NKG2D- RCD cell line P2 (E) Redirected lysis of P815 cells or Daudi cells by RCD cell line P3 in a 25:1 effector target ratio. Similar results were obtained with RCD cell line P1 and P2. (F) Esterase release for cell line P3 after incubation with plate-bound antibodies specific for NKG2D and/or DNAM-1, or an isotype-matched control antibody. Optical density (OD) values are shown. Similar results were observed for RCD cell line P1 and P2, except for the absence of NKG2D-induced esterase release by NKG2D- RCD cell line P2. All experiments shown are representative of at least three independent experiments.

of the NKG2D-specific antibody (Figure 3D), and only when using RCD lines expressing NKG2D (Figure 2). The CD103-specific antibody induced lysis of neither P815 cells nor Daudi cells (Figure 3D and 3E). Furthermore, combining the DNAM-1-specific antibody with a CD103-specific antibody showed that the integrin $\alpha_E\beta_7$ had no additional effect on the lysis of both P815 cells and Daudi cells (Figure 3E), confirming the notion that CD103 mediates adhesion to epithelial cells rather than effector cell activation. Consistent with the redirected lysis data, the DNAM-1 specific antibody was able to independently induce esterase release by the RCD cell lines (Figure 3F). Together, these results indicate that DNAM-1 is the dominant receptor triggering lysis of epithelial cells by the RCD cell lines. However, we cannot formally exclude that the DNAM-1-specific antibody was more efficient than the NKG2D-specific antibody in blocking epithelial cell lysis and in inducing redirected lysis.

Consistent with the importance of DNAM-1 in mediating lysis, the intestinal epithelial cell lines HT29, Caco2 and T84 expressed both DNAM-1 ligands CD112 and CD155[21;22] (Figure 4A). Masking of the individual DNAM-1 ligands with CD112- and CD155-specific antibodies had only a marginal effect on lysis of HT29 (Figure 4B), whereas simultaneous blocking of CD112 and CD155 resulted in partial inhibition of lysis (Figure 4B). Blocking with antibodies specific to CD112 and CD155 was never as efficient as blocking with anti-DNAM-1 (Figure 4B). These results indicate that the interaction of DNAM-1 with its ligands CD122 and CD155 indeed leads to lysis of intestinal epithelial cells. We cannot, however, exclude that an additional ligand for DNAM-1 is present on epithelial cells and is involved in the killing of intestinal epithelial cell lines.

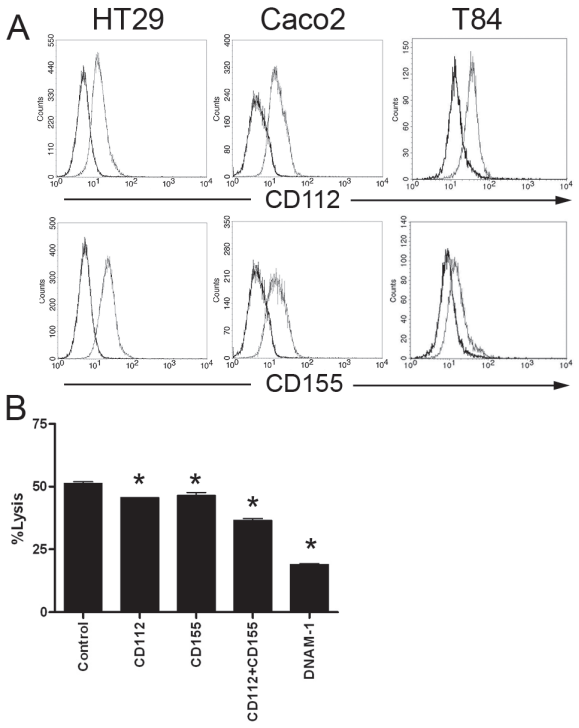

Figure 4. Blocking DNAM-1 ligands CD112 and CD155 on epithelial cell lines inhibits lysis. (A) FACS analysis of CD112 and CD155 on intestinal epithelial cell lines HT29, Caco2 and T84. The gray line in the histograms represents staining with the indicated antibody, the bold line the isotype-matched control. (B) Lysis of HT29 by RCD cell line P2 after pre-treatment with anti-DNAM-1 antibodies or after incubation in the presence of antibodies against CD112, CD155 or a combination of both (effector-target ratio 50:1). Similar results were observed for RCD cell lines P1 and P3. Each data point is the mean ± SEM of an experiment performed in duplicate. All experiments shown are representative of at least three independent experiments. *P < 0.05 versus the isotype control-matched antibody.

In conclusion, DNAM-1 contributes to the cytotoxicity of RCD cell lines against epithelial cells.

Small intestinal enterocytes express DNAM-1 ligands and can be lysed by RCD cell lines ex vivo

To explore the role of DNAM-1 *in vivo*, cells isolated from duodenal biopsies were analyzed by flowcytometry. Both CD112 and CD155 were consistently detected on enterocytes identified by the presence of epithelial specific antigen (ESA), although the expression of CD112 generally appeared to be higher than the expression of CD155 (Figure 5A, 5B). Enterocytes isolated from duodenal biopsies were also used as targets in a cytotoxicity assay. The RCD cell lines lysed enterocytes (Figure 5C), although the degree of cytotoxicity was substantially lower when compared to lysis of intestinal epithelial cell lines. In contrast, control T cell lines isolated from duodenal biopsies from the same patients displayed no cytotoxic activity against enterocytes (Figure 5C). These results indicate that RCD cell lines can destroy DNAM-1 ligand-positive enterocytes in vivo.

In vivo, DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients

To assess the expression of DNAM-1 on aberrant IEL lines from RCD II patients other than the three patients (P1, P2 and P3) studied thus far, cell lines from biopsies of 15 additional RCD II patients were generated. Three of these additional cell lines contained a substantial sTCR-CD3$^-$CD30$^+$ cell population (Figure 6A). These three sTCR-CD3$^-$CD30$^+$

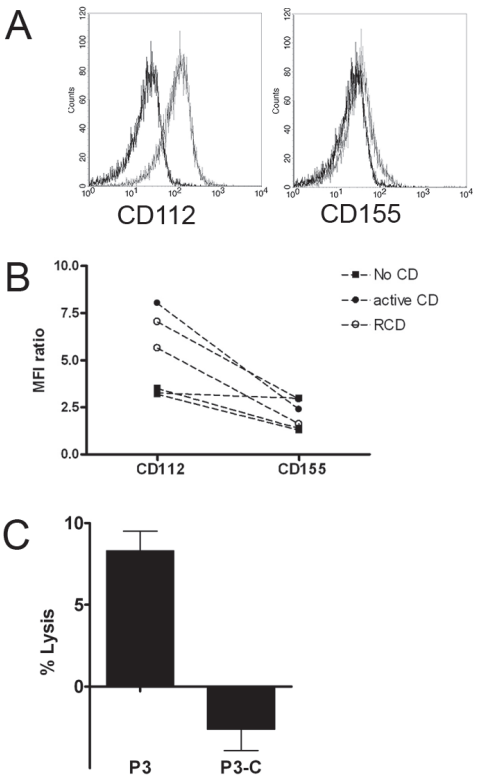

Figure 5. Enterocytes express DNAM-1 ligands and can be lysed by RCD cell lines. (A) FACS analysis of ESA$^+$ enterocytes isolated from a duodenal biopsy. The gray line represents staining with the indicated antibody, the bold line the isotype-matched control. (B) Mean Fluorescent Intensity (MFI) ratios (MFI-antibody of interest/ MFI isotype-matched control) for the expression of CD112 and CD155 on ESA$^+$ enterocytes from 6 independent donors. Closed squares represent individuals without CD, closed circles represent active CD and open circles represent RCD. (C) Cytotoxicity of RCD cell line P3 and control CD4$^+$ T cell clone P3-C, isolated from the same patient, to enterocytes isolated from duodenal biopsies in a 10:1 effector–target ratio. Similar results were obtained for RCD cell line P1 and a control CD8$^+$ T cell line isolated from the same patient. Data are representative of 5 independent experiments.

cell lines homogeneously expressed DNAM-1 (Figure 6B). The other cell lines were largely sTCR-CD3$^+$ and were, therefore, not considered aberrant IEL lines.
Previous studies indicated that DNAM-1 is present on the majority of T cells in peripheral blood[20]. In accordance with this, analysis of non-aberrant sTCR-CD3$^+$ IELs freshly isolated from biopsies of CD patients with active CD and those on a gluten-free diet indicated that these cells can be DNAM-1$^+$ (Figure 7A).

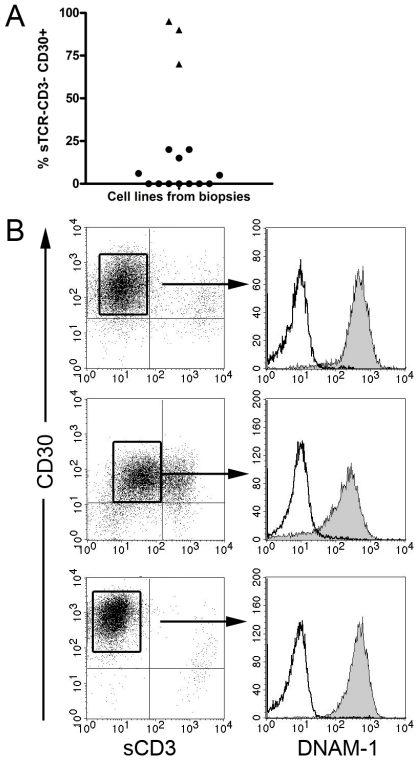

Figure 6. sTCR-CD3$^-$CD30$^+$DNAM$^+$ cell lines could be isolated from only a minority of RCD II biopsy specimens. Cell lines were isolated from small intestinal biopsies of 15 additional RCD II patients. (A) The percentage of sTCR-CD3$^-$CD30$^+$ cells within the total IEL population was determined with flowcytometry. Triangles represent the cell lines studied in figure 6B. (B) DNAM-1 expression on the three cell lines that contained a substantial sTCR-CD3$^-$CD30$^+$ population (triangles figure 6A). Analyses were performed within a live lymphocyte gate and quadrants were set based on staining with isotype-matched controls. Histograms are based on the sTCR-CD3$^-$CD30$^+$ population. The gray filled histogram represents staining with the indicated antibody, the bold line represents staining with the isotype-matched control.

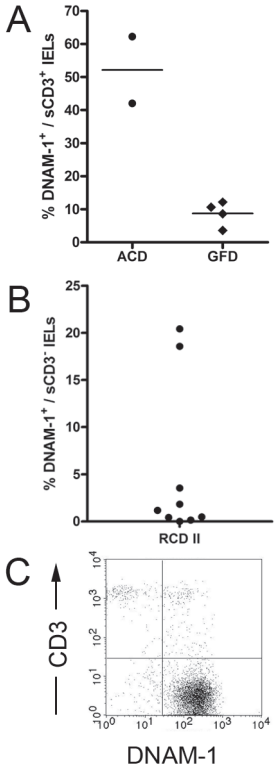

Figure 7. DNAM-1 is expressed on lymphoma cells and on aberrant IELs in a minority of RCD II patients. (A) FACS-analysis of IELs freshly isolated from biopsies of active CD patients (ACD) and CD patients on a gluten-free diet (GFD). The percentage of DNAM-1$^+$ cells within the sCD3$^+$ IEL population is depicted. (B) FACS-analysis of IELs freshly isolated from biopsies of 9 individual RCD II patients. The percentage of aberrant DNAM-1$^+$ cells within the sCD3- IEL population is depicted. (C) FACS analysis of lymphoma cells from ascitic fluid obtained from a patient suffering from invasive RCD-associated lymphoma. Analyses were performed within a live lymphocyte gate on CD45$^+$ cells and quadrants were set based on staining with isotype-matched controls.

In contrast to the aberrant IEL lines *in vitro*, on aberrant sTCR-CD3$^-$ IELs freshly isolated from duodenal biopsies, DNAM-1 was detected in only a minority of RCD II patients (Figure 7B). DNAM-1 was, however, uniformly present on sTCR-CD3$^-$ lymphoma cells directly isolated from ascitic fluid from a patient suffering from invasive RCD-associated lymphoma (Figure 7C). Taken together, DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients and part of a spectrum of receptors that aberrant IELs can deploy to lyse enterocytes.

Discussion

In active CD, epithelial damage is attributed to triggering of activating NK cell receptors on $CD8^+$ IELs[9;11]. This is presumably linked to the adaptive gluten-specific T cell response in the lamina propria as the damage is restored upon withdrawal of gluten in the diet[23]. In contrast, RCD II and the successive state of overt lymphoma are characterized by gluten-independent tissue damage and a monoclonal expansion of aberrant sTCR-$CD3^-$ IELs[24]. It has been shown that such aberrant IELs can be cytotoxic[5;11]. The availability of cell lines isolated from small intestinal biopsies from RCD II patients that exhibit the characteristic phenotype associated with aberrant IELs[13] allowed us to investigate the cytotoxic specificity of such cells and to identify the receptors involved.

The RCD cell lines used in this study closely resemble aberrant IELs as they have the characteristic surface $TCR^-CD3^-CD4^-CD8^-CD103^+$, intracellular $CD3^+$ phenotype and monoclonal TCR-γ gene rearrangements identical to aberrant IELs *in vivo*[13]. In accordance with the phenotype of aberrant IELs *in vivo*, they generally lack expression of NK cell marker CD56, with the exception of a small $CD56^+$ fraction in RCD cell line P3 (Figure 2). In contrast to the majority of aberrant sTCR-$CD3^-$ IELs *in vivo*, the RCD cell lines also express CD30 and DNAM-1, both uniformly expressed on RCD-associated lymphoma cells[25](Figure 7C). In addition, the expression of activating NK cell receptors differed between the three RCD cell lines, with RCD cell line P3 expressing a greater variety of NK cell receptors than RCD cell lines P1 and P2. Strikingly, RCD cell line P2 did not express NKG2D, even though this receptor is present on most cytotoxic IELs[11;26]. Indeed, RCD cell line P1 and P3 (Figure 2) as well as the three $CD8^+$ IEL lines and the additional cell lines described in figure 6 were $NKG2D^+$ (data not shown). As all cell lines tested in this study retained stable expression of surface markers throughout the study, it seems unlikely that RCD cell line P2 lost NKG2D expression during in vitro culture. Lack of sTCR-CD3 expression combined with expression of a subset of NK cell receptors is a feature that the RCD cell lines share with lymphoid tissue inducer cells[27] and with T cells that have undergone reprogramming into NK-like cells due to deletion of the Bcl11b gene[28]. The RCD cell lines, however, expressed neither RORC mRNA nor surface CD127 (data not shown), two characteristic markers for lymphoid tissue inducer cells[27]. Moreover, preliminary data indicate that Bcl11b is expressed by the RCD cell lines. The exact cellular origin of the RCD cell lines thus remains unclear and this will be the subject of further studies.

Our results indicate that the cytotoxicity of the RCD cell lines is directed preferentially at cells of epithelial origin, and that this epithelial cytotoxicity is mediated mainly by DNAM-1. The preference for epithelial cells is explained by the presence of CD103 ($\alpha_E\beta_7$) -an integrin widely expressed on IELs- on the RCD cell lines, as this integrin will interact with its ligand E-cadherin that is selectively expressed on epithelial cells. In contrast to previous studies in which RCD cell lines showed strong cytotoxicity against NK cell target K562[5], in our study only RCD cell line P3 displayed low-level cytotoxicity against K562. This indicates that RCD cell lines differ in their ability to lyse various target cells, which might reflect the differences in NK cell receptor repertoire expressed by these cell lines. While previous studies identified NKG2C and NKG2D as mediators of cytotoxicity directed against intestinal epithelial cells[9;11;12], we now show that DNAM-1 is a third receptor enabling epithelial lysis. It is striking that DNAM-1 can function autonomously on the RCD cell lines while it is known to act as a co-receptor on normal T and NK cells. However, the independent activity of DNAM-1 on the RCD cell lines is analogous to that

of NKG2D on activated IELs in active CD, as NKG2D can act TCR-independently upon exposure to IL-15[12]. All in all, this indicates that there is considerable diversity in RCD cell lines, which may be a reflection of the in vivo diversity of aberrant IELs.

Recently, the inhibitory receptor TIGIT was identified that can be co-expressed with DNAM-1 on T cells and NK cells and competes for binding to the ligands CD155 and CD112[29;30]. TIGIT was shown to have a 100-fold higher affinity for CD155 than DNAM-1[29], suggesting that, when co-expressed, TIGIT has the dominant function and can regulate the function of DNAM-1. Our study, however, indicates a dominant function for DNAM-1 on aberrant IELs in RCD II. This suggests that TIGIT, if present, does not function as a dominant negative regulator on these cells.

While it is well known that DNAM-1 can mediate lysis of tumor cells by $CD8^+$ T cells and NK cells[20;31], this is the first report describing DNAM-1-mediated cytotoxicity by (pre) malignant cells themselves. Various tumor cell types express DNAM-1 ligands CD112 and CD155[15;18;32] and can be lysed by DNAM-1 expressing $CD8^+$ T cells and NK cells. In marked contrast, the cell lines derived from RCD II patients do not express DNAM-1 ligands but instead express the DNAM-1 receptor which triggers specific lysis of intestinal epithelial cells (Supplementary Figure S3). Although lysis of freshly isolated enterocytes by the RCD cell lines was significantly lower compared to lysis of epithelial cell lines, this low degree

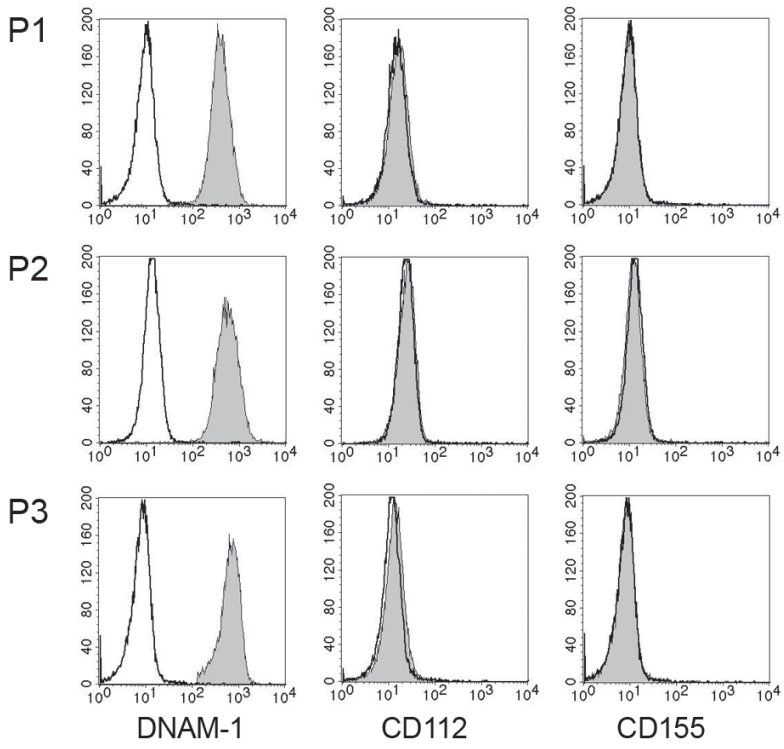

Figure S3. RCD cell lines do not express DNAM-1 ligands. RCD cell lines P1, P2 and P3 were stained with antibodies specific for DNAM-1, CD112 and CD155. The gray filled histogram represents staining with the indicated antibody, the bold line represents staining with the isotype-matched control. Analyses were performed within a live lymphocyte gate.

of lysis could lead to significant damage in vivo as this would be a continuous process in the patient. This might contribute to the gluten-independent intestinal damage in RCD II patients in which such cells are present and in patients suffering from RCD-associated lymphoma. As aberrant IELs in RCD II can disseminate to the entire gastrointestinal tract and RCD-associated lymphoma to extra-intestinal locations like skin, lung and liver[8;33], DNAM-1 mediated cytotoxicity of (pre)malignant cells against epithelium might contribute to tissue damage at various sites in the body.

In vivo, DNAM-1 was expressed on aberrant IELs in 2 out of 9 RCD II patients, and on lymphoma cells isolated from ascitic fluid from a patient suffering from invasive RCD-associated lymphoma. This indicates that DNAM-1 is expressed on aberrant IELs in a subset of RCD II patients. We speculate that these $sCD3^-DNAM\text{-}1^+$ IELs are the precursors of the $sCD3^-DNAM\text{-}1^+$ cell lines that grow out in vitro. However, we cannot exclude that culture conditions have induced DNAM-1 expression of the RCD cell lines.

In conclusion, this study provides the first evidence that in a subset of RCD II patients, aberrant IELs can acquire the ability to lyse epithelial cells via DNAM-1. Together with other activating NK cell receptors, DNAM-1 on aberrant IELs can contribute to the gluten-independent tissue damage in RCD II and RCD-associated lymphoma. This mechanism through which (pre)malignant cells can cause tissue damage might also apply to other tumors of lymphoid origin.

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