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*J Immunol* 2011; 186:6304-6312; Prepublished online 27 April 2011;
doi: 10.4049/jimmunol.1003382
http://www.jimmunol.org/content/186/11/6304

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Supplementary Material

http://www.jimmunol.org/content/suppl/2011/04/27/jimmunol.1003382.DC1

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

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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 TCR-CD3⁺ intraepithelial lymphocytes (IELs) that can progressively replace normal IELs and 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 demonstrate in this study 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, whereas 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. Although DNAM-1 on CD8⁺ T cells and NK cells is known to mediate lysis of tumor cells, this study provides, to our knowledge, 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. The Journal of Immunology, 2011, 186: 6304–6312.

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 type I and type II (RCD II), distinguished by the respective absence or presence of an aberrant IEL population lacking surface expression of the TCR–CD3 complex (surface TCR-CD3 [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 IL-15 that 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-y 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 costimulate 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⁻ 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 Ab 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 cytotoxicity of aberrant IELs.

In the current 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 (Mediglobe, Tempe, AZ) were taken from the second part of the duodenum (14). Biopsy specimens were used for culture, direct flow cytometric 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 DTT (Fluka, Buchs, Switzerland) and 0.75 mM EDTA (Merck, Darmstadt, Germany), biopsies were cultured in IMDM (Lonza, Verviers, Belgium) with 10% normal human serum (HNS) containing 10 ng/ml IL-15 (R&D Systems Europe, Abingdon, U.K.). The predominant population of CD3+CD4-CD8+ 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% HNS containing 10 ng/ml IL-15 and restimulated approximately every 4 to 5 wk with 1 μg/ml PHA, 10 ng/ml IL-15, and 1 × 10^6/ml irradiated allogeneic PBMCs 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% HNS containing 10 ng/ml IL-15 and restimulated every 2 wk. Fifteen 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 wk and maintained on IMDM with 10% HNS containing 10 ng/ml IL-15 and 20 Cetus units/ml IL-2 (Proliekin; Chiron, Emeryville, CA). Cell lines HT29, Caco2, T84, A549, Daudi, K562, P815, and EBV-BC107 were cultured in IMDM with 10% FCS. EBV-BC107 is an EBV B-lymphoblastoid cell line generated from a healthy blood bank donor (15).

Abs

Fluorochrome-conjugated anti-CD3, anti-CD226 (DNAM-1), anti-CD112, anti-CD30 anti-CD49d, and anti-perforin were purchased from BD Biosciences (San Jose, CA). Anti-CD155, anti-NKG2D, and 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 Ag (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 Abs from R&D Systems Europe, anti-CD103 and anti-CD155 from Beckman Coulter, and anti-CD112 from eBioscience (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, Eschwege, Germany) and 200 μg/ml DNAse II (Roche Diagnostics, Almere, The Netherlands). After rotating 1 h 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 min with fluorochrome-conjugated Abs. For cytotoxicity assays, three duodenal biopsies were treated with 1 mM DTT (Fluka) and 0.75 mM EDTA (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 [3H]Cr for 1 h at 37°C. After extensive washing, labeled target cells were cocultured with effector cells at E/T ratios between 50:1 and 1:5:1 for 4 h at 37°C. For blocking and redirected lysis experiments, effector cells were pretreated for 20 min at room temperature with 20 μg/ml indicated Abs. Masking ligands on target cells was achieved by the presence of 5 μg/ml mAbs in the effector-target mixture during the 4-h incubation period. Spontaneous chromium release and maximum chromium release by target cells was determined by addition of medium or 1% Triton X-100 (Pierce, Rockford, IL), respectively. The percentage of specific cytotoxicity was as follows: (%cpm experimental release – %cpm spontaneous release)/%cpm spontaneous release) × 100%.

N-benzyloxy carbonyl lysine thiobenzyl esterase assay

Ninety-six–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. A total of 1 × 10^5 cells from cell lines P1, P2, or P3 were added. After 4 h incubation at 37°C, supernatants were evaluated for esterase secretion using an N-benzyloxy carbonyl lysine thiobenzyl ester (Merek). OD values were obtained at an absorbance of 412 nm on a microplate reader (Bio-Rad, Veenendaal, The Netherlands).

Immunoelectron 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 granzyne B–specific mAb GB11 (Sanquin, Amsterdam, The Netherlands), followed by rabbit–anti-mouse IgG (DakoCytomation, Heverlee, Belgium) and 15 nm protein A–gold particles. Sections were evaluated with a Philips 410 electron microscope (Philips).

Statistical analysis

For cytotoxicity assays and N-benzyloxy carbonyl lysine thiobenzyl 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 Abs used in this study with their respective controls. A p value <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 overexpressed in the intestine of patients with RCD and lymphoma (5). Furthermore, they express granzyne B (Supplemental Fig. 1) 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 TCRγ 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 nonintestinal 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 nonepithelial cell lines Daudi and EBV-BC107 (Fig. 1A–D). RCD cell line P3 was the only cell line that induced lysis of NK cell target K562 (Fig. 1A–D), although lysis of K562 was much lower when compared with lysis of the epithelial cell lines (Fig. 1D). The nonintestinal epithelial cell line A549 was lysed as well (Fig. 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 (Fig. 1F), but efficiently lysed nonepithelial cell line K562 (Fig. 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 (αEβ7) (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 Ab (Fig. 1G, 1H), consistent with a role for the integrin αEβ7 in determining epithelial specificity.
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. Lysis of HT29, Caco2, T84, K562, and EBV-BC107 by: RCD cell line P1 (B), RCD cell line P2 (C), and RCD cell line P3 (D). 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-1^+ IEL lines in a 50:1 E:T ratio. G, Lysis of HT29 by RCD cell line P1 after incubation with an anti-CD103 Ab or with an isotype-matched control Ab. 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 Ab in a 50:1 E:T ratio. Similar results were obtained with P2 and P3. I and J, Cells from RCD cell lines P1, P2, and P3 were cultured for 72 h in the presence of 10 ng/ml IL-15 or in medium alone. After incubation, cells were viable and tested for cytotoxic activity against HT29, Caco2, T84, and K562. I, Cytotoxic activity against HT29 in a 50:1 E:T ratio. Similar results were obtained with the targets Caco2 and T84 (not shown). J, Cytotoxic activity against K562 in a 50:1 E:T 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. *p < 0.05.
Previous studies indicated that IL-15 potentiates effector functions of TCR-CD3+ cell lines (5, 6). In our hands, IL-15 indeed boosted the cytotoxicity of the RCD cell lines against intestinal epithelial cells (Fig. 1B). However, cells deprived of IL-15 for 3 d still lysed epithelial target cells, suggesting that cytotoxicity against epithelial cells is an intrinsic property of these cell lines (Fig. 1I). In contrast, 3-d IL-15 deprivation abrogated lysis of K562 nearly completely (Fig. 1I), 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 (Fig. 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 (Fig. 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 (Supplemental Fig. 2). 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 (Fig. 2).

**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 Ab strongly inhibited lysis of intestinal epithelial cells by all three RCD cell lines (Fig. 3A, 3B). In addition, although 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 (Fig. 3A, 3B). As expected, lysis by the NKG2D+ cell line P2 could not be inhibited by an Ab to NKG2D (Fig. 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 Ab was able to partially inhibit lysis of HT29 cells (5) (Fig. 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, preincubated with Abs specific for DNAM-1, CD103, NKG2D, or CD30 or with isotype-matched control Abs, were cocultured with Fc receptor-bearing P815 cells. Although neither the CD30-specific Ab nor the control Ab induced lysis of P815 cells, strong lysis was induced by the DNAM-1–specific Ab (Fig. 3D). Much weaker effects were observed in the case of the NKG2D-specific Ab (Fig. 3D) and only when using RCD lines expressing NKG2D (Fig. 2). The CD103-specific Ab induced lysis of neither P815 cells nor Daudi cells (Fig. 3D, 3E). Furthermore, combining the DNAM-1–specific Ab with a CD103-specific Ab showed that the integrin α3β1, had no additional effect on the lysis of both P815 cells and Daudi cells (Fig. 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 Ab was able to independently induce esterase release by the RCD cell lines (Fig. 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 Ab was more efficient than the NKG2D-specific Ab 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) (Fig. 4A).
Masking of the individual DNAM-1 ligands with CD112- and CD155-specific Abs had only a marginal effect on lysis of HT29 (Fig. 4B), whereas simultaneous blocking of CD112 and CD155 resulted in partial inhibition of lysis (Fig. 4B). Blocking with Abs specific to CD112 and CD155 was never as efficient as blocking with anti–DNAM-1 (Fig. 4B). These results indicate that the interaction of DNAM-1 with its ligands CD112 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 involved in the killing of intestinal epithelial cell lines.

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 flow cytometry. Both CD112 and

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) Abs directed against DNAM-1, NKG2D, CD103, CD30, or the appropriate isotype control Abs. Similar results were observed for targets Caco2 and T84. Each data point is the mean ± SEM of an experiment performed in duplicate. A, Lysis of HT29 by RCD cell line P1. B, Lysis of HT29 by RCD cell line P2 and P3 in a 25:1 E:T ratio. C, Lysis of HT29 by RCD cell line P1 in a 50:1 E:T 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 E:T 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 E:T ratio. Similar results were obtained with RCD cell line P1 and P2. F, Esterase release for cell line P3 after incubation with plate-bound Abs specific for NKG2D and/or DNAM-1 or an isotype-matched control Ab. 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. *p < 0.05 versus the respective isotype-matched control Ab.
CD155 were consistently detected on enterocytes identified by the presence of ESA, although the expression of CD112 generally appeared to be higher than the expression of CD155 (Fig. 5A, 5B).

Enterocytes isolated from duodenal biopsies were also used as targets in a cytotoxicity assay. The RCD cell lines lysed enterocytes (Fig. 5C), although the degree of cytotoxicity was substantially lower when compared with 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 (Fig. 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 (Fig. 6A). These three sTCR-CD3−CD30+ cell lines homogeneously expressed DNAM-1 (Fig. 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 nonaberrant 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+ (Fig. 7A).

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 (Fig. 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 (Fig. 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 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+, intracelular 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 (Fig. 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) (Fig. 2). 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 lines P1 and P3 (Fig. 2) as well as the three CD8+ IEL lines and the additional cell lines described in Fig. 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.

FIGURE 6. sTCR-CD3-CD30+DNAM-1+ 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 flow cytometry. Triangles represent the cell lines studied in B. B. DNAM-1 expression on the three cell lines that contained a substantial sTCR-CD3-CD30+ population (triangles, A). 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 Ab and the bold line represents staining with the isotype-matched control.

FIGURE 7. DNAM-1 is expressed on lymphoma cells and 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 nine 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.
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 (ε6β7), 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. Although 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 despite being known to act as a coreceptor 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 coexpressed 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 coexpressed, 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. Although 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, to our knowledge, 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 that triggers specific lysis of intestinal epithelial cells (Supplemental Fig. 3). Although lysis of freshly isolated enterocytes by the RCD cell lines was significantly lower compared with lysis of epithelial cell lines, this low degree 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 extraintestinal 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 two out of nine 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-1+ IELs are the precursors of the sCD3+DNAM-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, to our knowledge, 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.

Acknowledgments

We thank the Sanquin Blood Supply Foundation (Amsterdam, The Netherlands) for the Granzyme B Ab (GB11) and Jos Onderwater, Allan Thompson, Barbara Piccinini, and Frederike Schmitz for experimental support.

Disclosures

The authors have no financial conflicts of interest.

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