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CHAPTER 2:

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ACCEPTED MANUSCRIPT

CHAPTER 2
DENDRITIC CELLS PROMOTE EXPANSION AND SURVIVAL OF ABERRANT TCR-NEGATIVE INTRAEPITHELIAL LYMPHOCYTE LINES FROM REFRACTORY CELIAC DISEASE TYPE II PATIENTS

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ABSTRACT

Celiac disease (CD) patients who fail to respond to a gluten-free diet suffer from refractory celiac disease (RCD). A marked expansion of intraepithelial lymphocytes (IEL) lacking surface TCR/CD3 expression characterizes the RCD subtype II. In up to 50% of RCDII patients these so-called aberrant IEL (a-IEL) develop into lymphoma and can disseminate into other tissues. Elevated levels of Interleukin-15 (IL-15) in the intestine of CD and RCD patients likely contribute to the expansion of a-IEL. Here, we investigated if interactions with other cells might also influence a-IEL expansion. Similar to IL-15, cells from the monocyte lineage, particularly mature dendritic cells (DCs), promoted proliferation, prevented apoptosis and induced IFNγ secretion of a-IEL derived from RCDII biopsies (RCDII cell lines), which in turn induced CXCL10. In contrast to IL-15, mature DCs did not induce proliferation of regular TCR+/CD3+ IEL lines, generated from CD biopsies and IL-15-blocking antibodies did not inhibit DC-induced proliferation of RCDII cell lines. Furthermore, proliferation was dependent on cell-cell contact, but independent of the HLA-genotype of the stimulating cells. Our results suggest that contact with DC, either in the epithelium or upon dissemination, contributes to uncontrolled expansion of a-IEL in RCDII, independent of HLA-genotype and IL-15.

ABBREVIATIONS

CD- Coeliac Disease, RCD- Refractory Coeliac Disease, EATL- Enteropathy associated T cell Lymphoma, IEL- Intraepithelial Lymphocytes, T-IEL- TCR+/CD3+ Intraepithelial Lymphocytes, a-IEL- aberrant IEL
INTRODUCTION

Celiac disease (CD) is an enteropathy of the small intestine, caused by a strong immune response to dietary gluten that occurs in HLA-DQ2\(^+\) and/or HLA-DQ8\(^+\) individuals. Histological findings include villous atrophy, crypt hyperplasia, increased numbers of TCR-\(\alpha\beta\)\(^+\) and TCR-\(\gamma\delta\)\(^+\) intraepithelial lymphocytes (T-IEL)\(^2,3\) and elevated interleukin-15 (IL-15) levels in the epithelium and lamina propria\(^4\). Clinical symptoms include anemia, diarrhea and failure to thrive. CD is treated with a gluten-free diet, which usually leads to remission and amelioration of histological and serological findings. However, 2-5% of adult-onset CD patients develop refractory CD (RCD) with persisting epithelial damage and increased numbers of IEL\(^5,6\), despite adherence to a gluten-free diet.

RCD can be further subdivided into RCD type I (RCDI) and RCD type II (RCDII), based on the quantity and phenotype of IEL in duodenal biopsies. RCDII patients have >20% surface T-cell receptor-negative—also called aberrant-IEL (a-IEL) of the total IEL population\(^7\). In approximately 50% of RCDII patients these a-IEL gradually replace the normal IEL population and undergo malignant transformation\(^8\). These patients develop a severe life threatening condition, called enteropathy-associated T cell lymphoma (EATL) with a 2 year survival of less than 30\%\(^5,9\). a-IEL in RCDII contain clonal TCR rearrangements\(^6,8,10\) and display a unique phenotype: they express no lineage markers (CD3, CD14, CD19, CD56) (Lin) nor CD34 or CD127, but are positive for the T/NK cell marker CD7 and express intracellular CD3 (icCD3). a-IEL also express multiple NK cell markers and therefore share features with T and NK cells\(^7,8,10,11\).

While in non-celiac adults less than 10\% of IEL possess this ‘aberrant’ phenotype\(^11\), in RCDII patients a-IEL can make up more than 80\% of the IEL population. The combination of elevated IL-15 levels in the duodenal epithelium of RCD patients and greater IL-15 sensitivity of a-IEL compared to other IEL is likely to contribute to the selective expansion and survival of aberrant cells in RCDII\(^4,12,13\). The fact that cells with this ‘aberrant’ phenotype from non-celiac duodenal biopsies express the IL-2/15R\(\beta\) and become positive for the proliferation marker Ki67 upon incubation with IL-15\(^12\) suggests that the precursors of a-IEL in RCDII patients are also IL-15 sensitive.

Aberrant IEL in situ are located in the intraepithelial compartment and are therefore likely to closely interact with epithelial cells. In addition, contact with dendritic cells (DCs) could be made via DC protrusions extending into the epithelium\(^14-16\) and contact to DCs and other types of blood-borne cells could take place upon dissemination of a-IEL. We therefore investigated proliferation, apoptosis and cytokine production of a-IEL derived from RCDII biopsies (RCDII cell lines) after co-
culture with intestinal epithelial cell lines and several blood-borne cell types, including DCs.

MATERIALS AND METHODS

Small intestinal biopsy specimen

During upper endoscopy, large spike forceps biopsy specimens (Medi-Globe, Tempe, AZ) were taken from the second part of the duodenum. 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

RCDII lines P1 and P2 were isolated from duodenal biopsies of RCDII patients and maintained as described 13. TCR+IEL (T-IEL) lines were isolated from 4 CD patients, as described for RCDII lines. PBMC were isolated from buffy coats using standard ficoll gradient (PBMC layer). After ficoll separation the PBMC layer was removed, remaining cells were separately collected from the layer above the erythrocytes and termed neutrophils if CD16 was expressed or eosinophils if CD16 expression was not detected. Monocytes were isolated from fresh PBMC using CD14 beads (Miltenyi, Bergisch Gladbach, Germany) using LS MACS columns and the standard separation protocol (Miltenyi). Dendritic cells (DC) were generated by culture of these monocytes in RPMI/10% FCS medium supplemented with 1000 U/ml GM-CSF (R&D Systems, Abingdon, UK), 500 U/ml IL-4 (R&D Systems, Abingdon, UK) and macrophages were generated by culture in RPMI/10% FCS medium supplemented with 50 U/ml GM-CSF. On day 7, DC and/or macrophages were matured by incubation of 100 ng/ml LPS (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 10% FCS/IMDM medium for 48h. HT-29 cells were cultured in IMDM/10% FCS medium.

Cytotoxicity assay

One million target cells were labeled with 100 γCi [51Cr] for 1 h at 37°C. After extensive washing, labeled target cells were incubated with the RCDII lines as effector cells at an effector: target (E:T) ratio between 50:1 and 1.5:1 for 4 h at 37°C. 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 maximum release- cpm spontaneous release]) x 100%
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Proliferation assays

Responder cells were rested by culturing them in the absence of IL-15 for 4 days. 20,000 responder cells per well were subsequently cultured in triplicate in 96-well plates in the presence or absence of 10 ng/ml IL-15 or with irradiated (3000 Rad) stimulator cells for 3 days at 37°C, after which 0.5 μCi \( ^3 \)H-thymidine was added to every well. Stimulator cells were 1x10⁵/well PBMC, 2x10⁴/well DC/macrophages/monocytes, 2x10⁴/well HT29 and responder cells were 2x10⁴/well RCDII lines or T-IEL lines, unless stated otherwise. For the transwell experiments the inserts were discarded after 3 days co-culture and 0.5 μCi \( ^3 \)H-thymidine was added directly to the 96 well plate (HTS Transwell-96 system, 0.4μm pore size, Corning, MA, USA). \(^3\)H-thymidine incorporation was determined during the final 17h of the incubation.

Flowcytometry and antibodies

After co-culture, cells were harvested, washed in 0.5% FCS/PBS and stained with 7-AAD and Annexin PE using the apoptosis detection kit according to the BD Biosciences protocol (BD Biosciences, San Jose, CA). In short, 7-AAD and Annexin PE incubation was done for 15 min at room temperature (RT) in the supplied binding buffer and the labeled cells were acquired within 1h on a LSR II or FACS-Calibur (both from BD Biosciences). PBMC subsets were stained with CD14-PeCy7, CD19-PE, CD56-FITC, CD16-FITC (all BD Biosciences, San Jose, CA) and CD3-AlexaF750 (Invitrogen, NY, USA) and sorted with FACSariaIII (BD Biosciences). For proliferation assays 10 μg/ml IL-15 antibodies MAB 647 and 2 μg/ml MAB247 (both R&D systems Europe, Abingdon, UK) were used.

Cytokine production

IFNg and CXCL10 concentrations were measured using the Bio–Plex assay (BioRad, Hercules, CA, USA) according to the manufacturer’s protocol (www.biorad.com).

Data analysis

Flow cytometry results were analyzed with FACS DIVA 6.1.2 software. Data from cytotoxicity, proliferation and cytokine measurements were analyzed and computed in 4-GraphPad.
RESULTS

MONOCYTES INDUCE PROLIFERATION BUT NOT CYTOTOXICITY OF RCDII CELL LINES

In vivo, a-IEL are in direct contact with epithelial cells and likely also with blood-borne cells, either locally or upon dissemination. In vitro, a-IEL derived from RCDII biopsies (RCDII cell lines) efficiently lyse epithelial cell lines via the NK cell receptor DNAM-I\(^ {17} \), but it was not known whether the interaction between RCDII cell lines and other cell types could contribute to proliferation. To test this, we co-cultured RCDII lines with PBMC and the epithelial cell line HT-29. We observed that RCDII cell lines proliferated upon co-culture with PBMC, but not when co-cultured with the intestinal epithelial cell line HT-29 (Figure 1A). To investigate which cell type among PBMC induced RCDII line proliferation, we purified PBMC subpopulations by flow cytometry and co-cultured these with the RCDII lines. Monocytes (CD3\(^{-}\)CD14\(^{+}\)) induced proliferation of RCDII lines, but B cells (CD3\(^{-}\)CD19\(^{+}\)), NK cells (CD3\(^{-}\)CD56\(^{+}\)), T cells (CD3\(^{+}\)), neutrophils (CD3\(^{-}\)CD56\(^{-}\)CD16\(^{+}\)) and eosinophils (CD3\(^{-}\)CD56\(^{-}\)CD16\(^{-}\)) did not (Figure 1B). In contrast, RCDII lines lysed HT-29 but not monocytes (Figure 1C). Thus, monocytes induced proliferation but not cytotoxicity of RCDII lines, while the opposite was true for intestinal epithelial cells.

MATURE MONOCYTE-DERIVED DENDRITIC CELLS ARE SUPERIOR TO MONOCYTES IN INDUCING PROLIFERATION OF RCDII CELL LINES

Monocytes are recruited from peripheral blood to tissues, where they differentiate into macrophages and dendritic cells (DC). Macrophages and DC are found in the intestinal lamina propria and DC protrusions can be found in the epithelium\(^ {14-16} \). We therefore investigated the induction of proliferation by macrophages and DC differentiated in vitro from purified peripheral blood monocytes. While macrophages, monocytes, immature DC and LPS-matured DC induced proliferation of RCDII lines, the strongest responses were found with mature DC (Figure 2A). In contrast, T-IEL lines derived from CD patients did not proliferate when co-cultured with DC (Figure 2B). The interaction between RCDII lines and DC did not alter the expression of the maturation markers CD1a, HLA-DR, CD86, CD80, CD83 and DC-SIGN on DC (data not shown). Thus, RCDII lines proliferate upon interaction with cells of the monocyte lineage, particularly mature DC.
FIGURE 1. MONOCYTE-DERIVED CELLS INDUCED PROLIFERATION, BUT NOT CYTOTOXICITY OF RCDII LINES.

(A) Proliferation was measured after 3 days of co-culture with PBMC or HT-29 and RCDII cell lines. On the y-axis the 3H-incorporation (CPM) is depicted. (B) Proliferation (cpm) was measured after 3 days of co-culture with RCDII lines and monocytes, T cells, B cells, NK cells, eosinophils or neutrophils. Black bars indicate the proliferation when co-cultured with the RCDII line, white bars represent the background proliferation of the corresponding PBMC population. (C) Monocytes were sorted from fresh healthy PBMC by CD14+ MACS sort and used in a cytotoxicity assay. An effector:target ratio of 50:1 is depicted. On the y-axis the percentage of lysis is shown. Results are depicted for RCDII line P2. Similar results were obtained with RCDII line P1 for all tests. Graphs are representative of at least two independent experiments.
FIGURE 2. DENDRITIC CELLS INDUCED STRONGEST PROLIFERATION OF RCDII LINES WHEREAS T-IEL LINES SHOWED NO RESPONSE. (A) MACS sorted CD14+ monocytes (mono), monocyte-derived in vitro cultured macrophages (macro), immature dendritic cells (iDC) or 48h LPS-matured DC (mDC) were co-cultured with RCDII cell line P2 and proliferation (cpm) was measured after 3 days. Control 1 represents the mean background proliferation of monocyte-derived cells, control 2 the background proliferation of the RCDII line with an LPS-treated EBV line. Results were representative of 3 experiments. On the y-axis the 3H-incorporation (CPM) is depicted. (B) Proliferation (cpm) was measured after 3 days of co-culture with DC and a RCDII line P2 (white bars), or with DC and a representative T-IEL line (black bars). A plus or minus indicates whether DC or IL-15 as a control was added. The result is representative of 2 experiments. The T-IEL line used in this experiment is representative for four T-IEL lines tested, RCDII lines P1 and P2 showed similar results for all experiments depicted here.
PROLIFERATION OF RCDII LINES WAS INDEPENDENT OF IL-15 OR HLA GENOTYPE, BUT DEPENDENT ON CELL-CELL CONTACT

Since monocytes, macrophages and DC are known to produce Interleukin-15 (IL-15) \(^8\), we tested whether the proliferative effect seen in co-culture experiments was due to the IL-15 production and/or presentation by cells of the monocyte lineage. Blocking of IL-15 with a combination of IL-15 antibodies showed no effect on proliferation in monocyte RCDII line co-cultures, while they effectively blocked the response to soluble IL-15 (Figure 3A). These results indicate that it is unlikely that IL-15 is the factor responsible for the monocyte- and DC-induced proliferation of RCDII lines.

Activation of T and NK cells is modulated by receptors binding to Human Leucocyte Antigens (HLA). Since many of these receptors bind only to a selected set of HLA allele products, we investigated whether the proliferation of RCDII lines was affected by the HLA genotype of the stimulating cells. To address this, we used a panel of PBMC with divergent HLA-genotypes as a convenient source of monocytes. PBMC from 10 donors selected for divergence in HLA allotypes induced similar proliferation of RCDII lines (Figure 3B). What’s more, RCDII line P1 proliferated in response to autologous PBMC (Figure 3C). As observed for DC (Figure 2B), a control T-IEL line showed no response to PBMC. The HLA genotype of the stimulator cells therefore did not detectably influence the proliferative response of RCDII lines.

To address whether cell-cell contact was required for the induction of proliferation by DC, a transwell experiment was performed. In the absence of cell-cell contact, the proliferation of RCDII lines was severely reduced but did not disappear completely (Figure 3D). Therefore, cell-cell contact played a major role in the induction of proliferation, but soluble factors also contributed.
FIGURE 3. PROLIFERATION OF RCDII LINES WAS INDEPENDENT OF IL-15 OR HLA GENOTYPE, BUT DEPENDENT ON CELL-CELL CONTACT. Proliferation was measured by $^3$H incorporation (CPM) after 3 days of co-culture. (A) Co-culture of RCDII line P2 with monocytes (CD14), 10 ng/ml IL-15, IL-15 antibodies MAB647 (10 μg/ml) and MAB247 (2 μg/ml) (αIL-15) or 12 μg/ml of the isotype control (IgG1). (n=2) (B) Co-culture of RCDII line P2 and PBMC of divergent HLA-typed donors. The HLA-type is depicted for HLA-A, B and C on the x-axis to reflect the random HLA-type of the donors. Control 1 is the background of the representative RCDII line, control 2 stands for the representative background proliferation of PBMC used in the experiment. (C) Co-culture of RCDII line from patient 1 (P1) (black bar) and patient 2 (P2) (gray bar) or of a representative T-IEL (white bar) with PBMC from patient 1 (PBL(P1)). The legend on the x-axis indicates if autologous PBMC from patient 1 (PBL(P1)), or the corresponding cell lines P1, P2 or T-IEL (cells) were added to the co-culture experiment. IL-15, taken along as a positive control is not depicted here (162000 cpm for P1, 63000 cpm for P2 and 80000 cpm for T-IEL). (D) A transwell experiment was combined with a proliferation (cpm) assay. Cell-cell contact is depicted by the open triangle symbol, no cell-cell contact by the black square symbol. The round symbol represents the mean background.
proliferation of the RCDII line P2 and the DC. On the x-axis the amount of DC added to each well is depicted as x10³ cells. Results are representative of two independent experiments. RCDII line P1 and P2 showed similar results in all tests.

**DENDRITIC CELLS INHIBIT APOPTOSIS OF RCDII LINES**

IL-15 is known to induce proliferation and inhibit apoptosis of RCDII lines and other lymphoid cell types such as T-IEL ⁴ ¹². Since DC induced proliferation of RCDII lines, we also investigated whether apoptosis was inhibited. After 6 days of incubation in the absence of DC, the majority of RCDII lines underwent early (AnnexinV+/7-AAD-) and late (AnnexinV+/7-AAD+) stage apoptosis (Figure 4A). In the presence of DC the percentage of apoptotic cells was drastically reduced (Figure 4B). In line with previous observations ¹², IL-15 also drastically reduced apoptosis of RCDII lines. These data demonstrated that, similar to IL-15, DC prevented apoptosis of RCDII lines.
FIGURE 4. ANTI-APOPTOTIC EFFECTS OF DC ON RCDII LINES. Apoptosis induction was measured by 7-AAD and Annexin V staining by flow cytometry. (A) After 6 days incubation of RCDII line P2 with medium alone (Medium), after DC co-culture (+DC) or after 10 ng/ml IL-15 (+IL-15) cells were analyzed by flow cytometry. The percentages of Annexin V+ and 7-AAD+ cells are depicted in the graph. (B) Summarizing the data from co-culture experiments of 4, 6 and 7 days for RCDII line P2, as percentages of AnnexinV+ and 7-AAD+ RCDII cells with medium alone (-), after DC co-culture (DC) and after IL-15 (10 ng/ml) incubation (IL-15). RCDII line P1 and P2 showed similar results.
Previous studies showed that IL-15 triggered interferon-γ (IFNγ) production by RCDII lines. We therefore measured IFNγ secretion after co-culture of RCDII lines with DC or IL-15. In co-culture experiments with DC higher concentrations of IFNγ were measured in the supernatants compared to IL-15 incubation (Figure 5A). A control T-IEL line generated from a CD patient showed no IFNγ response to either stimulus, but did proliferate in response to IL-15 (Figure 5A). IFNγ is known to induce the secretion of CXCL10 from monocytes, macrophages and DC. Indeed, supernatants of DC and RCDII co-cultures contained high concentrations of CXCL10 (Figure 5B), which was mainly detected when cells had direct cell-cell contact (Figure 5C) and was not secreted after IL-15 incubation (Figure 5B). In contrast, co-culture of DC with a T-IEL line resulted in very little CXCL10 secretion, consistent with the absence of IFNγ in such co-cultures (Figure 5A and B).

In short, IFNγ is produced by RCDII lines upon co-culture with DC, which induces CXCL10 secretion by DC, especially after direct cell-cell contact.
FIGURE 5. IFNγ WAS DETECTED IN THE SUPERNATANTS OF DC-RCDII CO-CULTURES AND INDUCED THE SECRETION OF THE IFNγ-DEPENDENT CHEMOKINE CXCL10. Supernatants of co-culture experiments were analyzed after 3 days by Bioplex Human Cytokine assay. (A) In the upper graph the concentration of IFNγ in the supernatants of DC co-culture is depicted on the x-axis in pg/ml. Proliferation of cells was measured by 3H-incorporation (CPM) from the same experiment as depicted in the lower panel. Black bars indicate DC co-culture with RCDII line P2, gray bars with a representative T-IEL cell line. A plus or a minus in the legend on the x-axis indicates if DC, 10 ng/ml IL-15 or the corresponding cell line (cells) was added to the co-culture experiment. (B) The concentration of CXCL10 (in pg/ml) in supernatants of DC co-culture with RCDII line P2 (black bar) or T-IEL control lines (gray bar) is shown. The legend on the x-axis indicates whether DC, 10 ng/ml IL-15 or the corresponding cell lines (cells) was added. (C) The concentration of CXCL10 (in pg/ml) in supernatants of transwell experiments with DC and RCDII line P2 is depicted. The legend on the x-axis indicates if cell-cell contact was permitted (YES) or not (NO) and if the RCDII line or DC were added (+) or not (-). Similar results were obtained for both RCDII lines P1 and P2 and are representative of at least two independent experiments.
DISCUSSION

Interleukin-15 is generally believed to contribute to the outgrowth of α-IEL seen in RCDII patients, and its proliferation-inducing and anti-apoptotic effects on RCDII lines are well documented. Here we demonstrate that not only IL-15 but also monocytes, macrophages and especially dendritic cells (DC), prominent cell types of the innate immune system, can induce proliferation and inhibit apoptosis of RCDII lines. This effect on proliferation was specific to RCDII lines as regular T-IEL lines did not proliferate when co-cultured with monocyte-derived cells.

Although we were unable to identify the receptor-ligand interactions between DC and RCDII lines triggering proliferation, several important candidates could be excluded. First, since proliferation was drastically reduced in the absence of cell-cell contact, soluble mediators are unlikely to play a major role. Second, since RCDII lines (like α-IEL, their in vivo counterparts in RCDII) do not express TCR/CD3 complexes on the cell surface, TCR-dependent recognition of HLA-class I or II cannot be responsible for proliferation induced by DC. Third, co-culture experiments with a diverse panel of HLA-typed PBMC- including autologous PBMC- demonstrated that the HLA-genotype of the stimulator cells did not influence the observed proliferative response. This argues against HLA-allotype-specific recognition by NK cell receptors such as Killer-cell Immunoglobulin-like Receptors (KIR), which are expressed by the RCDII lines. Finally, IL-15-specific antibodies did not inhibit the induction of proliferation by DC, while they did block the response induced by soluble IL-15. In conclusion, recognition is most likely mediated by receptors on the RCDII lines that bind to non-polymorphic surface-expressed ligands on DC.

Contact between cells of the monocyte lineage and α-IEL can occur either in the epithelium in RCD patients or after dissemination into the lamina propria, the blood and other intestinal tissues reflecting ongoing malignant transformation and development into an enteropathy-associated T cell lymphoma. DC normally reside in the lamina propria, but can extend protrusions into the epithelial layer and thereby could contact α-IEL. Raki et al. reported increased numbers of dendritic cell subsets and macrophages in the lamina propria and in the basolateral membrane of epithelial cells in lesions of active celiac patients, reflecting the ongoing inflammatory responses of the untreated disease. While this has not been investigated in RCD patients, DC cell numbers may be similarly increased in these patients due to the ongoing severe inflammation in the intestine.

Our results indicate that IFNγ produced by RCDII lines leads to the secretion of CXCL10 by DC. In vivo, this would attract CXCL10-sensitive macrophages and DC to the lesion, thus creating a self-augmenting inflammatory process. It has been described that CXCL10 (Chirdo et al., personal
communication) and its receptor CXCR3 are more abundantly expressed in the mucosa of CD patients than in non-CD individuals, suggesting that this chemokine-receptor interaction is involved in ongoing inflammation in active celiac disease. Thus far, no data are available for refractory celiac disease. Nevertheless, high expression levels of CXCL10 have also been found in the mucosa of IBD patients and increased serum levels of CXCL10 have further been described in several autoimmune diseases and could be of value as a biomarker for inflammation. Thus, contact-dependent production of IFNγ and subsequently CXCL10 as a result of DC-lymphocyte interactions could help sustain persistent inflammation in refractory celiac disease and other intestinal and autoimmune diseases.

In conclusion, contact between α-IEL and cells of the monocyte lineage can protect the α-IEL from apoptosis and induce proliferation and IFNγ production. Since both IL-15 and DC quantities are increased in duodenal lesions, and since α-IEL are more sensitive to these stimuli than regular T-IEL, together they may contribute to the uncontrolled expansion of α-IEL in RCDII and support the development of an invasive lymphoma.

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