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Dendritic cells promote expansion and survival of aberrant TCR-negative intraepithelial lymphocyte lines from refractory celiac disease type II patients

Frederike Schmitz, Jennifer M.-L. Tjon, Jeroen van Bergen, Frits Koning*

Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

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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$^+$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.

1. 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 (Lundin et al., 1993), increased numbers of TCR-αβ$^+$ and TCR-γδ$^+$ intraepithelial lymphocytes (T-IEL) (Kutlu et al., 1993; Green and Cellier, 2007) and elevated interleukin-15 (IL-15) levels in the epithelium and lamina propria (Mention et al., 2003). 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 (Al-Toma et al., 2007; Cellier et al., 2000), 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 (Verbeek et al., 2008). In approximately 50% of RCDII patients these a-IEL gradually replace the normal IEL population and undergo malignant transformation (Malamut et al., 2009). These patients develop a severe life threatening condition, called enteropathy-associated T cell lymphoma (EATL) with a 2 year survival of less than 30% (Al-Toma et al., 2007; Delabie et al., 2011). a-IEL in RCDII contain clonal TCR rearrangements (Malamut et al., 2009; Cellier et al., 1998, 2000) 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 (Schmitz et al., 2013; Verbeek et al., 2008; Cellier et al., 1998; Malamut et al., 2009).

While in non-celiac adults less than 10% of IEL possess this 'aberrant' phenotype (Schmitz et al., 2013), 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 (Mention et al., 2003; Malamut et al., 2010; Tjon

Abbreviations: CD, coeliac disease; RCD, refractory coeliac disease; EATL, enteropathy associated T cell lymphoma; IEL, intraepithelial lymphocytes; T-IEL, TCR+/CD3+ IEL, intraepithelial lymphocytes; a-IEL, aberrant IEL.

* Corresponding author at: Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Tel.: +31 715266673; fax: +31 715265267.
E-mail address: F.Koning@lumc.nl (F. Koning).

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et al., 2008). The fact that cells with this 'aberrant' phenotype from non-celiac duodenal biopsies express the IL-2/15Rβ and become positive for the proliferation marker Ki67 upon incubation with IL-15 (Schmitz et al., 2013) 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 (Rescigno et al., 2001; Lelouard et al., 2012; Farache et al., 2013) 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.

2. Materials and methods

2.1. 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.

2.2. Cell lines and cell culture

RCDII lines P1 and P2 were isolated from duodenal biopsies of RCDII patients and maintained as described (Tjon et al., 2008). 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 48 h. HT-29 cells were cultured in IMDM/10% FCS medium.

2.3. Cytotoxicity assay

One million target cells were labeled with 100 γCi [^{51}Cr] 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]) × 100%.

2.4. 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 1×10^5/well PBMC, 2×10^4/well DC/macrophages/monocytes, 2×10^4/well HT29 and responder cells were 2×10^4/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, 0.4 μm pore size, Corning, MA, USA). ^{3}H-thymidine incorporation was determined during the final 17 h of the incubation.

2.5. 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 1 h 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.

2.6. 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).

2.7. 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.

3. Results

3.1. 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 (Tjon et al., 2011), 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 (Fig. 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 (Fig. 1B). In contrast, RCDII lines lysed HT-29 but not monocytes (Fig. 1C). Thus, monocytes induced proliferation but not cytotoxicity of RCDII lines, while the opposite was true for intestinal epithelial cells.