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Chronic Lymphocytic Leukemia: A Paradigm of Innate Immune Cross-Tolerance

Teresa Jurado-Camino,*1 Raúl Córdoba,*+1 Laura Esteban-Burgos,*1 Enrique Hernández-Jiménez,* Victor Toledano,* Jose-Angel Hernandez-Rivas,† Elena Ruiz-Sainz,* Teresa Cobo,* María Siliceo,* Rebeca Perez de Diego,†,‡ Cristobal Belda,** Carolina Cubillos-Zapata,* and Eduardo López-Collazo*+dl

Infections are a significant cause of morbidity and mortality in patients with chronic lymphocytic leukemia (CLL). The pathogenesis of infections is multifactorial and includes hypogammaglobulinemia, conventional therapy with alkylating drugs, and recently, purine analogs and mAb-associated T cells. Patients without these risk factors also suffer from infections, although the mechanism remains unknown. In a cohort of 70 patients with CLL, we demonstrated that their monocytes were locked into a refractory state and were unable to mount a classic inflammatory response to pathogens. In addition, they exhibited the primary features of endotoxin tolerance, including low cytokine production, high phagocytic activity, and impaired Ag presentation. The involvement of miR-146a in this phenomenon was suspected. We found miR-146a target genes, such as IRAK1 and TRAF6, were manifestly downregulated. Our study provides a new explanation for infections in patients with CLL and describes a cross-tolerance between endotoxins and tumors. The Journal of Immunology, 2015, 194: 719–727.
ET events in patients diagnosed with CLL. Our primary objective was to evaluate ET events in patients with CLL to provide new data that might explain the high risk for infections in these patients.

Materials and Methods

Patients

Patients with CLL were diagnosed according to the World Health Organization’s Classification of Tumors of the Hematopoietic and Lymphoid Tissues (21). The participants (n = 70) enrolled were from four centers in Madrid, Spain (La Paz Hospital, Infanta Leonor Hospital, El Tajo Hospital, Sureste Hospital). They were included in the study at their first diagnosis and were not receiving treatment at the time of inclusion. Exclusion criteria included an infection in the previous month, immune suppressor treatment, AIDS, hepatitis B or C, other cancers, diabetes mellitus, and other significant pathologies. As control subjects (n = 20), we included sex- and age-matched healthy volunteers (HVs) with no history of CLL or any other significant illness. The characteristics of the patients and HVs are summarized in Table I. A total of 30 ml blood was taken from a peripheral vein at the time of enrollment. To avoid circadian variability in cytokine production, all of the samples were drawn during the morning (between 8 and 11 AM). The data from the clinical stage, lactate dehydrogenase, β2-microglobulin level, ZAP70 status, cytogenetics, and history of infections and tumors were collected from the electronic medical history. The patients enrolled in this study were followed for 60 d after inclusion. The study protocol adhered to the ethical guidelines of the 1975 Declaration of Helsinki and received approval from the Ethics Committee of La Paz Hospital (PI-1277). All participants provided their written informed consent to participate in the study, and the Ethics Committee approved this consent procedure.

Reagents

DMEM (Invitrogen) was used for cell cultures. LPS from Salmonella abortus was a kind gift from Dr. C. Galanos (Max Planck Institute for Immunobiology, Freiburg, Germany). Hyaluronic acid (HA) was purchased from R&D Systems. GFP was located in a plasmid that was introduced by transformation into the Escherichia coli K12 lineage and selected in agar plates supplemented with ampicillin (100 µg/ml).

PBMCs, monocyte isolation, and cell cultures

PBMCs were isolated from the patients and controls. Monocytes were obtained by adherence, as we described previously (15). The purity of the monocyte cultures was tested by CD14 labeling and flow cytometry analysis (average 87% CD14+ cells). Other cell surface markers were tested (CD1a = 4.1%, CD89 = 89%, data not shown). The primary cultures of human fibroblasts were obtained as reported by Janssens et al. (22). All reagents used for the cell cultures were endotoxin free, as assayed with the Limulus amebocyte lysate test (Cambrex). In some experiments, we used CLL lymphocytes isolated from the supernatants of cultures of PBMCs from patients with CLL.

Cytometric bead array

The cytokine levels in the culture supernatants were determined using the CBA Flex Set, following the manufacturer’s protocol, and analyzed by flow cytometry using a BD FACSCalibur flow cytometer (both from BD Biosciences).

Intracellular TNF-α analysis

For cytokine staining, the cells were incubated for 16 h at 37°C in the presence of brefeldin A (1 µg/ml). The staining was performed after fixation using FACS Permeabilizing Solution 2 (BD Biosciences), according to the manufacturer’s instructions. The cells were washed with BD Perm/ Wash solution (BD Pharmingen) and stained with an allophycocyanin-conjugated anti–TNF-α mAb from Miltenyi Biotec at 4°C for 20 min. The cells were analyzed on a FACSCalibur (BD Biosciences).

Flow cytometry analysis

For surface marker staining, the cells were labeled with the following mAbs: allophycocyanin-conjugated anti-human CD14, PE-conjugated anti-human HLA-DQ, and allophycocyanin-conjugated anti-human HLA-DR (ImmunoTech), as well as anti-CD14-FITC, anti-CD16b-FITC, and anti-CD169-FITC (Serotec). Matched-isotype Abs were used as negative controls. The cells were incubated for 30 min at 4°C in the dark. The data were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

Phagocytosis assay

The monocytes from the patients and the controls were exposed to bacteria (GFP–E. coli K12) for 2 h. The cells were washed and kept in 300 µg/ml gentamicin (Laboratorios Normon) for 30 min. Phagocytosis was analyzed by flow cytometry of GFP+ cells, as reported previously (15, 23).

Proliferation assay

We followed a protocol described by Hernandez-Fuentes et al. (24).

HA and DeR3 quantification

Sera were isolated from EDTA-anticoagulated blood samples from patients and HVs and stored at −80°C until measurement. Serum HA was determined by ELISA (Hyaluronan DuoSet; R&D Systems), according to the manufacturer’s instructions. DeR3 detection was performed using ELISA (DeR3/TNFFRSF6B DuoSet; R&D Systems), according to the manufacturer’s instructions.

RNA and microRNA isolation, quantification, and analysis

After the cell culture, CD14 cells of high purity were recovered, using Dynabeads CD14 (Invitrogen), according to the manufacturer’s instructions. Total RNA was prepared using the RNAqueous-Micro Total RNA Isolation Kit (Ambion). RNA yield and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Thermo Scientific).

For mRNA expression analysis, cdNA was obtained by reverse transcription of 1 µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression levels were analyzed by real-time quantitative PCR using the LightCycler System (Roche Diagnostics); cdNA was obtained as described above. Quantitative PCR was performed using a QuantiMix Easy SYG Kit (Biotools) and specific primers. The results were normalized to the expression of β-actin, and cdNA copy number of each gene of interest was determined using a seven-point standard curve, as previously described (15, 20, 25–27). All primers were purchased from Biotools.

For microRNA (miRNA) analysis, 10 ng RNA from each sample was used for quantitative stem-loop reverse transcription and real-time PCR. Quantification of expression of the mature miRNAs was performed using the TaqMan microRNA RT kit, TaqMan Universal PCR MasterMix, and TaqMan miRNA assay primers of interest for human miRNAs (Applied Biosystems). The cycle threshold (Ct) values were determined, corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission. miRNA expression values were calculated using human RNU24 (Applied Biosystems) as an endogenous reference, following the 2−DDCt method (28). Standard curves for mature miR-146a were prepared by quantitative RT-PCR analysis using synthetic mature miRNA (Integrated DNA Technologies, Coralville, IA). Ct values were determined by quantitative RT-PCR analysis of the total RNA from the cell cultures and converted to miR-146a copy numbers using the standard curve.

Table I. Clinico-pathological characteristics of patients and HVs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CLL Patients</th>
<th>HVs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>Age (y; mean ± SEM)</td>
<td>71 ± 11</td>
<td>68 ± 12</td>
</tr>
<tr>
<td>Sex (male/female) [% (%)]</td>
<td>71.4 (50)/28.6 (20)</td>
<td>60 (12)/40 (8)</td>
</tr>
<tr>
<td>Binet stage [% [%]]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>87.2 (61)</td>
<td>NA</td>
</tr>
<tr>
<td>B</td>
<td>2.8 (2)</td>
<td>NA</td>
</tr>
<tr>
<td>C</td>
<td>10 (7)</td>
<td>NA</td>
</tr>
<tr>
<td>ZAP70% [% [%]]</td>
<td>22.8 (16)</td>
<td>NA</td>
</tr>
<tr>
<td>LDH &gt; upper limit of normal (234 U/l) [% [%]]</td>
<td>4.3 (3)</td>
<td>NA</td>
</tr>
<tr>
<td>β2-microglobulin &gt; 2 mg/l [% [%]]</td>
<td>37.1 (26)</td>
<td>NA</td>
</tr>
<tr>
<td>Cytogenetics [% [%]]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>del13q</td>
<td>22.9 (16)</td>
<td>NA</td>
</tr>
<tr>
<td>del17p</td>
<td>2.9 (2)</td>
<td>NA</td>
</tr>
<tr>
<td>TRISOMY12</td>
<td>7.1 (5)</td>
<td>NA</td>
</tr>
<tr>
<td>Normal karyotype [% [%]]</td>
<td>54.3 (38)</td>
<td>NA</td>
</tr>
<tr>
<td>No data</td>
<td>12.8 (9)</td>
<td>NA</td>
</tr>
<tr>
<td>Infections [% [%]]</td>
<td>27.1 (19)</td>
<td>0</td>
</tr>
<tr>
<td>Secondary malignancies [% [%]]</td>
<td>5.7 (4)</td>
<td>0</td>
</tr>
<tr>
<td>Death [% [%]]</td>
<td>5.7 (4)</td>
<td>0</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; NA, not applicable.
Statistical analysis

The number of experiments analyzed is indicated in each figure legend. The data are expressed as mean ± SD. The statistical significance was calculated using unpaired and paired t tests, depending on the specific assay. Statistical significance was set at p < 0.05, and statistical analyses were conducted using Prism 6.0 software (GraphPad).

Results

Patients

Seventy patients (71 ± 11 y) diagnosed with CLL and with no previous treatment were included in the study; 40 were men (57%), and 30 were women (43%). No patients were treated for $60 d after inclusion in the study. Of the included patients, 19 (27%) had infections within 60 d, 4 (6%) were diagnosed with secondary malignancies, and 4 (6%) died the following year. Twenty HVs with no clinical history of tumor and no immunosuppression were included as controls. The age and gender distributions were similar in the two groups (Table I). Interestingly, in our cohort of patients, we did not find any evident relationship between infection incidence and hypogammaglobulinemia (Table II).

LPS ex vivo challenge of isolated monocytes revealed an ET status in patients with CLL

During infections, monocytes are one of the primary effectors of innate immunity. However, throughout a refractory state the inflammatory response to an endotoxin challenge is severely blunted (14, 15). When circulating cells from patients with CLL were isolated and the monocytes were exposed to LPS ex vivo for 3 h, the production of soluble cytokines was significantly impaired (Fig. 1A–E). TNF-α also was downregulated at the intracellular level (Fig. 1F). An analysis of the mRNA expression of several cytokines and chemokines confirmed our findings (data not shown).

Table II. Hypogammaglobulinemia and infection incidence

<table>
<thead>
<tr>
<th>Ig</th>
<th>No. of Patients (n [%])</th>
<th>No. of Patients with Infections&lt;sup&gt;a&lt;/sup&gt; (n [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM &lt; 50 mg/dl</td>
<td>14 (20%)</td>
<td>5 (26.3%)</td>
</tr>
<tr>
<td>IgG &lt; 650 mg/dl</td>
<td>1 (1.4%)</td>
<td>0</td>
</tr>
<tr>
<td>IgA &lt; 40 mg/dl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgM &lt; 50 mg/dl and IgG &lt; 650 mg/dl</td>
<td>2 (2.85%)</td>
<td>0</td>
</tr>
<tr>
<td>IgM &lt; 50 mg/dl and IgA &lt; 40 mg/dl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgG &lt; 650 mg/dl and IgA &lt; 40 mg/dl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IgM &lt; 50 mg/dl, IgG &lt; 650 mg/dl, and IgA &lt; 40 mg/dl</td>
<td>2 (2.85%)</td>
<td>2 (10.5%)</td>
</tr>
<tr>
<td>Normal levels&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51 (72.9%)</td>
<td>12 (63.2%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Patients with infections reported within 60 d after admission to the study.

<sup>b</sup>IgM (50–300 mg/dl), IgG (650–1600 mg/dl), and IgA (40–350 mg/dl).

FIGURE 1. Cytokine production. Monocytes isolated from HVs and patients with CLL were cultured in the presence or absence of 10 ng/ml of LPS for 3 h. (A–E) The supernatant was collected, and cytokine production was quantified using a cytometric bead array. Levels of IL-1β (A), IL-6 (B), IL-10 (C), IL-12p70 (D), and TNF-α (E). HVs (n = 20), patients with CLL (n = 70). (F) Cells were harvested and stained intracellularly with anti–TNF-α and the appropriate isotype Ab to evaluate intracellular levels of TNF-α by flow cytometry in randomized selected cultures. Data are fold induction with respect to the corresponding nonstimulated culture. HV (n = 5), CLL (n = 7). O represents an outlier. *p < 0.05, **p < 0.01 versus HV.
shown). Basal levels of several cytokines were analyzed in sera from CLL patients; no significant difference was detected with respect to HVs (Supplemental Fig. 1).

In line with previously published data, the CLL monocytes exhibited a significant nonclassical subset (CD14<sup>+</sup>/CD16<sup>++</sup>, Fig. 2A) (29). These results suggested that the CLL monocytes were locked into a state of ET. Thus, we confirmed this hypothesis by assessing the primary features previously reported for ET: high phagocytic ability and poor Ag presentation (13–15).

In contrast to HVs, a significant expression of CD64 [a well-known marker for phagocytosis (30, 31)] was found in CLL monocytes (Fig. 2B). When we analyzed the monocytes’ ability to phagocytize in a standard assay using GFP-labeled bacteria, CLL monocytes exhibited higher levels of CD14<sup>+</sup>/GFP<sup>+</sup> cells when they were analyzed by flow cytometry (Fig. 2C). These data were corroborated by counting the CFU generated when the cytosol of these cells was spread on blood agar plates (data not shown). Thus, our findings established that enhanced phagocytic activity is a relevant feature of CLL monocytes.

In addition to a potent phagocytic function, CD64 is associated with a low Ag-presentation capacity (15, 30, 31). When we studied the cell surface levels of two important isotypes of MHC class II (HLA-DR and HLA-DQ) by flow cytometry in patients with CLL, they showed a significantly reduced expression after LPS challenge (Fig. 3A, 3B). Finally, we verified the functional impact of MHC class II downregulation via a lymphocyte-proliferation assay (15, 24, 32). In full agreement with the findings presented above, Ag presentation was significantly impaired in CLL monocytes (Fig. 3C). Although HLA-DR did not show a significant difference between HVs and CLL patients at baseline (no LPS challenge), the well-known M2 marker CD163 was significantly higher in CLL-CD14<sup>+</sup> cells prior to any endotoxin challenge (Supplemental Fig. 2).

These findings demonstrated that monocytes from patients with CLL exhibited a manifest ET status, given that they were unable to orchestrate an inflammatory response and showed high phagocytic activity and impaired Ag presentation (13–15). The purity of the monocyte population was confirmed as previously described (15).

Soluble factors with putative involvement in the observed ET status of CLL monocytes

Once we had established that ET takes place in CLL, we studied the factors that could cause this refractoriness. Several investigators reported that both the glycosaminoglycan HA and the decoy receptor DcR3 could be key players in tumor development (33–37). It was demonstrated that HA is released by tumor cells and blocks the inflammatory signaling in human monocytes (34, 37); DcR3 can be defined as an immunomodulator on the basis of its ne-
Potential intracellular signaling implicated in ET: The role of miR-146a

Signaling through the TLR pathways is a crucial mechanism for the detection of pathogens and their endotoxins by host immune cells, including monocytes/macrophages (38, 39). In particular, TLR4 has been described as the primary LPS sensor. Its activation leads NF-κB translocation into the nucleus and, subsequently, the transcription of inflammatory genes, such as TNFα, IL1β, IL6, and IL12 (40–43). Defects in TLR4 signaling have been observed at the level of the receptor, adaptors (MyD88 and TRIF) (44, 45), signaling molecules, and transcription factors. Several investigators suggested that ET in human monocytes and mouse macrophages is associated with decreased TLR4–MyD88–IRAK complex formation, impairment of IRAK-1 activity, and TRAF6 expression (45–49). In this regard, miRNAs have emerged as an important regulatory mechanism for gene expression of a number of TLR4 pathway components during ET (49); in particular, miR-146a is critical for ET development in human monocytes (48, 50–54). As shown in Fig. 5A, miR-146a was overexpressed in CLL monocytes in a randomly selected set of samples (n = 10) after 3 h of LPS stimulation. However, this overexpression was not observed in the basal state (Supplemental Fig. 4A). Remarkably, no miR-146a expression was found in CLL lymphocytes stimulated with LPS (Supplemental Fig. 4B).

In addition, mRNA levels of TNF-α, IRAK-1, and TRAF6 exhibited a potent downregulation (Fig. 5B–D); both IRAK-1 and TRAF6 were reported to be directed miR-146a targets (45–49). Furthermore, when human monocytes from HVs were exposed for 5 d to MEC-1 [a human cell line derived from CLL (55)] and later challenged with LPS (see the experimental design in Fig. 6A), cytokine production (Fig. 6B) and HLA-II (Fig. 6C) were reduced, whereas miR-146a was manifestly upregulated (Fig. 6D).

Discussion

Despite previous research revealing a certain connection between infection frequency and hypogammaglobulinemia in patients with CLL (4, 5, 10), a number of patients with standard production of IgG suffer from opportunistic pathogen colonization (3, 12). This apparent contradiction prompted us to analyze monocyte activity in patients with CLL. We showed that monocytes from 70 patients with CLL were unable to orchestrate a proper inflammatory response after an ex vivo endotoxin challenge. The production of soluble cytokines, such as IL-1β, IL-6, IL-10, and TNF-α, was significantly reduced in CLL monocytes, and IL-12p70 exhibited a marked downward trend after LPS stimulation. The same result was found after evaluating intracellular levels of TNF-α. Remarkably, IL-10, a well-known anti-inflammatory cytokine, also is...
downregulated. The expression and regulation of IL-10 are controversial in this context. Several investigators showed that IL-10 plays a crucial role in ET control, whereas others reported a weak effect of IL-10 in sepsis-induced tolerance (14). In addition, IL-10–knockout mice exhibited an ET phenotype when they were exposed twice to LPS.

As other investigators reported, our cohort of patients showed a significant increase in the nonclassical phenotype, given that 10% of them had CD14+/CD16++ profiles (29) (Fig. 2A). Collectively, these data revealed the presence of an ET status in patients with CLL that could explain the high frequency of reported infections. We also detected other previously established

FIGURE 5. miR-146a implication and target genes. Randomized selected HV and CLL cultures of monocytes were treated with 10 ng/ml LPS for 3 h. The cells were harvested, CD14+ cells were isolated, and both miRNA and mRNA were isolated. miR-146a and target genes were analyzed by real-time quantitative PCR. (A) Fold induction of miR-146a with respect to nonstimulated cells. Fold induction of TNF-α (B), IRAK-1 (C), and TRAF6 (D) with respect to nonstimulated cells. HV (n = 10), CLL (n = 10). *p < 0.05 versus HV.
ET features in CLL monocytes: overexpression of CD64 and CD163, high phagocytosis rate, poor HLA-DR and HLA-DQ expression, and low Ag presentation. These findings indicate the existence of a refractory state in these patients. Our data indicate an inherent risk for opportunistic colonization in patients with CLL that is not associated with either the treatment or the phenomenon of hypogammaglobulinemia. In addition, we did not find a patent relationship between hypogammaglobulinemia and the incidence of infection in our cohort of CLL patients. As shown in Table II, 63% of those patients who reported an infection within 60 d after inclusion in our study exhibited normal Ig levels.

Despite several previous studies that addressed the underlying mechanisms of ET, we still lack a complete understanding of the phenomenon. In most cases, ET is associated with previous endotoxin contact, which induces a refractory state during a second endotoxin challenge (56, 57). However, we also reported this phenomenon in “sterile” pathologies, such as myocardial infarction; mitochondrial DNA induce a manifest refractoriness in these patients (58).

We demonstrated an ET status in CLL monocytes caused by contact with tumor cells. In vitro assays indicate that monocytes from HVs were reprogrammed to an ET status after coculture with MEC-1. However, our findings indicated there is no “key” soluble factor in CLL serum that is able to induce a refractory state in monocytes. When HV monocytes were cultured in medium supplemented with 10% of patient sera, these cells did not exhibit primary ET features; these data were corroborated in other assays using Transwell chambers (data not shown). These latest findings apparently contradict the correlation found between levels of the glycosaminoglycan HA and the occurrence of infections in patients with CLL. Although HA emerges as a good predictor of infections in CLL, when cells from HVs were exposed to either patient serum with high HA levels or HA per se, we did not observe a shift in the response of monocytes toward a tolerant phenotype. Further studies are needed to clarify the role of HA in ET development in cancer.

CLL monocytes expressed significant levels of miR-146a after an LPS challenge. This microRNA has been identified as critical for endotoxin-induced tolerance (45, 48–54). Shao et al. (59) demonstrated the association between the miR-146a gene and severe sepsis in 226 septic patients. In this article, we report that previous contact with tumor cells increases its expression after endotoxin stimulation. We reached the same conclusion in ex vivo and in vitro assays. When monocytes from patients with CLL were exposed ex vivo to LPS, their miR-146a levels increased by 2–200-fold with respect to the basal level. In contrast, monocytes from HVs did not similarly upregulate miR-146a levels. Additionally, miR-146a target genes, such as TNF-α, IRAK1, and TRAF6, showed low levels in LPS-treated CLL monocytes. Downregulation of these factors could explain the poor inflammatory response observed after an endotoxin challenge. Similar findings were obtained with in vitro assays (Fig. 6). To our knowledge, this is the first time that a cross-tolerance between endotoxin and tumor has been reported.

Collectively, our data indicated that patients suffering from CLL were locked into a refractory state that prevented them from mounting a classic response to pathogens, regardless of their levels of Ig. As a result of the direct contact between tumor and monocytes, the latter reprogram their innate response and express high levels of miR-146a, which, in turn, might regulate the levels of critical factors in the inflammatory response, such as IRAK1 and TRAF6. These data provide a new explanation for the risk for infection in patients with CLL. Note that this refractory state should not be confused with immunosuppression. Although ET and immunoparalysis share some common characteristics (e.g., low proinflammatory cytokine production), the elevated phagocytosis rate, low apoptosis frequency, and the potential reversion from...
a refractory state to the standard immune response are specific features of ET and are very unusual during immunosuppression.

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Disclosures

The authors have no financial conflicts of interest.

References


