Recombinant Human IL-15 Trans-Presentation by B Leukemic Cells from Chronic Lymphocytic Leukemia Induces Autologous NK Cell Proliferation Leading to Improved Anti-CD20 Immunotherapy

Emilie Laprevotte, Gregory Voisin, Loïc Ysebaert, Christian Klein, Camille Daugrois, Guy Laurent, Jean-Jacques Fournie and Anne Quillet-Mary

*J Immunol* published online 30 August 2013

http://www.jimmunol.org/content/early/2013/08/30/jimmunol.1300187
Recombinant Human IL-15 \textit{trans}-Presentation by B Leukemic Cells from Chronic Lymphocytic Leukemia Induces Autologous NK Cell Proliferation Leading to Improved Anti-CD20 Immunotherapy

Emilie Laprevotte,*†‡§¶ Gregory Voisin,*†‡§¶ Loïc Ysebaert,*†‡§¶ Christian Klein,‡ Guy Laurent,*†‡§¶ Jean-Jacques Fournie,*†‡§¶ and Anne Quillet-Mary*†‡§¶

Recombinant human IL-15 (rhIL-15) is one of the most promising cytokines for antitumor immunotherapy. In physiology IL-15 \textit{trans}-presentation by accessory cells leads to pleiotropic activities, including activation of immune cells, such as NK cells. NK cells are largely involved in Ab-dependent cellular cytotoxicity mediated by therapeutic mAbs, such as rituximab, in chronic lymphocytic leukemia (CLL). Nevertheless, in CLL, Ab-dependent cellular cytotoxicity is relatively impaired by the low E:T ratio (NK/B leukemic cells). Thus, any strategy leading to an increase in NK cell number and activation status can offer new strategies for CLL treatment. To this end, we evaluated the effect of rhIL-15 on autologous NK cell stimulation in CLL samples. We show that rhIL-15 induces NK cell activation and proliferation, leading to improved B leukemic cell depletion. This phenomenon is significantly increased in the presence of anti-CD20 mAbs. In addition, the greater effect of obinutuzumab versus rituximab suggests a cooperative role between rhIL-15 signaling and CD16 signaling in the induction of NK cell proliferation. Moreover, rhIL-15-induced proliferation of autologous NK cells is strictly dependent on their interaction with B leukemic cells, identified in this study as new accessory cells for rhIL-15 \textit{trans}-presentation. Thus, rhIL-15 is able to promote NK cell–based activity in Ab immunotherapy of CLL. \textit{The Journal of Immunology}, 2013, 191: 000–000.

\textsuperscript{*}INSERM Unité Mixte de Recherche 1037, Cancer Research Center of Toulouse, BP3028 Centre Hospitalier de l’Université Purpan, F-31300 Toulouse, France; \textsuperscript{†}Équipe de Recherche Labelisée 5294 Centre National de la Recherche Scientifique, BP3028 Centre Hospitalier de l’Université Purpan, F-31300 Toulouse, France; \textsuperscript{‡}Université de Toulouse III Paul-Sabatier, F-31000 Toulouse, France; \textsuperscript{§}Institut Carnot Lymphome/CALYM, France; \textsuperscript{¶}Laboratoire d’Excellence Toulouse Cancer/TUCAN, France; \textsuperscript{‖}Centre Hospitalier de l’Université Purpan, Service d’Hématologie, F-31300 Toulouse, France; and \textsuperscript{¶}Roche Glycart AG, Roche Pharma Research and Early Development, CH-8952 Schlieren, Switzerland

Received for publication January 17, 2013. Accepted for publication July 25, 2013.

J.-J.F. and A.Q.-M.’s laboratory is supported by institutional grants from INSERM, Université de Toulouse III, and Centre National de la Recherche Scientifique, as well as by grants from Institut National du Cancer. E.L. was a recipient of grants from the Ministère Départemental à l’Enseignement Supérieur et à la Recherche and La Ligue Nationale contre le Cancer.

Address correspondence and reprint requests to Dr. Anne Quillet-Mary, INSERM Unité Mixte de Recherche 1037/Université Toulouse III/ERL5294 CNRS, Centre Hospitalier de l’Université Purpan, BP3028, 31024 Toulouse, France. E-mail address: anne.quillet-mary@insERM.fr

The online version of this article contains supplemental material.

Abbreviations used in this article: ADCC, Ab-dependent cellular cytotoxicity; CLL, chronic lymphocytic leukemia; DC, dendritic cell; h-IgG, control human IgG; rhIL-15, recombinant human IL-15; RTX, rituximab.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

IL-15 displays important physiological functions facilitating innate and adaptive immunity (7); it has an important role in the development, homeostasis, and activation of immune cells, such as T lymphocytes and NK cells (3, 8, 9). IL-15 exerts large pleiotropic activities on NK cells: survival, proliferation, differentiation, increase in cytotoxic functions, stimulation of cytokine production (IFN-γ, TNF-α, and GM-CSF), and regulation of NK/macrophage interactions (3, 8, 9). In addition, IL-15 was found to inhibit apoptosis of neutrophils and eosinophils, as well as Fas-mediated apoptosis of B or T cells, through upregulation of antiapoptotic proteins (8).

Several studies implicated IL-15 in infectious, autoimmune, and inflammatory diseases, as well as cancer malignancies (5, 7, 10). These included hematological pathologies, such as chronic lymphocytic leukemia (CLL), in which recombinant human IL-15 (rhIL-15) sustains the survival of leukemic cells in vitro (11). CLL, the most common leukemia in adults, is defined by the progressive accumulation of CD19+CD5+ B leukemic cells in blood and secondary lymphoid organs. The accumulation of B leukemic cells depends on both cell–cell interactions (12, 13) and CLL-released soluble factors (14, 15). In standard therapeutic regimens, mAbs, such as rituximab (RTX), contribute largely to improve response-rate, progression-free, and overall survival of CLL patients (16). Despite the clear therapeutic benefits of RTX in B cell malignancies (16), relapses occur frequently following treatment. In vivo, anti-CD20 Abs induce potent target cell lysis through complement-dependent cytotoxicity (17–19) and primarily Ab-dependent cellular cytotoxicity (ADCC) (17, 19–21). ADCC relies on the ability of CD16+ effectors, such as NK cells, to bind Abs via the FcγRIIa region. Obinutuzumab (GA101) is a glycoengineered, type II CD20 Ab that has higher affinity for the NK FcγRIIa than does RTX. GA101 is able to increase both...
direct and effector cell-mediated cytotoxicity (22) and shows higher activity than does RTX in B leukemic cell depletion (23, 24). Despite a low E:T ratio in patient samples, NK cells are the main effectors of RTX-mediated ADCC of CLL cells (21, 25). Moga et al. (26, 27) demonstrated that rhIL-15 promotes NK cell cytotoxic function from healthy donors against lymphoma cell lines or primary CLL cells. More recently, Suck et al. (28) demonstrated that rhIL-15 supports the generation of highly potent clinical-grade NK cells from healthy donors in long-term culture for use in targeting hematological diseases. These data suggest that rhIL-15 is a good candidate to promote NK cell activity in Ab-based immunotherapy. However, they did not explore rhIL-15 bioactivity in autologous NK cells from hematological diseases.

The aim of our study was to evaluate the impact of rhIL-15 on autologous NK cell function mediated by RTX or GA101 in CLL and to investigate cellular partners involved in NK cell stimulation by rhIL-15.

Materials and Methods

Cells and reagents

Peripheral blood samples from untreated CLL patients (n = 41, Table I) were obtained with informed consent and referenced at the Hemopathies Inserm Midi-Pyrénées cell bank. According to French law, Hémopathies Inserm Midi-Pyrénées cell bank has been registered with the Ministry of Higher Education and Research (DC 2008-307 collection 11) and obtained a transfer agreement (AC 2008-129) after being approved by an ethics committee (Comité de Protection des Personnes Sud-Ouest et Outremer II). Clinical and biological annotations of the samples have been reported to the Comité National Informatique et Libertés (the Data Processing and Liberties National Committee).

PBMCs were used immediately following separation by Ficoll gradient centrifugation. RTX and GA101 mAbs and RTX and GA101 F(ab')2 fragments were obtained from Hoffmann La Roche (Basel, Switzerland). Human IgG control (h-IgG) Ab was purchased from Beckman Coulter (Villepinte, France). rhIL-15 was purchased from Tebu-bio (Le Perray-en-Yvelines, France) and used at a final concentration of 10 ng/ml, as in previous studies (12, 27, 28).

For all experiments, cells were cultured at 37 °C and 5% CO2 in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Cergy Pontoise, France). To provide long-term viability, CLL cultures were performed at a high cell density (10 × 106 cells/ml) (29).

Cell isolation

B leukemic cells were purified by magnetic separation without CD43 depletion using an EasySep Human B Cell Enrichment Kit, according to the manufacturer's instructions (STEMCELL Technologies, Grenoble, France) on CD19+/CD5+ gated cells by flow cytometry, according to the manufacturer’s instructions.

NK cell activation assays from CLL samples

Fresh PBMCs from untreated CLL patients were seeded at 10 × 106 cells/ml in culture medium and treated with h-IgG (10 µg/ml), RTX (10 µg/ml), or GA101 (10 µg/ml) for 7 d. When appropriate, rhIL-15 was added at a final concentration of 10 ng/ml for 7 d.

The specific percentage of remaining B cells in anti-CD20–treated samples was calculated as (absolute number in treated samples/absolute number in control samples) × 100. For each condition, the absolute number of remaining B cells was calculated as total viable cell number (trypan blue exclusion determination) × % of viable CD19+/CD5+ lymphocytes (flow cytometry determination). Then, specific B leukemic cell depletion was calculated as follow: 100 − % specific remaining B cells.

Chromium-release cytotoxic assay in autologous CLL samples

Natural cytotoxicity of effector cells from four random CLL patients was tested using the classical chromium-release assay. Briefly, NK cells were purified from PBL samples and stimulated or not with rhIL-15 (10 ng/ml). Purified autologous B leukemic cells (target cells) were incubated in RPMI 1640 1% FCS for 1 h at 37 °C with [51Cr] Sodium Chromate; Perkin Elmer, Courtaboeuf, France) (100 µCi/106 cells). Cells were washed and plated at 106 well in round-bottom 96-well plates. Increasing amounts of effector cells were added to triplicate wells with an NK/target ratio ranging from 0.01:1 to 0.8:1. Control wells contained only target cells to measure spontaneous release or target cells plus 0.1% Triton X-100 to measure maximal release. After centrifugation, plates were incubated for 4 h at 37 °C, 5% CO2. A total of 50 µl was collected from each well and counted in a gamma counter. The percentage of specific lysis was calculated as follows: ([sample release − spontaneous release]/[maximal release − spontaneous release]) × 100.

NK cell proliferation assays

Freshly isolated PBMCs from CLL patients were labeled with 1 µM CFSE (Invitrogen/Molecular Probes, Carlsbad, California) for 10 min at 37 °C and washed with PBS, according to the manufacturer’s instructions. Labeled cells were cultured in complete medium with h-IgG (10 µg/ml), RTX (10 µg/ml), or GA101 (10 µg/ml) for 7 d. When appropriate, rhIL-15 was added at a final concentration of 10 ng/ml. Activation of NK cells was evaluated by flow cytometry detecting CD69 expression on CD3- /CD56+ gated cells.

Flow cytometry

The following mAbs were used for cell staining: FITC–anti-CD3, Pacific Blue–anti-CD3, and PE–Cy7–anti-CD5 (eBioscience, Paris, France); Pacific Blue–anti-CD19 and PE–Cy5–anti-CD16 (Ozyme, Saint-Quentin-en-Yvelines, France); PE–anti-CD69, PE–Cy7–anti-CD56, and PE–goat anti-human (Beckman Coulter, Roissy, France); PE–anti-CD11c (BD Biosciences, Pont de Claix, France); and isotype-matched control conjugates. Briefly, PBMCs or purified cells were washed with cold PBS containing 1% FCS, stained with the appropriate conjugated Abs on ice for 30 min, and then washed and analyzed using a BD LSR II cytometer (BD Biosciences) and DIVA software.

CD20 expression was quantified using the BD QuantiBRITE fluorescent assay (BD Biosciences, Le Pont de Claix, France) on CD19+/CD5+ gated cells by flow cytometry. The Ab bound per cell value represents the mean value of the maximum capability of each cell to bind the anti-CD20 Ab and was evaluated according to the manufacturer’s instructions.

The capacity of rhIL-15 to bind to B leukemic cells was evaluated using the Human IL-15 Biotinylated Fluorescein Kit (R&D Systems, Lille, France) on CD19+/CD5+ gated cells by flow cytometry, according to the manufacturer’s instructions.

In vitro B leukemic cell–depletion assays from CLL samples

Purified autologous B leukemic cells were incubated in RPMI 1640 1% FCS for 1 h at 37 °C with 106 cells/ml in culture medium and treated with h-IgG (10 µg/ml), RTX (10 µg/ml), or GA101 (10 µg/ml) for 7 d. When appropriate, rhIL-15 was added at a final concentration of 10 ng/ml for 7 d.

The specific percentage of remaining B cells in anti-CD20–treated samples was calculated as (absolute number in treated samples/absolute number in control samples) × 100. For each condition, the absolute number of remaining B cells was calculated as total viable cell number (trypan blue exclusion determination) × % of viable CD19+/CD5+ lymphocytes (flow cytometry determination). Then, specific B leukemic cell depletion was calculated as follow: 100 − % specific remaining B cells.

The capacity of rhIL-15 to bind to B leukemic cells was evaluated using the Human IL-15 Biotinylated Fluorescein Kit (R&D Systems, Lille, France) on CD19+/CD5+ gated cells by flow cytometry, according to the manufacturer’s instructions.

Downloaded from http://www.jimmunol.org/ on April 13, 2017
Statistics

Paired or unpaired Student t tests were used to determine differences between samples, as appropriate. Correlations were calculated using the Pearson test. The p values < 0.05 were considered statistically significant.

Results

Coactivity of rhIL-15 and anti-CD20 Abs in B leukemic cell depletion

We first determined whether rhIL-15 increases B cell depletion in blood samples from untreated CLL patients in an in vitro assay using mAbs. Bioactivity of rhIL-15 was assessed by B leukemic cell–depletion assays in PBMCs (n = 35), in combination with anti-CD20 Abs. Because they spontaneously die at low, but not high, density (13, 30), B cell cultures were performed at high concentrations (10 × 10^6 cells/ml) (29). Under these conditions, no significant effect of RTX- or GA101-induced B leukemic cell depletion was detected after 3 d of treatment (p = 0.08 for RTX, p = 0.07 for GA101, compared with day 0), but the effect of anti-CD20 mAbs was optimal after 7 d of treatment (p = 0.05 for RTX, p = 0.04 for GA101 compared with day 0; n = 10). Because long-term culture could lead to internalization of anti-CD20 mAbs or CD20 (31), we first analyzed, using flow cytometry, whether culture conditions altered RTX or GA101 binding to B leukemic cells. Compared with day 0, RTX binding, but not GA101 binding, significantly decreased after 7 d of culture in control conditions only (p = 0.01). This discrepancy could be due to the different recognition and affinity of the respective CD20 epitopes by RTX versus GA101. Nevertheless, after 7 d, anti-CD20 Abs remained bound to viable B leukemic cells in all culture conditions (Supplemental Fig. 1). Therefore, subsequent experiments were performed using a 7-d treatment.

In whole-PBMC samples, GA101 displayed a greater cytotoxic activity than did RTX with regard to CD19+/CD5+ cell depletion (Fig. 1A). This was not mediated by complement-dependent cytotoxicity, because the assay involved heat-inactivated FCS. B cell depletion was dependent upon the Fc portion of mAbs. No significant depletion was observed using F(ab’)2 fragments, despite a slight effect caused by GA101-F(ab’)2 compared with RTX-F (ab’)2 (p = 0.042) (Supplemental Fig. 2). Most importantly, RTX- or GA101-mediated B cell depletion was significantly increased in the presence of rhIL-15 (Fig. 1A).

 Altogether, our data suggest a cooperation of rhIL-15 with CD16 activation in anti-CD20–mediated leukemic cell depletion in CLL.

![FIGURE 1.](image)

![FIGURE 2.](image)

**Table I. Clinical characteristics of CLL patients**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binet stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (%)</td>
<td>53.7</td>
<td>22</td>
</tr>
<tr>
<td>B (%)</td>
<td>29.3</td>
<td>12</td>
</tr>
<tr>
<td>C (%)</td>
<td>17.0</td>
<td>7</td>
</tr>
<tr>
<td>Age (y; median [range])</td>
<td>66 (42–82)</td>
<td>41</td>
</tr>
<tr>
<td>Gender (male/female) (%)</td>
<td>61/39</td>
<td>41</td>
</tr>
<tr>
<td>Median leukocytosis/μl</td>
<td>72,500</td>
<td>41</td>
</tr>
<tr>
<td>Fluorescence in situ hybridization</td>
<td>13q deletion alone/normal (%)</td>
<td>41.5</td>
</tr>
<tr>
<td>11q deletion (%)</td>
<td>0.9</td>
<td>4</td>
</tr>
<tr>
<td>17p deletion (%)</td>
<td>4.8</td>
<td>2</td>
</tr>
<tr>
<td>Trisomy 12 (%)</td>
<td>1.2</td>
<td>5</td>
</tr>
<tr>
<td>Not determined (%)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>IgVH Mutated/unmutated (%)</td>
<td>40/60</td>
<td>30</td>
</tr>
<tr>
<td>Median NK/B ratio (range)</td>
<td>0.02 (0.002–0.11)</td>
<td>32</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>0.4 (0.1–3.3)</td>
<td>32</td>
</tr>
<tr>
<td>CD20 Ab bound per cell (range)</td>
<td>8154 (2538–6660)</td>
<td>24</td>
</tr>
</tbody>
</table>
They highlight a greater efficacy of the rhIL-15/GA101 combination in this depletion.

To identify which CD16+ effector cells were involved in B cell depletion from CLL samples, monocytes, DCs, and CD3+ lymphocytes were successively removed prior to the assay. The removal of monocytes/DCs, as well as monocytes/DCs/CD3+ lymphocytes, did not affect B cell depletion induced by RTX or GA101, with or without rhIL-15 (Fig. 1B), pinpointing the role of NK cells in anti-CD20–mediated B cell death. To reinforce the role of NK cells in B cell depletion mediated by RTX or GA101, PBMCs were depleted in NK cells (level of remaining cells was <0.1%, n = 8) and processed as above. In these conditions, RTX- or GA101-mediated B cell depletion was significantly inhibited compared with whole PBMCs (median B leukemic depletion: 7.85 and 11.5% for RTX and GA101, respectively, p < 0.005).

In the presence of mAbs, short-term interaction between NK cells and target cells leads to a decrease in the surface expression of CD16 in NK cells (32, 33). Whether this also occurs in long-term culture remains unclear. To address this, we tested whether CD16 could be modulated by the above-described 7-d culture conditions. Thus, CD16 expression in NK cells was analyzed by flow cytometry, in parallel with B cell–depletion experiments. Compared with day 0, the 7-d culture did not induce any noticeable change in the CD16 level in untreated controls. However, coincubation with mAbs in the presence or absence of rhIL-15 markedly decreased CD16 expression (Fig. 2). A CD16 decrease was also observed in h-IgG–treated control conditions, suggesting steric hindrance opposing CD16 labeling. This also could be due to CD16 modulation. Indeed, as described for short-term culture, anti-CD20 Abs strongly decreased CD16 levels after a 7-d culture without rhIL-15. This decrease was counteracted by rhIL-15 (Fig. 2). Altogether, our data show that, in long-term cultures, CD16 remains expressed at the surface of NK cells, allowing their ADCC function with anti-CD20 Abs.

Because rhIL-15 alone resulted in a slight B cell depletion (Fig. 1A), we analyzed fratricidal B cell death induced by IL-15, such as described for IL-21 treatment (34). No significant B cell death was observed in purified B cells treated with rhIL-15 in the presence or absence of h-IgG or anti-CD20 Abs (Supplemental Fig. 3). So we wondered whether rhIL-15 activated NK cell cytotoxicity against autologous B leukemic cells. In CLL samples, the median NK/B ratio was 0.02:1 (Table I). At this ratio, addition of rhIL-15 increased the natural cytotoxicity of purified autologous NK cells (% of specific cytotoxicity: 0.03 ± 0.1% for NK cells, 8.9 ± 1.9% for rhIL-15–stimulated NK cells, p = 0.004). Altogether, these data demonstrate that rhIL-15 increases both natural and Ab-directed cytotoxicity of NK cells against autologous CLL cells.

**NK proliferation induced by anti-CD20 and rhIL-15 in CLL samples**

Next we evaluated the combined effect of rhIL-15 and mAbs on NK cell activation and proliferation in 7-d CLL cultures. rhIL-15 strongly activated NK cells from healthy donors (3). Using random CLL samples (n = 26), NK activation was monitored by measuring

![FIGURE 3. NK cell proliferation induced by mAbs and rhIL-15 in CLL samples. PBMC, monocyte/DC-depleted, or monocyte/DC/CD3+-depleted samples from CLL were treated with h-IgG (10 µg/ml), RTX (10 µg/ml), or GA101 (10 µg/ml), with or without rhIL-15 (10 ng/ml), for 7 d and then analyzed by flow cytometry (>10,000 CD3+/CD56+ events acquired). (A) Activation of NK cell by mAbs and rhIL-15. Results represent the percentage of CD69+ NK cells among total NK cells after 7 d of culture (n = 26) in the presence or absence of mAbs and rhIL-15. Horizontal lines represent the median. (B) Representative experiment of CFSE dilution on NK cells in the presence or absence of mAbs and rhIL-15. (C) Percentage of proliferating NK cells among total NK cells after 7 d of culture in the presence or absence of mAbs and rhIL-15 (n = 17). Horizontal lines represent the median. (D) Mean percentage (± SEM) of proliferating NK cells among total NK cells in PBMC (●), monocyte/DC-depleted (○), or monocyte/DC/CD3+-depleted (*) samples after 7 d of culture (n = 9) in the presence or absence of mAbs and rhIL-15. **p < 0.01, ***p < 0.001.**
surface CD69 expression (Fig. 3A). A slight NK cell activation was observed after 7 d of culture in untreated samples. Incubation with RTX or GA101 led to an increase in activated NK cells compared with control. This effect was significantly higher with GA101 than with RTX (p < 0.01). The observed NK cell activation was dependent upon the Fc portion of mAbs, because no significant activation was observed using F(ab′)2 fragments (data not shown). Addition of rhIL-15 led to activation of all NK cells without synergistic or additive effects of RTX or GA101 (Fig. 3A).

The ADCC function of NK cells was related to their activation, as well as to the NK/target cell ratio. In CLL, the E:T ratio favors the leukemic fraction (median NK/B ratio = 0.02/1, Table I). Thus, any agent able to increase this ratio would be a good strategy to improve NK ADCC function and depletion of B leukemic cells. Therefore, we assessed the potential of rhIL-15 and anti-CD20 Abs to induce NK cell proliferation in CLL samples. As shown by CFSE dilution, we observed for the first time, to our knowledge, that both RTX and GA101 induced NK cell proliferation, demonstrating the involvement of the CD16-signaling pathway in this process (Fig. 3B, 3C). Furthermore, rhIL-15 alone also increased NK cell proliferation. Finally, the combination of rhIL-15 and mAbs induced a stronger NK cell proliferation (Fig. 3B, 3C). These data demonstrate a cooperation of rhIL-15 and CD16 in NK cell proliferation in CLL. It is noteworthy that NK cell proliferation correlates with B leukemic cell depletion (r² = 0.65, p = 0.048). Therefore, the consequence of NK cell expansion by rhIL-15 was an increase in RTX- and GA101-mediated B cell depletion.

Because IL-15 is trans-presented by accessory cells, we analyzed the involvement of monocytes or DCs in CLL samples. Despite the relatively low number of these cells in CLL samples as a result of the high excess of B leukemic cells (Table I), we explored their function in rhIL-15–induced NK cell proliferation using successive depletion. Monocyte/DC depletion or monocyte/DC/CD3⁺ depletion did not impair NK cell proliferation under any treatment conditions (Fig. 3D). The improved proliferation observed in the presence of mAbs could be attributed to a stronger interaction between B and NK cells via CD20 recognition, with subsequent FcγRIIIa cross-linking. It is noteworthy that rhIL-15 did not modulate CD20 expression on B leukemic cells (p = 0.36). These data suggest that the requirement of an interaction between B leukemic cells and NK cells is important to stimulate NK cell proliferation in the presence of rhIL-15.

**B leukemic cells as accessory for rhIL-15–induced NK cell proliferation**

We next explored the contribution of B leukemic cells to NK cell proliferation induced by rhIL-15. rhIL-15 treatment of purified B leukemic cells favored their survival but not their proliferation (Supplemental Fig. 4). Further, rhIL-15 did not induce NK cell proliferation of either purified NK cells or in Transwell condition (i.e., conditions without interactions between NK and B leukemic cells) (Fig. 4A). Importantly, similar to what was observed in the whole sample, rhIL-15 was able to induce NK cell proliferation in a reconstituted autologous system composed of only purified NK cells and purified B leukemic cells from the same donor (Fig. 4A). These results demonstrated that B leukemic cells serve as accessory cells in rhIL-15 trans-presentation, resulting in NK cell proliferation.

IL-15Rα is poorly expressed in the absence of stimulation in B cells from hematological diseases and is upregulated by in vitro stimulation with CD40L (11, 35). In random CLL samples, fluorescent rhIL-15 binds B leukemic cells, and this is decreased significantly in the presence of a blocking IL-15Rα mAb (Fig. 4B). These results suggest that B leukemic cells can promote rhIL-

**FIGURE 4.** B leukemic cells as accessory for rhIL-15–induced NK cell proliferation. (A) Control (PBMC) samples, purified NK cells, Transwell system (bottom: purified NK cells/top: purified B leukemic cells), or reconstituted system (purified NK cells + B leukemic cells from the same donor) were treated or not with rhIL-15 (10 ng/ml) for 7 d, as described in Materials and Methods, and then analyzed by flow cytometry. Results represent the fold increase in NK cells in rhIL-15–treated conditions compared with the untreated condition (n = 3). *p < 0.05. (B) rhIL-15 binding on B leukemic cell surface. The capacity of fluorescent rhIL-15 to bind IL-15Rα was evaluated by flow cytometry in the presence or absence of blocking anti-IL-15 or anti-IL-15Rα mAbs. Data are mean fluorescence intensity (MFI) ± SEM of fluorescent rhIL-15 binding on B leukemic cells (n = 5). *p = 0.03. (C) PBLs from CLL patients were treated or not with an Ab directed at IL-15Rα in the presence or absence of rhIL-15 (10 ng/ml) for 7 d and then analyzed by flow cytometry. Results represent the fold increase ± SEM of NK cells in rhIL-15–treated conditions compared with untreated conditions in the presence or absence of anti-IL-15Rα (n = 5). *p < 0.05. ns, Not significant.

15 trans-presentation to NK cells. Finally, rhIL-15–induced NK cell proliferation was evaluated in the presence of an IL-15Rα–blocking Ab in CLL samples. Blockade of IL-15 binding to B
leukemic cells using neutralizing Ab completely abrogated rhIL-15–induced NK cell proliferation (Fig. 4C).

Therefore, B leukemic cells act as accessory cells for rhIL-15 trans-presentation, leading to proliferation of autologous NK cells in CLL.

**Discussion**

This study demonstrates that NK cells from CLL patients can be stimulated and expanded by autologous B leukemic cells trans-presenting rhIL-15. In the presence of mAbs, especially GA101, this process is greatly amplified and leads to better B leukemic cell depletion.

Some immunotherapeutic strategies have aimed at increasing the cytotoxic E:T cell ratio. These approaches were essentially based on the use of IL-2 to activate and expand cytotoxic cell populations. Nevertheless, in vivo administration of high doses of IL-2 leads to systemic toxicity (36, 37). Other strategies have been developed using a combination of phosphoantigens and low doses of IL-2–targeting cytotoxic Tγδ lymphocytes. This concept was studied in vitro in CLL samples, and we previously showed a proliferation of cytotoxic Tγδ lymphocytes in some CLL patients (38); however, the amplification level and the cell surface expression of CD16 by these effectors were too weak to be of therapeutic interest.

More recently, IL-15 has emerged as a new candidate to improve immunotherapy (6, 39). In rhesus macaques, rhIL-15 can expand immune effector cells without the toxicity of IL-2 (40, 41). The use of rhIL-15 in human cancers is being evaluated in a phase I clinical trial in patients with refractory metastatic melanoma and metastatic renal cell cancer (http://clinicaltrials.gov/ct2/show/NCT01021059).

IL-15 plays a pleiotropic role in homeostasis and regulation of immune cell populations by stimulating tumor-reactive CD8+ T lymphocytes and NK cells. This point could prove crucial in CLL, in which either a deficit in CD8+ synaptic function or a low E:T ratio could contribute to immune escape (42, 43). In CLL, the E:T ratio (median NK/B ratio = 0.02:1) favors the leukemic cell population and could hamper the NK-mediated ADCC process (44). In this case, agents increasing this ratio could induce B leukemic cell depletion. We demonstrate in this study that rhIL-15 combined with therapeutic Abs induces NK cell activation and, especially, NK cell proliferation, leading to an increase in the NK/B ratio. This correlates with B leukemic cell depletion in vitro.

The prevailing mechanism of endogenous IL-15 action in vivo is its trans-presentation by accessory cells. The current theory is that intracellular IL-15/IL-15Rα complexes are shuttled to the cell surface of monocytes or DCs to stimulate neighboring cells expressing β/γ receptor chains, such as NK cells. In addition, a recent study demonstrated that in vitro coinubation of humanized IL-15Rα–IgG1-Fc mimicked IL-15 trans-presentation to healthy donors’ NK and CD8+ cells (45). In the context of CLL, B leukemic cells are not described to produce endogenous IL-15. In this study, we demonstrate that rhIL-15–induced NK cell proliferation is strictly dependent on NK cell–B leukemic cell interaction via IL-15Rα. To our knowledge, our results identify for the first time B leukemic cells as new accessory cells able to trans-present rhIL-15 to NK cells, leading to their proliferation. Moreover, this phenomenon is re-enforced in the presence of anti-CD20 Abs, highlighting the cooperative role of CD16- and IL-15-signal cascades in NK cell proliferation. rhIL-15 trans-presentation could also explain the more preserved CD16 expression in rhIL-15/ anti-CD20 Ab culture conditions.

How might such a low level of B cell surface IL-15Rα suffice for rhIL-15 trans-presentation to NK cells? One could hypothesize that the high level of B leukemic cells compensates for this low expression. Thus, the balance between NK cells and B leukemic cells could be restored in the presence of rhIL-15 and therapeutic Abs. This leads to better NK cell cytotoxic function. Therefore, we propose that the combination of rhIL-15 and anti-CD20 facilitates the immunological synapse between NK and B cells, thereby inducing NK cell proliferation and ultimately increasing CLL cell death.

Recently, Moga et al. (27) demonstrated that rhIL-15 could overcome the immunosuppressive effects of exogenous TGF-β in CLL samples in vitro, leading to better RTX–mediated ADCC. High levels of TGF-β activity were detected in CLL samples in vivo (46) and in vitro (47). Therefore, TGF-β could contribute to the immune deficit in CLL. In addition, TGF-β inhibits CD16-mediated IFN-γ production and ADCC in human NK cells from healthy donors (48). Thus, in vivo administration of rhIL-15 to CLL patients could activate and expand cytotoxic effectors, as well as inhibit the immunosuppressive effects of TGF-β on NK cells. This, combined with mAbs and/or chemotherapy (49), could induce a therapeutic benefit.

In summary, rhIL-15 increases B leukemic cell depletion mediated by mAbs. This involves both rhIL-15 NK cell activation and proliferation. This proliferation is strictly dependent on a new mechanism of rhIL-15 trans-presentation by B leukemic cells to autologous NK cells. This phenomenon is improved in the presence of anti-CD20 via IL-15R/CD16 cosignaling. Thus, rhIL-15 associated with ADCC-inducing therapeutic Abs, such as GA101, represents an attractive immunotherapy for CLL treatment.

**Acknowledgments**

We thank Christian Klein (Roche Glycart) for providing RTX and GA101 Abs, Maud Charley and Amandine Blanc for technical assistance, and Dr. K. Thorner and Dr. N. Jabrane-Ferrat for scientific English proofreading. We are grateful to the patients who participated in the study and their referring physicians.

**Disclosures**

C.K. is an employee of Roche Glycart, E.L. and A.Q.-M. are inventors on a patent application related to these findings and received support from Roche. The other authors have no financial conflicts of interest.

**References**


Supplemental Data 1: Anti-CD20 binding. PBMC samples from CLL were treated with control h-IgG (10μg/mL), RTX (10μg/mL) or GA101 (10μg/mL) with or without rhIL-15 (10ng/mL) for 1 hour (day 0) or 7 days. Anti-CD20 binding was analyzed by flow cytometry using a secondary PE-Goat anti-human antibody staining on gated remaining CD19+/CD5+ B leukemic cells (more than 10,000 CD19+/CD5+ events acquired; n=5); *p<0.05; a.u.: arbitrary units.
Supplemental Data 2: B leukemic cell depletion induced by monoclonal antibodies and rhIL-15 in CLL samples.

PBMC from CLL patients were treated with control h-IgG (10μg/mL), RTX (10μg/mL), GA101 (10μg/mL), RTX-F(ab’2) (10μg/mL) or GA101-F(ab’2) (10μg/mL) in the presence or not of rhIL-15 (10ng/mL) during 7 days, then analyzed by flow cytometry. Percentage of CD19+/CD5+ cell depletion compared to control h-IgG treated cells was assessed as described in Materials and Methods (n=7). Horizontal bars represent the median value (*p<0.05; **p<0.01)
Supplemental Data 3: Viability of purified B leukemic cells after 7 days’ treatment. Purified B leukemic cells from CLL patients were treated with control human IgG (10μg/mL), RTX (10μg/mL) or GA101 (10μg/mL) with or without rhIL-15 (10ng/mL) for 7 days and analyzed by flow cytometry. B-cell viability was assessed by 7AAD/Annexin-V staining (7AAD negative/Annexin negative CD19+/CD5+ cells) (n= 3).
Supplemental Data 4: rhIL-15 and monoclonal antibodies do not induce B leukemic cell proliferation in CLL samples. PBMC samples from CLL were treated with control human IgG (10μg/mL), RTX (10μg/mL) or GA101 (10μg/mL) with or without rhIL-15 (10ng/mL) for 7 days then analyzed by flow cytometry (more than 10,000 CD19+/CD5+ events acquired). Figure shows a representative experiment of CFSE dilution on B leukemic cells (n=21).