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Lymphocyte subpopulation imbalances, bone marrow hematopoiesis and histopathology in rituximab-treated lymphoma patients with late-onset neutropenia

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Several observations implicate the administration of rituximab in the development of severe late-onset neutropenia (LON) in some patients with lymphoma treated with rituximab ± chemotherapy.[1–5] The incidence of LON varies between series; this might be explained, at least in part, by the failure to detect some neutropenic episodes due to their short duration, the relatively long time to onset and the usually uncomplicated course. However, there is a gradual increase in the frequency of rituximab-related LON probably due to the widespread use of rituximab in the standard treatment of lymphomas and also due to the ongoing awareness for this event.

Several groups, including ours, have provided indirect evidence to implicate an immune-mediated mechanism in the pathogenesis of rituximab-associated LON.[1–3,6] However, published data are mainly based on small series and are often contradictory, perhaps due to the selective evaluation of isolated parameters. Our previous studies suggesting that LON in rituximab-treated lymphoma patients may be associated with T-cell large granular lymphocytic (T-LGL) proliferation[1–2] alluded to the possibility of altered T-cell responses associated with B-cell depletion induced by rituximab. To probe this hypothesis further, in the present study, we performed a detailed immunological and immunohistological study looking for quantitative changes and/or an activated profile of lymphocytes in rituximab-treated patients with LON, focusing on the possibility of T-cell-mediated suppression of bone marrow (BM) hematopoiesis.

The study included 12 patients (10 men and 2 women) with a median age of 48 years (range, 26–67 years) who developed unexplained LON after treatment with rituximab ± chemotherapy for diffuse large B-cell lymphoma (DLCL; $n=4$), chronic lymphocytic leukemia (CLL; $n=4$), mantle-cell lymphoma (MCL; $n=3$) or splenic marginal-zone lymphoma (SMZL; $n=1$). LON was defined as the unexplained reduction in neutrophil counts $\leqslant 1.0\times10^9\,l^{-1}$ (grade 3 according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC)) following neutrophil recovery after completion of the intended treatment with rituximab ± chemotherapy, without evidence of disease progression and before addition of chemotherapy. One DLCL patient developed neutropenia while on maintenance monotherapy with rituximab. The remainder ($n=11$) received rituximab in combination with CHOP (DLCL, MCL, SMZL) or fludarabine–cyclophosphamide (CLL). LON occurred at a median of 95 (range, 67–420) days after the last administration of rituximab. Neutrophil nadir during neutropenia episodes in each patient ranged from 0.01×10^9 to $0.85\times10^9\,l^{-1}$ (median $0.52\times10^9\,l^{-1}$). The recovery from neutropenia was observed at a median of 56 (range, 28–251) days. In all cases, clinical, serologic, virologic or ultrasonic investigation for any underlying cause for neutropenia was negative. Furthermore, all patients tested negative on extensive autoantibody screening (including direct antiglobulin test, rheumatoid factor, antiplatelet and antinuclear antibodies) and did not exhibit spleen enlargement on ultrasound scan of the abdomen.

The study also included two control groups: (i) healthy donors ($n=25$) for comparative analysis of BM functional parameters; (ii) non-Hodgkin lymphoma (NHL) patients treated with similar rituximab ± chemotherapy regimens without LON for comparative

evaluation of peripheral blood (PB) and BM lymphocyte subpopulations and BM pathology findings.

The methodologies applied in the present study are provided in the Supplementary Information file.

To explore whether the development of LON in rituximab-treated patients was associated with quantitative changes and/or an activated immunophenotypic profile of lymphocytes, we performed a detailed flow cytometric study of PB and BM cells. Patients with LON were followed up with flow cytometry analyses at regular intervals for up to 2 years after the onset of neutropenia (median number of tests: 3; range, 2–11). Lymphocyte subsets were also determined by flow cytometry in PB samples from a series of 38 patients with various types of NHL (CLL/SLL, $n=12$; DLCL, $n=8$; follicular lymphoma (FL), $n=10$; MCL, $n=3$; SMZL, $n=4$; lymphoplasmacytic lymphoma, $n=1$), who were treated with similar rituximab-containing regimens (mainly rituximab–fludarabine–cyclophosphamide for CLL/SLL and rituximab–CHOP for DLCL and a subset of five FL patients) or rituximab as monotherapy ($n=8$) but did not develop LON for a similar period of observation (control group). Control patients (NHL controls) have been followed up with flow cytometry analyses at regular intervals for up to 2 years after the initiation of the treatment (median number of tests: 3; range, 1–9).

Ten of 12 patients with LON and 33/38 controls (patients without LON) developed profound B-cell depletion (absence of $CD20^+$, $CD19^+$, $CD79\alpha^+$ cells); the remaining two cases (one each CLL and MCL) had residual neoplastic B cells (Supplementary Table 2). Inverted CD4/CD8 cell ratios in both PB and BM samples were observed in 10/12 patients with LON versus 13/38 controls ($P<0.01$); a rise in $CD8^+$ cell count above $1.0\times10^9\,l^{-1}$ was identified in 8/12 cases with LON versus 8/38 controls ($P<0.01$). T-LGL proliferation ($CD3^+CD8^+CD57^+$ cells $>30\%$) was observed in 7/12 cases with LON versus 11/38 controls ($P=0.064$); in all cases, the expanded T-LGL populations were negative for CD28. Increased numbers of $CD3^+CD8^+CD16^+$ cells were not observed in any case. In patients with LON, identical results were obtained by parallel analysis of both PB and BM samples.

The possibility that lymphocyte subset imbalances could be affected by the underlying disease and/or treatment regimen was suggested by the higher incidence of inverted CD4/CD8 ratios, increased CD8+ cells and T-LGL proliferations in CLL patients receiving combinations of rituximab and fludarabine. However, definitive conclusions cannot be drawn due to the low number of patients in each category. That notwithstanding, it is worth noting that no disease and/or treatment bias was evident in the subgroup of patients who developed LON.

Clonally expanded T cells with a restricted TCRβ variable gene usage have been shown to recognize tumor cells in patients with malignancies, including B-cell lymphomas. In particular, patients with CLL may spontaneously mount leukemia cell-specific CD4 and CD8 T-cell responses recognizing multiple leukemia cell-associated antigens.[7] In the present study, clonality assessment of T cells in cases with increased numbers of $CD3^+CD8^+$ and/or $CD3^+CD8^+CD57^+$ ($>30\%$) cells was performed by quantitative analysis of different variable regions of the *TCRβ* chain (Vβ repertoire) by means of flow cytometry using the IOTest Beta Mark kit (Beckman-Coulter, Marseille, France). Expanded Vβ subsets were identified in three cases with LON and six NHL controls. Multiplex PCR analysis did not detect a monoclonal *TCRβ* or *TCRγ* gene rearrangement in any of the above cases. Although detailed flow cytometry evaluation of T-cell subpopulations before initiation of rituximab was not performed for most patients included in the present study, the possibility of pre-existing immunodominant T-cell clones, which further expanded after rituximab-induced B-cell depletion, cannot be ruled out, at least for some patients.

Increased rate of apoptosis was observed in all stages of the granulocytic development in patients compared to healthy controls as shown by the proportions of apoptotic cells in the $CD34^+/CD33^+$, $CD34^-/CD33^+/CD15^+$ and $CD34^-/CD33^-/CD15^+$ cell compartments ($P<0.0001$, $P<0.01$ and $P<0.0001$, respectively; Supplementary Table 3). Similarly, increased proportions of apoptotic cells were found in the $CD34^+/CD71^+$, $CD36^+/GlycoA^+$ and $CD36^-/GlycoA^+$ sequential stages of the erythroid development, in patients compared to healthy controls ($P<0.01$, $P<0.0001$ and $P<0.0001$, respectively).

Activated as well as neoplastic T-LGLs express and secrete large amounts of Fas and FasL. Therefore, neutropenia in the context of T-LGL proliferations has been associated, at least in part, with neutrophil apoptosis through Fas/FasL pathway.[8] The BM $CD34^+$ cells express only very low levels of Fas under steady conditions but overexpress the antigen under the influence of tumor necrosis factor-α and/or interferon-γ, resulting in FasL-mediated apoptotic cell death. Remarkably, patients with LON, included in this study, displayed increased proportion of Fas-expressing cells within the $CD34^+/CD33^+$ cell compartment as well as increased FasL RNA expression in their $CD3^+$ cell population, compared to healthy donors (16.25 ± 9.20 versus $5.77\pm3.40\%$, respectively; $P<0.0001$), whereas no statistically significant difference was found in the proportion of Fas^+ cells within the $CD34^-/CD33^+/CD15^+$ and $CD34^-/CD33^-/CD15^+$ subpopulations. Interestingly, all patients but none of the healthy controls ($n=5$) displayed FasL mRNA expression ($P<0.001$) in purified $CD3^+$ cells. Similarly, interferon-γ mRNA expression was found in the BM $CD3^+$ cells from all patients but none of the control subjects ($n=5$) ($P<0.001$). Therefore, FasL production by expanded T-LGLs post-rituximab may induce apoptosis in granulocytic and erythroid BM progenitor cells that normally or abnormally express Fas under the influence of T-cell-derived interferon-γ and locally produced tumor necrosis factor-α.

Recent evidence suggests that $CD8^+CD57^+$ T-LGLs from healthy individuals may induce proinflammatory cytokine production and therefore suppress neutrophil precursors independent of Fas/FasL interactions.[9] Along these lines, we studied tumor necrosis factor-α and interleukin-1β levels in supernatants of patient long-term BM cultures that represent an approximation of BM microenvironment cells. Although the origin of the cytokine-producing cells in long-term BM culture adherent layers remains elusive, the remarkably increased tumor necrosis factor-α and interleukin-1β levels in LON patients (6.16 ± 5.31 and $2.32\pm1.65\,pg\,ml^{-1}$, respectively; $P=0.0144$) compared to normal controls (2.01 ± 1.56 and $1.14\pm0.73\,pg\,ml^{-1}$, respectively; $P=0.0388$) (Supplementary Figure 1) suggest an inflammatory milieu in patients' BM probably in the setting of a cross talk between lymphocytes and other immune cells that may further contribute to the associated cytopenias.

Flow cytometric analysis of BM and PB cells showed that patients with LON displayed significantly lower proportion of $CD34^+$ cells ($1.18\pm1.17\%$) compared to controls ($2.44\pm1.36\%$, $P=0.0008$) due to the lower percentage of the committed $CD34^+/CD38^+$ (1.00 ± 1.19 and $2.05\pm1.29\%$, respectively; $P=0.0062$) but not the primitive $CD34^+/CD38^-$ (0.18 ± 0.16 and $0.40\pm0.30\%$, respectively; $P=0.0711$) cells. Further analysis showed a significant decrease in the proportion of myeloid $CD34^+/CD33^+$ and erythroid $CD34^+/CD71^+$

progenitor cells in patients (0.30 ± 0.45 and $0.58 \pm 0.57\%$, respectively) compared to healthy controls (0.52 ± 0.31 and $0.89 \pm 0.50\%$, respectively; $P = 0.021$ and $P = 0.047$, respectively) but normal megakaryocytic $CD34^+/CD61^+$ progenitors ($P = 0.067$). Interestingly, a statistically significant increase was observed in the proportion of $CD36^+/GlycoA^+$ early erythroid precursor cells in patients ($42.65 \pm 16.06\%$) compared to healthy controls ($29.16 \pm 10.10\%$, $P = 0.0212$), which was associated with a significant decrease in the proportion of $CD36^-/GlycoA^+$ mature erythroid precursor cells in patients ($39.42 \pm 18.64\%$) compared to control subjects ($57.75 \pm 14.25\%$, $P = 0.0109$). The results from the clonogenic assays were in accordance with the flow cytometry data (Supplementary Figure 2). All the above suggest that patients with rituximab-associated LON display low reserves of granulocytic and erythroid progenitor cells in the BM but retain normal numbers of megakaryocytic progenitor cells. Our findings are in accordance with previous data in T-LGL leukemia patients presenting with neutropenia and anemia, who exhibit fewer granulocytic and erythroid precursors, respectively.[10] Furthermore, a similar specific inhibition of CFU-E and BFU-E by leukemic T-LGLs has been reported in T-LGL leukemia patients developing cyclic neutropenia and pure red cell aplasia, respectively.[10]

Because patients with T-LGL proliferations display a high incidence of autoimmune cytopenias, we evaluated patient sera for granulocyte-reactive antibodies. None of the patients studied displayed serum antineutrophil antibody activity as shown by both granulocyte immunofluorescence (GIFT) and agglutination (GAT) techniques.

Bone marrow biopsy samples from 10/12 patients with LON were examined morphologically and immunohistochemically at onset of neutropenia. A group of 27 patients who were treated with similar rituximab-containing regimens and did not develop LON over a 2-year observation period served as controls. This control group included seven patients with CLL, three patients with DLCL, three patients with FL, five patients with MCL, three patients with SMLZ and four patients with other pathologies. Mild (5%) to moderate (10–15%; one case: 30%) lymphocytic infiltration with predominantly nodular and/or interstitial, non-intrasinusoidal growth was observed in all LON cases (Supplementary Table 4). Although one could postulate residual or relapsed disease based on morphological evaluation, detailed immunohistochemical analysis showed that the lymphoid aggregates lacked $CD20^+$ and $CD79a^+$ B cells and were composed entirely of $CD3^+$, $CD45RO^+$ ($CD3 >> CD45RO$) and $CD43^+$ T cells. Interestingly, $CD8^+$ cells predominated over $CD4^+$ cells in 7/10 analyzed LON cases. In the control group, mild BM infiltration by T cells with similar morphological and immunohistochemical features as described above was observed in 18/27 cases. All nine cases without T-cell infiltrates as well as two cases with a mild T-lymphocytic infiltration had evidence for residual BM disease, as suggested by the detection of $CD20+CD79\alpha+$ cells (five cases) or exclusively $CD79\alpha+$ cells (four cases). On the basis of the above findings, histopathological evaluation of BM biopsies after rituximab administration should always include ancillary immunophenotypic studies so as to avoid a misdiagnosis of residual disease based on morphology alone.

Morphological and immunohistological examination of BM biopsy specimens showed a significant decrease of the granulocytic series in 7/10 samples, whereas three cases exhibited granulocytic hyperplasia. However, all samples were uniformly characterized by a moderate-to-pronounced shift to the left, with abnormal localization of immature precursors (ALIP)-like features (myeloperoxidase-positive) extending to maturation arrest in 6/10 cases. In stark contrast to the hypoplasia of the granulocytic series observed in LON cases, both in the present study and in all published reports, the great majority (21/27 cases; 78%) of similarly treated NHL cases without LON who were used as controls in the present study showed granulocytic hyperplasia; the remaining cases were either normal or showed granulocytic hypoplasia. As in the LON cases, the control NHL cases without LON exhibited different degrees of shift to the left of the granulocytic series, albeit always less pronounced than in LON cases. These findings generally correspond to our flow cytometry and clonogenic assay data on BM functional parameters in LON cases. They are also in agreement with sporadically published reports on BM biopsy findings in patients developing rituximab-associated LON.[4,6] Importantly, similar to our data, predominantly granulocytic hypoplasia with maturation arrest was a common finding in all studies. Therefore, clinicians should be aware of rituximab-induced LON and associated BM dysplasia to avoid a misdiagnosis of primary or secondary myelodysplastic syndrome (MDS).

Both the erythroid and megakaryocytic series were increased in all BM biopsy samples of LON cases and in most NHL controls without LON. However, for the group of patients with LON, given the reduction of the granulocytic series in the majority of cases, this increase may be relative, perhaps accounting for the apparent contrast between histological and flow cytometry/clonogenic assay results obtained for the erythroid and megakaryocytic lineages in LON cases. Finally, in all LON patients of the present study and most lymphoma control cases without LON, both the erythroid and the megakaryocytic series showed dyserythropoiesis and dysmegakaryopoiesis, respectively, with abnormal paratrabecular localization, suggestive of MDS. Dyserythropoiesis was also reflected by the accelerated rate of apoptosis within the BM erythroid cell populations, which was associated with increased rate of cell proliferation as indicated by the increased proportion of $CD36^+/GlycoA^+$ cells. Taken together, the above data provide evidence that rituximab-treated lymphoma patients show a spectrum of abnormalities of the myeloid series associated with dyshematopoiesis, very frequently in a setting of expanded T-LGL populations. Along these lines, similar MDS-like features have been reported in patients with T-LGL leukemia, often leading to a misdiagnosis of primary MDS, especially in patients with cytopenias and unremarkable BM lymphocytic infiltration by malignant T-LGLs ('insidious' T-LGL leukemia).

A possibility that should also be considered for the MDS-like changes reported in the present series is lymphoma-related dyshematopoiesis, which is observed in BM biopsies of lymphoma patients before treatment and often gets unrecognized. Furthermore, one might argue that the myelodysplastic features observed in our LON patients might be related to previous chemotherapy. However, both in the present study and in previous studies from our group, MDS-like changes were observed even in lymphoma patients treated with rituximab alone.[2] That notwithstanding, the possibility of secondary, treatment-related MDS could be excluded based on either negativity or very low numbers of $CD34+$ immature precursors. Also, of note, as revealed by examination of follow-up BM biopsy samples (available in 5/12 cases with LON and 24/27 cases of the control group), the observed MDS-like changes eventually disappeared.

In conclusion, the present study confirms recent data on expansions and imbalances of T-cell subsets in a setting of

abolished B–T-cell cross talk as a result of B-cell depletion in lymphoma patients treated with rituximab. Significantly more pronounced T-cell expansions/imbalances were observed in patients with LON who also exhibited extensive hypoplasia of the granulocytic series with concomitant dyshematopoiesis of moderate-to-severe degree affecting all myeloid series. Therefore, our study demonstrates a remarkable analogy between rituximab-associated LON and neutropenia associated with T-LGL leukemia, which is characterized by BM dyshematopoiesis and/or maturation arrest of the granulocytic series mimicking primary MDS. Of note, BM abnormalities were also evident in rituximab-treated patients without LON, who, in stark contrast to LON cases, were characterized by hyperplasia of all myeloid series albeit again associated with varying degrees of dyshematopoiesis. Taken together, our findings indicate that rituximab-associated LON is multifactorial and may perhaps represent the end of a spectrum of immunohematological sequellae in the context of T-LGL-mediated autoimmune myelopathy/myelodysplasia.

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Inhibition of MEK/ERK signaling synergistically potentiates histone deacetylase inhibitor-induced growth arrest, apoptosis and acetylation of histone H3 on p21^{waf1} promoter in acute myelogenous leukemia cell

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Histone deacetylase inhibitors (HDACIs) have emerged as a potentially promising new class of anticancer drugs.[1] These include the hydroxamic acid-derived suberoylanilide hydroxamic acid (SAHA, vorinostat), LBH589, tricostatin A, cyclic depsipeptide FR901228 and the benzamide MS-275.[2] HDACIs induce growth arrest and apoptosis of cancer cells by manipulating the transcription of genes involved in regulation of the cell cycle and apoptosis, as well as differentiation.[1] For example, we previously showed that SAHA induces growth arrest and apoptosis of human mantle cell lymphoma cells in association with induction of histone acetylation of the p21^{waf1} promoter region, resulting in upregulation of the p21^{waf1} protein.[3]

This study found that MS-275 induces growth arrest of human acute myelogenous leukemia (AML) HL-60 and NB4 cells, as well as freshly isolated leukemia cells from individuals with AML with concentration that induced 50% inhibition (IC_{50}) values less than 1 μM on day 2 of culture, as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and thymidine uptake, respectively (Figure 1a, Table 1). Western blot analyses showed that exposure of these cells to MS-275 downregulated levels of antiapoptotic molecules Bcl-2 and