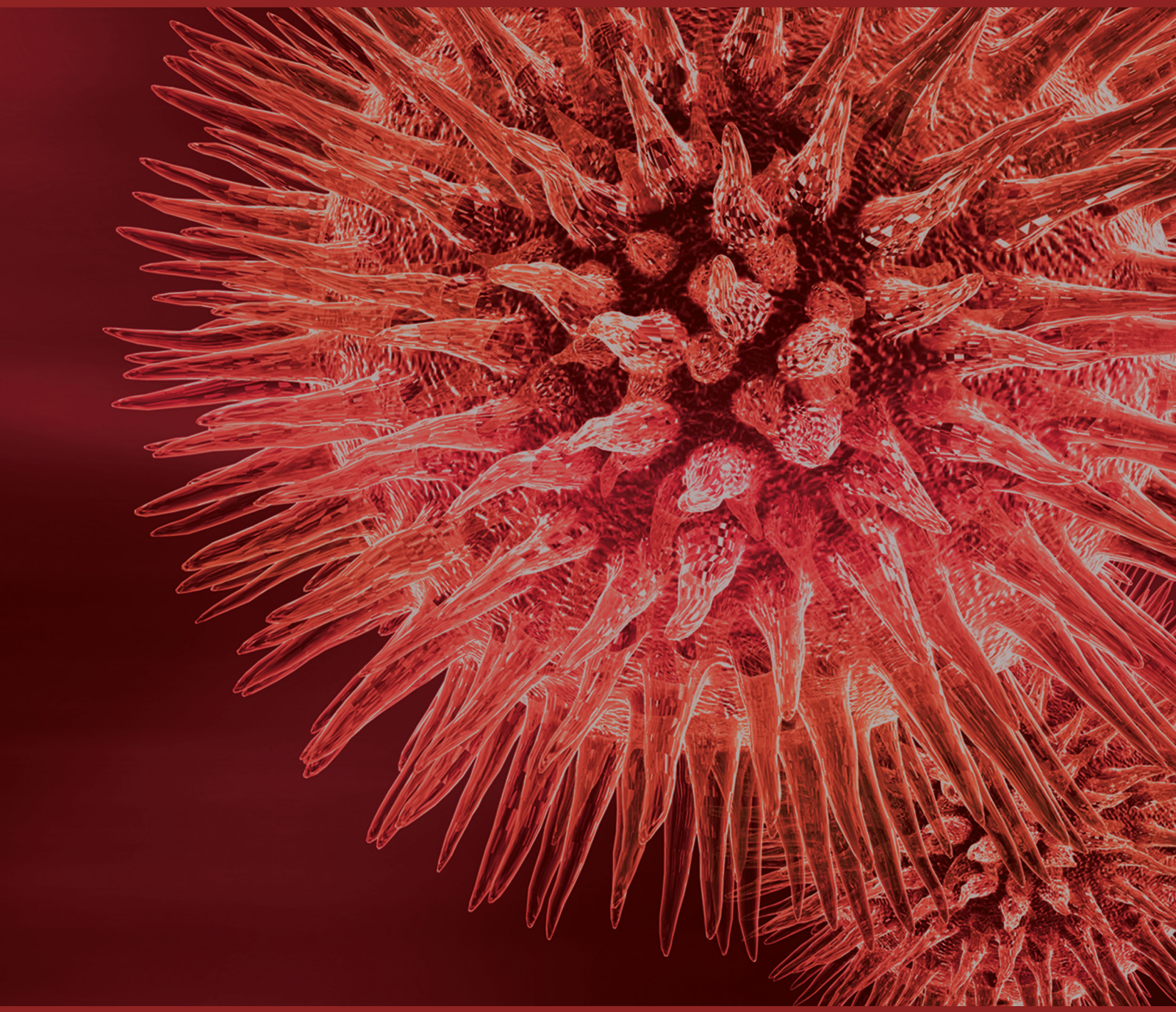
BioMed Research International

New Insights into Malignant B-Cell Disorders

Guest Editors: Marie-Christine Kyrtsonis, Kazuyuki Shimizu, Panayiotis Panayiotidis, and Gerassimos A. Pangalis



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Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 128084, 3 pages
http://dx.doi.org/10.1155/2015/128084

Editorial

New Insights into Malignant B-Cell Disorders

Marie-Christine Kyrtsonis,[1] Kazuyuki Shimizu,[2] Panayiotis Panayiotidis,[1] and Gerassimos A. Pangalis[3]

[1]*Hematology Section of First Department of Propaedeutic Internal Medicine, University of Athens Medical School, Laikon General Hospital of Athens, Athens, Greece*
[2]*The Japanese Society of Myeloma, Hematology Section of Internal Medicine, Tokai Chuo Hospital, Kakamigahara, Japan*
[3]*Department of Haematology, Athens Medical Center, Psychikon Branch, Athens, Greece*

Correspondence should be addressed to Marie-Christine Kyrtsonis; mck@ath.forthnet.gr

Received 8 December 2014; Accepted 8 December 2014

Mature B-cell disorders comprise numerous and very different entities including all B-cell lymphomas (NHL) subgroups, chronic lymphocytic leukemia (CLL), and plasma cell dyscrasias [1].

Growing knowledge on disease biology has led to the development of new drugs, while innovative diagnostic techniques have resulted in improvement of diagnosis, establishment of new prognostic factors, and the recognition of new entities.

Original research and review articles published in this special issue highlight some interesting aspects of this topic. Herein, we will briefly present these papers and the scientific background they rely on.

The treatment of symptomatic multiple myeloma (MM), a severe plasma cell dyscrasia, with considerable morbidity and a reported, until recently, disappointing median survival of 3–5 years, has incredibly changed over the last decade, for the benefit of patients. Prolonged remission duration and improved survival with a better quality of life can be now observed, while the notion of an eventual cure does not seem so improbable anymore [2, 3]. The new drugs that led to the aforementioned improvements were not "chemotherapeutic" agents in the classical sense of the term because they were all "biology-modulating" factors. The first three "so-called" new drugs that constitute today the backbone of MM treatment are (i) bortezomib, the first-in-class reversible proteasome inhibitor that downregulates the three major disease expansion mechanisms, namely, cell escape from apoptosis, neoangiogenesis, and osteoclast activation and (ii) thalidomide and (iii) thalidomide-analog lenalidomide that both commonly exert antiangiogenic, anti-inflammatory, and immunomodulatory functions. Today, next generation novel agents are already available and others under evaluation, while treatment with monoclonal antibodies (mAbs) against disease specific targets has been developed and appears promising [4].

mAb treatment alone or in combination with chemotherapy (immunochemotherapy) begun much earlier for the treatment of B-NHL and CLL with the introduction in the late 90s of a humanized monoclonal antibody against CD20, rituximab, resulting in a significant improvement of patients' response rates. Afterward, intense research efforts were made and are still ongoing, to exploit the better knowledge of disease pathogenesis and to develop biologically active drugs in the field of B-NHL and CLL.

With regard to CLL, which is a relatively common leukemia of the elderly in western countries, it behaves indolently in more than half of patients but may have more aggressive behavior that should be immediately and effectively treated. Conventional therapy included alkylators, purine analogs, and corticosteroids, while the introduction of rituximab constituted a significant improvement. Numerous novel agents and mAbs are on their way, of which ibrutinib, an oral inhibitor of Bruton's tyrosine kinase, that promotes apoptosis and inhibits proliferation, migration, and adhesion of CLL cells [5] is already available. The same agent has

shown activity in mantle cell lymphoma and other indolent B-NHL. Immunomodulatory agents, such as lenalidomide, that is currently routinely used for the treatment of relapsed MM showed also activity in relapsed/resistant CLL. In this context, it should be administered at much lower doses than in MM to avoid the dangerous flair phenomenon. The biologic background of new drugs activity is an interesting subject, and, intriguingly, variations in their mode of action may be observed among different B-cell entities.

Another therapeutic modality, high dose therapy with autologous hematopoietic stem cell (HSC) transplantation, although being a "classical and old" aggressive treatment option that can be applied to almost all B-cell disorders when it is for the benefit of patient to consolidate remission, had also improvements concerning stem cell mobilization. Knowledge of the regulation of HSC circulation and homing and adhesion to their surrounding milieu in the stem cell niche has led to the development of drugs that inhibit HSC adhesion and allow a better harvest.

Because of these therapeutic improvements, other fields of disease research had to move on. For example, the well-established prognostic factors and systems for MM, CLL, and B-NHL that mainly include respective staging, clinical and molecular adverse markers, could be shadowed in the era of biologic treatments. Reevaluation of these prognostic factors and the establishment of new ones is ongoing [6].

In the field of B-NHL, diffuse large B-cell lymphoma is the most frequently occurring entity and therefore has been better studied [7]. There are however some very rare entities, such as primary effusion lymphomas (PEL) and Waldenstrom's macroglobulinemia (WM), that remain largely understudied concerning the knowledge of their biology and optimal treatment's options. PEL-NHL is considered a very aggressive B-NHL caused by human herpesvirus type 8; it usually, but not always, occurs in HIV-infected patients. In HIV negative patients, its pathogeny is obscure, and in the absence of large series, there is no standard therapy [8]. Aggressive treatments have been administered to patients with conflicting results but also, in some instances, only drainage of the malignant effusions and pleurodesis with bleomycin (when the disease was located into the pleural cavity) produced prolonged remissions [9]. WM is an indolent lymphoplasmacytic lymphoma secreting an IgM component that may present resembling clinicolaboratory features and diagnostic overlap with other noncleaved small B-cell NHL without typical genetic features, such as marginal zone, mucosa associated, and small lymphocytic lymphomas [10]. Immunoglobulin heavy chain (IgH) gene rearrangement is the turning point of B-cell differentiation; likewise, IgH clonotypic sequence analysis may reveal the origin of the clone and/or preferential VH usage, helping differential diagnosis.

In addition, novel techniques and large multicenter studies have allowed the recognition of precursor B-cell disorders. It is now highly probable that all B-cell disorders are preceded by a premalignant condition. Few years ago, it became evident that all myelomas are preceded by monoclonal gammopathy of undetermined significance [11]. Then, "in situ" lymphomas were described. They are usually incidental findings and their risk of progression to clinically overt lymphoma is not known. Such lesions have been recognized for both follicular and mantle cell NHL and it could be assumed that they represent a preneoplastic condition that could regress by itself or on the contrary further evolve [12]. Another possible precursor condition of leukemic B-cell disorders, monoclonal B-cell lymphocytosis [13], is characterized by the presence of less than 5000×10^9/L circulating clonal B-cells that can be CD5+ or CD5−. Usage of multiparameter flow cytometry has revealed that it is not a rare finding in the general healthy population [14]. Its significance remains to be defined.

In conclusion, we indeed realize that there is an enormous amount of new information concerning B-cell disorders which was not approached; we however hope that the aspects developed in this special issue will be appealing to the reader, either haematologist or physician or scientist involved in the field.

Marie-Christine Kyrtsonis
Kazuyuki Shimizu
Panayiotis Panayiotidis
Gerassimos A. Pangalis

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 104912, 8 pages
http://dx.doi.org/10.1155/2015/104912

Review Article

Targeting of Prosurvival Pathways as Therapeutic Approaches against Primary Effusion Lymphomas: Past, Present, and Future

Marisa Granato, Roberta Santarelli, Roberta Gonnella, Antonella Farina, Pankaj Trivedi, Alberto Faggioni, and Mara Cirone

Department of Experimental Medicine, Istituto Pasteur Fondazione Cenci Bolognetti, Sapienza University, 00161 Rome, Italy

Correspondence should be addressed to Alberto Faggioni; alberto.faggioni@uniroma1.it
and Mara Cirone; mara.cirone@uniroma1.it

Received 28 February 2014; Accepted 29 October 2014

Academic Editor: Panayiotis Panayiotidis

Constitutively activated prosurvival pathways render cancer cells addicted to their effects. Consequently they turn out to be the Achilles' heels whose inhibition can be exploited in anticancer therapy. Primary effusion lymphomas (PELs) are very aggressive non-Hodgkin's B cell lymphomas, whose pathogenesis is strictly linked to Kaposi's sarcoma herpesvirus (KSHV) infection. Here we summarized previous studies from our and other laboratories exploring the cytotoxic effect of drugs inhibiting the main prosurvival pathways activated in PEL cells. Moreover, the immunogenicity of cell death, in terms of dendritic cell (DC) activation and their potential side effect on DCs, is discussed.

1. Primary Effusion Lymphomas' Biology

Primary effusion lymphomas (PELs) are very aggressive non-Hodgkin's B cell lymphomas with poor prognosis that frequently arise in immune-compromised patients [1]. PELs originate from postgerminal center B cells and exhibit indeterminate immune-phenotypes; that is, they express CD45, CD138, and activation-associated antigens (CD30, CD38, and HLA-DR) and lack surface expression of B cell markers (CD19, CD20, CD79a, and immunoglobulin) but exhibit clonal rearrangements and somatic hypermutation of the immunoglobulin genes. PELs are strictly associated with a DNA oncovirus, the Kaposi's sarcoma herpesvirus (KSHV), and, in the majority of cases, they are dually infected by a second oncovirus belonging to the gamma-herpesvirus family [2, 3], the Epstein-Barr virus (EBV) [4]. The low response to conventional therapies indicates that new therapeutic strategies are needed for PELs. PEL tumor growth relays on the presence of cytokines that, produced by the tumor itself [5], stimulate their own release with an autocrine positive feedback loop. An important role in PEL survival has been reported for interleukin- (IL-) 6, IL-10, vascular endothelial growth factor (VEGF), and Oncostatin M [6–8]. In addition to cellular cytokines, KSHV encodes its own viral cytokines such as IL-6 which is similar to human IL-6 and exerts an important prosurvival effect on PEL cells [6, 9] and macrophage inhibitory proteins (MIPs) that affect the immune system of the infected host [10].

2. Prosurvival Molecules in PELs

PEL cell survival relays on the constitutive activation of several pathways and also on the hyperexpression of several heat shock proteins (HSPs), a characteristic that is common among the cancer cells.

2.1. JAK2/STAT3 Pathway. By engagement of their specific cellular receptors, cytokines such as IL-6 and IL-10 and VEGF activate the JAK2/STAT3 transduction pathway that promotes the transcription of genes encoding for proteins involved in PEL resistance to apoptosis and contributes to tumor survival [11–13]. Consequently, the specific inhibition of STAT3 activation by AG490 has been reported to induce PEL cell death by downregulating survivin expression [14]. In addition, we have recently reported that AG490 is able to trigger an immunogenic apoptosis in PEL cells [15]. This aspect is of pivotal importance since it is now clear enough that,

without the contribution of the immune system, obtained by triggering an immunogenic cell death type and/or the reversion of the tumor induced immunosuppression [16], no complete eradication of cancer can be achieved by anticancer therapies [17, 18]. Moreover AG490 is able to revert immunosuppression caused by tumor-released factors or induced by tumor viruses such as KSHV, which correlates with STAT3 activation in immune cells [19, 20]. This immune-stimulating AG490 property further encourages its use in PEL anticancer therapy.

2.2. PI3K/AKT/mTOR Pathway. It is known that, in addition to STAT3, cytokines like IL-6 and IL-10, VEGF, and Oncostatin M [21] are able to activate other pathways such as PI3K/AKT and Ras/MAPK that govern fundamental processes [22], such as cell proliferation, differentiation, metabolism, and tumor survival. The PI3K/AKT is constitutively activated in PELs due to the effect mediated by PEL released cytokines and following KSHV infection [23], in particular as an effect of K1 viral protein expression [24]. Previous papers have reported that targeting PI3K/AKT pathway in PEL cells may represent an effective anticancer strategy [25, 26]. We have reported that KSHV infection hyperactivates AKT in THP-1 cells and, as a consequence, the response to proteasome inhibition is reduced. Moreover, AKT activation also leads to Glut1 membrane localization, which is known to promote cell survival by increasing the glucose uptake. On the other hand, we found that Glut1 translocation renders THP-1 cells more susceptible to the glycolysis inhibitor 2-deoxy-D-glucose (DG) effect [27]. This is another example of how a prosurvival effect turns out to be a prodeath effect in tumor cells. Finally, since PI3K/AKT activation leads to the phosphorylation of the downstream molecule mTOR, also its inhibition has been exploited in PEL therapy [28]. mTOR inhibition has been shown to reduce PEL cell survival by interfering with the release of cytokines known to be essential for PEL cell growth, and more recently it has been shown that mTOR inhibition can be effective also against a second KSHV-associated malignancy such as Kaposi's sarcoma.

2.3. MAPK Pathways. Mitogen activated protein kinases (MAPKs) including ERKs, JNKs, and p38 kinases control a vast array of physiological processes [29]. They can be activated in tumor cells by several stimuli such as stress conditions and inflammatory cytokines, also produced by tumor itself. Their inhibition can be potentially explorable in PEL therapy, considering that a cross-talk between these pathways and other prosurvival pathways, such as NF-κB and PI3K/AKT/m-TOR, has been reported [30]. In a recent study, we have shown that JNK inhibition enhances Bortezomib-induced cell death and that SP600125 JNK inhibitor is also able to induce PEL cell death to some extent [31]. A prosurvival role of JNK2 activation during the ER stress caused by tunicamycin treatment has also been shown in a different cell type [32]. In addition, p38 MAPK seems to be activated in PEL cells, in particular in those harbouring KSHV, in comparison with KSHV-negative PEL cells [4].

2.4. NF-κB Pathway. An essential role in PEL cell survival is known to be played by NF-κB, constitutively activated in PEL cells due also to the effect mediated by KSHV-encoded viral FLICE-inhibitory protein (v-FLIP) expression [33, 34]. It has been reported that NF-κB inhibition with Bay11-7082 exerts a strong reduction of PEL cell survival [33]. Moreover, the Bortezomib cytotoxic effect, previously observed on PEL cells, also involves NF-κB inhibition [35], even if its main cytotoxic effect is proteasome inhibition [36]. We have previously explored the immunogenicity of Bortezomib-induced cell death in PEL and showed that Bortezomib was able to induce damage associated molecular patterns (DAMPs) expression on the surface of apoptotic PEL cells, which then resulted in dendritic cell (DC) activation [15, 37]. Moreover, we have demonstrated that Bortezomib induces endoplasmic reticulum (ER) stress and a prosurvival autophagy in PEL cells, due to the accumulation of ubiquitinated proteins consequent to the inhibition of proteasomal degradation. The autophagic blockage, during Bortezomib treatment, further increased its cytotoxic effect. Thus this strategy could be explored in the cancer therapy against PELs [31].

2.5. HSPs. A further effect mediated by the activation of pathways such as Ras/MAPK and JAK/STAT3 is the increase of heat shock protein (HSP) expression [38, 39]. HSPs are known to help cancer cells to survive in the stressful conditions caused by their rapid growth and nutrient shortage. HSPs also counteract the cytotoxic effects induced by chemotherapeutic treatments. For this reason they represent a target for anticancer therapy [38], also considering that their expression, and consequently the cytotoxic effect of their inhibition, is low in normal cells compared with cancer cells. Accordingly, we have recently reported that the inhibition of HSP70, using the small molecule 2-phenylethynesulfonamide (PES), is a successful therapeutic strategy against PEL cells and that PES showed very low cytotoxic effect on normal B cells from which PEL cells arise [40]. PES induced lysosome permeabilization and a necroptotic cell death type in PEL cells with immunogenic properties toward DCs. It represents a valid strategy against this cancer and possibly against other tumors that, displaying oncosuppressor mutations, may be resistant to apoptosis inducing drugs. Also the inhibition of HSP90, another chaperone protein with the essential function in protein correct folding, has been reported to be effective against PELs [41, 42], as well as against multiple myeloma, which shares many similarities with PELs [43, 44].

3. Antiviral Strategies in PELs

PEL cells harbour KSHV, which persists in a latent state in the majority of the cells. Upon appropriate stimuli, latent infection can be switched into lytic productive infection that generally leads to cell lysis and spread of the viral particles. The possibility to kill tumor cells by inducing viral replication can be explored in the therapy against tumors latently infected with DNA viruses, especially because during viral replication tumor cells become sensitive to the effects induced by the

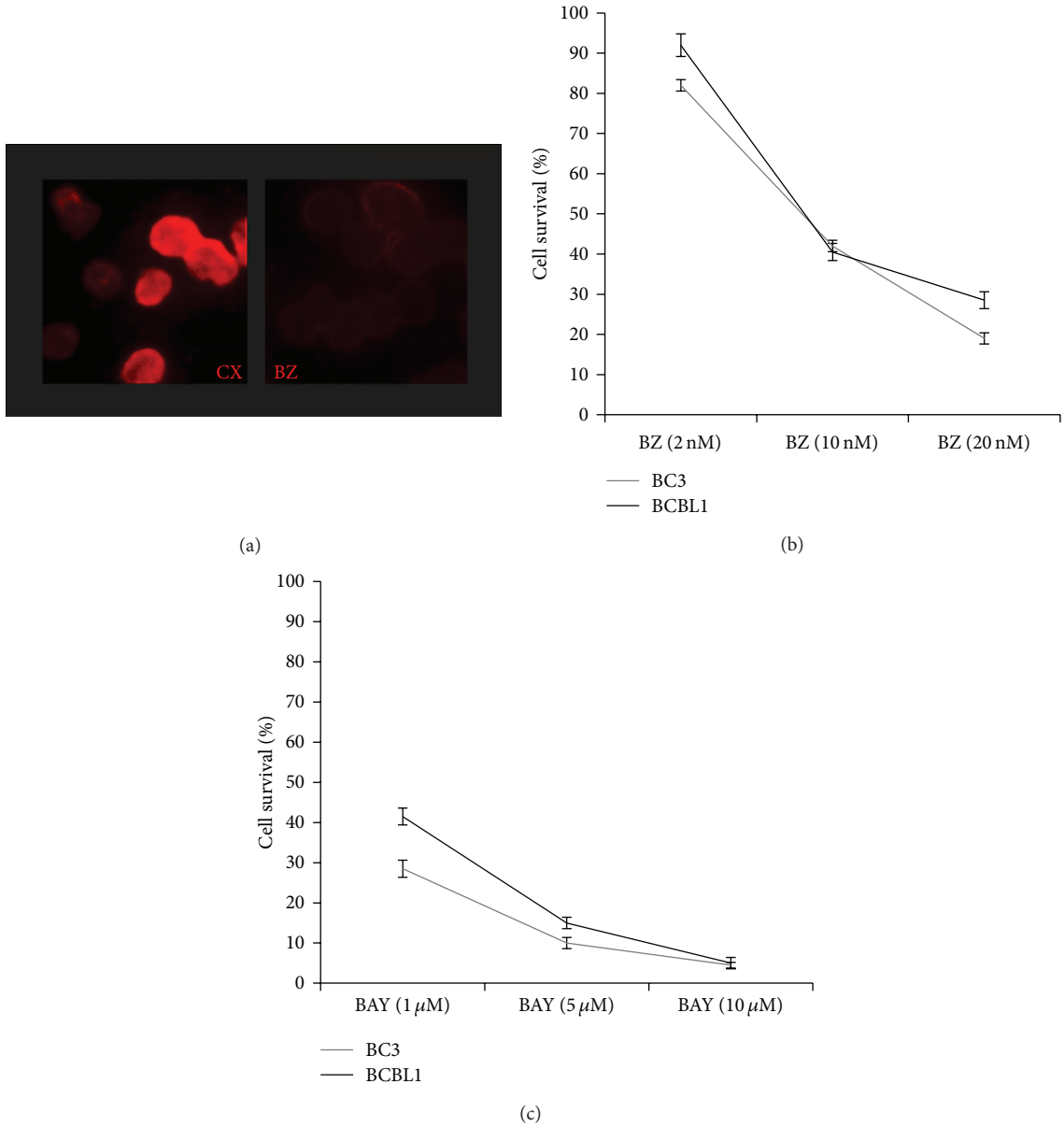

Figure 1: Effect of Bortezomib (BZ) (Santa Cruz) or Bay11-7082 (BAY) (Santa Cruz) on BC3 and BCBL1 PEL cells. (a) Effect on NF-κB p65 nuclear localization of Bortezomib in BC3 cells, in comparison to the untreated control cells, analyzed by immunofluorescence assay (IFA). A representative experiment out of three is shown. (b and c) Trypan blue exclusion assays of BC3 and BCBL1 PEL cells treated for 24 hours with Bortezomib (BZ) or Bay11-7082 (BAY), at the indicated doses. The mean ± SD of three independent experiments is reported.

antiviral drugs. For the above reasons, strategies that allow the manipulation of viral life cycle, reducing or promoting viral production, are potentially explorable in PELs. One of the strategies that allow manipulating the viral life cycle is the modulation of the cellular autophagy which has been shown to have a strong impact on KSHV replication [45].

4. Experimental Data

Here we have summarized our and other experimental evidences showing the cytotoxic effect mediated by the pharmacological inhibition of all these pathways in PEL cell lines. We first confirmed that indirectly inhibiting NF-κB with Bortezomib (Figure 1(a)) a dose-dependent reduction of PEL cell survival can be observed (Figure 1(b)), confirming our previous reported results [15]. Even stronger cytotoxic activity on PEL cells was obtained with Bay11-7082 NF-κB, specific inhibitor [46], that was indeed able to kill the majority of PEL cells at the concentration of 5 mM (Figure 1(c)). Next, given the importance of JAK2/STAT3 activation in PEL survival and in the reversion of immunosuppression, reported by us as well as by other groups [14, 15, 47], we performed a dose-response assay pharmacologically inhibiting STAT3 activation with AG490 and confirmed that it was very effective in reducing PEL cell survival. Besides its efficacy against PEL

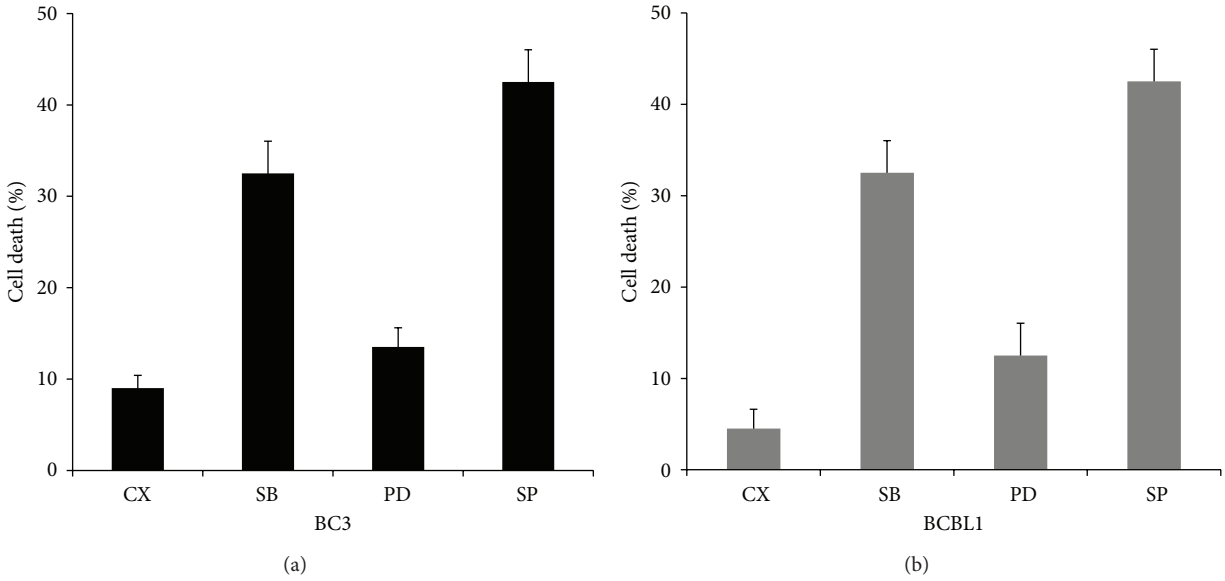

Figure 2: Effect on PEL cell cytotoxicity of p38, JNK, or ERK inhibition by SB203580, SP600125, and PD98059, respectively, based on trypan blue exclusion is reported. The treatment was performed for 24 hours at the concentration of 10 μM. All the reagents were purchased from Santa Cruz. The mean ± SD of three independent experiments is reported.

cells, the use of AG490 is also encouraged by our previous observations showing that it can induce an immunogenic cell death in these cells and has a low side effect on dendritic cell viability [15, 38]. Next, a dose-response treatment aimed at the targeting of PI3K/AKT pathway was then performed with AKT inhibitor LY294002 against BC3 and BCBL1 PEL cells. LY294002, used at the concentration of 20 mM, induced about 50% reduction of PEL cell survival, after 24 hours of treatment (Figure 2). One of the consequences of AKT activation is a change in the cell metabolism, such as an increase of cell resistance to glycolysis inhibitors. Thus, it will be interesting to investigate how treatment LY294002 would affect PEL cell resistance to glucose starvation. A low cytotoxic effect on PEL cells was observed with rapamycin mTOR inhibitor, used at 50 nM, for 24 hours. However, its combination with autophagy inhibitor 3-methyladenine (3-MA) resulted in a higher cytotoxic effect (unpublished data). This is in agreement with the notion that inhibition of mTOR induces autophagy that usually helps cells to survive during starvation or stressful conditions; thus its inhibition may increase cell death.

Additionally, the potential cytotoxic effect of the inhibition of JNK, p38, and ERK MAPK pathways was evaluated on PEL cells. The results shown in Figure 3 indicate that the higher cytotoxic effect was obtained by using SP600125 JNK inhibitor, confirming that this pathway plays an important prosurvival role in PEL cells, according to previously reported studies [31]. All MAPK inhibitors were used at the concentration of 10 mM and a low cytotoxicity was also observed with the inhibitor of p38 MAPK SB203580 and with the ERK inhibitor PD98059, according to a recent study [48] (Figure 3).

The activation of the above-mentioned prosurvival pathway leads to upregulation of HSPs, which are classified based on their molecular weight. They play multiple roles in cancer cells; for example, they ensure the correct protein folding, which is very important especially for cancer cells. Moreover, HSPs are required for the expression of some KSHV essential proteins [49, 50]. The major role in cancer cell survival is played by HSP70 and HSP90. The cytotoxicity of HSP70 inhibitor 2-phenylethynesulfonamide (PES) against PEL cells was previously reported by our group [40]. Here we compared the effect of PES with Benzisoxazole HSP90 inhibitor, considering that HSP70, besides its chaperone function, plays an important role in the maintenance of the lysosome membrane stabilization. The results shown in Figure 4 indicate that both HSP70 and HSP90 were essential for PEL cell survival. Trypan blue exclusion was used in all the dose-response cytotoxic assay performed in this study.

To further explore how cell death occurred in PEL cells by the inhibition of the above-reported prosurvival pathways, we then performed a western-blot analysis of the poly (ADP-ribose) polymerase (PARP) cleavage. PARP cleavage generally represents a final event of an apoptotic cell death and is mainly mediated by caspase activation [51]. We found that, except for PES, previously shown to induce a necroptotic cell death type [40], all the other drugs utilized in this study induced the cleavage of PARP in PEL cells (Figure 5). Besides considering the cytotoxic effect, it is important to evaluate the side effect that anticancer drugs could have on the immune cells and DCs in particular, being cells with a pivotal role in the immune system [52]. Preliminary data obtained by exposing monocyte-derived DCs to all the drugs used against PEL cells, at the concentrations able to reduce 50% of PEL cell survival, showed that all of them, except for Bortezomib and Bay11-7082, were almost completely safe towards DCs (data not shown).

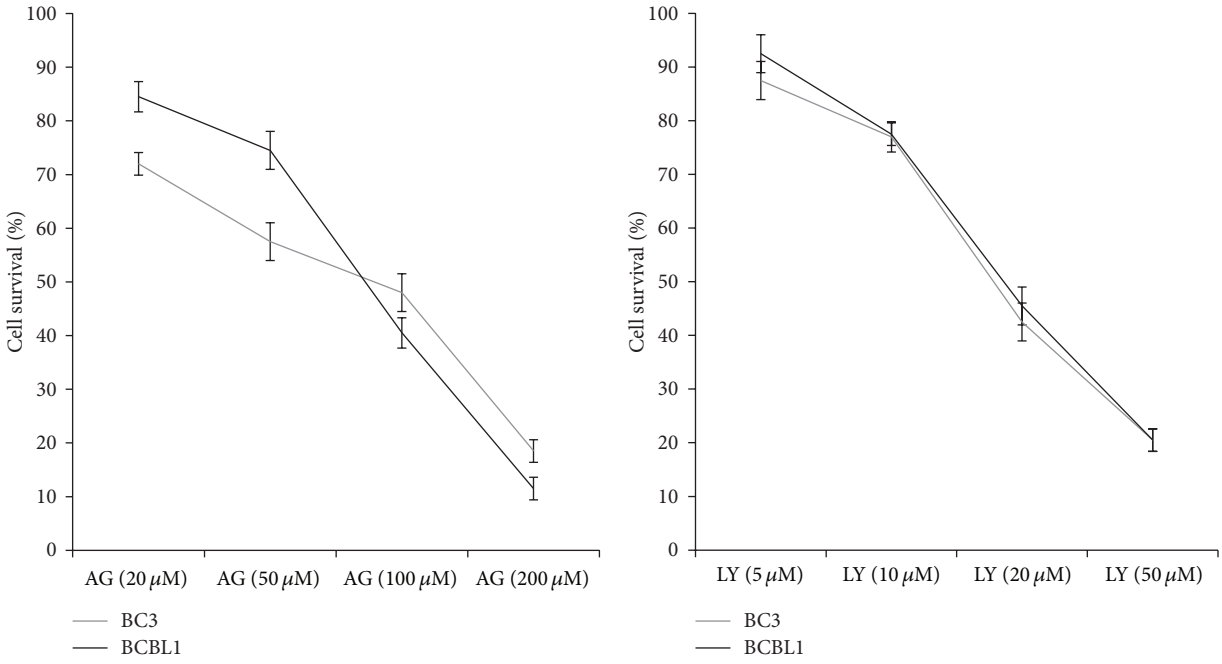

Figure 3: Dose-response cytotoxic assay of STAT3 or AKT inhibition in BC3 and BCBL1 PEL cells treated with AG490 (AG) (Calbiochem) or LY294002 (LY) (Santa Cruz), respectively, at the indicated doses. Trypan blue exclusion assays were performed after 24 hours of treatment. Mean ± SD of three experiments is reported.

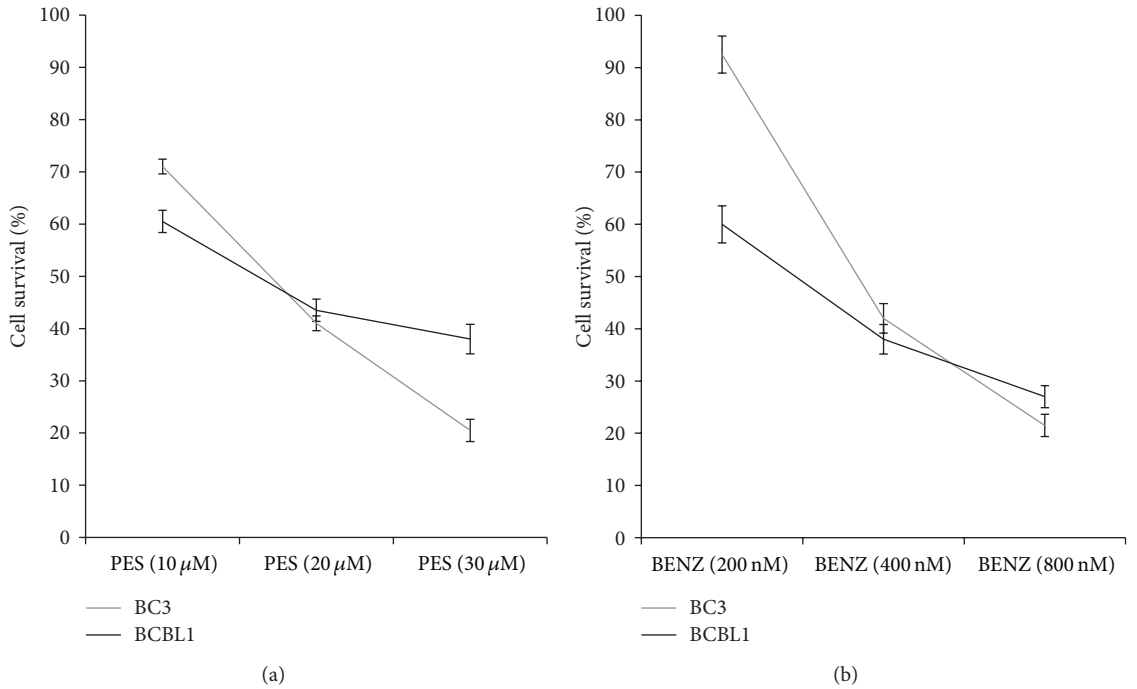

Figure 4: Dose-response cytotoxic assay of BC3 and BCBL1 PEL cells treated with PES (HSP70 inhibitor) (Calbiochem) and Benzisoxazole (BENZ) (HSP90 inhibitor) (Calbiochem), at the indicated doses. Trypan blue exclusion assays were performed after 24 hours of treatment and mean ± SD of three independent experiments is reported.

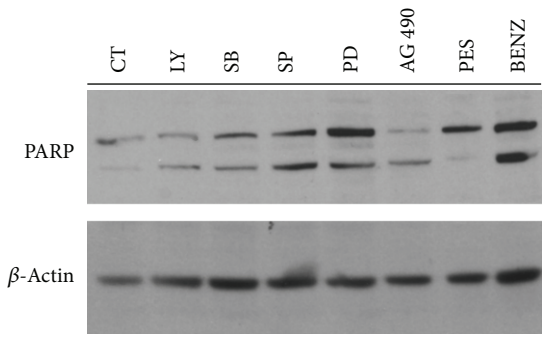

Figure 5: Western-blot analysis showing the PARP cleavage in BCBL1 cells treated for 24 hours with all the above reported cytotoxic drugs: LY294002 (LY), SB203580 (SB), SP600125 (SP), PD98059 (PD), AG490 (AG), PES, and Benzisoxazole (BENZ). β-Actin is included as loading control and a representative experiment out of three is reported. Antibodies against PARP (Cell Signaling) and β-actin (Sigma Aldrich) were diluted 1 : 1000 and 1 : 10000, respectively.

5. Conclusion

In conclusion, the use of drugs that target constitutively activated pathways in PEL cells could represent a valid alternative in translational therapies, especially considering the ability of most of them to induce an immunogenic cell death. In addition, their use is very promising because they have a low side effect towards the immune cells, and DCs in particular, rendering their use safer than conventional chemotherapies.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

This work was supported by MIUR, Associazione Italiana per la Ricerca (AIRC) no. 10265, and Pasteur Cenci-Bolognetti Foundation. The authors thank Sandro Valia, Marina Peddis, and Mariarosaria Filardi for technical assistance.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 265840, 11 pages
http://dx.doi.org/10.1155/2014/265840

Research Article

Lenalidomide Induces Immunomodulation in Chronic Lymphocytic Leukemia and Enhances Antitumor Immune Responses Mediated by NK and CD4 T Cells

Andrea Acebes-Huerta,[1] Leticia Huergo-Zapico,[1] Ana Pilar Gonzalez-Rodriguez,[2] Azahara Fernandez-Guizan,[1] Angel R. Payer,[2] Alejandro López-Soto,[1] and Segundo Gonzalez[1]

[1] *Department of Functional Biology, IUOPA, University of Oviedo, Facultad de Medicina, Julian Claveria sn, 33006 Oviedo, Spain*
[2] *Department of Hematology, Hospital Universitario Central de Asturias, C/Celestino Villamil s/n, 33006 Oviedo, Spain*

Correspondence should be addressed to Segundo Gonzalez; segundog@uniovi.es

Received 11 February 2014; Revised 21 July 2014; Accepted 5 August 2014; Published 17 September 2014

Academic Editor: Gerassimos A. Pangalis

Lenalidomide is an immunomodulatory drug with therapeutic activity in chronic lymphocytic leukemia (CLL). However, it has pleiotropic effects, and the mechanism of action responsible for its therapeutic activity has not been well defined yet. Herein, we show that lenalidomide treatment does not have an effect on the proliferation of leukemia cells, but it increases the proliferation of B cells from healthy donors. Lenalidomide did not exert a direct effect on the apoptosis of leukemia cells obtained from CLL patients, although it indirectly induced their apoptosis through the activation of nonmalignant immune cells. Thus, lenalidomide markedly increased the proliferation of NK and CD4 T cells. The effect of lenalidomide on NK cells was secondary to the induction of IL-2 production by CD4 T cells. Accordingly, depletion of T cells or blockade of IL-2 activity completely abrogated the proliferation of NK cells. Additionally, lenalidomide enhanced NK and NKT-like cell-mediated natural cytotoxicity against leukemia cells from CLL patients. Lenalidomide also upregulated CD20 expression on leukemia cells and, accordingly, it had a synergistic effect with rituximab on promoting antibody-dependent cell-mediated cytotoxicity against primary leukemia cells. Overall, these observations provide a support for combining lenalidomide with rituximab as a treatment in CLL.

1. Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease, with a clinical presentation ranging from indolent to advanced stage disease. A therapeutic intervention is scarcely required in patients with indolent disease, whereas chemotherapy treatment is frequently required in patients with advanced stage disease. However, CLL is generally considered as an incurable disease and, consequently, the development of new therapeutic strategies is a key goal in this malignancy [1].

Increasing evidence demonstrates that the tumor microenvironment plays a critical role in CLL progression and therapy efficiency. The immune system is able to prevent cancer development, either by eliminating cancer cells prior to tumors becoming clinically detectable or by attenuating tumor progression [2, 3]. NK and T cells may mediate antitumor responses, particularly in the initial stages of the disease, which may affect disease progression [4, 5]. However, advanced disease patients develop multiple immune defects, including hypogammaglobulinemia, deregulation of the cytokine network, or impairment of T and NK cells function [6]. Nevertheless, targeting the immune system may represent a promising therapeutic strategy in CLL. Thus, chemotherapy is often combined with an anti-CD20 monoclonal antibody (rituximab) in patients with advanced stage

disease, resulting in enhanced complete and overall response rates. The relevant mechanism of action of rituximab is the activation of NK cell-dependent antibody-dependent cell-mediated cytotoxicity (ADCC) against leukemia cells [7, 8].

Lenalidomide (Revlimid; Celgene) is an immunomodulatory drug that has shown a clinical effect in several hematological disorders including myeloma [9], myelodysplastic syndrome (MDS) [10], and CLL [11–14]. Lenalidomide displays a number of pharmacodynamic effects, but the main mechanism of action is not completely known and may vary depending on the disease. In multiple myeloma, lenalidomide exerts a direct cytotoxic effect on neoplastic plasma cells, inhibits cell adhesion, and induces changes in the bone marrow microenvironment [15]. In del(5q)MDS, lenalidomide directly affects erythroid progenitors [16]. In CLL, significant clinical responses, including molecular complete remissions in heavily pretreated patients, have been observed [12, 14]. It is noteworthy that lenalidomide does not directly induce the apoptosis of leukemic cells [17], but it regulates critical prosurvival and angiogenic cytokines (including IL-2, PDGF, and VEGF). Lenalidomide also stimulates antigen presentation, proliferation, and effector activity of T cells [18, 19] and may activate a minor cytotoxic population of T cells known as invariant or CD1d-restricted NKT cells [20, 21]. Furthermore, CLL cells incubated with healthy T cells inhibit immune synapse formation, where it is restored by lenalidomide treatment [22]. Additionally, lenalidomide increases NK cell proliferation, which correlates with clinical response [11, 23, 24] and augments NK cell-mediated ADCC against tumor cells [25, 26]. Likewise, clinical responses in CLL patients treated with lenalidomide correlated with a tumor flare reaction [18], which appears to be characteristic of this disease and may reflect a clinical manifestation of the enhancement of the immunogenic potential of tumors [14, 27].

The efficacy of lenalidomide in different malignant conditions may be explained by the existence of multiple mechanisms of action, different immune status, and specific pathogenesis of the disease. Unraveling the relevant mechanism of action is essential to optimize the treatment of patients and to develop new therapeutic strategies. Thus, in this study, we analyzed the mechanism of action underpinning the therapeutic activity of lenalidomide in CLL.

2. Material and Methods

2.1. Cell Isolation and Reagents. CLL patients ($n = 17$) fulfilling the diagnostic criteria for CLL [28] and healthy donors ($n = 10$) were analyzed in this study. These patients either were untreated or did not receive cytoreductive treatment within 6 months of the investigation. This study was approved by the ethics committee of our institution and informed consent was obtained from all patients and healthy donors.

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll gradient centrifugation from freshly isolated blood obtained from patients and donors. B cells were further purified using EasySep Human B Cell Enrichment Kit without CD43 Depletion (Stemcell Technologies) and NK cells were isolated from PBMCs by using the EasySep NK Cell Enrichment kit (Stemcell Technologies). The purity of B and NK cells (~90 to 95%) was assessed by flow cytometry.

PBMCs or purified immune cells were cultured in complete medium RPMI-1640 supplemented with 10% human AB serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma, St. Louis, MO) at 37°C in 5% CO_2. Lenalidomide was obtained from Celgene and was dissolved in dimethyl sulfoxide (DMSO), and fresh lenalidomide was replaced every 72 hours in cell cultures. In some experiments, cells were treated with recombinant human IL-2 (rhIL-2) (Peprotech), anti-human IL-2 receptor (IL-2 sRα) blocking antibody (R&D systems), or cyclosporine A (CsA) (Sigma, St. Louis, MO). In all experiments incubation with DMSO was used as a control.

2.2. Flow Cytometry. Diagnosis of CLL was confirmed for each patient by flow cytometry, revealing a typical $CD19^+$, $CD20^+$, $CD5^+$, $CD23^+$, and Ig light chain (κ or λ) restricted phenotype of leukemia cells (Becton Dickinson). To determine immune cells subsets, cells were stained with anti-CD3-FITC, anti-CD4-PerCP, anti-CD8-CFBlue, anti-CD56-APC, and anti-CD20-PE (all from Immunostep) and anti-CD3-PECy7 (eBioscience) and isotype-matched control conjugates. The populations of immune cells were defined as follows: CD4 T cells were defined as $CD3^+CD4^+$ and CD8 T cells as $CD3^+CD8^+$; NK cells were identified as $CD3^-CD56^+$, NKT-like cells as $CD3^+CD8^+CD56^+$, and leukemia cells as $CD5^+/CD19^+$. CD20 expression was quantified on $CD5^+/CD19^+$ cells.

To study the intracellular IL-2 production, PBMCs were incubated in complete culture media with GolgiStop (BD Biosciences) and with monensin (a protein transport inhibitor), for 4 hours. After that, cells were stained for surface antigens and, then, incubated with a fixation/permeabilization solution (BD Biosciences) prior to incubation with an anti-human IL-2-PerCP-Cy5.5 antibody (BD Biosciences).

Cells undergoing apoptosis were quantified by staining with annexin V-FITC according to the manufacturer's protocol (Immunostep). Cells were analyzed in a BD FACS Canto II cytometer and data were acquired and analyzed by using the FACS Diva Software.

2.3. Cell Proliferation Assay. Freshly isolated PBMCs from healthy donors and CLL patients were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma, St. Louis, MO) at 1 μM for 10 min at 37°C. Labeling was stopped with 5 volumes of complete media containing 10% fetal bovine serum (FBS). After 2 washes, cells were cultured at 2×10^6 cells/mL in complete media containing 10% of human AB serum, and 1 μM lenalidomide was added every 72 hours. After 3, 6, 9, 12, and 14 days of culture, cells were stained for CD19, CD3, CD4, CD8, and CD56 expression. Cell division was analyzed based on the decrease in CFSE staining, resulting from the dilution of the dye with each cell division.

2.4. NK Cell Cytotoxic Assays. CD107a lysosome-associated membrane protein-1 (LAMP-1) was used to measure NK-,

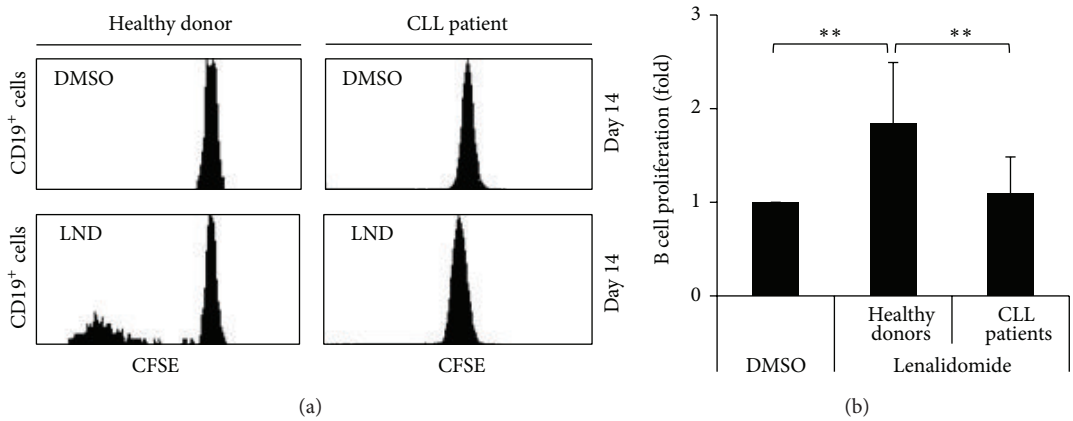

Figure 1: Effect of lenalidomide on the proliferation of B cells. (a) PBMCs obtained from healthy donors ($n = 10$) and CLL patients ($n = 17$) were labeled with CFSE and cultured with 1 μM lenalidomide or DMSO for 14 days. CFSE fluorescence in B cells (gated as $CD5^+/CD19^+$) was analyzed by flow cytometry. The histograms show a representative CLL patient and a healthy donor. (b) The figure shows the compilation of the results obtained from healthy donors and CLL patients. Results are expressed as the fold induction of the percentage of proliferating B cells in lenalidomide-treated cells relative to the vehicle-treated control ($^{**}P < 0.01$, Mann-Whitney U test).

NKT-like-, and CD8-cell cytotoxic activity. PBMCs from healthy donors or CLL patients stimulated with 1 μM lenalidomide for 14 days were incubated with target cells at an effector : target (E : T) ratio of 5 : 1 in complete media supplemented with human AB serum and BD GolgiStop (BD Biosciences). As a positive control of degranulation, PBMCs were stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL) (both from Sigma, St. Louis, MO). The anti-CD107a-PE antibody (BD Biosciences) was added to the plate during the incubation. Some experiments were made in the presence or absence of rituximab (20 μg/mL). After the incubation, samples were stained for CD3, CD4, CD8, and CD56 expression and analyzed by flow cytometry.

2.5. Statistical Analysis. Continuous variables were compared with Mann-Whitney U test. Correlations between continuous variables were analyzed by Spearman correlation test. The P values $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of Lenalidomide on Proliferation and Apoptosis of Leukemia Cells. The effect of lenalidomide on the proliferation of B cells was initially analyzed. PBMCs from healthy donors and CLL patients were cultured in presence of 1 μM lenalidomide for 14 days and the proliferation of B cells was assessed by CFSE assay. As shown in Figures 1(a) and 1(b), lenalidomide did not affect the proliferation of leukemia cells, but the proliferation of B cells from healthy donors was significantly increased in the presence of this drug.

Next, we studied the effect of lenalidomide on the apoptosis of leukemia cells. PBMCs from CLL patients containing variable amounts of leukemia cells (ranging from 70% to 95%) and nonmalignant immune cells were used. No significant increase of apoptosis of leukemia cells from CLL patients was observed after 48 hours of treatment with 1 μM lenalidomide (not shown). Nevertheless, after 7 days of treatment a significant effect of lenalidomide on the apoptosis of leukemia cells was detected (Figures 2(a), 2(b), and 2(c)). It is of note that the level of apoptosis on leukemia cells significantly correlated with the percentage of nonmalignant immune cells at day zero in CLL patients ($r = 0.87$, $P = 0.009$) (Figure 2(d)). Specifically, a strong correlation between apoptosis of leukemia cells and the percentage of NKT-like cells ($CD3^+CD8^+CD56^+$) was observed ($r = 0.84$, $P = 0.01$) (Figure 2(e)).

3.2. Lenalidomide Enhances the Proliferation of NK Cells. In addition to the effect observed on B cells, it is noticeable that the percentage of NK cells significantly increased after 14 days of culture of PBMCs in the presence of lenalidomide (Figures 3(a) and 3(b)). Moreover, such increase of NK cells was higher in patients than in healthy donors (9.8- versus 3.4-fold induction). To analyze whether the increase of NK cells was due to cell proliferation, PBMCs from patients and healthy donors were CFSE-stained and cultured in the presence of lenalidomide for 14 days, and the proliferation of NK cells was examined by flow cytometry at days 3, 6, 9, 12, and 14. Remarkably, no effect was observed on the proliferation of NK cells before 12 days of treatment (not shown). Nevertheless, after 14 days, lenalidomide significantly increased the proliferation of NK cells from both healthy donors and CLL patients (Figures 3(c) and 3(d)), although the level of the induction was higher in patients than in healthy donors (2.98- versus 2.19-fold induction). In addition, there was a significant interpatient variation in the response to lenalidomide (ranging from no response to a 9.2-fold induction).

Interestingly, the main subsets of NK cells ($CD56^{bright}$ and $CD56^{dim}$) proliferated in the presence of lenalidomide (not shown), although $CD56^{bright}$ NK cells were more potently induced by lenalidomide than $CD56^{dim}$ NK cells

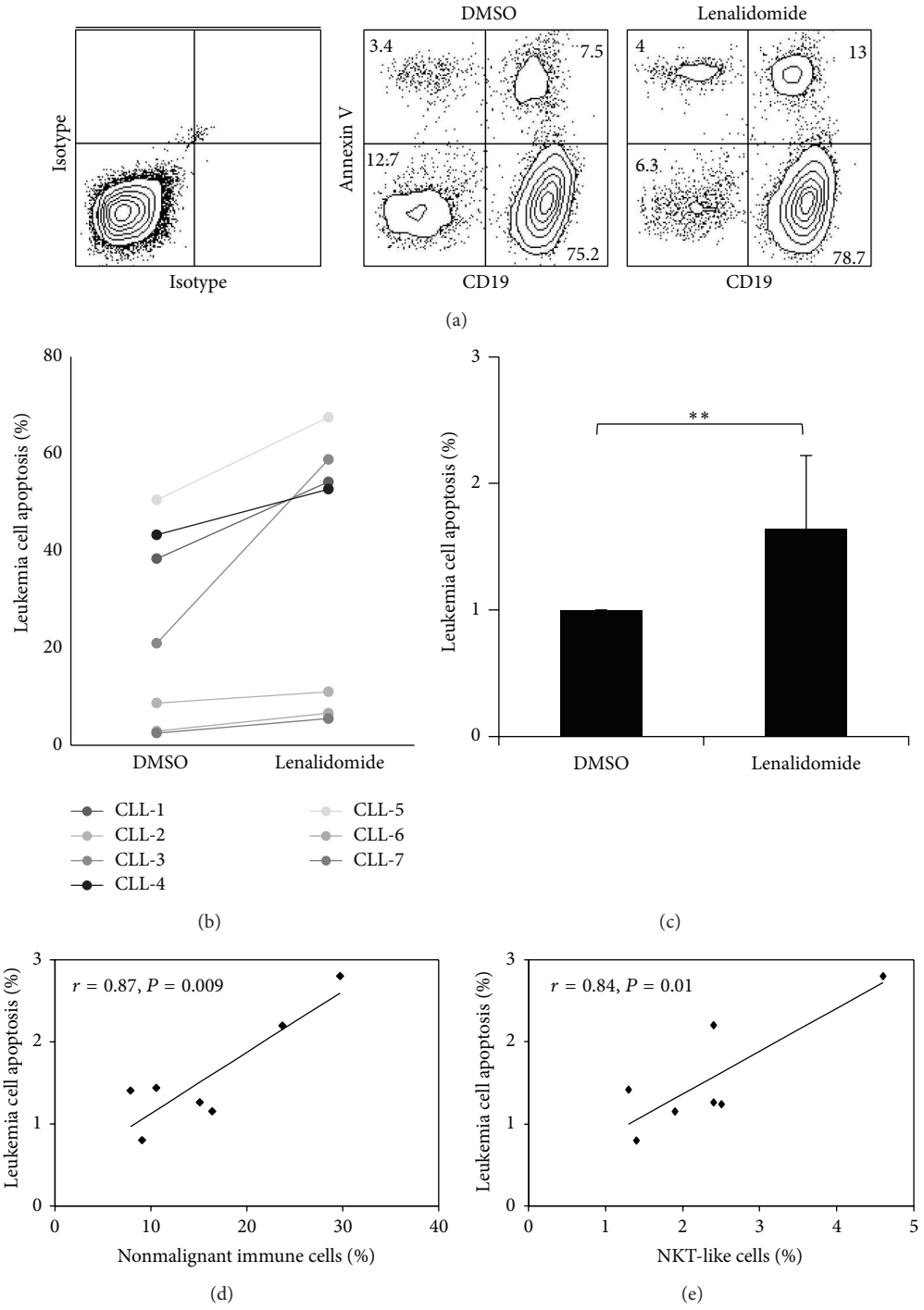

Figure 2: Effect of lenalidomide on the apoptosis of leukemia cells. (a) PBMCs from CLL patients ($n = 7$) were cultured in the presence of 1 μM lenalidomide or DMSO for 7 days and apoptosis was analyzed by staining with annexin V. The figure shows the flow cytometric profile of annexin V staining of leukemia cells from a representative patient after lenalidomide treatment (numbers in the dot plot represent the percentage of cells). (b) PBMCs (ranging from 70% to 95% of leukemia cells) from CLL patients ($n = 7$) were cultured as detailed in *a* and apoptosis of leukemia cells was studied by annexin V staining. The scatterplot represents the percentage of apoptosis in DMSO versus lenalidomide-treated cells. (c) The bars represent the mean and the standard deviation of the fold induction of annexin V-positive leukemia cells ($^{**}P < 0.01$, Mann-Whitney U test). (d) Correlation between annexin V staining of leukemia cells and the percentage of nonleukemia immune cells of CLL patients. (e) Correlation between annexin V labeling of leukemia cells and the percentage of NKT-like cells ($CD3^+CD8^+CD56^+$) of CLL patients.

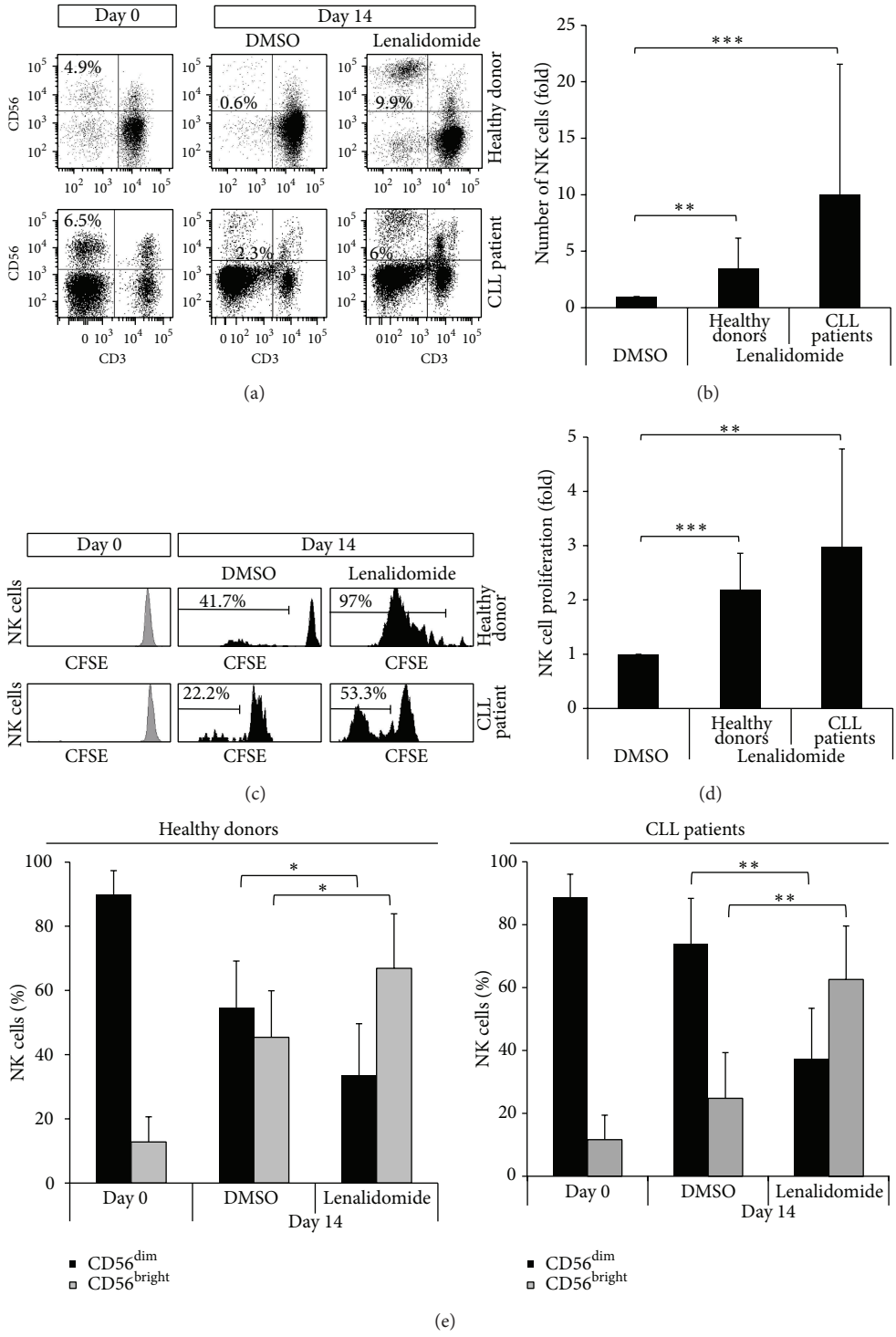

FIGURE 3: Effect of lenalidomide on NK cell proliferation. (a) Flow cytometric profile of NK cells (gated as $CD3^{-}CD56^{+}$) before and after stimulation with 1 μM lenalidomide for 14 days. One representative CLL patient and one healthy donor are shown. The numbers represent the percentage of NK cells. (b) The figure shows the compilation of the results obtained from CLL patients ($n = 17$) and donors ($n = 10$). Results are expressed as the fold induction of the percentage of NK cells of lenalidomide-treated cells relative to the vehicle-treated control ($^{**}P < 0.01$; $^{***}P < 0.001$, Mann-Whitney U test). (c) PBMCs labeled with CFSE were cultured with 1 μM lenalidomide or DMSO for 14 days and CFSE expression was examined in NK cells ($CD3^{-}CD56^{+}$) by flow cytometry. One representative CLL patient and one healthy donor are shown. (d) The figure shows the compilation of the results obtained from CLL patients ($n = 17$) and healthy donors ($n = 10$). Results are expressed as the fold induction of the percentage of proliferative NK cells of lenalidomide-treated cells relative to the vehicle-treated control ($^{**}P < 0.01$; $^{***}P < 0.001$, Mann-Whitney U test). (e) The figure shows the percentage of the two main subsets of NK cells ($CD56^{dim}$ and $CD56^{brigth}$) after the treatment with 1 μM lenalidomide or DMSO for 14 days. ($^{*}P < 0.05$; $^{**}P < 0.01$, Mann-Whitney U test).

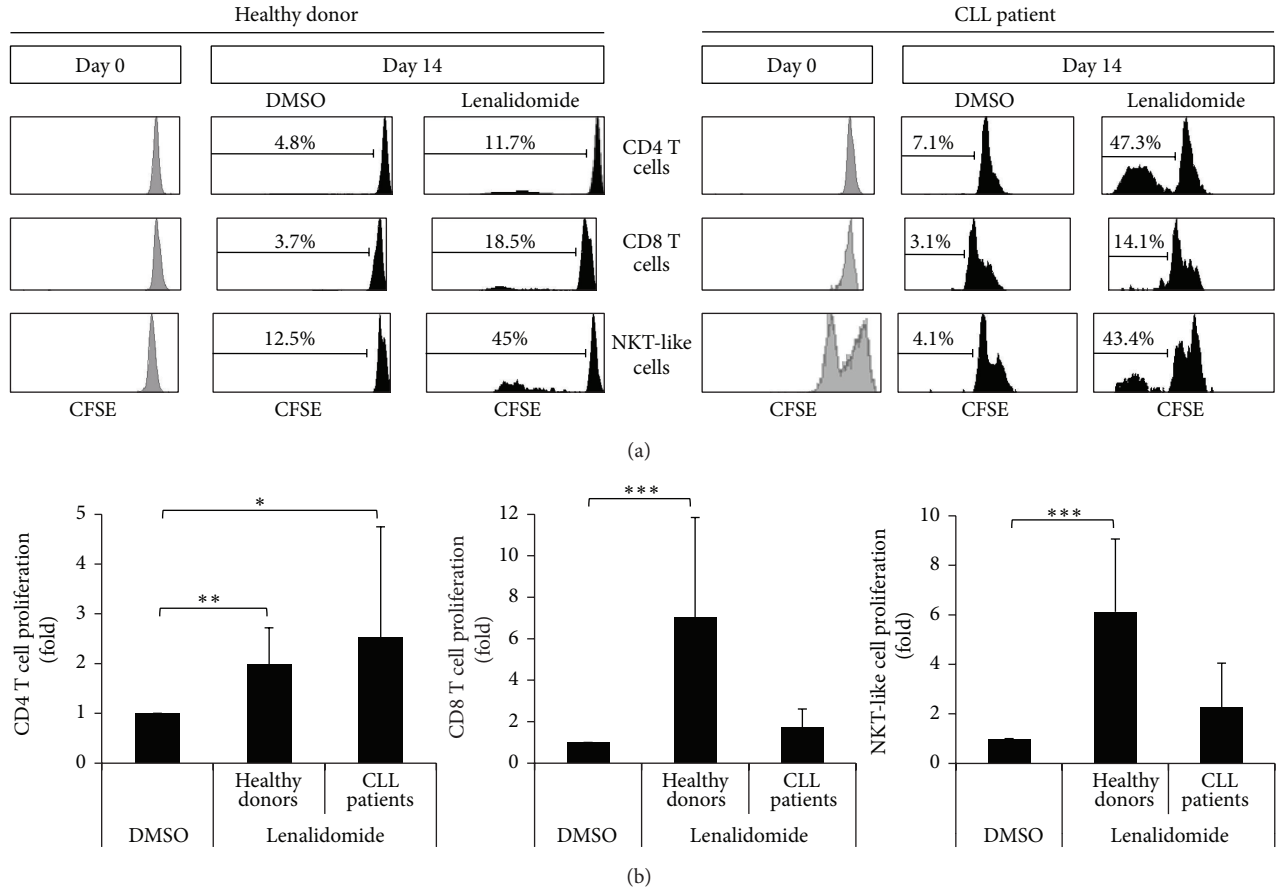

Figure 4: Effect of lenalidomide on the proliferation of T cell subsets. (a) The histograms show the CFSE expression of CD4 T cells, CD8 T cells, and NKT-like cells before and after stimulation with lenalidomide. PBMCs were labeled with CFSE and cultured with 1 μM lenalidomide or DMSO for 14 days. CFSE expression in CD4 T cells ($CD3^+CD4^+$), CD8 T cells ($CD3^+CD8^+$), and NKT-like cells ($CD3^+CD8^+CD56^+$) was examined by flow cytometry. One representative CLL patient and one donor are shown. (b) The figure shows the compilation of the results obtained from CLL patients ($n = 17$) and donors ($n = 10$). Results are expressed as the fold induction of the percentage of proliferative CD4 T cells, CD8 T cells, and NKT-like cells of lenalidomide-treated cells relative to the vehicle-treated control. ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Mann-Whitney U test).

(Figure 3(e)). Specifically, lenalidomide induced an inversion of the $CD56^{bright}/CD56^{dim}$ ratio from 0.82 to 1.99 in healthy donors and from 0.33 to 1.67 in CLL patients.

3.3. Lenalidomide Stimulates the Proliferation of T Cells. We next examined the effect of lenalidomide on the proliferation of T cells (Figures 4(a) and 4(b)). It is of note that CD4 T cells were the most proliferative subset of T cells in CLL patients. The proliferation of CD4 T cell after 14 days of culture in the presence of lenalidomide was higher in CLL patients than in donors (2.52- versus 1.98-fold induction). Similar to NK cells, there was a marked interindividual variability in the proliferation of CD4 T cells; and a significant correlation between the percentage of CD4 T cells of CLL patients after lenalidomide treatment and the proliferation of NK cells was observed ($r = 0.49$, $P = 0.04$).

CD8 T cells also proliferated in response to lenalidomide, but the level of induction was higher in donors than in patients (7.03- versus 1.72-fold induction) (Figures 4(a) and 4(b)). Specifically, CD8 proliferated in 100% of healthy donors and 66% of CLL patients. Similar results were obtained with NKT-like cells, a subset of CD8 T cells (Figures 4(a) and 4(b)).

3.4. Induction of IL-2 Production by CD4 T Cells Is Required for the Enhancement of NK Cell and NKT-Like Cell Proliferation by Lenalidomide. To unravel the mechanism of action underlying the induction of NK cell proliferation observed, we analyzed whether lenalidomide has a direct or an indirect effect on NK cell proliferation by comparing the effect of lenalidomide on whole PBMCs versus purified NK cells. The depletion of non-NK immune cells by negative selection completely abrogated the induction of NK cell proliferation in both CLL patients and donors (Figure 5(a)), suggesting that the effect of lenalidomide on NK cell proliferation was indirect. We next examined the effect of lenalidomide on the production of IL-2 by immune cells by intracellular staining and flow cytometry. As shown in Figure 5(b), lenalidomide treatment significantly induced the production of IL-2 by CD4 T cells. Furthermore, NK cell proliferation was abrogated in the presence of an anti-IL-2

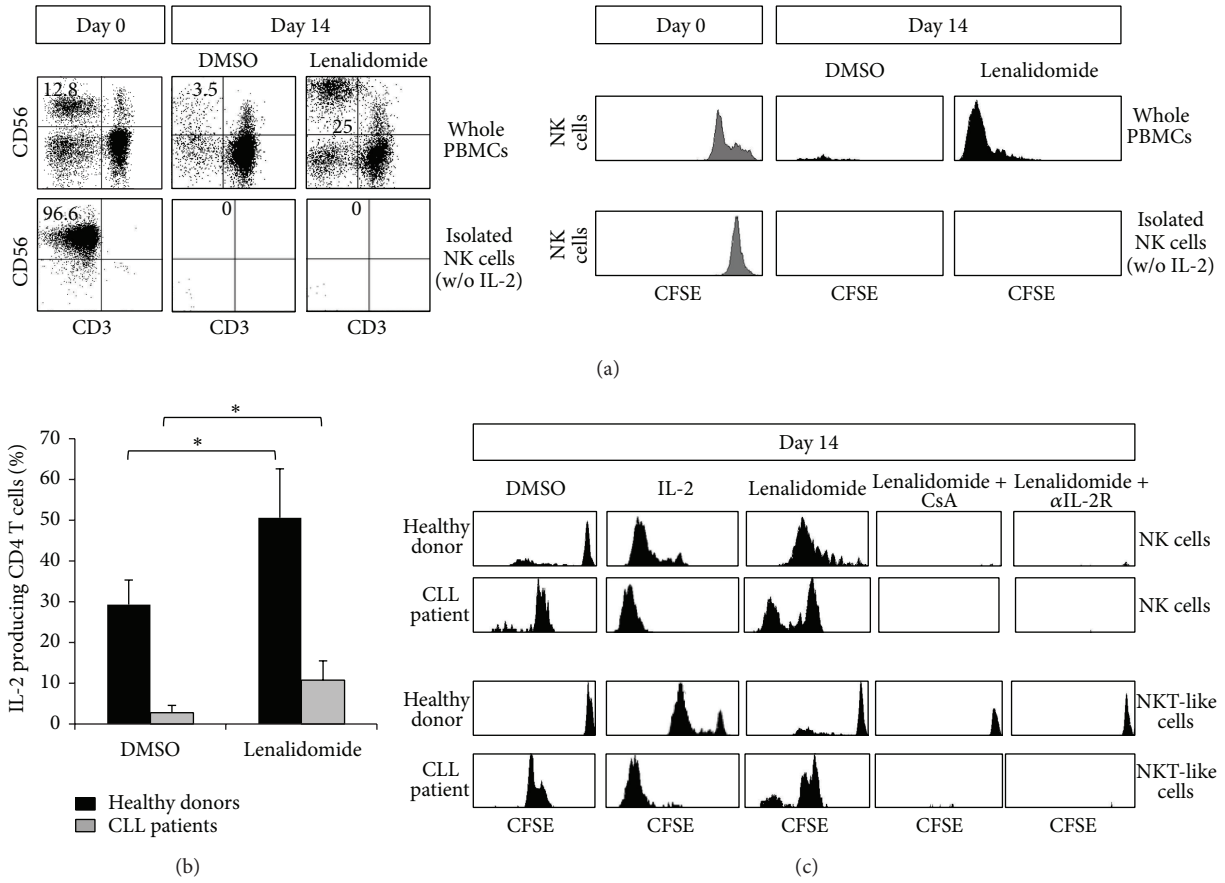

FIGURE 5: Production of IL-2 by CD4 T cells is required for lenalidomide-increased NK and NKT-like cells proliferation. (a) Whole PBMCs or purified NK cells (>95% of purity) from the same individual ($n = 4$) were labeled with CFSE and cultured with 1 μM lenalidomide or DMSO for 14 days (in the absence of recombinant IL-2, w/o). The proliferation of NK cells ($CD3^-CD56^+$) was assessed by flow cytometry. The figure shows the cytometric profile of NK cells (gated as $CD3^-CD56^+$) before and after the stimulation with lenalidomide. The numbers represent the percentage of NK cells. The histograms represent the expression of CFSE in NK cells from one representative donor. (b) PBMCs from healthy donors ($n = 4$) and CLL patients ($n = 4$) were cultured in the presence of lenalidomide for 14 days and the intracellular production of IL-2 by CD4 T cells ($CD3^+CD4^+$) was analyzed by flow cytometry. The bars represent the mean and the standard deviation of the percentage of IL-2-producing CD4 T cells ($^*P < 0.05$, Mann-Whitney U test). (c) PBMCs from healthy donors ($n = 4$) and CLL patients ($n = 4$) were stained with CFSE and cultured with DMSO, IL-2 (50 U/mL), or lenalidomide (1 μM) in presence or absence of cyclosporine A (CsA) (1 μM) or as anti-IL-2 blocking antibody (15 μg/mL) for 14 days. PBMCs treated with IL-2 (50 U/mL) were used as a positive control of proliferation. Baseline peaks of CFSE are the same as in Figures 3(c) and 4(a). The expression of CFSE on NK cells and NKT-like cells ($CD3^+CD8^+CD56^+$) was analyzed by flow cytometry. One representative healthy donor and one CLL patient are shown.

receptor blocking antibody or cyclosporine A, indicating that the production of IL-2 was required for the proliferation of NK cells (Figure 5(c)). Similarly, IL-2 was also involved in the proliferation of NKT-like cells. Finally, we demonstrated that NK and NKT-like cell proliferation was induced by the treatment of PBMCs obtained from both patients and donors with IL-2 (Figure 5(c)). Overall, these results indicate that the production of IL-2 by CD4 T cells is required for the induction of the proliferation of NK and NKT-like cells by lenalidomide. It is worth mentioning that IL-2 was also able to induce the proliferation of NK cells obtained from CLL patients who did not respond to lenalidomide treatment.

3.5. Lenalidomide Increases NK Cell-Mediated Natural Cytotoxicity and ADCC against Primary Leukemia Cells. First, the effect of lenalidomide on the expression of the main NK cell activating receptors was studied. To this end, PBMCs from patients and controls were incubated with lenalidomide for 7 days and the expression of NKG2D, DNAM1, NKp30, NKp44, and NKp46 was analyzed by flow cytometry. These experiments showed a significant increase in the expression of NKp30 on NK cells of CLL patients after lenalidomide treatment (Figure 6).

Next, we assessed whether lenalidomide modulates the cytotoxic activity of NK cells against purified leukemia cells. PBMCs from healthy donors incubated with lenalidomide or DMSO for 14 days were cocultured with purified leukemia cells obtained from CLL patients for 4 hours. Lenalidomide treatment significantly increased the cytotoxic activity of NK cells (1.5-fold induction) and NKT-like cells (2-fold

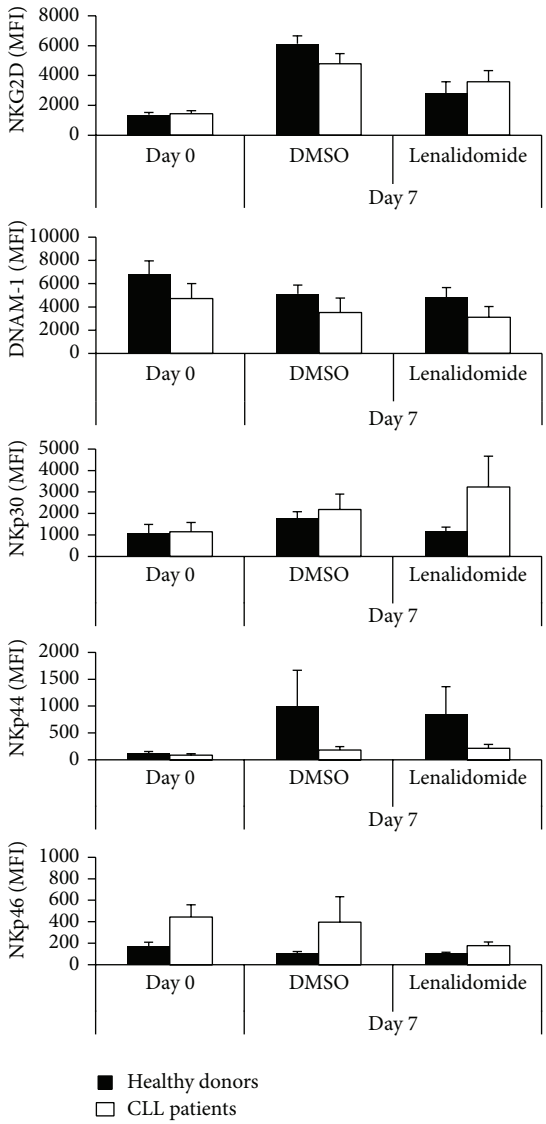

Figure 6: Effect of lenalidomide on the expression of NK cell activating receptors. PBMCs from healthy donors and CLL patients were treated with 1 μM lenalidomide or DMSO for 7 days and the expression of NKG2D, DNAM-1, NKp30, NKp44, and NKp46 on NK cells ($CD3^-CD56^+$) was analyzed by flow cytometry. The figure shows the compilation of the results obtained from patients ($n = 4$) and donors ($n = 3$) before and after lenalidomide treatment. The bars represent the mean and standard deviation of the MFI.

induction) against primary leukemia cells (Figures 7(a) and 7(b)), but no effect was observed on CD8 T cells (shown in Figure 7(a)). Similar results were obtained using IL-2 or IL-15 treatment (not shown). The cytotoxic activity was not further increased when leukemia cells were also treated with lenalidomide, suggesting that the major effect of lenalidomide was exerted on the activity of immune cells.

The recognition of leukemia cells by NK cells may be increased with the use of antileukemic monoclonal antibodies [7, 8]. Thus, treatment of leukemia cells with rituximab (anti-CD20) in the absence of lenalidomide significantly increased the cytotoxic activity of NK (4.3-fold) and NKT-like cells (1.7-fold) against primary leukemia cells (Figures 7(a) and 7(b)). Moreover, lenalidomide significantly increased the rituximab-mediated cytotoxic activity of NK cells (1.5-fold) and NKT-like cells (1.6-fold) against leukemia cells (Figures 7(a) and 7(b)), but no effect was observed on CD8 T cells (shown in Figure 7(a)).

To characterize the underlying mechanism involved in the cooperative effect between lenalidomide and rituximab, we analyzed the effect of lenalidomide on CD20 expression on leukemia cells ($CD5^+/CD19^+$) after 2, 7, and 14 days of treatment. No clear effect on CD20 expression was observed after 48 hours of treatment, but it is noteworthy that a significant increase of CD20 expression was observed after 7 and 14 days of treatment (Figures 7(c) and 7(d)).

Our findings suggest that lenalidomide indirectly promotes the proliferation and cytotoxic activity of NK and NKT-like cells against primary leukemia cells. Additionally, lenalidomide induces ADCC to rituximab-treated leukemia cells of CLL patients. Overall, these data indicate that NK and NKT-like cells are relevant mediators of lenalidomide-driven apoptosis of leukemia cells in CLL.

4. Discussion

The activation of the antileukemic immune response represents a promising therapeutic option in CLL, particularly for relapsed patients. In this regard, lenalidomide is an immunomodulatory drug with significant therapeutic activity in CLL. However, lenalidomide has a pleiotropic activity and the relevant mechanism of action responsible for its therapeutic activity has not been well defined [11–14]. Our study indicates that the antileukemic activity of lenalidomide is not due to the direct cytotoxicity against leukemia cells, but rather it may imply indirect mechanisms through the activation of nonmalignant immune cells, particularly NK and CD4 T cells.

Our study shows a pleiotropic effect of lenalidomide on different types of immune cells including NK, T, and B cells. It is noticeable that, unlike normal B cells, transformed B cells do not proliferate in the presence of lenalidomide, suggesting that leukemia cells lose the capacity to respond to lenalidomide. Nevertheless our data suggest that NK and CD4 T cells may play a relevant role in the antileukemic effect of lenalidomide in CLL. Thus, lenalidomide was able to induce the proliferation of NK cells, and this effect was higher in CLL patients than in healthy donors. In agreement with our data, the NK cell number in patients was increased by lenalidomide treatment *in vivo* and pretreatment levels of NK cells correlated with the response to therapy in CLL [11, 23, 24]. Additionally, we observed that lenalidomide has an important effect on the proliferation of T cells, particularly CD4 T cells. The proliferation of CD4 T cells in response to lenalidomide treatment was also higher in patients than in healthy donors, and the percentage of CD4 T cells of CLL patients after lenalidomide treatment significantly correlated with the proliferation of NK cells. Additionally, the depletion of T cells abrogated the proliferation of NK cells suggesting

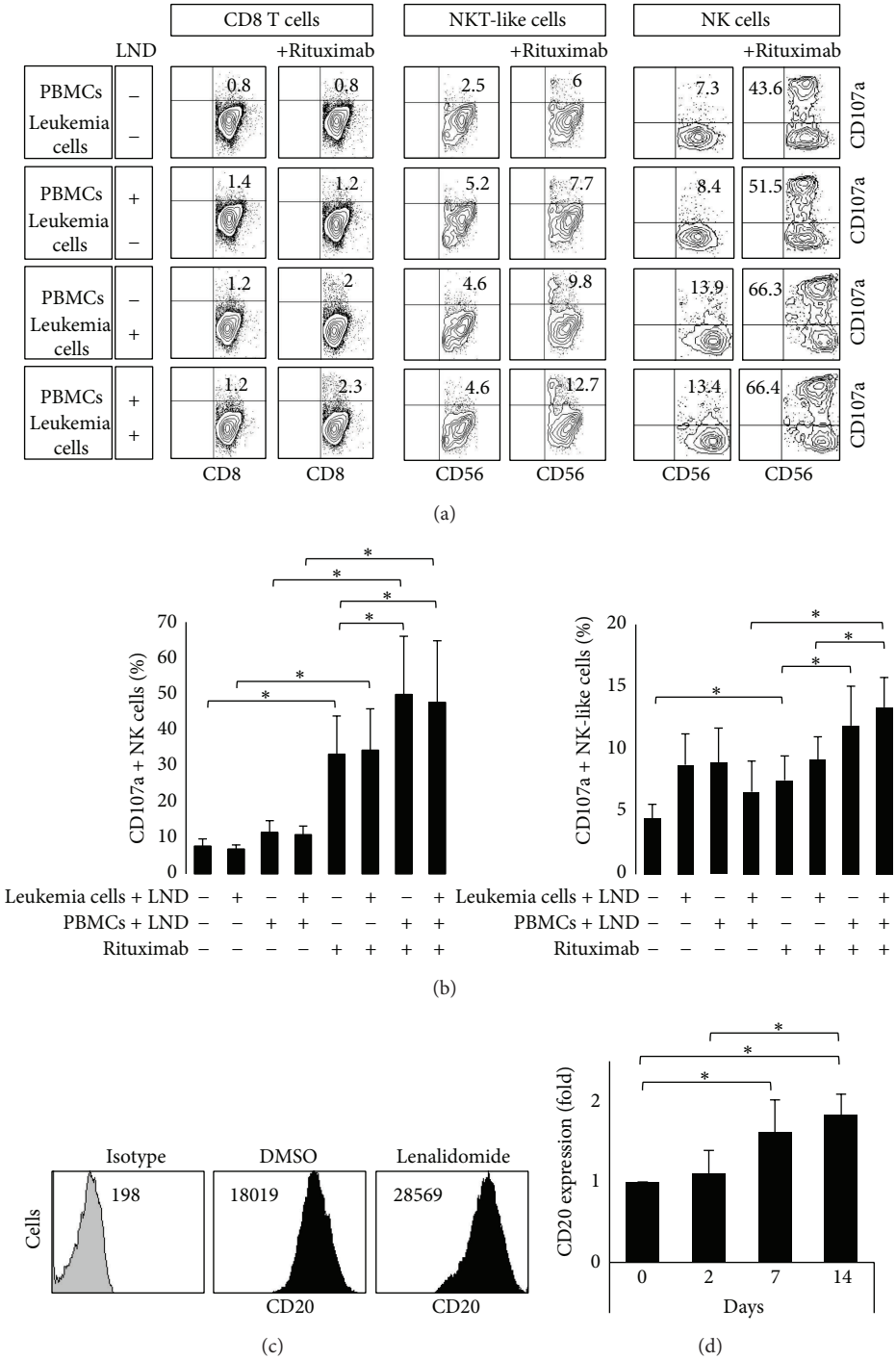

Figure 7: Lenalidomide enhanced natural cytotoxicity and ADCC against primary leukemia cells. (a) PBMCs from healthy donors ($n = 4$) and purified leukemia cells (>90% of purity) obtained from CLL patients ($n = 4$) were treated with 1 μM lenalidomide or DMSO for 14 days. Lenalidomide and DMSO-stimulated PBMCs were cocultured with both lenalidomide and DMSO-stimulated leukemia cells at 5 : 1 E : T ratio in the presence or absence of rituximab (20 μg/mL). The expression of CD107a was evaluated in NK cells ($CD3^-CD56^+$), CD8 T cells ($CD3^+CD8^+$), and NKT-like cells ($CD3^+CD8^+CD56^+$) by flow cytometry. Representative dot plots showing CD107a expression on CD8, NKT-like, and NK cells of one representative experiment are shown. The numbers represent the percentage of $CD107a^+$ cells for each subset analyzed. (b) The figure shows the compilation of the results obtained from patients and donors. The bars represent the mean and standard deviation of the percentage of $CD107a^+$ NK cells and NKT-like cell ($^*P < 0.05$; Mann-Whitney U test). (c) The histograms show the analysis of CD20 expression in leukemia cells after 14 days of lenalidomide treatment of a representative patient. Numbers in the histogram are the mean fluorescence intensity (MFI). (d) PBMCs from CLL patients ($n = 4$) were cultured with lenalidomide (1 μM) for 2, 7, and 14 days and the expression of CD20 was analyzed on B cells (gated as $CD5^+/CD19^+$) by flow cytometry. Results are expressed as the fold induction of CD20 MFI in lenalidomide-treated cells relative to the vehicle-treated control ($^*P < 0.05$; Mann-Whitney U test).

a potential link between both cell types. It has been reported that lenalidomide facilitates the nuclear translocation of NFAT and AP-1, via activation of PI3K signaling, which results in IL-2 secretion by T cells [29]. Accordingly, our experiments show that the proliferation of NK and NKT-like cells is mediated by the production of IL-2 by CD4 T cells in CLL. Thus, the blockade of IL-2 activity completely abrogated the proliferation of NK and NKT-like cells. However, a remarkable observation in our study is the existence of a significant interindividual variation in the NK cell response among patients. It is worth mentioning that, in those CLL patients who did not respond to lenalidomide, IL-2 was able to induce the proliferation and the cytotoxic activity of NK cells, suggesting that the level of induction of IL-2 production by CD4 T cells by lenalidomide may be involved in this marked variation of response. The analysis of the therapeutic consequences of this variation and the potential predictive value of the *in vitro* analysis of NK cell proliferation deserves further investigation.

We observed that lenalidomide enhanced NK cell-mediated natural cytotoxicity against leukemia cells. This effect was mainly due to the activation of immune cells, since no further effect was achieved when leukemia cells were also treated with lenalidomide. Lenalidomide treatment increased the expression of NKp30 on NK cells from CLL patients, suggesting a role of this receptor in the increase of NK cell cytotoxicity observed in response to lenalidomide. It is also remarkable, but not unexpected, that lenalidomide had only a modest effect on promoting the antileukemic activity of NK cells against primary leukemia cells. In fact, leukemia cells express low levels of ligands of NK cell activating receptors, probably due to immune evasion mechanisms, being highly resistant to NK cell-mediated lysis [1, 30]. To increase the cytotoxic activity against leukemia cells it is necessary to favor the recognition of leukemia cells by NK and NKT-like cells. In line with this idea, lenalidomide is an attractive agent for combination with rituximab [25]. Our experiments showed a synergistic effect of lenalidomide with rituximab in promoting ADCC against leukemia cells, and this effect is supported by the fact that lenalidomide upregulated CD20 expression on leukemia cells. Overall, our findings provide a support for the combined use of lenalidomide with rituximab in the treatment of CLL patients and suggest that other treatments that increase the immunogenicity of tumor cells, for instance, by inducing the expression of ligands of NK cell receptors on leukemia cells such as histone deacetylase inhibitors [30], may be an attractive therapeutic strategy to be combined with lenalidomide. This clearly warrants further investigation.

In conclusion, our study indicates that the activation of CD4 T and NK cells is a key process underlying the therapeutic effect of lenalidomide in CLL, thus providing a rational support for optimizing and improving the efficacy of lenalidomide treatment in CLL patients.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This work was supported by the Spanish grants of Fondo de Investigaciones Sanitarias (Instituto Carlos III) PS09/00420 and PI12/01280 and by the Celgene Corporation.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 258917, 11 pages
http://dx.doi.org/10.1155/2014/258917

Review Article

New Insights into Monoclonal B-Cell Lymphocytosis

Christina Kalpadakis,[1] Gerassimos A. Pangalis,[2] Sotirios Sachanas,[2] Theodoros P. Vassilakopoulos,[3] Stavroula Kyriakaki,[1] Penelope Korkolopoulou,[4] Efstathios Koulieris,[2] Maria Moschogiannis,[2] Xanthi Yiakoumis,[2] Pantelis Tsirkinidis,[2,5] Marie-Christine Kyrtsonis,[6] Georgia Levidou,[4] Helen A. Papadaki,[1] Panayiotis Panayiotidis,[6] and Maria K. Angelopoulou[3]

[1] *Department of Haematology, University Hospital, University of Crete, P.O. BOX 1352, 71110 Heraklion, Crete, Greece*
[2] *Department of Haematology, Athens Medical Center-Psychikon Branch, 11525 Athens, Greece*
[3] *Department of Haematology, University of Athens, Laikon General Hospital, 11527 Athens, Greece*
[4] *Department of Pathology, University of Athens, 11527 Athens, Greece*
[5] *Department of Haematology, 401 Military Hospital, 11525 Athens, Greece*
[6] *1st Department of Propedeutics, University of Athens, Laikon General Hospital, 11527 Athens, Greece*

Correspondence should be addressed to Christina Kalpadakis; xkalpadaki@yahoo.gr

Received 17 April 2014; Accepted 21 July 2014; Published 11 September 2014

Academic Editor: Kazuyuki Shimizu

Monoclonal B-cell lymphocytosis (MBL) is a premalignant condition characterized by the presence of less than 5000/μL circulating clonal B cells in otherwise healthy individuals. Three subcategories have been identified according to the immunophenotypic features: CLL-like, CD5(+) atypical, and CD5(−) MBL. CLL-like MBL is by far the most frequent and best studied category and further divided in low-count [LC] and high-count [HC] MBL, based on a cutoff value of 500/μL clonal B cells. LC-MBL typically remains stable and probably does not represent a truly premalignant condition, but rather an age-related immune senescence. On the other hand, HC-MBL is closely related to CLL-Rai0, bearing similar immunogenetic profile, and is associated with an annual risk of progression to CLL requiring therapy at a rate of 1.1%. Currently there are no reproducible factors for evaluating the risk of progression to CLL. CD5(−) MBL is characterized by an immunophenotype consistent with marginal zone origin and displays many similarities with marginal zone lymphomas (MZL), mainly the splenic MZL. The cutoff value of 5000/μL clonal B cells cannot probably be applied in CD5(−) MBL, requiring a new definition to describe those cases.

1. Introduction

Multiparameter flow cytometry analysis dramatically increased the sensitivity for detection of small B-cell clones in otherwise healthy individuals [1–7]. A variety of terms have entered the literature to designate this finding. In 2005 the International Familial CLL consortium summarized the literature, proposed certain diagnostic criteria to define this entity, and established the term "monoclonal B-cell lymphocytosis" (MBL) [8]. MBL is characterized by an asymptomatic monoclonal expansion of $<5.0 \times 10^9$/l circulating B cells in apparently healthy individuals without any other feature diagnostic of a B-lymphoproliferative disorder. MBL is classified into three groups according to the immunophenotype of the clonal population. The majority of MBL cases (75%) have the immunophenotype of chronic lymphocytic leukemia (CD19(+), CD5(+), CD23(+), $CD20^{dim}$, and sIg^{dim}) and are classified as typical CLL-like MBL, while the remaining MBL cases are classified as atypical CLL (CD5(+) clonal population not meeting the criteria for typical CLL and not meeting the criteria for mantle cell lymphoma) and as CD5(−) non-CLL MBL [4, 5, 8].

2. CLL-Like MBL

2.1. Prevalence. The reported prevalence of MBL ranges widely from <1% to more than 18%, depending on the

Table 1: Prevalence of CLL-type MBL in some of the larger reported series.

Population studied	Median age, Y (range)	n	Number of colours	Events $\times 10^3$	All ages in study, %	CLL-type MBL > 60 y, %	MBL counts (median)
US residential population [9]	53 (40–76)	1926	2	N.R.	0.6	>0.6	88/μL
US blood donors [10]	45 (18–79)	5141	2	N.R.	0.14	0.9	NR
UK hospital outpatients [4]	57 (40–90)	910	4	200	3.5	5.0	0.013×10^9
Italy primary care [5]	74 (65–98)	500	4	200	5.5	5.5	0.114×10^9 (mean)
UK hospital outpatients with normal ALC [11]	74 (62–80)	1520	4	200	5.1	5.1	NR
UK hospital outpatients with lymphocytosis [11]	71 (39–99)	2228	4	200	13.9	NR	NR[)]
Italy residential population [12]	55 (18–102)	1725	5	500	5.2	8.9	0.034×10^9
Spain primary care [3]	62 (40–97)	608	8	5000	14.3	>20	$0.17 \times 10^{9'}$
US blood donors [13]	57 (45–91)	2098	6	500	4.8	10.7*	0.01×10^9(10/μL)
1st-degree relatives of sporadic CLL [14]	62 (18–84)	167	4	300	4.2	15.6	NR
1st-degree relatives of familial CLL [15]	NR	33	2-3	NR	18	NR	NR
1st-degree relatives of familial CLL [16]	47 (23–86)	59	4	NR	13.6	>20	5/μL

NR: not reported.

sensitivity of flow cytometry and the populations tested (Table 1). The absence of standardized flow cytometry methods for MBL diagnosis complicates determination of the true prevalence. Early population studies indicated an MBL prevalence of 0.1% to 3% by screening for B-cell clones with 1- to 3-color flow cytometric analysis [9, 10]. Studies using 4- to 5-color protocols revealed a higher prevalence, ranging from 3% to more than 6% [4, 5, 12]. In the most sensitive study published to date, Nieto et al. utilized the highest sensitivity flow cytometry approaches available with the use of 8-color staining panels and the analysis of 5 million B cells per subject and identified an MBL prevalence of 14% among 608 healthy adults aged more than 40 years [3]. So, as the sensitivity of flow cytometry increases by the use of multicolor techniques and the analysis of greater numbers of B cells per subject, the prevalence of MBL increases over time.

MBL prevalence also varies based on the studied population. In the general population studies MBL is detectable in approximately 4% to 5% of adults when typical flow cytometric techniques are used (4-color with a detection sensitivity of 1 : 10000 events) [4, 5, 12]. General trends across studies indicate a greater risk of MBL among those with increasing age and among men, similar to CLL [4–6]. In the Spanish study, a CLL-like MBL was detected in more than 1 in 5 individuals over 60 years old [3, 7]. Furthermore, the same group, based on the analysis of 638 healthy adults (>44 years old), investigated the association between the frequency of CLL-like MBL and the volume of sample analyzed. Based on this model, they suggested that the frequency of MBL cases would rise to 100% for subjects older than 70 years when more than 45 mL of peripheral blood was analyzed [7]. In a recent study in 2098 healthy blood donors with the use of 6-color flow cytometry and the analysis of at least 500000 events yielded an overall prevalence of 7.1% [13]. The prevalence was higher in men than in women and increased with age in both genders. There was ~1.4-fold increase for every 10 years in

Table 2: Representative studies on the risk of progression of HC-MBL and suggested cutoff values of clonal B cells for predicting the risk of progression.

STUDY	Pts n	MEDIAN FUP (years)	Range	CLL/SLL requiring therapy	B-cell count cutoff value
Shim et al., 2007 [9]	$N = 185$ Clinical MBL	6.7	0.2–11.8	1.1% per year	<1900/μL no progression
Shanafelt et al., 2009 [17]	$N = 459$ MBL = 190	1.5	0.3–7.9	1.4% per year	<11000/μL predict better TFS and OS
Rossi et al., 2009 [18]	$N = 123$ Clinical MBL	3.6	NS	4% per year for the first 7 years and then 0%	<1200/μL low risk progression
Scarfo et al., 2012 [19]	$N = 184$ Clinical MBL	3.75	0–306	1.5%	10374 2.4 versus 8.5 5000 1.5 versus 5.2

the MBL prevalence among men, starting from 6.0% in the 45–64-year-old group and reaching 12.2% in the 65-year or older group.

MBL is more common in first-degree relatives of patients with familial CLL, with a reported prevalence of 12% to 18% [14–16]. This elevated MBL risk is particularly evident in young adults aged 16 to 40 years with a 17-fold relative risk, suggesting that there is an inherited abnormality that increases susceptibility to development of MBL at a much earlier age than the general population. In relatives of patients with sporadic CLL the overall prevalence has been reported in the range of 4%, similar to that detected in the general population. However, in individuals aged more than 60 years from families with sporadic CLL, the MBL risk seems to be significantly increased (approximately 16%) similar to that seen in relatives of familial CLL cases [14].

In outpatient series the prevalence of MBL is almost the same as in the general population as long as patients with normal blood counts are encountered [4, 5]. However in one study on 2228 outpatients aged 39–99 years who were referred for investigation of lymphocytosis, the prevalence of MBL was much higher, in the range of 13.9% [11].

2.2. Classification of MBL into Low and High Count. In the general population studies the majority of MBL cases have very low numbers of clonal B cells, typically in the range of 0.1–10/μL, with a median number of clonal B cells 0.001×10^9/L [20]. In contrast, MBL cases identified after investigation of absolute lymphocytosis typically have clonal B-cell counts above 450/μL while the median absolute clonal B-cells count is 2,939/μL [11, 18].

Based on this heterogeneity of the size of the B cell clone, MBL is now subdivided into two categories: high-count MBL (also known as clinical MBL) and low-count MBL [20, 21]. A cutoff value of 0.5×10^9 clonal B cells/L has been proposed by the literature for discriminating LC-MBL and HC-MBL [20, 21]. This cutoff value seems to be of clinical relevance, since the majority of MBL cases carry either very low-count clones (<56 clonal B cells/μL) or more than 1500/μL clonal B cells, with different risk of progression [20].

Of note, both low- and high-count MBL cases usually display multiclonality, in contrast to CLL in which multiclonality is much less common [22, 23].

2.3. Clinical or High-Count MBL. HC-MBL is closely related to CLL-Rai0, as it has been shown by several series [11, 18, 20, 21, 24]. Current data indicate CLL is more likely to develop in individuals with clinical MBL than in those with low-count MBL [20]. In the study by Rossi et al. 123 HC-MBL cases were compared to 154 CLL Rai stage 0 cases [18]. Demographics were similar. MBL cases were characterized by lower percentage of bone marrow infiltration, better preserved immune function, more favorable genetic profile, slower disease kinetics, and longer TFS compared to CLL-0, suggesting that HC-MBL is a distinct entity. However, another study by Kern et al. compared 298 MBL cases with 356 CLL patients with regard to biological characteristics and cytogenetics and suggested that these two entities largely overlap and represent closely related stages of the same disease that differ only in tumour mass and that the separation of MBL and CLL by a threshold of 5,000/μL clonal B cells cannot be reproduced by genetic aspects [25]. Studies including cases with clinical MBL have shown that the annual risk of progression to CLL requiring therapy is 1% to 2% (Table 2) [11, 17, 26, 27]. In the Leeds study 185 CLL-like MBL cases were followed for a median of 80 months and 1.1% per year required treatment for disease progression [20]. Similar results were observed in the Mayo Clinic studies which reported on 302 CLL-like MBL cases and observed a 1.4% annual risk of progression to CLL requiring therapy with a median follow-up time of 18 months [17]. In the Italian study, 123 clinical CLL-like MBL cases were monitored for a median of 43 months and it was observed that 4% per year required therapy in the first 7 years and decreased thereafter to 0% [18]. These studies also showed that the absolute B-cell count is the most important determinant of progression to CLL. However there is not any established cutoff point of B-ALC to predict the risk of progression to CLL (Table 2). In the Italian study, a B-ALC greater than 3.7×10^9 B cells/L predicted the highest risk of developing CLL or SLL, whereas a B-ALC at presentation below 1,200/L was the optimal cutoff point to predict a stable

lymphocyte count [18]. In the UK study cutoff points were similar to the Italian one [20]. A B-ALC below 1,200/L and above 4,000/L was the optimal cutoff point for predicting a stable and a rising lymphocyte count, respectively [20]. A Mayo Clinic study by Shanafelt et al. included patients with MBL and Rai stage 0 CLL and observed that a threshold of 11,000/L B-ALC at diagnosis optimally predicted treatment-free survival as well as overall survival [28]. A similar cutoff point of 10,000/L B-ALC has been reported by Molica et al. as well as by Scarfo et al. as a predictor for time to first therapy [19, 29, 30]. In the latter study, by using a cutoff level of 10,374/μL clonal B cells, the difference in terms of annual risk of progression became larger (2.4 versus 8.5% per year) compared with the difference based on the traditional cutoff level of 5,000/μL clonal B cells (1.1% versus 5.2% per year) [19].

The above data indicate that, by using a higher cutoff level of B-ALC instead of the currently used (5,000/μL) for discriminating MBL from CLL Rai stage 0, it seems that it can better assess the risk of progression. However studies are ongoing investigating the best cutoff value for discriminating MBL into LC-MBL and HC-MBL, with distinct biological and clinical significance, as well as for discriminating HC-MBL from CLL Rai 0. Given the fact that B-cell count is a continuous variable, it is rather impossible to establish any specific B-cell count threshold to precisely identify MBL cases with no risk of progression [19].

Assessing the B-cell count seems not to be enough to sharply demarcate the lowest and the highest risk categories. Biological parameters may be needed to best stratify patients [19]. Several factors have been assessed for predicting outcome of MBL, but with conflicting results. Biological factors that have been proposed include IGHV homology, CD38, CD49d, and ZAP-70 expression, and FISH karyotype [18, 20, 21, 28]. Cytogenetic parameters as well as mutation status seem to have the best prognostic power for predicting the risk for progression to CLL [18, 25].

Another important finding of these studies is that progression to CLL did not plateau over a long follow-up time, indicating that clinical MBL always progresses to CLL if given sufficient time [31]. However, only a small number of patients will actually experience disease progression due to the low progression rates per year and the age of subjects.

Besides the risk for progression to CLL requiring therapy, HC-MBL carries also a higher risk of serious infection than the general population. Based on the Mayo Clinic database, Moreira et al. reported that HC-MBL is at ~3-fold higher risk of hospitalization with infection compared with a control population and that this risk is fourfold greater than the risk for progression to CLL requiring therapy [32]. Interestingly, this increased susceptibility to infections was not associated with hypogammaglobulinemia.

2.4. Low-Count or Population Screening MBL. Low-count MBL can be detected only by applying highly sensitive flow cytometry techniques in otherwise healthy individuals. Data on the outcome of low-count MBL is limited. Fazi et al. reported on 76 patients with LC-MBL and observed that, after a median follow-up time of 34 months, 90% of the cases persisted over time in contrast to only 44.4% and 66.7% of atypical CLL and CD5(−) MBL, respectively [33]. Furthermore they reported that most of the LC-MBLs remained stable without progression to clinically overt disease, suggesting that the potential risk of progression into overt CLL is exceedingly rare and definitely less than that of clinical MBL. These findings confirm the hypothesis that the natural history of LC-MBL differs from that of HC-MBL and LC-MBL does not represent a true preleukemic condition, in contrast to HC-MBL. These results are in line with the molecular and biologic differences observed between the two subgroups of MBL [12, 34, 35].

Since the prevalence of LC-MBL is much higher in the elderly population with a peak of 75% in persons above 90 years of age, it seems that LC-MBL may represent an epiphenomenon of immunosenescence, observed in the elderly [33, 34]. In accordance with this hypothesis is the finding of increased clonal T-cell populations [33]. In more than one-half of the cases multiple T-cell clones were identified, compared with the general population suggesting a widespread deregulation of the immune system in MBL. Clonal expansions of T cells are frequently observed in elderly individuals [36]. Moreover, it has been shown that LC-MBL is associated with reduced numbers of normal B-cell subsets, mainly of immature and naïve B cells [37].

2.5. Bone Marrow Histopathology. Bone marrow examination is not required for the establishment of MBL diagnosis. Therefore there is limited data on bone marrow findings in MBL [38]. Our group has recently evaluated the histopathological and immunohistochemical findings of bone marrow biopsies (BMB) in a series of 48 cases (data unpublished). The median percentage of bone marrow infiltration was 28% (range, 5–85%). The pattern of infiltration was interstitial or mixed (nodular and interstitial) in the majority of the cases, 88%. There was no correlation between the extent of BM infiltration and the absolute number of peripheral blood monoclonal B cells.

2.6. Cytogenetic and Molecular Features of MBL. Significant progress has been made recently regarding the molecular and cytogenetic aspects of MBL. Reports of chromosomal abnormalities in CLL-type MBL indicate that both LC-MBL and HC-MBL carry the same cytogenetic aberrations associated with good prognosis CLL [3, 11, 17, 18, 25, 35, 39, 40]. The frequency of 13q deletions, the most favourable cytogenetic subgroup in CLL, is similar to that observed in newly diagnosed CLL, since it has been detected in more than one-third of CLL-like MBL, in both subcategories (low and high count). Even in the Salamanca series, del13q was detected in 36% of the cases, whereas in the Italian series by Fazi et al. del13q was evident in 43.8% of the LC-MBL cases [3, 33]. This suggests that 13q deletion occurs early in the natural history of CLL-like MBL and is probably not associated with the disease progression [41]. A similar finding is seen for trisomy 12 which has been detected in 8–22% of cases among different series including both HC-MBL and LC-MBL [3, 11, 17, 25, 33, 35]. However deletions of 11q and 17p, which are seen in CLL in association with a poor

prognosis, are infrequent in HC-MBL and LC-MBL [3, 11, 17, 25, 33, 35]. Overall, LC-MBL shows a lower frequency of genetic alterations associated with CLL than HC-MBL and CLL. Furthermore, coexistence of ≥2 cytogenetic alterations is less frequent in MBLs than in CLL [35].

LC-MBL and HC-MBL have similar somatic hypermutation status, since more than two-thirds of the cases carry somatically mutated IgHV genes [12, 25, 35, 39]. In the study by Vardi et al. [34], unmutated IgHV genes were used in approximately 25% of LC- and HC-MBL cases, similarly with CLL-0, while CLL > 0 cases displayed unmutated IgHV genes in a significantly higher frequency (~45%). Significant differences have been identified regarding the usage of IgHV genes as well as the Ig gene repertoire between LC-MBL and HC-MBL [12, 34, 35]. The IGHV gene repertoire of LC-MBL displays pronounced differences compared with HC-MBL and CLL-0, such as suppressed frequency of the IGHV1-69, *IGHV4–34*, and *IGHV3–23* genes and overrepresentation of the IGHV4–59/61 genes [12, 34]. The latter genes are found with increased frequency in elderly individuals, confirming the notion that LC-MBL may represent an aspect of the age-related immune senescence [42]. In contrast to LC-MBL, the IGHV repertoire of HC-MBL closely resembles that of mutated CLL, since the *IGHV3–07*, *IGHV3–23,* and *IGHV4–34* genes are used in around half of the cases [12, 34]. A recent study by Orfao's group based on 78 CLL-like MBL and 117 CLL clones showed that certain patterns of IGHV gene usage are associated with specific genetic alterations [35]. Based on these findings the authors identified three different groups: a group including mainly LC-MBL commonly expressing the VH3-23 gene with no or isolated good prognosis cytogenetic alterations, another group which mainly consisted of HC-MBL and advanced-stage CLL with a common usage of the VH1-69 gene along with the presence of poor prognosis cytogenetic alterations, and a third group with intermediate features. Another important difference between LC-MBL and HC-MBL is the finding that BcR stereotypy is exceedingly rare in LC-MBL, in contrast to HC-MBL and CLL, in which BcR stereotypy is a distinctive feature being present in almost one-third of patients [34, 43].

All the above findings further support the notion that LC-MBL does not represent a truly preleukemic condition, but more possibly a physiological process of age-related immune senescence, despite being clonal. HC-MBL, on the other hand, displays significant similarities with CLL-0 at both the clinical and the biological level [18, 25]. In the largest series published today including 333 CLL-like MBLs, HC-MBL exhibited the same frequency of unmutated rearrangements (~25%) with CLL-0 [34]. Furthermore, both HC-MBL and CLL-0 cases presented similar IGHV gene repertoire, which differed from that of LC-MBL and CLL > 0. Another important observation from the aforementioned study is the presence of BcR stereotypy in the same frequency as in CLL-0 (~20%), further supporting the possibility that these two entities have common immunogenetic profile.

Most CLL-like MBL cases display an indolent and stable clinical course. However a small proportion of HC-MBL cases will eventually progress to CLL. On the other hand, it has been shown that virtually all CLL are preceded by an MBL [3]. Since only a minority of MBLs will actually progress to CLL requiring therapy, it is of significant importance to be able to discriminate at diagnosis these rare cases which will eventually progress into a malignant disorder. Understanding the key mechanisms involved in the expansion of the MBL clones may help in a better understanding of the natural history of the disease and to modify our strategies for correctly managing B-cell premalignant states. Given the fact that cytogenetic abnormalities are commonly detected even in LC-MBLs, there is a possibility that their role may be very limited or even nonexistent in the early phases of MBL development [34, 41]. The underlying mechanisms responsible for the development and evolution of MBL into CLL are not known yet. It is of interest that LC-MBL remains stable despite the fact that the proliferative stimuli, such as persistent Ag-induced activation of the BCR, lead to the acquisition of genetic aberrations. On the contrary, in HC-MBL there is probably a genetic predisposition leading to quick transit from the LC-MBL to the HC-MBL/CLL phase [34]. Autonomous genetic abnormalities (e.g., a single mutation) affecting the same or parallel critical signaling pathways may further support and amplify the initial clonal expansion [41]. Among these multiple pathophysiologic mechanisms for the progression of MBL to CLL, antigen stimulation seems to play a critical role. Evidence exists in the literature for a link between infections and increased risk to develop CLL [44, 45].

Next generation sequencing technologies have allowed the identification of several genomic alterations in CLL with prognostic significance, such as mutations of ATM, p53, NOTCH1, SF3B1, and BIRC3 genes that might represent new biomarkers of potential clinical relevance [46–48]. In MBL these secondary lesions are found in a significantly lower frequency (0%–3%) than in CLL [23, 49, 50]. However a recent multicenter trial by the Gruppo Italiano Studio Linfomi reported a much higher prevalence of NOTCH 1 mutations in 100 MBL cases (11%) by using a highly sensitive method [51].

Recently it has been proposed that microRNAs (miRs) are involved in the transition from monoclonal B-cell lymphocytosis (MBL) to CLL [52]. In a study, Ferrajoli et al. tested miR-15a/16-1 cluster, miR-21, and miR-155 expression in purified B cells of normal individuals, individuals with MBL, and patients with CLL, found that miR-155 was overexpressed in B cells from individuals with MBL and even more so in B cells from patients with CLL, when compared with B cells from normal individuals, and supported the use of cellular and plasma levels of miR-155 as biomarkers for the risk of progression in individuals with MBL [53].

Several studies have shown the existence of multiclonality in a significant proportion of MBL cases (up to 20%) [22]. A recent study showed that the B-cell receptor of B-cell clones from multiclonal cases presented a slightly higher degree of HCDR3 homology than B-cell clones from monoclonal cases, in association with unique hematological (e.g., lower B-lymphocyte counts) and cytogenetic (e.g., lower frequency of cytogenetically altered clones) features usually related to earlier stages of the disease. Based on these findings, the authors supported the antigen-driven nature of such

multiclonal B-cell expansions, with potential involvement of multiple antigens/epitopes [54].

The conclusion from the above studies is that the progression of MBL to CLL probably involves multiple pathophysiologic mechanisms including critical gene mutations and microenvironmental stimulation along with a CLL-prone genetic background [41].

3. Practical Aspects Of MBL Diagnosis: Unresolved Issues

3.1. Establishment of Reproducible Prognostic Factors: Drawing the Line between MBL and CLL-0. There is active ongoing research in the field of molecular pathogenesis and progression of MBL to CLL. Significant advances have been recorded so far in order to better understand the clinical aspects of this entity. Currently, it is still a matter of debate which are the best B-cell cutoff value and biological or cytogenetic factors for predicting the risk of progression. Further studies are required in order to determine factors with prognostic significance that would allow us to identify, early, MBL cases with high risk for progression to CLL requiring therapy. On the other hand, among CLL-0 cases, a significant proportion would not progress. Ideally, in the future the category of clinical MBL could be applied for all these cases (MBL and CLL-0) with no risk for progression (irrespectively of their MBL count) and stratify into the group of CLL-0 only those cases with a tendency to progress [41].

3.2. Staging Procedures. According to the guidelines for MBL evaluation at diagnosis, no imaging studies are required [8]. This raises concerns about the possibility that at least a proportion of cases classified as MBL could in fact represent small lymphocytic lymphoma [55] with a nodal burden not evident by physical examination (e.g., intra-abdominal lymphadenopathy). There is limited data evaluating this topic. In the series by Scarfo et al. the vast majority (155/165) of the HC-MBL cases imaging studies were negative, arguing against this hypothesis [19]. Further studies are required to clarify the role of imaging studies in non-CLL-like MBL.

3.3. MBL in Blood Donors. Several of the general population studies for the identification of MBL have been performed in blood donors [10, 13]. Recently, Shim et al. report that MBL is a surprisingly common finding in healthy blood donors in the range of 7.1% [13]. This finding raises concerns regarding the potential risk of transfer of a premalignant condition to recipients of blood transfusion [56]. Given the fact that there is an increased risk for development of a B-cell malignancy, particularly in CLL, with blood transfusions [57], it is very important to clarify this issue. Since LC-MBL is not typically associated with a risk of progression, blood products from such donors are probably acceptable. The potential risk mainly involves blood donors with HC-MBL. So the question is whether donors with mild lymphocytosis should be screened for MBL. In order to answer this question further studies are required. Until then, the recommendation of Shim et al. for a conservative approach to blood transfusions is warranted [13].

3.4. MBL in Transplant Donors. The implications for allogeneic transplantation are significantly more complicated than blood donation, especially if the transplant is for a CLL patient. MBL prevalence is especially high in relatives of familial CLL cases reaching 18% in some studies [15]. Given this high incidence, it is probably reasonable to recommend that potential HLA-matched sibling donors for CLL patients be tested for the presence of MBL or early CLL [58]. Currently, there is limited data evaluating the risk of MBL transfer to recipients and the impact on overall survival after transplantation.

3.5. Ethical Considerations. Clinicians usually face difficulty on how to inform and follow up an individual with a diagnosis of MBL. The most sensible strategy, for the HC-MBL cases, is to reassure them that MBL is not a malignant entity and that the risk of progression to CLL is low, but not negligible, indicating a yearly hematologic consultation with a complete blood cell count and physical examination [31, 41]. For LC-MBL, which is identified in general population studies after the application of high-sensitivity flow cytometry methods, the risk of progression to CLL is very low, if any. Based on these data, it would be appropriate not to inform individuals for having MBL and not to prompt any monitoring [34, 41].

4. Commentary on CLL-Like MBL

Based on the above data the following comments can be made. CLL-like MBL is a quite common condition, being at least 100 times more frequent than CLL. Based on the absolute clonal B-cell count, MBL is further divided into low- and high-count MBL. Not all MBL carry the same risk of clinical progression. The absolute clonal B lymphocyte count is the only well-established prognostic factor so far for the detection of the risk for the progression of MBL into CLL. New data on the biology of MBLs may help to better discriminate the subset of MBLs which are more likely to progress from those cases with no propensity to progression. LC-MBL (<500/μL clonal B cells) is identified in healthy adults during screening-population studies, while clinical or HC-MBL is usually identified during evaluation of lymphocytosis. Based on the current data, LC-MBL is a condition with no clinical relevance and do not require any monitoring. It appears to represent a physiological process of age-related immune senescence rather than a truly premalignant condition. Finally, HC-MBL closely resembles CLL-Rai0 and research is ongoing to identify factors that could help in discriminating those cases with high risk for progression. Table 3 summarizes the main differences in clinical, cytogenetic, and molecular characteristics between HC-MBL, LC-MBL, CLL-0, and CLL > 0.

5. CD5(−) MBL

Data on the clinical aspects and biological significance of non-CLL-like MBL is limited. Non-CLL-like MBL has generally been subdivided into two major groups: CD5(−) MBL and atypical CD5(+) MBL. Atypical MBL displays CD5

Table 3: Main clinical, cytogenetic, and molecular features of CLL-0, CLL>0, HC-MBL, and LC-MBL.

Characteristics	CLL>0	CLL-0	HC-MBL	LC-MBL
		%		
Annual risk of progression		5.2	1.1	0
Del 13q	50	~40	~40	~30
Trisomy 12	16	~20	~20	~10
Del 11q	18	~5	~5	0
Del 17p	7	2-3	0–3	0
Unmutated IGHV genes	~45	~25	~25	~25
VH1-69	~13	~5	~8	~3
VH4-59/61	<5	~5	<5	~20
BCR stereotypy	~5	~20	~20	~30
NOTCH 1 mutation	>15	13	11	0

positivity along with higher expression of CD20 and other immunophenotypic features different from typical CLL-like MBL cells [3–5], resembling the immunophenotype of mantle-cell lymphoma. In most CD5(−) MBL cases, clonal B cells display either an unclassifiable or a marginal zone lymphoma- (MZL-) like immunophenotype. However we should take into account that although most marginal zone lymphoma clones are CD5(−), occasionally CD5 is positive.

The frequency of non-CLL-like MBL is significantly lower than that of CLL-like MBL, comprising less than 20% of MBL cases. In the general population studies the prevalence ranges from less than 1% to 2%, depending mainly on the sensitivity of the flow cytometry used [4, 5]. The frequency of non-CLL-like MBLs increases with age. Nieto et al. found a progressively higher frequency of non-CLL-like MBL cases in the general population with increasing age ranging from 0.4% among subjects aged 40–59 years to 5.4% among individuals over 80 years of age with a male predominance [59].

The clinical course of low-count MBL is usually indolent with no evidence of progression to overt lymphoma. A study by Fazi et al., which included only low-count MBL cases, showed that CD5(−) MBL tended to be transient in a significant proportion of cases (3/9) [33]. These results differ from those published in a series of 12 atypical CLL and CD5(−) MBL reevaluated 12 months after the first immunophenotypic analysis [59]. All clones were confirmed and even showed a significant increase in the median concentration of clonal B cells, without however progression to overt lymphoma. Furthermore in the latter study it was shown that a significant proportion of non-CLL-like MBL cases (4/13) showed biclonality.

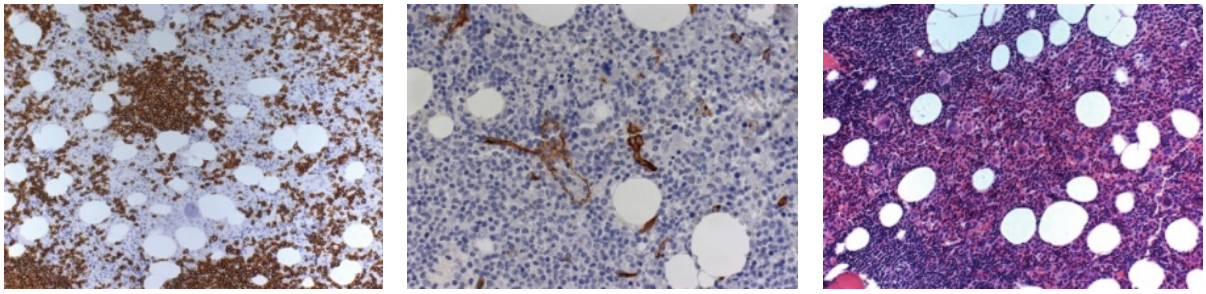
The biology and clinical significance of clinical non-CLL-like MBL has only recently been investigated. There is only a limited series of patients addressing this issue. Amato et al. reported on 7 cases with CD5(−) non-CLL-like clonal B-cell lymphocytosis with an absolute lymphocyte count ranging from 3,600 to 9,400/μL [60]. The clonal population accounted for 95% to 99% of B cells. The mean age at diagnosis was 72.6 years. Somatic hypermutations of the IGHV gene were found in 6 of 7 cases with different VH gene repertoire from that of CLL. Furthermore, cytogenetic aberrations were found in 5 of 6 cases: 2 cases bearing isochromosome 17q that resulted in loss of p53 and 2 other cases that displayed clones with 7q abnormalities. These latter cases had no evidence of an underlying splenic marginal zone lymphoma. During a follow-up period of 4 to 16 years there was no indication of progression to overt lymphoma. The lymphocytosis was persistent but no progressing.

In 2010 our group described an entity presenting with bone marrow infiltration and blood involvement by CD5(−) lymphocytes of marginal zone origin without any other disease localization, which was named primary bone marrow MZL (PBMMZL) [61]. A total of 23 cases were analyzed; 16 of them presented with lymphocytosis. All patients included in this study had undergone bone marrow evaluation, whole-body CT scan, and gastroscopy. Blood lymphocytes were heterogeneous, consisting mainly of small lymphocytes admixed with medium size lymphocytes with nuclear indentations and monocytoid and villous lymphocytes in various proportions. Blood immunophenotype disclosed a clonal B-cell population with strong expression of CD20 and CD79a and moderate to strong expression of surface light chain; approximately half of the cases also expressed the CD23 and CD11c markers, while all cases were negative for the CD5 antigen. Paraproteinemia was observed in almost half of the cases, mainly of the IgG or IgM type at various levels. Bone marrow was always infiltrated but there was a highly variable degree of infiltration (10–90%, median 25%). The findings of the above study disclosed that CD5(−) MBL displays many similarities with marginal zone lymphomas, mainly the splenic form.

In an attempt to further evaluate these results we performed a comparative study of splenic marginal zone lymphoma (SMZL) and CD5(−) MBL, which disclosed that CD5(−) MBL displays similar features with SMZL regarding morphology of lymphomatous cells, bone marrow infiltration pattern, and immunophenotypic findings [62].

Based on this analysis, we soon came up with a difficulty to characterize and stratify cases with clonal B-cell counts more than 5000/μL, which cannot be characterized as MBL, since there is not currently any recognized entity to include such cases.

In an effort to further investigate this issue we performed an analysis on 44 cases with CD5(−) clonal B cells [63].



(a) Nodular pattern (b) Intrasinusoidal pattern (c) Mixed pattern

Figure 1: Different patterns of bone marrow infiltration in CD5(−) MBL cases.

22 of them presented with less than 5000/μL clonal B cells (the median absolute count of clonal B cells was 1123/μL) and could be formally characterized as MBL, while the other 22 cases presented with more than 5000/μL clonal B cells (median count was 6096/μL) and could not be classified under a well-recognized entity. In order to describe these cases, we adopted the term "CD5-monoclonal B-cell lymphoproliferation." No difference was noticed between the two groups regarding morphology of lymphomatous cells, immunophenotype, pattern, and extent of bone marrow infiltration. Bone marrow evaluation revealed a variable extent of infiltration (median 25%, range 5–80%), while the pattern of infiltration was mixed in the majority of the cases and intrasinusoidal infiltration was evident in one-third of the cases (Figures 1(a), 1(b), and 1(c)). Immunohistochemistry showed a strong expression of CD20 along with negativity for CD5 in all cases, while DBA-44 was positive in approximately half of the cases. Analysis of the IgHV mutation status and of the VH gene usage was performed in 20 cases. Unmutated VH genes were noticed in only 25% of the cases, while the VH4-34∗01 was the most commonly used VH gene. Differences were observed only regarding the clinical course of these two subcategories: in cases with <5000/μL clonal B cells 19/22 (86%) remained stable after a median follow-up time of 27 months, while 2 presented a gradual increase of ALC, without cytopenias or any other features consistent with a lymphoproliferative disorder. In one case MBL regressed a year later. Among MBL cases with >5000/μL clonal B cells after a median follow-up of 48 months (range, 7–154), 10 had stable CBC, 9 had a gradual increase of ALC, 3 progressed, and 2 had resolution of lymphocytosis 6 and 24 months after diagnosis, respectively. Two out of the 3 progressing patients developed cytopenias 57 and 79 months after diagnosis, respectively, while 1 patient developed minimal splenomegaly and pancytopenia 130 months later. These 3 patients were treated with rituximab and achieved complete response. This data showed that CD5-MBL displays many similarities with marginal zone lymphomas and that cases with more than 5000/μL clonal B cells may display a more aggressive clinical course than the typical MBL cases, probably representing a distinct entity. Lymphoplasmacytic lymphoma and IgM-like MGUS are two other entities which may overlap with CD5(−) MBL, at least in a proportion of cases presenting with plasmacytic differentiation and paraproteinemia.

Berger et al. used the term leukemic form in an attempt to characterize lymphomas with MZL features, which could not be classified in any of the subcategories of MZL [64]. Our group had used the term primary bone marrow marginal zone lymphoma in order to describe those cases.

A recent collaborative study on a larger series of patients thoroughly evaluated the clinical aspects, biology, and outcome of 102 non-CLL-like clonal B-cell lymphocytosis cases [65]. The median age at presentation was 70 years with a wide range from less than 40 to more than 90 years, with no sex predilection. Absolute lymphocyte counts ranged from 3,000 to 37,100/μL. Lymphocyte morphology was characterized by heterogeneity, comprised of small lymphocytes, monocytoid-like admixed with variant proportion of villous lymphocytes and lymphocytes with plasmacytoid features. Paraproteinemia was evident in approximately one-third of the cases. The immunophenotype was consistent with a marginal zone derivation since the clonal B cells displayed strong expression of B-cell markers (CD20), moderate to strong expression of surface Ig, and negativity for CD10, with a Matutes score <2. Other markers were positive in a proportion of the cases: CD5 in 18.6%, CD23 in 15.6%, and CD38 in 11.3%. CD79b and FMC-7 were positive in the majority of the cases (>80%). CD49d was positive in all 35 studied cases along with low coexpression of CD38. This was the first published study evaluating the bone marrow histopathology and immunohistochemistry of CBL. In most cases a mixed pattern of infiltration was noticed, mainly interstitial along with intrasinusoidal or nodular. Lymphocytes usually were of small size, while there was a wide variation of the percentage of BM infiltration ranging from less than 10% to more than 70%, with no correlation between the extent of bone marrow infiltration and the absolute number of circulating clonal B cells. Plasmacytic differentiation was presented in some cases. Immunohistochemistry was characterized by expression of CD20 and CD79a, while DBA44 was positive in approximately one-third of the cases. CD5 and CD23 were positive in one and four cases, respectively, but with no coexpression, while cyclin-D1 was always negative.

Cytogenetic analysis revealed an abnormal karyotype in the majority of the cases (~70%), while complex karyotype was found in 23%. The chromosomes most frequently involved included 3, 12, 17, and 7. A high incidence (27%) of aberrations involving chromosome 7 was observed. Del7q

which is a typical abnormality in splenic marginal zone lymphoma was detected in 12.5% of MBL cases. Isochromosome 17q was identified in 16.6%. The majority of CBL cases (>70%) carried somatically mutated IGHV genes, with predominance of IGHV4–34 gene. MYD-88 L265P mutation was negative in all of the 45 studied cases. The clinical course varied, with a median follow-up of 5 years; 85 patients remained with isolated lymphocytosis, while 17 cases progressed to an overt lymphoma. Fifteen of them developed splenomegaly, one developed a gastric MALT lymphoma, and one developed diffuse large B-cell lymphoma (DLBCL) of the skin. No difference was found between stable and progressing cases regarding clinical and laboratory characteristics at diagnosis, degree of marrow infiltration, IGHV gene repertoire, and mutational status, while the cytogenetic profiles of the two groups were distinct. Deletions of chromosome 7q were confined to the stable group and complex karyotypes were more frequent in the progressing one. Based on the above study, CBL is closely related to marginal zone lymphomas and in particular to the splenic form: the presence of villous lymphocytes, lymphocytes with plasmacytoid differentiation, intrasinusoidal pattern of bone marrow infiltration, cytogenetic abnormalities of chromosome 7q34, and development of splenomegaly in a significant proportion of cases. According to this study a new term has been initiated which is clonal B-cell lymphocytosis with marginal zone features (MZ-CBL), in order to include cases with CD5(−) clonal B cells irrespective of the absolute number, which fulfill otherwise all the established criteria for MBL diagnosis and proposed to be included in the WHO classification as a provisional entity.

Further studies are required in order to better characterize this entity, identify the relationship with SMZL, and distinguish the majority of cases of MZ-CBL that will remain clinically stable from those destined to progress. Furthermore, no clear recommendations can be made regarding the staging procedures at diagnosis. CT scanning or ultrasonography to exclude nodal and especially splenic enlargement is mandatory, while routine screening for extranodal lymphomas (e.g., gastroscopy) in the absence of specific symptoms is not generally required. Bone marrow examination is mandatory since it provides additional diagnostic information. Since disease progression can occur, often many years after presentation, MZ-CBL cases require long-term follow-up [65].

6. Commentary on CD5(−) MBL

On the basis of the aforementioned data the following comments can be made: CD5(−) MBL displays features consistent with a marginal zone origin. The cutoff value of less than 5000/μL CD5(−) clonal B cells cannot be applied in non-CLL-like MBL, since in contrast to CLL, there is not currently a defined entity to include cases with more than 5000/μL CD5(−) clonal blood B cells. The term MZ-CBL can better describe non-CLL-like cases with clonal B-cell lymphocytosis, irrespective of the absolute number of clonal B cells, and it may be regarded as a provisional entity, probably under the name of "primary bone marrow marginal zone lymphoma." The majority of MZ-CBL present an indolent and stable clinical course. Nevertheless, a proportion of such cases may progress into an overt lymphoma, usually SMZL. Finally, further studies are required in order to better define this entity.

Conflict of Interests

The authors report that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 835138, 11 pages
http://dx.doi.org/10.1155/2014/835138

Review Article

New Insights in the Mobilization of Hematopoietic Stem Cells in Lymphoma and Multiple Myeloma Patients

Maria K. Angelopoulou,[1] Pantelis Tsirkinidis,[2] Georgios Boutsikas,[1] Theodoros P. Vassilakopoulos,[1] and Panayiotis Tsirigotis[3]

[1] *Department of Hematology and Bone Marrow Transplantation, Laikon General Hospital, National and Kapodistrian University of Athens, 17 AgiouThoma, Goudi, 11527 Athens, Greece*
[2] *Department of Hematology, 401 Army Forces Hospital, 138 Mesogeion Avenue, 11525 Athens, Greece*
[3] *2nd Propedeutic Department of Internal Medicine, National and Kapodistrian University of Athens, 1 Rimini Street, Chaidari, 12462 Athens, Greece*

Correspondence should be addressed to Maria K. Angelopoulou; mkangelop@gmail.com

Received 18 April 2014; Accepted 12 July 2014; Published 14 August 2014

Academic Editor: Gerassimos Pangalis

Following chemotherapy and/or the administration of growth factors, such as granulocyte-colony stimulated factor (G-CSF), hematopoietic stem cells (HSC) mobilize from bone marrow to peripheral blood. This review aims to systematically present the structure of the HSC "niche" and elucidate the mechanisms of their mobilization. However, this field is constantly evolving and new pathways and molecules have been shown to contribute to the mobilization process. Understanding the importance and the possible primary pathophysiologic role of each pathway is rather difficult, since they share various overlapping components. The primary initiating event for the mobilization of HSC is chemotherapy-induced endogenous G-CSF production or exogenous G-CSF administration. G-CSF induces proliferation and expansion of the myelomonocytic series, which leads to proteolytic enzyme activation. These enzymes result in disruption of various receptor-ligand bonds, which leads to the disanchorage of HSC from the bone marrow stroma. In everyday clinical practice, CXC chemokine receptor-4 (CXCR4) antagonists are now being used as mobilization agents in order to improve HSC collection. Furthermore, based on the proposed mechanisms of HSC mobilization, novel mobilizing agents have been developed and are currently evaluated in preclinical and clinical studies.

1. Introduction

Autologous hematopoietic stem cell transplantation (ASCT) is a widely used therapeutic strategy in the treatment of multiple myeloma and relapsed/refractory lymphomas. It can offer long-term disease control or even cure in a substantial proportion of patients. The prerequisite of ASCT is a successful and adequate stem cell mobilization and collection. Initial observations regarding the steady state circulation of hematopoietic stem cells (HSC) in the blood led to the study of HSC kinetics after the administration of chemotherapy with or without growth factors. Thus, nowadays, collection of HSC from the bone marrow (BM) has been neglected at least in the autologous transplantation setting and has been largely replaced by peripheral blood stem cell collection through cell separators. Patients who fail to collect $\geq 2.0 \times 10^6$ $CD34^+$ stem cells/kg of body weight cannot undergo ASCT and thus experience its benefits. The deep knowledge and understanding of HSC mobilization will give insight into the mechanisms of "poor mobilization" and moreover may help in developing new mobilizing agents.

2. The Stem Cell Niche

The term HSC was introduced for the first time by Alexander Maximov in 1909 [1]. HSC are primitive undifferentiated cells capable of giving rise to all mature cells of the hematopoietic system through proliferation, differentiation, and maturation. Moreover, they have a self-renewal capacity and the

balance between their quiescence and proliferative potential is under strict control. This in part succeeded through asymmetrical cell division. One HSC gives rise to 2 daughter cells, one of which remains as a pluripotent stem cell and the other migrates to the main bone marrow compartment, where it differentiates to its progenies [2, 3]. The specialized environment, where this fine balance is maintained, is described as the "stem cell niche" and was introduced by Ray Schofield in 1978 [4].

Anatomically, the niche is located in close proximity to the endosteum and is supported by a variety of cells and molecules referred as "stroma." The main representatives of the stroma are bone tissue cells (osteoblasts (OB), osteoclasts (OC), osteomacrophages (OMAC), chondrocytes, fibroblasts, and fat cells), reticuloendothelial cells (dendritic cells, lymphocytes, and macrophages), endothelial cells, as well as mesenchymal stem cells (MSC), myocytes, and cells of the autonomous nervous system. Noncellular stromal elements include the extracellular matrix (ECM), collagen, and minerals [5].

Three types of niches have been recognized: the endosteal (osteoblastic), the reticular, and the vascular (endothelial). The former is located at the endosteum and consists mainly of the "spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic cells" (SNO) [5]. The SNO are supported by the OMAC [6, 7]. The reticular niche is diffusely developed in the BM as a "data network" and consists of specialized reticuloendothelial cells, called "CXCL12-abundant reticular cells" (CAR), which are in close contact with immune cells (B-lymphocytes, plasma cells, plasmacytoid dendritic cells, and NK-lymphocytes), sinusoidal endothelial cells, and Nestin+ MSC[8]. The third niche type refers to a microenvironment rich in oxygen, with low calcium content, consisting of the vascular sinusoidal endothelial cells. Among them, the BM-derived endothelial cells (BMEC) are in close proximity to Nes+ MSC and CAR [7–10]. HSC represent 0.005% of all BM cells, while the multipotent progenitors (MPP) are approximately 0.1%. Human HSC are CD34$^+$, CD38$^-$, CD45RA$^-$, and CD90$^+$. However the ultimate proof of their "stemness" comes from experimental in vivo assays, such as long-term repopulating (LTRA), competitive repopulation unit (CRU), SCID repopulating cell (SRC), and limiting dilution assays [11].

3. Mechanisms of Quiescence and Self-Protection of HSC

The stem cell niche is essential for the quiescence of HSC. More than 70% of them are in the G_0 phase of the cell cycle, while only 10% of their progenies are quiescent. It has been shown that approximately 30% of the quiescent HSC divide every 145–193 days, while a more active subpopulation does so every 28–36 days [12]. These two different subpopulations represent the long-term HSC (LT-HSC), capable of sustaining life-long hematopoiesis, and the short-term HSC (ST-HSC), giving growth to hematopoiesis lasting for several weeks, respectively.

LT-HSC protect themselves from DNA damage by limiting the number of their cellular divisions. The main DNA-repair mechanism of HSC is the nonhomologous end joining, NHEJ [13]. In addition, HSC are capable of transporting damaging agents outside of the cell by ATP-dependent cell transporters, such as BCRP-1 [14]. A variety of other mechanisms contributing to their quiescence are active, such as molecular regulation of the cell cycle through cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors, signaling through thrombopoietin and transforming growth factor β, the ubiquitin/proteasome pathway, and signaling pathways through ATM, P13K-Akt, mTOR, Mdm2/p53, osteopontin, and Notch and Wnt [15–21]. In addition, the hypoxic microenvironment of the BM compared to blood is thought to play a role in the balance between quiescence and differentiation of HSC [22, 23].

It is obvious that the description of the aforementioned three types of niches is undertaken for understanding purposes. They do not represent different anatomical compartments, since bone and vessels are in close proximity to each other [23]. The "niche" is an integrated specialized microenvironment unit that aims to sustain a stable number of HSC at any time, allowing only for a minimal release of HSC to the circulation under steady state conditions. Under "stress," like after recovery from chemotherapy, a massive release of HSC into the blood is observed. This phenomenon may last from hours to few days and is described as HSC mobilization. The underlying mechanisms of chemotherapy/growth factor-induced mobilization have been extensively studied. However, it is not known, whether the same mechanisms are active in steady state mobilization [23, 24]. Due to the wide application of HSC transplantation in every day clinical practice, it is important to get a thorough insight in the mechanisms of HSC mobilization.

4. Mechanisms of HSC Mobilization

A concise presentation of the main cells, receptors, and ligands that interact with each other during the process of mobilization is shown in Table 1. Most of the data regarding mechanisms of HSC mobilization come either from in vitro or from in vivo animal studies; thus it is uncertain whether the exact same mechanisms are functional in the human organism. Moreover, several pathways described thereafter are overlapping and many aspects of this complex cascade of events are still obscure. Mobilization of HSC involves an active interplay between cytokines, chemokines, adhesion molecules, and proteolytic enzymes, leading to the breakdown of HSC anchorage on their niches and their subsequent egress to the blood.

In 1976 Richman et al. described an increase of HSC in the blood of patients who had undergone chemotherapy [25]. Later, a similar increase was observed after the administration of recombinant growth factors [26]. Sequentially, it was proven that both granulocyte-colony stimulating factor (G-CSF) and chemotherapy result in HSC mobilization through the same mechanisms, since chemotherapy leads to endogenous G-CSF increase [27]. Thus the main initiating event of HSC mobilization is either exogenous or endogenous G-CSF or the combination of both.

Table 1: Receptors, ligands, and adhesions molecules involved in the homeostasis of the HSC niche.

Expression from HSC		Expression from BM stroma
Tie2	$\Longleftrightarrow$	Ang-1
Mpl	$\Longleftrightarrow$	TPO
c-kit (CD117)	$\Longleftrightarrow$	SCF
CXCR4	$\Longleftrightarrow$	SDF-1 (CXCL12)
TGF-βR	$\Longleftrightarrow$	TGF-β
FGFR1-4	$\Longleftrightarrow$	FGF
Notch	$\Longleftrightarrow$	Jagged-1
GRP78	$\Longleftrightarrow$	Cripto
		PTH-R
HIF-1α	$\Longleftrightarrow$	Cripto
N-cadherin	$\Longleftrightarrow$	N-cadherin
BMP-R2A	$\Longleftrightarrow$	BMP
VLA4	$\Longleftrightarrow$	VCAM-1
Frizzled	$\Longleftrightarrow$	Wnt (ECM)
FLT-3 (CD135)	$\Longleftrightarrow$	FLT3 ligand
CaR	$\Longleftrightarrow$	Ca^{++} (ECM)
HCAM (CD44)	$\Longleftrightarrow$	Hyaluronan (ECM)
LFA-1	$\Longleftrightarrow$	ICAM-1
VEGF	$\Longleftrightarrow$	VEGFR
Agrin-R	$\Longleftrightarrow$	Agrin proteoglycan (ECM)
$S1P_1$	$\Longleftrightarrow$	S1P

Ang-1: angiopoietin-1, SCF: stem cell factor, SDF-1: stromal-derived factor-1, FGF: fibroblast growth factor, PTH-R: parathormone receptor, Cripto: teratocarcinoma derived growth factor-1/TDGF-1, BMP: bone morphogenic protein, VLA4: very late antigen 4, VCAM-1: vascular cellular adhesion molecule-1, FLT-3: fms-like tyrosine kinase 3, HCAM: hyaluronan binding-cellular adhesion molecule, LFA-1: lymphocyte function-associated antigen-1, VEGF: vascular endothelial growth factor, S1P: sphingosine-1-phosphate, and ECM: extracellular matrix.

4.1. G-CSF. G-CSF promotes proliferation and maturation of the myeloid series, while at the same time it induces substantial changes in the bone marrow stroma, leading to the rise of HSC in the circulation by 60 times compared to baseline [28]. Only cells of the myelomonocytic series, including macrophages and OMAC, express the G-CSF receptor (CD114), whereas HSC do not [29]. Thus mobilization through G-CSF is indirect, through the following proposed pathways.

(a) It directly activates OMAC and macrophages, a fact that downregulates neighboring SNO, Nes+ MSC, and CAR, leading to reduced production of stromal-derived factor-1 (SDF-1) by these latter cells [7, 30]. Reduced SDF-1 leads in turn to loosening of the SDF-1/CXCR4 bond of HSC on BM stroma.

(b) The hyperplastic myelomonocytic series (through G-CSF) secrete a large variety of proteases, which induce proteolytic cleavage/clearance of SDF-1, leading to the release of the CXCR4 receptors on HSC and their subsequent liberation from the BM stroma. The activity of the proteases is further assisted by the cleavage of protease inhibitors [28]. The most widely studied protease is metalloproteinase-9 (MMP-9) [31]. However the dipeptidase CD26 may be more important, since it inactivates SDF-1. Moreover, CD26−/− mice display impaired mobilization [32]. Other proteases implicated in this process are cathepsins G and K and neutrophil elastase [33]. Mobilization through G-CSF is associated with a decrease in heparanase levels and a concomitant increase of MMP-9 and cathepsins [34]. The exact role of other proteins, such as complement [35] and the fibrinolysis/plasminogen [36] system, has not been elucidated yet.

(c) The same proteolytic G-CSF-induced mechanism is responsible for the degradation of VCAM-1, fibronectin, and OPN, leading to reduced cellular adhesion of HSC through their receptor VLA-4 to BM stroma [28, 37].

(d) G-CSF evokes a shift of HSC to more central locations in BM, close to the vascular endothelium due to increased oxygenation needs of the HSC [38]. HSC move towards higher oxygen concentrations, a fact that precedes their migration to the periphery.

(e) G-CSF reacts as a potent noradrenalin reuptake inhibitor. An additive effect of G-CSF and tricyclic antidepressants in HSC mobilization has been shown [39]. Vice versa chemical sympathectomy with beta blockers results in impaired HSC mobilization in mice [40]. Moreover, the sympathetic nervous system results in reduced SDF-1 production and directly reacts with HSC, since the latter express beta-adrenergic and dopaminergic receptors, which activate after G-CSF administration through Wnt signaling [41]. In addition, beta-2 adrenergic signaling augments the expression of vitamin D receptors on SNO, a crucial event for G-CSF mobilization [42].

The two basic G-CSF-induced mechanisms of SDF-1/CXCR4 and VCAM-1/VLA4 disruption have a synergistic effect. Coadministration of G-CSF and CXCR4 inhibitors or G-CSF and anti-VLA4 antibodies results in additive and more potent HSC mobilization compared to G-CSF alone [28].

4.2. SDF-1/CXCR4. SDF-1 (CXCL12) is a CXC chemokine, secreted by various BM stromal cells, such as CAR, Nes+ MSC, osteoblasts, and endothelial cells. The interaction of SDF-1 with its receptor CXCR4 on HSC plays a key role in HSC retention and trafficking. The expression of CXCR-4 on HSC is enhanced through a signaling cascade involving cAMP, phosphatidylinositol-4,5-biphosphate 3 kinase (PI3K), a number of GTPases, and atypical protein kinase C isoform ζ (PKC-ζ) [43]. PKCζ induces motility, adhesion and survival of $CD34^+$ cells and MMP-2, and MMP-9 secretion. In addition, CXCR4 expression is dependent on stem cell factor (SCF) [44]. SDF-1 is the most powerful HSC chemoattractant and survival regulator. Peled et al. were among the pioneers investigating the crucial role of SDF-1/CXCR4 interaction in HSC homing. They showed that treatment of human cells with a CXCR-4 antibody prevented their engraftment in a xenotransplantation model [44]. As

already emphasized, the disruption of the SDF-1/CXCR4 axis represents the major mechanism by which HSC are released from their niche. This event is initiated by exogenous or endogenous G-CSF. The ultimate proof of the pivotal role of SDF-1/CXCR-4 axis has been shown from experiments, where HSC mobilization was inhibited by neutralizing anti-SDF-1 or anti-CXCR-4 antibodies [33].

The recently introduced mobilizing agent CXCR4 inhibitor plerixafor (AMD3100) disrupts the SDF-1/CXCR4 axis in a synergistic way to G-CSF. The following hypotheses have been proposed for its mechanism of action [45]. (a) The inhibition of CXCR4 on HSC leads to loss of their sensitivity to SDF-1. As a result, HSC are attracted to the circulation through a positive signal, most likely the sphingolipid sphingosine-1-phosphate (S1P). Alternatively HSC passively move to the blood. (b) Plerixafor targets SDF-1 that is produced by BM stromal cells, causing a decrease of its levels and leading ultimately to mobilization. Only a minor to moderate decrease in SDF-1 levels is sufficient for the migration of HSC from BM to blood. (c) Bonig and Papayannopoulou have suggested that plerixafor does not actually cause mobilization from the BM, but it traps HSC in the circulation by binding on CXCR4 and leading to loss of chemoattraction to SDF-1. This hypothesis requires a rapid steady state HSC turnover between marrow and other tissues and may explain the high HSC numbers observed in the blood within 30 minutes after plerixafor administration [23].

Comparing the differences between G-CSF- and plerixafor-induced mobilization, it should be noted that CXCR4 antagonists lead to a more rapid mobilization. Additionally, CXCR4 antagonists do not induce either myeloid hyperplasia or proteolysis. It has been shown from experiments in primates that myeloid hyperplasia is not necessary for mobilization [27]. Recent studies have evaluated possible differences in cell composition between G-CSF and plerixafor-mobilized grafts. Plerixafor, G-CSF, and plerixafor plus G-CSF mobilize different types of $CD34^+$ cells. The proportion of the most primitive HSC ($CD34^+$ $CD133^+$ $CD38^-$) has been reported higher in grafts from patients with lymphomas mobilized with chemotherapy/G-CSF plus plerixafor [46]. Fruehauf et al. found increased expression of several antiapoptosis, cell cycle, DNA repair, cell motility, and oxygen transport genes in plerixafor plus G-CSF-mobilized $CD34^+$ cells [47]. Donahue et al. studied HSC collected from primates and show that plerixafor plus G-CSF mobilizes a greater proportion of B- and T-cell precursors than either G-CSF or plerixafor alone. Moreover they suggest that G-CSF-mobilized $CD34^+$ cells may contain a wider range of lineage progenitors and hypothesize that $CD34^+$ cells mobilized with G-CSF may result in faster neutrophil recovery, while those mobilized with plerixafor or plerixafor plus G-CSF may have more rapid B-cell and T-cell recovery [48]. Prospective studies focusing on the reconstitution of different cellular subpopulations are needed in order to draw firm conclusions. Regarding, other cellular populations, plerixafor-mobilized grafts have increased absolute numbers of T cells, helper T- and suppressor T-cell subsets, as well as interferon-gamma and tumor necrosis factor-alpha secreting T-lymphocytes compared to G-CSF-mobilized grafts [46, 49].

4.3. Sphingolipids, Prostaglandins, and Eicosanoids. The SDF-1/CXCR4 axis seems to be influenced by sphingolipids, with the main representative being sphingosine-1-phosphate (S1P), which is abundantly produced by red blood cells, activated platelets, and endothelial cells [50]. Currently S1P is extensively studied and represents a "hot" topic in HSC mobilization [51]. There is evidence that HSC possess receptors for S1P. Through interaction with its receptors, it may control the chemotaxis of HSC between BM, blood, and tissues. Characteristically, it increases in blood and decreases in BM during mobilization, while it also inhibits SDF-1 through the p38/Akt/mTOR pathway [51]. Experimental elimination of $S1P_1$ receptors leads to impaired mobilization after CXCR4 inhibitors in mice, while intravenous administration of $S1P_1$ agonists induces the opposite effect [52, 53]. The motility of HSC depends on the equilibrium between S1P levels in the circulation and SDF-1 in the bone marrow. While SDF-1 results in the adherence of HSC in the niche, S1P is a major chemoattractant in the blood. This balance is directed towards the egress of HSC to the blood during stress. SDF-1 and S1P are both regulated by specificity-protein-1 (SP1). SP1 acts as a circadian-regulated transcription factor of SDF-1, but it also induces the biosynthesis of S1P [54]. Thus both molecules, which act antagonistically, are closely regulated. Apart from sphingolipids, endocannabinoids induce decreased expression of various adhesion molecules and CXCR4, leading to synergy with G-CSF mobilization [55]. On the contrary, prostaglandin E_2 (PGE_2) regulates the expression of CXCR4 on HSC and aids SDF-1 in attracting them to the BM [56].

4.4. Adhesion Molecules. Adhesion molecules are surface antigens that facilitate cell-cell and cell-extracellular matrix (ECM) interactions, through their respective receptors. They play a major role in inflammation, mediating trafficking, endothelial rolling, adhesion, and extravasation of leukocytes and lymphocytes. They are essential for cellular immune responses, normal hematopoiesis, and differentiation, as well as for the organization of cells within tissues during ontogenesis. They are widely distributed on hematopoietic and nonhematopoietic cells [57]. According to their structure, they are classified in integrins, molecules belonging to the immunoglobulin superfamily, and selectins. HSC express several types of adhesion molecules, responsible for their interaction with bone marrow stroma.

(a) The VLA-4/VCAM-1 axis: the β1 integrin very late antigen-4 (VLA-4) is expressed by HSC and facilitates their adhesion on BM stroma through interaction with vascular cellular adhesion molecule-1 (VCAM-1), fibronectin, and OPN. Studies in mice, primates, and humans have shown that the administration of the anti-VLA-4 monoclonal antibody (natalizumab) or blockade of its ligands leads to a potent mobilization effect [23, 28, 58]. However, the disruption of the VLA-4/VCAM-1 axis is induced by both G-CSF and CXCR4 inhibitors. Thus it cannot be viewed as an independent/primary mobilization mechanism.

(b) CD44 (HCAM): CD44 has many isoforms. Among these, CD44s, the smallest one, is the most common one expressed on HSC. The major ligand of CD44 is hyaluronic acid (HA), a component of ECM. The most HA-rich regions of the bone marrow stroma are the endosteum and the sinusoidal endothelium, the sites that are considered the niches of the most primitive HSC. CD44/HA interactions are essential for homing of HSC in the BM. Membranal CD44 cleavage on HSC is associated with mobilization and depends on the equilibrium of a membrane-bound proteolytic enzyme (membrane-type-1 metalloproteinase-MT-1 MMP) and its inhibitor RECK. G-CSF administration leads to increased expression of MT-1 MMP on $CD34^+$ cells, which in turn results in membranal CD44 cleavage and HSC egress from the BM [59, 60]. Homing of HSC is impaired after the administration of anti-CD44 antibodies in mice, highlighting its crucial role for SDF-1/CXCR4 binding [60].

4.5. SCF/c-kit. Stem cell factor (SCF) is an important chemokine produced by SNO and CAR. Its active form binds to c-kit on the surface of HSC. C-kit (CD117) is a type III tyrosine kinase receptor and is also expressed by endothelial progenitor cells and MSC. The SCF/c-kit axis plays an important role in embryonic hematopoiesis and cellular differentiation. SCF has been shown to augment engraftment in mice through increased CXCR-4 expression on HSC [44]. Experimental elimination of c-kit in mice mobilized with plerixafor or anti-VLA-4 resulted in a negative effect of mobilization [61]. In these c-kit-mutant mice, c-kit activity is defective, but c-kit levels and its binding ability to SCF are normal. Thus the c-kit kinase activity appeared to be crucial for mobilization [61]. However its exact role has not been elucidated, and conflicting results have been reported. In another experiment, the functional blockade of c-kit with a neutralizing antibody was associated with HSC migration from the BM to the blood [62].

4.6. Osteoblasts/Osteoclasts (OB/OC). It is nowadays widely accepted that bone tissue plays an important role in maintaining and regulating the HSC niche. Bone remodeling is a complex process and its consequences on HSC retention and mobilization are an evolving field [63].

Receptor activator of NF-κB ligand (RANKL) is a cytokine that belongs to the tumor necrosis factor superfamily. It is produced by OB, other stromal cells, and activated T-lymphocytes. It stimulates osteoclastic differentiation, maturation, and activation. RANKL upon binding to its receptor RANK (on OC) stimulates osteoclastic activity. RANKL may also bind to osteoprotegerin (OPG). OPG is also produced by OB and acts as a soluble decoy receptor of RANKL that prevents the latter from binding to RANK. Thus the regulation of osteoclastic activation is dependent on RANKL/OPG balance.

The administration of RANKL in mice resulted in an expected increase of osteoclastic activity, but also a simultaneous HSC mobilization, was observed [64]. During the same experiment, the inhibition of OC by exogenous calcitonin led to a decrease of HSC egress from the BM. Thus, it has been proposed that OC produce proteolytic enzymes, such as MMP-9 and cathepsin K, which disrupt important HSC/niche interactions, promoting the release of HSC from the BM [63]. G-CSF has been shown to induce reduction of the number and function of endosteal OB leading to reduced SDF-1 transcription, while it causes an increase of activated osteoclasts [63]. However, experimental data in humans indicate that the augmentation of osteoclastic activity and bone resorption occur a few days later than the typical peak of 4-5 days after G-CSF mobilization [65]. Moreover, inhibition of OC through the administration of bisphosphonates or RANKL neutralizing antibodies does not negatively affect the mobilization of HSC in mice [7]. In line with these findings, osteoporotic mice show impaired HSC mobilization, while increased bone mass is associated with an increase in the HSC pool [66]. Thus the role of OB/OC interactions in regulating mobilization seems more complicated and remains enigmatic.

In an effort to elucidate the role of bone tissue in human HSC mobilization, we undertook a study of various biochemical markers of bone remodeling, osteoclast/osteoblast regulators, and angiogenic cytokines in the process of HSC mobilization. We studied 24 patients, 10 with multiple myeloma, 5 with Hodgkin's Lymphoma, and 9 with non-Hodgkin's lymphoma who were mobilized with G-CSF +/− chemotherapy [67]. We found that both soluble RANKL and OPG increased significantly between premobilization and HSC collection, but the increase in sRANKL was more prominent than OPG, leading to an increased sRANKL/OPG ratio, indicating stimulation of osteoclastic activity during mobilization. However, impressively, we did not observe a respective increase in bone resorption. On the contrary we found a significant increase of bone specific alkaline phosphatase (bALP), which is considered the most sensitive marker of bone formation. In parallel we showed that the ratio of angiopoietin-1/angiopoietin-2 was significantly reduced indicating vessel destabilization. Our results suggest that increased osteoblastic activity and endothelial vessel destabilization are the two major events during human HSC mobilization. Osteoblasts are the orchestrating cells, while osteoclasts are stimulated, but not fully active. Moreover, high levels of bone resorption markers (C-terminal and N-terminal telopeptides) as well as low levels of angiopoietin-1 before the initiation of mobilization are reliable predictors of poor HSC collection.

4.7. CD45. CD45, known as leukocyte common antigen, is expressed by all leukocytes, including HSC. It is a transmembrane protein tyrosine phosphatase. Through dephosphorylation of Src kinase, it is involved in signal transduction pathways, regulating several cell processes. CD45 has been mainly studied in lymphocyte maturation, proliferation, and activation. Recent data have demonstrated a pivotal role of

CD45 in HSC motility and microenvironmental regulation in both mice and human. From CD45 knockout mice experiments and CD45 treated human HSC in xenotransplantation experiments, it has been shown that CD45 is essential for HSC mobilization. G-CSF leads to increased expression of CD45 in bone marrow mononuclear cells, which in turn correlates with HSC release from the bone marrow. HSC from mice lacking CD45 show reduced mobilization, impaired ability to cross extracellular matrix barriers, and a hyperadhesive phenotype. In addition CD45 is required for normal osteoclastic development and function. CD45 negative osteoclasts display abnormal morphology and have a reduced ability to form multinucleated cells. Moreover they secrete lower amounts of MMP-9 and have a reduced bone resorbing activity. CD45 negative osteoclasts display a reduced response to RANKL resulting in poor HSC mobilization. Thus CD45 is a paradigm of the concurrent regulation of hematopoiesis and the microenvironment [68, 69].

4.8. Hypoxia. The main regulator of hypoxia in the endosteal niche is HIF-1, consisting of HIF-1α and HIF-1β. Under conditions of adequate oxygenation, HIF-1α is hydroxylated and is recognized by Von Hippel-Lindau protein, leading to its proteasome-mediated degradation [70]. Under hypoxic conditions (which are prevalent during G-CSF mobilization), the hydroxylation of HIF-1α is prevented, and HIF-1α is stabilized. The full heterodimeric HIF-1 binds to hypoxia response elements of the genome, resulting in the transcription of several genes, including VEGFA in the BM sinusoids [71]. VEGF results in vasodilatation and HSC mobilization.

It has been shown that activation of m-TOR and the resultant increase of reactive oxygen species (ROS) in the BM lead to the egress of HSC, whereas the inhibition of mTOR with rapamycin has the opposite effect. It appears that a "critical" level of ROS and HIF-1α are necessary for mobilization [72].

5. Clinical Applications of HSC Mobilization Mechanisms

As already stated, the main initiating molecule of the HSC mobilization process is exogenous or chemotherapy-induced endogenous G-CSF. For many years, the administration of G-CSF with or without chemotherapy has been the mainstay of mobilization in every day clinical practice. Ifosfamide-containing (IGEV and ICE) or platinum-containing (ESHAP and DHAP) regimens with G-CSF are usually applied in lymphomas [73–75]. These regimens have a triple role: they offer disease control, they test tumor chemosensitivity, and they result in excellent HSC mobilization. In multiple myeloma, either cyclophosphamide at high or intermediate doses in combination with G-CSF or G-CSF alone may be used [76].

The endpoint of every mobilization attempt is the collection of adequate numbers of HSC, reflected practically by the absolute number of $CD34^+$ cells collected per kg of body weight. Adequate number is considered the dose of HSC that ensures rapid and sustained long-term hematopoiesis after the administration of myeloablative chemotherapy and HSC infusion. The optimal dose of HSC is 5×10^6/kg, with little clinical benefit with doses $5–8 \times 10^6$/kg and no further improvement with grafts containing $>10 \times 10^6$/kg $CD34^+$ cells [77]. The lowest acceptable dose is 2×10^6/kg.

Still, there are a substantial proportion of patients who fail to mobilize and collect adequate numbers of HSC and consequently cannot proceed to autologous stem cell transplantation. The inability of adequate HSC mobilization is associated with HSC reserve exhaustion and disruption of BM stroma [78]. However, even 5% of healthy donors fail to mobilize adequate numbers of HSC. Genetic polymorphisms in molecules associated with the BM niche, such as VCAM-1 and SDF-1, have been implicated in this [79].

Risk factors for poor mobilization include age >65 years, bone marrow cellularity <30%, bone marrow infiltration, multiple previous chemotherapy regimens, especially alkylating agents, fludarabine, >4 cycles of lenalidomide, and previous extended field radiotherapy including the pelvis [80, 81].

There is not a widely accepted definition of the "poor mobilizer." For this purpose, the Italian group of apheresis has proposed criteria not only for the definition, but also for the early recognition of the poor mobilizer [81]. According to these criteria a patient is considered as a proven poor mobilizer if he/she collected $<2 \times 10^6$/kg $CD34^+$ cells through three consecutive days of apheresis, after the administration of an adequate mobilization regimen (G-CSF monotherapy at a dose of $\geq 10\,\mu$g/kg/day or chemotherapy + G-CSF $\geq 5\,\mu$g/kg/day) [82]. However patients who fail to achieve a maximum peak of circulating $CD34^+$ cells $< 20/\mu$L on the day of the expected peak are also considered poor mobilizers [83]. The day of the expected peak is dependent on the mobilizing regimen. Thus with G-SCF monotherapy at a dose of 10 μg/kg, peak levels of $CD34^+$ cells are expected on the 5th-6th day after the initiation of G-CSF. After ifosfamide-containing regimens + G-CSF, the peak day is usually the 12th-13th day since chemotherapy initiation, whereas the corresponding day for platinum-containing chemotherapy ranges between the 15th–17th day.

6. Plerixafor in HSC Mobilization

Plerixafor is a reversible CXCR4 antagonist and its mechanisms of action have been already described. It has been approved in both the US and the EU for the mobilization of patients with lymphoma and myeloma. With its use in combination with G-CSF, approximately 70% of poor mobilizers may succeed in the collection of adequate numbers of HSC in order to proceed to ASCT [84]. The superiority of plerixafor in combination with G-CSF over G-CSF alone was established by several phase II [85–89] and two phase III multicenter randomized double-blinded placebo controlled studies [84, 90]. In the lymphoma trial [84] a significantly higher proportion of patients (59.3%) achieved optimal $CD34^+$ cell collection ($\geq 5 \times 10^6$/kg) in the G-CSF plus plerixafor arm compared to the G-CSF plus placebo arm (19.6%). In addition 86.7% of the patients mobilized with G-CSF plus plerixafor reached the minimum target of $\geq 2 \times$

10^6/kg $CD34^+$ cells versus 47.3% of those who received G-CSF plus placebo. The number of apheresis sessions was also significantly lower in the combination arm. The median fold increase in circulating $CD34^+$ cell count from day 4 to day 5 was 5.0 in the plerixafor treated patients and 1.4 in the placebo treated patients. Similarly in the myeloma study [90] 71.6% of the G-CSF plus plerixafor treated patients collected $\geq 6 \times 10^6$/kg $CD34^+$ cells in ≤ 2 apheresis procedures versus 34.4% of the G-CSF plus placebo group. Plerixafor has an excellent safety and tolerability profile.

The high cost associated with plerixafor requires a stringent protocol with well-defined criteria for its proper use [84, 88, 91]. Plerixafor can be applied with three strategies: (i) identification of patients with risk factors for poor mobilization and upfront use of plerixafor. This strategy is still outside the current approved indications and is not recommended; (ii) combination of G-CSF and plerixafor in a second mobilization attempt for patients who have already failed a prior HSC collection. This is the current approved usage of the drug; G-CSF is given at a dose of 10 μg/kg/day $\times$ 4 days and plerixafor is administered at a dose of 240 μg/kg on the fourth day, 9–11 hours before collection on the 5th day; however with this strategy, significant time is lost between the mobilization attempts, which might be crucial for some patients with aggressive disease; in addition, the repetition of the whole procedure is cumbersome for the personnel and inconvenient for the patient; (iii) the preemptive—"just in time"—use of plerixafor seems to be the most cost-effective strategy: the proper mobilization regimen according to the patient's disease status is chosen. If CD34 counts in peripheral blood on the day of the expected peak are suboptimal ($<20/\mu$L), plerixafor is administered prior to HSC collection. With this latter strategy >90% of the patients can achieve grafts with $>2 \times 10^6$/kg $CD34^+$ cells [92]. Another practical issue with plerixafor is the optimal timing of its administration. Current data indicate that the peak of circulating $CD34^+$ cells can be achieved in 3–8 hours after its administration, which may be more convenient for organizing the procedure of apheresis [93].

Although plerixafor rescues a significant proportion of patients from failing collection of HSC, there is still a 35% possibility of its failure. Towards this aim, new mobilizing agents are being tested either in preclinical or clinical studies.

7. Novel Agents Tested in Human Clinical Studies

(i) Agents targeting the SDF1/CXCR4 axis: POL6326 (Polyphor, Allschwil, Switzerland) is a potent selective CXCR4 inhibitor, which has shown considerable efficacy as monotherapy in newly diagnosed multiple myeloma patients [94]. Its excellent safety and tolerability profile renders it a promising agent and is currently tested in further dose escalation. BKT140 (Biokine Therapeutics, Rehovot, Israel) is another promising high affinity CXCR4 inhibitor that has been tested in a phase I/IIa trial in multiple myeloma patients in combination with intermediate dose cyclophosphamide and G-CSF. BKT140 resulted in a significant collection success that reached a mean absolute of 20.6×10^6/kg $CD34^+$ collected cells for the highest dose used. Moreover the number of apheresis procedures was reduced with the highest doses. This agent warrants further evaluation, since it also shows antimyeloma activity [95, 96]. Other molecules belonging to this category are TG-0054 (Taigen Biotechnology, Taipei, Taiwan) [97] and NOX-A12 (NOXXON Pharma, Berlin, Germany) [98]. The latter is a structured mirror-image RNA oligonucleotide, a so-called Spiegelmer that binds SDF-1, thereby inhibiting its activity.

(ii) Proteasome inhibitors: bortezomib is nowadays considered among the leading therapeutic drugs in multiple myeloma. Surprisingly, in animal studies, bortezomib proved to be a potent mobilizing agent, increasing significantly CFU-Cs compared to placebo. In addition, a synergistic effect of bortezomib with G-CSF, plerixafor, and chemotherapy was evident [99]. The most likely mobilizing mechanism of bortezomib is the disruption of VLA-4/VCAM-1 axis. In a recent phase II study, the addition of bortezomib to cyclophosphamide and G-CSF during mobilization resulted in a median $CD34^+$ yield of 23.2×10^6/kg in a median of 1 apheresis session [100].

(iii) Parathormone (PTH): Brunner et al. showed that PTH induces a significant increase of progenitor cells in the peripheral blood (1.5- to 9.8-fold) of mice. The authors postulated that this activity relates to endogenous release of G-CSF [101]. PTH was evaluated in a phase 1 study in escalating doses in combination with G-CSF in patients who had failed prior mobilization attempts: 47% and 40% of the patients who had failed one and two prior mobilization attempts, respectively, succeeded in reaching the mobilization criteria of the study [102].

8. Novel Agents Evaluated in Animal Studies

(i) *VLA-4/VCAM-1 Inhibitors*. The importance of VLA-4/VCAM-1 axis has been already extensively described. Papayannopoulou and Nakamoto were among the pioneers in studying the effect of the VLA-4/VCAM-1 axis disruption in HSC mobilization. They showed that the administration of anti-VLA-4 antibodies resulted in the mobilization of HSC into the circulation [103]. Moreover, the concurrent inhibition of VLA-4 and CXCR-4 has a synergistic mobilization effect in primates [104]. Although, anti-VLA-4 antibodies have not been tested in human mobilization, there are data indicating that it has a mobilizing effect in the human as well. Natalizumab is a humanized monoclonal antibody against the α4 subunit of VLA-4, approved for treatment of multiple sclerosis (MS) and Crohn's disease. In patients with MS treated with Natalizumab, a gradual increase

in circulating $CD34^+$ cells has been observed with a peak being present in 3-4 days after treatment. However the persistence of $CD34^+$ cells in the circulation for an extended time period may be problematic in clinical practice [58, 105]. The study of other small inhibitors of the VLA-4/VCAM-1 axis is ongoing.

(ii) Other novel agents currently evaluated in preclinical studies are FG-4497 that stabilizes HIF-1 through inhibition of its hydroxylation [106], Groβ (CXCL2), a chemokine whose exact mechanism is unclear [107], and S1P agonists [52].

9. Conclusions

The mechanisms of HSC mobilization are overlapping and not fully elucidated yet. Experimental studies have given light into many aspects of this cascade of events. However, we are still far away from establishing an integrated model of the mechanisms that control the equilibrium of HSC between quiescence and mobilization. Laborious research is mandatory for the development of newer agents that might render HSC mobilization and collection possible for all patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 809103, 6 pages
http://dx.doi.org/10.1155/2014/809103

Research Article

Clonotypic Analysis of Immunoglobulin Heavy Chain Sequences in Patients with Waldenström's Macroglobulinemia: Correlation with *MYD88* L265P Somatic Mutation Status, Clinical Features, and Outcome

Loizos Petrikkos, Marie-Christine Kyrtsonis, Maria Roumelioti, George Georgiou, Anna Efthymiou, Tatiana Tzenou, and Panayiotis Panayiotidis

Hematology Section of First Department of Propaedeutic Medicine, National and Kapodistrian University of Athens Medical School, Laikon Hospital, Agiou Thoma 17, 11527 Athens, Greece

Correspondence should be addressed to Loizos Petrikkos; lpetrikkos@gmail.com

Received 18 April 2014; Accepted 12 July 2014; Published 14 August 2014

Academic Editor: Gerassimos Pangalis

We performed *IGH* clonotypic sequence analysis in WM in order to determine whether a preferential *IGH* gene rearrangement was observed and to assess *IGHV* mutational status in blood and/or bone marrow samples from 36 WM patients. In addition we investigated the presence of *MYD88* L265P somatic mutation. After *IGH* VDJ locus amplification, monoclonal VDJ rearranged fragments were sequenced and analyzed. *MYD88* L265P mutation was detected by AS-PCR. The most frequent family usage was *IGHV3* (74%); *IGHV3-23* and *IGHV3-74* segments were used in 26% and 17%, respectively. Somatic hypermutation was seen in 91% of cases. *MYD88* L265P mutation was found in 65,5% of patients and absent in the 3 unmutated. These findings did not correlate with clinical findings and outcome. Conclusion. *IGH genes*' repertoire differed in WM from those observed in other B-cell disorders with a recurrent *IGHV3-23* and *IGHV3-74* usage; monoclonal *IGHV* was mutated in most cases, and a high but not omnipresent prevalence of *MYD88* L265P mutation was observed. In addition, the identification of 3 patients with unmutated *IGHV* gene segments, negative for the *MYD88* L265P mutation, could support the hypothesis that an extra-germinal B-cell may represent the originating malignant cell in this minority of WM patients.

1. Introduction

Waldenström macroglobulinemia (WM) is an uncommon B-cell lymphoproliferative disorder characterized by bone marrow lymphoplasmacytic infiltration and by the presence of a monoclonal IgM immunoglobulin in the serum [1]. It belongs to the lymphoplasmacytic lymphoma type [2]. Clinical manifestations of WM include lymphoma-related lymphadenopathy, organomegaly, fatigue, disease related fever, or symptoms related to bone marrow failure (cytopenias) and IgM-related cryoglobulinemia, cold agglutinin syndrome, demyelinating neuropathy, and symptomatic hyperviscosity [3]. The monoclonal IgM is produced by malignant B-cells harboring a unique clonotypic rearrangement of immunoglobulin heavy chain variable genes (*IGH*), the VDJH rearrangement, associated with a specific constant region [4, 5]. On immunophenotype WM lymphoplasmacytes are usually CD5−, CD10−, CD23−, CD19+, CD20+ but numerous variations can be observed and in addition there is no characteristic pathognomonic genetic alteration; thus, differential diagnosis with other entities that can secrete IgM, share the same immunophenotype, and present lymphoplasmacytic differentiation may be sometimes difficult [6].

Immunoglobulin heavy chain gene (*IGH*) sequence analysis can provide useful clues in the investigation of B-cell lymphoproliferative disorders. It provides evidence regarding the maturation status of specific B-cell entities. Disorders characterized by germline immunoglobulin genes

are likely to be derived from naive B cells, which have not encountered antigen. Most of B-cell lymphoproliferative disorders, however, exhibit somatic hypermutation (SHM) of immunoglobulin variable genes and are, therefore, derived from cells that have encountered antigen in germinal center. In addition, in chronic lymphocytic leukemia (CLL), marginal zone lymphoma (MZL), and mantle cell lymphoma (MCL), biased usage of *IGHV* genes and stereotyped clusters of immunoglobulin receptor support the role of antigen-driven mechanisms in their pathogenesis [7, 8]. The WM *IGHV* gene repertoire is completely different from other B-cell lymphoproliferative disorders like CLL and MZLs, as it is characterized by an overrepresentation of *IGHV3-23* genes with high *IGHV* mutation rates [8–13]. These features indicate that the transformation leading to WM occurred in postgerminal center B-cells that bear SHM and have been submitted to T-dependant antigen selection.

Recently, whole genome sequencing in WM patients revealed a highly recurrent somatic mutation (*MYD88* L265P) in these patients [8, 14–20]. It was furthermore suggested that *MYD88* L265P mutation possibly constitutes the initiating event, responsible for disease transformation [21, 22]. Furthermore its detection could constitute a valuable differential diagnosis tool.

In the present study we characterized *IGH* genes rearrangements and somatic hypermutations (SHM) in a cohort of WM patients and we investigated any eventual correlation with patients' clinical features. The frequency of the *MYD88* L265P mutation was also investigated and correlated with the *IGH* genes rearrangements in an attempt to reveal new insights in WM pathogenesis and the nature of WM B-cell.

2. Materials and Methods

2.1. Patients. A cohort of 36 WM patients was studied retrospectively. Diagnostic workout included physical examination, hematological and laboratory parameters, chest radiographs, and computed tomography scans of the thorax, abdomen, and pelvis. Bone marrow smears and biopsy as well as immunophenotype were performed in all patients, and lymph node histology was additionally performed in the cases with lymphadenopathy. International diagnostic criteria were used for the diagnosis of WM. Patients' characteristics are shown in Table 1.

Forty-five percent, 39% and 16%, of patients were staged 1, 2, and 3, respectively, according to IPSS [23]. Seventy-two percent were symptomatic and required treatment; median time to treatment and overall survival were 13 and 61 months, respectively.

The study was approved by the local ethical committee.

2.2. Specimens and DNA Extraction. We analyzed genomic DNA extracted from patients' blood and/or bone marrow samples (bone marrow mononuclear cells, bone marrow smears, and bone marrow biopsies).

Genomic DNA was extracted by standard protocols using QIAmp DNA Mini kit (QIAGEN) according to the manufacturer's recommendations.

Table 1: Clinical and laboratory findings for the study's WM patients.

	Mean value (median value)		Range
Age (years)	65,5 (64)		42–84
Gender (male/female)			
IPSS stage		19/17	
1		45%	
2		39%	
3		16%	
Bone marrow involvement	46,7% (40%)		5–100%
Lymphadenopathy		21%	
Splenomegaly		19%	
Hepatomegaly		9%	
Extranodal sites		3%	
IgM (mg/dL)	2777,2 (2500)		138–7870
Hb (g/dL)	10,9 (11,1)		6–14,3
Platelets ($\times 10^9$/L)	233,2 (234)		60–472
WBC ($\times 10^9$/L)	7,1 (6,7)		2,1–16,8
B_2M (mg/dL)	4,1 (3,4)		1,9–10,4
Abnormal (high) LDH		27%	

2.3. Sequencing of IGHV Gene Sequences and Analysis of IGHV Sequences. Immunoglobulin heavy chain VDJ locus was amplified by PCR using the Biomed-2 strategy with FR1 primers as previously described [24]. It was possible to confirm monoclonality of the PCR product in 26/36 samples studied, by using capillary electrophoresis in Agilent 2100 Bioanalyzer using Agilent DNA 1000 kit (Agilent Technologies) according to the manufacturer's recommendations. PCR products were directly sequenced on both strands with the same primers using Sanger's chain-termination method and fluorescent dideoxynucleotides with GenomeLab DTCS Quick start kit in Beckman-Coulter CEQ 8000 sequencer platform.

In order to confirm monoclonality in the remaining 10/36 samples, cloning techniques were used as follows: (1) ligation of the PCR product to pCRII-TOPO vector (Invitrogen) using TOPO TA Cloning Kit (Invitrogen) according to manufacturer's recommendations, (2) transformation of One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen) by insertion of plasmids, (3) selection of 8–10 colonies of transformed *E. coli* cells followed by liquid culture (in LB medium for 12–14 hours at 37°C), and (4) plasmid DNA purification using PureLink HiPure Plasmid DNA Purification Kit (Invitrogen) according to manufacturer's recommendations. Plasmid DNA was sequenced, as described above, and sequences (8–10) were aligned using DNASTAR SeqMan Pro software in order to confirm monoclonality by detecting the same *IGH*-VDJ rearrangement in at least 3 out of 8 sequences.

Each clonal DNA *IGHV* sequence was aligned with the closest germline sequence using the international immunogenetics information system (IMGT, http://www.imgt.org/).

Sequences were translated into amino acids in order to identify the functional *IGHV* gene rearrangement. The percentage of homology between the functional *IGHV* segment used in the tumor and the germline sequence was then calculated (excluding primer sequences). Somatic hypermutation was defined as a >2% deviation from germline (as per convention) [25]. The length of CDR3 regions was determined according to IMGT numbering.

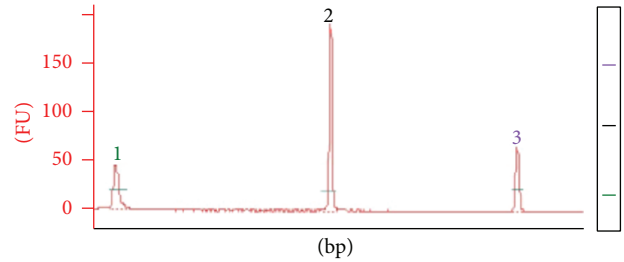
2.4. Screening for MYD88 L265P Mutations. Samples from 31 patients were also investigated for detection of *MYD88* L265P mutation by allele specific PCR (AS-PCR). Two PCRs were performed for each sample, one for wild type *MYD88* and one for possible detection of mutated *MYD88* by using primers as previously described [18]. PCRs were carried out by using a HotStarTaq DNA Plus Master Mix kit (QIAGEN). PCR consisted of an initial denaturation step of 15 minutes at 95°C, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension step of 5 minutes at 72°C. Agarose gel (1,5%) electrophoresis was performed to visualize the PCR products (220 bp).

2.5. Statistical Analysis. The SPSS software v.15 was used. Correlations between molecular findings and clinical parameters were assessed by the Mann-Whitney or by the chi-square test. Pictorial representation of survival curves was done by the Kaplan-Mayer method and their comparison by the log-rank test.

3. Results and Discussion

3.1. IGHV Usage and Mutation Analysis. Thirty-six WM patients were studied. Two out of the 36 patients were excluded from the analysis, as in these two patients genomic DNA was extracted from peripheral blood and not bone marrow. Although these two patients had not lymphoma cells in blood (by morphology or immunophenotype analysis), monoclonality of *IGHV*-PCR product was confirmed in both samples (Figure 1 illustrates the electropherogram of *IGHV6*-PCR product in one of these two patients after capillary electrophoresis). Mutated *IGHV* genes were detected in these two patients (one *IGHV3-74* and one *IGHV6-1*).

We observed an *IGHV3* overrepresentation (74,3%), as high as described in previous studies [8, 12] but lower than reported by others [11, 26]. The distinctive *IGHV* gene segments usage in our patients is presented in Table 2. It should be mentioned that in one patient two different productive *IGH* VDJ sequences were identified (two different B-cell clones). There was an overrrepresentation of *IGHV3-23* gene usage (25,7%), as expected according to previous studies; while *IGHV3-74* gene -another member of the *IGHV3* family- was also overrepresented (17,1%), which is not reported in other studies [12, 26]. The repertoire of *IGHV* genes in WM, as presented in this study and previous studies, has some similarities with IgM-MGUS *IGHV* genes' repertoire [12, 13], but it is absolutely different from the ones observed in CLL/SLL [7], MCL [27], and MZL [8, 28, 29]. This is important because the aforementioned B-cell malignancies



Peak	Size (bp)	Conc. (ng/μL)	Molarity (nmol/L)	Observations
1	15	4.20	424.2	Lower marker
2	335	5.41	24.5	
3	1,500	2.10	2.1	Upper marker

Figure 1: Electropherogram—after capillary electrophoresis in Agilent 2100 Bioanalyzer using Agilent DNA 1000 kit (Agilent Technologies)—of *IGHV6*-PCR product in one of the two patients of whom genomic DNA was extracted from blood sample and not bone marrow. Monoclonality of *IGHV*-PCR product is obvious (peak number 2) and was further confirmed by direct sequencing.

Table 2: Distinctive *IGHV* gene segments usage in present study.

Segment	Number of patients	%
IGHV1-2	1	2,86
IGHV1-8	1	2,86
IGHV2-5	1	2,86
IGHV3-7	3	8,57
IGHV3-23	9	25,71
IGHV3-30	3	8,57
IGHV3-33	2	5,71
IGHV3-48	1	2,86
IGHV3-64	1	2,86
IGHV3-73	1	2,86
IGHV3-74	6	17,14
IGHV4-34	3	8,57
IGHV4-59	2	5,71
IGHV5-51	1	2,86

can secrete a monoclonal IgM and be in some cases misdiagnosed as WM or vice versa.

SHM was seen in all but three cases (91,4%). Mean percentages of mutations in all cases, *IGHV3* family, *IGHV3-23*, and *IGHV3-74* segments were 7,5%, 8%, 9,4%, and 7,5%, respectively (Table 3). These findings are in agreement with previous studies [11, 12, 26] and suggest that WM cells are derived from postgerminal center memory B cells that have been submitted to T-dependant antigen selection. It should be mentioned that in this study unmutated *IGHV* genes (≤2% deviation from germline homolog gene) were detected in 3 cases, as it was described in previous studies [10–12, 26], while in some other studies [8, 9, 13] all (100%) were mutated. In detail, 3 (8,6%) unmutated IGHV genes were detected (two *IGHV3-33* and one *IGHV5-51*), and one of the three was 100% homolog to germline gene. It is remarkable that none of these three genes belonged to the highly represented

Table 3: Mean (median) of somatic mutations' percentage in different groups.

	Mean (median) somatic mutations' percentage	Range (%)
In all 35* cases	7,5% (7,3%)	0–16,1
In 32 cases with mutated genes (<98% homology)	8,1% (7,6%)	2,83–16,1
In *IGHV3* cases' group (27 cases)	8% (8,3%)	0–14,46
In *IGHV3-23* cases' group (9 cases)	9,4% (9,7%)	2,83–14,46
In *IGHV3-74* cases' group (6 cases)	7,5% (8,1%)	4,02–9,65

*34 patients, 1 with two clones; *IGHV3-74* and *IGHV4-59*.

IGHV segments in WM (*IGHV3-23* and *IGHV3-74*). The existence of unmutated *IGHV* genes could mean that the transformation leading to WM does not target exclusively postgerminal center B-cells that bear SHM and have been submitted to T-dependent antigen selection. Even higher percentages of unmutated *IGHV* genes have been observed in resembling diseases such as splenic MZL (SMZL) [30–32]. Indeed an erroneous diagnosis can never be excluded in this disease although the 3 patients presented typical WM features. CDR3 length was short (≤17 amino acids) in 80% of all cases as previously described [8, 10–12, 33], although in the three unmutated cases the mean of CDR3 length was 22,3 amino acids.

The above-mentioned findings were compared with patients' physical and routine laboratory workup results and no correlations were found nor was it the case with time to treatment or survival. Although in CLL the *IGHV* genes mutational status is one of the most important independent prognostic factors [34–37], this was not the case for our WM patients.

3.2. MYD88 Mutation Analysis. Since *MYD88* has been reported to be mutated (L265P) in the large majority of WM patients, we next looked for this mutation. Two out of the 31 patients investigated for detection of *MYD88* L265P mutation were excluded from the analysis, as in these two patients genomic DNA was extracted from blood and not bone marrow. However, in these samples monoclonality of *IGHV*-PCR product was confirmed. These two samples were negative for *MYD88* L265P mutation. Nineteen out of 29 patients (65,5%) were positive for the *MYD88* L265P mutation. This percentage is high and in accordance with the study of Gachard et al. [8]; however it is lower compared to other studies reporting a *MYD88* L265P mutation prevalence of 79% to 100% [14–16, 18, 20]. These variations could reflect differences in methodology followed by each study. The use, in our study, of BM unselected tissue for *MYD88* L265P AS-PCR assay could have contributed to the seemingly lower detection rate as has been raised by others. Cases not exhibiting *MYD88* L265P mutation had a statistically significant lower bone marrow infiltration by lymphoplasmacytes ($P < 0.005$).

The findings were also compared with patients' physical and routine laboratory workup results and no correlations were found nor was it the case with time to treatment or survival, as also described by Jiménez et al. [16]. We have also seen a difference in *MYD88* L265P detection based on bone marrow involvement, with a higher BM involvement in *MYD88* L265P positive patients.

Finally it should be mentioned that the mean of SHM levels of *IGHV* genes in *MYD88* L265P positive patients was 8,3%, while in *MYD88* L265P negative patients it was 5,4%. Five of seven patients with *IGHV3-23* who were tested for *MYD88* L265P and five out of five patient with *IGHV3-74* were positive for this mutation, which suggests that *IGHV3-23* and *IGHV3-74* are represented more in *MYD88* L265P positive patients. This may reinforce the concept that there are biological differences between the patients with and without the *MYD88* L265P mutation. This is further supported by an additional observation in this study: all three cases of unmutated *IGHV* genes were negative for *MYD88* L265P. These findings imply that a different (extra-germinal) B-cell represents the origin of the malignant cell in a minority of patients. Such hypothesis of the origin of the malignant cell in some WM patients is also described by Sahota et al. [38]. Larger studies may support further this concept.

In addition, the landscape is still unclear in this field as *MYD88* L265P mutation was recently found in other lymphoma entities [16, 18] while it was negative in lymphoplasmacytic lymphoma not secreting IgM [39]. Further studies are needed.

4. Conclusions

WM *IGH* genes repertoire, as expected, differs from that observed in normal B-cells and other B-cell diseases such as MZL, MCL, and B-CLL/SLL.

In addition to the known hyperrepresentation of the *IGHV3-23* gene, another member of the *IGHV3* family, the *IGHV3-74* gene is also overrepresented in WM, as shown in the present study. The high *IGHV* mutation rate supports a derivation of WM cells from postgerminal center memory B cells in the majority (91,4%) of WM patients. However, the identification of a minority of patients (3 of 34) with unmutated *IGHV* gene segments, negative for the *MYD88* L265P mutation, supports the hypothesis that they represent a subgroup of WM not arising from postgerminal B cells with a different disease pathogenesis. Finally, consensus and guidelines for *MYD88* L265P detection's methodology are needed, as it is quite obvious that this mutation could be both helpful in the diagnosis of WM and a potential therapeutic target in WM patients.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors thank D. Palaiologou, E. Staikou, and A. Stefanou for excellent technical assistance.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 159632, 5 pages
http://dx.doi.org/10.1155/2014/159632

Research Article

Serum Soluble TACI, a BLyS Receptor, Is a Powerful Prognostic Marker of Outcome in Chronic Lymphocytic Leukemia

Marie-Christine Kyrtsonis,[1] Katerina Sarris,[1] Efstathios Koulieris,[1] Dimitrios Maltezas,[1] Eftychia Nikolaou,[1] Maria K. Angelopoulou,[1] Vassiliki Bartzis,[1] Tatiana Tzenou,[1] Maria Dimou,[1] Mariana P. Siakandaris,[1] Nora A. Viniou,[1] Sotirios Sachanas,[2] Christina Kalpadakis,[2] Petros P. Sfikakis,[1] Gerassimos A. Pangalis,[2] and Panayiotis Panayiotidis[1]

[1] *Hematology Section of the First Department of Propedeutic Internal Medicine, Laikon University Hospital, Agiou Thoma 17, 11527 Athens, Greece*
[2] *Hematology Clinic of Athens' Medical Center, Andersen 1, Psychiko, 11525 Athens, Greece*

Correspondence should be addressed to Marie-Christine Kyrtsonis; mck@ath.forthnet.gr

Received 17 April 2014; Accepted 20 July 2014; Published 6 August 2014

Academic Editor: Kazuyuki Shimizu

BLyS is involved in CLL biology and its low soluble serum levels related to a shorter time to first treatment (TFT). TACI is a BLyS receptor and can be shed from cells' surface and circulate in soluble form (sTACI). We investigated the impact of serum BLyS and sTACI levels at diagnosis in CLL patients and their relationship with disease parameters and patients' outcome. Serum BLyS was determined in 73 patients, while sTACI in 60. Frozen sera drawn at diagnosis were tested by ELISA. sTACI concentrations correlated with BLyS ($P = -0.000021$), b2-microglobulin ($P = 0.005$), anemia ($P = -0.03$), thrombocytopenia ($P = 0.04$), Binet stage ($P = 0.02$), and free light chains ratio ($P = 0.0003$). Soluble BLyS levels below median and sTACI values above median were related to shorter TFT ($P = 0.0003$ and 0.007). During a ten-year followup, sTACI levels, but not BLyS, correlated with survival ($P = 0.048$). In conclusion, we confirmed the prognostic significance of soluble BLyS levels with regard to TFT in CLL patients, and, more importantly, we showed for the first time that sTACI is a powerful prognostic marker, related to parameters of disease activity and staging and, more importantly, to TFT and OS.

1. Introduction

Chronic lymphocytic leukemia (CLL) is an indolent leukemic B-cell lymphoproliferative disorder, defined in the WHO classification of neoplasms as a neoplasm of mature B-lymphocytes involving peripheral blood, bone marrow, spleen, and lymph nodes [1]. It is the most common form of leukemia in the elderly in the Western world. For diagnosis a minimum of 5×10^9/L absolute blood lymphocyte count is required; these typically coexpress CD5, CD19, CD20, and CD23 antigens with dim CD20, CD79b, and surface immunoglobulin expression [2, 3]. Patients usually enjoy a relatively favourable outcome and the majority of them are asymptomatic and may not need any treatment for years. However, some patients have a more aggressive course and shorter survival; clinical manifestations, when present, include anaemia, peripheral lymphadenopathy, splenomegaly, and autoimmune manifestations. Treatment should be immediately started in presence of a lymphocyte doubling time of less than 6 months, very enlarged or rapidly growing lymph nodes or spleen, anemia, thrombocytopenia, and B-symptoms. For symptomatic patients, adverse prognostic factors include classical staging (according to Rai or Binet), unmutated *VH* genes, ZAP-70 and CD38 expression, and cytogenetic alterations such as deletion of 11q22, deletion of 17p, and/or presence of a *TP53* mutation [4]. Additional prognostic factors are needed for asymptomatic patients to predict whether they will remain stable for years or not.

Table 1: Patients' characteristics at the time of diagnosis.

Age, median (range)	60 years (37–82)	
	For BLyS measurements	For sTACI measurements
N	73	60
	%	
Sex, M/F	61/39%	66/34%
Binet stage		
1	59%	60%
2	34%	32%
3	7%	8%
Lymphadenopathy	60%	60%
Splenomegaly	18%	15%
Haemoglobin <10 g/dL	5.5%	8.3%
Platelet counts $<100 \times 10^9$/L	2.7%	3.3%
Abnormal LDH	15%	15%
BM infiltration >50%	59%	50%
β2-Microglobulin >3.5 mg/L	30% (12/39 pts)	27% (9/33 pts)
Abnormal κ-sFLC (normal 3.3–19.4 mg/L)	30% (20/67 pts)	30%(17/56 pts)
Abnormal λ-sFLC (normal 5.71–26.3 mg/L)	8% (3/67 pts)	5% (3/56 pts)

FLC: free light chain.

B-lymphocyte stimulator (BLyS) is a cytokine, member of the TNF-superfamily, that is involved in CLL biology and was shown to regulate B-CLL cells proliferation and survival [5]. Furthermore, serum BLyS levels were found decreased in CLL patients and their low concentrations related to a shorter time to first treatment (TFT) but not to overall survival (OS) [6]. BLyS is produced by myeloid cells, monocytes, dendritic cells, and osteoclasts [7]. It may be cleaved from cells' surface and circulate in body fluids in a soluble form [8]. BLyS actions concern almost exclusively cells of lymphoid lineage and are exerted through its receptors [9]. TACI (transmembrane activator and CAML interactor) is one of the 3 BLyS (BAFF) receptors and is expressed by B and T cells. It can also bind APRIL. TACI can also be shed from cells' surface and circulate in its soluble form [10, 11]. Very few studies so far investigated soluble TACI (sTACI) serum levels in CLL.

The purpose of this study was to investigate possible relationship between serum BLyS and sTACI concentrations at diagnosis in CLL, as well as eventual correlations of their respective levels with disease parameters and patients' outcome.

2. Patients and Methods

Seventy-three CLL patients were studied. Their characteristics are shown in Table 1.

Sera from patients were drawn at diagnosis and aliquots were kept frozen and retrospectively tested to determine BLyS and sTACI concentrations. Frozen sera from 14 healthy individuals (HI) for BLyS and sTACI, respectively, were also tested as controls.

42 patients were or became symptomatic and in need of treatment during disease course. Patients' median OS was 79 months (range 18–174) while median TFT was 34 months (range 1–157).

Serum BLyS levels were determined by ELISA (R&D Quantikine KIT) in all 73 patients while sTACI (R&D Quantikine, DuoSet) in 60 of them, according to the manufacturer's instruction; briefly (1) for BLys determination, the specified amount of patients' or HI serum was added to a 96-cell microplate already coated with a capture antibody. After incubation the plate was washed, the detection antibody was added, and after another incubation and wash Streptavidin-HRP and Tetramethylbenzidine in H_2O_2 were added, and finally H_2SO_4 was added to stop the reaction. (2) For sTACI determination, a 96-cell microplate was coated with the specified amount of capture antibody and, after being incubated overnight and washed, Reagent Diluent was added to block the plate and after another wash the specified amount of patients' or controls' sera was added. The subsequent procedure was almost identical (washing, addition of detection antibody, incubation, washing, and addition of Streptavidin-HRP followed by Tetramethylbenzidine in H_2O_2 solution and then stop solution). In order to determine the values of both BLys and sTACI, the optical density of each cell was determined at 450 + 620 nm by using a photometer. On a biaxial system where x-axis was the serum concentration of the cytokine and y was optical density, the sera cytokine concentration was determined using a curve plotted based on the optical density of known serial concentrations.

Statistical analysis was performed using the SPSS v.15 software. Nonparametric variables were compared by the Mann-Whitney test. TFT and OS curves according to BLyS or sTACI levels were assessed and plotted by the Kaplan-Meier analysis and then compared by the log-rank test. P values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Serum BLyS and sTACI Levels. In the present study, median serum BLyS levels were 65 pg/mL (undetectable −680) while

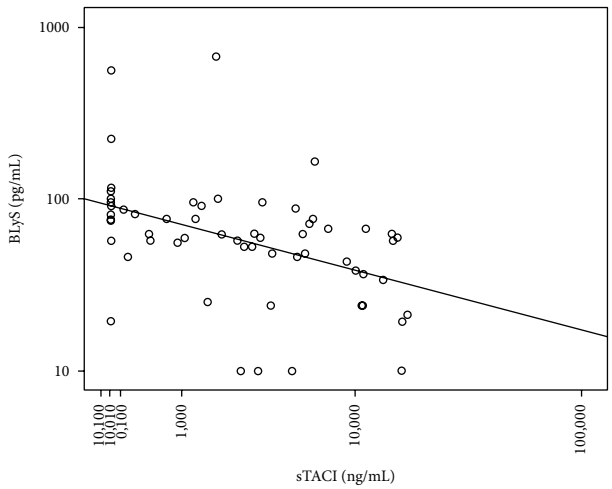

Figure 1: Inverse correlation between serum soluble BLyS and sTACI.

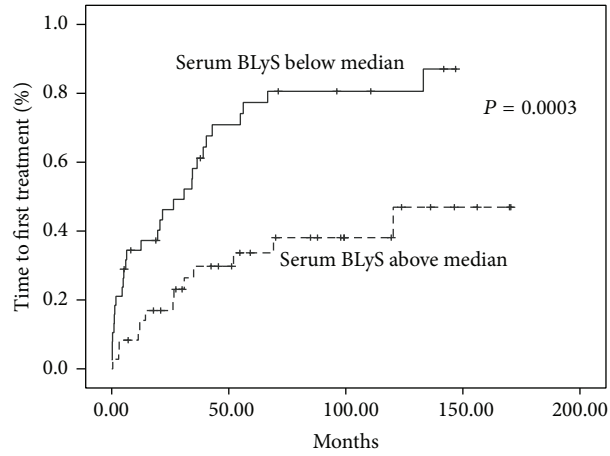

Figure 2: Time to first treatment according to serum soluble BLyS.

sTACI's ones were 2,52 ng/mL (undetectable −17) in CLL patients. The corresponding median concentrations in HI sera samples were 183 pg/mL (undetectable −381) and 0.86 ng/mL (undetectable −4,14) for BLyS and sTACI, respectively. The difference between levels in patients and HI is significant for both ($P < 0.01$). In addition, sTACI concentrations strongly correlated inversely with soluble BLyS ($P = 0.000021$), as shown in Figure 1.

In accordance with our results, serum BLyS levels were reported lower in CLL patients as compared to HI, in several previous studies [12–14]. However, with regard to sTACI levels in CLL, that we found higher in patients than in HI, there are no reports in medical literature, so far, to our knowledge.

As an attempt to explain the low serum BLyS levels found in CLL patients, Molica et al. [14] suggested that in aggressive CLL high amounts of soluble BLyS are bound by its receptors on B-cell surface, thus sequestrating it from circulation. This theory was reinforced by another study that showed that serum BLyS concentrations increased in follicular lymphoma patients after rituximab administration, possibly because it is unbounded by its surface receptors [15]. In view of our finding of a strong inverse correlation between BLyS and sTACI serum concentrations, one could assume that binding of soluble BLyS to sTACI results in hiding the BLyS epitope that is detected by ELISA measurements and that BLyS levels are falsely low. This supposition remains indeed to be proved but if it was the case, it would have significant therapeutical implications, given that anti-BLyS or anti-BLyS receptors antibodies have been manufactured for adjuvant treatment in B-cell lymphoproliferative disorders [16, 17] but are not considered for CLL for which caution is needed until BLyS contribution to disease biology is fully understood.

3.2. Correlations between BLyS or sTACI Levels and Disease Parameters. Serum BLyS levels correlated inversely with absolute lymphocyte count ($P = 0.01$, Spearman's rho = −0.303). Furthermore an inverse correlation was demonstrated for BLyS values above median and bone marrow lymphocyte infiltration greater than 50% ($P = 0.031$, chi square) while sTACI concentrations correlated with b2-microglobulin ($P = 0.005$), inversely with anemia ($P = 0.03$), thrombocytopenia ($P = 0.04$), Binet stage ($P = 0.02$), and free light chains ratio ($P = 0.0003$). In Molica et al.'s study higher BLyS levels were associated with younger age, higher platelet count, mutated IgVH, normal cytogenetic profile or presence of 13q deletion, and low ZAP-70 and CD38 expression. Unfortunately, in our series mutational status and cytogenetic abnormalities were determined in only 15% of patients rendering statistical analysis impossible; ZAP-70 expression was not determined and there was no correlation with CD38 expression. With regard to sTACI levels, given that this is the first report, we cannot compare with other studies.

3.3. Time to First Treatment and Overall Survival according to BLyS and sTACI Serum Concentrations. In CLL patients serum BLyS values below median were related to a significantly shorter TFT compared to values above median ($P = 0.0003$) (Figure 2) but no correlation was found with overall survival. In addition, serum sTACI values above median were also related to a shorter TFT compared to values below median ($P = 0.007$) and, importantly, also to OS ($P = 0.048$, HR: 2.789, 95% CI: 0.967–8.047), as shown in Figure 3. Serum sTACI values above median maintained their negative prognostic impact in the Cox Regression model when tested with known disease parameters such as Rai and Binet stadium, b2-microglobulin, and LDH.

Lower soluble BLyS levels have already been associated with a shorter time to first treatment [14, 18] but not with overall survival, most probably because of the indolent nature of the disease, requiring a very long follow-up time, in order to show survival advantage. Of special interest are our findings concerning the prognostic significance of sTACI with regard to both TFT and OS; such findings have not been reported so far in medical literature. Because the vast majority of our patients were not in need of treatment and low staged at diagnosis, our results suggest that serum sTACI could be a new, easily assessed, marker for predicting CLL behaviour in low staged patients. Such prognostic factors are missing for

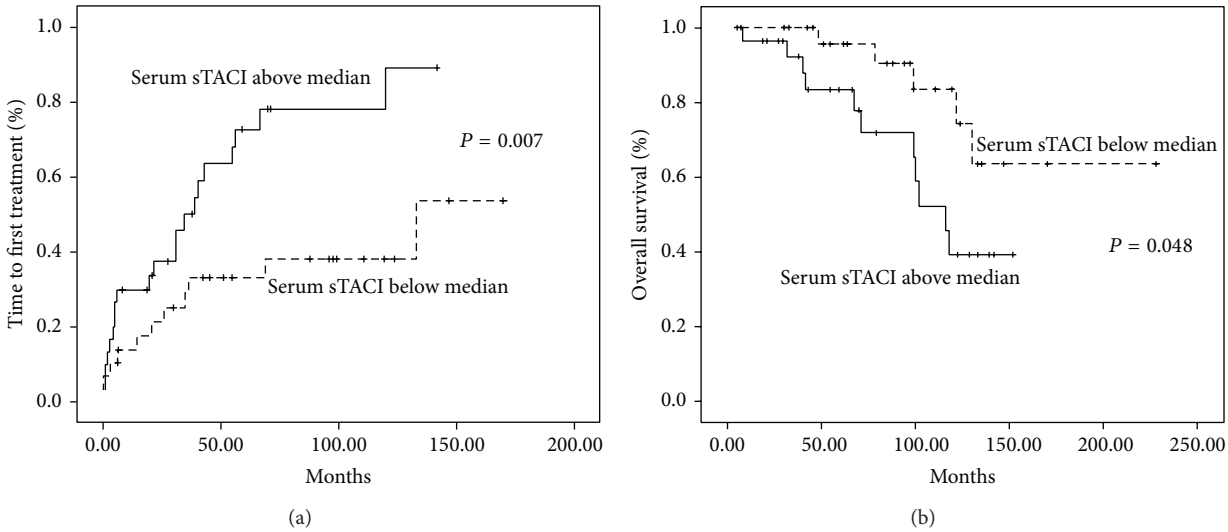

Figure 3: Time to first treatment (a) and overall survival (b) according to serum sTACI.

asymptomatic patients although in this context serum free light chain measurements appear very promising [19–21].

4. Conclusions

We confirmed that low soluble BLyS are associated with a shorter time to first treatment while in addition we found that sTACI serum concentrations at diagnosis constitute a powerful prognostic marker in chronic lymphocytic leukemia; sTACI is related to disease activity parameters and the stage of CLL and more importantly, sTACI levels above median predicted a shorter time to first treatment and worse outcome for the patients. Indeed these results are preliminary and concern a relatively short series, although with a very long follow-up, and further researches are needed. However, if confirmed, our results suggest that sTACI could be a valuable prognostic marker in CLL while, in addition, they could open interesting therapeutical applications.

Ethical Approval

The study was approved by the local ethical committee.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 106713, 6 pages
http://dx.doi.org/10.1155/2014/106713

Research Article

PARP1-Driven Apoptosis in Chronic Lymphocytic Leukemia

Panagiotis T. Diamantopoulos, Maria Sofotasiou, Vasiliki Papadopoulou, Katerina Polonyfi, Theodoros Iliakis, and Nora-Athina Viniou

Department of Internal Medicine, Hematology Unit, Laikon General Hospital, National and Kapodistrian University of Athens, 11527 Athens, Greece

Correspondence should be addressed to Panagiotis T. Diamantopoulos; pandiamantopoulos@gmail.com

Received 30 March 2014; Accepted 19 July 2014; Published 3 August 2014

Academic Editor: Marie-Christine Kyrtsonis

Chronic lymphocytic leukemia (CLL) is considered a malignancy resulting from defects in apoptosis. For this reason, targeting apoptotic pathways in CLL may be valuable for its management. Poly [ADP-ribose] polymerase 1 (PARP1) is the main member of a family of nuclear enzymes that act as DNA damage sensors. Through binding on DNA damaged structures, PARP1 recruits repair enzymes and serves as a survival factor, but if the damage is severe enough, its action may lead the cell to apoptosis through caspase activation, or necrosis. We measured the *PARP1* mRNA and protein pretreatment levels in 26 patients with CLL and the corresponding posttreatment levels in 15 patients after 3 cycles of immunochemotherapy, as well as in 15 healthy blood donors. No difference was found between the pre- and posttreatment levels of PARP1, but we found a statistically significant relative increase of the 89 kDa fragment of PARP1 that is cleaved by caspases in the posttreatment samples, indicating PARP1-related apoptosis in CLL patients after treatment. Our findings constitute an important step in the field, especially in the era of PARP1 inhibitors, and may serve as a base for future clinical trials with these agents in CLL.

1. Introduction

The poly [ADP-ribose] polymerases (PARPs) are a family of nuclear enzymes comprising 17 members. Their main function is to bind to DNA breaks, serving as a signal to other DNA-repairing enzymes, in order to fix the damage. Binding of PARPs to DNA leads to their polymerization, and by poly [ADP-ribosylation], a posttranslational modification of proteins playing a crucial role in many cell processes, they participate in DNA repair and gene transcription [1, 2].

Among the members of the PARP family, PARP1 is the most abundant and plays a role in the repair of single-strand DNA (ssDNA) and double-strand DNA (dsDNA) breaks. Inhibition of PARP1 activity leads to reduced ssDNA break repair, eventually leading to cell death. The molecular structure of PARP1 consists of 4 domains, an N-terminal double zinc finger DNA-binding domain, a nuclear localization signal, a central automodification domain, and a C-terminal catalytic domain [3]. PARP1 has a low enzymatic activity, which is stimulated by allosteric activators, such as damaged DNA (single- and double-strand breaks, crossovers, cruciform, and supercoils), undamaged DNA structures, nucleosomes, and some protein-binding partners. Binding of PARP1 with such molecules boosts its enzymatic activity that targets core histones, histone H1 and transcription-related factors [4–8]. Upon binding to these allosteric activators, PARP1 recruits various proteins involved in the DNA damage response to the sites of DNA damage [3], and this means that PARP1 acts essentially as a DNA damage sensor [4]. Low level DNA damage seems to trigger detection and repair of the DNA damage. In that case, PARP1 acts as a survival factor. On the other hand, high levels of DNA damage may lead to cell death by either apoptosis or necrosis through PARP1 overactivation [9].

PARP1 may induce apoptosis, through apoptosis inducing factor (AIF) activation, as well as necrosis. The cell type and the type, strength, and duration of the stimuli are presumed to be factors determining the cell death pathway. It has been shown that actively proliferating cells (such as malignant cells) are more sensitive to PARP1 activation and die by necrosis, while nonproliferating cells are resistant to cell death under the same conditions [10], a fact that is mainly determined by the availability of ATP in the cell [11]. Strong stimuli, such as severe DNA damage, may lead to necrosis through

overactivation of PARP1 which causes depletion of the NAD+ and ATP pool of the cell [12, 13].

During the execution phase of apoptosis, caspases cleave several proteins that are necessary for the cell function and survival. Among them, PARP1 is cleaved by caspases 3 and 7 into a ~25 kDa N-terminal fragment containing the DNA-binding domain (DBD) and a ~85 kDa C-terminal fragment that retains basal enzymatic activity but cannot be stimulated by DNA damage [14]. This cleavage is necessary to eliminate PARP1 activation in response to DNA fragmentation, protecting the cells from ATP depletion and subsequent necrotic death, and preventing futile attempts of DNA repair. Through these processes, PARP1 cleavage may help to commit cells to the apoptotic pathway [15]. Thus, PARP1 plays a central role in apoptosis determining the cell fate [16].

CLL is a highly heterogeneous disease in terms of biology and hence clinical presentation. The clinical course of CLL can vary from asymptomatic and indolent for several years to severely symptomatic since diagnosis, requiring treatment. Clinical staging, age, and performance status remain the major factors defining prognosis and need for treatment. New prognostic factors include cytogenetic analysis, immunoglobulin mutation analysis, and expression of 70 kDa zeta associated protein (ZAP-70) and CD38 [17, 18]. Several studies have identified the signal transduction pathways that contribute to antiapoptotic signaling in CLL cells, and CLL is considered a malignancy resulting from defects in apoptosis [19].

Among other genetic defects, defects in the ds-DNA break response have been implicated in the pathogenesis of CLL. Impairment of the DNA damage response has been correlated to aggressive CLL [20], unresponsiveness to standard therapy, and adverse clinical outcomes of patients with CLL [21].

A recent study showed that reduced expression of PARP1 is associated with an impairment of CLL responsiveness to cell death [22]. This is, to our knowledge, the only study on PARP1 expression in CLL.

As PARP1 inhibitors are currently under study in the context of phase II [23, 24] and phase III clinical trials [25], mostly for advanced or relapsed breast and ovarian cancer, the need to further understand the role of PARP1 in hematological malignancies is mandatory. This study tries to shed light on the possible role of PARP1 in the pathways that drive apoptosis in CLL. The aim of our study is to determine the levels of PARP1 expression in patients with CLL before and after immunochemotherapy as well as to compare them with those of healthy individuals.

2. Patients and Methods

2.1. Patients. Twenty-six patients with B-cell chronic lymphocytic leukemia (CLL) were included in the study. Informed consent was obtained from all patients. The diagnosis of CLL was established in each case using morphological, histopathological, and immunophenotypic criteria. All patients had immunophenotypically confirmed disease by peripheral blood at the time of first sample collection. Fifteen patients among them received treatment with rituximab based immunochemotherapy according to common clinical practice after the first sample collection, and a second sample was obtained after 3 treatment cycles. We also obtained blood samples from 15 healthy blood donors, to be used as a control group.

We obtained from all patients and healthy controls peripheral whole blood samples that were collected in ethylenediaminetetraacetic acid (EDTA). All samples were processed within 6 hours from collection. Following RNA extraction and cDNA synthesis, the samples were kept at −80°C. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed in order to measure *PARP1* mRNA levels. Moreover, PARP1 protein was detected by an immunoblotting assay following protein extraction, as described below.

2.2. Methods

2.2.1. RNA Extraction and Reverse Transcription. The Trizol protocol (Invitrogen, Carlsbad, CA, USA) was used to extract and purify total RNA from peripheral whole blood samples. Reverse transcription was performed using MMLV-derived reverse transcriptase enzyme (M-MLV RT, Invitrogen), according to standard protocols.

2.2.2. Primer Design for Real-Time PCR. Primers for PARP1 and β-actin were designed with the help of the Primer3 software (University of Massachusetts, USA), using the relevant annotated cDNA sequences from NCBI BLAST (NM_001618.3 for PARP1 and NM_001101.3 for β-actin)—primer sequences: for PARP1 forward, CCTGATCCCCCACGACTTT; reverse, GCAGGTTGTCAAGCATTTC and for β-actin forward, AGGATGCAGAAGGAGATCACT; reverse GGGTGTAACGCAACTAAGTCATAG.

2.2.3. Real-Time PCR. Real-time PCR was performed with the use of 2X iTaq Universal SYBR GREEN Supermix (Bio-Rad, California, USA) on a CFX96 Real-Time PCR system (Bio-Rad, California, USA) using the following cycling conditions for both PARP1 and β-actin: 5″ at 95°C, 15″ at 59°C, and 5″ at 72°C, all steps repeated for 40 cycles. Relative quantitation of PARP1 and β-actin transcripts was performed with the standard curve method. PARP1 expression was in fact compared between samples as a ratio of PARP1/actin transcript levels.

2.2.4. Immunoblotting. Total cellular protein was obtained from about 107 cells from each sample, using RIPA buffer. Lysates were incubated on ice for 15 minutes and then centrifuged for 10 minutes at 14,000 rpm. Protein extracts were then separated by SDS-PAGE electrophoresis on acrylamide 5% stacking and 7.5% separating gels, using the Mini-Protean electrophoresis cell (BioRad), according to standard procedures. Molecular weight values were estimated using prestained protein markers (Full Range Rainbow Marker, GE Healthcare). Proteins were transferred from the gel to PVDF

Table 1: Patient characteristics: epidemiology, disease characteristics, treatment, and response.

Characteristic	All patients	Subset of patients that received treatment
Number of patients, *N* (%)	26 (100)	15 (100)
Median age, years (range)	74 (51–87)	73 (51–82)
Male to female ratio	1.5	1.4
Peripheral blood lymphocytes, $\times 10^9$/L (range)	29.4 (3.9–81.0)	26.7 (3.9–81.0)
LDH/ULN at presentation, mean (range)	1.2 (0.9–3.1)	1.1 (0.9–2.7)
Previous treatment, *N* (%)	2 (7.7)	2 (13.3)
Disease stage (Binet) (15.24), *N* (%)		
A	10 (38.5)	0 (0)
B	9 (34.6)	8 (53.3)
C	7 (26.9)	7 (46.7)
Immunochemotherapeutic regimen, *N* (%)	NA	
R		8 (53.3)
R, Ch		3 (20.0)
FCR		4 (26.7)
Response to treatment, *N* (%)	NA	
Complete response		3 (20.0)
Partial response		10 (66.7)
Stable disease		2 (13.3)
Disease progression		0 (0)

ULN: upper limit of normal; R: rituximab; Ch: chlorambucil; F: fludarabine; C: cyclophosphamide.

membrane (Immun-blot, Biorad), according to the manufacturer's instructions. Membranes were then incubated in blocking solution (5% w/v BSA in TBS-T, i.e., Tris-buffered saline/0.1% Tween 20) for 1 hour at room temperature and the primary antibody was added at a dilution 1/1000 (PARP rabbit mAb, Cat. number 9542, Cell Signal, or β-actin rabbit polyclonal Ab, Cat. number 4967, Cell Signal, when membranes were reprobed for loading control). After overnight incubation at 4°C, the membrane was washed 3x in TBS-T and incubated with secondary antibody at a dilution 1/4000 in blocking buffer for 1 h at room temperature (anti-rabbit IgG, HRP conjugated, Cat. number 7074, Cell Signal). After 3x washes in TBS-T, signal was detected with ECL Blotting reagent (GE Healthcare) and X-OMAT LS-1 film (Kodak).

2.3. Statistical Analysis. For the statistical analysis of the results we used IBM SPSS statistics, version 19.0. The Related Samples Wilcoxon Signed Rank test was used for comparisons involving pre- and posttreatment values, while the Independent Samples Mann-Whitney *U* test was used to compare the levels of *PARP1* mRNA and protein between patients and healthy controls.

3. Results

Whole blood samples were obtained from 26 patients with CLL before treatment and from 15/26 following 3 cycles of immunochemotherapy. Whole blood samples were also obtained from 15 healthy volunteers. The patients' characteristics are shown in Table 1. Data is presented for the total population (26 patients) as well as for the subset of 15 patients from whom samples were obtained both before and after treatment. The vast majority (13/15, 86.6%) of this subset of patients were treatment naïve at the time of first sample collection, while the rest (2/15, 13.3%) had not received any treatment for at least 6 months. None of the above patients had been treated with rituximab in the past. The programmed and eventually administered treatment schemes are shown in Table 1.

The pretreatment levels of *PARP1* mRNA (ratio of *PARP1* to *ACTB* mRNA levels) were found to be 0.088 (0.001–3.490), while the posttreatment value was 0.055 (0.003–0.535). The two values did not differ in a statistically significant level ($P = 0.51$). Moreover, the pretreatment levels of *PARP1* mRNA did not differ significantly from those of the control group ($P = 0.364$), although the control group levels were slightly higher (0.241; range 0.024–1.762).

The used PARP1 antibody detects the endogenous levels of full length PARP1 (116 kDa), as well as the large fragment (89 kDa) of PARP1 resulting from caspase cleavage. We detected the pre- and posttreatment levels of both molecules (full length and large fragment) and we calculated the ratio of their expression (i.e., 116/89). This ratio was used as an indicator of caspase activation. Specifically, a decrease of this ratio would imply a relative increase of the 89 kDa fragment that results from caspase activation in comparison to the full length molecule. On the contrary, an increase of this ratio would mean a relative reduction of the caspase derived fragment.

The 89 kDa fragment was detected in all samples (pre- and posttreatment), while the 116 kDa molecule was detected in 22/26 pretreatment samples and in 12/15 posttreatment samples. For these patients, the 116/89 ratio was not calculated,

Table 2: PCR and immunoblotting results.

	All patients	15 patients (pretreatment)	15 patients (posttreatment)	P^*
PARP1-mRNA, median (range)[1]	0.094 (0.001–3.490)	0.088 (0.001–3.490)	0.055 (0.003–0.535)	0.507
116 kDa fragment, median (range)[2]	0.532 (0–1.808)	0.528 (0.263–0.673)	0.551 (0.311–0.864)	0.308
89 kDa fragment, median (range)[2]	0.665 (0.202–2.097)	0.647 (0.202–1.002)	0.607 (0.162–0.992)	0.875
116/89 ratio, median (range)[†]	1.182 (0.754–1.589)	1.245 (0.754–1.589)	1.095 (0.444–1.554)	0.026
	All patients	Healthy donors		
PARP1-mRNA, median (range)[1]	0.094 (0.001–3.490)	0.24 (0.024–1.762)		0.364

[1]PARP1/ACTB ratio; [2]PARP1/ACTB expression ratio.

*Correlation between pre- and posttreatment levels was performed using the related samples Wilcoxon Signed Rank test.

†Four (4/26) patients did not have a measurable 116 kDa molecule. One of them was in the 15-patient group that was given treatment. Following treatment, 3/15 patients did not have a measurable 116 kDa molecule. For these patients the calculation of 116/89 ratio was not performed, and they were excluded from the relevant statistical analysis.

and they were excluded from the statistical analysis. The pre- and posttreatment levels of both the full length molecule and the large fragment of PARP1 did not differ significantly. On the contrary, the pretreatment 116/89 ratio was higher than its posttreatment value (1.245 (0.754–1.589) versus 1.095 (0.444–1.554)), and this difference was statistically significant ($P = 0.026$). The results are presented in detail in Table 2. Figure 1 shows the immunoblotting results of two patients before and after immunochemotherapy.

The full length molecule of 116 kDa was detected in only one (1/15) of the healthy subjects, while the caspase derived 89 kDa fragment was detected in all of them. The median level of the 89 kDa fragment in the control group was 0.494 (0.172–0.985) and was lower than the pretreatment levels of the patients ($P = 0.036$). Due to the absence of the 116 kDa molecule in the vast majority (14/15) of the healthy controls, the 116/89 ratio was not calculated; thus further correlations were not possible between the control and the patient groups.

Multivariate analysis did not reveal statistically significant differences in the mRNA and protein levels in correlation to the stage of disease, the peripheral blood lymphocyte count, the LDH levels, and the response to treatment. More specifically, there was no statistically significant correlation of the difference of the pre- and posttreatment 116/89 ratios with the response to treatment ($P = 0.378$).

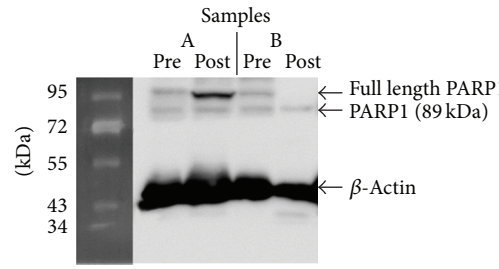

Figure 1: Pre- and posttreatment immunobloting results for 2 patients (A and B). Patient A expresses different levels of the full length (116 kDa) and the 89 kDa fragment of PARP1. Patient B expresses both the full length and the 89 kDa fragment of PARP1 before treatment but loses the full length molecule after immunochemotherapy.

4. Discussion

Physiological apoptosis is a process that controls cell numbers, as well as tissue and organ morphology, and removes injured and mutated cells [26]. Dysregulation of apoptotic pathways may result in cancer or other hyperproliferative disorders [27, 28]. The caspases are highly specialized proteases that, when activated, incite one of the more common apoptosis pathways. Upon caspase activation, cell death is initiated through cleavage of several key proteins required for cellular function and survival [29]. Cleavage of PARP1 is considered to be a hallmark of apoptosis [14]. All members of the caspase family may modify PARP1, but caspases 3 and 7 tend to cleave PARP1 in a way that results in the formation of two fragments with specific functions: an 89 kDa catalytic fragment and a 24 kDa DNA binding domain [30]. The 89 kDa fragment has a greatly reduced DNA binding capacity and is released from the nucleus into the cytosol [31]. The 24 kDa fragment binds irreversibly to the DNA strand breaks and inhibits DNA repair enzymes (including PARP1) leading to attenuation of DNA repair [32]. Although the main role of PARP1 is to detect and repair DNA damage, a severe DNA damage could result in high NAD+ and ATP consumption through PARP1 overactivation, leading to depletion of the cell ATP pool. This activity would inevitably lead to necrotic cell death, a process that is blocked by the rapid cleavage and inactivation of PARP1 by the caspases [33, 34]. Thus, when the damage is "too severe to handle" the action of caspases may shift the cell, through enhanced PARP1 cleavage, from necrosis to apoptosis.

We detected, in our samples, the *PARP1* mRNA using a PCR and the corresponding protein (the full length molecule as well as the cleaved by caspases 89 kDa fragment of PARP1), using an immunoblotting technique. By measuring the levels of PARP1 in both RNA and protein levels, we managed to crosscheck our results and most importantly to measure both the "production" and the "usage" of PARP1.

We did not detect any differences in the level of *PARP1* mRNA yield before and after treatment, but we found a statistically significant difference in the ratio of the full length molecule to the 89 kDa fragment before and after immunochemotherapy, indicating caspase activation as reflected by the relatively higher levels of the 89 kDa fragment in the

posttreatment samples. Moreover, we found that PARP1 driven apoptosis is probably lower in healthy persons, as indicated by the lower levels of the 89 kDa fragment, in comparison to patients with CLL, a fact that is compatible with the basic speculation that PARP1 driven apoptosis is an indicator of DNA damage which is fundamental in the pathogenesis of CLL and neoplasia in general.

Our results suggest a possible role of PARP1 induced apoptosis in patients with CLL that are treated with rituximab based immunochemotherapy. This preliminary result could serve as a clinical basis for further research in this field and for the use of PARP1 inhibitors in patients with CLL in the context of clinical trials.

Our finding is of significant value for two major reasons. Firstly, it confirms the results of other investigators who measured the levels of PARP1 before and after irradiation treatment of CLL cells [22]. The results of their study indicate that PARP1 is downregulated in nonresponder versus responder samples and that its basal expression is positively correlated with PARP1 cleavage after irradiation. Secondly, our study is the first—to our knowledge—to measure the levels of PARP1 in patient samples before and after "in vivo" treatment administration, and this fact increases the importance of the finding and correlates it more directly to the possible results of the administration of PARP1 inhibitors in CLL.

A drawback of this study is that, due to the rather small study population, no further analysis could be made for the several prognostic factors such as p53 mutation and the immunoglobulin variable (IgVH) region mutation status. Moreover, due to the small number of patients (4/15) treated with fludarabine containing regimens, no correlations of PARP1 expression could be made between patients treated with more or less aggressive regimens.

The molecular mechanisms involved in balancing survival and death of B lymphocytes in CLL triggered by PARP1 activation are highly complex and incompletely understood. According to our results, the regulative action of caspases on PARP1 seems to be important in CLL. We consider this finding of significant value, because it helps to further understand the pathophysiology of the disease and to define the apoptotic pathways that are defective in CLL. Because CLL is considered a malignancy resulting from defects in apoptosis, targeting apoptotic pathways in CLL is a valuable weapon in the treatment of the disease, and our preliminary results could guide future research on whether PARP1 serves as a treatment target in CLL. The extension of this study can provide more detailed information about the role of PARP1 and caspases in several subsets of patients, based on their genetic profile, and could help formulate a plan about the possible use of PARP1 inhibitors in CLL.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 965384, 7 pages
http://dx.doi.org/10.1155/2014/965384

Review Article

Targeted Therapy for HM1.24 (CD317) on Multiple Myeloma Cells

Takeshi Harada[1] and Shuji Ozaki[2]

[1] *Department of Medicine and Bioregulatory Sciences, University of Tokushima Graduate School of Medical Sciences, 3-18-15 Kuramoto, Tokushima 770-8503, Japan*
[2] *Department of Hematology, Tokushima Prefectural Central Hospital, 1-10-3 Kuramoto, Tokushima 770-8539, Japan*

Correspondence should be addressed to Shuji Ozaki; ozaki@tph.gr.jp

Received 15 March 2014; Accepted 2 July 2014; Published 17 July 2014

Academic Editor: Marie-Christine Kyrtsonis

Multiple myeloma (MM) still remains an incurable disease, at least because of the existence of cell-adhesion mediated drug-resistant MM cells and/or continuous recruitment of presumed MM cancer stem cell-like cells (CSCs). As a new alternative treatment modality, immunological approaches using monoclonal antibodies (mAbs) and/or cytotoxic T lymphocytes (CTLs) are now attracting much attention as a novel strategy attacking MM cells. We have identified that HM1.24 [also known as bone marrow stromal cell antigen 2 (BST2) or CD317] is overexpressed on not only mature MM cells but also MM CSCs. We then have developed a humanized mAb to HM1.24 and defucosylated version of the mAb to adapt to clinical practice. Moreover, we have successfully induced HM1.24-specific CTLs against MM cells. The combination of these innovative therapeutic modalities may likely exert an anti-MM activity by evading the drug resistance mechanism and eliminating presumed CSCs in MM.

1. Introduction

Multiple myeloma (MM) is a plasma cell neoplasm in the bone marrow and is likely to present with hypercalcemia, renal failure, anemia, bone resorption (CRAB), and/or immunodeficiency [1]. Treatment approaches in the management of MM have made a remarkable progress in the recent decades and are comprised of high-dose chemotherapy (melphalan) followed by autologous peripheral blood stem cell transplantation (PBSCT) and novel therapies using proteasome inhibitors and immunomodulatory drugs (IMiDs) [2, 3]. These strategies have improved overall survival of MM patients. However, most patients eventually relapse even after the achievement of complete response [4]. Therefore, other novel therapeutic approaches are strongly needed to further improve the outcome of MM.

Treatment with monoclonal antibody (mAb) has demonstrated the efficacy in several hematological malignancies such as CD20-positive malignant lymphomas and chronic lymphocytic leukemia [5, 6]. The principal mechanisms of its cytotoxic activity are derived from antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [7]. ADCC is induced when mAb binds to the specific antigen on the surface of malignant cells followed by binding of the Fc domain of the mAb to the Fc receptors on the surface of effector cells. The binding affinity between the Fc domains and the Fc receptors is related to control of fucosylation of N-linked oligosaccharides within the immunoglobulin heavy chain Fc regions [8, 9]. To enhance the binding affinity of mAbs to Fc receptors, defucosylated versions of the mAbs have been developed [9].

In MM, several mAbs with confirmed cytotoxic activity have been developed over the past years [10–12]. The targeted molecules of the mAbs include CS1 [13, 14], CD38 [15], CD138 [16], and CD40 [17]. We have identified a new plasma cell-specific antigen, HM1.24, and developed a humanized anti-HM1.24 mAb (AHM). To enhance the cytotoxic activity of the AHM, we have developed a defucosylated version of the AHM and antibody-drug conjugates (ADC).

In addition, to explore the relevance of cellular immunity against HM1.24, we have investigated the activity of HM1.24

peptide-specific cytotoxic T lymphocytes (CTLs) by using peripheral blood mononuclear cells (PBMCs) and peripheral blood stem cells (PBSC) harvested from MM patients.

In this review, we summarize the targeted therapies for HM1.24 and discuss the perspectives of these new targeted therapies in MM.

2. HM1.24 Antigen (CD317)

HM1.24 was originally identified as a cell-surface protein that is preferentially overexpressed on MM cells [18]. Later, this protein was found to be identical to bone marrow stromal cell antigen 2 (BST2) and was designated as CD317 [19–22]. This antigen is a type II transmembrane glycoprotein consisting of 180 amino acids with a molecular weight of 29 to 33 kD and is expressed as a homodimer by the disulfide bond (Figure 1). Regarding the topology of HM1.24, the N-terminus is located in the cytoplasm and the transmembrane domain is present near the N-terminus [23]. The cytoplasmic domain contains a Tyr-(X)-Tyr-$(X)_3$-Pro-Met sequence motif, which is conserved in mouse, rhesus, and human. The extracellular domain bears two N-linked glycosylation sites, and the C-terminus is modified with a glycosylphosphatidylinositol (GPI) membrane anchor. In addition, HM1.24 is a lipid raft-associated glycoprotein traversing between the cell surface and the Golgi apparatus [23–25].

The HM1.24 gene is located on chromosome 19p13.2 [19]. The promoter region of HM1.24 gene contains the interferon-(IFN-) stimulated response elements such as IFN related factor (IRF)-1/2 and IFN-stimulated gene factor (ISGF) 3, and therefore, the expression of HM1.24 can be upregulated by IFNs especially IFN-α [20, 26].

The expression of HM1.24 mRNA is upregulated on both normal and neoplastic plasma cells, and the expression level is increased in symptomatic MM when compared with monoclonal gammopathy of undetermined significance (MGUS) or smoldering MM [27] (http://amazonia.transcriptome.eu/expression.php?geneId=Hs.118110&zone=Hematology-MM). Although the mRNA expression levels vary among primary MM cells [28–30] (http://amazonia.transcriptome.eu/expression.php?geneId=Hs.118110&zone=Hematology-MM), more than 1×10^4 molecules/cell of HM1.24 are detected at the surface of MM cells in more than 85% of patients [31].

During the normal plasma cell differentiation, HM1.24 mRNA is expressed at the highest level in plasmablasts as well as in early plasma cells compared with mature plasma cells [30, 32] (http://amazonia.transcriptome.eu/expression.php?geneId=Hs.118110&zone=PlasmaCell). These findings support the idea that HM1.24 is an intriguing target molecule for immature MM cells or MM cancer stem cells. In fact, we have observed that side population (SP) of MM cells including MM cancer stem cell-like cells (CSCs) expressed HM1.24 at high levels [33].

Several studies have shown that HM1.24 is also expressed on a variety of human tissues and organs such as hepatocytes, pneumocytes, salivary glands, kidney, and vascular endothelium both at the mRNA and protein levels [19, 21, 34]. However, the expression profiles at the protein level in normal tissues have not been clarified yet. In addition, we and other researchers have found that HM1.24 is overexpressed on various cancer cells isolated from breast, lung, kidney, endometrium, and skin [26, 35–43].

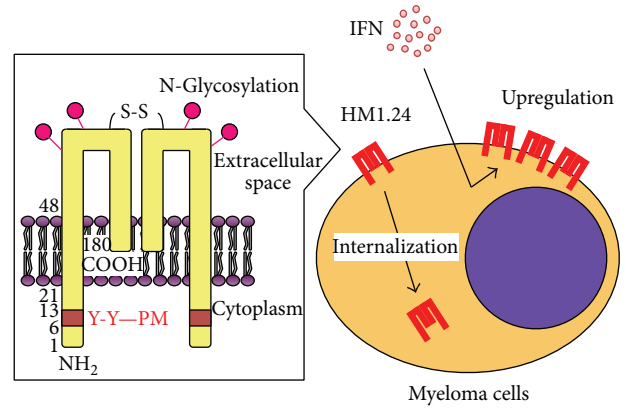

Figure 1: The schema of the structure of HM1.24. HM1.24 is a type II transmembrane glycoprotein that is selectively overexpressed on MM cells as a homodimer with a unique topology. HM1.24 internalizes and localizes to the Golgi apparatus. In the promoter region of HM1.24, there are several *cis*-elements for transcription factors such as IRF-1/2 and ISGF3, and the expression levels of HM1.24 can be upregulated by IFN.

The physiological role of HM1.24 remains unclarified; however, recent studies have shown that HM1.24 directly binds to immunoglobulin-like transcript 7 (ILT7) protein and initiates signaling via the ILT-7-FcεRIγ complex [44, 45]. HM1.24 is now termed "tetherin" as a molecule that tethers outgoing virions to the infected cell surface preventing their dissemination [46–48]. However, its biological role in MM cells has not been clarified yet.

3. The Development of Anti-HM1.24 mAb Therapy

3.1. Mouse Anti-HM1.24 mAb. We first developed a mouse anti-HM1.24 mAb (IgG2a-κ) by immunizing Balb/c mice with human MM cells [18]. After fusing spleen cells collected from the immunized mice with myeloma cells and cloning of these fused cells, mAbs that react with the cell surface antigens were obtained after screening the hybridomas by flow cytometry.

To evaluate the specificity of the mouse anti-HM1.24 mAb *in vivo*, we employed a mouse xenograft model using severe combined immunodeficiency (SCID) mice. After establishing subcutaneous tumors of human plasmacytoma (RPMI 8226 cells) in SCID mice, the radiolabeled mouse anti-HM1.24 mAb was injected intravenously, and the biodistribution of the mAb was studied [49, 50]. Our results have shown that the mouse anti-HM1.24 mAb selectively accumulates in the xenograft tumors, suggesting that the anti-HM1.24 mAb has a sufficient specificity for targeting human MM cells *in vivo*.

We next studied the antitumor activity of the mouse anti-HM1.24 mAb. Our *in vitro* experiments have shown that the mouse anti-HM1.24 mAb induces ADCC in the presence of effector cells obtained from mice spleen and CDC in the presence of baby rabbit serum [51]. We next evaluated the *in vivo* efficacy of the mouse anti-HM1.24 mAb using human myeloma xenograft models in SCID mice [51]. The treatment with the mouse anti-HM1.24 mAb has resulted in a decrease of the serum levels of M-proteins and the size of the tumors and has resulted in not only a prolonged survival of the mice but also a cure in some of them.

3.2. Humanized Anti-HM1.24 mAb (AHM). Because the mouse anti-HM1.24 mAb exerted a marked anti-MM activity through the operation of ADCC and CDC machineries, we have established a humanized anti-HM1.24 mAb (AHM, IgG1-κ) by grafting the complementary-determining regions [52, 53]. AHM induced ADCC in the presence of human PBMCs against both MM cell lines and MM cells from MM patients, but not CDC in spite of the presence of human serum [31, 52]. The ADCC activity of AHM was increased in a dose-, an effector to target (E/T) ratio-, and HM1.24 expression-dependent fashion. In addition, our *in vivo* experiments have shown that AHM kills MM cells through ADCC [54].

Based on these results, the safety and efficacy of AHM were investigated in a phase I/II clinical study in patients with relapsed or refractory MM in the UK. [55]. Although adverse events were very modest and manageable, the response rate was relatively low in the study. This was considered probably due to the diminished activity of effector cells in this heavily pretreated patient population.

3.3. Defucosylated Versions of AHM. In the context of ADCC activity, it has been shown that physiological levels of human serum IgG strongly inhibit the ADCC activity of therapeutic antibodies administered [56]. In addition, a genetic polymorphism of Fcγ receptor (FcγR) IIIa influences the binding affinity between Fc domains of mAb and FcγRIIIa of effector cells [57–59]. The polymorphism of FcγRIIIa is present on position 158 [valine (V) or phenylalanine (F)], and patients with homozygous 158 F/F or heterozygous 158 V/F alleles of FcγRIIIa have been shown to have a lower response rate to rituximab treatment [58, 59]. On the other hand, the binding affinity between the two is controlled by fucosylation in N-linked oligosaccharides within immunoglobulin heavy chain Fc regions [8, 9]. Therefore, defucosylated mAbs might overcome the impaired ADCC activity in terms of a low E/T ratio and a low FcγRIIIa affinity. To overcome cellular immune deficiency in MM, we have established a defucosylated version of AHM (YB-AHM) with a higher binding ability to FcγRIIIa [60]. We have found that YB-AHM elicits ADCC more effectively than the parental AHM even with low E/T ratios. Similarly, Tai et al. have shown that Fc-engineered AHM with two amino acid substitutions (S239D/I332E) in the IgG1 Fc portion strongly induces anti-MM activity *in vitro* and *in vivo* [61].

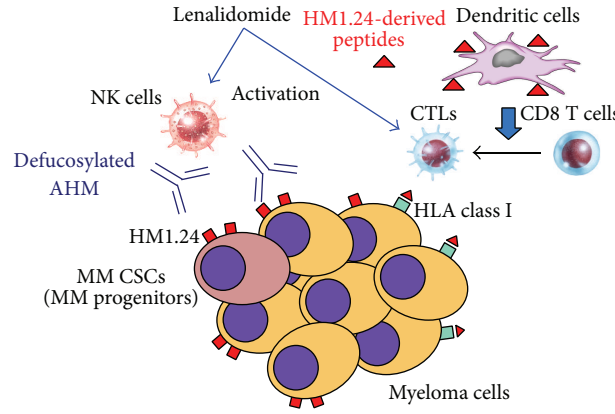

Figure 2: HM1.24-targeted therapy with mAbs and CTLs. Defucosylated AHM induces ADCC activity against MM cells including cancer stem cell-like cells (CSCs) in the presence of human effector cells such as NK cells. On the other hand, functional dendritic cells and HM1.24 peptide-specific CTLs can be induced from PBMCs or PBSC harvests, and these CTLs have the cytotoxic activity against MM cells. Len augments the activity of these cellular immunities.

4. Augmentation of ADCC Activity by Lenalidomide (Len)

Len, one of the IMiDs, induces not only direct cytotoxic effects on MM cells but also immunomodulatory, anti-inflammatory, and antiangiogenic effects on the cells surrounding and supporting MM cells in the bone marrow [62]. In particular, Len stimulates the activity of T, NKT, and NK cells and enhances the ADCC activity (Figure 2). For these reasons, Len has been combined with various mAbs including anti-CS1 [63, 64], anti-CD38 [65], and anti-CD20 [66] to enhance the therapeutic efficacy of them.

Tai et al. and our group severally studied the ADCC activity of Fc-engineered AHM or YB-AHM in combination with Len against MM cell lines and MM cells obtained from bone marrow mononuclear cells of MM patients [61, 67]. The results have shown that Len can enhance the ADCC activity of both defucosylated versions of AHM and Fc-engineered AHM.

MM cancer stem cell-like cells (CSCs) have been proposed as responsible for drug resistance and relapse although they are not properly defined yet [68]. Side population (SP) cells have been identified as a drug resistant fraction that contains CSCs in MM [33]. We have found that HM1.24 is highly expressed on the surface of SP cells and that the combination of YB-AHM plus Len effectively reduces the number of SP fractions in MM cell lines [67]. Furthermore, this combination inhibited the clonogenic potential of MM CSCs *in vitro* [67]. Thus, the combination therapy with YB-AHM plus Len might become an effective strategy to target putative MM CSCs (Figure 2).

With respect to targeting therapy, the number and function of effector cells are important for eliciting ADCC activity. Therefore, YB-AHM therapy could be a suitable strategy as consolidation and/or maintenance therapy because MM cells have already been reduced in number and the number of

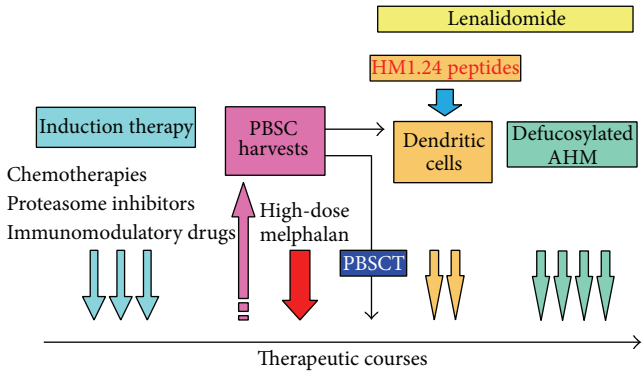

Figure 3: The combination strategy with HM1.24-targeted therapies and the current therapeutic regimen in MM. Induction therapy containing proteasome inhibitors and/or IMiDs and consolidation therapy with high-dose chemotherapy followed by autologous PBSCT induce favorable therapeutic effects; however, the existence of minimal residual disease or MM CSCs is related to relapse and refractoriness. To overcome the drug resistance of MM cells, active immunotherapy with HM1.24-derived peptides and dendritic cells from autologous PBSC harvests and passive immunotherapy with defucosylated AHM might be effective approaches along with lenalidomide in the treatment of MM.

effector cells has recovered (relatively high E/T ratio) by this phase (Figure 3).

5. HM1.24 mAb-Conjugated ADC

ADC is another approach to enhance the efficacy of mAb therapy. Several ADCs have been developed by the conjugation of mAb with either cytotoxins or radiation emitters to increase the antitumor effect. HM1.24 is a suitable element of ADC because this antigen is internalized from MM cell surface into the Golgi apparatus. Therefore, we have manufactured ADC by using an internalizing mAbs specific to HM1.24 [69]. One of the fully human anti-HM1.24 mAbs, b-76-8, is rapidly internalized after cell surface binding. Thus, ADC consisted of b-76-8 and the analog of the cytotoxic drug maytansine, DM1 [N2′-deacetyl-N2′-(3-mercapto-1-oxopropyl)-maytansine], has been developed. Our results have shown that this ADC significantly elicits the cytotoxic activity against MM cells without effector cells *in vitro* and *in vivo*. Recently, Staudinger et al. have established a novel single-chain immunotoxin, HM1.24-ETA′, by genetic fusion of a HM1.24-specific single chain Fv antibody and a truncated variant of *Pseudomonas aeruginosa* exotoxin A (ETA′) [70].

6. HM1.24 Peptide-Specific CTLs

Besides anti-HM1.24 mAb therapy, we have also examined the possibility of HM1.24-specific CTL therapy against MM cells (Figure 2). We selected four HM1.24-derived peptides that possess binding motifs for HLA-A2 or HLA-A24 by using two computer-based algorithms and developed the methods inducing HM1.24 peptide-specific CTLs from PBMCs of healthy donors or PBSC harvests from MM patients in the presence of HM1.24 peptide-pulsed dendritic cells [71]. The experiments *in vitro* have shown that HM1.24 peptide-induced CTLs have the direct cytotoxic activity against MM cells. Several investigators have reported similar results by using HM1.24-derived peptides [72, 73].

Notably, Len has been reported to augment the cytotoxic activity of CTLs in MM [74] including HM1.24-specific CTLs [75]. On the other hand, Herth et al. have recently reported that thalidomide maintenance therapy compromises the HM1.24-specific CTL immunity in MM patients who underwent PBSCT [76]. These results indicate that the cellular immunotherapy targeted for HM1.24 could also be effective in MM, and further studies are warranted to determine whether the IMiDs maintenance therapy with Len or pomalidomide could augment antigen-specific T cell activity.

High-dose chemotherapy followed by autologous PBSCT is considered the most effective consolidation therapy for younger patients with MM. For this procedure, PBSCs are harvested and cryopreserved together with peripheral lymphocytes and monocytes. Therefore, we have investigated the possibility for active CTL therapy by using residual PBSC products after PBSCT. Tarte et al. have previously reported the generation of functional dendritic cells using apheresis products from MM patients [77]. Our results have confirmed that frozen PBSC harvests are useful source for dendritic cells and also for HM1.24-specific CTLs [71]. Thus, we consider that HM1.24-specific cellular immunotherapy could be applied to increase the therapeutic efficacy of autologous PBSCT (Figure 3).

7. Conclusion

HM1.24 is an overexpressed antigen on MM cells, and HM1.24-targeted therapies might provide alternative strategies in the management of MM. With regard to mAb therapy, the defucosylated versions of AHM have been established and the synergistic effects have been shown when combined with Len. To further enhance the cytotoxic activity of mAbs, several types of ADC have been developed. Moreover, CTLs specific for HM1.24 have been successfully induced from PBSC harvests obtained from MM patients, and the activity could further be augmented by Len. Most importantly, *in vitro* experiments have shown that some of these approaches are effective for the eradication of MM CSCs.

The treatment paradigm of MM has been dramatically changed since the introduction of autologous PBSCT and novel agents such as thalidomide, lenalidomide, and bortezomib. HM1.24-targeted therapies can be combined with the current therapeutic approaches (Figure 3). Further studies are needed to determine whether these strategies could improve the outcome of MM patients.

Conflict of Interests

The authors declare no competing financial interests related to this work.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (C) for Shuji Ozaki from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors thank Dr. Tetsuya Goto for his pioneering work in establishing the anti-HM1.24 mAb and for his continued support for our research projects. They also thank Dr. Kazuyuki Shimizu for his thorough review of the paper.

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