Toll-like receptor signaling-deficient mice are easy mobilizers: evidence that TLR signaling prevents mobilization of hematopoietic stem/progenitor cells in HO-1-dependent manner

Hematopoietic stem/progenitor cells (HSPCs) are mobilized from bone marrow (BM) into peripheral blood (PB) in steady-state conditions in a circadian rhythm-dependent manner as well as in pathological processes related to tissue/organ injury and inflammation. The number of HSPCs circulating in PB is also enhanced by administration of certain drugs, such as granulocyte colony-stimulating factor (G-CSF) or a CXCR4 receptor antagonist (AMD3100). The pharmacologically induced mobilization by G-CSF or AMD3100 is a strategy currently employed in the clinic to obtain HSPCs from mobilized PB for hematopoietic transplantation.1–5

Evidence has accumulated that elements of innate immunity, such as the complement cascade (ComC), neutrophils, monocytes/macrophages and naturally occurring IgM antibodies, have a crucial role in mobilization of HSPCs into PB, which can be considered as a response to pro-inflammatory stimuli.6,7 Toll-like receptors (TLRs) that have a key role in the innate immune system, and are expressed by granulocytes and macrophages have been reported to be engaged in crosstalk with complement C3a and C5a receptors and thus are important modulators of inflammatory responses in vivo. This crosstalk reportedly augments ComC-mediated synthesis of several pro-inflammatory cytokines.7,8

In general, TLRs recognize structurally conserved pathogen-associated molecular pattern molecules (PAMPs), such as endotoxin or lipopolysaccharide (LPS), which are derived from invading microbes. However, at the same time they also recognize certain endogenous ligands belonging to the family of danger-associated molecular pattern molecules (DAMPs), such as extracellular ATP, fibrinogen, heat-shock proteins, high-mobility group box 1 protein (HMGB1), extracellular matrix components and self DNA produced by activated cells in the organism.9 In mice, 11 TLRs (TLR1–11) have been described, and all of them except TLR3 use MyD88 as an adapter signaling protein.9 It has been reported that TLRs are also expressed by HSPCs and directly regulate some of their biological functions.10 As recently proposed, G-CSF regulates hematopoietic stem cell activity, in part, through activation of TLR signaling.11

For many years our group has pursued the role of the ComC and other elements of innate immunity in the mobilization of HSPCs. As has been reported, the ComC is activated in BM after pharmacological mobilization by G-CSF or AMD3100.2,6 On the other hand, the ComC is also strongly activated by certain PAMPs, including LPS and zymosan. Thus, mobilization of HSPCs can be considered as a response of the BM microenvironment and stem cell niches to inflammation and tissue/organ injury.2 We also recently found that heme oxygenase 1 (HO-1) is a negative regulator of ComC activation and inhibits cell migration and mobilization of HSPCs.12,13

As TLRs are expressed by both HSPCs and accessory cells that promote the mobilization process (for example, granulocytes and macrophages) as well as endothelial cells lining the BM sinuses, we became interested in the role of TLRs in the egress of HSPCs from BM into PB. To address this question, we performed mobilization studies employing G-CSF, AMD3100, LPS and zymosan in MyD88 mice that lack the adapter signaling protein associated with all TLRs except TLR3. Furthermore, to address the role of TLR3 in MyD88-independent signaling, we also employed TLR3-KO mice in our mobilization studies. Supplementary Figure 1 shows that, under steady-state conditions, MyD88-KO animals have (i) PB counts, (ii) erythropoietic parameters, (iii) the number of HSCs and (iv) the number of clonogenic CFU-GM, BFU-E and CFU-Meg progenitors in BM that are similar to wild-type (WT) control mice. Similarly, we did not observe any abnormalities in the evaluated hematopoietic parameters under steady-state conditions in TLR3-KO animals (data not shown).

In our studies, MyD88-KO, TLR3-KO and WT mice were mobilized by G-CSF (100 μg/kg daily for 6 days), AMD3100 (5 mg/kg), LPS (200 ng/mice) or zymosan (0.5 mg/mice). Mobilization efficiency in response to mobilizing agents was evaluated in MyD88-KO, TLR3-KO and WT control animals by determining the number of (i) circulating white blood cells (WBCs), (ii) Sca-1-c-kit+Lin- (SKL) cells, (iii) Sca-1+CXCR4+HSCs and (iv) clonogenic CFU-GM in the PB of mobilized animals. We found that MyD88-KO mice (Figure 1) as well as TLR3-KO mice (Supplementary Figure 2) are more easily mobilized by G-CSF and AMD3100 than WT littermates. Moreover, the enhanced mobilization status of MyD88-KO mice and TLR3-KO mice corresponded with low activation of HO-1, as measured at the mRNA and protein levels in BMMNCs and SKL cells isolated from mutant animals, compared with WT control mice (Figure 2 and Supplementary Figure 2).

Mobilization after administration of G-CSF or AMD3100 in the context of TLR signaling may depend on the crosstalk between activated ComC receptors and TLRs and could be potentiated by direct stimulation of TLRs by certain endogenous DAMP molecules released from activated BM cells (for example, heat-shock...
proteins, HMGB1, extracellular matrix components and self DNA). To our surprise, however, we found that expression of TLRs is not obligatory to augmenting mobilization of HSPCs, as MyD88-KO and TLR3-KO animals exhibited enhanced, not decreased, egress of HSPCs into PB in our hands.

Next, we employed PAMPs such as LPS and zymosan, which are potent ligands for TLRs. Specifically, although LPS is a potent activator of TLR4, zymosan preferentially activates TLR2. As mentioned above, both TLR2 and TLR4 depend on the MyD88 adapter protein for proper signaling. As with G-CSF and AMD3100, we again observed that MyD88-KO mice are easily mobilized by administration of LPS or zymosan compared with WT animals and that this enhanced mobilization correlated again with lower activation of HO-1 in BMMNCs and SKL cells (Supplementary).

Figure 1. MyD88-KO mice are easily mobilized by G-CSF and AMD3100. MyD88-deficient and WT mice (as control) were treated with G-CSF (for 6 days at 100 μg/kg per day, subcutaneous injection, upper panel) and AMD3100 (single dose at 5 μg/kg, intraperitoneal injection, lower panel). Experimental mice were killed 6 h after the last G-CSF injection and 1 h after AMD3100 mobilization, and the numbers of WBCs, SKL (Sca-1+ c-kit− Lin−) cells, HSCs (Sca-1+ CD45− Lin−) and CFU-GM clonogenic progenitors from PB were evaluated. Results from two separate experiments are pooled together (n = 8 mice per group). *P < 0.05.

Figure 2. The level of HO-1 was evaluated in BMMNCs, and sorted Sca-1+ cells collected from WT and MyD88-KO mice were stimulated with G-CSF (140 ng/ml) or AMD3100 (140 ng/ml). Left panel: expression of HO-1 at the mRNA level by real-time PCR. Results from three independent experiments are pooled together and shown as the percentage of expression in WT mice. *P ≤ 0.05. Right panel: expression of HO-1 by western blotting in BMMNCs and Sca-1+ cells purified from WT and MyD88-KO mice. As shown here, the cells from MyD88-KO mice show downregulation of HO-1 compared with WT-derived cells. The experiment was carried out twice with similar results, and a representative blot is shown.
Figure 3). Interestingly, as TLR2 and TLR4 are classical receptors for zymosan and LPS, respectively, our data indicate involvement of MyD88-independent signaling pathways in these cells.

The enhanced mobilization state in TLR signaling-deficient mice could be the result of intrinsic TLR-mediated properties of hematopoietic cells or could depend on a TLR defect in non-hematopoietic BM cells (for example, stroma or endothelium). To address this question, we created irradiation chimeras by transplanting WT mice with BM from MyD88-KO mice and, vice versa, by reconstituting MyD88 mice with BM from WT animals. To track the chimerism status of transplanted mice, we used matched CD45.1 and CD45.2 murine congenic strains for cross-transplantation, and 3 months after transplantation, chimerism was demonstrated by employing anti-CD45.1 and anti-CD45.2 FACS-based immune identification of the chimeric mice. Both groups of chimeric mice as well as control WT mice transplanted with BM cells from syngeneic WT mice were mobilized by G-CSF or AMD3100, and we evaluated the mobilization efficiency by evaluating the number of WBCs, SKL cells, HSCs and circulating CFU-GM clonogenic progenitors in PB as described above. Supplementary Figure 4 shows that enhanced mobilization was observed in WT mice that were transplanted with MyD88-KO BM cells and not in MyD88-KO mice transplanted with WT cells. This finding supports the conclusion that enhanced mobilization in TLR signaling-deficient mice depends on lack of TLRs expressed in hematopoietic cells and not on non-hematopoietic cells in the BM microenvironment.

The results presented in this short report are important for several reasons. First, it is evidence that although TLRs are part of innate immunity and may have crosstalk with CsAR and CsAR, they do not promote, but instead prevent, mobilization of HSPCs into PB.7,8 This finding is supported by our observation that TLR signaling-deficient mice are easy mobilizers. This negative effect of TLRs on mobilization of HSPCs may somehow balance the mobilization process and may correlate with intracellular expression of HO-1, as TLR-KO mice display a lower level of HO-1 in response to G-CSF, AMD3100, LPS or zymosan. Because HO-1 is a negative regulator of HSPC trafficking, this finding negatively affect the migration of HSPCs by enhancing HO-1 expression. Our results have been supported by other reports showing that LPS–TLR4 signaling in macrophages, in fact, increases intracellular HO-1 activity.14

An open question remains: what are the receptors responsible for egress of HSPCs, because TLR-KO mice are easily mobilized? It is well-known that PAMPs and DAMPs are also recognized by soluble pattern-recognition receptors (PRRs), such as mannan-binding lectin (MBL) and ficolins, and that the MBL pathway and PRRs are crucial for activation of the ComC during mobilization.15 Therefore, it is most likely that activation of this pathway leads to enhanced activation of the ComC and, as a consequence, more efficient mobilization of HSPCs. Our results are somewhat supported by a recent report demonstrating enhanced trafficking of HSPCs from BM to spleen in MyD88-KO, TLR2-KO and TLR4-KO mice.11 Comparing differences in the mobilization of HSPCs in mice treated with antibiotics to eliminate gut commensal microbiota as an endogenous source of the low level of LPS in PB and mutant mice, the authors concluded that trafficking of HSPCs in these mice is TLR-independent. To explain this finding, we propose that MBL and ficolins are most likely the PRRs involved in this process.

In conclusion, these results shed new light on the role of TLRs in the trafficking of HSPCs. We report that TLR signaling involving MyoD88 has a negative role in egress of HSPCs from BM into PB as TLR signaling enhances expression of HO-1 in hematopoietic cells.14 Moreover, our results from mobilizing irradiation chimeras support the conclusion that this effect of TLRs-associated MyD88 depends on its expression in hematopoietic cells. On the basis of this finding, we propose that, despite the fact that TLRs have crosstalk with ComC receptors, it is not TLRs but rather soluble PRRs, such as MBL and/or ficolins, that recognize PAMPs and DAMPs, and are likely to have a role in triggering activation of the ComC and the mobilization process of HSPCs.1,5

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Evidence of shared epitopic reactivity among independent B-cell clones in chronic lymphocytic leukemia patients

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Chronic lymphocytic leukemia (CLL) is a neoplastic disease of mature B cells that express a functional immunoglobulin (Ig) B-cell receptor (Ig-BCR) on the cell surface. The Ig-BCR includes the binding site (idiotype) for the epitope of cognate antigen, which results from the stochastic and productive Ig variable genes rearrangement and possible somatic hypermutation. Individual B-cell clones differ from one another because of the diverse amino acid sequences within the idiotope of the expressed Ig-BCR, so that the epitopic specificity is shared in a given clonal B-cell population.

Several studies have recently highlighted two peculiar characteristics that distinguish CLL among other mature B-cell malignancies. First, the Ig-BCR repertoire of CLL is biased, as it is restricted toward the preferential usage of certain Ig heavy- (IgVH) and light (IgL) -chain variable genes, and unusually similar, stereotyped heavy-chain complementarity determining region 3 (VH CDR3) amino acid sequences. The skewed Ig-BCR could be owing to an Ig-BCR-driven selection mechanism initiated by specific antigens that promote the expansion and possibly the maintenance of the cognate CLL clone. Consistent with this hypothesis, several studies have demonstrated the reactivity of CLL Ig-BCR against foreign antigens, self-antigens, peptides and intrinsic IgVH motifs. Second, CLL is not always a monoclonal disorder, as two or multiple CLL clones have been found in 2–5% of CLL patients. Furthermore, the monoclonal B-lymphocytosis precursor state, which precedes the clinically relevant leukemic phase in virtually all CLL patients, sometimes involves multiple B-cell clones.

It is still unknown whether two or more CLL clonotypes within the same patient potentially associate with the same antigenic reactivity, or alternatively arise as stochastic and antigen-independent events, fostered by the accumulation of oncogenic abnormalities in the preleukemic state. To answer this question, here we have characterized the epitope recognition profiles of CLL clonotypes by coupling the genetic analysis of Ig variable genes and the epitopic reactivity at single-cell level.

We isolated single CD5+ B cells from peripheral blood of six newly diagnosed untreated CLL patients, randomly referred to the Medical Oncology Unit—University Magna Gracia of Catanzaro. CLL patients displayed the typical CLL immunophenotype, with no evidence of separate/aberrant B-cell populations (Supplementary Table S1). We determined the V_HJH and VLJL complementary DNA (cDNA) sequence of at least 20 single leukemic cells per patient (Supplementary Table S2). All cDNA sequences showed a productive rearrangement at the heavy- and light-gene loci. In particular, we detected single and distinct V_HJH and VLJL rearrangements in CLL#1, CLL#2 and CLL#3, indicating the presence of a single clonotype (Table 1, Supplementary Table S2). Differently, CLL#4, CLL#5 and CLL#6 exhibited two different V_HJH rearrangements, each of them pairing with a unique and

### Table 1. Characteristics of CLL clonotypes and mimotopes

| Patient | Clonotype | mlgCLL* | V_HJH | VLJL | CDR3 length | VH CDR3 IMGT aa sequence | Freq. (%) | Stereotypic subset | Mimotope name | Mimotope aa sequence | K_D (nM) |
|---------|-----------|---------|------|------|-------------|--------------------------|-----------|-------------------|----------------|----------------------|----------|
| CLL#1   | CLL1      | mlgCLL1 | V3-I1-D3-10/J6 xV1-39(1D-39)/J2 17 | 100 90 | pCLL1 | CSPA| KELGgc | |  | | | | | |
| CLL#2   | CLL2      | mlgCLL2-1 | V37-D3-3/J6 xV3-31(1D-31)/J3 20 | 80 20 | pCLL2-1 | CNTYSVSLc | CD | | | | | | | |
| CLL#3   | CLL3      | mlgCLL2-2 | V3-23-D3-22/4 xV1-8(1D-8)/J3 | 20 60 | pCLL2-2 | CKSYSVSLc | CD | | | | | | | |
| CLL#4-1| CLL#4-2  | mlgCLL3-1 | V2-D3-22/4 xV1-8(1D-8)/J3 17 | 90 90 | pCLL3-1 | CPPQSVTEc | CD | | | | | | | |
| CLL#5-1| CLL#5-2  | mlgCLL4-1 | V4-D3-27/72 xV1-33(1D-33)/J3 | 70 70 | pCLL4-1 | CTPNAPSc | CD | | | | | | | |
| CLL#6-1| CLL#6-2  | mlgCLL4-2 | V4-D6-19/12 xV2-28(2D-28)/J1 | 21 20 | pCLL6-1 | CVLWWSPSc | CD | | | | | | | |
| CLL#7-1| CLL#7-2  | mlgCLL5-1 | V4-D3-23/24 xV1D-12/1J | 20 57 | pCLL5-1 | CFSDDDEWc | CD | | | | | | | |
| CLL#8-1| CLL#8-2  | mlgCLL6-1 | V4-D3-10/J6 xV3D-10/J6 22 | 65 65 | pCLL6-1 | CNQFWKHCc | CD | | | | | | | |
| CLL#9-1| CLL#9-2  | mlgCLL6-2 | V2-46-D3-10/J6 xV1D-17/1J | 24 24 | pCLL6-2 | CTTVIPERC | CD | | | | | | | |

*Name of the CLL-derived monoclonal IgY1 antibodies expressing the indicated V_HJH and VLJL rearrangements. aNumber of amino acids of the VH CDR3. bVH CDR3 amino acid sequence according to the International Immu- nogenetics information system (http://www.imgt.org). cPercentage of analyzed cells expressing the indicated VH CDR3. dStereotypic subset according to Agathangelidis et al.*

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Letters to the Editor

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