

increased expression and increased activity of PTPN22 could play a role in the pathogenesis of CLL. It will be interesting to investigate in future studies whether these 2 mechanisms operate in the same or different patients. Given the possibility of targeting PTPN22 or PTPN22-regulated pathways, this issue could be of considerable importance.

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**Conflict-of-interest disclosure:** The author declares no competing financial interests.

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## To the editor:

### CLL B-cell receptors can recognize themselves: alternative epitopes and structural clues for autostimulatory mechanisms in CLL

Chronic lymphocytic leukemia (CLL) may be driven by antigen recognition through the B-cell receptor (BCR).<sup>1-7</sup> A recent paper in *Nature*<sup>8</sup> suggested a new mechanism for such antigenic drive by functionally characterizing an epitope previously identified by our group as being recognized by virtually all CLL BCRs within a large cohort of CLL patients.<sup>9</sup> This epitope has now been shown to be part of the CLL BCR itself, thus conferring apparently autonomous signaling of BCRs within the cell membrane that may promote growth and survival of the leukemic cell. This provides an entirely new view on CLL pathobiology and its mechanism of disease that appears to apply to the majority of CLL patients.<sup>8</sup>

We describe herein an additional epitope involved in self-recognition of CLL BCRs. We screened random phage display peptide libraries for peptides specifically binding to the CLL BCR (expressed as a Fab fragment) of a randomly chosen patient (Fab007). We identified peptides mimicking the epitope recognized by Fab007 and validated their strong binding to this BCR (data not shown). The consensus amino acid sequence of this epitope (YYC) is homologous to Ig heavy and light chains of different gene families used by Igs of the IgG, IgM, IgA, IgE, and IgD isotype, including the respective CLL Fab007 itself (data not shown). Binding of the CLL Fab007 to the Fab part of Igs was verified in a protein array (Figure 1A) and in ELISAs (Figure 1B). Further binding assays indicated that CLL Fab007 recognizes itself and other CLL BCRs even in patients (eg, CLL Fab005) in whom the recently described epitope VRQ<sup>8</sup> is not present (Figure 1C). A large binding study on primary CLL samples indicated that approximately 50% of CLL cases interact with the YYC motif, most of them showing also considerable reactivity with the VRQ motif,<sup>9</sup> suggesting redundant recognition of alternative epitopes. Interestingly, our new epitope, which is located in framework region 3 of the variable part of Igs, is sterically adjacent and builds a structural continuum with the VRQ epitope located in framework region 2 that was recently suggested as driving autonomous BCR signaling in most CLL patients<sup>8</sup> (Figure 1D bottom right panel). This remarkable colocalization of different epitopes mediating self-recognition of CLL BCRs may explain how autonomous signaling can occur even at a

single-cell level<sup>8</sup> (Figure 1D blue circles) and not just in the context of 1 CLL cell recognizing receptors in adjacent cells (Figure 1D red circles). This is because the region containing both described epitopes is (1) structurally exposed on the surface of the protein and (2) approximately the same distance from the cell surface as the CDR3 region mediating this autorecognition (Figure 1D). This allows interaction of adjacent BCRs within the membrane of the same CLL cell. Our data support the recently established theory of autostimulatory mechanisms in CLL pathobiology and point out redundant recognition profiles and structural explanations that provide the basis for self-recognition of the BCR and self-antigen binding despite the low-affinity typically attributed to CLL BCRs.

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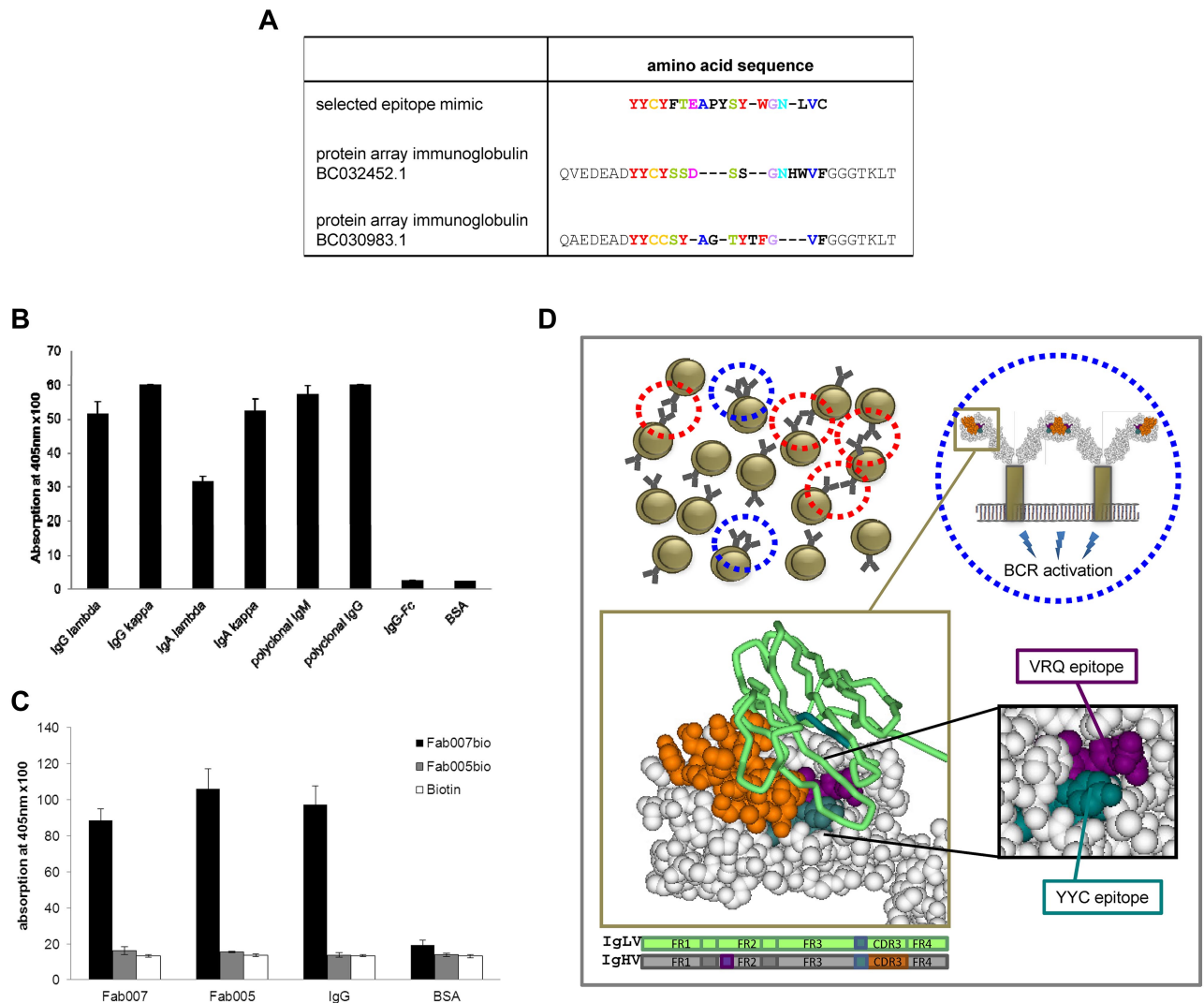
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**Figure 1. BCR self-recognition in CLL via alternative epitopes.** (A) Alignment of the insert sequence of the selected epitope mimicking phage YYCYFTEAPYSYWGN-LVC with 2 species of Ig identified by protein array screening. Fab007 bound specifically to 2 distinct Igs, BC032452.1 and BC030983.1, on a protein array (ProtoArray human protein microarray; Invitrogen). Sequence homology of the phage insert sequence YYCYFTEAPYSYWGNLVC selected on Fab007 with an epitope within the variable region of Igs BC032452.1 and BC030983.1 is shown. Homologous sequences (single-letter amino acid code) are colored. (B) CLL BCR Fab007 binds to immobilized Igs of the IgG, IgA, and IgM isotype. All Igs and control BSA were coated and incubated with Fab007. Binding of Fab007 was detected by ELISA with an HRP-conjugated anti-His-tag antibody. Data are shown as means from triplicate experiments ( $\pm$  SEM). Sources of Igs: monoclonal Igs of the IgG ( $\kappa$  and lambda) and IgA ( $\kappa$  and lambda) isotype were purified from the sera of multiple myeloma patients by protein-A chromatography for IgG and jacalin chromatography for IgA. Polyclonal IgG and IgM were from Octapharma or USB products, respectively; the Fc fragment of human IgG1 (IgG-Fc) was from R&D Systems (P-Selectin/Fc-gamma, #137-PS). (C) CLL BCR Fab007 displays "self-reactivity." Fab007 was labeled with biotin and tested for its binding to immobilized unlabeled receptor in an ELISA assay. Bound biotinylated Fab007 (Fab007bio) was detected using alkaline phosphatase-conjugated avidin. Unrelated Fab005 was used as a control (note that this BCR contained the YYC epitope presented herein but not the VRQ epitope described in Dühren-von Minden et al<sup>8</sup>). Data are shown as means from triplicate experiments ( $\pm$  SEM). (D) Illustration of the structural basis of potential autostimulatory mechanisms in CLL. Red circles mark potential interactions between BCRs on 2 different CLL cells; blue circles mark potential interactions between BCRs on the same CLL cell. The bottom panels show the structure of Ig heavy chains (gray space-filling model) and Ig light chains (green tube model) with the spatially related VRQ epitope (depicted in violet) and YYC epitope (depicted in dark green). The heavy chain CDR3 region is depicted in orange. Below, the exact positions of both epitopes are shown within the framework regions of Ig heavy (IgHV) and Ig light (IgLV) chains (VRQ epitope in violet; YYC epitope in dark green). For the structural Ig model, a random crystallized Fab fragment from the RCSB database ([www.rcsb.org](http://www.rcsb.org)) was chosen (identifier 3GBM).

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**Acknowledgments:** The authors thank Dr Nicholas Chiorazzi and Dr Charles Chu (The Feinstein Institute for Medical Research, Manhasset, NY) for kindly supplying BCRs as recombinant proteins, and Barbara Gösch, Mareile Cordes, and Janina Rahlf for expert technical assistance. This work was

supported by the José Carreras Leukemia Foundation (grant R10/36f to M.T. and M.B.) and the German Research Foundation (Deutsche Forschungsgemeinschaft, grant TR448/6-1 to M.T.).

**Contribution:** M.B. and M.T. designed the study; M.B., F.M., M.F., C.W., and F.S. acquired the data; M.B., F.M., B.L., H.V., R.M., and M.T. analyzed and interpreted the data; and M.B. and M.T. wrote the article.

**Conflict-of-interest disclosure:** The authors declare no competing financial interests.

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## To the editor:

### Antibodies against neutrophil LY6G do not inhibit leukocyte recruitment in mice in vivo

Ly6G is a marker of neutrophils, but the function of this GPI-linked receptor has remained mysterious. Wang et al provided data implicating Ly6G in regulating leukocyte migration.<sup>1</sup> These investigators found that low doses of antibodies (10 µg total administration) directed against Gr-1 or Ly6G reduced experimental arthritis, an effect not due to neutrophil depletion. Instead, anti-Gr-1 or anti-Ly6G antibodies were proposed to inhibit neutrophil recruitment, leading to a reduction in arthritis by acting on the β2-integrins CD11a and CD11b and on ICAM-1 binding levels.

Our laboratory uses intravital imaging to assess the entire leukocyte recruitment cascade directly in real time in vivo. Although granulocyte depletion is achieved at high anti-Gr-1 antibody doses (150-250 µg), we visualize leukocyte recruitment using lower IV doses (1-40 µg) of fluorochrome-labeled anti-Gr-1 (clone RB6-8C5) or anti-Ly6G (clone 1A8). Exploiting these antibodies as imaging tools, we have characterized rolling, adhesion, intravascular crawling, transmigration, emigration, phagocytosis, and tissue NETosis in multiple tissues including skin, liver, brain, and muscle.<sup>2-6</sup> Despite not observing defects in leukocyte recruitment with these antibodies, we performed new experiments to compare neutrophil recruitment directly in transgenic *LysM-eGFP* mice, in which peripheral blood neutrophils are visualized without antibodies to Ly6G, with *LysM-eGFP* mice treated with a fluorochrome-labeled anti-Gr-1 antibody (Figure 1 and supplemental Video 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Using a mouse model of *Staphylococcus aureus* cellulitis,<sup>6</sup> we imaged neutrophil recruitment directly using spinning disk confocal intravital microscopy. *LysM-eGFP* mice received an intradermal injection of live *S aureus* and fluorochrome-conjugated anti-Gr-1 antibody (5 µg IV) or Ly6G clone 1A8 (data not shown), followed immediately by intravital imaging. Neutrophils were visualized rolling and adhering within the dermal microvasculature in both the *LysM-eGFP* (green) laser channel and the anti-Gr-1 (yellow) laser channel immediately after infection (Figure 1A,D and supplemental Video 1). Within 60 minutes of infection, *LysM-eGFP/anti-Gr-1* double-positive peripheral blood neutrophils transmigrated into the parenchyma (Figure 1B,E) and continued to chemotax through the skin (Figure 1C,F). Therefore, we conclude that the entire recruitment cascade, from rolling to chemotaxis, occurs in anti-Gr-1 antibody-treated animals.

To ensure that the anti-Gr-1 antibody did not have subtle quantitative effects on neutrophil recruitment, we compared *LysM-eGFP* mice with and without anti-Gr-1 antibody (Figure 1G-L). The number of emigrated neutrophils was similar between the 2 groups (Figure 1G). Once emigrated, neutrophils from each group crawled with identical velocities (Figure 1H), meandering index (a measurement of the ability of a cell to move in a straight line; Figure 1I), and displacement (Figure 1J). Leukocyte crawling tracks are shown for tissue neutrophils in *LysM-eGFP* mice (Figure 1K) and *LysM-eGFP* mice treated with anti-Gr-1 (Figure 1L). These data demonstrated that no quantifiable difference in leukocyte recruitment and behavior occurred after anti-Gr-1 administration. A fluorochrome-conjugated anti-Ly6G antibody (1A8 clone) did not disrupt any of the leukocyte recruitment measurements (data not shown).

Our ability to visualize all aspects of the leukocyte recruitment cascade directly in vivo demonstrates that low-dose anti-Gr-1 antibodies do not interfere with neutrophil migration. Anti-Gr-1 administration in vitro inhibited stimulation-induced up-regulation of β2-integrins above baseline; however, cell-surface levels continued to be high, as were ICAM-1-binding levels. It is likely that these levels continue to mediate normal recruitment under in vivo physiologic models. The abrogation of arthritis reported by Wang et al is an interesting observation that highlights the need to better characterize Ly6G. However, given our data, we do not believe that the mechanism proposed by the authors adequately explains their observations. We demonstrate that fluorochrome-conjugated anti-neutrophil antibodies against Gr-1 can be used to investigate leukocyte recruitment without interfering with cell migration.

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