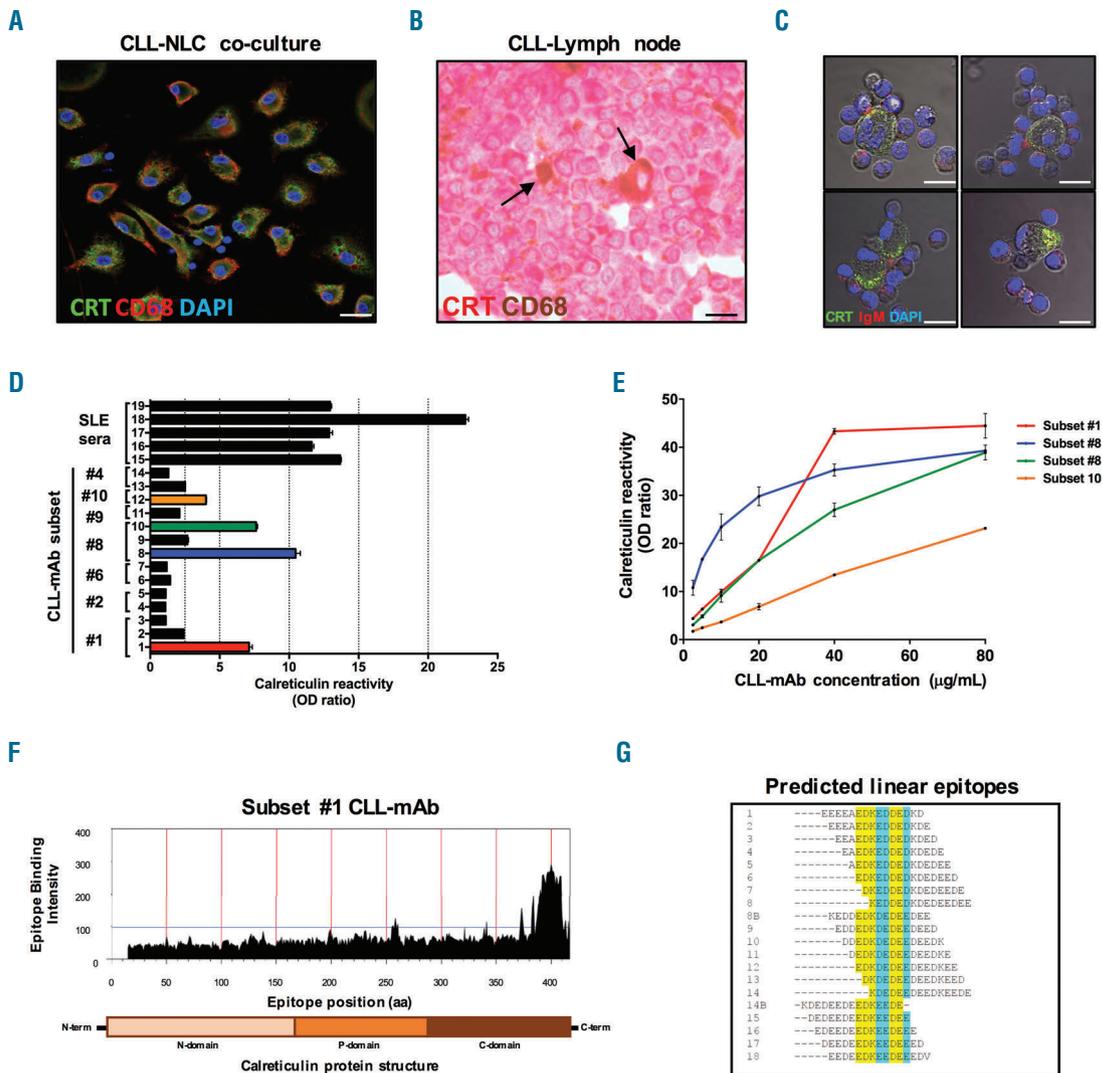


## Calreticulin as a novel B-cell receptor antigen in chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature monoclonal B lymphocytes with a distinct immune-phenotype (CD19<sup>+</sup>CD5<sup>+</sup>CD23<sup>-</sup>), and a markedly heterogeneous clinical course, ranging from indolent disease to more aggressive presentations. B-cell receptor (BCR) signaling plays a central role in disease pathogenesis and is primarily activated in secondary lymphatic tissues.<sup>1</sup> The mutational status of the immunoglobulin heavy chain variable region

(IGHV) genes of the BCR, which distinguishes patients with mutated IGHV from those with unmutated IGHV (M-CLL or U-CLL, with  $\geq 2\%$  deviation or  $<2\%$  deviation from the germline sequence, respectively), is associated with BCR signaling capacity and major differences in disease progression.<sup>2</sup> Patients with high BCR signaling responsiveness typically belong to the U-CLL subset, and present with more aggressive disease, resulting in an inferior prognosis, while patients with M-CLL generally have more indolent disease and better prognosis.<sup>3,4</sup> Approximately 30% of patients with CLL, in both the M-CLL and U-CLL subgroups, express quasi-identical sur-



**Figure 1. Calreticulin is a putative CLL-BCR antigen.** (A) Representative confocal imaging analysis of CLL-NLC co-cultures stained with DAPI (blue) for nuclear staining, anti-CRT (green), anti-CD68 (red). The merge of the three colors and the scale bar of 40µm is indicated in the image. (B) Representative immunohistochemistry staining of a CLL lymph node section stained with anti-CD68 antibodies (brown) and with anti-CRT antibodies (red). Black arrows indicate CD68<sup>+</sup> macrophages. Scale bar, 20µm. (C) Surface immunofluorescence staining of CLL-NLC preparations from 2 U-CLL (upper panels) and 2 M-CLL cases (lower panels) with anti-CRT antibodies (green), anti-IgM antibodies (red) and DAPI (blue) for nuclear staining. Scale bar, 20µm. (D) Calreticulin reactivity, expressed as OD ratio of negative control wells of 14 stereotyped CLL-mAbs, derived from CLL patients belonging to distinct subsets (i.e., U-CLL subsets #1, #2, #6, #8, #9, #10 and M-CLL subset #4), and tested by ELISA at a concentration of 20 µg/mL. Sera from systemic lupus erythematosus (SLE) were tested in a 1:100 dilution, as positive control. CLL-mAbs with positive reactivity to Calreticulin (OD sample at least 2.5 fold of OD blank) were color-coded: Subset #1 (red), Subset #8 (blue and green), Subset #10 (yellow). (E) Titration of Calreticulin reactivity of the 4 CLL-BCRs for which we detected high binding activity, when tested at increasing concentrations (µg/mL) in ELISA assays. The color code is consistent to the one in Figure 1D. (F) Epitope binding intensity of one subset #1 CLL-mAb to 15-mer linear epitopes spanning the full Calreticulin protein. ELISA-based signal intensity (in mAU) is displayed. A schematic representation of full-length Calreticulin is depicted underneath. (G) Curated alignment of the 18 peptides bound by the subset #1 CLL-mAb. Fully conserved residues are highlighted in yellow, conservative mutations are highlighted in cyan. Peptides 8 and 14 are present twice in this alignment (second incidence indicated by B).

face BCRs with stereotyped CDR3 regions, which are commonly classified into subsets (*reviewed in 5*). Interestingly, similarities between cases belonging to distinct stereotyped subsets link BCR immunoglobulin sequences with shared genetic and biological characteristics and clinical behavior. For example, stereotyped subsets #1, #2, #6 and #8 often present as more aggressive CLL.<sup>6</sup> Furthermore, independent studies demonstrated frequent associations between specific genetic aberrations in CLL patients and stereotyped subsets.<sup>7-9</sup> For example, subset #1 cases frequently harbor *NOTCH1* and *NFKB1E* mutations,<sup>10</sup> subset #2 patients often carry *del(11q)* and *SF3B1* mutations, and subset #8 patients present with trisomy 12, *NOTCH1* mutations and Richter's transformation.<sup>11</sup> In contrast, subset #4 patients are characterized by relatively young age at diagnosis, an indolent disease course, and a rare need for therapy.<sup>6</sup> The mechanisms that trigger BCR activation in CLL have not been fully elucidated, although BCR activation in the lymphoid tissues by autoantigens and microbial antigens is the most plausible mechanism, together with homotypic interaction of BCR-binding epitopes within the heavy and light chain of selected stereotyped BCRs.<sup>12</sup> Relevant antigens for CLL have been characterized, particularly for BCRs from patients with U-CLL, including self-antigens, such as non-muscle myosin heavy chain IIA, vimentin, dsDNA, oxidized lipoproteins<sup>15-16</sup> and fungal antigens.<sup>17</sup>

BCR signaling is primarily activated in secondary lymphoid organs, presumably by interactions between CLL cells and the microenvironment, resulting in activation of key survival pathways for CLL cells, including c-MYC and NF- $\kappa$ B proteins.<sup>18</sup> Importantly, when CLL cells are cocultured in the presence of monocyte-derived nurse-like cells (NLCs), an *in vitro* model system for the lymphoid tissue microenvironment, gene signatures associated to BCR signaling are recapitulated,<sup>19</sup> and associated with activation of BCR-associated kinases and IgM internalization, suggesting engagement of the BCRs by antigenic determinants.<sup>20</sup> Based on these notions, we hypothesized that NLC may carry surface proteins, which can be recognized by the CLL-BCRs, thereby initiating BCR signaling activation in the CLL lymph node microenvironment.

We focused our work on the protein Calreticulin (CRT), a known autoantigen for ulcerative colitis (UC), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA),<sup>21</sup> which was recently described to be expressed on the surface of tumor-associated macrophages (TAMs), facilitating cancer cell phagocytosis.<sup>22</sup> Under physiologic conditions, CRT is an endoplasmic-reticulum (ER) resident chaperone, involved in protein folding, antigen presentation and calcium homeostasis.<sup>23</sup> CRT is over-represented in the plasma of CLL patients with aggressive disease,<sup>24</sup> and is upregulated on the surface of apoptotic CLL cells,<sup>25</sup> although its functional role in CLL has not been defined. Provided the importance of autoantigen stimulation in CLL biology, and given the fact that NLC represent CLL-associated TAMs,<sup>26</sup> we hypothesized that CRT could be a putative CLL-BCR antigen, expressed in the context of CLL-NLC interactions.

We analyzed CRT expression by immunofluorescence staining of 4 CLL-NLC preparations after a 14-day coculture with CLL cells, and noted diffuse CRT expression, predominantly on CD68<sup>+</sup> NLC (Figure 1A and *Online Supplementary Table S1*). We also detected CRT expression on CD68<sup>+</sup> macrophages (Figure 1B) of a CLL lymph node biopsy. In this context, both CLL cells (red staining) and macrophage-like cells (double signal in red for calreti-

culin and granular brown for CD68) displayed strong immunoreactivity for CRT. We further asked whether CRT could be exposed on the surface of NLC in the context of CLL-NLC interactions, and tested its expression by surface immunofluorescence staining (sIF), using IgM as a marker for CLL cells (Figure 1C and *Online Supplementary Table S1*). We were able to detect CRT expression on the surface of NLC on CLL-NLC cocultures of 2 U-CLL and 2 M-CLL cases. Surface expression of CRT was further confirmed by Western Blot analysis of 3 NLC membrane preparations (*Online Supplementary Figure S1A and Table S1*), and flow cytometry analysis of 4 cases (*Online Supplementary Figure S1B and Table S1*).

We then explored CRT reactivity of 14 different recombinant monoclonal antibodies (mAbs) derived from CLL patients carrying BCRs from various different stereotyped subsets, representative of the most common IGHV genes used by CLL-BCRs (Figure 1D, *Online Supplementary Table S2*), and sera from 5 patients with systemic lupus erythematosus (SLE), whose antibodies have known reactivity against CRT, as positive control. Of the 14 CLL antibodies, one mAb belonging to subset #1, two belonging to subset #8, and one to subset #10 (a minor subset enriched for trisomy 12 with no definitive correlation with clinical outcome)<sup>27</sup> showed robust CRT binding (Figure 1D). Lower, but detectable binding, was also observed when testing one M-CLL subset #4 antibody (Figure 1D, mAb #13). We further tested the four strongest binders for dose-dependent reactivity to CRT and confirmed their protein binding properties (Figure 1E). Next, we characterized CLL-BCR specific binding epitopes within CRT. We assessed binding specificity of three CLL-mAbs [1 (subset #1), 8 (subset #8) and 12 (subset #10) from Figure 1D and *Online Supplementary Table S2*] to linear epitopes of the full CRT protein, and identified high binding of the subset #1 CLL-mAb to eighteen 15-mers of a region comprising aminoacids 376-409 of the C-terminal domain of CRT protein (Figure 1F). Interestingly, the predicted linear epitopes included a common aminoacidic core EDK(D/E)(D/E)DE(D/E) (Figure 1G), which has also been described as a putative target region for SLE antibodies.<sup>21</sup> Discrete binding of the subset #8 and subset #10 CLL mAbs to linear epitopes of CRT was not identified (*Online Supplementary Figure S2*), indicating that such mAbs may preferentially recognize conformational epitopes or post-translational modifications of CRT protein.

Taken all together, these results support the concept that CRT may function as a putative CLL-BCR antigen for selected U-CLL patients belonging to clinically more aggressive subsets. We also cannot exclude CRT binding to M-CLL cases, in particular those belonging to stereotyped subset #4. Further functional analysis in selected CLL primary cell subsets is required to fully understand the contribution of these interactions to CLL survival. Since CRT is overexpressed in the plasma of CLL patients,<sup>24</sup> upregulated in CLL cells undergoing apoptosis<sup>25</sup> and exposed on the surface of NLCs, conceivably CRT-BCR interactions could occur not only in the lymph node microenvironment, but also to some extent in the peripheral blood, particularly in relation to treatment-induced apoptosis mainly associated with cytotoxic therapies.

This evidence poses the basis for development of novel combinatorial therapies, whereby monoclonal antibodies directed against CRT (or its specific BCR-binding epitopes) could be employed in combinatorial treatments together with BCR signaling inhibitors. Overall, our findings provide insight into subset-specific recognition of

CLL-BCR antigens, with implications for differential BCR activation profiles of CLL cells *in vivo* and optimization of novel BCR-subset specific therapeutic strategies.

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