The role of antigen in the development of B-cell chronic lymphocytic leukemia
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A mutated B-cell chronic lymphocytic leukemia subset that recognizes and responds to fungi

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Abstract

B-cell chronic lymphocytic leukemia (CLL), the most common leukemia in adults, is a clonal expansion of CD5⁺CD19⁺ B lymphocytes. Two types of CLL are being distinguished carrying either unmutated or somatically mutated immunoglobulins, which are associated with unfavorable and favorable prognoses, respectively. Over 30% of CLL can be grouped based on expression of stereotypic B-cell receptors (BCRs), strongly suggesting that distinctive antigens are involved in the development of CLL. Unmutated CLL, carrying immunoglobulin heavy chain variable (IGHV) genes in germline configuration, express low-affinity poly- and self-reactive BCRs. However, the antigenic specificity of CLL with mutated IGHV-genes (M-CLL) remained elusive. Here, we describe a new subset of M-CLL expressing stereotypic BCRs highly specific for β-(1,6)-glucan, a major antigenic determinant of yeasts and filamentous fungi. β-(1,6)-glucan binding depended on both the stereotypic immunoglobulin heavy and light chains, as well as on a distinct amino acid in the IGHV-CDR3. Reversion of IGHV-mutations to germline configuration reduced the affinity for β-(1,6)-glucan, indicating that these BCRs are indeed affinity-selected for their cognate antigen. Moreover, CLL cells expressing these stereotypic receptors proliferate in response to β-(1,6)-glucan. This study establishes a class of common pathogens as functional ligands for a subset of somatically mutated human B-cell lymphomas.
Introduction

B-cell chronic lymphocytic leukemia (CLL), the most common leukemia in adults in the western world, is a clonal expansion of mature CD5⁺CD19⁺ B lymphocytes. Two types of CLL are being distinguished carrying either unmutated (U-CLL) or somatically mutated immunoglobulins (M-CLL), which are associated with unfavorable and favorable prognoses, respectively. Despite this difference in clinical behavior, U-CLL and M-CLL share a highly similar gene expression profile.

Many studies allude to a role for BCR-derived signals in the pathogenesis of B-cell non-Hodgkin’s lymphomas. These signals are either antigen-independent, such as in diffuse large B-cell lymphomas harboring activating mutations in CD79a and CD79b, or antigen-dependent as proposed for CLL. The immunoglobulin heavy chain variable (IGHV) gene repertoire in CLL is biased to frequent usage of IGHV1-69, IGHV3-7 and IGHV4-34 and over 30% of CLL can be grouped based on similarities of the amino acid sequences in the highly variable complementary determining region 3 (CDR3). These stereotypic IGHV display biased patterns of somatic hypermutations and are often paired with distinct immunoglobulin (Ig) light chains. Altogether, these observations suggest that distinctive antigens are involved in the development of CLL.

The majority of U-CLL express low-affinity BCRs that are polyreactive, recognizing self- and exo-antigens, such as DNA, LPS, insulin, apoptotic cells, oxidized LDL, and the cytoskeletal antigens myosin and vimentin. In contrast, M-CLL BCRs are generally not polyreactive. Recently, two stereotypic subsets of M-CLL were identified by us and by others with specificity for the Fc-tail of IgG, so-called rheumatoid factors, a specificity that is commonly found among Mucosa-Associated Lymphoid Tissue (MALT)-lymphomas, Splenic Marginal Zone Lymphomas and Hepatitis C Virus (HCV)-associated lymphomas. In general, the specificity of M-CLL with stereotypic BCRs remained unknown. It has been hypothesized that chronic antigenic stimulation drives CLL development, as was also proposed for MALT lymphomas. For MALT lymphomas, this hypothesis is supported by the observation that Helicobacter pylori-associated MALT lymphomas of the stomach can be eradicated by antibiotic treatment alone. Nevertheless, it was demonstrated that gastric MALT lymphoma cells themselves were not specific for Helicobacter pylori. To our knowledge, expression of BCRs with high-affinity for pathogen-derived antigens has not been reported for any lymphoma entity. In this study, we provide evidence that a subset of somatically mutated lymphomas is selected for an antigenic determinant of a major class of pathogens and that these cognate ligands can drive tumor expansion.
Results

Identification of a novel subset of CLL expressing mutated BCRs
To study the antigen-specificity of M-CLL, we collected 82 CLL expressing IGHV3-7-encoded BCRs. IGHV3-7 is overrepresented in CLL and is mutated somatically in the majority of cases\(^{10}\). Among these IGHV3-7-expressing CLL, we noted four with exceptionally short CDR3 sequences of 5 to 6 amino acids (Figure 1B), as compared to an average of ~15 amino acids for normal B cells\(^{33}\). In addition, these four cases, which we designated V3-7Short (V3-7Sh), shared a glutamic acid at codon 106 in the CDR3 instead of the highly conserved arginine present at that position in the vast majority of IGHV genes, including IGHV3-7 (Figure 1A). This characteristic glutamic acid at position 106 was not found in 78 other IGHV3-7-expressing CLL cases nor in 534 unique IGHV3-expressing B-cell clones from healthy donors, indicating that the frequency of this feature in the normal repertoire is less than 1% (Figure 1C). The four V3-7Sh also harbored subset-specific replacement mutations Y37H and S40H (Figure 1C and 1D), suggesting that V3-7Sh are selected for a shared epitope. In support, these CLL also expressed near-identical IGKV2-24-encoded Ig light chains (Figure 1A). We conclude that these four CLL form a thus far unidentified stereotypic subset, which represents approximately 0.3% of CLL.

V3-7Sh BCRs recognize yeasts and filamentous fungi
To address the BCR-specificity of V3-7Sh, we produced recombinant soluble IgM (sIgM) from three V3-7Sh CLL (designated V3-7Sh-1, -2 and -3). V3-7Sh sIgM were not polyreactive in tissue microarrays containing 21 different tissues from healthy human donors (Figure 2A). In contrast, recombinant sIgM from two U-CLL stained various cell types in all tissues, in accordance with reported self- and polyreactivity\(^{20,22,23}\). V3-7Sh sIgM did not stain apoptotic lymphocytes as analyzed by flow cytometry (data not shown). In ELISAs containing 36 different auto- and exo-antigens, no binding was found of V3-7Sh sIgM, except for V3-7Sh-2, which showed some reactivity with vimentin (data not shown). V3-7Sh-1 and V3-7Sh-3 did not bind vimentin, indicating that this is not a general feature of this subset.

Next, we tested the reactivity of these CLL-derived sIgM towards 33 microbial species by flow cytometry (Table S1). Interestingly, V3-7Sh sIgM brightly stained strains of the commensal yeast species of Candida, Trichosporon, Malassezia and Saccharomyces (Figure 2B and Table S1), whereas 19 bacterial species were negative (Table S1). The specificity of V3-7Sh for fungi was confirmed in ELISAs (Figure 2C) and was not seen with any of 23 other recombinant CLL BCRs, including two IGHV3-7-expressing CLL with CDR3 sequences distinct from V3-7Sh (listed in Table S2). V3-7Sh sIgM specifically stained hyphae in cytological specimens of
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human cervical smears and yeasts in hair-follicles in paraffin-embedded human skin biopsies (Figure 2D). In addition, specific staining by V3-7Sh slgM was observed of the filamentous fungus *Aspergillus* in paraffin-embedded human lung tissue (Figure 3A) and a subset of spores and conidia of *Aspergillus fumigatus*, *Penecilium chrysogenum*, *Fonsecaea pedrosoi*, and *Rhizopus oryzae* (Figure 3B–E), suggesting that V3-7Sh bind epitopes conserved in ascomycetous and basidiomycetous fungi. V3-7Sh slgM weakly stained wild-type *Cryptococcus neoformans*, whereas a mutant strain that lacks a capsule was stained brightly (Figure 2E), suggesting that the epitope is part of the cell wall and shielded from detection. V3-7Sh slgM also bound
Figure 2: Recombinant V3-7Sh sIgM are specific for yeasts

(A) Staining of tissue microarrays with sIgM of V3-7Sh, two non-subset M-CLL and two polyreactive U-CLL. Displayed are stainings of kidney, duodenum, liver, and spleen tissues of healthy human donors. Stainings are representative for at least two independent experiments.

(B) Flow cytometry staining of yeast species and zymosan with V3-7Sh sIgM (blue histograms) or CLL81, a non-subset IGHV3-7-encoded control sIgM (red histograms). Displayed stainings are representative for three V3-7Sh sIgM.

(C) ELISA for C. albicans with 23 CLL-derived recombinant sIgM. Data are representative for three independent assays.

(D) Staining of hyphae in cytological specimens of cervical smears (upper panel) and staining of yeast in hair follicles of paraffin-embedded skin biopsies (lower panel). Displayed stainings are representative for all three V3-7Sh sIgM.

(E) Flow cytometry staining of wild-type C. neoformans (red histogram) and a mutant strain that lacks a capsule (blue histograms) with V3-7Sh sIgM. Displayed staining is representative for all three V3-7Sh sIgM.

(F) Flow cytometry staining of wild-type S. pombe cells (red histogram) and zymolyase-treated S. pombe cell walls (blue histogram) with V3-7Sh sIgM. Displayed staining is representative for all three V3-7Sh sIgM.

(G) Flow cytometry staining of zymosan, a cell wall preparation of S. cerevisiae with V3-7Sh sIgM (blue histograms) or control sIgM (red histograms). Displayed staining is representative for all three V3-7Sh sIgM.
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zymolyase-treated cell walls of Schizosaccharomyces pombe (Figure 2F) and zymosan, a cell wall preparation of S. cerevisiae (Figure 2G), confirming that the antigen recognized is a cell wall component.

V3-7Sh BCRs bind beta-(1,6)-glucan

The three V3-7Sh sIgM displayed strong reactivity to dot-blotted alkali-soluble fractions of zymosan and pustulan (β-(1,6)-glucan) (Figure 4A). A weaker reactivity was observed to preparations of mannan and curdlan (β-(1,3)-glucan) and no binding was detected to α-glucans. By ELISA we confirmed reactivity to pustulan, mannan, and laminarin (β-(1,3)-glucan) preparations (data not shown). As all these antigen preparations are enriched but impure, we assayed their ability to block the binding of V3-7Sh sIgM to zymosan. Pustulan inhibited zymosan binding by 50% (IC50) at concentrations of approximately 0.2 μg/ml (Figure 4B and 4C), whereas the IC50...
Figure 4: Recombinant V3-7Sh sIgM bind β-(1,6)-glucan

(A) Binding of V3-7Sh sIgM to serial dilutions of zymosan, pustulan (β-(1,6)-glucan), curdlan (β-(1,3)-glucan), mannan and amylose (α-(1,4)-glucan) on dot blot. Blots are representative for three independent experiments. (B) Binding of V3-7Sh sIgM to zymosan in the presence of indicated concentrations of pustulan (blue histograms). Red histogram represents an unstained control. Staining is representative for three independent experiments. (C) Binding of V3-7Sh sIgM to zymosan in the presence of indicated concentrations of pustulan (●), mannan (■), and laminarin (β-(1,3)-glucan) (▲). Displayed graph is representative for all three V3-7Sh sIgM. (D) Binding of V3-7Sh sIgM to zymosan in the presence of indicated concentrations of gentiobiose, a β-(1,6)-glucose disaccharide (blue histograms). Red histogram represents an unstained control. Staining is representative for three independent experiments. (E) Binding of V3-7Sh sIgM to zymosan in the presence of indicated concentrations of gentiobiose (●), laminaribiose (β-(1,3)-glucose disaccharide) (▼), cellobiose (β-(1,4)-glucose disaccharide) (▲), isomaltose (α-(1,6)-glucose disaccharide) (■), and salicin (♦). Displayed graph is representative for all three V3-7Sh sIgM.
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values of laminarin and mannan were at least ten-fold higher (Figure 4C), strongly suggesting that \( \beta-(1,6) \)-glucan is the antigen recognized by V3-7Sh. In accordance, binding to the mannan preparations in ELISA was reduced after enzymatic removal of \( \beta-(1,6) \)-glucan (data not shown) and binding to carbohydrate preparations on dot blot was abolished by periodate treatment, which degrades \( \beta-(1,6) \)-linkages, but leaves \( \beta-(1,3) \)-linkages intact, as confirmed by a mouse anti-\( \beta-(1,3) \)-glucan antibody (data not shown). In addition, the alkali-soluble fraction from a temperature-sensitive \( kre5-ts2 \) mutant strain of \( S. \) cerevisiae grown at restrictive temperature stained weaker by V3-7Sh sIgM on dot blot (data not shown), in line with a reported reduction in \( \beta-(1,6) \)-glucan levels in this strain\(^3\)\(^4\). Importantly, competition by micromolar concentrations of the \( \beta-(1,6) \)-glucose disaccharide gentiobiose inhibited binding to zymosan (Figure 4D and 4E). We did not detect inhibition with salicin, a structural analogue of gentiobiose. Neither glucose disaccharides with alternative linkages (laminaribiose, cellobiose, isomaltose) nor a mannose disaccharide (2α-mannobiose) inhibited binding of V3-7Sh sIgM (Figure 4E), demonstrating that V3-7Sh are highly specific for \( \beta-(1,6) \)-glucan, a structure that is not expressed by human cells.

**V3-7Sh BCRs are affinity-selected for \( \beta-(1,6) \)-glucan**

Binding to zymosan was abolished when either the endogenous Ig heavy chain or the endogenous Ig light chain was replaced with those of unrelated M-CLL (Figure 5A), showing that the combination of heavy and light chains in V3-7Sh CLL is essential for \( \beta-(1,6) \)-glucan binding. Binding of V3-7Sh sIgM to pustulan was also detected.
by surface plasmon resonance (Figure 6). Association occurred very rapidly and
dissociation could not accurately be detected, showing that V3-7Sh slgM have very
high affinities for β-(1,6)-glucan (K_D <0.1 nM). Reversion of the somatic mutations
to \textit{IGHV}3-7 \text{germline} resulted in slower association of pustulan to V3-7Sh slgM (Figure 6),
indicating that the somatic mutations contribute to β-(1,6)-glucan affinity and that V3-
7Sh are affinity-selected for β-(1,6)-glucan. Of note, the germline V3-7Sh revertants
did not show polyreactive staining on tissue arrays (data not shown), suggesting that
V3-7Sh are not derived from a polyreactive precursor, in contrast to a report showing
polyreactivity for a majority of reverted M-CLL22. Substitution of the characteristic

Figure 6: V3-7Sh BCR are selected for β-(1,6)-glucan affinity
(A) Surface plasmon resonance curves of binding of pustulan (0, 0.3, 1 and 3 μg/ml) to V3-7Sh slgM
(left) and V3-7Sh slgM after reversion of somatic mutations (V3-7Sh germline) (right). The response
curves were fitted to a 1:1 binding model (orange lines). Curves are representative for two independent
experiments. (B) Kinetic constants for pustulan binding to V3-7Sh slgM. k_a in 10^4 sec^{-1}M^{-1}, k_d in 10^{-5}
sec^{-1}, K_D in pM. Kinetic constants were calculated with data from at least five different anti-IgM coated
spots. The error value is the deviation in the kinetic constants between different coated spots. For
calculations, an estimated average molecular weight of 20 kDa was used for Pustulan. C) Association
constants (k_a) of pustulan binding to V3-7Sh slgM with somatic mutations (black bars) and after reversion
of somatic mutations to \textit{IGHV}3-7 \text{germline} determined by surface plasmon resonance (white bars). Data
is representative for two independent experiments.
A mutated CLL subset is specific for fungi glutamic acid in the CDR3 to an arginine (E106R) resulted in a 3.2-fold and 2.1-fold reduction in binding to zymosan for V3-7Sh-1 and V3-7Sh-2, respectively. A combination of E106R with reversion of somatic mutations to IGHV3-7 germline abrogated binding completely (Figure 5B), indicating that both the glutamic acid at position 106 and the somatic mutations determine β-(1,6)-glucan specificity and affinity.

**V3-7Sh CLL cells proliferate in response to β-(1,6)-glucan**

V3-7Sh CLL cells bound zymosan, confirming that membrane bound V3-7Sh BCRs are capable of binding β-(1,6)-glucan (Figure 7A). Peripheral blood B cells from healthy donors and six control M-CLL, including two non-subset IGHV3-7-expressing CLL, did not bind zymosan (Figure 7B). As the natural ligands of CLL are typically not known, direct evidence for antigen-dependent CLL growth was not available. Now able to address this issue, we cultured primary CLL cells from three V3-7Sh...
patients on CD40L-expressing cells. In this system, addition of pustulan induced proliferation of tumor cells (Figure 7C and 7D). The proliferating cells were clonal and expressed the V3-7Sh CDR3 as demonstrated by CDR3-length spectratyping (data not shown). Pustulan did not induce proliferation of three control M-CLL, whereas BCR-crosslinking with anti-Ig light chain antibodies induced proliferation in both V3-7Sh CLL and control M-CLL (Figure 7C and 7D).

Stimulation of V3-7Sh cells with pustulan yielded slightly more proliferating tumor cells as compared to V3-7Sh cells stimulated with anti-Ig light chain beads (Figure 7C), suggesting that V3-7Sh CLL cells may also receive signals through innate immune receptors recognizing fungal antigens. To date, no innate immune receptors have been described that detect β-(1,6)-glucan. However, as pustulan also contains traces of fungal antigens other than β-(1,6)-glucan (data not shown), other innate immune receptors may also be stimulated. To analyze this possibility, we measured the expression of several innate receptors by flow cytometry. Expression of TLR2, TLR4 and DC-SIGN was not detected in V3-7Sh CLL, whereas Dectin-1, Dectin-2 and the mannose receptor were expressed (at the surface of V3-7Sh CLL cells), suggesting that V3-7Sh CLL cells may indeed receive additional signals through innate immune receptors. Expression of Dectin-1, Dectin-2 and the mannose receptor was low as compared to dendritic cells and not higher than in the three control CLL used in our experiments (data not shown), indicating that control CLL potentially received the same amount of signals. Control CLL cells however did not proliferate in response to pustulan stimulation, demonstrating that signaling by innate fungal immune receptors alone is insufficient to drive proliferation.

Discussion

In this study, we have identified a new subset of M-CLL, designated V3-7Sh, expressing stereotypic BCRs specific for the fungal antigen β-(1,6)-glucan. V3-7Sh slgM did not show polyreactivity and it is unlikely that V3-7Sh are cross-reactive with a self-antigen, as structures containing β-(1,6)-linked glucoses have not been identified in humans. In accordance, no specific staining was seen in any of 21 different tissues of healthy human donors in a tissue microarray. The β-(1,6)-glucan specificity of V3-7Sh BCRs depends on both the stereotypic Ig heavy and light chains, therewith excluding that this reactivity is due to superantigen-like binding. β-(1,6)-glucan binding also depends on the characteristic glutamic acid in the IGHV-CDR3, indicating that the binding capability for this antigen accounts for the BCR stereotypy. The observation that the somatic mutations increase the affinity for β-(1,6)-glucan demonstrates that V3-7Sh BCRs are affinity-selected for β-(1,6)-glucan. We provide unambiguous evidence that different members of a mutated CLL
subgroup recognize an identical pathogen-derived epitope, supporting the hypothesis that BCR stereotypy in CLL results from stringent antigenic selection and affinity maturation. In stark contrast, Dühren-von Minden et al have recently claimed that the growth of mutated and unmutated CLL is driven by antigen-independent, cell-autonomous Ca\textsuperscript{2+} signaling due to self-recognition of an intrinsic IGHV motif, based on signaling studies of retrovirally expressed CLL BCRs in mouse cells\textsuperscript{35}. In our view, the observations by this group, although interesting, do not explain why there are subgroups of CLL patients expressing highly similar BCRs with shared somatic mutations. Importantly, we found that primary CLL cells did not proliferate in vitro in the absence of extrinsic BCR crosslinking. In agreement, we found that rheumatoid-factor-expressing primary CLL cells proliferated in response to aggregated IgG\textsuperscript{24}. These observations are difficult to reconcile with an antigen-independent driven growth of CLL cells. Rather, antigen-independent elevation of Ca\textsuperscript{2+}-levels caused by BCR self-recognition might be indicative of CLL anergy\textsuperscript{36,37}. Recently, an elegant study by Zikherman et al showed that encounter of endogenous antigen during development fine-tunes the responsiveness of mouse B cells to BCR stimulation and that the level of responsiveness inversely correlates with the level of self-antigen exposure\textsuperscript{37}. Along these lines, it may be hypothesized that, due to superior cross-linking characteristics, extrinsic cognate antigens are essential to overcome self-antigen-induced anergy of CLL cells.

The BCR specificity of CLL cells may withhold clues concerning the B-cell type of origin. Two B-cell subsets have been proposed, i.e. natural antibody (Nab)-producing cells and marginal zone (MZ) B-cells\textsuperscript{38}. Nabs are usually germline-encoded and capable of binding many antigens with low-affinity. In recent years, it has become clear that U-CLL express poly- and self-reactive BCRs of low affinities\textsuperscript{20,22,23}, suggesting that U-CLL are derived from Nab-producing cells. M-CLL by contrast usually are monospecific, indicating that M-CLL are not derived from Nab-producing cells. In support, we show that V3-7Sh CLL cells are monospecific and affinity-selected. In addition, the finding that reversion of somatic mutations in V3-7Sh did not result in polyreactivity indicates that these CLL do not originate from a polyreactive precursor. β-(1,6)-glucan is a polyvalent carbohydrate antigen. In general, polyvalent antigens induce MZ B-cell responses\textsuperscript{39}, which may point towards a MZ B-cell origin for V3-7Sh CLL.

It is noteworthy that the natural ligand of a M-CLL subset is a major antigenic determinant shared by an extensive group of pathogens. The V3-7Sh CLL patients studied here, do not have a history of persistent or recurrent fungal infections. However, fungi are ubiquitous\textsuperscript{40,41} and likely to chronically or intermittently stimulate V3-7Sh-expressing clones, even without clinically overt infections. We here show
a crucial role for a microorganism in the pathogenesis of CLL, apparently similar to the dependence of gastric MALT lymphomas on *H. pylori*. However, whereas CLL growth is driven by pathogen-specific BCR signaling, MALT lymphomas were demonstrated not to be pathogen-specific but to recognize self-antigens present within the inflammatory microenvironment induced by *H. pylori* infection\textsuperscript{11,27,32,42}. It is conceivable that B cells that are specific for common pathogens are particularly at risk for gradual genetic derailment during chronic or intermittent immune reactions. A major challenge now is to assess the specificity of other M-CLL subsets. Our finding that CLL cells are induced to proliferate in response to their natural ligand may reflect a general principle of somatically mutated low-grade B-cell lymphomas. It is tempting to speculate on the possibilities for antigen- or pathogen-targeted therapies for this group of patients, but one might anticipate that this approach is especially challenging in case of antigens derived from commensal microorganisms, for which complete eradication may be hampered by their ubiquitous and persistent nature.

**Materials and methods**

**Patients and IGHV sequences**

We obtained 21 and 40 *IGHV3-7* sequences subjected to mutation status analysis according to the BIOMED protocol\textsuperscript{43} from the Academic Medical Center, Amsterdam and the Erasmus Medical Center, CLL patient cohorts. An additional 21 CLL *IGHV3-7* BCR sequences were obtained from GenBank. This study was conducted in accordance with the ethical standards in our institutional medical ethical committee (Medisch Etische Toetsingscommissie AMC) on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983. Accordingly, all patients signed informed consent.

**Recombinant immunoglobulin production**

Following RNA isolation from peripheral blood mononuclear cells (PBMCs) using Trizol Reagent (Invitrogen), cDNA was synthesized using Pd(N)\textsubscript{6} random primers. *IGHV* and *IGKV* genes were amplified using *IGHV-, IGKV-, IGHJ- and IGKJ*-specific primers. Recombinant, soluble IgM (slgM) of 23 CLL patients were produced using plgH(\(\mu\)) and plgL(\(\kappa\)) expression vectors as described previously\textsuperscript{44}. In brief, the rearranged *IGHV* and *IGKV* genes of each of these leukemias were cloned into the plgH(\(\mu\)) and plgL(\(\kappa\)) vectors, respectively. For production of recombinant antibodies, 10 \(\mu\)g plgH(\(\mu\)) and 10 \(\mu\)g plgL(\(\kappa\)) was linearized with PvuI and co-transfected into SP2/0 myeloma cells by electroporation, which produce soluble pentameric IgM, as confirmed by SDS-PAGE (data not shown). Subsequently, transfected cells were selected in geneticin-containing medium. To screen supernatants for slgM, 4 \(\mu\)g/
ml of anti-human Ig\(\kappa\) is coated to Costar EIA 96-wells plates (Corning) in carbonate buffer overnight at 4\(^\circ\)C, followed by blocking of the wells with 1% BSA in PBS. Subsequently, serial dilutions of supernatants containing the sIgM are incubated 1h at room temperature. Bound sIgM was detected by horseradish peroixidase (HRP)-conjugated anti-human IgM and developed as described previously \(^{45}\). The pIgH(\(\mu\)) and pIgL(\(\kappa\)) expression vectors were provided by J. van Es and T. Logtenberg (Utrecht Medical Center, Utrecht, The Netherlands). Heavy and light chains were exchanged by co-transfection of non-endogenous combinations of heavy and light chains as described above.

To generate recombinant IgM of V3-7Sh with \(\text{IGHV3-7}\) in germline configuration, naive B-cells (CD19\(^+\)CD27\(^-\)) were sorted from a tonsil. Subsequently, the \(\text{IGHV3-7}\) germline was amplified by PCR using an \(\text{IGHV3}\)-specific primer and a reverse \(\text{IGHV3-7}\) primer located in FR3. The CDR3s of V3-7Sh-1 and V3-7Sh-2 were amplified with an \(\text{IGHV3-7 FR3}\) specific forward primer and an \(\text{IGHJ}\) reverse primer. V3-7Sh-1 does not contain any replacement mutations in the IGHV-CDR3, whereas V3-7Sh-2 contains few somatic mutations in the IGHV-CDR3, which we did not revert. The \(\text{IGHV3-7}\) germline was then ligated to the V3-7Sh CDR3 by PCR and produced as described above. V3-7Sh-1 and V3-7Sh-3 express \(\text{IGLV}\) in germline configuration, indicating that the SHM in the \(\text{IGLV}\) are not necessary for binding \(\beta\)-(1,6)-glucan. Therefore, somatic mutations present in the \(\text{IGLV}\) were not reverted. The glutamic acid at position 106 was substituted into an arginine using specific primers and the QuickChange II site-directed XL mutagenesis kit from Stratagene, according to the manufacturer’s instructions.

**Microorganisms**

Bacteria, yeasts, and filamentous fungi (Table S1) were grown overnight on agar plates, supplemented with medium appropriate for the species. S. pombe cell walls were treated with zymolyase as described previously \(^{46}\). S. cerevisiae kre5-ts2 mutant was a kind gift from Dr. Vishukumar Aimanianda (Institut Pasteur, Paris, France) \(^{34}\) and was cultured in YPAD-medium at a permissive temperature of 22\(^\circ\)C and at a restrictive temperature of 37\(^\circ\)C. Cells were lysed with glass beads and total cell lysates were adjusted to 400 \(\mu\)g/ml protein in 0.75 M NaOH.

**Immunohistochemistry**

Fungal mycelium was separated from agar culture plates with a scalpel, chopped, and mounted on glass slides in 0.005% Tween-80 in PBS. Slides were air-dried and fixed with 5% PFA in PBS for 30 minutes. Cytological specimen of cervical smears, paraffin-embedded skin and lung biopsies and healthy donor tissues were obtained.
from the department of Pathology at the AMC, Amsterdam, The Netherlands. Healthy donor tissues were assembled in tissue microarrays (TMA) using a manually operated TMA device (Beecher Instruments, Silver Spring, MD). After antigen retrieval in Citrate buffer (20 min at 120°C), slides were stained overnight with slgM at 1 μg/ml in PBS. Mouse anti-human IgM (Clone MH15/1, Sanquin) was used as secondary antibody. Subsequently staining was visualized using Powervision® (ImmunoVision Technologies).

**Dot blot**

Zymosan and total cell lysates were dissolved in 1 M NaOH and incubated at 75°C for 1 hour to extract the alkali-soluble fraction as described previously. Pustulan (Calbiochem), curdlan (Sigma), C. albicans mannan (NIBSC) and amylose (Sigma) were dissolved in 1 M NaOH and 100, 20, 4 and 0.8 ng was spotted on PVDF-membranes and dried for 30 min. When indicated, dot blots were treated overnight with 50 mM periodic acid. Membranes were blocked with 5% milk for 1 hour and subsequently stained with 2 μg/ml of slgM or mouse β-(1,3)-glucan antibody (Biosupplies) for 30 min at room temperature. Anti-human IgM-HRP (Southern Biotech) and rabbit anti-mouse Ig-HRP (Dako) were used as secondary antibodies. Staining was visualized with ECL (GE Healthcare). To analyze binding of V3-7Sh slgM to the alkali-soluble fraction of the kre5-ts2 mutant total lysates, serial dilutions were spotted and stained as described above.

**ELISA**

Single colonies of yeasts were isolated from agar plates, suspended in PBS and 1.5 x 10^6 yeast cells/well were coated overnight at 4°C in PBS in Costar EIA 96-wells plates (Corning). 10 μg/ml of purified C. albicans mannan, laminarin (Sigma) and pustulan were coated in carbonate buffer overnight at 4°C. After blocking with 1% BSA for 30 minutes at 37°C, wells were incubated with 0.4 μg/ml slgM for 1 hour at room temperature, followed by incubation with HRP-conjugated mouse anti-human IgM (Clone MH15/1, Sanquin) and developed as described previously. Absorbance at 450 nm is plotted without subtraction of background.

**Flow cytometry**

Single colonies of bacteria and yeasts were isolated from agar plates and suspended in DMEM medium. 5x10^6 bacteria, 1.5 x 10^6 yeast or approximately 1 x 10^6 zymosan particles (Sigma-Aldrich) were stained with 2 μg/ml of slgM and subsequently with anti-IgM F(αb')_2-PE (Dako). For inhibition experiments, approximately 1 x 10^6 zymosan particles were stained in presence of ≤ 10 μg/ml pustulan, mannan, or
A mutated CLL subset is specific for fungi laminarin and ≤ 5 mM of gentiobiose (Sigma), laminaribiose (Seikagaku), cellobiose (Sigma), isomaltose (Sigma), 2α-mannobiose (Sigma), or salicin (Sigma).

For zymosan staining of PBMCs, 2 x 10^5 cells were incubated with anti-CD19-APC (BD Biosciences), anti-CD5-PE (BD Biosciences) and zymosan-FITC (Invitrogen). To generate apoptotic cells, Nalm-6 cells were irradiated by γ-irradiation (20 gray) and cultured overnight in IMDM supplemented with 10% FCS. 2 x 10^5 cells were incubated with 2 μg/ml slgM and subsequently with anti-IgM F(ab')_2-PE (Dako) and 7-AAD (eBioscience). Staining was visualized on a FACS Canto II (BD Biosciences) and analysis was done using FlowJo software (Tree Star).

**Surface plasmon resonance (SPR)**

Serial dilutions (0.1 - 10 μg/ml) of mouse anti-human IgM (MH15/1, Sanquin) were spotted on an amine-specific Easy2Spot gold-film gel-type SPRchip (Ssens), using a Continuous Flow Microspotter (Wasatch Microfluidics). Pustulan binding was analyzed on an IBIS MX96 (IBIS Technologies) by performing cycles of concatenated injections of recombinant slgM and pustulan on the anti-IgM coated chip. In each concatenated injection, first slgM was captured on the anti-IgM coated spots for 30 min. Subsequently, pustulan (0.3 - 3 μg/ml in binding buffer, 0.1 % BSA and 0.03 % Tween-20 in PBS) was injected and incubated for 30 min, followed by thorough washing with binding buffer to detect pustulan dissociation. Injections with binding buffer were used as reference. After each concatenated injection, the chip was regenerated with 10 mM glycine-HCl, pH 2.0. Experimental data was processed with SPRint software (IBIS Technologies) and kinetic parameters were determined using Scrubber2 software (BioLogic).

**Cells isolation and cell culture**

Peripheral blood mononuclear cells were isolated using Ficoll Hypaque (GE Healthcare) according to manufacturer’s instructions. Cells were stained with carboxyfluorescein succinimidyl ester (CFSE) supplied by Invitrogen. 4 x 10^5 cells were cultured in the presence of CD40L expressing 3T3 fibroblasts in wells coated with pustulan (Calbiochem) or in the presence of biotinylated anti-kappa or anti-lambda F(ab)_2 (Southern Biotech) coupled to anti-biotin antibody-coated Microbeads (Miltenyi Biotec) (40 μg/ml). After 8 days, CFSE staining was measured in combination with CD19-APC (BD biosciences) and CD5-PE (BD Biosciences) by flow cytometry. Precursor frequency, defined as the proportion of cells that have responded in the original population, was calculated by FlowJo software as described previously. The precursor frequency of cells cultured with CD40L expressing fibroblasts was subtracted as background.
Supporting information
Table S1 lists the microorganisms used in this study and Table S2 lists IGHV- and IGLV-rearrangements of the CLL used in this study.

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References
A mutated CLL subset is specific for fungi

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A mutated CLL subset is specific for fungi

Supplementary tables

Table S1: List of microbial species tested for reactivity with V3-7Sh sIgM

<table>
<thead>
<tr>
<th>Genus</th>
<th>Gram</th>
<th>Strain</th>
<th>Reactivity</th>
</tr>
</thead>
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<td>IIIB66</td>
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<tr>
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<td>-</td>
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<td><strong>Klebsiella pneumonia</strong></td>
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**Table S2: IGHV-, IGKV- and IGLV-rearrangements of control CLL**

**Mut: somatic mutations in percentage of germline**