3. Methods

3.1. Antibody production

3.1.1. B cell preparation and purification

All samples were collected after signed informed consent in accordance with Institutional Review Board-reviewed protocols. Peripheral blood was collected from two healthy donors (ED, KR). V-preB$^+$L$^+$ B cells were obtained from the peripheral blood mononuclear cells (PBMC) using a combination of magnetic bead cell selection (MACS), followed by two rounds of cell sorting (Figure 3) [60]. In brief, V- preB$^+$L$^+$ B cells were enriched from PBMCs by negative selection against non-B cells followed by positive selection using anti-VpreB antibody labeled with phycoerythrin (PE) [63] and MACS anti-PE microbeads. The anti V-preB antibody was produced against a region of V-preB that shows no Ig homology, i.e., extensive measurements failed to detect cross reactivity with Ig light or heavy chains [64]. Conventional V-preB$^-$L$^+$ B cells were B cell-enriched PBMC that did not bind to the MACS column. After the initial enrichment the cells were stained with FITC human anti-κ, anti-λ, and allophycocyanin (APC) anti-CD19. B cell viability was determined by propidium iodide staining. V-preB$^+$L$^+$CD19$^+$ B cells and conventional V- preB$^-$L$^+$CD19$^+$ B cells were purified by sorting on a FACS-Vantage^{TM} [60]. After double sorting the fractions reached 99% purity of V- preB$^+$L$^+$ and V- preB$^-$L$^+$ B cells (Figure 3). These fractions were single cell sorted into 96 well plates filled with 4 µl Lysis solution containing 2 µl RNAse free PBS, 1 µl RNAse free H2O, 8 U of RNAsin, 0.4 U 5‘-3‘ RNAse Inhibitor, 0.4 µl 100 mM DTT and immediately frozen on dry ice. All samples were stored at –70 ^{o}C.

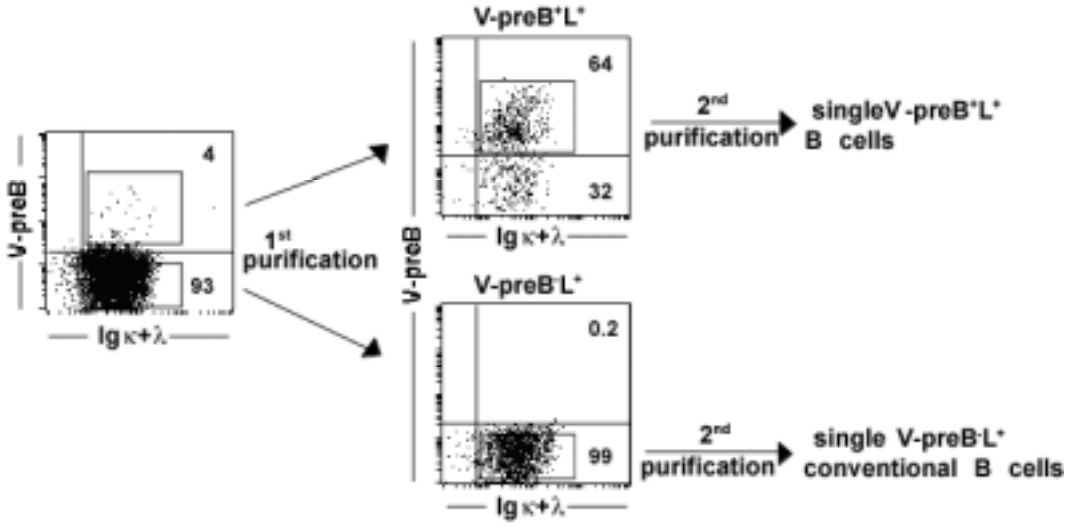

Figure 3. V-preB$^+$L$^+$ B cell purification scheme. Dot plots show V-preB and Igk and Igλ expression on B cells pre-enriched for V-preB expression using magnetic beads (left), and after the first purification for V-preB$^+$L$^+$ B cells (upper right) or conventional V-preB$^-$L$^+$ B cells (lower right). These populations were subsequently sorted into 96-well plates during a second round of cell purification.

Human rheumatoid patient sera were obtained from Dr. L. Ivashkiv (Hospital for Special Surgery at Weill Cornell Medical School, 10021 New York, USA). All patients met the revised American College of Rheumatology Criteria for the diagnosis of rheumatoid arthritis. Control sera of healthy donors were collected by employees of Rockefeller University Hospital (Rockefeller University, 1230 York Avenue, New York, N.Y. 10021).

3.1.2. cDNA synthesis

The following steps including cDNA synthesis and single cell polymerase chain reactions (PCR) were performed in RNA and DNA free area to avoid contaminations.

7 µl of Random Hexamer Primer mix (containing: 150 ng random hexamer primer ($pd(N)_6$), 1 µl NP-40 10%, 1 U Prime RNAse Inhibitor and 4 µl RNAse free H2O) were added to each well (placed on dry ice), and to allow the cells to release their RNA the plate was heated to 65^o C for 1 minute and cooled down on ice afterwards. 14 µl of reverse transcriptase (RT) mix (containing: 5 µl of 5X RT buffer, 0.5 µl dNTP-Mix (10 mM each nucloetide), 2 µl 0.1 M

DTT, 4.25 µl RNAse free H2O, 10 U RNAsin, 1 U Prime RNAse Inhibitor and 50 U RT Superscript) were added to each well and incubated at room temperature for 10-15 minutes. Following incubation steps using a thermocycler were performed: 42 ^{o}C for 5 minutes, 25 ^{o}C for 10 minutes, 37 ^{o}C for 45 minutes for RT reaction and 94 ^{o}C for 5 minutes for RT inactivation.

3.1.5. PCR amplification of human VH, Vκ, and Vλ gene rearrangements from individual B cells

Polymerase chain reaction:

Polymerase Chain Reactions (PCR) were performed with instruments from Perkin Elmer. The melting temperature of the primer T_m was calculated as Tm= 4x (G/C) + 2x (A/T). The annealing step was performed at 3°C below the melting temperature, using the lowest melting temperature of the two to six oligonucleotides used for the different reaction.

To add restriction sites to a PCR product, oligonucleotides fused to restriction site sequences were used. Several extra bases were added to their 5' end to generate a fully functional site for restriction enzymes to cut.

For the separation of DNA fragments horizontal agarose gels were used. The concentration of agarose varied between 0.7 % (separation of fragments >3,000 bps) and 2 % (separation of fragments <500 bps). The concentration of ethidium bromide in the gel was 0.3 mg/ml; 0.5x TAE served as a buffer system. Before loading the samples to the gel, 5x loading buffer was added. Electrophoresis was performed at 80 V.

First and secondary amplification:

For the primary amplification of the heavy-, κ- and λ-chain Ig genes a mix of different external leader consensus 5'V_H/Vκ/Vλ and 3'Cµ/Cκ/Cλ primer were used. For the secondary amplification heavy- and λ-chain rearrangements were amplified using consensus 5'V_H/λ and 3'Cµ/λ primer already fused to the required restriction site. The strategy for the second amplification κ-chain genes differed a bit because of the wide range of specific Vκ genes. Therefore an internal consensus 5'Vκ primer was created to amplify a PCR product to determine the sequence. Knowing the sequence of the rearranged Vκ gene, a PCR using

specific 5'AgeI-Vκ and consensus 3'BsiWI-Jκ primer was performed on the PCR product of the primary amplification.

3,5 µl of cDNA were used to amplify IgH, Igλ or Igκ transcripts by two rounds of PCR. The cDNA was added to 35 µl of a PCR master mix, containing 1.2 U HotStar Taq DNA polymerase, 5 µl 10X buffer, 0,05 µg Yeast tRNA, 5 µl 25 mM dNTP's, 30 µl RNAse free H2O and 20 pg of each primer as listed (Table I). The first round of PCR was performed for 50 cycles at 94 ^{o} C for 30 s to denature, 57 ^{o} C for 30 s to anneal, 72 ^{o} C for 1 min to elongate including a previous 3 min heating at 95 ^{o} C to unfold the cDNA and a final 10 minutes extension at 72 ^{o} C. For the second amplification 3,5 µl of the primary PCR product was amplified for 40 cycles as described for first amplification.

3.1.6. Sequencing

DNA were extracted and purified by using QUIAquick PCR purification Kit and 6 pg of following primer were added:

- for IgH chain genes: 5'AgeI-VH1,2,3,4,5,6 primer mix
- for Igκ chain genes: 5'Vκ consensus
- for Igλ chain genes: 3'XhoI- Cλ

Sequencing of DNA fragments was performed at the Sequencing Facility, Rockefeller University, 1230 York Avenue, New York, N.Y. 10021. All sequences were analyzed by IgBLAST comparison with GenBank.

3.1.7. Generation of the IgH, Igκ, and Igλ expression vectors

The original expression vector was modified for this experiment as follows:

For the insertion of sequences encoding different IgH, Igκ and Igλ chains, a linker sequence was designed to create a cloning cassette for heavy or light chain genes. Oligonucleotides for linker are:

Sense (85-mer):

5'AATTCCACCATGGGATGGTCATGTATCATCCTTTTTCTAGTAGCAACTGCAACC
GGTGTACACTCGAGCGTACGGTCGACGCGTA-3'

Antisense (85-mer):
5’AGCTTACGCGTCGACCGTACGCTCGAGTGTACACCGGTTGCAGTTGCTACTAGA
AAAAAGGATGATACATGACCATCCCATGGTGG-3’

In order to generate the leader peptide-encoding sequence 5’-*CTGCAACCGGTGTACATTCA*-3’ was added to the 5’end of the V region insert. The addition of several nucleotides in italics are included as buffer sequence for the restriction site.

To generate the constant region of the antibody, the sequence corresponding to human IgG Cγ1 region was inserted into the expression cassette.

After cloning, the resulting recombinant DNA was inserted into bacteria and the presence of the inserted IgH or IgL encoding sequence was checked by the PCR. The PCR reaction was performed in 25 µl PCRmix for 27 cycles at 94 ^{o}C for 30 s, 58 ^{o}C for 30 s, 72 ^{o}C for 1 min with preaheating to 95 ^{o}C for 3 min and with a final extention at 72 ^{o}C for 10 minutes. The PCR mix contained 20 pg of the following primers:

- for IgH chain plasmid: 5‘ Ab-sense and 3‘ IgG1-325 antisense
- for Igκ chain plasmid: 5‘Ab-sense and 3‘Cκ cons
- for Igλ chain plasmid: 5‘Ab-sense and 3’ Cλ-1

The size of the inserted DNA was analyzed by 2 % agarose gel electrophoresis followed by fragment isolation and sequencing to exclude the presence of PCR-introduced mutations and to select clones (analysed by IgBLAST) with inserts identical to the original PCR product.

Table I: Primer used for immunoglobulin gene amplification

IgH	Sense		Antisense	
First PCR	5' L-VH1	ACAGGTGCCCACTCCCAGGTGCAG	3' Cμ CH1	GGGAATTCTCAGAGGAGACGA
	5' L-VH3	AAGGTGTCCAGTGTGARGTGCAG		
	5' L-VH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG		
	5' L-VH5	CAAGGAGTCTGTTCCGAGGTGCAG		
Second PCR	5' AgeI VH1/5	CTGCA**ACCGGT**GTACAT TCCGAGGTGCAGCTGGTGCAG	3' SalI JH1/2	TGCGAA**GTCGAC**GCCTGAGGAGACGGTGACCAG
	5' AgeI VH3	CTGCA**ACCGGT**GTACATTCTGAGGTGCAGCTGGTGGAG	3' SalI JH3	TGCGAA**GTCGAC**GCTGAAGAGACGGTGACCATTG
	5' AgeI VH4	CTGCA**ACCGGT**GTACATTCCCAGGTGCAGCTGCAGGAG	3' SalI JH4/5	TGCGAA**GTCGAC**GCTGAGGAGACGTGACCAG
	5' AgeI VH3-23	CTGCA**ACCGGT**GTACATTCTGAGGTGCAGCTGTTGGAG	3' SalI JH6	TGCGAA**GTCGAC**GCTGAGGAGACGGTGACCGTG
	5' AgeI VH4-34	CTGCA**ACCGGT**GTACATTCCCAGGTGCAGCTACAGCAGTG		
Igλ	Sense		Antisense	
First PCR	5' L Vλ1	GGTCCTGGGCCCAGTCTGTGCTG	3' Cλ	CACCAGTGTGGCCTTGTTGGCTTG
	5' L Vλ2	GGTCCTGGGCCCAGTCTGCCCTG		
	5' L Vλ3	GCTCTGTGACCTCCTATGAGCTG		
	5' L Vλ4/5	GGTCTCTCTCSCAGCYTGTGCTG		
	5' L Vλ6	GTTCTTGGGCCAATTTTATGCTG		
	5' L Vλ7	GGTCCAATTCYCAGGCTGTGGTG		
	5' L Vλ8	GAGTGGATTCTCAGACTGTGGTG		
Second PCR	5' AgeI Vλ1	CTGCT**ACCGGT**TCCTGGGCCCAGTCTGTGCTGACKCAG	3' XhoI Cλ	CTCCTCA**CTCGAG**GGYGGGAACAGAGTG
	5' AgeI Vλ2	CTGCT**ACCGGT**TCCTGGGCCCAGTCTGCCCTGACTCAG		
	5' AgeI Vλ3	CTGCT**ACCGGT**TCTGTGACCTCCTATGAGCTGACWCAG		
	5' AgeI Vλ4/5	CTGCT**ACCGGT**TCTCTCTCSCAGCYTGTGCTGACTCA		
	5' AgeI Vλ6	CTGCT**ACCGGT**TCTTGGGCCAATTTTATGCTGACTCAG		
	5' AgeI Vλ7/8	CTGCT**ACCGGT**TCCAATTCYCAGRCTGTGGTGACYCAG		
Igκ	Sense		Antisense	
First PCR	5' L Vκ1/2	ATGAGGSTCCCYGCTCAGCTGCTGG	3' Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
	5' L Vκ3	CTCTTCCTCCTGCTACTCTGGCTCCCAG		
	5' L Vκ4	ATTTCTCTGTTGCTCTGGATCTCTG		
Second PCR	5' Pan Vκ	ATGACCCAGWCTCCABYCWCCCTG	3' Cκ 494	GTGCTGTCCTTGCTGTCCTGCTC
Specific PCR	5' AgeI Vκ 1-5	CTGCA**ACCGGT**GTACATTCTGACATCCAGATGACCCAGTC	3' BsiWI Jκ1/2/4	GCCAC**CGTACG**TTTGATYTCCACCTTGGTC
	5' AgeI Vκ 1-9	TTGTGCTGCA**ACCGGT**GTACATTCAGACATCCAGTTGACCCAGTCT	3' BsiWI Jκ3	GCCAC**CGTACG**TTTGATATCCACTTTGGTC
	5' AgeI Vκ 1D-43	CTGCA**ACCGGT**GTACATTGTGCCATCCGGATGACCCAGTC	3' BsiWI Jκ5	GCCAC**CGTACG**TTTAATCTCCAGTCGTGTC
	5' AgeI Vκ 2-24	CTGCA**ACCGGT**GTACATGGGGATATTGTGATGACCCAGAC		
	5' AgeI Vκ 2-28	CTGCA**ACCGGT**GTACATGGGGATATTGTGATGACTCAGTC		
	5' AgeI Vκ 3-11	TTGTGCTGCA**ACCGGT**GTACATTCAGAAATTC		
	5' AgeI Vκ 3-15	CTGCA**ACCGGT**GTACATTCAGAAATAGTGATGACGCAGTC		
	5' AgeI Vκ 3-20	TTGTGCTGCA**ACCGGT**GTACATTCAGAAATTGTGTTGACGCAGTCT		
	5' AgeI Vκ 4-1	CTGCA**ACCGGT**GTACATTCGGACATCGTGATGACCCAGTC		

Restriction sites are shown in bold

3.1.8. Antibody production

A293 human embryonic kidney fibroblasts were cultured in DMEM containing 10 % ultra-low IgG FCS, 1 % Glutamine, 1 % Pyruvate and 1 % Penicillin-Streptomycin at 37 ^{o}C with 5 % CO2. A293 cells were co-transfected by calcium-phosphate precipitation with 12.5 µg IgH chain, 12.5 µg IgL chain plasmid, 2.5 µg of "helper" plasmid and 5 µg of a plasmid containing a GFP encoding sequence. 8-12 hours after co-transfection cells were washed with serum-free DMEM and cultured in serum-free DMEM supplemented with 1 % Nutridoma SP.

Supernatants were collected after 7 days of culture and span down at 1000 rpm for 30 minutes to remove dead cells. The resulting cleared supernatants were frozen down at –20 ^{o}C in 20 % (volume/volume) of glycerol.

The concentrations of antibodies (Ab) in the supernatants were determined by ELISA using a goat anti-human IgG Fcγ antibody. ELISA microtiter plates were incubated at room temperature overnight with 50 µl/well 1x PBS containing 4 µg/ml goat anti-human IgG Fcγ Ab. The plates were then washed three times with deionized water and incubated with 150 µl/well of blocking buffer for 1 hour at room temperature. After three washes the wells were incubated with 50 µl/well supernatants with unknown concentration of Ab at dilutions with 1x PBS of 1:0, 1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625. As a standard to determine the concentration, a human monoclonal IgG1 κ Ab was used at the same dilutions, starting with 10 µg/ml. After 2 hour incubation at room temperature the plates were washed three times and 50 µg/well blocking buffer containing 1 µl/ml (concentration of 1:1000) goat anti-human IgG Fcγ Ab HRP labeld were added and incubated again for 2 hours at room temperature. After washing and blocking again, the samples were then developed by adding 100 µl of developing reagent (9 parts of solution A and 1 part of solution B, HRP Substrate Kit, BIORAD) and analyzed using a microplate reader at 405 nm after 10 minutes incubation.

The quality of the produced antibodies was controlled by SDS-gel electrophoresis. Aliquots of 50 µl Protein G were washed three times with 1x PBS and incubated with 5 ml of Ab containing supernatant at 4 ^{o}C for at least 4 hours or overnight. After washing twice with 1x PBS the Protein G beads were mixed with 2x buffer (4x sample buffer mixed 1:1 with 50 % Glycerol) 1:1 and boiled in 95 ^{o}C for 5 minutes to denature the proteins. The samples were loaded on 4-20 % gel in 1x loading buffer and after 30 min at 120 V the gel was removed and incubated for 1 hour in Coomasie blue on a shaker. Afterwards the gel was washed with wash buffer until the bands were visible.

3.2. Analysis of the antibody specificity

3.2.1. Anti-nuclear antibody (ANA) analysis

To purify the antibodies 250 ml of the culture supernatants were incubated with 1 ml Protein G beads over night at 4 ^{o}C (gently rotating). After washing with 1x PBS the beads were transferred into a column and eluted using 0.1 M Glycine buffer pH 3.0 in at least 10 fractions (0.5 ml each). The fractions were collected into eppendorf tubes containing 50 µl 1M TRIS pH 8.0. The protein concentrations were measured by photometric analysis at 280 nm and by ELISA, which was performed (antibody start dilution 1:25) and analyzed as described. For further purification Micropore centrifugal filter YM-50 were used.

Purified antibodies were tested for antinuclear reactivity performing an anti-nuclear antibody (ANA) ELISA by using Anti-Nuclear Antibody Screen. The ANA-ELISA detects antibodies against clinically relevant nuclear antigens such as: dsDNA, histones, Sm/RNP and PCNA (associated with SLE), ssDNA (associated with SLE and RA), Ro (SS-A) and La (SS-B) (associated with SLE and Sjögren's syndrome), Scl-70 (associated with Scleroderma), centromere (associated with Raynaud's syndrome and CREST). The screen also detects diagnostically important cytoplasmic antigens such as Jo-1 (associated with Myositis) as well as unidentified self-antigens from human epithelial cell line (HEp-2) lysate [2]. The purified antibodies were used at 100 µg/ml and three 1:4 dilutions in PBS. V-pre^{+}L^{+} and V-pre^{-}L^{+} B cell derived antibodies were compared to provided strong positive, weak positive, and negative controls (sera from patients and healthy individuals respectively) using a microplate reader at 405 nm. To be considered reactive the results for any given antibody had to be confirmed in at least two independent experiments.

The purified antibodies were also tested for antinuclear reactivity by immunofluorescence assays using HEp-2 cells coated slides (Anti-Nuclear Test System Kit) according to the protocol provided by the manufacturer. HEp-2 cells coated slides were incubated at room temperature with purified antibodies at 50-100 µg/ml for 30 min, washed with PBS and visualized with FITC labeled goat anti-human IgG by fluorescent microscopy. The staining pattern was analyzed and compared to provided positive and negative controls. Antibodies

that exhibit a characteristic ANA staining pattern with a fluorescent intensity of 1+ or greater compared to the negative controls were considered to be positive.

3.2.2. Polyreactivity assay

The polyreactivity of antibodies was determined by ELISA technique. Microtiter plates were incubated overnight at room temperature with 50 μl/well 1x PBS containing one of the listed antigens: 5 μg/ml BSA (used as a negative antigen control), 5 μg/ml chitine, 5 μg/ml Type II collagen from bovine tracheal cartilage, 10 μg/ml dsDNA, 10 μg/ml ssDNA, 5 μg/ml GPI Type XI from rabbit muscle, 10 μg/ml histone, 5 μg/ml chimeric human IgG2 λ, 5 μg/ml human IgG1 λ, 5 μg/ml human IgM, 5 μg/ml recombinant IL-1β, 5 μg/ml human recombinant insulin, 5 μg/ml phosphocholine-hapten, 5 μg/ml LPS, 2,5 μg/ml myoglobulin from human heart, 1 μg/ml tetanus toxoid and 5 μg/ml human thyroglobulin. As a negative sample control serum-free DMEM containing Nutridoma was used, the same in which the cells grew for 7 days. As a positive control for polyreactivity, mAb55, a well-characterized polyreactive human antibody[65, 66], was used after being cloned and expressed in the previously described system. Originally produced as IgM antibody, mAb55 remained its polyreactive activity after switching to IgG [66] which allowed the comparison to the V-preB$^+$L$^+$ antibodies, also switched from IgM to IgG. The wells were incubated with 50 μl/well 1x PBS containing 1 μg/ml of secreted Ab and three 1:4 dilutions in PBS and the ELISA was performed as described previously. A goat anti-human IgG Fcγ HRP labeld antibody was used at a 1:1000 dilution as secondary antibody. To investigate the binding activity against human IgG1 λ and IgG2 λ by V–preB$^+$L$^+$ cells derived IgG1 κ antibodies, a goat F(ab')2 anti-human κ HRP labeled antibody was used at a 1:1000 dilution as a secondary antibody instead. Positivity in this assay was defined as an A_{405} value in the range of mAb55 and above the mean of the normal V-preB$^-$L$^+$ control values at the same antibody concentrations.

3.3. Analysis of the antigenic specificity of the rheumatoid arthritis patient sera

Sera of 59 RA patient and 23 control sera from healthy individuals were diluted 1:50 and the ELISA assays for GPI, ssDNA, dsDNA and insulin were performed and analyzed as described. The previously described mAb55 was used as positive control for polyreactive activity. To detect reactive antibodies of different isotypes (IgA, IgD, IgE, IgG, IgM), a polyclonal goat F(ab')2 anti-human κ (HRP) 1:1000 dilution was used as secondary antibody (data not shown). Notably, using this approach the activity of the Igλ clone fraction is not detectable.

To test whether GPI reactivity detected in the serum of rheumatoid arthritis was attributed to specific or polyreactive antibodies, the anti-GPI antibody were affinity purified from the patients sera by using beads coupled to GPI. Antibodies, purified by using BSA-coupled beads, were used as negative control. To prepare these beads, 1g of CNBR-activated Sepharose ^{TM} 4 Fast Flow beads were incubated with 5 mg of either GPI or BSA at 4 ^{o} C gently rotating over night. The beads were washed sequentially with 25 ml Borate (0.1 M), NaCl (1 M, pH 8), 25 ml Acetate (0.1 M), NaCl (0.5 M, pH 4), $EtNH_2$ (1 M, pH 9) and 25 ml PBS. The antigenic specificities of antibodies eluted from GPI or BSA coupled beads were tested as described (see 3.2.2.). To determine the isotypes of polyreactive antibodies in the serum of individual patients, a polyclonal goat F(ab')2 anti-human IgG (HRP) 1:1000 dilution and anti-human IgM (HRP) 1:200 dilution were used in separate assays as secondary antibodies. For each assay human IgG1 κ and human IgM were coated at the same dilutions (starting with 10 μg/ml), which were, depending on the secondary antibody, either used as a standard to determine the concentration or as a negative control.

3.5. Transgenic mice

The V(D)J_H exon from V-pre$^+$L$^+$ B cell ED45 was cloned into a human IgM transgene vector [31] and microinjected into CBxC57B1/6F2 pronuclei. For FACS analysis 12-week old F1 offsprings from both transgenic founders were compared to non-transgenic littermate controls. Total bone marrow, spleen cells, or MACS CD19 microbeads enriched spleen B cells were stained with FITC, PE, APC, and biotin conjugated monoclonal antibodies (anti-CD43, anti-human IgM, anti-B220, anti-CD21, and anti-CD23), and visualized with PE-Cy7 conjugated

streptavidin (Caltag laboratories, Burlingame, CA). Intracellular staining was performed with cytofix/cytoperm TM kit. Data were collected on a FACSalibur and analyzed using CellQuest software.

3.6. Statistical analysis

P values were calculated using (two-tailed) Fisher Exact Test and student t-test. P values smaller than 0.05 were considered to be statistically relevant.