4. Results

4.1. General strategy for the analysis of the antibody repertoire expressed by V-preB$^+$L$^+$ B cells

Initial analysis of the immunoglobulin repertoire of V-preB$^+$L$^+$ B cells has revealed increased frequencies of the expression of IgH and IgL chains with potential self-reactive features [59, 60]. To investigate whether the ability to produce self-reactive antibodies is a common feature or reflects a clonal expansion of few auto-reactive V-preB$^+$L$^+$ B cells, analysis of the antibody repertoire at the single cell level is required. A common technique is based on in vitro immortalization of individual B cells by Epstein-Barr virus (EBV) transformation [67] followed by individual antibody sequencing. In the case of V-preB$^+$L$^+$ B cells, however, preliminary experiments using this method were not efficient. Sequencing of a large number of individual EBV transformed V-preB$^+$L$^+$ B cell clones revealed only two different IgH and IgL chains (data not shown), which may be due to insufficient transformation of V-preB$^+$L$^+$ B cells by EBV. The failure of this approach underscored the necessity of a non-biased method for the analysis of the antibody repertoire of individual V-preB$^+$L$^+$ B cells. Therefore, a new method to analyze the antibody sequence of individual V-preB$^+$L$^+$ B cells as well as the antigenic specificity of their antibodies needed to be established. The latter employed expression of cDNA encoding IgH and IgL chains from individual peripheral V-preB$^+$L$^+$ B cells in fibroblasts followed by production of antibodies in quantities sufficient for the analysis of their antigenic specificity (Figure 4). The overall efficiency of the expression of individual antibodies in fibroblasts was greater than 50 %.

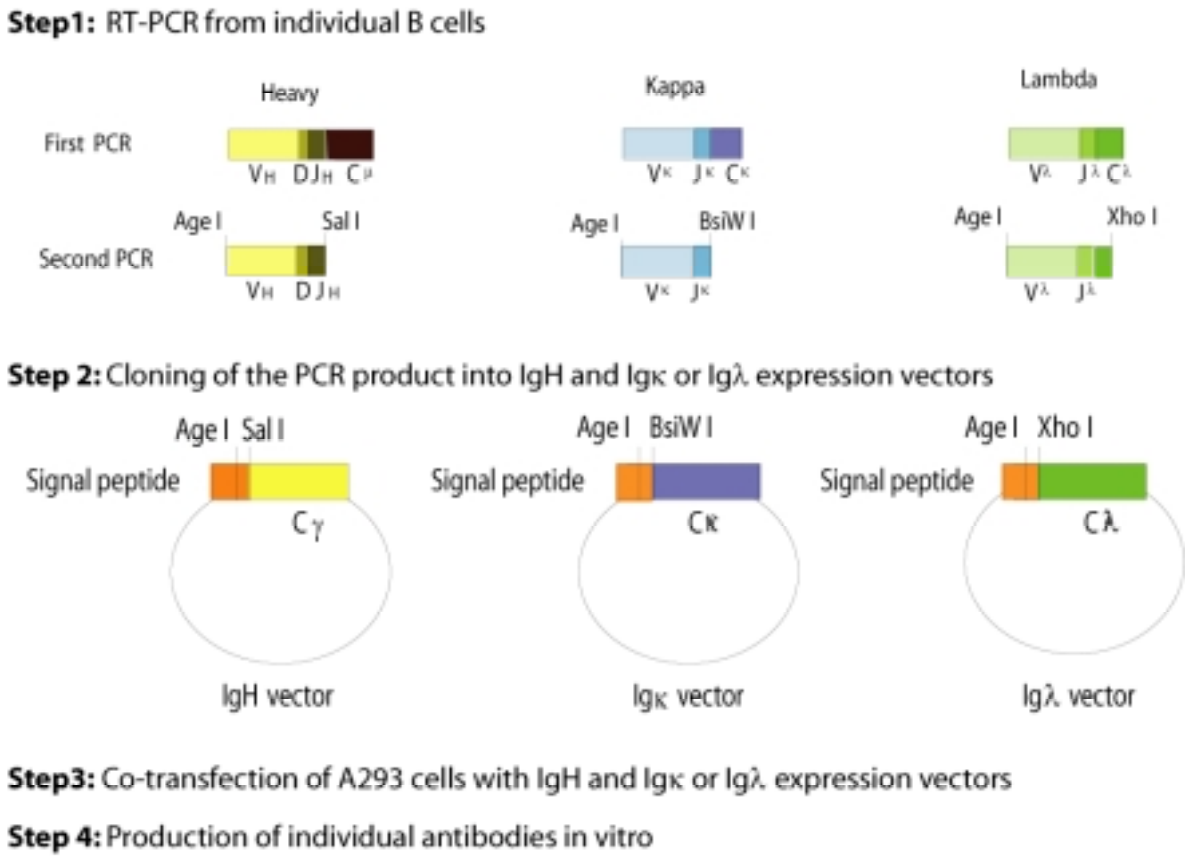

Figure 4. Individual antibody production scheme. After individual B cell purification, mRNA was extracted and used for cDNA preparation. cDNA was amplified by PCR and PCR products were sequenced. Restriction sites were introduced by PCR to clone the DNA products into the expression vectors. A293 fibroblasts were co-transfected with IgH and corresponding IgL chain vector and sufficient amount of individual antibodies were obtained.

4.1.1. Sequence analysis of single cell-derived IgH and IgL gene rearrangements

Comparison of the IgH genes derived from V-preB$^+$L$^+$ B cells to V-preB$^-$L$^+$ B cells revealed a frequent usage of the JH6 gene segment (20.7 % in V-preB$^+$L$^+$ compared to "not present" in V-preB$^-$L$^+$; p=0.0266) and significant longer CDR3s (15.3 aa in V-preB$^+$L$^+$ vs 12.2 aa in V-

preB$^-$L$^+$ B cells; p=0.005) among V-preB$^+$L$^+$ B cells. In addition, the IgH CDR3s of the V-preB$^+$L$^+$ B cells showed an increased prevalence of basic residues (Arginine (R), Histidine (H) and Lysine (K)) (Figure 5), whereas no differences in the frequency of acidic residues (Aspartate (D) and Glutamate (E)) were observed (data not shown).

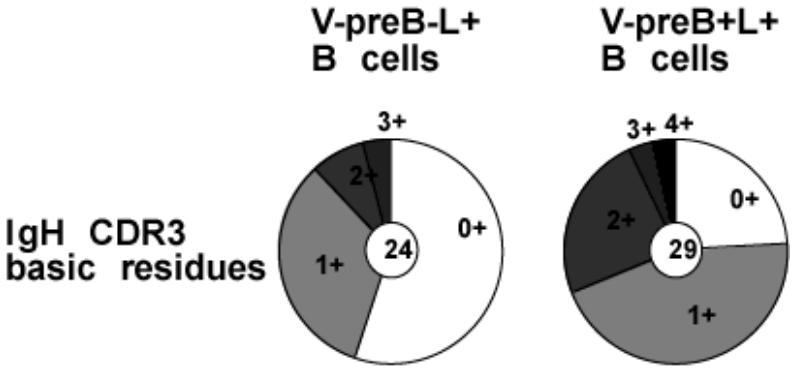

Figure 5. Increased frequency of positively charged amino acids in IgH CDR3s from VpreB$^+$L$^+$ B cells. Pie charts show segment size proportional to the number of clones from V-preB$^-$L$^+$ (left) and V-preB$^+$L$^+$ B cells (right) displaying 0, 1, 2, 3, and 4 or more positively charged amino acids per IgH CDR3. The number of sequences analyzed in each group is indicated in the center.

The analysis of the VκJκ rearrangements isolated from V-preB$^+$L$^+$ B cells showed a bias towards the usage of the Jκ3, Jκ4, and Jκ5 segments located at the 3' end of the Jκ locus (27,5 % of V-preB$^+$L$^+$ compared to 16.5 % of V-preB$^-$L$^+$ B cell population). Notably, 50 % of V-preB$^+$L$^+$ B using Jκ3, Jκ4 and Jκ5 gene segments were associated with upstream Vκ genes suggesting that they might have had undergone receptor editing (Table III). V-preB$^+$L$^+$ B cells also displayed increased incidence of Vκ4-1, which has been associated with anti-DNA antibodies (13,6% in V-preB$^+$L$^+$ B cells as compared to 0% in V-preB$^-$L$^+$ B cells) [68], and presented 11 amino acid residues long Igκ CDR3s, which correlated with an increase in N addition in Igκ genes in these cells (Figure 6 and Tables II and III).

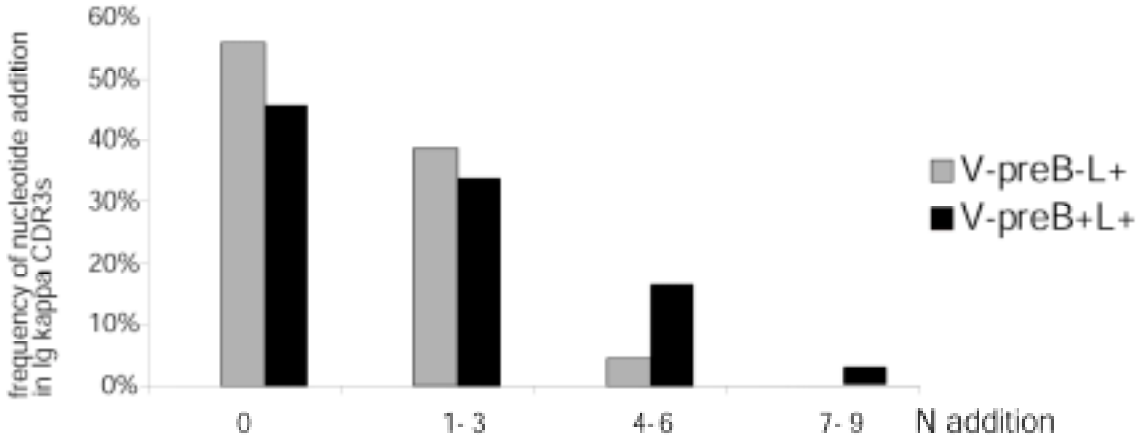

Figure 6. Increased N-addition in Igκ CDR3s from V-preB$^+$L$^+$ B cells. The frequency of nucleotide addition (0, 1-3, 4-6 or 7-9) in Igκ CDR3s is shown for V-preB$^+$L$^+$ B cells (black bars) compared to V-preB$^-$L$^+$ B cells (grey bars).

Comparison of the IgH gene sequences derived from V-preB$^+$L$^+$ B cells to the IgH germline sequences did not reveal the presence of somatic mutations and confirmed the germline configuration of the sequences (Tables II and III).

Table II. Immunoglobulin repertoire* of V-preB^{-}L^{+} B cells

Ig	HEAVY						LIGHT				
	VH	D	RF	JH	CDR3 (aa)	Length	Vk	Jk	CDR3 (aa)	Length	N
EDV-7	3-49	4-17	3	4	VGPTVTTPAFDY	12	2-28	2	MQALQTPYT	9	3
EDV-8	1-69	3-10	2	5	GASYGSGSYSRGDWFDP	17	3-20	3	out of frame		
EDV-13	4-34	/	/	4	VDHAPDY	7	1-17	1	LQHNSYPWT	9	0
EDV-15#	3-7	/	/	4	SLALPGISRRTASSVFDY	18	2-24	1	MQATQFPRT	9	0
EDV-21	1-69	2-2	2	2	DADCSSTSCYTSWYFDL	17	3-20	1	QQYGSSLGT	9	3
EDV-29	5-51	1-1	3	3	FIYSVGTTGTTTVLNAFDI	19	1-39	1	QQSYSTPWT	9	0
EDV-30	3-7	6-13	3	4	GGIAAAGITDY	11	1-33	4	QQYDNLPLT	9	0
EDV-36	3-64	6-19	3	4	GYVRGGRGSIAVAPFDY	17	1-5	2	QQYNSYSEYT	10	3
EDV-40	3-30	/	/	4	PNY	3	1-5	1	QQYNSYSWG	9	6
EDV-43	3-30	1-7	1	3	GPFPLELRANAFDI	14	3-15	2	QQYNNWPLYT	10	2
EDV-44#	1-8	6-13	2	1	AGYSSSWYGARTEREYFQH	19	1D-43	1	QQYYSTPWT	9	0
EDV-47	3-23	5-5	2	4	TRGYSYGGIDY	11	3-11	2	QQRSNWPPT	9	0
EDV-48	3-30	/	/	4	DLRAEPVYFDY	11	3-11	2	QQRSNWPRT	9	3
EDV-74	3-11	/	/	4	EVDY	4	1-17	3	LQHNSRFT	8	2
EDV-85	1-2	/	/	5	AGVDNWFDP	9	3-20	1	QQYGSSPWT	9	0
EDV-92	3-49	2-15	3	4	DLSLAADDY	9	1-33	2	QQYDNLPPT	9	0
KRV-18	5-51	6-19	3	4	VAVAGDFNY	9	3-20	1	QQYGSSPRT	9	2
KRV-21	3-33	/	/	3	GSEFWVEVDITGDVFDI	17	1-16	4	QQYNSYPLT	9	0
KRV-24#	4-39	6-13	2	4	RSSWAGAFDY	10	3-15	2	QQYNNWYT	8	0
	VH	D	RF	JH	CDR3 (aa)	Length	Vλ	Jλ	CDR3 (aa)	Length	N
EDV-16	3-23	6-6	2	4	KSGSSSPFDY	10	1-40	3	QSYDSSLSGSV	11	0
EDV-22	3-43	/	/	4	DIGGGLDY	8	1-47	7	AAWDDSLSGAV	11	0
EDV-27	1-18	3-22	2	5	NQALYYYDSSGSKFSWFDP	19	1-44	3	AAWDDSLNGPVWV	13	1
EDV-93	3-21	/	/	4	GPWVASFDY	9	1-51	2	GTWDSSLSAGQRV	13	3
KRV-9	3-15	6-13	3	4	GLAAAAPDPFDY	12	3-1	2	QAWDSSVV	8	0

* information about individual V(D)J gene segments is provided. For IgH chain V_H, D, and J_H family names, D reading frame (RF), IgH CDR3 amino acid (aa) sequence and length are presented. For IgL chains the name of Vκ/λ and Jκ/λ family, IgL CDR3 aa sequence, CDR3 length and nucleotide addition (N) are displayed. # indicates clones that failed to be expressed *in vitro*

Table III. Immunoglobulin repertoire* of V-preB$^+$L$^+$ B cells

Ig	HEAVY						LIGHT				
	VH	D	RF	JH	CDR3 (aa)	Length	Vκ	Jκ	CDR3 (aa)	Length	N
ED 3	3-48	2-2	3	4	LGGIEDIVVVPAAVRTPRGDY	21	1-33	3	QQYDNLPLT	9	2
ED 6	4-31	3-22	2	4	GPDTNYYDSFLPPLKV	16	4-1	2	QQYYSTYT	8	0
ED 8	4-31	4-17	3	4	ESTVIRGLDY	10	3-15	2	QQYNNWPRGDT	11	6
ED 11	1-2	4-17	3	4	MNAATVTTDY	10	4-1	4	QQYYSTPQLT	10	1
ED 13	4-59	5-5	1	3	KGRIQLWLGAFDI	13	3-20	1	QQYGSSLWT	9	1
ED 16	3-23	1-26	3	4	RSTRIVGAPFDY	12	3-11	2	QQRSNWVPMYT	11	6
ED 17	3-64	3-3	3	4	DFGVVIGWRYYY	12	1-39	5	QQSYSTSIT	9	2
ED 19	3-74	3-3	2	5	DYDFWSGYPRGFDP	14	3-11	1	QQRSNWPPT	9	0
ED 24	3-21	3-22	2	4	DGGAWPSNYDSSGYYSS	17	3-20	2	QQYGSSPYT	9	0
ED 29	1-24	3-22	2	4	HYYDSSGPPAYYFDY	15	1-33	4	QQYDNLPLT	9	0
ED 30	3-64	3-22	2	4	ALYDSSGYYHDY	12	3-11	2	QQRSNWPRT	9	3
ED 33	3-21	6-19	2	4	DSRHSSGWRSPHFDY	15	3-11	3	QQRSNWPVT	9	2
ED 37	3-33	3-22	2	3	AGGYYYDSSGYRRGAFDI	18	1-17	2	LQHNSYPPT	9	0
ED 38	1-69	6-6	3	6	DPRIAARPLYYYYGMDV	17	3-15	2	QQYNNWPPYT	10	4
ED 40	3-23	6-19	2	3	PPPPYSSGWYLDFYAFDI	18	3-15	1	QQYNNWPPWT	10	0
ED 44	5-51	5-5	2	3	HDESSYGFMWGAFDI	15	3-20	4	QQYGSSPLT	9	0
ED 45	3-21	3-3	1	6	LYGHHFLYYYGMDV	14	1-9	1	QQLNSYPRT	9	0
ED 46	3-15	/	/	3	DTGSSGDAFDI	11	4-1	1	QQYYSTPRT	9	0
KR 3	3-21	6-13	2	6	DSSSWYTFGVSYYYGMDV	18	3-20	2	QQYGSSPMCS	10	9
KR 8	5-51	6-6	1	3	RLEQLEAFDI	10	3-20	1	QQYGSSLTWT	10	4
KR 9	4-39	5-24	1	5	HGEFVRRWLQTGVGWFDP	18	1-5	1	QQYNSYWT	8	0
KR 13	4-39	6-13	2	5	RGSSSWYAWFDP	12	3-20	1	QQYGSYPRT	9	6
	VH	D	RF	JH	CDR3 (aa)	Length	Vλ	Jλ	CDR3 (aa)	Length	N
ED 1	3-74	2-15	2	5	EGGEDCSGGSCYNEAENWFDP	21	3-25	2	QSADSSGTSVV	11	2
ED 7	3-7	2-15	2	4	RCSGGTCHPVES	12	1-51	1	GTWDSGLSAYV	11	0
ED 20	3-74	6-13	2	6	VSSSWHYYYYYGMDV	15	3-21	1	QVWDSSSDHYV	11	0
ED 26#	3-21	5-5	1	5	YYLSIQLWLAGNWFDP	16	1-44	3	AAWDDSLNAWV	11	2
ED 27	3-23	3-9	2	4	SPPRPDYDILTGYLEGFDY	19	3-25	2	QSADSSGTYVV	11	0
ED 34	3-30	3-9	1	6	GGIRYFDWSPPSEYYYYYGMDV	22	3-1	2	QAWDSSIVV	9	2
ED 41	3-30	2-2	3	6	DIVVVPAAMTRVLEYYYYGMDV	22	1-44	3	AAWDDSLNGWV	11	1

*as described for Table II, # indicates clones that failed to be expressed *in vitro*.

4.2. Self-reactivity/ polyreactivity of antibodies generated by individual V-preB$^+$L$^+$ cells

4.2.1. V-preB$^+$L$^+$ B cell derived antibodies recognize nuclear antigens

To determine the specificity of the antibodies produced by the V-preB$^+$L$^+$ B cells, the antibody-producing fibroblasts were used to generate sufficient amounts of individual antibodies. Antibodies from 28 V-preB$^+$L$^+$ and 21 V-preB$^-$L$^+$ B cells could be analyzed following this procedure. A commercially available enzyme-linked immunoassay (ELISA) for antinuclear antibodies (ANA) was used as an initial screen for self-reactivity. ANAs are abundant in the serum of patients with rheumatic diseases and represent one of the most common self-reactive antibodies. The assay allowed the detection of a broad spectrum of antibodies against different nuclear and cellular antigens. In this ANA screen, 68 % of V-preB$^+$L$^+$ derived antibodies (19/28) showed reactivity against the HEp-2 cell lysates compared to 14.5 % of antibodies (3/21) isolated from V-preB$^-$L$^+$ B cells (Figure 7a; p=0.0004).

To characterize further the specificity of the ANAs derived from V-preB$^+$L$^+$ B cells, an ANA specific immunofluorescence assay was performed. Overall, 54 % V-preB$^+$L$^+$ B cells derived antibodies (15/28) yielded positive results using this assay. Forty-three percent of the antibodies recognized a diverse spectrum of nuclear antigens, including those associated with the nucleolus (KR9) and mitotic spindle apparatus (ED11) (Figure 7b). Notably, three of the V-preB$^+$L$^+$ B cell derived antibodies tested positively for ANA showed cytoplasmic specificity and bound stress fiber (ED16), vinculin (ED19) and vimentin (ED37) (Figure 7b). In contrast, none of the 21 antibodies cloned from V-preB$^-$L$^+$ B cells bound nuclear antigens. The difference in the frequencies of ANA among V-preB$^+$L$^+$ and V-preB$^-$L$^+$ B cells measured by ELISA and immunofluorescence is likely to be attributed to differences in sensitivity of these methods. Collectively these data show the abundance of ANAs among the antibodies produced by the V-preB$^+$L$^+$ B cells. Thus, the ability of V-preB$^+$L$^+$ B cells to express ANA clearly distinguished these cells from V-preB$^-$L$^+$ B cells.

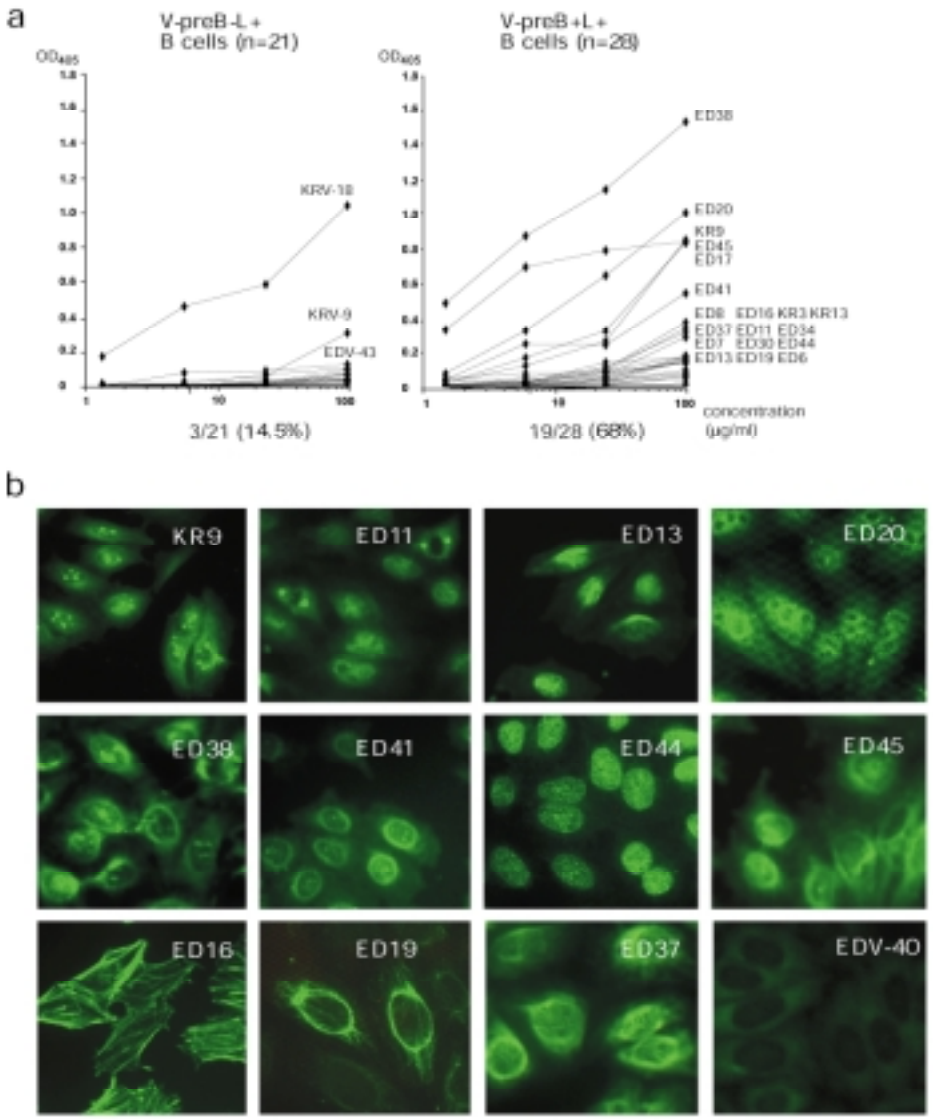

Figure 7. V-preB$^+$L$^+$ B cells express self-reactive antibodies. a) Antibodies from V-preB$^+$L$^+$ B cells react against HEp-2 cell lysates. Recombinant antibodies from 21 V-preB$^-$L$^+$ (left), and 28 V-preB$^+$L$^+$ B cells (right) were titrated and tested by Anti-Nuclear Antibody Screen (Invitrogen) ELISA for anti-HEp-2 cell reactivity and analyzed by microplate reader at 405nm. The percentage of autoreactive clones for each fraction is indicated (p=0.0004). **b)** V-preB$^+$L$^+$ antibodies express anti-nuclear antibodies (ANA**)**. Recombinant antibodies were tested for antinuclear reactivity by using Anti-Nuclear Test System Kit (BION Enterprises) according to the protocol provided by the manufacturer. Antibodies from V-preB$^+$L$^+$ B cells showed various patterns of ANA including nucleolar (KR9), mitotic spindle apparatus (ED11), speckled (ED20, ED44), and other uncharacterized patterns (ED13, ED38, ED41, ED45) and cytoskeletal reactivity against stress fiber (ED16), vinculin (ED19) and vimentin (ED37). Antibodies from V-preB-L+ B cells including EDV-40 did not stain the nucleus.

4.2.2. Anti-GPI activity of VpreB$^+$L$^+$ B cells

Given the abundance of VpreB$^+$L$^+$ B cells in the joints of RA patients and the production of autoreactive antibodies by VpreB$^+$L$^+$ B cells, it seemed conceivable that some of these auto-reactive antibodies might recognize GPI. To test this hypothesis, the anti-GPI reactivity of antibodies produced by individual VpreB$^+$L$^+$ B cells was analyzed. Among the antibodies produced by VpreB$^+$L$^+$ B cells 32% (9/28) were positive for anti-GPI, whereas none of the 21 antibody samples produced by VpreB$^-$L$^+$ B cells possessed anti-GPI activity (Figure 8; p=0.0063). Thus, the recognition of GPI by VpreB$^+$L$^+$ B cells derived antibodies underscores the potential significance of VpreB$^+$L$^+$ B cells in the pathogenesis of RA.

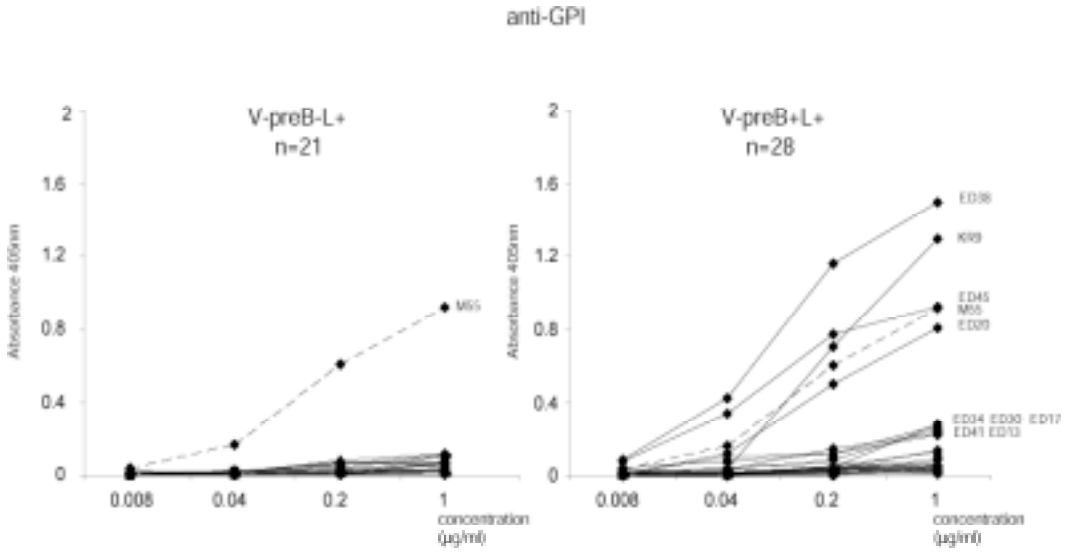

Figure 8. V-preB$^+$L$^+$ antibodies recognize GPI. Recombinant antibodies from conventional V-preB$^-$L$^+$ (left) and V-preB$^+$L$^+$ B cells (right) were titrated (1µg/ml) and tested for anti-GPI reactivity by ELISA (p=0.0063). Human mAb 55 (M55, dotted line) was included as a positive control [66].

4.2.3. Polyreactivity of antibodies derived from individual V-preB$^+$L$^+$ B cells

Simultaneous binding of the V-preB$^+$L$^+$ B cell derived antibodies to nuclear antigens and GPI raised the question about the possible polyreactive nature of these antibodies. It was previously shown that antibodies found in the serum of RA patients bind to GPI Type XI from rabbit muscle [11], Type II Collagen [69] and Citrullin [70] (data not shown). To further investigate the polyreactivity of V-preB$^+$L$^+$ B cell derived antibodies, binding to a variety of antigens, such as histones (data not shown), human insulin, thyroglobulin and myoglobulin [66] (data not shown), ssDNA and dsDNA, recombinant IL-1β (data not shown), LPS, carbohydrated structure (Chitin) and tetanus toxoid (data not shown) was tested. Human IgG1 and IgM was used to detect antibodies against the Fc fragment of a human antibody, also called rheumatoid factors (RF), that are frequently found in patients with RA and other autoimmune diseases [71].

Among the antibodies expressed by V-preB$^+$L$^+$ B cells 43% (12/28) recognized at least one of these antigens and 32% (9/28) bound to two or more antigens (ssDNA, dsDNA, IgM, insulin and LPS were selected to be shown representatively; Figure 9). In contrast, only 4.8% (1/21) of the antibodies expressed from conventional V-preB$^-$L$^+$ B cells were polyreactive (Figure 9; $p=0.0297$). Therefore a significant fraction of antibodies expressed by V-preB$^+$L$^+$ B cells is polyreactive.

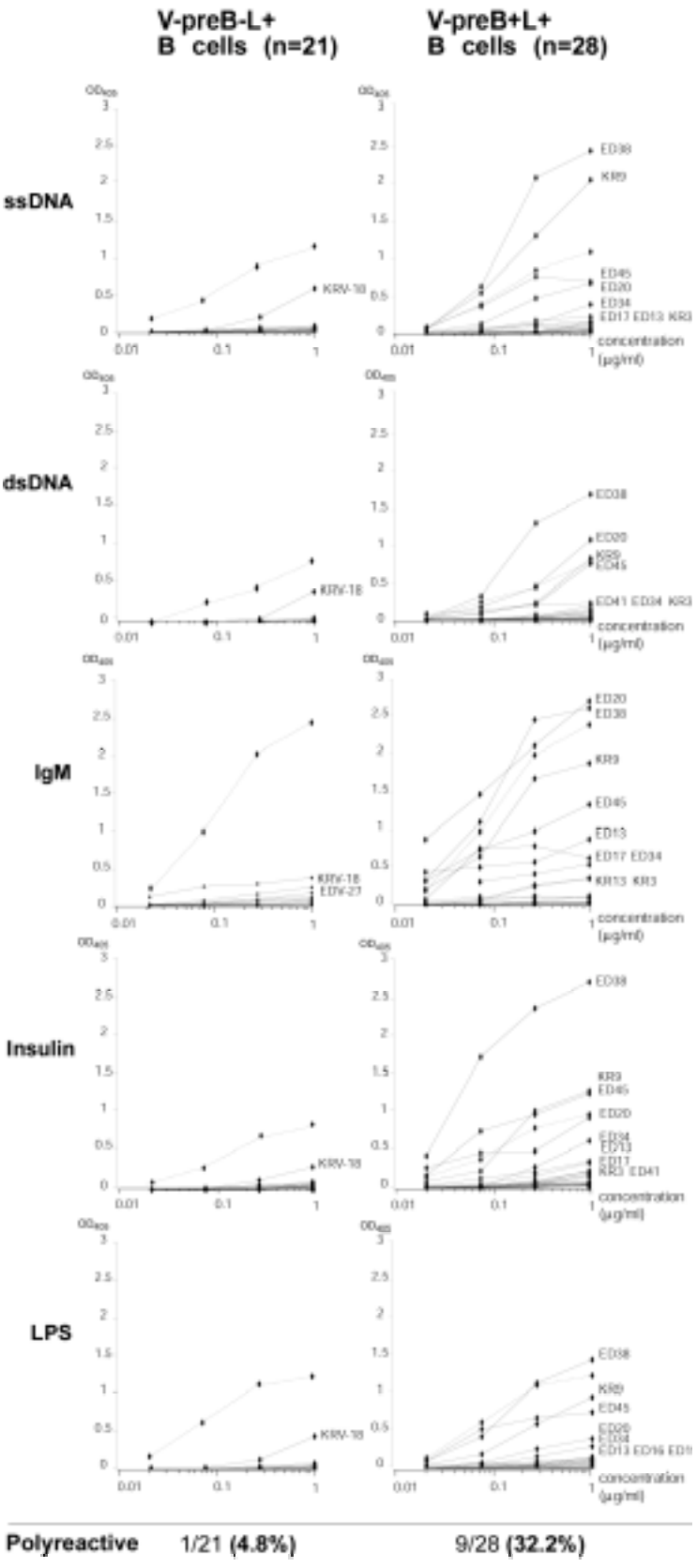

Figure 9. V-preB$^+$L$^+$ antibodies are polyreactive. Recombinant antibodies from conventional V-preB$^-$L$^+$ (left) and V-preB$^+$L$^+$ B cells (right) were titrated (1µg/ml) and tested for reactivity against single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), IgM, insulin, and lipopolysaccharide (LPS) by ELISA. Human mAb55 (M55, dotted line) was included as a positive control [66]. The percentage of polyreactive clones for each fraction is indicated ($p=0.0297$).

4.3. Antibodies against GPI or polyreactive antibodies: linkage to human rheumatoid arthritis?

4.3.1. Anti-GPI activity in sera of rheumatoid arthritis patients

The binding of polyractive antibodies produced by V-preB$^+$L$^+$ B cells to GPI raised the question about a possible polyreactivity of the anti-GPI antibodies present in the serum of RA patients [11]. In our studies 25% (15/59) of the patients with RA showed variable levels of anti-GPI activity in the serum (data not shown). All 23 samples derived from healthy donors were negative for GPI reactivity ($p=0.0083$). These results may reflect the presence or accumulation of polyreactive antibodies producing B cells in these patients. Alternatively, the presence of anti-GPI as well as of the other specific autoreactive antibodies could be due to a general defect in the negative selection of distinct autoreactive B cell clones.

The specificity of anti-GPI antibodies in the sera of RA patients was studied by the analysis of anti-GPI antibodies obtained from these patients. The anti-GPI antibodies were purified from the serum samples of seven RA patients by affinity chromatography on CNBR-activated Sepharose TM 4 Fast Flow beads coupled to GPI. An antibody fraction of the serum that binds to BSA-coupled beads was used as a negative control. The specificity of eluted antibodies was analyzed for GPI, ssDNA and insulin. Purified anti-GPI antibodies bound ssDNA (Figure 10) and insulin (data not shown), whereas BSA-binding antibodies purified from the same patient did not bind to any of the tested antigens (Figure 10). This result together with the finding that these antibodies are IgM and not IgG isotypes (data not shown) supports the hypothesis that the anti-GPI antibodies present in the serum of RA patients are polyreactive.

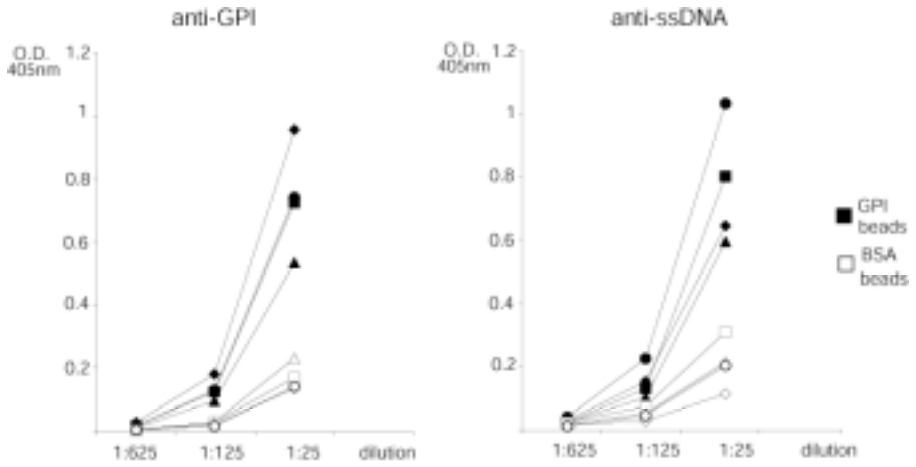

Figure 10. Anti-GPI antibodies of RA patients are polyreactive. Purified anti-GPI antibodies from RA patient (black squares) and BSA-binding antibodies from the same patient as a control (open squares) were eluted and titrated. The antibodies were tested against GPI and ssDNA reactivity by ELISA. Purified anti-GPI antibodies recognize GPI as well as ssDNA, whereas BSA-binding antibodies from the same patient do not recognize GPI or ssDNA.

4.4. Transgenic mice carrying a particular V-preB$^+$L$^+$ B cell IgH chain gene display a block in B cell development

The self-reactivity of antibodies derived from V-preB$^+$L$^+$ B cells pointed to inefficient negative selection of these cells during B cell development. One possible mechanism may lie in the ability of the co-expressed pre-BCR to prevent negative B cell selection, which would allow self-reactive V-preB$^+$L$^+$ B cell migration to the periphery. In this case expression of any given V-preB$^+$L$^+$ specific BCR in the absence of pre-BCR (V-preB and λ-like) should lead to negative B cell selection. Conversely, co-expresssion of BCR and pre-BCR should result in accumulation of autoreactive B cells.

To test this hypothesis, transgenic mice were generated carrying one of the V-preB$^+$L$^+$ B cell-specific IgH chain genes (ED45H-tg, Table II). ED45 IgH chain was chosen because of the previously detected self-reactivity of the antibody. The analysis of B cell subpopulations from ED45 transgenic mice showed a block in the development of B220$^+$CD43$^+$ pro-B cells to B220$^+$CD43$^-$ pre-B cells in the bone marrow (Figure 11a) and no B220$^+$ B cells were found in the spleen (Figure 11b). Thus, transgenic B cells expressing the self-reactive V-preB$^+$L$^+$

ED45 IgH chain in the absence of the pre-BCR undergo negative selection. This phenotype is similar to what has been described for mice that express transgenic self-reactive antibodies specific for MHC class-I, membrane HEL, or DNA [72-74]. These results provided a further prove for the autoreactive nature of the V-preB^{+}L^{+} B cell derived antibodies and highlight the importance of peripherally expressed pre-BCR in the regulation of B cell tolerance.

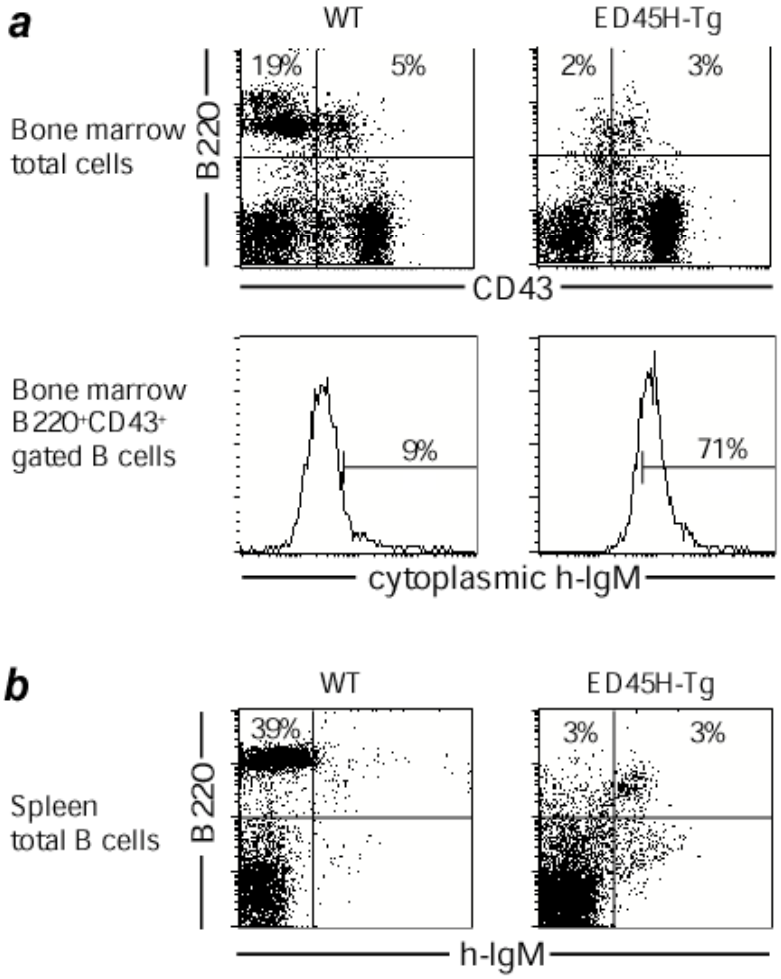

Figure 11. **B cell development in ED45H-tg mice**. **a) Deletion of transgenic B cell precursors.** Bone marrow B cells from wild type (WT, left) and ED45H-transgenic (ED45H-tg) mice (right) were stained with anti-CD43 and anti-B220 (top). Intracellular cytoplasmic human Igμ (cytoplasmic h-IgM) expression was analyzed after gating on permeabilized B220^{+}CD43^{+} pro-B cells (bottom). **b) Deletion of transgenic B cells in the spleen.** Total spleen B cells from wild type (WT, left) and ED45-transgenic (ED45H-tg) mice (right) were stained with anti-B220 vs. anti-human IgM. Numbers indicate percentages of gated B220^{+}/ h-IgM^{-} B cells and B220^{+}/ h-IgM^{+}B cells.