5. Discussion

B-lymphocytes that co-express BCR and the surrogate light chain comprise a small subset (0,5-1%) of peripheral B cells in humans [59, 60]. These cells are significantly enriched in the joints of 33% of patients with RA, indicating a possible role of V-preB$^+$L$^+$ B cells in the pathogenesis of RA. The aim of the present study was to investigate whether V-preB$^+$L$^+$ B-lymphocytes express self-reactive antibodies including those that are important for the development of RA.

Comparative analysis of the IgH gene repertoire in V-preB$^+$L$^+$ B cells and conventional peripheral B cells reveled characteristic and distinct features of IgH expressed by the individual V-preB$^+$L$^+$ B cells. The IgH of the V-preB$^+$L$^+$ B cells are characterized by extended hydrophobic CDR3s, carrying tyrosine residues at increased frequencies. Enrichment in the aromatic residues is likely to increase the flexibility of the IgH CDR3 loop thereby enhancing antibody binding to a number of different self-antigens [75-77]. In addition, an increased length of IgH CDR3s was previously shown to correlate with the ability of antibodies to bind to self-antigens. Long CDR3s are a hallmark of self-reactive antibodies in human and mice [21, 55, 77-79]. Potentially autoreactive features of the antibodies generated by V-preB$^+$L$^+$ B cells are also supported by an increased frequency of Vκ4-1 usage that is thought to confer DNA specificity to the antibodies [68, 80]. Additionally, the bias towards basic (positively charged) amino acids, especially arginine, in the heavy chain CDR3 confers antibody specificity to double- and single-stranded DNA (negatively charged) [81, 82]. Whereas forming of ion pairs is thought to be the main mechanism for this antibody-DNA interaction, arginine side-chains from CDR3s appear to contribute to oligo recognition by helping to maintain the structural integrity of the combining side [83]. The Igκ chains of antibodies found in rheumatoid synovia of RA patients resemble those produced by V-preB$^+$L$^+$ B cells by increased usage of rare 11 amino acid long Igκ CDR3s and frequent N addition [84]. In contrast to those B cells, V-preB$^+$L$^+$ B cells lack any evidence for somatic hypermutation.

The features of IgH and IgL expressed in V-preB$^+$L$^+$ B cells may explain the presence of V-preB/λ-like on the surface of these cells. Increased usage of D reading frames that encode hydrophobic amino acids, which are counterselected in normal V-preB$^-$L$^+$ B cells, might affect the structure of the antigen-binding region and create improper heavy and light chain pairing. Hence the abundance of non-paired heavy chains may favor their surrogate light chain association. In mice, DNA binding antibodies that carry positively charged residues in IgH CDR3s are neutralized by light chains that have CDR3s with low isoelectric points [85]. Human V-preB, which has a very low isoelectric point of 5.67, may act as an "neutralizer" and substitute conventional editing by light chain gene replacement through neutralization of positively charged IgH CDR3s expressed by V-preB$^+$L$^+$ B cells.

The presence of V-preB/λ-like as a part of the pre-BCR on V-preB$^+$L$^+$ B cells may alter the signaling capacity of the co-expressed BCR and influence V-preB$^+$L$^+$ B cells selection in the bone marrow. The signaling through the pre-BCR is not well characterized, but is likely to be ligand-independent. Recent results indicate that the non-Ig portion of λ5 is needed for signal transduction and that signaling of the pre-BCR could be cell autonomous [86]. Hence the expression of pre-BCR on V-preB$^+$L$^+$ B cells may generate a constitutive signal supporting B cell survival and expansion regardless of the expression and/or specificity of the BCR. In addition, expression of V-preB/λ-like may "dilute" the signal derived from the BCR along the mechanisms that have been described for B cells expressing two different BCR [87]. Co-expression of two BCRs with distinct specificities on B cells reduces the expression levels of individual BCRs and may lead to the reduction in strength of signaling mediated by a BCR of a given specificity. Both of the described hypothetical aberrations in signaling may hamper the negative selection of autoreactive V-preB$^+$L$^+$ cells during the bone marrow development and in the periphery.

The lack of somatic mutation in Ig genes derived from V-preB$^+$L$^+$ cells suggests a low-affinity of the self-specific BCRs expressed by V-preB$^+$L$^+$ cells [77, 88-91]. This in turn may spare V-preB$^+$L$^+$ B cells from negative selection. Under physiological conditions low-affinity self-reactive B cells in adults are thought to be silenced by anergy before leaving the bone marrow, whereas V-preB$^+$L$^+$ B cells sexpress normal BCR surface levels and therefore do not resemble anergic B cells [60]. V-preB/λ-like expression of V-preB$^+$L$^+$ B cells may help to escape the anergic stage by dilution of the amount of self-reactive BCRs on the cell surface.

This might be a distinct escape mechanism to prevent the population of low affinity polyreactive V-preB$^+$L$^+$ B cells from negative selection by deletion and anergy.

Inefficient negative selection of V-preB$^+$L$^+$ B cells by self-antigens does not preclude their positive selection by the same ligands. Indeed, long IgH and Igκ CDR3s in V-preB$^+$L$^+$ B cells may be a sign of selection. The enrichment in aromatic residues in IgH CDR3s caused by a JH6 bias of the antibody repertoire may also reflect selection of these cells but could alternatively result from secondary rearrangements between a cryptic V(D)J$_H$ RSS and JH6 [92]. Additionally, the shift in Jκ usage in combination with the low expression of RAG resembles features found in autoreactive B cells of mice that undergo receptor editing [45, 93, 94]. Human V-preB$^+$L$^+$ B cells might therefore represent low affinity, polyreactive B cells undergoing receptor editing in the periphery, as recently shown for mice [95].

Fifty-five percent of early immature B cells found in the human bone marrow express ANAs and polyreactive antibodies [55]. Early immature B cells resemble V-preB$^+$L$^+$ B cells as they display long IgH CDR3s with increased frequency of positively charged residues [55]. Most of these self-reactive B cells are removed from the repertoire during immature B cell stage in the bone marrow and in the transition between immature B cell and naïve B cell stage in the periphery. Thus, very few B cells producing self-reactive antibodies and ANAs are found in the mature B cell compartment [55]. In contrast, they were highly enriched in the V-preB$^+$L$^+$ B cell subpopulation of the present study. To address the specificity of antibodies expressed by V-preB$^+$L$^+$ B cells, the heavy and light chain genes derived from single-cell sorted V-preB$^+$L$^+$ B cells were expressed in A293 fibroblasts. This novel approach allowed the production of individual V-preB$^+$L$^+$ B cell derived antibodies in quantities sufficient for the analysis of the antigenic specificity. In agreement with putative polyreactive nature of the V-preB$^+$L$^+$ expressed antibodies, a significant fraction of the antibody samples bound to different self- and non-self antigens including anti-nuclear and unspecified antigens within the cell nuclei and on the membrane of apoptotic T cells (data not shown).

Whereas ANA are present in the serum of patients with SLE, the ability of the V-preB$^+$L$^+$ B cells to produce antibodies that bind GPI, a prime target for the antibody-induced RA [10, 11], pointed towards a possible involvement of V-preB$^+$L$^+$ B cells in RA pathogenesis. The ability of V-preB$^+$L$^+$ B cells to produce anti-GPI antibodies may contribute to the

pathogenesis of RA along the mechanism suggested recently by Matsumoto et al. [96], i.e., extracellular GPI attached to the cartilage surface could serve as a target for the anti-GPI antibody followed by the immune complex induced joint inflammation.

It remains unclear whether the anti-GPI antibody produced by V-preB$^+$L$^+$ B cells are as pathogenic as the anti-GPI antibodies found in the serum of K/BxN mice or RA patients. Surprisingly, we found that the affinity purified anti-GPI antibodies of RA patients were polyreactive and able to recognize multiple antigens. It therefore seems plausible that poly-reactive antibodies produced by V-preB$^+$L$^+$ B cells might constitute the fraction of polyreactive antibodies in the serum of RA patients. Nevertheless, RA could also be caused by highly specific, high affinity anti-GPI antibodies, which represent a fraction of the total pool of anti-GPI reactivity in the serum of RA patients.
In view of the presence of V-preB$^+$L$^+$ B cells in every healthy individual it seems likely that the initiation of the V-preB$^+$L$^+$ B cell mediated autoimmunity reflects not the presence of these cells but rather their state of activation, which could depend on various factors, e.g., loss-of-function mutations in genes encoding repressors of B cell signaling for infection-induced non-specific B cell activation.

Expression of autoreactive antibodies on developing B cells led to the efficient elimination of these B cells upon their encounter with the antigen including MHC class-I, membrane HEL, and DNA [72-74]. To prove that V-preB$^+$L$^+$ B cell derived antibodies are indeed autoreactive and can trigger tolerance in the absence of V-preB/λ-like expression, we generated transgenic mice carrying one of the V-preB$^+$L$^+$ B cell-specific IgH chain genes. In these mice the transgenic B cells were mostly deleted during the early B cell development in the bone marrow. Deletion was not simply due to incompatibility of human IgH and mouse IgL chains because a similar human IgH transgene with a different VDJ_H gene segment displaying an equally long IgH CDR3 including JH6 showed normal B cell development [31, 97]. These data provided functional prove for the autoreactive nature of the V-preB$^+$L$^+$ B cell expressed immunoglobulins. We propose that sustained expression of V-preB protein might possibly cause autoreactive receptor dilution similar to dual receptor expression in autoreactive mouse B cells [87] and therefore be able to prevent negative selection. The rescue of peripheral B

cells in ED45H-tg mice by co-expression with human V-preB/λ-like protein would be implied by this proposal.

In conclusion, the presented data suggest a possible causal role of V-preB^{+}L^{+} B cells in the pathogenesis of autoantibody-induced RA. Further experiments have to address the escape mechanism of these cells from self-antigen induced tolerance and to what extent these cells are involved in the pathogenesis of RA in human.