1. Introduction

1.1. Autoimmune diseases

Autoimmune diseases occur when a specific adaptive immune response, as a result of failed self-tolerance, is mounted against self-antigens. Autoimmune diseases caused by activated self-reactive T and B cells are relatively common in human population. As reviewed in Samter's Immunologic Diseases, altogether 5% of the human population suffers from some form of autoimmunity [1]. Patients with systemic autoimmune disorders such as systemic lupus erythematosus (SLE) account for as many as one in 500 African-American or Asian women living in westernized societies. SLE is characterized by a female predominance of 10:1. Autoimmune diabetes type 1A affects 1 out of 400 adolescents among the general population with onset peak at the age of 15-24. The prevalence of rheumatoid arthritis (RA), a chronic systemic autoimmune inflammatory disease of the joints, accounts in most of the population groups of the world for about 0.5% to 1 % with a peak onset in the fourth decade of life. Like many other autoimmune diseases RA is more common in woman than in man, the accepted ratio is 3:1 (reviewed in [1, 2]).

1.1.1. Autoimmune nature of rheumatoid arthritis

The etiology and pathogenesis of RA are not well understood [3]. The primary role of auto-reactive B cells in RA has been recently suggested by findings in K/BxN T-cell receptor (TCR) transgenic mice. These mice were generated by breeding NOD mice to KRN/C57Bl/6 TCR transgenic mice and are characterized by early onset of spontaneous RA [4]. The arthritogenic activity in K/BxN serum resides in the IgG antibody fraction specific for glucose-6-phosphate isomerase (GPI) [5]. GPI is a ubiquitously expressed, largely cytosolic enzyme, which is essential for basic carbohydrate metabolism. GPI also exhibits extra-cellular functions that include cytokine and growth factor activities [6-9]. Recent studies revealed high concentrations of GPI on the surface of the hypertrophic joint synovia of RA patients [10, 11]. In addition, high concentrations of anti-GPI specific antibodies have recently been described in sera of patients with RA [11]. As evidence for the disease-provoking role of autoantibodies,

injection of purified IgGs against GPI into healthy mice induced arthritis [12]. The similarities between the described mouse model of arthritis, and RA in human imply a defective negative selection or activation of normally dormant but potentially pathogenic autoreactive B cells in the pathogenesis of RA.

1.2. B cells

The immune system generates 2 x 10^7 B lymphocytes per day with each of them recognizing a different antigen. The unique antigen specificity of B lymphocytes is defined by the primary structure of the immunoglobulin heavy and light chains comprising the surface expressed antigen receptor [13]. The specificity of the B cell antigen receptors is achieved through the rearrangement of the immunoglobulin genes [14]. In mice, random rearrangement of V_H, D_H and J_H genes and junctional diversity allows generation of 11.000 possible variants of IgH chain. Similarly, rearrangement of the Vk and Jk genes attributes to the generation of 320 different IgL chains. In contrast to humans where Igκ is used to an equal extent as Igλ, Igλ chains are barely used in mice. Pairing of distinct IgH and IgL chains can potentially yield 3,5x10^6 different antibody specificities per mouse, and even more in humans. Although there are differences between mouse and human B cell rearrangement, as for example the frequency of Igλ usage, the main principal is the same (reviewed in [15]).

1.3. B cell development in the bone marrow

B lymphocyte progenitors derive from hematopoietic stem cells (Figure 1). The earliest stage of a B cell lineage commitment is, based on the Philadelphia nomenclature, defined as a pre-pro-B cell [16, 17], which is characterized by the cell-surface expression of the general B cell marker B220 (isoform of CD45). Pre-pro-B cells express low levels of the recombination activating genes (RAG-1 and RAG-2) [18, 19], and they do not express components of the B cell antigen receptor (BCR) [20]. These very early B-lineage progenitors are immediate precursors of the pro-B cells and rarely have any immunoglobulin gene rearrangements.

The V(D)J recombination is initiated at the pro-B cell stage of B cell development and a significant fraction (30%) of pro-B cells contains rearranged IgH genes [16]. The

rearrangement of IgH genes is accomplished in two steps: (1) the recombination of a diversity (D_H) segment to a junction (J_H) segment, and (2) the recombination of a variable (V_H) gene to the joined DJ_H gene complex. Because of random nucleotide loss and addition, D segments can be joined to J_H gene segments in any one of three reading frames. Most of the mature B cells in humans have D_H segments in reading frame 2 (RF2) that encodes for hydrophilic amino acids, one third of B cells produces DJ_H joints in RF3, which frequently encodes hydrophobic amino acids, and the remaining number of B cells carry D_H segments in RF1, which often contains stop codons [21]. After DJ_H rearrangement, V_H genes become accessible to the V(D)J recombination machinery and complete heavy chain transcription units are assembled [22]. Following translation, the IgH is transported to the cell surface where it becomes associated with the surrogate light chains VpreB and λ-like (VpreB1/2 and λ5 in mice) [23-25]. Together with the transmembrane signaling proteins Igα and Igβ, which are essential for the initiation of the IgH-dependent signal, this pre-B cell receptor controls the differentiation of pro-B cells into pre-B cells and their clonal expansion [26-30].

Signals derived from pre-BCR arrest further IgH rearrangements (allelic exclusion) and initiate IgL gene rearrangement [31, 32]. Successful light chain VJ rearrangement leads to replacement of surrogate light chains VpreB and λ-like by Igκ or Igλ that together with the IgH generate the unique B cell antigen receptor (BCR). Expression of BCR leads to the generation of immature B cells and marks the transition to the antigen dependent phase of B cell development.

Immature lymphocytes display mainly surface IgM and remain in the immature compartment for an average of 3.5 days [33]. During this time the B cells undergo antigen-induced BCR mediated negative selection (see below).

1.4.. Antigen-dependent B cell selection

The clonal selection theory proposes that each B lymphocyte produces a single antibody and that individual lymphocytes undergo clonal expansion upon encounter with a specific antigen [34, 35]. The mass production of lymphocytes with virtually unlimited specificities has a potential drawback due to the generation of large numbers of lymphocytes that potentially recognize self-antigens. Ehrlich and others proposed that the consequences of formation of

self-antibodies were so severe that the immune system stringently prohibited its occurrence [36]. Defects in negative selection of self-reactive B lymphocytes can lead to immune cell-mediated destruction of various organs. The causal role of auto-antibodies in immune cell-mediated injury was proven by Harrington who showed that the transfer of sera derived from patients with idiopathic thrombocytopenic purpura caused trombocytopenia in the injected individuals [37]. Despite overwhelming evidence for the causal role of autoantibodies in the development of various autoimmune diseases, the molecular and cellular mechanisms preventing the development and accumulation of the autoreactive B cells remain largely elusive. So far three different mechanisms called negative selection, anergy, and receptor editing are described to guarantee B cell tolerance (Figure 1).

1.4.1. Clonal deletion and anergy

Most of the generated self-reactive B cells, however either die of clonal deletion or become functionally incapacitated (anergized) upon encounter with self-antigens. Clonal deletion is defined as self antigen-induced death of autoreactive B cells during early B cell development or at later stages of B cell maturation. Overall, the efficiency of clonal deletion is likely to be higher in the bone marrow than in the periphery due to the high susceptibility of immature B cells to BCR-induced apoptosis [38].

Incubation of immature B cells with anti-IgM antibodies in vitro revealed a direct correlation between the degree of the BCR cross-linking and the rate of cell death [39], i.e., B cells cultured in the presence of high concentrations of anti-μ antibodies die at a high rate. In contrast, BCR cross-linking at intermediate levels due to lower concentrations of anti-μ does not cause B cell death but prevents B cell proliferation and antibody production upon subsequent mitogen exposure. This non-responsive or anergic state of self-antigen specific B cells was extensively characterized in mice co-expressing the transgenic BCR specific for hen egg lyzozyme (HEL) and transgenic soluble HEL [40]. B cell anergy also occurs in mice that express BCR specific for the single-stranded DNA (ssDNA) [41, 42]. Thou, the state of anergy seems to be reached by B cells with a low affinity self-reactive BCR whereas high affinity anti-self-reactivity leads to clonal deletion.

Anergic B cells are short lived and unable to enter anatomical niches, e.g., lymphoid follicles, in the presence of normal, wild type mice derived B cells [43, 44]. Chronic encounter of

soluble HEL by HEL-binding B cells leads to changes in the B cell phenotype characterized by low expression levels of IgM and CD23, but relatively high surface expression levels of CD5 as compared to B cells of wild-type mice. Compared to the anergic anti-HEL specific B cells, the anergic ssDNA specific B cells are functionally potent but fail to differentiate into plasma cells. It seems possible, that differences in anergy-related features of B cells may reflect the affinity of the self-antigen-BCR interaction as well as other less defined antigen-determined changes in B cell signaling. Characterization of the anergic state of B cells is further complicated by the lack of well defined anergy-specific markers that precludes identification of anergic cells within peripheral B cell population. Therefore, the frequency of these cells in non-transgenic mice as well as in human could not be determined so far.

1.4.2. Receptor editing

The frequency of autoreactive B cells during B cell development and maturation can be reduced significantly by secondary light chain gene recombination ("receptor editing"). Elimination of self-reactive B cells through receptor editing was first described for transgenic B cells specific for double-stranded DNA (dsDNA) or major histocompatibility complex (MHC) class I [45, 46]. In mice, about 25% of auto-reactive B cells change their specificity through secondary IgL gene rearrangement [47]. In contrast to anergy and deletion, this recently described mechanism of “social correction" of autoreactive B cells spares the initially autoreactive B cells by replacing self-specific BCR by an "unharmful" one. The high numbers of antibody molecules that are produced by gene replacement as shown by Casellas et al. [47] suggest that receptor editing represents a major force in shaping the antibody repertoire under physiological conditions.

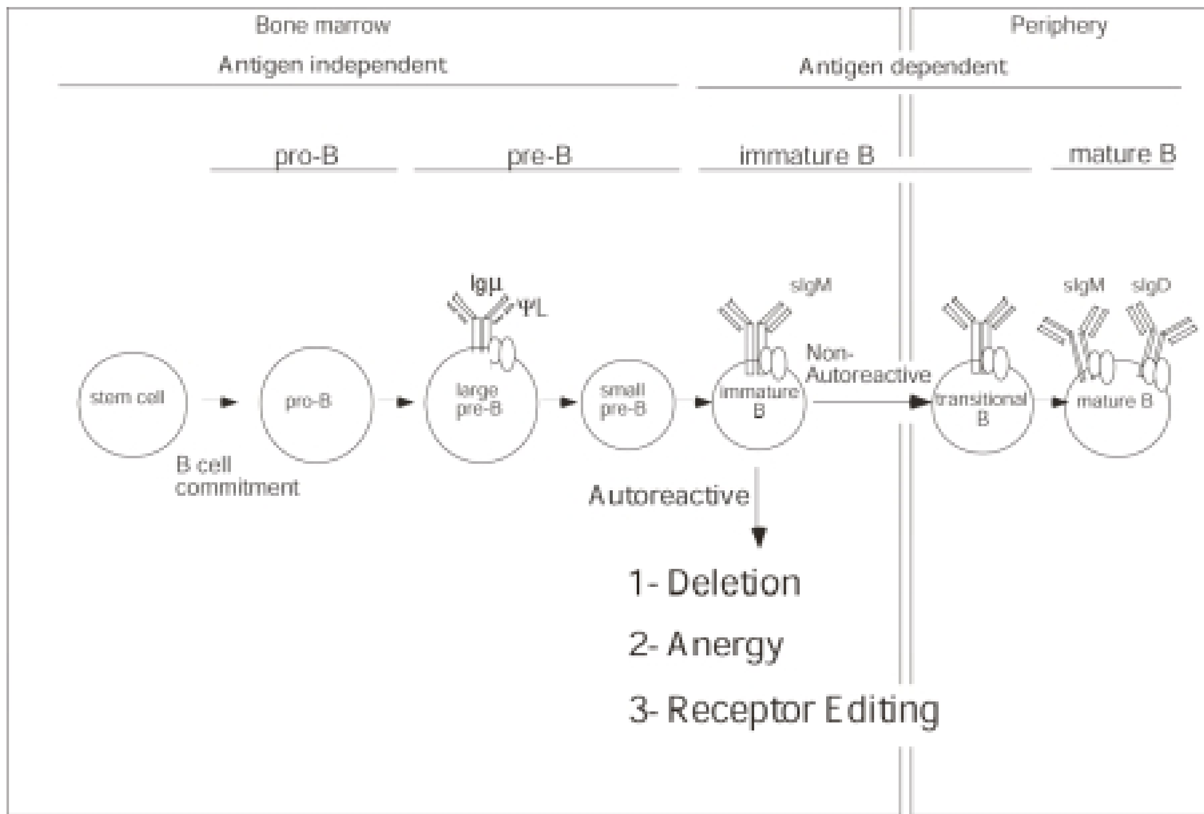

Figure 1. B cell differentiation. Developmental stages of B cell lymphopoiesis with cell surface expression of pro-B, pre-B (Igμ, VpreB/λlike and Igα-Igβ) or B (Igμ, Igκ or Igλ and Igα-Igβ) cell receptors is shown**.**

1.5. Peripheral B cell maturation

Immature B cells that have passed selection in the bone marrow and carry a non-self-reactive BCR on the cell surface, migrate to the peripheral lymphoid tissues where they form a population of transitional B cells (Figure 1). Kinetic studies have shown that only 10 to 30% of all daily produced immature B cells (2 x 10^7), differentiate into the long-lived mature B cells [48-50]. Mature B cells can be distinguished from transitional B cells by expression of IgM and increased expression of IgD, in combination with the expression of complement receptors (CR1/CR2). Compelling evidence for selection of peripheral B cells relies on differences found in the antibody repertoire of pre-B, immature, and mature B cells [51-55], which strongly support an antigen-dependent selection of peripheral mature B cells.

1.6. Self-reactive B cells

Besides high affinity autoreactive B cells that are caused by insufficient self tolerance in the cause of autoimmune disease, there are different B cell populations in healthy human and mice that express self-reactive B cell receptors, e.g., B-1 cells that comprise about 5% of the peripheral B cell pool and express self-/polyreactive antibodies. In mice and human, low-affinity IgM auto-antibodies produced by B-1 cells presumably neutralize various self and non-self antigens prior to the development of specific immune responses [56, 57]. However, the expansion of autoreactive B-1 cells may lead to the development of autoimmunity in mice and man [58].
Another recently described B cell population that may also contribute to the pathogenesis of RA in human is characterized by co-expression of B cell antigen receptor and surrogate light chain VpreB (V-preB$^+$L$^+$ B cells, Figure 2) [59, 60]. As previously mentioned, differentiation of pro-B into pre-B cells in the bone marrow is governed by the pre-B cell receptor that is comprised of the V-preB and λ-like proteins (surrogate light chains) associated with Ig heavy chain (IgH). Further developmental progression of pre-B cells into immature B cells is associated with the silencing of the V-preB and λ-like genes. As a consequence V-preB and λ-like proteins are therefore not expected to be present on immature or mature B cells in the periphery [61, 62]. The exception is this small population of B cells, accounting for 0.5-1% of all peripheral B cells in healthy human donors (Figure 2), that is found to be accumulated in joints of RA patients [59, 60]. Moreover, these V-preB$^+$L$^+$ B cells display an unusual heavy and light chain repertoire formerly seen in autoreactive B cells [59, 60], which suggests a pathogenic role of antibodies produced by V-preB$^+$L$^+$ B cells in the pathogenesis of RA.

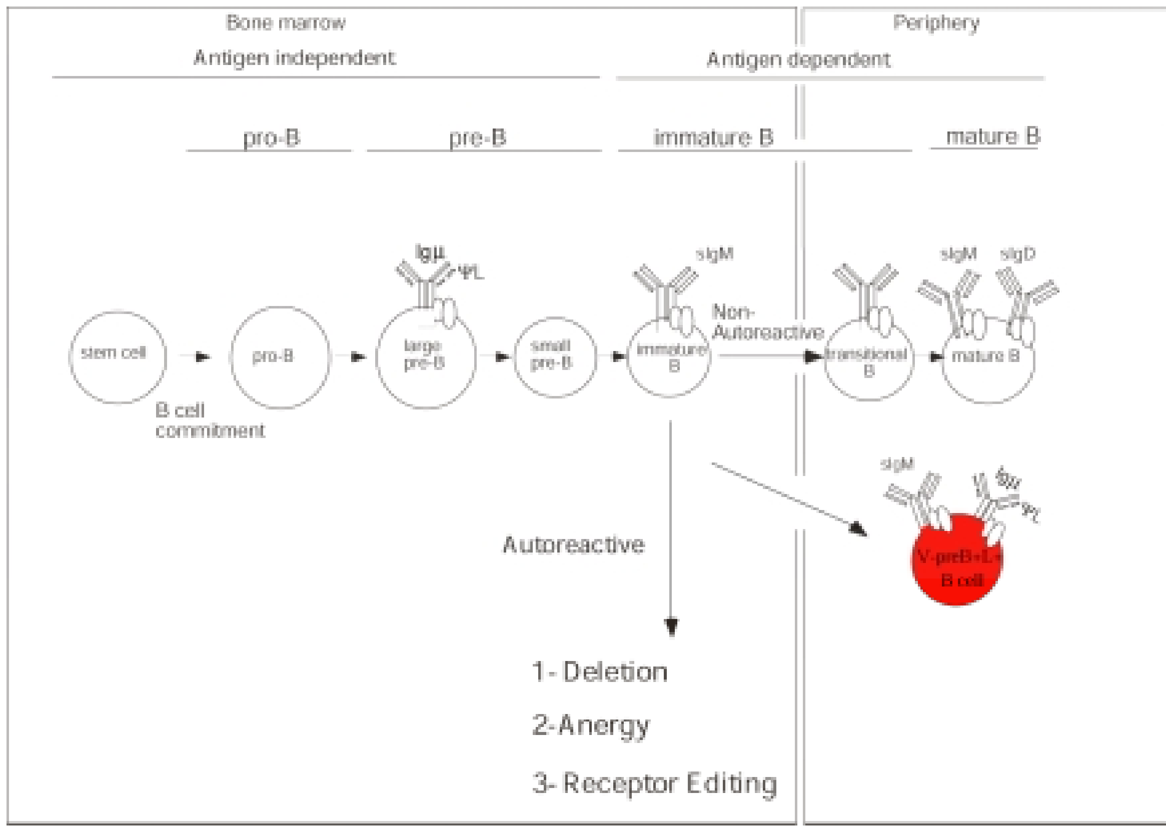

Figure 2. V-preB^{+}L^{+} B cells in developmental stages of B cell lymphopoiesis. Cell surface expression of pro-B, pre-B (Igμ, VpreB/λlike and Igα-Igβ), or B (Igμ, Igκ or Igλ and Igα-Igβ) cell receptors is shown**.** Peripheral V-preB^{+}L^{+} B cells (red) co-express pre-BCR and BCR on the surface.

1.7. Objectives

The hypothesis underlying the present study was that V-preB^{+}L^{+} B cells might be involved in the pathogenesis of rheumatoid arthritis in human by the production of autoreactive, arthritogenic antibodies. The specific aims of this work were:

1. Establishment of a method to analyze the immunoglobulin repertoire and antibody specificity of individual V-preB^{+}L^{+} B cells.

2. Analysis of the antigenic specificity of antibodies expressed by individual V-preB^{+}L^{+} B cells:

- Do antibodies expressed by individual V-preB^{+}L^{+} B cells display autoreactive activity?
- Do antibodies expressed by individual V-preB^{+}L^{+} B cells recognize antigens with presumably specific artritogenic activity?

3. Does the co-expression of, as revealed by the objectives, autoreactive BCR and pre-BCR on V-preB^{+}L^{+} B cells prevent negative B cell selection?
Generation of transgenic mice that carry one of the characteristic V-preB^{+}L^{+} B cell IgH chain genes to test the hypothesis whether in the absence of the pre-BCR (V-preB and λ-like) these cells undergo negative selection.