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The selection of mature B cells is critically dependent on the expression level of the co-receptor CD19

Lilly von Muenchow[a], Corinne Engdahl[a], Klaus Karjalainen[b], Antonius G. Rolink[a,*]

[a] Developmental and Molecular Immunology, Department of Biomedicine, University of Basel, Mattenstraße, Basel 284058, Switzerland
[b] School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore

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ABSTRACT

CD19 plays a crucial role in mature B cell development as best exemplified by the finding that CD19 deficient mice have severely reduced mature B cell compartments (Engel et al., 1995; Rickert et al., 1995). In the present study we show that the transition into the mature B cell compartments is heavily dependent on the correct amount of CD19 expression. Thus, Nup-98–HoxB4 immortalized hematopoietic stem cells (HSCs) over-expressing CD19 show upon transplantation an impaired pro/pre B to immature B cell transition in the bone marrow, whereas Nup-98–HoxB4 HSCs expressing a shRNA that down-modulates CD19 expression show upon transplantation a strongly reduced mature B cell compartment. Overall our findings indicate that too high CD19 expression might result into too strong BCR signaling in the bone marrow and therefore causing negative selection. Too low CD19 expression might result into too little BCR signaling and thereby preventing the B cells to enter the mature pool (absence of positive selection).

1. Introduction

Early stages of B cell development take place in the bone marrow (BM), whereas final maturation takes place in the spleen. In order to get selected into the long-lived mature compartment B cells have to pass several crucial selection steps. Signal strength delivered from the B cell receptor (BCR) seems to be a key determination factor. This is best exemplified by the large number of BCR signaling mutants which show a developmental arrest [1]. Among the different modulators of BCR signaling, CD19 is known as positive regulator. Together with CD21, CD81, and CD225 it builds the co-receptor of the BCR [2,3]. CD19 has been shown to lower the threshold for B cell activation and decreases the amount of anti-IgM that is needed to induce DNA synthesis [4]. Upon ligation the conserved tyrosine residues of the cytoplasmic domain become phosphorylated and serve as binding sites for SH2 domains of regulatory proteins, such as PI3K and Lyn, thereby acting on BCR signaling [5,6]. However, also ligand-independent "tonic" signaling activity has been described for CD19, which is possibly involved in generating a basal signaling level essential for positive selection [7,8]. CD19 is a 95 kDa transmembrane protein which is first detectable on the surface of D_H–J_H rearranged proB cells and remains expressed until terminal differentiation into memory B cells. However, antibody secreting plasma cells are CD19 negative [9,10]. CD19 deficient mice have been described previously [11,12]. In these mice early stages of B cell development do not seem to be affected. However, they have reduced numbers of mature B cells suggesting that the transition from immature to mature B cells is impaired. Also mice over-expressing human CD19 have been generated [13]. The earliest stages of B cell development were not affected. However, beginning with IgM^+ immature cells in the bone marrow total numbers were reduced. Taken together these findings strongly suggest that CD19 plays a crucial role in the transition of immature to mature B cells.

In the present study we analyzed this phenomenon in more detail. In order to do this, we employed the recently described method of immortalizing multipotent hematopoietic cells with a retrovirus encoding a Nup98–HoxB4 fusion protein [14]. It was shown that upon injection into irradiated recipients these cells are able to give rise to all hematopoietic lineages [14]. We introduced in these cells a retrovirus encoding CD19 and a retrovirus containing a shRNA directed against CD19. Our *in vivo* findings with these cells show that early stages of B cell development are independent of the CD19 expression level, whereas only those immature B cells that express amounts of CD19 comparable to non-transduced B cells can be selected into the long-lived mature compartment.

Abbreviations: BCR, B cell receptor; FoB, follicular B cell; HSC, Hematopoietic stem cell; Ig, Immunoglobulin; MZB, marginal zone B cell; OV.EX. CD19, over-expression of murine CD19; shCD19 shRNA, directed against murine CD19 mRNA.

* Corresponding author. Tel.: +41 61 267 16 31; fax: +41 61 695 30 70.
E-mail address: antonius.rolink@unibas.ch (A.G. Rolink).

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2. Materials and methods

2.1. Mice

RAG-2 deficient mice were first described by Shinkai et al. and CD3ε deficient mice by Malissen et al. [15,16]. Animals were bred under pathogen-free conditions at the Center for Biomedicine at the University of Basel. Experiments were carried out within institutional guidelines with the permission of national or local authorities (permission numbers 1886 and 1888).

2.2. Generation of Nup98–HoxB4 transduced multipotent hematopoietic progenitor cells from CD3ε$^{-/-}$ mice

In order to "immortalize" hematopoietic progenitor cells of CD3ε$^{-/-}$ mice using the Nup98–HoxB4 construct we employed a method, that was first described by Sauvageau et al., and then further improved by Ruedl et al. [14,16,17]. In short, bone marrow of mice, treated with 5-Fluorouracil (5-FU), was transduced with the pMYc-IP vector containing a Nup98–HoxB4 fusion protein. After selection for transduced cells using the puromycin-resistance (1 μg/ml), cells were kept in culture by addition of 3% (v/v) IL-6 supernatant and 100 ng/ml stem cell factor (SCF).

2.3. Cell lines and cell culture

The CD19 positive Abelson virus-transformed preB cell line 40E1 was grown in IMDM supplemented with 5×10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (w/v) Primatone (Quest; Naarden, NL), 100 U/ml penicillin, 100 μg/ml streptomycin and 5% (v/v) fetal bovine serum [18]. Once established, Nup98–HoxB4 transduced hematopoietic progenitor cells were maintained in the same IMDM, additionally supplemented with 3% (v/v) IL-6 supernatant and 100 ng/ml SCF.

2.4. Plasmids

Complete murine CD19 cDNA was cloned into the pMigR1-IRES-GFP expression vector. To decrease CD19 protein levels a shRNA (5′-CTCGAGAAGGTATATTGCTGTTGACAGT GAGCGCTCTGAGAAGCTGGCTTGGTATTAGTGAAGCCACAGATGTAA TACCAAGCCAGCTTCTCAGAATGCCTACTGCCTCGGAATTC-3′) targeting the beginning of exon 2 of murine CD19 mRNA was introduced into the MSCV-LTRmiR30-PIG (LMP) vector. The pMYc-NUP98–HOXB4-IP retroviral vector was a kind gift from Christiane Ruedl and Klaus Karjalainen. Its preparation was described before [14].

2.5. Transfection of phoenix-eco cells, retroviral transduction and sorting

Phoenix-eco cells were seeded in a 6-well plate at a density of 2×10^5 cells/well one day before transfection [19]. Medium was removed and transfection conducted using FuGENE HD (Promega; Fitchburg, WI) according to the manufacturer's protocol. Two days after transfection virus supernatant was collected and used immediately or stored at −80 °C. For transduction 10^6 target cells were resuspended in virus supernatant and spun 3 h at 1157 rcf and 33 °C. Successfully transduced cells were sorted using a FACSAria (BD Biosciences; Franklin Lakes, NJ) to >98% purity.

2.6. Transplantation

Mice were sublethally irradiated (4 Gy). For reconstitution 10×10^6 cells were injected intravenously. At around 5 weeks after transfer, mice were sacrificed and organ cell suspensions were prepared by mechanical disruption. Bone marrow was aspirated from the femur.

2.7. Antibodies and flow cytometry

Anti-CD19-PE (6D5) was purchased from BioLegend (San Diego, CA). Anti-B220-PE (RA3-6B2), anti-CD23-PE (B3B4), anti-CD19-PECy7 (1D3) and anti-Ter119-PE (TER-119) were purchased from BD Bioscience (Franklin Lakes, NJ). Anti-B220 (RA3 6B2), anti-IgM (M41) and anti-CD21 (7G6) were purified from hybridoma culture supernatants on protein G-Sepharose columns (Pharmacia) as recommended by the supplier. Purified monoclonal antibodies were conjugated with Alexa647 according to standard protocols. Staining of cells was performed as previously described [20]. Propidium iodide (Sigma–Aldrich; St. Louis, MO) was used at 0.5 μg/ml. Flow cytometry was performed using a FACSCalibur (BD Biosciences) and data were analyzed using the FlowJo (Tree Star; Ashland, OR) software.

3. Results

3.1. Generation of retroviruses encoding murine CD19 and containing shRNA directed against CD19

To analyze the effect of the CD19 "dose" on various B subpopulations, plasmids containing either complete murine CD19 cDNA or a shRNA directed against CD19 mRNA were generated. In both the sequence of interest was linked to a GFP cassette via an IRES sequence. In this way a broad spectrum of CD19 protein expression could be covered and easily monitored by GFP expression levels. Functionality of the various retroviruses was tested *in vitro* through transduction of the CD19$^+$ Abelson virus-transformed preB cell line 40E1 [18]. Non-transduced cells expressed identical levels of CD19 compared to the ones infected with the empty pMigR1 vector. In marked contrast, shRNA transduced GFP positive cells expressed about an order of magnitude less CD19 on the surface than non-transduced controls. On the other hand, GFP positive cells transduced with CD19 encoding retrovirus expressed about a 10 fold higher level of CD19 than non-transduced cells (Fig. 1A).

3.2. Generation of multipotent progenitors expressing CD19 or containing shRNA for down-modulation of CD19 expression

Recently it was shown that BM cells with hematopoietic stem cell properties (hereafter called HSCs) can be immortalized by expression of a Nup98–HoxB4 fusion protein [14]. Here we employed these cells in order to analyze the effect of over and/or under expression of CD19 on B cell development. Therefore, HSCs from CD3ε deficient mice were generated [16]. In brief, BM cells were collected from 5-Fluorouracil treated mice and cultured for 3 days in medium containing IL-6 and SCF. Thereafter, the cells were infected with a retrovirus encoding a Nup98–HoxB4 fusion protein and containing a puromycin resistance cassette. By day 3 of culture puromycin was added (1 μg/ml). After 3 days of culture with puromycin cells were harvested and infected with retroviruses containing the empty pMigR1-IRES-GFP expression vector, the CD19 encoding pMigR1-IRES-GFP expression vector and the MSCV-LMP vector containing the CD19 shRNA and GFP. After 4 days of reculture GFP expressing cells were FACS sorted and further propagated. As shown in Fig. 1B all transduced HSCs expressed robust levels of GFP. Moreover, HSCs transduced with the CD19 encoding pMigR1-IRES-GFP expression vector expressed homogenous levels of CD19 on their surface (Fig. 1B).