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Transcriptome analysis in primary B lymphoid precursors following induction of the pre-B cell receptor

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Abstract

Pre-BCR signals are part of a checkpoint where early precursor (pre-) B cells with a pairing Ig μH chain (μHC) are clonally expanded before they differentiate into IgL-rearranging, resting pre-B cells. A pre-BCR consists of two μHCs, two surrogate L chains and the signal transducer Igα/Igβ. The molecular circuits by which the pre-BCR controls proliferation and differentiation of pre-B cells are poorly characterized. Therefore, we identified the differential transcriptome by genome-wide expression profiling in progenitor (pro-) B cells from a Rag2-deficient mouse, in which the expression of a transgenic μHC and thus a pre-BCR as well as pre-BCR-mediated clonal expansion can be controlled by tetracycline (μHC-inducible mouse). This analysis revealed that pre-BCR signals upregulate components of the BCR signalosome, open the IgL chain (LC) locus and induce the *krüppel*-like transcription factor KLF2, a key regulator of quiescence and lymphocyte migration. Hence, pre-BCR signals establish the molecular network for BCR signaling even before the production of an IgLC and induce the expression of KLF2, a candidate for controlling clonal expansion and migration of functional pre-B cells.
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1. Introduction

The establishment of a large antibody repertoire through the assembly of functional exons encoding the variable regions (V) and thus the antigen binding site of an B cell receptor (BCR) from V, D and J gene segments first at the IgH and subsequently at the IgL locus is the hallmark of B cell development (Alt et al., 1984). In this scenario, signals provided by an immature Ig-like transmembrane complex, the so-called pre-B cell receptor, are part of a critical checkpoint where precursor B (pre-B) cells with a functional, i.e., an IgL chain-pairing μH chain (μHC) are clonally expanded (Bradl et al., 2007; Burrows et al., 2002; Keyna et al., 1995a,b; Kline et al., 1998; Martensson et al., 2002; Meffre et al., 2000; Muljo and Schlissel, 2000; Vettermann et al., 2006). Although all descendants from a clonally expanded pre-B cell produce the same V_H idiotype, each of these cells will synthesize a different IgL chain (LC) at a later stage of differentiation and thus a different antigen specificity. Hence, pre-BCR signals are critical for increasing the diversity of the primary antibody repertoire by expanding the pool of pre-B cells that produce a functional μHC.

The extent of clonal expansion of a pre-B cell is determined by the strength of signal provided by the pre-BCR. This receptor consists of two μHCs, two surrogate light chains (SLCs), which are composed of the invariant and non-covalently associated Ig-like polypeptides VpreB and λ5, and the signal transducer Igα/Igβ. As pre-B cells pass the pre-BCR checkpoint, they undergo first a limited clonal expansion phase with two to six cell divisions and differentiate into small resting pre-B cells, which subsequently assemble a V and J gene segment at the IgL-locus into a V_L exon (Bradl et al., 2007; Decker et al., 1991; Hendriks and Middendorp, 2004; Hess et al., 2001; Rolink et al., 2000; Vettermann et al., 2006) Molecular circuits by which the pre-BCR controls the limited clonal expansion of early pre-B cells and rearrangements at the IgL locus are still poorly understood.

Therefore, we investigated the effect of pre-BCR signals on the transcriptome and the opening of the Igκ locus in the

Abbreviations: FC, fold change; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KLF2, *krüppel*-like factor 2; μHC, IgμH chain; LC, IgL chain; pre-BCR, precursor-BCR; pro-B, progenitor B; SLC, surrogate L chain; Tet, tetracycline; tTA, tetracycline-controllable transactivator

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μHC-inducible mouse (Hess et al., 2001; Schuh et al., 2003), a double-transgenic (dTg) mouse that allows us to induce *de novo* the expression of a μHC and thus a pre-BCR by tetracycline (Tet) in freshly isolated primary pro-B cells. *Affymetrix*-microarray analyses identified 231 genes that were significantly up- or downregulated upon pre-BCR expression. Besides known pre-B cell signature genes like CD2, CD25, c-kit and TdT we found that pre-BCR signals also opened the Igκ locus and upregulated signature genes of immature B cells, such as CD20 and molecules involved in BCR signaling and antigen presentation. Hence, pre-BCR signals establish the molecular network for BCR signaling even before IgL rearrangement has been completed. Most importantly, we identified a large group of novel pre-BCR target genes, including LKLF/KLF2, a transcription factor of the *krüppel*-like family known to be a key regulator in controlling quiescence and migration of T cells (Carlson et al., 2006; Kuo et al., 1997a). KLF2 as well as its target gene sphingosine-1-phosphate receptor-1 (S1P1) (Carlson et al., 2006) were both strongly upregulated after pre-BCR induction; as expected, the highest expression of KLF2 and S1P1 was detected in freshly isolated and primary small, resting pre-B cells. Hence, KLF2 is a new pre-BCR target, which may play a critical role in controlling clonal expansion and cell migration of functional pre-B cells.

2. Materials and methods

2.1. Animals

Rag2$^{-/-}$/dTg mice, Rag2$^{-/-}$/tTA mice (Hess et al., 2001; Schuh et al., 2003) and C57Bl/6 mice (Charles River, Sulzfeld, Germany) were maintained under pathogen-free conditions in the animal facility of the Nikolaus-Fiebiger-Center (Erlangen, Germany). Transgenic animals were genotyped by PCR using DNA from tail biopsies.

2.2. Flow cytometry

For surface staining, 5×10^5 cells were stained with the respective antibodies as described (Schuh et al., 2003). For cytoplasmic staining, 5×10^5 cells were fixed in 1% paraformaldehyde/PBS, permeabilized with 0.5% Tween-20 in PBS, stained with fluorochrome-conjugated antibodies and analyzed with a FACS-Calibur (BD, San Diego, CA) equipped with Cell Quest software. PE-conjugated monoclonal rat antibodies against CD22, CD25 and c-kit, FITC-conjugated monoclonal rat antibody against CD19 and pan-MHCII were from BD (San Diego, CA). Cy5-conjugated goat anti-mouse μHC-specific antibodies were purchased from Southern Biotechnology (Birmingham, AL). FITC-conjugated monoclonal rat antibody against CD2 was obtained from Caltag (Burlingame, CA).

2.3. Cell sorting and cell culture

Pro-B cells were isolated by magnetic cell sorting with CD19-coated magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) from the bone marrow of either Rag2$^{-/-}$/dTg or Rag2$^{-/-}$/tTA mice that had received Tet (Sigma, Deisenhofen, Germany; 200 μg/ml) in the drinking water for 7 days. Cells were cultured for 72 h in RPMI 1640 (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum, pyruvate, glutamine and IL-7 in the presence and absence of Tet as previously described (Hess et al., 2001; Schuh et al., 2003). For isolation of primary pro-B as well as small and large pre-B cells, bone marrow cells of C57Bl/6 mice were membrane stained for either c-kit and CD19 or CD25, CD19 and surface μHC. Stained cells were sorted in a MoFlo high speed cell sorter (Dako cytomation, Glostrup, Denmark) to a purity >98%.

2.4. RNA-isolation, RT-PCR and Affymetrix microarray analyses

For microarray and RT-PCR analyses, total RNA was isolated from sorted B lymphoid populations and cell lines with the TRIZOL-Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA was cleaned up using the RNeasy-Kit (Qiagen, Hilden, Germany). RNA was processed as described (Wittmann et al., 2006) and microarray analyses using MOE430_2.0 *Affymetrix* DNA Chips were performed at the BioChip Facility of the University of Essen (Dr. L. Klein-Hitpass, Essen, Germany). For RT-PCR analyses, 0.5 μg of total RNA was reverse-transcribed using the Superscript-RT II Kit (Invitrogen, Karlsruhe, Germany). The cDNA was then amplified by PCR using transcript specific primers (Table 1).

2.5. Western blot analysis

For Western blot analysis, cells were lysed in NET/Triton X-100 buffer as described (Bradl and Jäck, 2001). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were developed with HRP-conjugated goat antibodies against mouse μHC (Southern Biotechnologies, Birmingham, AL) or with rabbit polyclonal antibodies against actin (Sigma, Deisenhofen, Germany) and HRP-conjugated goat anti-rabbit IgG sera (Biorad, Munich, Germany). A polyclonal serum against mouse KLF2 was obtained by immunizing rabbits with a KLF2-specific peptide (ERWPRNEPEAGGTDE) coupled to KLH (Pineda Antibody Services, Berlin, Germany). Anti-KLF2 serum was affinity-purified on a peptide column and the specificity of the serum was confirmed by Western blot analysis in KLF2-transduced fibroblasts and B-lymphoid cells, which resulted in the detection of two anti-KLF2-reactive bands (data not shown).

3. Results

3.1. Experimental system: primary pro-B cells with Tet-regulated expression of a transgenic μHC

To determine the impact of pre-BCR signals on the mRNA transcriptome in B-lymphoid precursors, we isolated CD19$^+$ B-lymphoid cells from the bone marrow of Rag2$^{-/-}$ mice, in which the expression of a transgenic μHC (tetop-μHC) can be

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