Requirement for CD100–CD72 interactions in fine-tuning of B-cell antigen receptor signaling and homeostatic maintenance of the B-cell compartment

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Abstract

Co-receptors on the B-cell surface regulate B-cell antigen receptor (BCR) signaling; however, it remains unclear how BCR signals are coordinated to maintain immune homeostasis. CD72, a negative regulator of B-cell responses, has immunoreceptor tyrosine-based inhibitory motifs within its cytoplasmic region, and the tyrosine phosphatase SHP-1 binds these sites. The natural ligand of CD72, CD100/Sema4D, belongs to the semaphorin family and induces the dissociation of SHP-1 from CD72, thereby switching off the negative signals of CD72. In the absence of CD100, BCR signals are significantly suppressed due to the constitutive association of SHP-1 with CD72, resulting in B-cell hyporesponsiveness. Here we show that CD100 regulates the sensitivity of the BCR by preventing the association of the CD72 with BCR, and this interaction is required for proper B-cell homeostasis. Consequently, as CD100-deficient mice age, they accumulate marginal zone B cells and develop high auto-antibody levels and autoimmunity. Collectively, our findings indicate that the strength of BCR signals is strictly tuned by the interaction of CD100 with CD72, and this interaction is essential for maintaining immunological homeostasis as well as generating a proper immune response.

Introduction

B-cell antigen receptors (BCRs) play central roles not only in the development of antibody responses but also in the regulation of B-cell development and homeostasis (1–4). Because the strength of BCR signals can generate many variable outcomes including cell death, survival, proliferation and differentiation, the threshold of BCR sensitivity is controlled by both stimulatory and inhibitory factors (4–6). In particular, inhibitory receptors such as FcγRIIB, CD22 and CD72, which have immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic region, are thought to be critical for preventing excessive and sometimes harmful antibody responses (6). CD72 negatively regulates BCR signals by recruiting a tyrosine phosphatase, SHP-1, to its ITIM (7), and CD72-deficient B cells are hyperresponsive following BCR stimulation (8). The transmembrane semaphorin CD100/Sema4D, a natural ligand for CD72, enhances B-cell responses by blocking negative signals arising from CD72 (9, 10). Indeed, in CD100-deficient B cells, SHP-1 is constitutively associated with CD72, resulting in impaired B-cell responses (9, 11). Consequently, CD100-deficient mice exhibit several immunological defects including impaired humoral immunity (11), and this phenotype is nearly the opposite of that seen in CD72-deficient mice (8). However, it remains to be determined how and to what extent the CD100–CD72 interaction regulates BCR signals and B-cell homeostasis.

In this study, we show that CD100 fine-tunes the strength of BCR signals by regulating the association of CD72 with the BCR, and the absence of the CD100–CD72 interaction affects B-cell survival, resulting in reduced B-cell turnover and the accumulation of marginal zone B cells. Consequently, CD100-deficient...
mice develop autoimmunity with age, demonstrating an essential role for the interaction of CD100 with CD72 in the maintenance of homeostasis in the immune system.

**Methods**

**Mice**

CD100-deficient mice were crossed for more than eight generations with C57BL/6 mice (11, 12). All mice were maintained in our animal facility and cared for in accordance with institutional guidelines for animal welfare.

**Antibodies**

Anti-CD72 (H-96), anti-phosphotyrosine (PY99), anti-SHP-1 (C-19), anti-CD79b (V-18), anti-CD79a (V-20), anti-Syk (N-19) and anti-BLNK (2B-11) antibodies were purchased from Santa Cruz Biotechnology, and goat anti-mouse F(ab')2 fragment to mouse IgM and intact anti-mouse IgM antibodies were purchased from ICN Pharmaceuticals. Anti-FcγRIIB mAb (2.4G2) was purchased from PharMingen. Anti-phospho-Erk (E10) was purchased from Signal Transduction Laboratories.

**Immunoprecipitation and western blotting**

B cells were negatively prepared against anti-Thy-1, anti-CD11c and anti-CD11b using MACS (Miltenyi Biotech). B cells (2 × 10⁷ cells) from wild-type or CD100-deficient mice (6–10 weeks of age) were stimulated with F(ab')2 anti-μ (10 μg ml⁻¹) in the absence or presence of mCD100-Fc (40 μg ml⁻¹) (9) for 1 min and lysed in buffer containing 1% Nonidet P-40 or digitonin, 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM Na₂VO₄, 0.5 mM phenylmethylsulfonylfluoride, 5 μg ml⁻¹ aprotinin and 5 μg ml⁻¹ leupeptin. For immunoprecipitation, cell lysates were pre-cleared with protein G-Sepharose beads (Amersham Pharmacia Biotech), followed by incubation with protein G-Sepharose beads plus anti-CD72, anti-CD79a, anti-CD79b, anti-Syk, anti-BLNK or anti-FcγRIIB for 3 h at 4°C. After washing with lysis buffer four times, immunoprecipitates were subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose membranes. Membranes were immunoblotted with anti-CD72, anti-CD79a or anti-phosphotyrosine antibodies, and the blots were developed by enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) following the manufacturer's protocol.

**Calcium measurement**

B cells (1 × 10⁷ cells ml⁻¹) in PBS containing 20 mM HEPES, pH 7.2, 5 mM glucose, 0.025% BSA and 1 mM CaCl₂ were loaded with 3 μM Fura-2AM at 37°C for 45 min. Cells were washed twice and adjusted to 1 × 10⁶ cells ml⁻¹. Emission at 510 nm was monitored under excitation of the cell suspension at two different wavelengths (340 and 380 nm) with a fluorescence spectrophotometer (mode RF-1500; Shimazu, Kyoto, Japan) at 37°C.

**B-cell proliferation assay**

B cells were prepared from splenocytes using MACS. B cells (1 × 10⁶ ml⁻¹) were cultured in a 96-well plate in the presence of the intact or F(ab')2 forms of rabbit IgG to mouse IgM. For B-cell proliferation assays, cultures were pulsed with 2 μCi of [³H]thymidine ([³H]TdR) for the last 16 h of culture period, and [³H]TdR incorporation was measured.

**Flow cytometry and antibodies**

One million cells from the indicated tissues were stained with the following antibodies: FITC-conjugated anti-B220 (RA3-6B2), anti-CD21 (B3B4), PE-conjugated anti-Thy-1 (30H12), anti-CD21 (7G6) or biotinylated anti-CD21 plus allophycoerythrin-conjugated streptavidin. These antibodies and reagents were purchased from PharMingen. Data analysis was performed using FlowJo software (Tree Star).

**Titers of serum antibodies**

Rheumatoid factor (RF) for mouse Ig of both IgM and IgG classes, anti-dsDNA antibodies and anti-ssDNA antibodies were measured using commercial ELISA kits (Shibayagi, Japan) as described (13).

**Histology**

Tissues were fixed for light microscopy in formalin solution for 24 h and embedded in paraffin. Sections were stained with hematoxylin–eosin. For immunofluorescence staining, frozen sections were incubated with FITC-conjugated anti-CD45R/B220 (RA3-6B2), PE-conjugated anti-CD11c (HL-3) and biotinylated anti-Thy-1 (30H12) followed by Cy5-conjugated streptavidin (Jackson Laboratory). Deposition of immune complexes at glomeruli was detected with FITC-conjugated goat anti-mouse IgG antibody (DAKO). Slides were analyzed with a confocal laser scanning microscope (Zeiss).

**Cell death assay**

Apoptotic cells were evaluated by the annexin V–FITC apoptosis detection kit (BD Biosciences) following the manufacturer’s protocols, and phosphatidylinositol was used for detecting dead cells by flow analysis. The percentage of dead cells was determined by flow cytometry.

**5-Bromo-2-deoxyuridine assay**

Mice were injected intra-peritoneally with 1 mg 5-bromo-2-deoxyuridine (BrdU) (Sigma) dissolved in PBS and were fed with drinking water containing 1 mg ml⁻¹ BrdU for different periods of time (14). The BrdU-containing drinking water was light protected and exchanged every 4 days. Cells were then prepared from the spleen at indicated periods and stained with PE-conjugated B220. Thereafter, the cells were fixed in 70% ethanol for 30 min. For determining the percentage of BrdU-positive cells, cells were washed twice in PBS and resuspended in 2 N HCl/0.5% Triton-100 and left for 30 min to denature DNA. Cells were collected by centrifugation and were then neutralized by re-suspension in 0.1 M borate buffer, pH 8.5. Sample tubes were then filled with PBS/0.5% Tween 20, and the cells were washed twice with the same buffer. Cells were stained with FITC-conjugated anti-BrdU (PharMingen) and analyzed by flow cytometry.

**Results and discussion**

We previously showed that CD100 negatively regulates both tyrosine phosphorylation of CD72 and its association...
with SHP-1 in anti-μ-stimulated B cells (9). However, the mechanism by which CD100 induces dephosphorylation of CD72 and how the binding of CD100 to CD72 affects BCR signals remain unknown. Therefore, we initially analyzed the effect of CD100 on the physical interaction of CD72 with the BCR complex. As shown in Fig. 1(A), the association of CD72 with CD79α, a component of the BCR complex, was clearly observed in wild-type B cells following BCR stimulation. However, in CD100-deficient B cells, CD72 was found to be associated with CD79α even before BCR stimulation, and BCR stimulation enhanced this association significantly. We next examined the effect of exogenous CD100 on CD72–BCR association. As shown above, anti-μ stimulation induced the association of CD72 with CD79α in wild-type B cells, and this was inhibited by inclusion of recombinant CD100-Fc protein (Fig. 1B). Collectively, these results imply that CD100 binding induces the sequestration of CD72 from kinase-rich BCR signalosomes, and this may lead to the dephosphorylation of CD72 and dissociation of SHP-1. We then analyzed the phosphorylation of signaling molecules downstream of the BCR in CD100-deficient B cells. Following BCR stimulation, CD79b, Syk, BLNK, FcγRIIB and Erk-2 were rapidly tyrosine phosphorylated in wild-type B cells (5) (Fig. 1C). In contrast, their phosphorylation was severely impaired in CD100-deficient B cells (Fig. 1C). Additionally, Ca2+ mobilization induced by BCR stimulation was also reduced in CD100-deficient B cells (Fig. 1D). Taken together, these results suggest that constitutive negative signaling by CD72 attenuates BCR signals in CD100-deficient B cells.

BCR signaling in CD100-deficient B cells was clearly altered. We next examined the cellular consequences of this altered signaling by analyzing anti-μ-induced cell death and proliferation in these cells. Hypercrosslinking of the BCR by immobilized anti-IgM antibodies induced cell death in a large proportion of wild-type B cells (Fig. 2A) (4). In contrast, CD100-deficient B cells were resistant to anti-IgM-induced cell death (Fig. 2A). Moderate cross-linking of the BCR by soluble F(ab′)2 fragments against IgM induced proliferative responses in wild-type B cells, while these responses were consistently reduced in magnitude in CD100-deficient B cells (Fig. 2B). When B cells were stimulated with intact anti-IgM capable of cross-linking the BCR and FcγRIIB, wild-type B cells failed to proliferate due to the negative feedback arising from FcγRIIB (Fig. 2C) (15). However, CD100-deficient B cells underwent substantial proliferation (Fig. 2C), indicating a defective inhibitory signal from FcγRIIB. Consistent with this hypothesis, we found poor phosphorylation of FcγRIIB in CD100-deficient B cells stimulated with anti-IgM (Fig. 1C). Collectively, these results suggest that reduced signals downstream of the BCR affect not only antigen-induced proliferation and cell death but also negative feedback through FcγRIIB in CD100-deficient B cells.

Altered signals downstream from antigen receptors can affect lymphocyte turnover in vivo. To examine cell turnover, wild-type and CD100-deficient mice were fed BrdU, and the proportion of labeled B cells in the spleen was measured (Fig. 3A). Splenic BrdU uptake in CD100-deficient mice was slower than that seen in wild-type mice during continuous feeding of BrdU (pulse). Conversely, BrdU-positive B cells disappeared more slowly from CD100-deficient mice compared with wild-type animals (chase). Thus, the overall turnover of B cells in CD100-deficient mice was reduced compared with wild-type mice. However, the overall number of spleen cells did not differ between wild-type and CD100-deficient mice, although they fluctuated among aged mice (data not shown). We next monitored the cell-surface phenotypes of spleen cells in CD100-deficient mice (Fig. 3B). Interestingly, as CD100-deficient mice aged, the proportion of CD21highCD23low marginal zone B cells gradually increased (Fig. 3B). Although the precise mechanism regulating this expansion remains unclear, reduced BCR signal strength may affect the homeostatic control of B-cell compartments. The requirements for BCR signals differ among B-cell subsets. For instance, the expansion of marginal zone B cells is sometimes observed in mice with defective BCR signals (4, 16–18). In contrast, the development of B1 cells is highly dependent on BCR signals, as clearly shown by increased numbers of B1 cells in mice lacking inhibitory receptors such as CD22 and CD72 and a decrease in

**Fig. 1.** Impaired BCR-mediated signals in CD100-deficient B cells. (A) CD72 is constitutively associated with the BCR in CD100-deficient B cells. (B) The association of CD72 with the BCR is blocked by CD100. (C) Impaired phosphorylation of BCR-mediated signaling in CD100-deficient B cells. (D) Reduced calcium mobilization following F(ab′)2 anti-μ stimulation in CD100-deficient B cells. For (A) and (B), wild-type or CD100-deficient B cells were stimulated with anti-μ F(ab′)2 fragment in the absence or presence of CD100-Fc. Cell lysates (1% Nonidet P-40) were immunoprecipitated with anti-CD72 or anti-CD79a and blotted with anti-CD72 or CD79a (A and B) or immunoprecipitated with anti-CD79b, anti-Syk, anti-BLNK or anti-FcγRIIB and blotted with anti-phosphotyrosine antibodies (C). For Erk-2, cell lysates were blotted with anti-pErk-2. For (D), emission at 510 nm was monitored with a fluorescence spectrophotometer.
CD100-deficient mice (8, 11, 19, 20). These findings strongly suggest that a higher BCR-signaling threshold promotes the development or survival of marginal zone B cells but is detrimental for the development of B1 cells in CD100-deficient mice.

To our surprise, the apparent expansion of marginal zone B cells was accompanied by the production of a variety of auto-antibodies, including anti-ssDNA, anti-dsDNA, RFs (Fig. 4A), anti-Sjogren’s Syndrome A and anti-ribonucleoprotein (Supplementary Table 1, available at International Immunology Online), in CD100-deficient mice, although such auto-antibodies were not detectable by 25 weeks of age. Furthermore, histological examination of aged CD100-deficient mice showed marked perivascular leukocytic infiltration in several tissues, including the salivary gland (75%, n = 20), liver (30%, n = 20) and kidney (25%, n = 15) (Fig. 4B). Additionally, glomeruli from these mice exhibited thickened glomerular capillary basement membranes with accompanying periodic acid silver-positive material (Fig. 4C). We also observed prominent glomerular staining with antibodies to mouse IgG (Fig. 4C), suggesting that these lesions may arise from circulating auto-antibodies. Finally, mice lacking both CD100 and CD72 had no evidence of autoimmune disease (Fig. 4A) or expansion of marginal zone B cells (data not shown). Although B cells from CD72-deficient mice are hyperresponsive, they did not develop auto-antibodies up to 6 months of age, and a limited number of animals produced substantial amounts of auto-antibodies accompanied by the significant accumulation of B1 B cells over 1 year of age (data not shown). These findings strongly suggest that the constitutive association of CD72 with the BCR might be responsible for the development of autoimmunity in aged CD100-deficient mice. Immunohistochemical analysis revealed that the majority of infiltrating cells in the salivary glands were B cells (Supplementary Figure 1, available at International Immunology Online), although T cells and dendritic cells were also observed. Interestingly, the infiltrating B cells in the salivary gland were CD21highCD23low (Fig. 4D). In addition, when
CD21<sup>high</sup>CD23<sup>low</sup> marginal zone or CD21<sup>low</sup>CD23<sup>high</sup> follicular B cells purified by cell sorting were cultured in vitro, the marginal zone B cells predominantly produced auto-antibodies (Supplementary Figure 2, available at International Immunology Online), suggesting that the large population of marginal zone B cells was the primary source of auto-antibodies in CD100-deficient mice. We did not observe any significant differences in the expression of T-cell activation markers, including CD25, CD69 and CD45RB (data not shown). To further examine the possible involvement of T-cell autoreactivity in the development of autoimmunity, we transferred wild-type or CD100-deficient T cells into nude mice. Two months after transfer, no elevations of serum auto-antibody levels were seen in mice receiving either wild-type or CD100-deficient T cells into nude mice. Two months after transfer, no elevations of serum auto-antibody levels were seen in mice receiving either wild-type or CD100-deficient T cells. Although mice receiving CD25-depleted T cells either from wild-type or CD100-deficient mice had elevations of serum auto-antibodies (Supplementary Figure 3, available at International Immunology Online) (21). In addition, CD25<sup>+</sup> T cells from CD100-deficient mice exhibited regulatory activity comparable to those from wild-type mice (Supplementary Figure 3, available at International Immunology Online), thus excluding the possible involvement of increased T-cell autoreactivity in CD100-deficient mice. Impaired FcγRIIB-mediated inhibitory signals might directly promote the development of autoimmunity in CD100-deficient mice. Indeed, the absence of FcγRIIB results in autoimmunity (22, 23). However, this phenotype is strain dependent and is not seen in Balb/c or 129 strains of mice, whereas CD100-deficient mice on the Balb/c background still develop autoimmunity (data not shown). In addition, FcγRIIB-deficient mice do not have an accumulation of marginal zone B cells. Given this data, impaired inhibitory signals mediated by FcγRIIB are likely not a direct explanation for the development of autoimmunity in CD100-deficient mice.

Here we clearly demonstrate that the interaction of CD100 with CD72 regulates BCR signal strength to maintain B-cell homeostasis. Both the loss of negative signaling molecules and the increased activity of positive co-receptors such as CD19 are associated with the development of autoreactivity (6, 24, 25). When the balance between positive and negative signals is shifted, increased BCR signal strength has the potential to evoke autoimmunity. In contrast, we here present evidence that reduced strength of BCR signals can do the same. Our findings, therefore, imply that a basal level of CD100 on B cells during quiescence is critical for the fine-tuning of BCR signals by the CD100-FcγRIIB axis. Consequently, it appears that CD100, the expression of which...
Development of autoimmunity in CD100-deficient mice is significantly up-regulated on the surface of T cells and B cells following stimulation, suppresses an inhibitory signal from CD72 to reduce the signaling threshold of various receptors including the BCR and CD40. In this context, CD100 seems to be necessary for both eliciting immune responses and maintaining immunological homeostasis. Our findings provide new insights into the regulation of immune homeostasis.

**Supplementary data**

Supplementary data are available at International Immunology Online.

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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>BCR</td>
<td>B-cell antigen receptor</td>
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<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
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<tr>
<td>[3H]TdR</td>
<td>[3H]thymidine</td>
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<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
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<td>RF</td>
<td>Rheumatoid factor</td>
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**References**