

## Modulation of Peripheral B Cell Tolerance by CD72 in a Murine Model

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**Objective.** B cells play a dominant role in the pathogenesis of several autoimmune diseases, including systemic lupus erythematosus. It is not well understood how B cell signaling contributes to autoantibody production. The goal of this study was to elucidate the role of CD72 in modulating B cell receptor (BCR)–mediated tolerogenic signaling and peripheral B cell tolerance.

**Methods.** A mouse model utilizing hen egg lysozyme (HEL) “anergic” B cells was studied. CD72-deficient mice carrying the BCR-specific  $Ig^{HEL}$  and/or soluble HEL ( $sHEL$ ) transgenes were generated by breeding  $Ig^{HEL}$ -transgenic MD4 mice and/or  $sHEL$ -transgenic ML5 mice with congenic, CD72-deficient C57BL/6J mice. Normal and anergic B cells were isolated for analyses of B cell signaling. Aged wild-type and CD72-deficient mice were also examined for autoimmune phenomena.

**Results.** In the absence of CD72, anergic B cells inappropriately proliferated and survived in response to stimulation with self antigen. Biochemical analyses indicated that in anergic B cells, CD72 dominantly down-regulated BCR signaling to limit the antigen-induced elevation in  $[Ca^{2+}]_i$  and the activation of NFATc1,

NF- $\kappa$ B, MAPK, and Akt. Mechanistically, CD72 was associated with, and regulated, the molecular adaptor Cbl-b in anergic B cells, suggesting that Cbl-b may play a role in mediating the negative effects of CD72 on BCR signaling. Moreover, in aged CD72-deficient mice, spontaneous production of antinuclear and anti-double-stranded DNA autoantibodies and features of lupus-like autoimmune disease were observed.

**Conclusion.** CD72 is required to maintain B cell anergy and functions as a regulator of peripheral B cell tolerance. Thus, altered CD72 expression may play a role during the development of systemic lupus erythematosus.

B cell self tolerance is maintained by several mechanisms, including deletion, receptor editing, and anergy (1–4). Mechanisms of peripheral tolerance are thought to be important, because a considerable proportion of self-reactive B cells escape central tolerance mechanisms and emerge into the periphery (5). Breakdown of peripheral B cell tolerance results in autoantibody production, which contributes to autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis, Sjögren’s syndrome, and rheumatoid arthritis.

SLE is an autoimmune disorder characterized by multiorgan inflammation and deregulated production of autoantibodies, including antinuclear antibodies (ANAs) and anti-double-stranded DNA (anti-dsDNA) antibodies (6). B cell-targeted therapies have shown great promise in the treatment of SLE (7). B cell coreceptors, such as CD19, CD22, CD72, Fc $\gamma$  receptor type IIb (Fc $\gamma$ RIIb), CD45, and CD5, tightly regulate B cell receptor (BCR) signaling to fine-tune B cell responses in encounters with antigens in the periphery (8,9). In fact, several of these coreceptors, including CD22, Fc $\gamma$ RIIb, and CD45, have been directly linked to SLE (10–13).

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CD72, a 45-kd type II transmembrane glycoprotein, is constitutively expressed on developing and mature B cells, but not on terminally differentiated plasma cells. The cytoplasmic domain of CD72 contains an immunoreceptor tyrosine-based inhibition motif (ITIM) that binds SH2-containing protein tyrosine phosphatase 1 (SHP-1), and an ITIM-like motif that binds Grb2 (14). Our recent data indicate that CD72 plays a negative role in regulating BCR-induced signaling in primary mature B cells (9), but CD72 may also transmit a positive signal independent of the BCR (15). CD100, a ligand for CD72, can turn off the negative effect of CD72 by inhibiting phosphorylation of CD72 and, in turn, disrupting the SHP-1/CD72 complex (16,17). Although a link between CD72 and SLE has been suggested (18–20), the role of CD72 in autoimmunity is not well defined and the molecular mechanisms by which CD72 might influence B cell tolerance are unknown.

Cbl-b, an E3 ubiquitin ligase, has been shown to regulate B cell tolerance. Cbl-b-deficient B cells are hyperreactive to BCR ligation, and mice lacking Cbl-b produce anti-dsDNA autoantibodies (21). Clinically, Cbl-b-deficient mice are susceptible to spontaneous and induced autoimmune diseases (22–24), and mice with B cell-specific ablation of both Cbl and Cbl-b manifest SLE-like autoimmune disease (25), suggesting that Cbl-b modulates peripheral T cell and B cell tolerance. However, the proteins that anchor Cbl-b to the membrane in close proximity to the BCR signalosome and that regulate Cbl-b function in B cells have not been identified.

In the present study we examined the direct role of CD72 in peripheral B cell tolerance and in the development of autoimmunity. Our findings indicate that CD72 is an essential negative regulator of BCR signaling in self-reactive B cells, and that CD72 interacts with and regulates Cbl-b. As indicated by multiple criteria, CD72 functions as a physiologic regulator of B cell anergy, and inappropriate regulation of the CD72-signaling network may contribute to the development of autoimmune diseases such as SLE.

## MATERIALS AND METHODS

**Mice.** A well-defined mouse model of hen egg lysozyme (HEL) “anergic” B cells was utilized (26). CD72-deficient (CD72<sup>-/-</sup>) mice carrying the *Ig<sup>HEL</sup>* and/or soluble *HEL* (*sHEL*) transgenes were generated by breeding *Ig<sup>HEL</sup>*-transgenic (Tg) MD4 mice and/or *sHEL*-Tg ML5 mice with congenic, CD72<sup>-/-</sup> C57BL/6J mice (27). All CD72<sup>-/-</sup> mice studied were backcrossed onto the C57BL/6 strain for at least 8 generations. Experiments were performed in accordance with institutional and national guidelines. All mice used in this study were 8–12 weeks of age.

**Cell preparation.** For determination of calcium flux, B cells were obtained from total splenocytes. For all other in vitro assays, mature B cells were purified from either the spleen or lymph nodes by magnetic-activated cell sorting using CD43 microbead negative selection (Miltenyi Biotec, Auburn, CA). The purity of the B cell preparations was always higher than 95%.

**Antibodies.** Phosphorylated Akt (Ser<sup>473</sup>) antibodies were from Cell Signaling (Beverly, MA). Antibodies to NFATc1 (7A6), Ig $\beta$  (HM79b), CD40 (HM40-3), and Bcl-x<sub>L</sub> were from BD PharMingen (San Diego, CA). Antibodies to CD72 (H-96), cyclin D2 (M-20), p27<sup>KIP</sup> (C-19), Cbl-b (C-20), Syk (N-19, LR), and Myc (N-262) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies (mAb) for detection of tyrosine phosphorylation (expressed as pY levels) were from Upstate Biotechnology (4G1 mAb; Charlottesville, VA). Anti-CD72 mAb (K10.6) were from Caltag (Burlingame, CA). Rabbit serum for analyses of Ig $\alpha$  was kindly provided by John C. Cambier (National Jewish Medical and Research Center, Denver, CO).

**Detection of ANAs and kidney immune complex deposition.** For analyses of glomerular immune complexes, frozen kidney sections were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA). More than a dozen glomeruli on each kidney section were evaluated. For detection of ANAs, HEp-2 cells were incubated with mouse serum, followed by staining with FITC-conjugated anti-mouse IgG antibodies. Sera from lupus-prone MRL-lpr/lpr mice were used as a positive control. To determine in vitro production of anti-HEL IgM antibodies, purified naive mature (CD43<sup>-</sup>) B cells were incubated with or without HEL antigen (0.5  $\mu$ g/ml) for 3 days, and the culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). Levels of anti-dsDNA antibodies in 9-month-old mice and serum levels of anti-HEL IgM antibodies in 10-week-old mice were determined by ELISA, as previously reported (28,29).

**Analyses of cell proliferation, apoptosis, cell cycle, and calcium flux.** B cell proliferation was determined as the rate of <sup>3</sup>H-thymidine incorporation. In addition, flow cytometry was used to assess bromodeoxyuridine (BrdU) incorporation (BrdU Flow kit; BD PharMingen) as previously described (9). For detection of apoptosis, purified splenic B cells (5  $\times$  10<sup>5</sup>/well) were stimulated with HEL (0.5  $\mu$ g/ml) with or without interleukin-4 (IL-4) (10 ng/ml) or anti-CD40 (10 ng/ml) for 24 hours. For cell cycle analyses, staining with annexin V and propidium iodide (PI) was performed according to the manufacturer's protocol (BioVision, Mountain View, CA). For determination of the cell cycle and antiapoptotic protein expression, purified B cells (3  $\times$  10<sup>6</sup>/well) were stimulated with HEL (0.5  $\mu$ g/ml) in various culture conditions, and analyzed by Western blotting. For determination of surface CD72 expression, purified naive mature (CD43<sup>-</sup>) B cells were stimulated with HEL antigen (0.5  $\mu$ g/ml) for varying times, and analyzed with fluorescence-activated cell sorting. Measurement of Ca<sup>2+</sup> mobilization (referred to as [Ca<sup>2+</sup>]<sub>i</sub>) was performed as described previously (9).

**ELISAs for NFATc1 and NF- $\kappa$ B p65 activity.** The DNA binding activities of activated NFATc1 and activated NF- $\kappa$ B were measured as described previously (9). Briefly, to determine NFATc1 activity, purified B cells from the lymph nodes were stimulated with HEL (1  $\mu$ g/ml) for 1 minute or 1.5

minutes at 37°C. Nuclear protein was then extracted and assayed by ELISA (TransAm NFATc1; Active Motif, Carlsbad, CA). To determine NF- $\kappa$ B p65 activity, purified splenic B cells were stimulated with HEL (1  $\mu$ g/ml) for 5 minutes or 15 minutes at 37°C. Nuclear protein was then assayed by ELISA (TransAm NF- $\kappa$ B Chemi; Active Motif).

**Kinase assays for ERK and Akt.** The activities of ERK and Akt were determined using a kinase assay kit according to the manufacturer's protocol (Cell Signaling). For analysis of ERK, mature splenic B cells ( $3.2 \times 10^7$ /ml) were stimulated with HEL (2  $\mu$ g/ml) and lysed. Activated ERK was then immunoprecipitated with immobilized phospho-ERK antibodies (Thr<sup>202</sup>/Tyr<sup>204</sup>) overnight at 4°C, followed by assessment with the kinase assay. For analysis of Akt, mature splenic B cells ( $3 \times 10^7$ /ml) were stimulated with HEL (3  $\mu$ g/ml at 37°C for 5 minutes) and lysed. Activated Akt was immunoprecipitated with immobilized Akt antibodies (Ser<sup>473</sup>) for 16 hours at 4°C, followed by assessment with the kinase assay. The reactions were terminated with sodium dodecyl sulfate sample buffer, and the protein samples were analyzed by Western blotting.

**Western blotting.** To detect phosphorylated or dephosphorylated NFATc1, mature splenic B cells were stimulated with HEL. The cells were then analyzed with specific mAb (7A6) on Western blot.

CD72, Cbl-b, Syk, Ig $\alpha$ , I $\kappa$ B $\alpha$ , and pY antibody levels were determined by Western blotting and an enhanced chemiluminescence system. Purified B cells were stimulated with HEL (1.5–3  $\mu$ g/ml), and the target proteins were then immunoprecipitated with specific antibodies for 4–5 hours at 4°C and denatured for further analyses. For experiments involving preligation of CD72, purified B cells were incubated with anti-CD72 mAb (K10.6) (5–10  $\mu$ g/ml) on ice for 10 minutes before stimulation with HEL antigen (1.5  $\mu$ g/ml).

**Histopathology.** Immediately after the mice were killed with CO<sub>2</sub>, tissue samples were collected and fixed in 10% neutral buffered formalin. The tissue was routinely processed into 4 $\mu$  sections in paraffin, and stained with hematoxylin and eosin. All slides were examined by a board-certified veterinary pathologist (CRD), in a blinded manner, using light microscopy. Organs were evaluated for the extent of changes in specific histopathologic features of inflammation, where 0 = no change, 1 = minimal to mild change, 2 = moderate change, and 3 = severe change.

**Genotyping analysis.** Genotyping was carried out by polymerase chain reaction (PCR) analyses of genomic DNA, using microsatellite markers of simple sequence-length polymorphisms distributed among the 19 autosomal chromosomes. Control DNA for PCR analysis of each marker was obtained from a C57BL/6ByJ mouse and from E14.1 embryonic stem cells.

**Statistical analysis.** Student's *t*-test was used to determine statistically significant differences between groups. *P* values less than or equal to 0.05 were considered significant.

## RESULTS

**Phenotypic characterization of CD72-deficient anergic B cells.** An HEL anergic B cell model in mice (26) was utilized to investigate whether CD72 plays a role in B cell anergy. C57BL/6 mice carrying Ig<sup>HEL</sup>

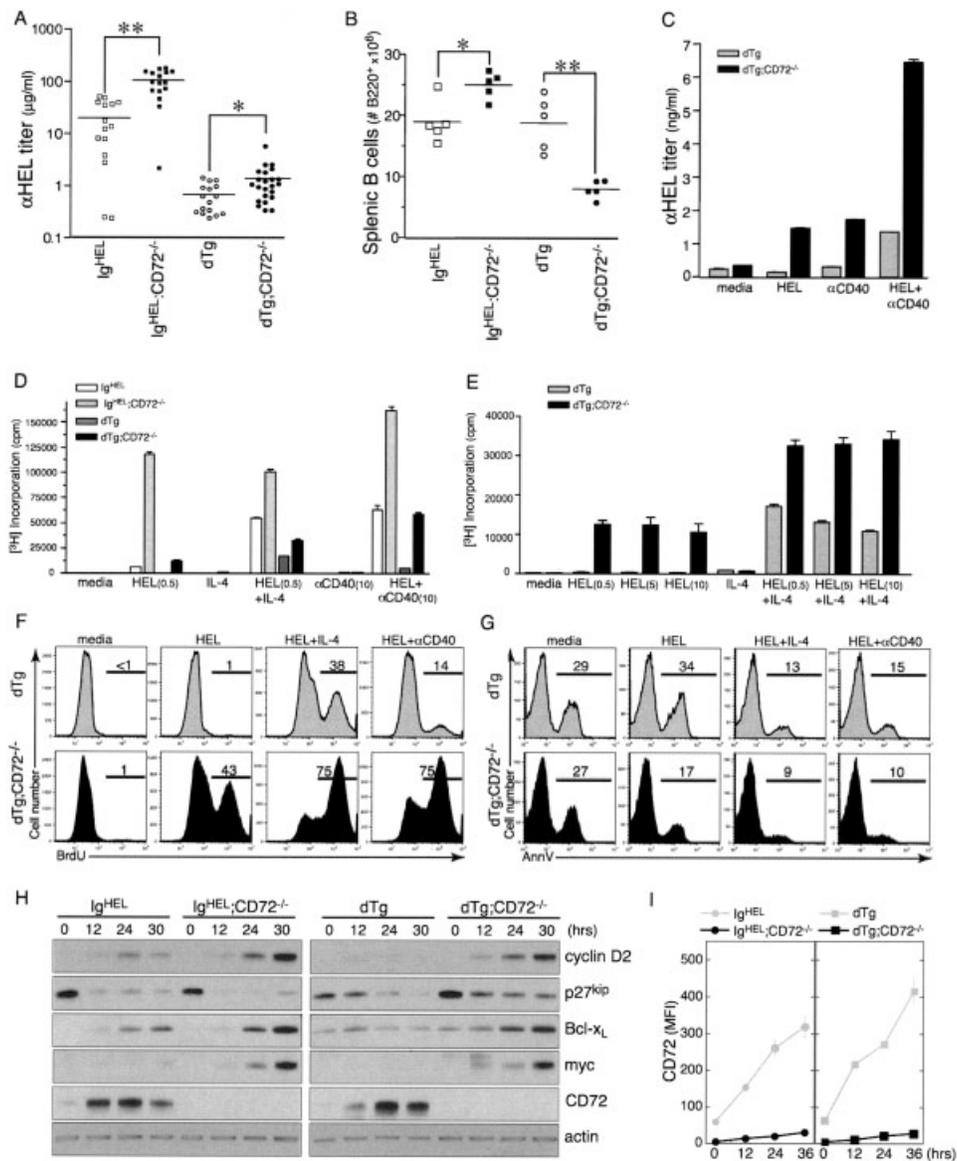
transgenes (BCR specific to HEL) and *sHEL* transgenes were bred with CD72<sup>-/-</sup> mice to assess the function of CD72 in the presence and absence of a defined self antigen (HEL). Control and CD72<sup>-/-</sup> mice expressing Ig<sup>HEL</sup> alone (Ig<sup>HEL</sup>-Tg) or expressing both Ig<sup>HEL</sup> and *sHEL* transgenes (double-transgenic [dTg]) were used to study the role of CD72 in regulating self tolerance. B cells from Ig<sup>HEL</sup>-Tg mice are capable of being immunogenically activated by HEL antigen, whereas dTg B cells show tolerance as a result of continual interaction with the self antigen (30). Moreover, anergic B cells from dTg mice fail to increase [Ca<sup>2+</sup>]<sub>i</sub> or to activate NF- $\kappa$ B, and also are unable to proliferate and differentiate after short-term stimulation with self antigen (30,31).

To examine the phenotypic similarities and differences in B cells between the control and CD72<sup>-/-</sup> groups of Ig<sup>HEL</sup>-Tg and dTg mice, staining for cell surface markers was performed. Compared with B cells from the control and CD72<sup>-/-</sup> Ig<sup>HEL</sup>-Tg mice, B cells from the respective groups of dTg mice were observed to be IgM<sup>low</sup> and CD5<sup>high</sup>, consistent with the published phenotype of anergic B cells in this dTg mouse model (32,33). However, B cells from the dTg CD72<sup>-/-</sup> mice, as compared with the dTg control mice, had, in addition, a mild decrease in the levels of IgM and IgD antibodies as well as a reduction in the BCR-binding capacity of HEL, but had unaltered expression of B220, CD5, CD16/32, CD19, and CD22 (results not shown).

To determine whether CD72 affects antibody production in this HEL model, we examined anti-HEL antibody production in vivo and in vitro (Figures 1A–C). In the serum, anti-HEL antibody levels were significantly higher in the dTg CD72<sup>-/-</sup> mice than in the dTg control mice (1.5  $\mu$ g/ml versus 0.6  $\mu$ g/ml; *P* < 0.04) (Figure 1A).

To determine whether the difference in anti-HEL antibody production could be attributable to a difference in B cell numbers in vivo, we examined the percentages and absolute numbers of splenic B cells in the mice. Ig<sup>HEL</sup>-Tg CD72<sup>-/-</sup> mice had an increased percentage and absolute number of B cells compared with Ig<sup>HEL</sup>-Tg control mice. Surprisingly, dTg CD72<sup>-/-</sup> mice, compared with dTg control mice, had a decreased percentage of B cells (23% versus 32%) (results not shown) and decreased absolute number of B cells ( $8 \times 10^6$  versus  $19 \times 10^6$ ; *P* < 0.001) (Figure 1B). Thus, it appears that, given an equivalent number of B cells in vivo, dTg mice with CD72 deficiency will have a >6-fold increase in anti-HEL autoantibody production as compared with dTg control mice.

Antibody production was also examined in vitro. Ig<sup>HEL</sup>-Tg CD72<sup>-/-</sup> B cells stimulated with HEL in vitro



**Figure 1.** Analyses of B cell proliferation and B cell death in vitro in *Ig<sup>HEL</sup>*-transgenic (Tg) and double-transgenic (dTg) *CD72<sup>-/-</sup>* mice compared with *Ig<sup>HEL</sup>*-Tg and dTg control mice. **A**, Serum levels of anti-hen egg lysozyme (anti-HEL) antibodies ( $n \geq 15$  per group). **B**, Total numbers of splenic B cells ( $n = 5$  per group). Horizontal bar indicates the mean. \* =  $P < 0.04$ ; \*\* =  $P < 0.001$ . **C**, Anti-HEL autoantibody production in mature splenic dTg B cells after stimulation with HEL antigen with or without anti-CD40. Bars show the mean and SEM of duplicate cultures. **D**, Assessment of B cell proliferation, using  $^3\text{H}$ -thymidine incorporation, after stimulation with HEL (0.5  $\mu\text{g}/\text{ml}$ ) with or without interleukin-4 (IL-4) or anti-CD40 (10 ng/ml each). **E**, Proliferation of dTg B cells after antigen stimulation with various concentrations of HEL (0.5  $\mu\text{g}/\text{ml}$ , 5  $\mu\text{g}/\text{ml}$ , or 10  $\mu\text{g}/\text{ml}$ ) with or without IL-4. Bars in **D** and **E** show the mean and SD of triplicate cultures. **F**, Flow cytometric analysis of bromodeoxyuridine-positive (BrdU<sup>+</sup>) cells after stimulation with HEL with or without IL-4 or anti-CD40. Values over bars are the percentage of BrdU<sup>+</sup> cells. **G**, Flow cytometric analysis of cell death using annexin V (AnnV) staining. Values over bars are the percentage of annexin V<sup>+</sup>-positive cells. **H**, Cytoplasmic expression of cyclin D2, p27<sup>kip</sup>, Bcl-x<sub>L</sub>, Myc, and CD72. Actin was used to show equal loading. **I**, Cell surface expression of CD72 after stimulation with HEL in vitro. Bars show the mean  $\pm$  SD representative results from 1 of 2 independent experiments in triplicate cultures, with values expressed as the mean fluorescence intensity (MFI).

produced 10-fold more anti-HEL antibodies than did  $Ig^{HEL}$ -Tg control B cells (46.75 ng/ml versus 4.35 ng/ml) (results not shown). Similarly, dTg  $CD72^{-/-}$  B cells stimulated with HEL in vitro produced 10-fold more anti-HEL autoantibodies than did dTg anergic B cells from control mice (1.5 ng/ml versus 0.15 ng/ml) (Figure 1C), indicating that dTg  $CD72^{-/-}$  anergic B cells undergo a partial breakdown in tolerance when stimulated with self antigen in vitro. Collectively, these results indicate that antigenic stimulation of dTg anergic B cells from  $CD72^{-/-}$  mice, but not those from control mice, leads to augmented autoantibody production both in vitro and in vivo.

**Enhanced proliferation and decreased cell death of  $CD72$ -deficient anergic B cells.** To examine whether  $CD72$  plays a role in the maintenance of B cell tolerance, we analyzed the in vitro proliferation and survival of B cells from dTg control and dTg  $CD72^{-/-}$  mice. Consistent with the results from our previous study (9),  $Ig^{HEL}$ -Tg B cells from  $CD72^{-/-}$  mice showed a greater extent of proliferation than did  $Ig^{HEL}$ -Tg control B cells (Figure 1D). Similarly, dTg B cells from  $CD72^{-/-}$  mice displayed enhanced  $^3H$ -thymidine incorporation after stimulation with HEL, whereas dTg anergic B cells from control mice did not (Figures 1D and E). In fact, dTg  $CD72^{-/-}$  B cells incorporated  $^3H$ -thymidine to an extent comparable with that of  $Ig^{HEL}$ -Tg control B cells, after stimulation with HEL. Moreover, dTg B cells from  $CD72^{-/-}$  mice were not hyperproliferative in response to IL-4 alone or to anti-CD40 alone, but showed enhanced  $^3H$ -thymidine incorporation when either of these stimuli was combined with HEL antigen (Figures 1D and E).

To determine whether  $CD72$  deficiency affects the proliferation and/or death of anergic B cells, in vitro assays of BrdU incorporation and cell viability were performed. Similar to the differences observed between  $Ig^{HEL}$ -Tg control and  $Ig^{HEL}$ -Tg  $CD72^{-/-}$  mice (9), B cells from dTg  $CD72^{-/-}$  mice showed a dramatic increase in the percentage of BrdU+ cells after stimulation with HEL, as compared with that in anergic B cells from dTg control mice (Figure 1F). Consistent with the  $^3H$ -thymidine incorporation assay results,  $CD72$  deficiency in dTg mice also increased anergic B cell proliferation in response to stimulation with HEL plus IL-4 or HEL plus anti-CD40, as measured by BrdU incorporation (Figure 1F).

In analyses of the role of  $CD72$  in regulating B cell death, we found that addition of HEL antigen, with or without IL-4 or anti-CD40, induced fewer dTg  $CD72^{-/-}$  B cells to become annexin V positive (Figure 1G). Consistent with the BrdU incorporation and cell

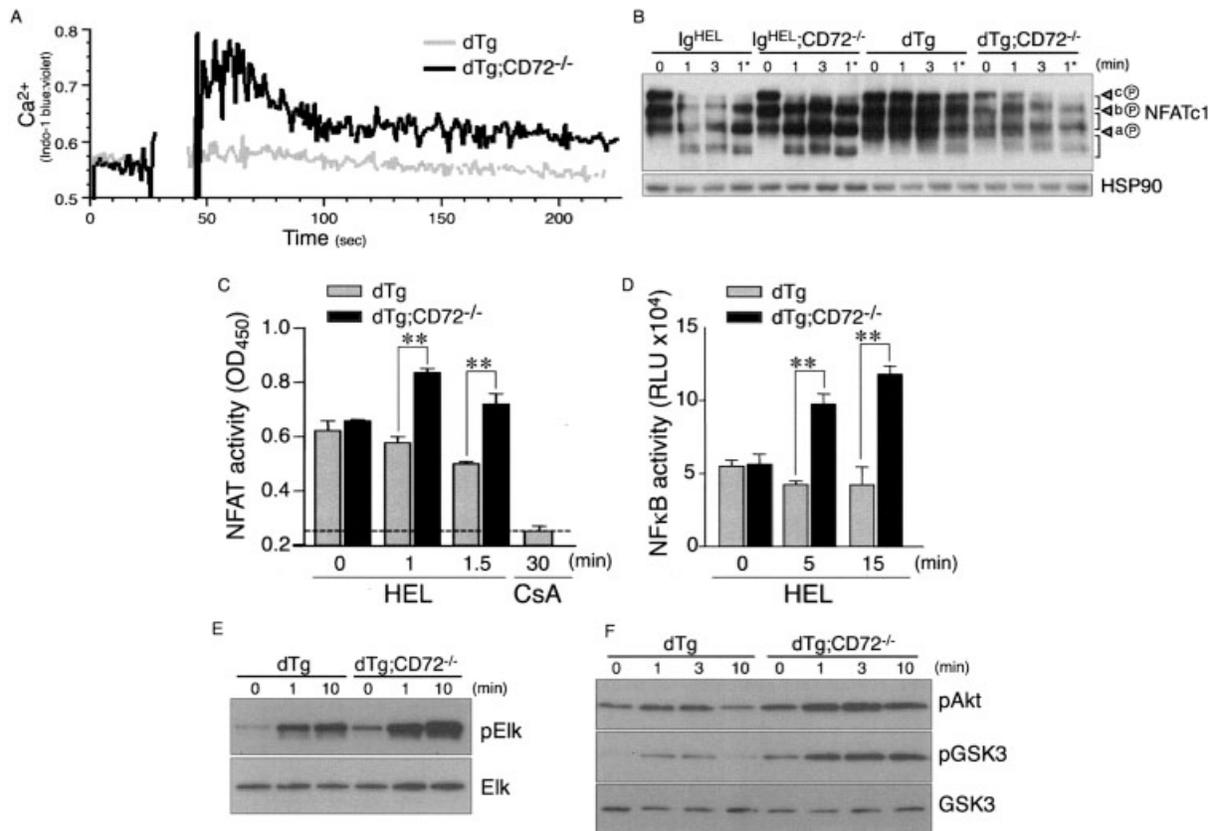
viability assay results, analyses of the cell cycle using PI staining showed that dTg  $CD72^{-/-}$  B cells had an increased percentage of cells in the S phase and  $G_2/M$  phase and a decreased number of apoptotic cells (DNA content  $<2n$ ) after stimulation (results not shown). Collectively, these results indicate that  $CD72$  negatively regulates BCR-induced proliferation of anergic B cells and enhances apoptosis of anergic B cells after stimulation.

To analyze the molecular basis of the increased proliferation and survival of dTg  $CD72^{-/-}$  B cells, we examined the expression of proteins playing a key role in cell cycle and survival, after antigenic stimulation of control and  $CD72^{-/-}$  B cells. After stimulation with HEL antigen, B cells from  $Ig^{HEL}$ -Tg control mice up-regulated cyclin D2 and down-regulated p27<sup>kip</sup>, which allowed entry into the S phase.  $Ig^{HEL}$ -Tg  $CD72^{-/-}$  B cells up-regulated cyclin D2 to a greater extent than did  $Ig^{HEL}$ -Tg control B cells (Figure 1H), consistent with our observations of the increased proliferation of these  $CD72^{-/-}$  B cells in vitro (Figures 1D and E).

Anergic B cells from the dTg control mice did not up-regulate cyclin D2, which limited the rate of phosphorylation of retinoblastoma (Rb) protein and, thus, limited the rate of entry into the S phase. In contrast, HEL-stimulated dTg B cells from  $CD72^{-/-}$  mice up-regulated cyclin D2 to levels comparable with those observed in HEL-stimulated  $Ig^{HEL}$ -Tg control B cells (Figure 1H). B cells from the control and  $CD72^{-/-}$  groups of dTg mice down-regulated p27<sup>kip</sup> to comparable levels after antigen stimulation, indicating that in anergic B cells,  $CD72$  functions specifically to regulate the BCR signal intensity such that cyclin D2 is not induced by antigen encounter.

Survival of antigen-stimulated B cells is intimately controlled by induction of Bcl-x<sub>L</sub> and Myc (34). Both  $Ig^{HEL}$ -Tg control and  $Ig^{HEL}$ -Tg  $CD72^{-/-}$  B cells up-regulated Bcl-x<sub>L</sub> and Myc after stimulation with HEL (Figure 1H). The induction of these pro-survival proteins was greater in  $Ig^{HEL}$ -Tg  $CD72^{-/-}$  B cells, which is consistent with the decreased apoptosis of these  $CD72$ -deficient B cells after antigen stimulation (9). B cells from dTg  $CD72^{-/-}$  mice, but not those from dTg control mice, up-regulated Bcl-x<sub>L</sub> and Myc after BCR stimulation (Figure 1H). These results indicate that the induction of these factors may mediate the enhanced survival of the antigen-stimulated B cells in dTg  $CD72^{-/-}$  mice.

To verify the genotypes used for these studies, we probed Western blots for  $CD72$ . Consistent with the findings from a previous gene microarray analysis (35), the levels of  $CD72$  total protein were robustly induced by short-term BCR signaling in  $Ig^{HEL}$ -Tg B cells (Figure

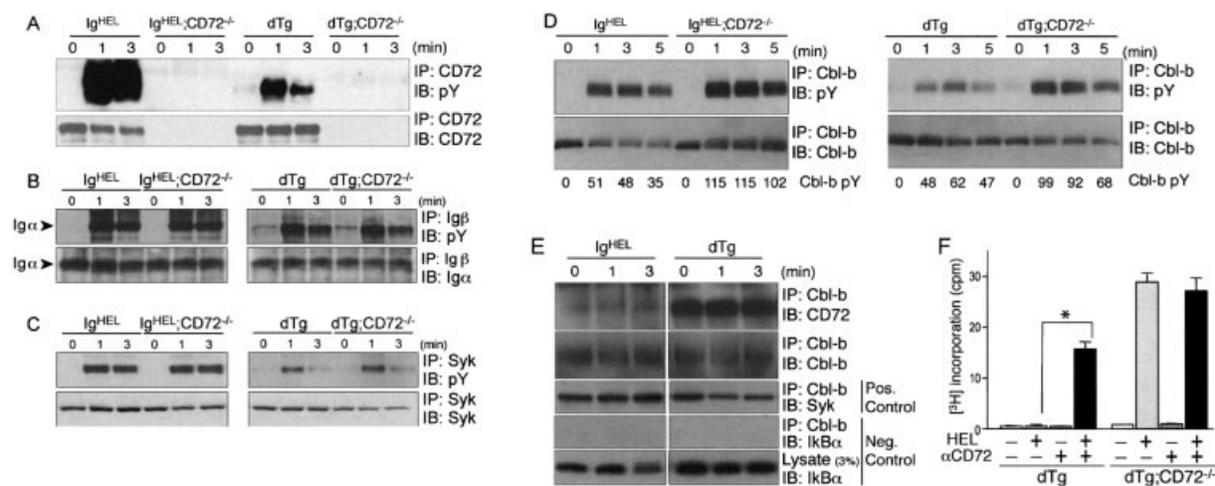


**Figure 2.** Regulation of B cell receptor signaling by CD72 in B cells from *Ig<sup>HEL</sup>-Tg* and dTg CD72<sup>-/-</sup> mice compared with *Ig<sup>HEL</sup>-Tg* and dTg control mice. **A**, Calcium flux in splenic B cells after stimulation with HEL antigen. Results are expressed as Ca<sup>2+</sup>, calculated as the ratio of indo-1 blue to violet. **B**, Dephosphorylation of NFATc1 after stimulation with HEL. Hsp90 was used to show equal loading. Lanes marked with the asterisk indicate incubation with HEL plus anti-CD40 (1 μg/ml). Arrowheads and brackets indicate the phosphorylated (P) and dephosphorylated NFATc1 isoforms, respectively. **C**, NFATc1 nuclear binding activity after stimulation with HEL. B cells treated with cyclosporin A (CsA) (100 ng/ml) for 30 minutes were used as a negative control. Broken line indicates the cutoff for positivity. Bars show the mean and SD optical density at 450 nm (OD<sub>450</sub>). **D**, NF-κB activity after stimulation with HEL. Bars show the mean and SD relative light units (RLU). \*\* = *P* < 0.01. **E**, ERK activity after stimulation with HEL. Results were obtained by in vitro kinase assay using Elk as the substrate. Total Elk was used to show equal loading. **F**, Akt phosphorylation and Akt kinase activity. Results were obtained by in vitro kinase assay using glycogen synthase kinase 3 (GSK3) as the substrate. Total GSK3 was used to show equal loading. Results in **E** and **F** are representative of at least 2 independent experiments. See Figure 1 for other definitions.

1H). With the use of flow cytometry, we quantified the increase in CD72 cell surface expression after antigen stimulation. Both *Ig<sup>HEL</sup>-Tg* and dTg B cells up-regulated cell surface expression of CD72 after antigen stimulation. However, at later time points after stimulation with HEL, dTg B cells had a substantially enhanced CD72 expression as compared with *Ig<sup>HEL</sup>-Tg* B cells (Figure 1I).

**Negative regulation of BCR signaling by CD72 in anergic B cells.** To investigate the influence of CD72 on B cell signaling during tolerance induction and maintenance, dTg control and dTg CD72<sup>-/-</sup> B cells were stimulated with HEL, and the downstream signaling

pathways were examined. Similar to the differences observed between control and CD72<sup>-/-</sup> *Ig<sup>HEL</sup>-Tg* B cells, dTg B cells from CD72<sup>-/-</sup> mice had an overall greater signaling intensity than did dTg control B cells, indicating that CD72-deficient anergic B cells have higher Ca<sup>2+</sup> flux and stronger activation of the NFATc1, NF-κB, MAPK, and Akt pathways. Although these signaling pathways are known to be induced to a greater extent in dTg CD72<sup>-/-</sup> B cells, the increased signal does not reach the same level as that detected in stimulated *Ig<sup>HEL</sup>-Tg* B cells (9). In general, CD72 deficiency rescues signaling by restoring the signal intensity to a level that is one-quarter to one-half of that in *Ig<sup>HEL</sup>-Tg* cells (9).



**Figure 3.** Regulation of Cbl-b phosphorylation by CD72 in B cells from  $Ig^{HEL}$ -Tg and dTg  $CD72^{-/-}$  mice compared with  $Ig^{HEL}$ -Tg and dTg control mice, and effects of short-term inhibition of CD72 on anergic B cell proliferation. **A**, Tyrosine phosphorylation of CD72 after stimulation with HEL antigen. Results are expressed as pY levels. CD72 was identified by immunoprecipitation (IP), and pY levels were determined by immunoblotting (IB). **B**, Tyrosine phosphorylation of Ig $\alpha$  and Ig $\beta$  after stimulation with HEL. Ig $\beta$  was identified by IP, and pY levels were determined by IB. Bands for Ig $\alpha$  were determined on the basis of reactivity with anti-Ig $\alpha$  antibodies and the molecular weight, which differs from that for Ig $\beta$ . Total Ig $\alpha$  is shown as a control. **C**, Tyrosine phosphorylation of Syk after stimulation with HEL. Syk was identified by IP, and total and tyrosine phosphorylated Syk were analyzed by IB. Total Syk is shown as a control. **D**, Tyrosine phosphorylation of Cbl-b after stimulation with HEL. IP and IB results for Cbl-b were equal. The pY levels were normalized to those for total Cbl-b at time 0 for all time points, due to the reduced ability of the anti-Cbl-b antibody to recognize phosphorylated Cbl-b. **E**, Constitutive interaction of CD72 with Cbl-b. Syk and I $\kappa$ B $\alpha$  are shown as positive (Pos.) and negative (Neg.) controls, respectively, for Cbl-b interaction. IP and IB results for Cbl-b were equal. **F**, Effects of preligation of CD72 with anti-CD72 on proliferation and self tolerance, with or without stimulation with HEL. Bars show the mean and SD representative results from 1 of at least 2 independent experiments in triplicate cultures. \* =  $P < 0.01$ . See Figure 1 for other definitions.

As shown in Figure 2A, dTg  $CD72^{-/-}$  B cells, but not dTg control anergic B cells, increased the calcium flux when stimulated with HEL. Consistent with this sustained rise in  $[Ca^{2+}]_i$ , NFATc1 was rapidly dephosphorylated and translocated to the nucleus, as indicated by the results from Western blotting and the increase in NFATc1 DNA binding activity of dTg  $CD72^{-/-}$  B cells after stimulation with HEL (Figures 2B and C). Consistent with the findings in a previous report (30), nuclear translocation of NF- $\kappa$ B was not induced in dTg anergic B cells from control mice after stimulation with HEL. However, dTg  $CD72^{-/-}$  B cells had elevated NF- $\kappa$ B p65 nuclear translocation and increased nuclear DNA binding activity after stimulation with HEL (Figure 2D).

Mitogenic signaling requires the activation of MAPK signaling pathways. Both the basal ERK activity and the BCR-induced ERK activity were increased in dTg  $CD72^{-/-}$  B cells (Figure 2E). The basal activity of JNK was higher in dTg anergic B cells from  $CD72^{-/-}$  mice than in those from control mice. Moreover, after stimulation with HEL antigen, JNK activity was also induced to a greater extent in dTg  $CD72^{-/-}$  B cells (results not shown). Similarly, p38 kinase activity was

induced to a higher level in the absence of CD72 (results not shown). These results indicate that MAPK signaling appears to be generally increased in dTg anergic B cells that lack CD72-mediated negative regulation.

Akt signaling contributes to the survival of many primary and transformed cell types. B cells from the dTg  $CD72^{-/-}$  mice, which had increased survival after stimulation, also had increased Akt phosphorylation and higher Akt kinase activity (Figure 2F). Taken together, these results indicate that CD72 likely influences BCR-induced proliferation and survival of dTg anergic B cells at the early stage of BCR signaling as downstream effectors are increased in the absence of CD72 (8).

**Interaction of CD72 with Cbl-b and regulation of Cbl-b phosphorylation.** We next examined which proteins might be involved in the CD72-mediated negative regulation of BCR signaling. Since phosphorylation of CD72 is essential for the recruitment of SHP-1, we first examined the extent of tyrosine phosphorylation of CD72 (expressed as pY levels) after stimulation of  $Ig^{HEL}$ -Tg and dTg B cells with HEL antigen. As shown in Figure 3A, CD72 was tyrosine phosphorylated rapidly after stimulation with HEL antigen in both  $Ig^{HEL}$ -Tg and

dTg B cell types, although CD72-specific pY levels were lower in dTg than in  $Ig^{HEL}$ -Tg B cells. This finding indicates that CD72 is phosphorylated in dTg anergic B cells after self-antigen stimulation.

We next determined which downstream signaling molecules were affected by CD72 deficiency in mature B cells. It was previously reported that CD72 down-regulates phosphorylation of  $Ig\alpha$  and Syk in a CD72-transfected myeloma cell line (36). To investigate whether this also occurs in primary B cells, we examined pY levels for  $Ig\alpha$  and Syk after stimulation with HEL antigen. As shown in Figures 3B and C, pY levels for  $Ig\alpha$  and Syk were not affected in the absence of CD72, either in  $Ig^{HEL}$ -Tg or in dTg primary B cells. (It is noteworthy that the protein levels and the pY levels for both  $Ig\alpha$  and Syk were lower in dTg compared with  $Ig^{HEL}$ -Tg B cells.) In contrast, for Cbl-b, we found that stimulation with HEL induced stronger pY levels in both  $Ig^{HEL}$ -Tg and dTg CD72<sup>-/-</sup> B cells as compared with their control counterparts (Figure 3D).

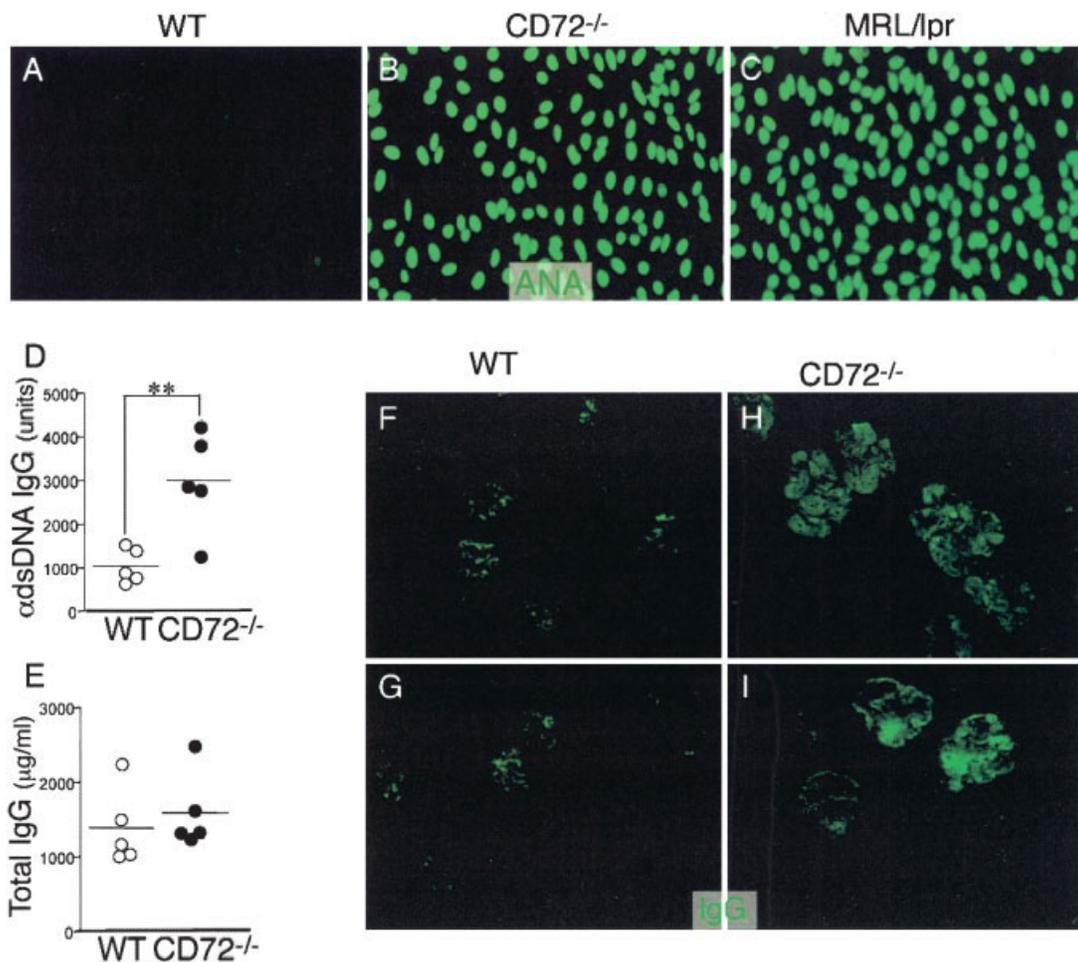
To further confirm the correlation in pY levels between CD72 and Cbl-b, preligation of CD72 with anti-CD72 mAb (clone K10.6), which mimics CD100 binding to CD72 (37), was used to sequester CD72 from the BCR and to impair tyrosine phosphorylation of CD72 after antigen stimulation. We found that preligation of CD72 with K10.6 diminished the pY levels for CD72 and enhanced the pY levels for Cbl-b to an extent similar to that observed in CD72<sup>-/-</sup> B cells after stimulation with HEL (results not shown). The observation that both CD72-deficient B cells and B cells treated with anti-CD72 mAb had enhanced tyrosine phosphorylation of Cbl-b suggests that the CD72/SHP-1 complex specifically targets Cbl-b for dephosphorylation, and that Cbl-b may be an immediate target of the CD72/SHP-1 complex after antigen stimulation.

Since Cbl-b-deficient B cells are hyperreactive to BCR ligation, and mice lacking Cbl-b produce anti-dsDNA autoantibodies and develop severe autoimmune disease (21), we further investigated the connection between Cbl-b and CD72. To determine whether CD72 forms a complex with Cbl-b, coimmunoprecipitation experiments were performed. Anti-Cbl-b coimmunoprecipitated CD72 and Syk before and after antigenic stimulation, both in  $Ig^{HEL}$ -Tg and in dTg B cells (Figure 3E). This finding indicates that Cbl-b constitutively interacts with CD72, either directly or indirectly. Notably, despite equal total levels of CD72 and Cbl-b, more CD72 interacted with Cbl-b in dTg anergic B cells than in  $Ig^{HEL}$ -Tg B cells (Figures 3A and E), suggesting that the interaction between CD72 and Cbl-b might play a direct role in maintaining B cell tolerance.

**Required role of CD72 in the maintenance of B cell tolerance.** Although our results indicated that CD72 is important for proper B cell tolerance, CD72 could also be functioning during the induction of B cell tolerance and/or be required for the maintenance of tolerance when B cells re-encounter self antigen. To determine whether removal of the inhibitory function of CD72 could influence anergic B cell proliferation, we examined dTg B cells stimulated with HEL in the presence of anti-CD72 mAb (clone K10.6). Preligation of CD72 with K10.6 has been shown to result in impaired tyrosine phosphorylation of CD72 (16,17,37), enhanced and sustained Ca<sup>2+</sup> flux (9), and enhanced ERK activation and I $\kappa$ B $\alpha$  phosphorylation after antigen stimulation (9). Similar to the differences observed between  $Ig^{HEL}$ -Tg control and  $Ig^{HEL}$ -Tg CD72<sup>-/-</sup> B cells (9), incubation of dTg anergic B cells with anti-CD72 and HEL showed that CD72 ligation indeed uncoupled the inhibitory effects of CD72 on cell cycle progression and survival, resulting in significant proliferation of the anergic B cells and blockade of the self tolerance of these cells (Figure 3F). These results indicate that the presence of CD72 is continuously required to negatively regulate BCR signaling for the maintenance of B cell tolerance.

**Production of lupus-associated autoantibodies in vivo in CD72-deficient mice.** Since CD72 is required to maintain B cell anergy in vitro (Figure 3F), we next investigated the role of CD72 in autoimmunity in vivo, by investigating the levels of ANAs and anti-dsDNA antibodies and the extent of deposition of glomerular immune complexes in aged CD72<sup>-/-</sup> C57BL/6 mice. To detect ANAs, HEp-2 cells from the sera of 9-month-old wild-type (WT) and CD72<sup>-/-</sup> mice were stained. Serum from WT mice displayed minimal reactivity, whereas serum from CD72<sup>-/-</sup> mice reacted with the nuclei in HEp-2 cells (Figures 4A–C). Although the total IgG levels were comparable between WT and CD72<sup>-/-</sup> mice, the levels of IgG specific for dsDNA were significantly increased in CD72<sup>-/-</sup> serum as compared with WT serum (Figures 4D and E). Aged CD72<sup>-/-</sup> mice also appeared to have increased IgG immune complex deposition in the kidneys, because the majority of the glomerular deposits in the CD72<sup>-/-</sup> mice were substantially increased in size compared with those in the WT mice (Figures 4F–I). These results indicate that CD72 plays a role in preventing autoantibody production and autoimmunity. Experiments involving large-scale autoantigen microarrays to elucidate the autoantibody targets in CD72<sup>-/-</sup> mice are ongoing (38).

**Development of lupus-like autoimmune disease in vivo in CD72-deficient mice.** To determine whether CD72<sup>-/-</sup> mice develop lupus-like autoimmune disease, we



**Figure 4.** Autoantibody production in vivo in CD72-deficient mice. **A–C**, HEP-2 cells were stained (1:100) for antinuclear antibodies (ANAs) in the sera of 9-month-old wild-type (WT) and CD72<sup>-/-</sup> mice; MRL/lpr mice were used as a positive control. Results are representative of 1 of 5 samples from each genotype. **D** and **E**, Levels of anti-double-stranded DNA (anti-dsDNA) IgG autoantibodies (**D**) and total IgG (**E**) were determined in WT and CD72<sup>-/-</sup> mice (n = 5 per group). Horizontal bar indicates the mean. \*\* = *P* < 0.007. **F–I**, IgG immune complex deposition was determined in the glomeruli of WT mice (**F** and **G**) and CD72<sup>-/-</sup> mice (**H** and **I**) by assessing >12 glomeruli on each kidney section. Two representative samples from each genotype are shown.

performed histopathologic analyses of the organs from 1-year-old mice. CD72<sup>-/-</sup> mice developed variable degrees of glomerulitis, characterized by glomerular enlargement, segmental mesangial thickening, dilation and filling of capillaries with amorphous eosinophilic material, and occurrence of small numbers of neutrophils and pyknotic cells in the tuft (Figures 5A, B, G, H, and M). The Bowman's capsule was frequently hyalinized and/or fibrotic, and the capsular epithelium was hyperplastic and multifocally adherent to the glomerular tuft.

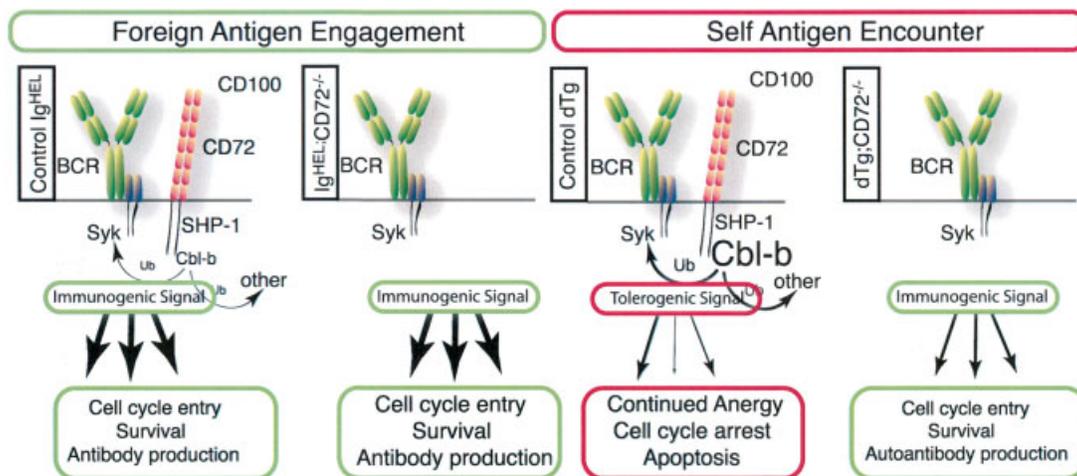
Staining with periodic acid-Schiff and Masson's trichrome showed basement membrane condensation in the glomerular tuft and fibrosis of the tuft and the Bowman's capsule in CD72<sup>-/-</sup> mice (Figures 5C–F). CD72<sup>-/-</sup> mice also had multifocal and coalescing lym-

phoplasmacytic and histiocytic infiltrates in the interstitium of the kidneys, salivary glands, and lungs (Figures 5G–L and N–P). These results, along with the differences in production of ANAs and anti-dsDNA autoantibodies, demonstrate that CD72 may play an important role in preventing autoimmune disease in C57BL/6 mice.

#### **Lack of influence of the 129/Sv genetic background on the autoimmune phenotype of CD72-deficient mice.**

Susceptibility to murine lupus has a polygenic background, and several disease-associated loci have been mapped (39). A recent report defined a locus linked to the development of anti-dsDNA IgG antibodies located on chromosome 4, ~25 cM telomeric to the CD72 gene (40). To exclude the possibility that the CD72-deficient phenotype is influenced by 129 alleles at





**Figure 6.** Working model of the role of CD72 in maintaining B cell anergy. CD72 is essential for the maintenance of B cell tolerance through the interaction with Cbl-b and in the negative regulation of B cell receptor (BCR) signaling. During foreign antigen engagement (immunogenic signaling) in the presence of CD72, some Cbl-b is recruited to the immunogenic signalosome. This results in moderate Cbl-b-mediated inhibition of BCR signaling, thereby attenuating cell cycle entry and survival of mature B cells. In the absence of CD72, less or no Cbl-b is recruited to the immunogenic signalosome, resulting in uninhibited BCR signaling and enhanced cell cycle entry and survival. The effects of CD72 deficiency on cell cycle entry and survival are consistent with the enhanced antigen-induced in vitro proliferation of  $Ig^{HEL}$ -transgenic  $CD72^{-/-}$  B cells (see Figure 1). During self antigen engagement (tolerogenic signaling), self-reactive B cells transduce low-intensity signals, leading to an anergic phenotype and functional nonresponsiveness. In the presence of CD72, Cbl-b is recruited to the tolerogenic signalosome, which, through induced proximity and direct activation, allows Cbl-b to inhibit BCR signaling (45,46). This attenuated self antigen-directed signaling (tolerogenic signaling) is not sufficient to induce cell cycle entry, promote survival, or result in autoantibody production. Thus, B cell anergy is maintained. In the absence of CD72, Cbl-b is not recruited to the tolerogenic signalosome, resulting in high-intensity (immunogenic) BCR signaling, which leads to cell cycle entry, cell survival, and autoantibody production (see Figure 1). Thus, binding to self antigen is misinterpreted by the anergic B cells in the absence of CD72, which results in the development of autoimmunity. HEL = hen egg lysozyme; dTg = double-transgenic; SHP-1 = SH2-containing protein tyrosine phosphatase 1; Ub = ubiquitination. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

NF- $\kappa$ B, MAPK, and Akt in anergic B cells. Mechanistically, CD72 is associated with Cbl-b, suggesting that Cbl-b may mediate the negative effects of CD72 on BCR signaling in anergic B cells.

CD72 plays a role in discriminating tolerogenic signaling from immunogenic signaling. Normally, foreign antigens elicit an immunogenic response while self antigens elicit a tolerogenic response by mature B cells. The mature B cells from both  $Ig^{HEL}$ -Tg control and  $Ig^{HEL}$ -Tg  $CD72^{-/-}$  mice transduce an immunogenic signal resulting in B cell activation after antigen stimulation (9). In the present study we used the dTg anergic model to elucidate the function of CD72 in regulating B cell anergy and tolerogenic signaling. In anergic B cells, CD72 constitutively regulates BCR-induced signaling to limit proliferation and survival. As a result of a lack of cyclin D2 up-regulation and Rb phosphorylation, dTg anergic B cells have a near complete block in proliferation and fail to enter the S phase (Figures 1D–G). B cells from dTg  $CD72^{-/-}$  mice misinterpret self-antigen binding as an immunogenic signal, which results in a breakdown of B cell self tolerance and leads to B cell activation. The up-regulation of cyclin D2 and Bcl- $x_L$  in

antigen-stimulated dTg  $CD72^{-/-}$  B cells offers a molecular explanation for their altered proliferation and survival (Figure 1H).

Anergic B cells from the dTg model have been shown to be defective in the induction of the calcineurin/NFAT and NF- $\kappa$ B signaling pathways (30,31). CD72 deficiency allows dTg anergic B cells to activate both of these immunogenic pathways. Our data directly assessing the activity of these pathways are supported by the induction of cyclin D2, the expression of which requires both calcineurin/NFAT and NF- $\kappa$ B signaling (41,42). MAPK and Akt signaling are fundamental regulators of cell cycle and survival, and these pathways are also induced to a greater extent by self-antigen binding to dTg  $CD72^{-/-}$  B cells.

CD72 may modulate the B cell tolerance via binding to its ligand, CD100. CD72 negatively regulates BCR-induced signaling, but binding of CD72 to CD100 can release this negative regulation (16). CD100 is expressed on several immune cell types, including T cells, B cells, and antigen-presenting cells. It is likely that CD100 fine-tunes CD72-mediated negative regulation during T cell-dependent responses, which is consistent

with the reduced T cell-dependent responses in CD100-deficient mice (17). The observation that CD72<sup>-/-</sup> mice develop autoimmunity may appear at odds with a recent report that CD100-deficient mice develop autoantibodies and autoimmune disease (43). However, CD100 is expressed on several different cell types, has signaling properties of its own, and has ligands other than CD72, so the mechanisms that contribute to the development of autoimmune disease in aged CD100<sup>-/-</sup> mice may be complex.

By utilizing the ability of an anti-CD72 mAb to mimic CD100 binding to surface CD72, we demonstrated that anergic B cells continuously require CD72 to maintain B cell tolerance. As shown in Figure 3F, cocubation of anti-CD72 mAb and HEL in B cell cultures revealed that CD72 ligation indeed releases the inhibitory effects of CD72 on cell cycle progression and survival, resulting in breakdown of self tolerance in anergic B cells. These findings suggest that proper regulation of CD72 is important to maintain B cell tolerance in the periphery.

CD72 down-regulates BCR signaling through Cbl-b to maintain B cell anergy. CD72 may anchor Cbl-b to the membrane in close proximity to the signalosome, and thus allow the appropriate regulation of Cbl-b function in anergic B cells (Figures 3D and E). Syk tyrosine kinase has been shown to be a target of Cbl-mediated ubiquitination upon antigen stimulation in mature B cells (44,45). Preliminary findings (Li DH, et al: unpublished observations) indicate that Syk ubiquitination was significantly enhanced in WT B cells as compared with CD72-deficient B cells after antigen stimulation. This finding implies that Cbl-b may increase its E3 ubiquitin ligase activity after dephosphorylation by CD72 and, in turn, enhance the ubiquitination of Syk. The enhanced ubiquitination of Syk may augment turnover of the active phospho-Syk associated with Ig $\alpha$ , thereby decreasing Syk kinase activity (25,45). Based on our observations, we propose a working model for CD72 function in immunogenic and tolerogenic signaling, as shown in Figure 6.

Collectively, these results show that aged CD72-deficient mice develop lupus-like autoimmune disease, and that in the absence of CD72, anergic B cells inappropriately proliferate and survive in response to self-antigen stimulation in vitro. Biochemical analyses indicate that in anergic B cells, CD72 down-regulates BCR signaling. Thus, CD72, which normally functions to reduce mature B cell proliferation and differentiation, is an important modulator of peripheral B cell anergy. The enhanced understanding of cell surface receptors that critically maintain immune tolerance will aid in the

development of targeted therapies to treat autoimmune disorders.

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## AUTHOR CONTRIBUTIONS

Drs. Li and Parnes had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

**Study design.** Li, Parnes.

**Acquisition of data.** Li, Winslow, Cao, Chen, Davis.

**Analysis and interpretation of data.** Li, Winslow, Davis, Mellins, Utz, Crabtree, Parnes.

**Manuscript preparation.** Li, Winslow, Mellins, Parnes.

**Statistical analysis.** Li, Winslow.

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