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A Bacterial Artificial Chromosome Transgene with Polymorphic Cd72 Inhibits the Development of Glomerulonephritis and Vasculitis in MRL-Faslpr Lupus Mice

Hisashi Oishi,*,†,‡ Takahito Tsubaki,†,‡ Tatsuhiko Miyazaki,† Masao Ono,‡ Masato Nose,†,‡,§ and Satoru Takahashi*

Systemic lupus erythematosus is considered to be under the control of polygenic inheritance, developing according to the cumulative effects of susceptibility genes with polymorphic alleles; however, the mechanisms underlying the roles of polygenes based on functional and pathological genomics remain uncharacterized. In this study, we substantiate that a CD72 polymorphism in the membrane-distal extracellular domain impacts on both the development of glomerulonephritis and vasculitis in a lupus model strain of mice, MRL/MpJ-Faslpr, and the reactivity of BCR signal stimulation. We generated mice carrying a bacterial artificial chromosome transgene originating from C57BL/6 (B6) mice that contains the Cd72b locus (Cd72B6 transgenic [tg]) or the modified Cd72b locus with an MRL-derived Cd72b allele at the polymorphic region corresponding to the membrane-distal extracellular domain (Cd72B6/MRL tg). Cd72B6 tg mice, but not Cd72B6/MRL tg mice, showed a significant reduction in mortality following a marked improvement of disease associated with decreased serum levels of IgG3 and anti-dsDNA Abs. The number of splenic CD4+CD8− T cells in Cd72B6 tg mice was decreased significantly in association with a reduced response to B cell receptor signaling. These results indicate that the Cd72 polymorphism affects susceptibility to lupus phenotypes and that novel functional rescue by a bacterial artificial chromosome transgenesis is an efficient approach with wide applications for conducting a genomic analysis of polygene diseases. The Journal of Immunology, 2013, 190: 000–000.

Systemic lupus erythematosus (SLE) is a representative prototype of a systemic autoimmune disease in which the lethal hallmark is lupus nephritis and, occasionally, systemic vasculitis, associated with hypergammaglobulinemia and the production of Abs directed against a broad range of self-Ags (1). Etiologically, SLE is a disease of polygenic disorders that develop according to the cumulative effects of multiple genes with poly-

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Abbreviations used in this article: B6, C57BL/6; BAC, bacterial artificial chromosome; BUN, blood urea nitrogen; C3H-Faslpr, C3H/Hej-Faslpr; FO, follicular zone; MRL-Faslpr, MRL/MpJ-Faslpr; MZ, marginal zone; SLE, systemic lupus erythematosus; tg, transgenic.

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stitutions may cause considerable structural differences compared with the other alleles, which likely leads to functional alterations. CD72 negatively regulates BCR signaling by recruiting tyrosine phosphatase SHP-1 to its ITIM at the cytoplasmic region (15). The negative-regulatory role of CD72 causes CD72-deficient B cells to be hyperresponsive following BCR stimulation (16). However, it is still unclear whether the CD72 polymorphism is involved in the development of lupus-like diseases in MRL-\(Fas^{low}\) mice.

To identify the genes associated with complex disease traits, linkage analyses and subsequent congenic mapping were most effective (17). However, generating recombinant congenic strains of mice with particular phenotypes in conjunction with recombination at desired loci is challenging. In the current study, we performed complementation rescue of the autoimmune phenotypes in MRL-\(Fas^{low}\) mice using transgenesis of a bacterial artificial chromosome (BAC) carrying the \(Cd72^{\delta}\) allele without any additional enhancers. We generated two strains of BAC transgenic (tg) MRL-\(Fas^{low}\) mice: one carrying the \(Cd72^{\beta}\) locus originating from the genomic DNA of B6 mice (\(Cd72^{\beta}\)tg mice) and another carrying the modified \(Cd72^{\delta}\) locus in which polymorphic exon 8 was replaced by MRL-derived exon 8 of the \(Cd72^{\delta}\) allele (\(Cd72^{\delta}\)tgMRL1 tg mice). We then examined lupus phenotypes in \(Cd72^{\beta}\)tg and \(Cd72^{\delta}\)tgMRL mice. We demonstrate that transgenesis of \(Cd72^{\beta}\)tg mice, but not \(Cd72^{\delta}\)tgMRL mice, rescues MRL-\(Fas^{low}\) mice from autoimmune diseases, strongly suggesting that CD72 plays a crucial role in the development of autoimmune diseases in these mice.

**Materials and Methods**

Generation of BAC tg mice

MRL-\(Fas^{low}\) mice were purchased from Charles River Laboratories (Shizuoka, Japan). The RP23-195K8 BAC clone (178,592 bp), which is constructed from the female B6 mouse, includes the upstream 110 kbp and downstream 60 kbp of the \(Cd72^{\beta}\) gene and was purchased from Invitrogen (Carlsbad, CA). For the modification of the membrane-distal extracellular domain of \(Cd72^{\beta}\) on the BAC DNA sequence from intron 7 to exon 8, the genomic DNA of the MRL-\(Fas^{low}\) mouse kidney was amplified by PCR method. The following primers in the RP23-195K8 BAC sequences were used for the transgene PCR analysis using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), according to the manufacturer’s protocol. Assays were performed in duplicate with the ABI PRISM 7700 system (Applied Biosystems). The oligonucleotide primers for transgene-derived \(Cd72^{\beta}\) were 5’-GGACAGTCTTGATGAAAGTTGCTAGATCGTT-3’ (forward; \(Cd72^{\delta}\)ex6 Fw) and 5’-GGATGAACACGCATTCAATGACCCTC-3’ (reverse; \(Cd72^{\delta}\)ex7 Rv2). The primers for endogenous \(Cd72^{\beta}\) were 5’-GGACAGTCTTGATGAAAGTTGCTAGATCGTT-3’ (forward; \(Cd72^{\delta}\)ex6 Fw) and \(Cd72^{\delta}\)ex7 Rv2. The primers for total \(Cd72^{\beta}\) were \(Cd72^{\delta}\)ex6 Fw, \(Cd72^{\delta}\)ex6 Fw, and \(Cd72^{\delta}\)ex7 Rv2. The primers for hypoxanthine phosphoribosyltransferase (Hprt) were 5’-CAAAGGTGGCATTTCCCTGTT-3’ (forward; \(Hprt\) F) and 5’-CAAGGGCATATCCCACTGCCCACAAGC-3’ (reverse; \(Hprt\) R).

**Quantitative real-time RT-PCR analysis**

Total RNA was prepared from the MACS-sorted splenic CD19\(^{+}\) B cells of each tg line at 6–10 wk of age, and cDNA was synthesized. Real-time RT-PCR analysis was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), according to the manufacturer’s protocol. Assays were performed in duplicate with the ABI PRISM 7700 system (Applied Biosystems). The oligonucleotide primers for transgene-derived \(Cd72^{\beta}\) were 5’-GGACAGTCTTGATGAAAGTTGCTAGATCGTT-3’ (forward; \(Cd72^{\delta}\)ex6 Fw) and 5’-GGATGAACACGCATTCAATGACCCTC-3’ (reverse; \(Cd72^{\delta}\)ex7 Rv2). The primers for endogenous \(Cd72^{\beta}\) were 5’-GGACAGTCTTGATGAAAGTTGCTAGATCGTT-3’ (forward; \(Cd72^{\delta}\)ex6 Fw) and \(Cd72^{\delta}\)ex7 Rv2. The primers for total \(Cd72^{\beta}\) were \(Cd72^{\delta}\)ex6 Fw, \(Cd72^{\delta}\)ex6 Fw, and \(Cd72^{\delta}\)ex7 Rv2. The primers for hypoxanthine phosphoribosyltransferase (Hprt) were 5’-CAAAGGTGGCATTTCCCTGTT-3’ (forward; \(Hprt\) F) and 5’-CAAGGGCATATCCCACTGCCCACAAGC-3’ (reverse; \(Hprt\) R).

**Transgene copy number analysis of genomic DNA and transgene genotyping**

The transgene copy number was determined by a real-time quantitative PCR method. The following primers in the RP23-195K8 BAC sequences were used for the transgene 5’-GAACACGCATTGGCTACTTCT-3’ (for-
ward), 5′-TCACTGGCAAGTGGCCAATC-3′ (reverse) and the refer-
ence gene (ml)2, 5′-CTAGGCCCAAGAATTGGAAGATC-3′ (forward),
5′-GATGGGAATGACAGCTCCAC-3′ (reverse). The Platinum SYBR
Green qPCR SuperMix UDQ (Invitrogen) was used for the real-
time amplification, according to the manufacturer’s protocol. The assays
were performed in duplicate on an ABI PRISM 7700 system (Applied Bio-
systems). Transgene-positive mice were identified by PCR using specific
primers for the pBeloBAC11 vector. The oligonucleotide primers were 5′-
AGGTGCTACCTAAATAGCTTG-3′ (forward) and 5′-CAGTACTGGCAF-
GAGTGGCA-3′ (reverse).

Histopathologic and immunohistologic examinations
Tissue samples were fixed with 10% formalin in 0.01 mol/l phosphate
buffer (pH 7.2) and embedded in paraffin. They were stained with HE for
histological examination by light microscopy. The severity of the diseases
was evaluated according to the previously proposed criteria for glomeru-
lonephritis and renal vasculitis (10–12). In brief, glomerulonephritis was
estimated as follows: grade 0 is normal; grade 1 has limited segmental
mesangial proliferation, grade 2 has endocapillary proliferation with wire
loop and/or hyaline thrombotic lesions, and grade 3 has dominant sclerosing
and/or hyalinosis of the lesions in grade 2. The renal vascular lesions were
graded as follows: grade 0 is normal-to-minimal perivascular lympho-
phocytes infiltration, grade 1 is moderate perivascular cell infiltration
associated with destruction of external elastic lamina, and grade 2 is the
above findings plus intimal thickening with the destruction of internal
elastic lamina. Frozen renal sections for the immunofluorescence analyses
were stained with FITC-labeled anti-mouse IgG, IgM, IgG3, and C3 Abs
(Inc Pharmaceutica, Cleveland, OH). The average fluorescence intensity
≥ 20 glomeruli from each kidney section was quantified by analyzing the
fluorescence microscopy images using ImageJ software (http://rsbh.
info.nih.gov/j). Serum levels of IgG, anti-dsDNA autoantibody titer,
creatinine, and blood urea nitrogen
Total serum IgG was determined by the Mouse Ig ELISA Quantitation kits
(Bethyl Laboratories, Montgomery, TX) and the TMB Microwell Peroxidase
Substrate system (KPL, Gaithersburg, MD), according to the manufacturer’s
protocols. Anti-dsDNA autoantibody titers were measured by ELISA, as
described previously (19). Arbitrary units were calculated using the linear
ranges of the dilution and the standard curves generated with the pooled sera
of old MRL-Faspr mice. The concentration of serum creatinine and blood
urea nitrogen (BUN) was measured using a BD LSR.

Abs and flow cytometry
We used the following mAbs for flow cytometric analysis: FITC-, PE-
PerCP-, or biotin-conjugated anti-CD3, anti-CD5, anti-B220, anti-CD21,
anti-CD23, anti-Thy1.2, anti-CD4, anti-CD8, and anti-CD43. All Abs
were from BD Pharmingen. For the expression analysis of CD72, an anti-
CD72 Ab (CT-724; Cedarlane) was used. Cell surface staining was
performed according to standard techniques, and the flow cytometric
analysis was done with a FACSCalibur using CellQuest software (BD
Biosciences, San Jose, CA).

Isolation of splenic B cells and viability assays
Splenic B cells from 6–8-wk-old mice were purified by negative selection
using a MACS system with a B cell isolation kit (Miltenyi Biotec, Auburn,
CA). The resulting splenic B cells (1 × 107 cells/ml) were stimulated by
15 µg/ml F(ab′)2 goat anti-IgM Ab, 10 µg/ml anti-CD40, 15 µg/ml F(ab′)2
(goat anti-IgM Ab + 10 µg/ml anti-CD40, 10 µg/ml LPS, and 10 U/ml II–4 (BD Biosciences) in flat-bottom 96-well microtiter plates for 3 d.
After 68 h, the cells were pulsed with 20 µL/well of the vital dye Cell-
Titer96 AQueous One Solution Reagent (Promega). B cell viability was
determined by measurement of light absorbance at 490 nm, according to
the manufacturer’s manual.

Intracellular calcium response
Calcium mobilization of the splenic cells was measured using a BD LSR
(Becton Dickinson). Erythrocyte-depleted splenocytes were loaded with
5 µM Fluo-4 AM ester (Dojindo, Kumamoto, Japan). Cells (1 × 106 cells)
were stained with PE-conjugated CD19 Ab and stimulated with 20 µg/ml
F(ab′)2 goat anti-IgM Ab (Jackson ImmunoResearch Laboratories). The
increases in intracellular calcium mobilization were presented as increased
Fl-1 fluorescence intensity following Ab treatment.

Kinetics of phosphorylation of ERK
Mouse lymphocytes from 6–8-wk-old mice were positively sorted for
CD19+ B cells using MACS CD19 microbeads. CD19+ B cells (5 × 106
cells) were stimulated by adding 10 µg/ml F(ab′)2 goat anti-IgM Ab. The
cells were incubated for 0, 3, or 5 min at 37°C and were fixed by 2% formaldehyde, followed by 90% methanol. The cells were washed and
loaded with Alexa Fluor 488–conjugated anti-phospho-p44/p42 MAPK
Ab (E10; Cell Signaling Technology, Beverly, MA) and stained with a PE-
conjugated anti-B230 Ab. The kinetics of p-ERK from the splenic cells
were measured using a BD LSR.

Statistical analysis
Data are expressed as mean ± SEM. The comparison of two groups was
performed using a one-way ANOVA, and a post hoc Bonferroni correction
was used for multiple comparisons. The unpaired Student’s t test was used
to compare two groups. The comparisons of survival rates were performed
with the Kaplan-Meier method, with differences in the survival curves
evaluated with a log-rank sum testing. A p value < 0.05 was considered
statistically significant.
Results

Generation and molecular characterization of Cd72B6/MRL and Cd72B6 BAC tg mice

A Cd72 polymorphism is accumulated in exon 8 encoding the C-terminal half of the C-type lectin-like domain in the extracellular region (Fig. 1A, 1B). Therefore, we replaced the region covering exon 8 in Cd72 in RP23-195K8 plasmid with that of Cd72 obtained from MRL mice using homologous recombination (Supplemental Fig. 1), which resulted in the BAC clone encoding a chimeric CD72 (CD72b/c). This CD72b/c protein consists of the N-terminal region (amino acid positions 1–305) derived from Cd72B6 and the C-terminal region (amino acid positions 306–355) derived from Cd72MRL where a 7-aa deletion and 13 aa substitutions exist (Fig. 1B). The original and modified BAC DNAs were microinjected into MRL-Faslpr fertilized oocytes to generate tg mice designated as Cd72B6 tg, lines 771 and 775, and Cd72B6/MRL tg, lines 360 and 518 (Supplemental Fig. 2). Littermates negative for the transgene (non-BAC tg mice) were described as MRL-Faslpr mice. Quantitative PCR using genomic DNA revealed the copy numbers of the tg BACs in the 771, 775, 360, and 518 lines to be two, two, three, and five, respectively.

We first examined the expression of tg CD72 in splenic CD19+ B cells. When mice of these lines were 6–10 wk old, quantitative RT-PCR analysis was performed with primers specific for the transgene-derived, endogenous, and total Cd72 transcript (Fig. 1C, Supplemental Fig. 3). Transgene-derived Cd72 was expressed in all tg lines, and its expression levels were almost compatible with those of endogenous Cd72. In the FACS analysis of splenic B cells, the expression level of a transgene-derived Cd72 product identified in mice of every tg line and the expression levels in mice of lines 518 and 771 were relatively high (Fig. 1D). Endogenous Cd72MRL (CD72c) was not detected by the anti-Cd72 Abs used in this analysis. These Abs may recognize an epitope on the polypeptides derived from Cd72B6 (CD72b) at amino acid positions 1–305 but not at amino acid positions 306–354 (Fig. 1B). In addition, the expression levels of Cd72b on spleen cells of B6 and Cd72B6 tg mice were almost the same in FACS analysis (data not shown).

Reduced mortality and splenomegaly as a result of Cd72B6 BAC transgenesis

Next, we analyzed the effects of Cd72B6 and Cd72B6/MRL transgenes on survival in MRL-Faslpr mice using a Kaplan–Meier analysis (Fig. 2A). The life spans of both lines 771 and 775 Cd72B6 tg mice were significantly longer than were those of MRL-Faslpr mice, whereas the life spans of both lines of Cd72B6/MRL tg mice were comparable to MRL-Faslpr mice. Further studies were performed

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**FIGURE 3.** Decreased severities of glomerulonephritis and vasculitis in Cd72B6 tg mice. (A) Representative histological manifestations of glomerulonephritis (upper panels) and renal vasculitis (lower panel) at 20 wk of age. H&E, original magnification ×100. (B) Semiquantitative analysis of the nephritis score. Data are average ± SEM. (C) Semiquantitative analysis of the vasculitis score. Data are average ± SEM. (D) Representative glomerular deposits of IgG, IgM, IgG3, and C3 in MRL-Faslpr, Cd72B6/MRL, and Cd72B6 tg mice. IgG, IgG3, and C3 deposits were only suppressed in Cd72B6 tg mice compared with MRL-Faslpr mice. Original magnification ×400. (E) Semiquantitative analysis of the immunofluorescence. Data are average ± SEM. (F) Serum BUN and serum creatinine levels in MRL-Faslpr, Cd72B6/MRL, and Cd72B6 tg mice. Data are average ± SEM. *p < 0.05, **p < 0.01.
using Cd72<sup>B6</sup>MRL tg, Cd72<sup>B6</sup>tg, and MRL-Fasl<sup>pr</sup> mice as a combination of lines 360 and 518, lines 771 and 775, and transgene-negative littersmates, respectively.

Furthermore, the spleen weights of Cd72<sup>B6</sup>tg mice, but not Cd72<sup>B6</sup>MRL tg mice, at 20 wk of age were significantly reduced compared with those in MRL-Fasl<sup>pr</sup> mice (Fig. 2B, 2C). Therefore, Cd72<sup>B6</sup>, but not Cd72<sup>B6</sup>MRL, transgenesis reduces both mortality and splenomegaly in MRL-Fasl<sup>pr</sup> mice. In contrast, no significant differences were observed in axillary lymph node weight at 20 wk of age among strains (Fig. 2D).

Cd72<sup>B6</sup> BAC transgenesis reduces the severity of glomerulonephritis and renal vasculitis

Renal dysfunction is the major cause of death of MRL-Fasl<sup>pr</sup> mice. A histopathological analysis of renal sections obtained at 20 wk of age revealed that the severity of both glomerulonephritis and vasculitis was markedly reduced in Cd72<sup>B6</sup>tg mice, consistent with extended life spans (Fig. 3A). Scoring of the severity of both diseases revealed significant reductions in Cd72<sup>B6</sup>tg mice compared with that observed in MRL-Fasl<sup>pr</sup> mice, although the diseases were not completely inhibited (Fig. 3B, 3C). We then examined the deposits of IgG, IgM, IgG3, and C3 in the renal glomeruli using immunofluorescence staining (Fig. 3D). A semiquantitative analysis showed that IgG, IgG3 and C3 deposits, but not IgM deposits, were significantly decreased in Cd72<sup>B6</sup>tg mice, but not in Cd72<sup>B6</sup>MRLtg mice, compared with that observed in MRL-Fasl<sup>pr</sup> mice (Fig. 3E). Furthermore, the serum BUN levels of Cd72<sup>B6</sup>tg mice were significantly lower than those in MRL-Fasl<sup>pr</sup> mice and Cd72<sup>B6</sup>MRLtg mice at 20 wk of age (Fig. 3F). The serum creatinine levels of Cd72<sup>B6</sup>tg mice were also lower than MRL-Fasl<sup>pr</sup> and Cd72<sup>B6</sup>MRLtg mice, although the differences were not statistically significant. Therefore, the expression of Cd72<sup>B6</sup>, but not Cd72<sup>B6</sup>MRLtg, ameliorates glomerulonephritis with immune complex deposition in MRL-Fasl<sup>pr</sup> mice. Interestingly, the presence of vascular lesions in Cd72<sup>B6</sup>tg mice was limited only in perivascular mononuclear cell infiltration, whereas that in MRL-Fasl<sup>pr</sup> and Cd72<sup>B6</sup>MRLtg mice was associated with the destruction of the arterial external elastic lamina and media, thus indicating vasculitis (Fig. 3A, 3C). Although Cd72<sup>B6</sup> BAC transgenesis ameliorated glomerulonephritis and vasculitis in MRL-Fasl<sup>pr</sup> mice, other autoimmune manifestations, such as sialadenitis and arthritis, were not improved histopathologically (data not shown). This is consistent with the fact that quantitative trait loci for sialadenitis and arthritis of MRL-Fasl<sup>pr</sup> mice were not mapped at a centromeric region of chromosome 4 in our previous studies using MRL-Fasl<sup>pr</sup×C3H-Fasl<sup>pr</sup> crosses (20, 21).

Reduced expansion of lpr T cells and enhanced B cell maturation in Cd72<sup>B6</sup>tg spleens

We next analyzed changes in the lymphocyte population in relation to any improvements in disease phenotypes in Cd72<sup>B6</sup>tg mice. The total spleen cell number in Cd72<sup>B6</sup>tg mice was significantly reduced compared with that in MRL-Fasl<sup>pr</sup> and Cd72<sup>B6</sup>MRLtg mice (Table I). Thymocyte cellularity and T cell development in Cd72<sup>B6</sup>tg mice were comparable to that observed in MRL-Fasl<sup>pr</sup> mice, as assessed by the percentages of CD4 and CD8 expression (Table I). However, in Cd72<sup>B6</sup>tg mouse spleens, the numbers of T cells, especially those of CD4<sup>+</sup>CD8<sup>−</sup> T cells, so-called “double-negative” lpr T cells, were reduced compared with those observed in MRL-Fasl<sup>pr</sup> mouse spleens (Cd72<sup>B6</sup>tg 7.40 ± 3.9%, n = 5; MRL-Fasl<sup>pr</sup> 81.7 ± 0.9%, n = 7; Cd72<sup>B6</sup>MRLtg 78.7 ± 4.1%, n = 5; p < 0.05) (Fig. 4B). Reductions in the expansion of lpr T cells appear to be involved in the improvement of macroscopic splenomegaly in Cd72<sup>B6</sup>tg mice.

We next examined B cell components in Cd72<sup>B6</sup>tg mice at 20 wk of age. In the bone marrow of Cd72<sup>B6</sup>tg mice, the fre-

### Table I. Cellular phenotypes in MRL-Fasl<sup>pr</sup>, Cd72<sup>B6</sup>MRLtg, and Cd72<sup>B6</sup>tg mice

<table>
<thead>
<tr>
<th>Population</th>
<th>MRL-Fasl&lt;sup&gt;pr&lt;/sup&gt; (n = 3–9)</th>
<th>Cd72&lt;sup&gt;B6&lt;/sup&gt;MRLtg (n = 3–5)</th>
<th>Cd72&lt;sup&gt;B6&lt;/sup&gt;tg (n = 3–6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cell number (×10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>1.1 ± 0.17</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cell number (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>3.9 ± 0.2</td>
<td>4.1 ± 0.9</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt;DP (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;SP (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;S&lt;sup&gt;-&lt;/sup&gt;P (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>7.1 ± 0.8</td>
<td>5.9 ± 0.4</td>
<td>7.0 ± 0.7</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cell number (×10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>3.3 ± 0.7</td>
<td>3.8 ± 0.8</td>
<td>1.7 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Thy1.2&lt;sup&gt;+&lt;/sup&gt;B22&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>2.6 ± 0.5</td>
<td>2.9 ± 0.5</td>
<td>1.7 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;SP (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>3.6 ± 0.6</td>
<td>4.2 ± 1.2</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt;SP (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.0 ± 0.1</td>
<td>1.8 ± 0.5</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;−&lt;/sup&gt;DN (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>2.4 ± 0.5</td>
<td>2.3 ± 0.4</td>
<td>0.9 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B22&lt;sup&gt;+&lt;/sup&gt;CD3&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>5.6 ± 2.0</td>
<td>4.5 ± 1.6</td>
<td>5.4 ± 1.1</td>
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<tr>
<td>T1&lt;sup&gt;+&lt;/sup&gt;T2&lt;sup&gt;+&lt;/sup&gt; (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.6 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>FO (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.7 ± 0.4</td>
<td>2.4 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MZ (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>2.3 ± 0.9</td>
<td>5.8 ± 0.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.1 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total cell number (×10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>1.3 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Peritoneal cavity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-1a (×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.4 ± 0.4</td>
<td>N.D.</td>
<td>0.9 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data for bone marrow and thymus were acquired when the mice were 8 wk of age; data for spleen, lymph nodes, and peritoneal cavity were acquired at ~20 wk of age.

Data are mean ± SEM.

<sup>a</sup>According to one-way ANOVA and post hoc Bonferroni correction, p < 0.05 versus MRL-Fasl<sup>pr</sup> mice or Cd72<sup>B6</sup>MRLtg mice.

<sup>b</sup>ANOVA and post hoc Bonferroni correction yielded statistically significant differences of p < 0.05 versus MRL-Fasl<sup>pr</sup> mice.

<sup>c</sup>No statistically significant differences were found by unpaired Student t test.

B-1a, IgM<sup>+</sup>CD5<sup>+</sup> DN, double negative; DP, double positive; FO, follicular zone B cell; B22<sup>+</sup>CD3<sup>+</sup> CD21<sup>low</sup>/CD23<sup>low</sup>; MZ, marginal zone B cell; B22<sup>+</sup>CD3<sup>+</sup>CD21<sup>high</sup>/CD23<sup>high</sup>; N.D., no data; SP, single positive; transitional 1 (T1) + transitional 2 (T2); B22<sup>+</sup>CD3<sup>+</sup>CD21<sup>low</sup>/CD23<sup>low</sup>.
frequency of the pro-B cell subset (B220+CD43+) was decreased slightly compared with that observed in MRL-Faslpr mice; however, the frequency of the more differentiated B cell subset (B220+CD43−) was not different among the three genotypes (Fig. 4C). In spleens, the number of B220+CD3− cells did not differ between MRL-Faslpr and Cd72B6tg mice (Table I). However, the percentage of follicular zone (FO) B cells (CD21low CD23high) was increased in Cd72B6tg mice compared with that observed in MRL-Faslpr mice (Cd72B6tg 58.3 ± 8.1%, n = 4; MRL-Faslpr 36.3 ± 7.6%, n = 7; Cd72B6/MRL 42.7 ± 17.2%, n = 4; p < 0.05) (Fig. 4D). The percentage of marginal zone (MZ) B cells (CD21highCD23low/−) was increased slightly in Cd72B6tg mice (Cd72B6tg 12.6 ± 3.3%, n = 4; MRL-Faslpr 7.6 ± 3.6%, n = 6; Cd72B6/MRL 9.8 ± 4.9%, n = 4; NS) (Fig. 4D). Accordingly, the absolute numbers of FO and MZ B cells were significantly increased in Cd72B6tg mice compared with those in MRL-Faslpr mice (Table I). The frequency of peritoneal B-1a cells was not different between Cd72B6tg mice and MRL-Faslpr mice (Table I). In humans, it is reported that CD72 suppresses naive B cell differentiation to plasma cells by downregulating XBP-1 (22). However, in our study, the percentage of CD138+ cells in the mouse spleens and the Xbp1 expression levels in splenic CD19+ B cells showed no differences among the genotyped groups of mice (Supplemental Fig. 4). These results indicate that Cd72B6 transgenesis improves splenomegaly by reducing the number of lpr T cells and augmenting B cell maturation to FO and MZ B cells in the periphery while not affecting B cell differentiation to plasma cells.

Reduction of serum levels of IgG3 and anti-dsDNA Abs in Cd72B6tg mice

The serum levels of Igs and autoantibodies were measured (Fig. 5). The IgG3 levels and anti-dsDNA autoantibody titers decreased significantly in Cd72B6tg mice but not in Cd72B6/MRLtg mice. These results are consistent with our previous finding that the production of IgG3 in MRL-Faslpr mice is a major factor responsible for the development of glomerulonephritis (19).

Reduced survival and BCR signaling responses in B cells obtained from Cd72B6tg mice

To examine proliferation of Cd72B6 and Cd72B6/MRLtg B cells in response to various mitogenic stimuli, we purified splenic B cells using negative selection with magnetic beads and stimulated the cells with anti-IgM F(ab′)2 Abs in the presence or absence of anti-CD40 Abs or with LPS. Cell viability responding to these stimuli was significantly reduced in Cd72B6tg, but not Cd72B6/MRLtg, B cells compared with that observed in MRL-Faslpr B cells (Fig. 6A).

To examine BCR signaling, we compared BCR-induced intracellular Ca2+ increases and kinetics of MAPK activation among the three groups of mice, because B cells from MRL-Faslpr mice show hyperactivity to BCR stimulation compared with that observed in other nonautoimmune mice (23). B cells from Cd72B6tg mice showed decreased and shortened influxes of Ca2+ after stimulation with anti-IgM F(ab′)2 Abs compared with those observed in B cells from MRL-Faslpr mice (Fig. 6B), whereas the Ca2+ response in Cd72B6/MRLtg B cells was similar to that in MRL-Faslpr B cells. Furthermore, the levels of phosho-p44/42
MAPK, one of the major downstream pathways of BCR, were decreased in Cd72<sup>tg</sup> mice, but not in Cd72<sup>homo/MRL</sup>tg B cells compared with MRL-Fasl<sup>pr</sup> B cells (Fig. 6C). These results indicate that Cd72<sup>tg</sup>, but not Cd72<sup>homo/</sup>MRC<sup>tg</sup>, BAC transgenesis downregulates B cell responses to BCR ligation and other stimuli.

Discussion

To examine the impact of polymorphic Cd72 as a positional candidate gene for autoimmune diseases in MRL-Fasl<sup>pr</sup> mice, we generated transgenic MRL-Fasl<sup>pr</sup> mice carrying a Cd72<sup>homo</sup> locus by means of BAC, which is usually >100 kbp in length and reproduces a spatio-temporal expression pattern similar to that of the endogenous locus independently of its integration site (24). The BAC clone used in this study includes the upstream 110 kbp and downstream 60 kbp of the Cd72 gene, suggesting that the expression of BAC-derived Cd72 could be induced and distributed in tissues and stages of development in the tg mice in the same manner as endogenous Cd72 under the control of the cis- and trans-elements. To adjust the expression levels of Cd72, as well as other involved genes on the BAC transgene, we compared tg MRL-Fasl<sup>pr</sup> mice carrying a non-modified and modified Cd72<sup>homo</sup> locus in BAC DNA, both of which were identical in the DNA sequence, with the exception of the introduced mutation at the polymorphic sites of interest. Moreover, pronuclear injection of transgene into fertilized oocytes of MRL-Fasl<sup>pr</sup> mice was performed to maintain a complete host genetic background, thereby preventing any issues related to genetic background. Although there might be a concern about the effect of integration sites of BAC on function, especially when multiple copies have integrated, a genomic-integration site of the transgene is typically confined to a single genomic site, regardless of the copy numbers (25). Furthermore, we revealed two independent tg lines of each group to avoid the risk that a DNA-integration site might reflect on different phenotypes in each group, which showed similar autoimmunity and mortality. Therefore, the results strongly suggest that phenotypic differences between Cd72<sup>homo/MRC</sup> mice and Cd72<sup>tg</sup> mice are dependent on the mutations within the BAC and not on the transgene copy numbers and their integration sites. Thus, this method enabled us to directly identify the role of the Cd72 polymorphism in the development of autoimmune diseases in MRL-Fasl<sup>pr</sup> mice.

Cd72 is a negative regulator of BCR signaling (15, 26). The strength of the BCR signal is regulated by both positive and negative regulators for B cell homeostasis (27). In this study, we demonstrated that the Cd72<sup>+</sup> allele was a hypofunctional polymorphism of B cell signaling compared with the Cd72<sup>−/−</sup> allele; the B cells of Cd72<sup>−/−</sup>tg mice showed a lower BCR signal strength than did Cd72<sup>homo/MRL</sup>tg mice, as demonstrated by the decrease in cell survival upon various stimuli, intracellular Ca<sup>2+</sup> influx, and MAPK activation. This was remarkable, even subtracting for the possibility of the effects of endogenous Cd72<sup>+</sup> of MRL-Fasl<sup>pr</sup> mice and/or the dominant negative effects of the heterodimeric Cd72 composed of Cd72<sup>+</sup> and Cd72<sup>−/−</sup>. These effects may result in improved autoantibody production and lupus phenotypes in conjunction with reductions in mortality. The ligand binding sites of Cd72 have not been mapped; however, the membrane-distal extracellular domain is considered to have an important role in ligand binding (28, 29). Therefore, the emphasized protective effects of BCR signaling in Cd72<sup>−/−</sup>tg mice might depend on the receptor’s affinity for its ligand.

The serum IgG3 levels in Cd72<sup>−/−</sup>tg mice were significantly reduced compared with those in MRL-Fasl<sup>pr</sup> and Cd72<sup>homo/MRL</sup>tg mice, although other IgG isotype expression levels were comparable among the three groups of mice. Production of IgG3 in MRL-Fasl<sup>pr</sup> mice was shown to be a major factor responsible for the development of glomerulonephritis (19). The IgG3 expression levels in Cd72<sup>−/−</sup>tg mice were similar to those in wild-type mice, whereas Cd72<sup>−/−</sup> mice showed significantly lower levels of serum IgG3 to the T cell–dependent secondary responses (16). In contrast to IgM- and IgD-specific negative regulation by CD22, Ig class–specific regulation by CD72 was not detected in vivo (30). However, it may be possible that Cd72 has unknown effects on the regulation of IgG3 specific in the MRL genetic background.

Several anti-DNA Abs have been produced by MZ B cells in other murine models of lupus including NZB/W F1 mice (31, 32). The autoimmunity of MRL-Fasl<sup>pr</sup> mice was also reported to be involved in MZ B cells or B-1 cells as shown by the enlarged splenic MZ compartment and anti-Sm Ab transgenic study (33, 34). In addition, Cd72<sup>−/−</sup> mice showed an accumulation of pre-B cells in bone marrow and a reduction in the number of mature B cells in the periphery in combination with a slightly higher percentage of MZ B cells and increased numbers of B-1 cells (16). Whereas, in the current study, the proportion of MZ B cells and B-1 cells in Cd72<sup>−/−</sup>tg mice were not decreased compared with MRL-Fasl<sup>pr</sup> mice. Therefore, the disease improvement in Cd72<sup>−/−</sup>tg mice seems to be independent of MZ B cells and B-1 cells. Indeed, a recent study indicates that MRL-Fasl<sup>pr</sup> mice with a mutant B cell activating factor receptor showed decreased FO or MZ B cell subsets; however, this did not contribute to glomerulonephritis (35). Taken together, the pathological B cell population in MRL-Fasl<sup>pr</sup> mice is different from that in NZB/W F1 mice.

Other quantitative trait loci associated with autoimmune disorders, such as Lbw2 and Ste2, were mapped on chromosome 4, which were derived from other lupus-prone mice, NZB/W F1 and NZB2410, respectively (36, 37). However, NZB and NZW mice have the same Cd72<sup>+</sup> allele, suggesting that Cd72 is not a common susceptibility gene in lupus-like phenotypes in NZB/W F1 and other related mice. In MRL-Fasl<sup>pr</sup> mice, the polymorphisms of Cd22, Fcgr2b, Spp1, Ili10ra, and Coro1a were reported to be associated with autoimmune phenotypes (11, 38–41). Of particular interest, the epistatic interaction between Cd72 and Fcgr2b in the...
FIGURE 6. Hypoproliferation, decreased kinetics of intracellular Ca\(^{2+}\) response, and decreased MAPK activation of splenic B cells from Cd72\(^{tg}\) mice. (A) Survival of MACS-sorted splenic B cells from MRL-Faslpr\(^{tg}\), Cd72\(^{Cd72B6/MRL}\), and Cd72\(^{Cd72B6}\) mice at 20 wk of age in response to various stimuli. All assays were performed in triplicate, and data are average \pm SEM. A total of 15 \(\mu\)g/ml F(ab\(^{9}\))2 goat anti-IgM Ab, 10 \(\mu\)g/ml anti-CD40 Ab, 15 \(\mu\)g/ml F(ab\(^{9}\))2 goat anti-IgM Ab + 10 \(\mu\)g/ml anti-CD40, 10 \(\mu\)g/ml LPS, and 10 U/ml IL-4 were used. (B) Intracellular Ca\(^{2+}\) response of splenic cells from MRL-Faslpr\(^{tg}\), Cd72\(^{Cd72B6/MRL}\), and Cd72\(^{Cd72B6}\) mice, following IgM cross-linking. Splenocytes were stimulated with 20 \(\mu\)g/ml of F(ab\(^{9}\))2 goat anti-IgM Ab and then examined by flow cytometry for FL-1. Data are representative of three independent experiments that had similar results. The time of addition of stimuli is indicated by the arrow. (C) Flow cytometric analysis of B cells from MRL-Faslpr\(^{tg}\), Cd72\(^{Cd72B6/MRL}\), and Cd72\(^{Cd72B6}\) mice, using a p-ERK Ab after the BCR stimulation [10 \(\mu\)g/ml F(ab\(^{9}\))2 goat anti-IgM Ab] for 0, 3, or 5 min at 37 \(^{\circ}\)C. The values in parentheses are mean fluorescence index. Data are representative of four independent experiments with similar results. \(*p < 0.05.\)

development of autoimmune diseases has been observed in both humans and mice (42, 43). The occurrence of polygenic diseases has been explained in a threshold-liability model, in which individuals develop the disease when the total number of disease-susceptibility genes exceeds a given threshold. Therefore, it will be valuable to analyze the interactions of Cd72 with other susceptible genes to elucidate the mechanisms of polygenic inheritance.

In this study, we demonstrated that a CD72 polymorphism in the membrane-distal extracellular domain is associated with glomerulonephritis and vasculitis in an autoimmune-prone mouse, owing to the hypofunctional allele of Cd72. These results suggest that CD72 is a target molecule for the therapeutic management of these diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary figure 1

Construction of recombinant CD72 gene on bacterial artificial chromosome (BAC). A, Sequential homologous recombinations—cointegration and resolution—on BAC to generation for the exon replacement between the CD72 allotypes: Cd72b (white square) and Cd72c (black square). B, Electrophoresis profile of EcoRI-digested ‘cointegrated’ (lanes 1 to 5) and original BAC (B6) clones. M, λ HindIII-digested. C, Southern blot analysis of cointegrated (lanes 1 to 5) and original BAC (B6) clones probed with exons 8 and 9 of Cd72c gene. Expected signals as in (A)—2.3 kb and 10 kb—are shown in lanes 2 to 5, indicating successful cointegration occurred in these clones. D, Electrophoresis profile of EcoRI-digested ‘resolution’ (lanes a to e) and original BAC (B6) clones. M, λ HindIII-digested. E, Southern blot analysis of cointegrated (lanes a to e) and original BAC (B6) clones probed as in (C) a single signal—8.4 kb—at each lane indicates successful resolution occurred in all the clones.
Establishment of the bacterial artificial chromosome (BAC) transgenic mice by pronuclear microinjection of MRL-\(Fas^{lp}r\) fertilized oocytes. Each two independent lines of \(Cd72^{B6/MRL}\) and \(Cd72^{B6}\) BAC transgenic mice were established, and maintained with a complete MRL-\(Fas^{lp}r\) genetic background.

<table>
<thead>
<tr>
<th>transgene</th>
<th>total number of done:</th>
<th>total number of injection</th>
<th>total number of transfer</th>
<th>birth</th>
<th>founder</th>
<th>line</th>
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<tr>
<td>(Cd72^{B6/MRL})</td>
<td>54</td>
<td>438</td>
<td>377</td>
<td>45</td>
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<td>2</td>
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<td></td>
<td>(8.1 cells / mouse)</td>
<td>(86.1%)</td>
<td>(11.9%)</td>
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<tr>
<td>(Cd72^{B6})</td>
<td>99</td>
<td>712</td>
<td>547</td>
<td>34</td>
<td>2</td>
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<td>(7.2 cells / mouse)</td>
<td>(76.3%)</td>
<td>(6.2%)</td>
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</tbody>
</table>

| total             | 153                   | 1150                      | 924                      | 79    | 7       |      |
|                   | (7.5 cells / mouse)  | (80.3%)                   | (8.5%)                   |       |         |      |

**Supplementary figure 2**
Cd72 gene expression in splenic CD19+ B cells. Transgene- and endogenous-specific Cd72 expression in cDNA from MACS-sorted CD19+ B cells by a RT-PCR analysis. The PCR analyses, using primer pairs specific for the Cd72^{B6} allele (upper) and the Cd72^{MRL} allele (lower), were appropriately performed.
Flow cytometric analyses of plasmablast development and X-box binding protein 1(Xbp-1) expression. A, Plasmablast development of each genotyped group at 20 weeks of age was determined by the percentage of CD138-positive cells in the spleen. Cell surface staining using an anti-CD138 antibody (BD Pharmingen) was performed according to standard techniques, and the flow cytometric analysis was performed with a FACSCalibur using the CellQuest software program (BD Biosciences). The values in the histograms indicate the percentage of CD138 positive cells. The plamablast development was not significantly different among the three genotyped groups of mice. B, A real-time RT-PCR analysis of Xbp-1 gene expression levels in splenic CD19+ sorted B cells at 6-10 weeks of age of mice. The Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) was used according to the manufacturer's protocol. Assays were performed in duplicate on the ABI PRISM 7700 system (Applied Biosystems). The oligonucleotide primers are 5’- TGG ATC CTG ACG AGG TTC CA -3’ (forward) and 5’- TGA CAG GGT CCA ACT TGT CC -3’ (reverse). The expression levels were not different among the three genotype groups of mice.

Supplementary figure 4