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Cyclin-Dependent Kinase Inhibitor Cdkn2c Regulates B Cell Homeostasis and Function in the NZM2410-Derived Murine Lupus Susceptibility Locus Sle2c1

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Sle2c1 is an NZM2410- and NZB-derived lupus susceptibility locus that induces an expansion of the B1a cell compartment. B1a cells have a repertoire enriched for auto-reactivity, and an expansion of this B cell subset occurs in several mouse models of lupus. A combination of genetic mapping and candidate gene analysis presents Cdkn2c, a gene encoding for cyclin-dependent kinase inhibitor p18INK4c (p18), as the top candidate gene for inducing the Sle2c1-associated expansion of B1a cells. A novel single nucleotide polymorphism in the NZB allele of the Cdkn2c promoter is associated with a significantly reduced Cdkn2c expression in the splenic B cells and peritoneal cavity B1a cells from Sle2c1-carrying mice, which leads to a defective G1 cell cycle arrest in splenic B cells and increased proliferation of peritoneal cavity B1a cells. As the cell cycle is differentially regulated in B1a and B2 cells, these results suggest that Cdkn2c plays a critical role in B1a cell self-renewal and that its impaired expression leads to an accumulation of these cells with high autoreactive potential. The Journal of Immunology, 2011, 186:6673–6682.

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CD70+ T cells (7). This confers a greater significance to the understanding of the mechanisms that regulate the size and functions of this B cell subset. We have shown that the Sle2-mediated expansion of B1a cells is B cell intrinsic and results from a combination of factors, which includes an increased spontaneous proliferation of B1a compared with B2 cells (8). Using congenic recombinants, we mapped the expansion of B1a cells to three regions of Sle2, Sle2a, Sle2b, and Sle2c, but the major contribution was provided by the telomeric sublocus Sle2c (9).

The repertoire of B1a cells is autoreactive (10), and an expanded B1a cell population has been consistently associated with lupus, but how these cells contribute to autoimmune pathology has been controversial (4). Recent studies have shed a new light on the topic, strongly suggesting a direct pathogenic role of B1a cells. The (NZB × NZW)F1 B1a cells migrate to inflamed tissues, notably the kidneys, where they class-switch and produce anti-dsDNA IgG (11). In addition, B1a cells favor the differentiation of CD4+ T cells into Th17 cells (12). Th17 cells have been directly implicated in lupus in that they provide help to autoreactive B cells in mice (13) and lupus patients (14) and contribute to the inflammatory cascade in lupus nephritis (15). Therefore, B1a cells contribute to lupus pathology through either Ab production or Ag-presentation, or both. A complementation analysis of the Sle1.Sle3 combination failed, however, to show that Sle2c contributed to autoimmune pathology (9). This study was complicated, however, by the complex structure of Sle2c, which contains, in addition to the B1a-promoting locus named Sle2c1, a suppressor locus named Sle2c2 (16). In the absence of Sle2c2, Sle2c1 dramatically increased the severity of lupus nephritis induced by Fas-deficiency by enhancing the differentiation of CD4+ T cells into Th17 cells and promoting their infiltration of the kidneys (17). These results support the hypothesis that the expansion of the B1a cell compartment by Sle2c1 contributes to autoimmune pathogenesis at least through their induction of inflammatory T cells.

The current study was conducted to identify the gene in the Sle2c1 interval that is responsible for the expansion of the B1a cell compartment. A combination of genetic mapping and candidate gene analysis presents Cdkn2c, a gene encoding for cyclin-
dependent kinase inhibitor p18\textsuperscript{Nkx,c} (p18), as the top candidate gene for inducing the Sle2c1 phenotype. A novel single nucleotide polymorphism (SNP) in the NZB allele of the \textit{Cdkn2c} promoter is associated with a significantly reduced \textit{Cdkn2c} expression in the splenic B cells and B1a cells from Sle2c1-carrying mice, which leads to defective G1 cell cycle arrest in splenic B cells and increased proliferation of Pbc B1a cells.

Materials and Methods

Mice

The B6.Sle2, B6.Sle2c1, and B6c3Lmj (B6c3Tc) congenic strains have been previously described (2, 9, 18). B6.Sle2c1 recombinants obtained from a backcross to B6 were identified and fine-mapped with a panel of microsatellite markers and SNPs that are polymorphic between NZB and B6 (Fig. 1). These recombinants were then expanded in out-crosses to B6 and bred to homozygosity to generate three Sle2c1 recombinant strains. B6, NZM2410, NZB, and NZW mice were originally obtained from The Jackson Laboratory. The phenotypes of B6.Sle2c1 and its recombinants were analyzed in both males and females at the age indicated. Cohorts of (NZB × B6.Sle2c1)F1 (NZB × B6c3Lmj)F1 (B6c3Tc)F1 and (NZB × B6)F1 (B6 × NZW)F1 females were aged to 12 mo. Kidney histopathology was performed and glomerulonephritis (GN) was scored as previously described (9). Severity and a description of the type of lesion (in order of severity: hyalinosis was performed and glomerulonephritis (GN) was scored as previously described (21)). Briefly, plates were coated with NP-BSA (Biosearch Technologies) at 10 \mu g/ml. After blocking, serially diluted sera were incubated in duplicate, and the NP-bound Abs were detected by alkaline phosphatase-conjugated goat anti-mouse IgG (Chemicon) or IgM (SouthernBiotech). Each immunization was performed in duplicate with three to seven mice per strain. For in vitro measurement of Ab production, \textit{sB} cells were stimulated with 8 \mu g/ml of either LPS (Sigma) or anti-IgM Fab\textsubscript{ab} (Abcam) for up to 72 h, and Pbc cell cultures were stimulated with 5 \mu g/ml LPS for 48 h. Total IgM in supernatants diluted up to 1:250 was measured by sandwich ELISA on plates coated with goat anti-mouse IgM (ICN/Cappel) at 1 \mu g/ml and revealed with alkaline phosphatase-conjugated goat anti-mouse IgM (SouthernBiotech).

\textit{p18} immunoprecipitation and Western blot

Cells were lysed in RIPA lysis buffer (0.1% Nonidet P-40, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM \mu l/m of antiprotein, 2 mM PMSF, 10 \mu g/ml leupeptin, and 2 \mu g/ml pepstatin A in PBS; all reagents from Sigma) on ice for 60 min. After centrifugation, the supernatants were assayed for protein concentration (Bradford assay; Bio-Rad). Total proteins (250–500 \mu g) were immunoprecipitated with p18 mouse mAb (sc-56453; Santa Cruz Biotechnology) and protein-G beads (Amerham) (Amersham) at 1 \mu g/ml and revealed with alkaline phosphatase-conjugated goat anti-mouse IgG (Thermo Scientific). Blots were developed using an ECL detection system (Amersham).

Cdkn2c sequencing and promoter analysis

The \textit{Cdkn2c} promoter, 5'UTR, exons, and 3'UTR were sequenced from genomic DNA extracted from B6 and B6.Sle2c1 mice. The PCR primers used for sequencing are listed in Table I. Genomic DNAs from the Sle2c1 recombinant strains and parental strains were genotyped for the –74 C → T SNP with the following primers: 5'-AGCCTCTAAGGCCAAGCGCAGC-3' and 5'-GCAACTGTGCTACGGTGTCG-3'. Reporter constructs were generated by cloning into the firefly luciferase reporter plasmid pGL3

Ab measurements

Anti-dsDNA IgG was measured as previously described (2) in sera from the NZB crosses diluted 1:100. Relative units were standardized using a B6. TC serum, arbitrarily setting the reactivity of a 1:100 dilution of this control serum to 100 U. Mice were immunized with 100 \mu g NP-KLH (Biosearch Technologies) emulsified in alum (Pierce Biotechnology) at a 1:1 ratio as previously described (21)). Primary immune responses were measured 7 d after. For secondary responses, mice were boosted with the same dose of NP-KLH and alum 3 wk after the primary immunization. Mice were sacrificed 10 d after the secondary immunization. Anti-nitrophenylacyl (NP) IgM and IgG were quantified by ELISA as previously described (21)).
These results map Sle2c1 transcripts. (Supplemental Table I), as well as 14 anonymous protein-coding between rs28132547 and D4MIT278 (Fig. 1). This interval B Sle2c1 mice, were similar to that of B6. Sle2c1.Rec1a recombinants (Fig. 1) for expanding the Pc B1a cell subset, we generated three B6. The percentage and absolute number of Pc B1a cells, because most of the few SNPs in this region that are published as (NZW or NZB)-derived intervals are indicated by the solid boxes, with the area of recombination between the NZM2410 and B6 genomes indicated by the lines on each side. Intervals associated with an expansion of the Pc B1a cell phenotype are shown in black, and the Pc B1a phenotype is indicated in gray. Overall, these results resulted in an accelerated production of anti-dsDNA Ab. But Sle2c1 enhances NZB autoimmune phenotypes To determine the contribution of Sle2c1 to autoimmune pathogenesis, we compared cohorts of N×Sle2 and N×Sle2c1 females, in which the entire Sle2 or only Sle2c1 was expressed as a homozygous locus (N×Sle2 and N×Sle2c1, respectively) to N×B mice in which these loci were heterozygous, all on an NZB × B6 heterozygous background. This complementation analysis previously determined that Sle1 (22) and Sle1c (23) significantly enhanced the autoimmune phenotypes of the NZW and NZB heterozygous genotypes, respectively. At 12 mo of age, both N×Sle2 and N×Sle2c1 mice showed a significantly increased splenomegaly (Fig. 3A). In addition, N×Sle2c1 mice showed an increased percentage of B1a cells in the spleen (Fig. 3B) and an expansion of the transitional T1 B cell subset at the expense of follicular B cells (Fig. 3C) compared with N×B mice. These two phenotypes were even more accentuated in N×Sle2 mice. However, the Pc B1a cell compartment was not expanded in N×Sle2c1 mice, as it was in N×Sle2 mice (Fig. 3D). Both N×Sle2 and N×Sle2c1 mice produced significantly more anti-dsDNA IgG than that of N×B mice at 7 mo of age (Fig. 3E), but this difference was not maintained at either 9 or 12 mo of age (data not shown). The expression of the whole Sle2 locus on the NZB background significantly enhanced renal pathology compared with N×B (comparison of the distribution of GN scores, \( \chi^2 = 26.84, p < 0.001 \)), but there was no difference between the renal pathology of N×Sle2c1 and N×B mice (Fig. 3F). Furthermore, the amount of glomerular immune complex deposits was not different between these two latter stains (data not shown). Overall, these results show that Sle2c1 homozygosity enhanced NZB cellular phenotypes that are associated with autoimmune, namely splenomegaly, and expanded transitional B cells and splenic B1a cells and resulted in an accelerated production of anti-dsDNA Ab. But...
Sle2c1 expression was not sufficient to induce strong autoimmune phenotypes, including renal pathology, as was observed for the entire Sle2 locus. This suggests that Sle2c1 contribution to autoimmune pathogenesis requires the presence of other susceptibility loci.

Cdkn2c expression is significantly decreased in B6.Sle2c1 B cells

To gain insight into the mechanisms by which Sle2c1 expands the Pc B1a cell compartment, we performed microarray analyses on Pc B1a cells and sB cells obtained from B6.Sle2c1 and B6 mice. Five hundred thirty-four genes for Pc B1a cells and 120 genes for sB cells were differentially expressed between the two strains \( (p \leq 0.001) \). Among these genes, the Cyclins and Cell Cycle Regulation pathway was differentially expressed with least square permutation \( p \) values of \( 8 \times 10^{-4} \) for Pc B1a cells and \( 10^{-3} \) for sB cells (Fig. 4A, 4B). The specific genes within this pathway that were differentially expressed between the two strains were not identical between the two cell types, which corroborates the existence of a B1a-specific cell cycle regulation (24). However, the

FIGURE 2. Mapping the Pc B1a cell expansion in the Sle2c1 recombinants. A, Representative FACS plots of B220 and CD5 staining of Pc lymphocytes in B6 and B6. Sle2c1 and its recombinants showing the B1a (solid line) and B2 (dashed line) gates. B, Percentages and absolute numbers of Pc B1a cells and B1a/B2 cell ratios in the B6.Sle2c1 strain and its recombinants compared with B6. The graphs show means and SEM for 10–25 mice per strain at 5–6 mo of age. \( *** p < 0.001 \) (statistical significance of Dunnett’s multiple comparison tests with B6 values).

FIGURE 3. Sle2c1 homozygosity enhanced splenic B1a and T1 B cell expansion but not renal pathology on an NZB heterozygous background. Comparisons between NxB, N\times Sle2c1, and N\times Sle2 spleen weight (A), percentage of splenic B1a cells (B), AA4.1 IgM+ CD21hi CD23lo follicular B cell (T1/FOB) ratio (C), Pc B1a/B2 cell ratio (D), serum anti-dsDNA IgG at 7 mo of age (E), and GN score distribution (F). Graphs A–E show individual mice and means plus SEM. \( * p < 0.05, ** p < 0.01, *** p < 0.001 \). Graph E shows the distribution of GN scores in ten 12-mo-old mice from each strain. Scores were grouped according to the type of lesion (H, hyaline; MM, mesangial matrix; MC, mesangial cellular; and P, proliferative) and the severity score (all scores were either 3 or 4).
expression of one gene, *Cdkn2c*, was ∼4-fold lower in B6.*Sle2c1,* than in B6 for both Pc B1a and sB cells (Fig. 4A, 4B). *Cdkn2c* is located within the *Sle2c1* interval (Fig. 1B). RT-PCR confirmed a significantly lower *Cdkn2c* expression in B6.*Sle2c1* and *Sle2c1*-expressing B6.TC sB cells compared with B6 sB cells (Fig. 4C, D). *Cdkn2c* message expression in sB cells from B6, B6.*Sle2c1*, B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6.TC mice. *E*, Representative p18 expression in B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6 sB cells. IgG expression was used as control. *F*, *Cdkn2c* message expression in Pc B1a cells from B6, B6.*Sle2c1*, B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6.TC mice. *G*, Representative p18 expression in Pc B1a cells from B6.*Sle2c1* and B6 mice immunized with NP-KLH (right) and unimmunized B6 and B6.TC mice (left). The densitometry analysis normalized to IgG expression is shown on the graph on the right. In *D* and *F*, qRT-PCR data were normalized to Gapdh and expressed as fold difference with one B6 value. Individual mouse values and means plus SEM are represented.

FIGURE 4. *Cdkn2c* expression in B cells from B6.*Sle2c1* recombinant mice. Cyclins and Cell Cycle Regulation pathway genes differentially expressed (*p* < 0.05) in Pc B1a (black) or sB (white) cells between B6 and B6.*Sle2c1* mice, showing the *p* values (*A*) and the ratios of geometric means (five mice per strain), in which negative values indicate genes underexpressed in B6.*Sle2c1* B cells (*B*). The double arrow between *A* and *B* points to *Cdkn2c*. C, Representative *Cdkn2c* and β-actin (*Actb*) expression in B6.*Sle2c1*, B6.TC, and B6 sB cells. *D*, *Cdkn2c* message expression in sB cells from B6, B6.*Sle2c1*, B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6.TC mice. *E*, Representative p18 expression in B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6 sB cells. IgG expression was used as control. *F*, *Cdkn2c* message expression in Pc B1a cells from B6, B6.*Sle2c1*, B6.*Sle2c1.Rec1a*, B6.*Sle2c1.Rec1b*, and B6.TC mice. *G*, Representative p18 expression in Pc B1a cells from B6.*Sle2c1* and B6 mice immunized with NP-KLH (right) and unimmunized B6 and B6.TC mice (left). The densitometry analysis normalized to IgG expression is shown on the graph on the right. In *D* and *F*, qRT-PCR data were normalized to Gapdh and expressed as fold difference with one B6 value. Individual mouse values and means plus SEM are represented.

expression of one gene, *Cdkn2c*, was ∼4-fold lower in B6.*Sle2c1* than in B6 for both Pc B1a and sB cells (Fig. 4A, 4B). *Cdkn2c* is located within the *Sle2c1* interval (Fig. 1B). RT-PCR confirmed a significantly lower *Cdkn2c* expression in B6.*Sle2c1* and *Sle2c1*-expressing B6.TC sB cells compared with B6 sB cells (Fig. 4C). qRT-PCR showed that B6.*Sle2c1*, B6.*Sle2c1.Rec1a*, and B6.TC sB cells expressed significantly less *Cdkn2c* than B6 whereas B6.*Sle2c1.Rec1b* sB cells expressed a similar level as B6 cells (Fig. 4D). Correspondingly, p18 protein was expressed at a low level in B6.*Sle2c1.Rec1a* sB cells in comparison with B6 and B6.*Sle2c1.Rec1b* cells (Fig. 4E). *Cdkn2c* message expression was also low in B6.*Sle2c1.Rec1a* and B6.TC Pc B1a cells and high in B6.*Sle2c1.Rec1b* and B6 Pc B1a cells (Fig. 4F). A decreased expression of p18 protein was found in both B6.*Sle2c1* and B6.TC Pc B1a cells compared with B6 cells (Fig. 4G). Expression of the other cyclin-dependent kinase inhibitors was also examined by qRT-PCR in sB and Pc B1a cells (Supplemental Fig 1). Although the results were inconclusive for *Cdkn1a*, this analysis confirmed the microarray data for *Cdkn1b* expression, which was decreased in *Sle2c1*-expressing B1a cells, and for *Cdkn2d*, which was increased in sB cells and decreased in Pc B1a cells expressing *Sle2c1*. Overall, these results showed an association between a low level of *Cdkn2c* expression in B cells and elevated numbers of Pc B1a in the *Sle2c1* congenic recombinants (this report) and in B6.TC mice that carry the *Sle2c1* locus (2).

p18, the cyclin-dependent kinase inhibitor encoded by *Cdkn2c*, prevents the activation of cyclin D2 and D3 by CDK4 and CDK6 and leads to early G1 cell cycle arrest (25). p18-mediated G1 arrest is necessary for differentiation into functional Ab-secreting plasma cells, and p18-deficient mice display a defective Ab production in response to immunization (26). We hypothesized that a functional consequence of a low p18 expression in the *Sle2c1* recombinants should be a defective Ab response to immunization with NP-KLH. *Cdkn2c* message expression was significantly
lower in the spleens of immunized B6.Sle2c1 and B6.Sle2c1.Rec1a than that in B6 and B6.Sle2c1.Rec1b mice (Fig. 5A). As previously reported (26), Cdkn2c expression increased in B6 mice upon secondary immunization, but it remained unchanged in B6.Sle2c1 mice. This result was confirmed at the protein level, with p18 being barely detectable in the spleen of B6.

p18 expression increased in B6 mice after secondary NP-KLH immunization. IgG expression was probed as control. Quantitation of the bands by densitometry is shown on the right. C and D, Anti-NP IgM from B6, B6.Sle2c1.Rec1a, B6.Sle2c1.Rec1b, and B6.Sle2c1 mice after primary NP-KLH immunization in sera diluted 1:100 (C) and in serially diluted sera (D). E and F, Anti-NP IgG from B6, B6.Sle2c1.Rec1a, B6.Sle2c1.Rec1b, and B6.Sle2c1 mice after secondary NP-KLH immunization in sera diluted 1:100 (E) and in serially diluted sera (F). G, Anti-NP IgM and IgG in 1:100 diluted sera from B6 and B6.Sle2c1 mice after secondary NP-KLH immunization. The graphs show means and SEM of at least five mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 (statistical significance of non-parametric tests with B6 values with multiple test correction when appropriate). In E, the difference was not significant between B6 and B6.Sle2c1 mice with a multiple comparison test but was different (p = 0.02) with a Mann–Whitney U test. In D and F, the tests were performed between the B6.Sle2c1.Rec1a and B6 values for each dilution.

lower in the spleens of immunized B6.Sle2c1 and B6.Sle2c1.Rec1a than that in B6 and B6.Sle2c1.Rec1b mice (Fig. 5A). As previously reported (26), Cdkn2c expression increased in B6 mice upon secondary immunization, but it remained unchanged in B6.Sle2c1 mice. This result was confirmed at the protein level, with p18 being barely detectable in the spleen of B6.Sle2c1 mice after secondary immunization (Fig. 5B). Similar data were obtained in the primary immunization, although the overall level of p18 expression was much lower than in the secondary immunization (data not shown). Serum anti-NP IgM (Fig. 5C, 5D) and IgG (Fig. 5E, 5F) were significantly lower in B6.Sle2c1 and B6.Sle2c1.Rec1a than in B6 and B6.Sle2c1.Rec1b mice 7 d after immunization, indicating that p18 expression affects functional plasma cell differentiation in the Sle2c1 recombinants as it did in the p18-deficient mice (26). As expected, B6.Sle2c1 mice also produced less anti-NP Ab than B6 mice after secondary immunization (Fig. 5G). The number and frequency of NP^* CD138^* plasma cells was, however, not affected by Sle2c1 expression (data not shown). Notably, B6.Sle2c1 heterozygous mice showed an intermediate level of Cdkn2c and p18 after secondary immunization (Supplemental Figs. 2A, 5B). The anti-NP IgM and IgG production was, however, similar between B6.Sle2c1 homozygous and heterozygous mice (Supplemental Fig. 2B, 2C). This suggests that the Sle2c1 allele controls p18 expression in a dose-dependent manner, but that a 50% reduction of p18 expression is sufficient to significantly impair plasma cell differentiation. Overall, these results show that mice expressing the NZB allele of Sle2c1 mount a lower Ab response to T-dependent immunization compared with that of mice expressing the B6 allele of Sle2c1, which is consistent with the respective levels of Cdkn2c expression found in the sB cells of these mice.

To compare the effect of low Cdkn2c expression between sB and Pc B1a cells, purified cells were stimulated in vitro. Cdkn2c expression increased overtime in sB cells stimulated with LPS in all strains, but it remained significantly lower in B6.Sle2c1 and B6.Sle2c1.Rec1a than in B6 and B6.Sle2c1.Rec1b mice (Fig. 6A). Accordingly, a significantly lower amount of IgM was obtained from LPS-stimulated B6.Sle2c1 and B6.Sle2c1.Rec1a sB cells than from B6 and B6.Sle2c1.Rec1b sB cells (Fig. 6B). Similar results were obtained with sB cells stimulated with anti-IgM (Fig. 6C and data not shown). These results are consistent with those obtained with the T-dependent immunization showing an impaired
Ab production from sB cells expressing low levels of Cdkn2c. PC B1a cells from B6, Sle2c1, Rec1a and B6, Sle2c1, Rec1b mice expressed a lower level of Cdkn2c than PC B1a cells from B6 or B6, Sle2c1, Rec1b mice without significant change in the presence or absence of LPS stimulation (Fig. 6D). However, LPS-stimulated B6, Sle2c1 and B6, Sle2c1, Rec1a B1a cells secreted significantly more IgM than B6 or B6, Sle2c1, Rec1b B1a cells (Fig. 6E). These data corroborate earlier studies that have shown that B1a cells do not undergo G1 arrest for Ab secretion (27). Therefore, high IgM secretion occurs in B1a cells expressing the NZB allele of Sle2c1, despite low Cdkn2c expression. Finally, we have previously shown that B6, Sle2c1 PC B1a cells proliferated more than B6 PC B1a cells, either spontaneously or in response to LPS, whereas no difference was observed for CD5− B cells (8). In vitro LPS stimulation indicated that this phenotype mapped to Sle2c1: In both spleen and PC, more B6, Sle2c1 CD5+ B cells proliferated than B6 CD5+ B cells, but there was no difference for CD5− cells (Fig. 6F).

Assessment of the spontaneous B cell proliferation status ex vivo found a significantly higher number of Ki67+ proliferating PC B1a cells in the strains expressing the NZB allele of Sle2c1 (B6, Sle2c1 and B6, Sle2c1, Rec1a) compared with the B6 allele (B6 and B6, Sle2c1, Rec1b) (Fig. 6G). Overall, these results show that Cdkn2c,
a gene located within the Slev2cI critical interval, is expressed at lower levels in B6.Slev2cI B cells. This low-level expression impaired functional plasma cell differentiation in sB cells but not in Pc B1a cells, and it increased B1a but not B2 cell proliferation.

A novel promoter SNP regulates Cdkn2c transcription

The sequence of the Cdkn2c exons, 5′ UTR, and 3′ UTR regions was identical between B6 and B6.Slev2cI mice (data not shown). We identified, however, a novel SNP at position −74 in the promoter in which the B6 allele is C and the Slev2cI allele is T (Table I, Supplemental Fig. 3A). This region is highly conserved in the Cdkn2c promoter in mammals (Supplemental Fig. 3B). An in silico analysis (28) predicted that the −74 C → T transition results in the loss of binding sites for the NRF2 and Hunchback transcription factors, whereas it creates a binding site for YY1 that is adjacent to the already existing YY1 site. YY1 can either negatively or positively regulate transcription depending on the context of the adjacent transcription factors (29). Therefore, a detailed analysis of the contribution of each transcription factor binding at this site will be necessary to understand how they impact Cdkn2c expression. No other mutation was found in the Slev2cI allele of the Cdkn2c promoter. We genotyped the Slev2cI recombinant strains as well as the parental strains for this SNP (Table II) and found a perfect concordance between 1) the imputed genomic origin of the region (B6.Slev2, NZM2410, NZB, all carrying the T allele) and 2) the expansion of the Pc B1a cell compartment. Indeed, B6.Slev2cI.Rec1b mice carry the C allele and show B6-like levels of Pc B1a cells, whereas all the strains with T allele have been shown to expand this compartment (Refs. 3, 9, 30 and this report). Notably, NZW mice have elevated numbers of Pc B1a cells (Ref. 31 and L. Morel, unpublished observations), but they carry the −74 C allele.

The impact of the −74 C/T SNP on Cdkn2c transcription was determined by comparing the expression of a luciferase reporter gene driven by each allele. Three promoter–luciferase constructs were produced within the region that has been shown to induce maximal transcription (32), with two constructs (−1209 and −279) containing the SNP and one construct (−52) corresponding with the minimal promoter (33) not containing the SNP. As expected, the −52 construct induced a low luciferase expression that was similar between the two alleles (Fig. 7). For both constructs containing the SNP, however, the C allele induced a significantly higher level of transcription than that of the T allele. Cdkn2c expression is regulated by the E2F1 transcription factor (30), and the −1209 and −279 constructs contain two sites that loosely fit the consensus DNA sequence of E2F-binding sites (34) (Supplemental Fig. 3A). As a preliminary to a detailed analysis of the Cdkn2c promoter, we extended these experiments to determine whether the difference of expression between the Slev2cI and B6 allele is maintained in the presence of E2F1. E2F1 increased the activity of the Cdkn2c promoter constructs −1209 and −279 by 190- and 225-fold, respectively (Fig. 7B). Notably, even in the presence of this strong transcriptional induction, the C allele induced a significantly higher level of transcription than that of the T allele for both constructs. A decreased transcriptional activity of the −279 Slev2cI construct was also observed in Raji cells (Fig. 7C). These results strongly suggest that the −74 C → T transition is responsible for the low level of p18 found in the B cells expressing the NZB allele of Cdkn2c.

Discussion

Slev2cI is the major locus contributing to the expansion of Pc B1a cells in the NZM2410 mouse model of lupus. Genetic analysis mapped Slev2cI to a 6 Mb NZB-derived region of chromosome 4 based on the analysis of two new recombinant intervals, Slev2cI.Rec1a and Slev2cI.Rec1b. An expansion of both Pc B1a cells and splenic NKT cells was mapped by another group to a larger interval on the NZB chromosome 4 that includes Slev2cI (35). The phenotype of the two recombinant intervals analyzed in that study are consistent with our results, and we predict that the NZBc4S interval that has a B6-like phenotype is telomeric to Slev2cI. In addition, B6.Slev2cI and B6.Slev2e mice have a normal NKT cell level (data not shown), suggesting that the increased NKT cell number is an NZB phenotype that maps to the NZW-derived part of Slev2. The Slev2cI critical interval contained too many genes, most of them with known expression in B cells, for a direct candidate gene analysis. Gene expression profiling of both sB and Pc B1a cells provided the leading cues by identifying among the many genes differentially expressed a significant involvement of the cell cycle regulation pathway. Furthermore, the expression of one of its members, Cdkn2c, was 4-fold lower in Slev2cI B cells of both types and mapped to the Slev2cI interval. We have confirmed the difference in Cdkn2c expression with qRT-PCR. Moreover, we have identified a unique promoter polymorphism for which there is a complete concordance between, on one hand, the C allele and B6-like Pc B1a levels, and on the other hand, the T allele and an enlarged Pc B1a compartment. The only exception is the NZW strain, indicating that the accumulation of B1a cells in that strain is sustained through a different mechanism, which is not surprising because a large number of genetic alterations have already been found to affect the size of the B1a cell compartment (10).

We have initially reported that Slev2e did not increase clinical disease in B6.Slev1.Slev3 mice (9). This experiment was, however, conducted with a long Slev2e interval, in which we have identified, in addition to Slev2cI, a strong Slev2c2 suppressor locus (16). Such “gene masking” resulting from the proximity of susceptibility and suppressor loci has been reported in the NOD mouse model of diabetes (36) and is likely to represent a frequent occurrence in the genetic architecture of complex traits. When Slev2cI contribution was analyzed separately from Slev2c2, we found that it promoted a severe renal and skin pathology in B6. lpr mice by favoring TH17 differentiation (15). In this study, we showed an allele-dose

<table>
<thead>
<tr>
<th>Cdkn2c Regions</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>Amplicon*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter and exon 1</td>
<td>AGCCCTCTAAAGGCCTCCGC</td>
<td>GCAACTGCTGTACGTTGCC</td>
<td>−423 to 45</td>
</tr>
<tr>
<td>Promoter and exon 1</td>
<td>TGTCGGCCTGTAGTGGAGA</td>
<td>GCCCTCGATGATGAGAAGGT</td>
<td>−81 to +417</td>
</tr>
<tr>
<td>Exon 1</td>
<td>GCCGGAGGAGGCCAACAAGAC</td>
<td>TCTCCGGAGGAGGCTGGTGG</td>
<td>+271 to +707</td>
</tr>
<tr>
<td>Exon 1</td>
<td>TCTGCCGTACACGGTTCAG</td>
<td>TGTACGTCAGACAACCCAGG</td>
<td>+607 to +1075</td>
</tr>
<tr>
<td>Exon 1</td>
<td>TTCCTGCCATCCCTCGTCG</td>
<td>CGCGCCCTCGATTTAC</td>
<td>+629 to +1137</td>
</tr>
<tr>
<td>Exon 1</td>
<td>CAGCGCTGTCGACGCTTTTC</td>
<td>CCGGAGGAGGCTGGTGGT</td>
<td>+1386 to +1652</td>
</tr>
<tr>
<td>Exon 3</td>
<td>CCTTCGCGATTGACGCAAGAC</td>
<td>GCCGTTAGCAAGTAAAC</td>
<td>+1627 to +2377</td>
</tr>
<tr>
<td>3′UTR</td>
<td>TCGACTTGGCCAGGTTCTAT</td>
<td>CACACTACACCAGGCTTC</td>
<td>+1928 to +2510</td>
</tr>
</tbody>
</table>

*All the locations were calculated relative to +1 transcription start site.
Table II. Genotype at the −74 C→T SNP in the B6.Sle2c1 recombinant and parental strains as well as the relative expansion of the Pc B1a cell compartment relative to B6.

<table>
<thead>
<tr>
<th>Strain</th>
<th>jPc B1a</th>
<th>−74 C→T</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>−</td>
<td>C</td>
</tr>
<tr>
<td>B6.Sle2</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>B6.Sle2c1</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>B6.Rec1</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>B6.Rec1a</td>
<td>+</td>
<td>T</td>
</tr>
<tr>
<td>B6.Rec1b</td>
<td>−</td>
<td>C</td>
</tr>
<tr>
<td>NZB</td>
<td>++</td>
<td>T</td>
</tr>
<tr>
<td>NZW</td>
<td>+/−</td>
<td>C</td>
</tr>
<tr>
<td>NZM2410</td>
<td>++</td>
<td>T</td>
</tr>
</tbody>
</table>

−, B6-like; +/− to ++, increased levels of Pc B1a cells.

The effect of Sle2c1 expression on a heterozygous (NZB × B6)F1 background, with the homozygous expression of Sle2c1 inducing lymphocyte expansion and an increased number and percentage of splenic B1a and T1 B cells, as well as an accelerated production of anti-dsDNA IgG Abs. These phenotypes have all been implicated in lupus pathogenesis but were not sufficient to induce renal pathology as we observed when the entire Sle2 locus was expressed on an NZB heterozygous background. Therefore, the Sle2c1 locus is associated not only with B1a cell homeostasis but also with a subset of phenotypes leading to lupus pathogenesis, the severity of which depends on the nature of the other susceptibility loci it interacts with.

It is well established that cell cycle regulation differs between B1a and conventional B cells (24). Cyclins D2 and D3 are both expressed in B cells with overlapping functions that are not totally defined. Cyclin D2 is required for B1a but not B2 cell development (37), and cyclin D3 deficiency reduces the number of follicular B cells (38) and severely impairs the development of germinal center B cells (39) but does not affect B1a cell numbers or functions (38). However, temporal inactivation of cyclin D3 complexes in late G1 phase blocks B1a cell proliferation (38). In addition, phorbol ester (PMA) stimulation is sufficient to induce proliferation in B1a but not in B2 cells. This difference has been attributed to the activation of cyclin D3–CDK4 complexes that phosphorylate the retinoblastoma gene product (pRb) in PMA-stimulated B1a but not B2 cells (40). Overall, these results emphasize the crucial role of cyclin D2 and D3 complexes in B1a cell proliferation and predict that gene products such as p18, which fine-tune the amount of activated D2 and D3 complexes, would impact the accumulation of the self-renewing B1a cells. It is not surprising that a decreased level of p18 has a different outcome on B1a and B2 cells due to the differences in cell cycle regulation between these two cell types. The reduced p18 level in Sle2c1 sB cells reproduced the impaired T-dependent humoral response of p18-deficient B cells due to the p18-deficient G1 arrest in plasma cells (26). The impaired response to T-dependent immunization that we have previously reported in B6.TC mice (21) could be due at least to their expression of Sle2c1 and p18 deficiency. Other genetic factors positively regulating the development of plasma cells in the NZM2410 model (41) could compensate for this defect and allow the robust differentiation of autoreactive plasma cells that we have observed in B6.TC mice (21, 41). It should be noted that according to recent results obtained in the BWF1 mice, some of these anti-dsDNA plasma cells should be of B1a origin (11) and may therefore bypass the G1-arrest requirement. The proliferation of Sle2c1 B2 cells was not affected, suggesting that p18 plays a nonessential role in the cell cycle regulation of this cell type until terminal differentiation, which is corroborated by the normal number of splenic B cells in B6.Sle2c1 mice. In contrast, Ab production was not impaired in Sle2c1 B1a cells, but their proliferation was increased. This latter phenomenon provides an explanation for the age-dependent accumulation of B1a cells observed in these mice.

The identification of p18 as a key regulator of B1a cell homeostasis is novel, and it should open new avenues to understand the specificity of cell cycle regulation in the various lymphocyte subsets. In addition, the NZB mouse has long been recognized as a model for chronic lymphocytic leukemia (CLL) (42). Defects in p18 expression have been associated with multiple tumors, including multiple myeloma (43), but not CLL to date. As Sle2c1 represents a major locus responsible for NZB B1a cell accumulation, a better analysis of cell cycle regulation in Sle2c1 B1a cells may shed light on CLL induction.

An association between a dysregulated cell cycle due to a deficiency in a cyclin kinase inhibitor and lupus has been established for Cdkn1alp/p21CIP1/WAF1. p21-deficient mice develop a lupus-like disease through the accumulation of activated/memory T cells (44, 45), and human CDKN1A polymorphisms leading to decreased p21 levels have been associated with systemic lupus erythematosus (46). Contrary to Cdkn2c, Cdkn1a expression does not seem to be significantly affected in B cells expressing Sle2c1. In addition, it was very recently shown that B cell homeostasis is regulated by the RAPL-mediated translocation of Cdkn1b/p27kip1 to the nucleus and that the sequestration of p27 in the cytoplasm leads to a lupus-like phenotype (47). Cdkn2c/p18 has never been associated with lupus or any autoimmune phenotype, and to our knowledge, the −74 C→T SNP is the first naturally occurring polymorphism that has been identified to regulate the size of the
REGULATES B CELLS IN LUPUS MICE

B1a cell compartment. The recent developments that have uncovered new mechanisms by which B1a cells can contribute to autoimmunity, either directly through the production of pathogenic Abs or indirectly thought their promotion of Th17 differentiation, identify Cdc2n2a/p18 as a novel type of lupus susceptibility gene whose characterization is likely to unravel emerging functional pathways contributing to this complex disease.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1

Message expression of Cdkn1a, Cdkn1b, and Cdkn2d in sB (left) and Pc B1a (right) cells collected from B6, B6.Sle2c1 and B6.TC mice. The graphs show means and SEM.
Supplemental Figure 2

A

Supplemental Figure 2. Secondary immunization of B6-Sle2c1 heterozygote mice as compared to B6-Sle2c1 homozygote and B6 mice. A. Col6a2 expression in splens of mice of the indicated strains after secondary immunization with NP-KLH. qRT-PCR Col6a2 expression was normalized to Gapdh and expressed relatively to an unimmunized B6 value set at 1. B and C. Anti-NP IgM and IgG Ab produced by these mice. Means and SEM of 3-4 mice per strain are shown. For B and C, the statistical significance corresponds to values at the lowest dilutions. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
Supplemental Figure 3. The Sle2c1/NZB allele of the Cdkn2c promoter contains a SNP in a highly conserved region. **A.** The -74 SNP in the Cdkn2c promoter with C allele for B6 and a T allele for Sle2c1/NZB is shown (bold underlined) as well as the E2F (grey boxes) and the YY1 binding sites. The common YY1 binding site is indicated with a dashed arrow, the YY1 binding site created by the C allele is shown by a bold arrow. **B.** The region surrounding the -74 C/T SNP is highly conserved in mammals.
### Supplemental Table 1. Gene list in the Sle2c1 interval between rs28132547 and D4MIT278

<table>
<thead>
<tr>
<th>Start</th>
<th>End</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Expressed in B cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>108673260</td>
<td>108734382</td>
<td><em>Nrd1</em></td>
<td>nardilysin, N-arginine dibasic convertase, NRD convertase 1</td>
<td>+</td>
</tr>
<tr>
<td>108734260</td>
<td>108874821</td>
<td><em>Osbp9</em></td>
<td>oxysterol binding protein-like 9</td>
<td>++</td>
</tr>
<tr>
<td>108907090</td>
<td>108927174</td>
<td><em>Calr4</em></td>
<td>calreticulin 4</td>
<td>+</td>
</tr>
<tr>
<td>108952906</td>
<td>109060255</td>
<td><em>Eps15</em></td>
<td>epidermal growth factor receptor pathway substrate 15</td>
<td>++</td>
</tr>
<tr>
<td>109125504</td>
<td>109149588</td>
<td><em>Rnf11</em></td>
<td>ring finger protein 11</td>
<td>-</td>
</tr>
<tr>
<td>109333481</td>
<td>109339262</td>
<td><em>Cdkn2c</em></td>
<td>cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)</td>
<td>++</td>
</tr>
<tr>
<td>109349299</td>
<td>109634646</td>
<td><em>Faf1</em></td>
<td>Fas associated factor</td>
<td>++</td>
</tr>
<tr>
<td>109650630</td>
<td>109656289</td>
<td><em>Dmrt2</em></td>
<td>doublesex and mab-3 related transcription factor like family A2</td>
<td>+</td>
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<tr>
<td>109876327</td>
<td>110024419</td>
<td><em>Elavl4</em></td>
<td>embryonic lethal, abnormal vision, Drosophila-like 4 (Hu antigen D)</td>
<td>-</td>
</tr>
<tr>
<td>111392615</td>
<td>111501789</td>
<td><em>Spata6</em></td>
<td>spermatogenesis associated 6</td>
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<tr>
<td>111547980</td>
<td>111575523</td>
<td><em>S SCP5a9</em></td>
<td>solute carrier family 5 (sodium/glucose cotransporter), member 9</td>
<td>+</td>
</tr>
<tr>
<td>111591997</td>
<td>113917633</td>
<td><em>Skint1-11</em></td>
<td>Skint gene cluster</td>
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<tr>
<td>114578887</td>
<td>114581478</td>
<td><em>Foxd2</em></td>
<td>forkhead box D2</td>
<td>+</td>
</tr>
<tr>
<td>114597752</td>
<td>114598618</td>
<td><em>F oxe3</em></td>
<td>forkhead box E3</td>
<td>+</td>
</tr>
<tr>
<td>114633245</td>
<td>114659751</td>
<td><em>Cmpk</em></td>
<td>cytidine monophosphate (UMP-CMP) kinase 1</td>
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<tr>
<td>114672805</td>
<td>114715476</td>
<td><em>Stil</em></td>
<td>Scl/Tal1 interrupting locus</td>
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<tr>
<td>114729031</td>
<td>114744360</td>
<td><em>Tal1</em></td>
<td>T-cell acute lymphocytic leukemia 1</td>
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<tr>
<td>114761328</td>
<td>114766498</td>
<td><em>Pdzk1ip1</em></td>
<td>PDZK1 interacting protein 1</td>
<td>-</td>
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<tr>
<td>114778928</td>
<td>114806886</td>
<td><em>CYP4x1</em></td>
<td>cytochrome P450, family 4, subfamily x, polypeptide 1</td>
<td>+</td>
</tr>
</tbody>
</table>

* Based on BioGPS (http://biogps.gnf.org)