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Cyclin-Dependent Kinase Inhibitor Cdkn2c Deficiency Promotes B1a Cell Expansion and Autoimmunity in a Mouse Model of Lupus

Hari-Hara S. K. Potula,*1 Zhiwei Xu,*1 Leilani Zeumer,*, Allison Sang,*, Byron P. Croker,*† and Laurence Morel*

The lupus-prone NZM2410 mice present an expanded B1a cell population that we have mapped to the Sle2c1 lupus susceptibility locus. The expression of Cdkn2c, a gene encoding for cyclin-dependent kinase inhibitor p18\(^{\text{INK4c}}\) and located within Sle2c1, is significantly lower in B6.Sle2c1 B cells than in B6 B cells. To test the hypothesis that the B1a cell expansion in B6.Sle2c1 mice was due to a defective p18 expression, we analyzed the B1a cell phenotypes of p18-deficient C57BL/6 mice. We found a dose-dependent negative correlation between the number of B1a cells and p18 expression in B cells, with p18-deficient mice showing an early expansion of the peritoneal B1a cell pool. p18 deficiency enhanced the homeostatic expansion of B1a cells but not of splenic conventional B cells, and the elevated number of B6.Sle2c1 B1a cells was normalized by cyclin D2 deficiency. These data demonstrated that p18 is a key regulator of the size of the B1a cell pool. B6.p18\(^{−/−}\) mice produced significant amounts of anti-DNA IgM and IgG, indicating that p18 deficiency contributes to humoral autoimmunity. Finally, we have shown that Sle2c1 increases lpr-associated lymphadenopathy and T cell–mediated pathology. B6.p18\(^{−/−}\).lpr mice showed a greater lymphadenopathy than B6.Sle2c1.lpr mice, but their renal pathology was intermediate between that of B6.lpr and B6.Sle2c1.lpr mice. This indicated that p18-deficiency synergizes, at least partially, with lpr-mediated pathology. These results show that Cdkn2c contributes to lupus susceptibility by regulating the size of the B1a cell compartment and hence their contribution to autoimmunity. The Journal of Immunology, 2012, 189: 000–000.

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1a B cells represent a first line of defense in the serous cavities by secreting germine encoded IgM, which is referred to as natural Ab. Unlike conventional B2 cells, most B1a cells have a fetal origin and are maintained through self-renewal (1), which is achieved through a specific regulation of the cell cycle (2). Cyclin D2 is required for B1a but not B2 cell development (3), while cyclin D3-deficient mice show normal B1a cell numbers and functions (4). Cyclin D3 is involved in B1a cell proliferation, which is blocked by the temporal inactivation of cyclin D3 complexes in late G1 phase (4). In addition, phorbol ester (PMA) stimulation induces B1a but not B2 cell proliferation by activating cyclin D2-CDK4 complexes to phosphorylate the retinoblastoma gene product (pRb) (5). These results suggest that the regulation of the G1 phase through cyclin D2 and D3–CDK complexes is critical for B1a cell proliferation and consequently the size of the B1a cell pool.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ANA, anti-nuclear autoantibody; B6, C57BL/6; BM, bone marrow; DN, CD4\(^{−}\)CD8\(^{−}\) double-negative; ICl, interstitial chronic inflammation; MZ, marginal zone; NZB, New Zealand Black; NZW, New Zealand White; p18, p18\(^{\text{INK4c}}\); PAS, periodic acid–Schiff; Pe, peritoneal cavity; SLE, systemic lupus erythematosus; Sp, splenic; Treg, Foxp3\(^{+}\) regulatory CD4\(^{+}\) T cell.

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The New Zealand Black (NZB) × New Zealand White (NZW) F1 and the NZM2410 mouse models of systemic lupus erythematosus (SLE) present a greatly enlarged B1a cell pool (6), which has been proposed to contribute to autoimmunity through several mechanisms: B1a cells produce polyreactive autoantibodies and home to CXCR5-producing inflamed tissues where they can class-switch and secrete anti-dsDNA IgG (7, 8). B1a cells are also highly efficient APCs (9), and they favor the polarization of CD4\(^{+}\) T cells into Th17 cells (10). Recently, CD27/CD43/CD70\(^{+}\) B cells have been identified as the human functional equivalent to the murine B1a cells on the basis of spontaneous IgM secretion, tonic BCR signaling, and ability to activate T cells (11). B1 cells are expanded in the peripheral blood of SLE patients and possess a greatly enhanced T cell activation ability (12), suggesting that this function, rather than their autobody production, may contribute to autoimmune pathology.

In the NZM2410 model, we have shown that the expansion of the B1a cell compartment is B cell intrinsic (13). The largest genetic contribution to this expansion was mapped to the NZB-derived Sle2c1 lupus susceptibility locus (14, 15). Sle2c1 enhances autoimmune pathology either in combination with the NZB genome (15) or with Fas deficiency (16). The Sle2c1 locus contains the Cdkn2c gene, which encodes for the cyclin-dependent kinase inhibitor p18\(^{\text{INK4c}}\) (p18). p18 fine-tunes the relative amount of activated complexes formed between cyclin D2 or D3 on one hand and cyclin-dependent kinases CDK4 or CDK6 on the other hand (17). p18 has been shown to be involved both in early and late B cell differentiation. p18 facilitates B cell differentiation from hematopoietic stem cells, and its expression can be compensated partially by p27\(^{\text{kip1}}\) (18). At the final stage of B cell differentiation, p18 expression is responsible for the G1 cell cycle arrest that characterizes plasma cells (19, 20). The expression of Cdkn2c in B cells is 4-fold lower in mice expressing the Sle2c1 allele than...
the B6 allele, and this low expression level segregated with a high number of B1a cells in Sle2c1 recombinants (15). At the molecular level, the Sle2c1 and B6 Cdkn2c alleles differ by a single nucleotide polymorphism in the promoter (-74 C/T) that replaces an Nfr2 by a YY-1 binding site adjacent to the existing YY-1 binding site common to both alleles (21).

Based on these results, we tested the hypothesis that Cdkn2c was the gene responsible for the B1a cell expansion in mice carrying the Sle2c1 locus by comparing the phenotypes of p18-deficient C57BL/6 (B6.p18<sup>−/−</sup>) and B6.Sle2c1 mice. B6.p18<sup>−/−</sup> mice showed in an early expansion of the B1a cell subset corresponding to a preferential B1a cell homeostatic expansion. Furthermore, B6.p18<sup>−/−</sup> mice produced autoantibodies, including anti-dsDNA IgG and anti-nuclear autoantibody (ANA). The magnitude of these phenotypes was greater in p18-deficient mice than in B6.Sle2c1 mice, demonstrating that p18 limits the size of the B1a cell compartment in a dose-dependent manner. In addition to expanding the B1a cell compartment, Sle2c1 greatly enhances lymphadenopathy and the autoimmune pathology induced by Fas-deficiency (16). In this study, we showed that p18 deficiency accounts for the enhanced lymphadenopathy and IL-17 production in B6.Sle2c1.lpr mice. P18 deficiency, however, only partially contributed to the increased T cell activation characterized by the production of CD4<sup>+</sup> CD8<sup>+</sup> double-negative (DN) T cells and activated memory T cells, as well as the concomitant decreased production of Foxp3<sup>+</sup> regulatory T cells (Tregs). Moreover, the renal pathology of B6.p18<sup>−/−</sup>.lpr mice was intermediate between that of B6.lpr and B6.Sle2c1.lpr mice. Overall, these results suggest that Cdkn2c is the Sle2c1 gene that regulates the size of the B1a cell compartment and therefore their contribution to autoimmune pathology. In addition, our results suggest the existence of a Sle2c1 modifier gene closely linked to Cdkn2c that accentuates the effects of p18 deficiency when combined with Fas deficiency.

### Materials and Methods

#### Mice

B6.p18<sup>−/−</sup> mice (20) and B6.Con2<sup>−/−</sup> (B6.D2<sup>−/−</sup>) (22) were provided by Dr. Yue Xiong (University of North Carolina) and by Dr. T. Rothstein (Feinstein Institute, respectively). Screening of the genetic background of each of these two strains with 1449 single nucleotide polymorphisms (www.darmouse.org) revealed a 9.39% and 7.52% non-B6 contribution, respectively. The B6.Sle2c1.Re1c1 mice used in this study (referred to as B6.Sle2c1) have been described previously (15). This strain carries an ~10-Mb genomic interval that includes the Cdkn2c gene derived from the NZB genome on a B6 background. B6, B6.lpr, and B6.Rag1<sup>−/−</sup> mice were originally obtained from the Jackson Laboratory. The B6.Sle2c1.D2<sup>−/−</sup> and B6.p18<sup>−/−</sup>.lpr strains were generated by intercrossing the parental strains and selecting for homozygosity at both loci. B6 and B6.lpr mice were used as controls for Fas-sufficient and Fas-deficient mice, respectively. Both female and male mice were used at the ages indicated. All experiments were conducted according to protocols approved by the University of Florida Institutional Animal Care and Use Committee.

#### Flow cytometry

Peritoneal cavity (Pc) lavages and lymph node and splenic (Sp) single-cell suspensions were prepared by lysing RBCs with 0.83% NH₄Cl. Cells were first blocked with saturating amounts of anti-CD16/CD32 (2.4G2) and then stained with a FITC-PE-, or biotin-conjugated mAbs: B220 (RA3-2C5), CD3e (145-2C11), CD4 (RM4–4), CD5 (53-7.3), CD8a (53-6.7), CD9 (KMC8), CD11b (M1/70), CD19 (ID3), CD21 (7G6), CD23 (B3B4), CD24 (M1/69), CD40 (3/23), CD43 (S7), CD69 (1H4), CD80 (16-10A), CD86 (GL1), CD93 (AA4.1), CCRX5 (2G8), and IgM (IgM<sub>H</sub>), all purchased from BD Pharmingen. Biotinylated mAbs were revealed by Streptavidin-PerCP-Cy5.5. Mononuclear live cells were gated on the basis of forward and side scatter characteristics. In vivo lymphocyte proliferation was analyzed either 18 h after an injection of 1.5 mg BrdU or 7 d after ad libitum exposure to BrdU in the drinking water (0.8 mg/ml). Proliferating cells were detected with a FITC-conjugated anti-BrdU Ab (BD Pharmingen).

#### Ab measurements

Serum anti-ssDNA IgM and anti-dsDNA IgG were measured as previously described (23). Relative units were standardized to a 1:100 dilution of a B6.TC serum set to 100 U. Based on age-matched B6 values, sera containing >15 U of anti-dsDNA IgG were considered to be positive for this specificity. Total IgM and IgG were measured in sera diluted 1:5000 by sandwich ELISA on plates coated with goat-anti mouse IgM or IgG (ICN/Cappel) at 1 μg/ml, and revealed with AP-conjugated goat-anti mouse IgM or IgG (Southern Biotech). Sera from B6.Rag1<sup>−/−</sup> mice received the same number of Pc cells were diluted 1:10 for total IgM measurements. ANA stains were conducted on slides containing fixed HEp-2 cells (Inova Diagnostics) with mouse sera diluted 1:40 revealed with FITC-conjugated anti-mouse IgG (Southern Biotech) at 1:50 dilution. Staining intensity was calculated with image analysis software (Metamorph, Molecular Devices) for a standardized level of green fluorescence on 10–20 randomly selected HEp-2 cells per sample. Samples with an average staining intensity ≥1 were considered positive. For ELISPOT and in vitro Ab measurements, Pc B1a cells were purified as Thy1<sup>+</sup> CD5<sup>+</sup> and Sp B cells were purified as CD43<sup>−</sup> cells with magnetic beads as described previously (15). The purity of B1a was near 90% for both strains. Total IgM and ssDNA IgM ELISPOTs were performed as described previously (24). Cells (2 × 10<sup>5</sup>) were cultured in medium alone or with 5 μg/ml LPS or PMA for 5 d. Total IgM was measured by ELISA in supernatants diluted 1:10 and 1:100.

#### Pc cell adoptive transfers

Pc lavages were obtained from 5–7-mo-old mice and depleted from adherent cells by a 2-h culture at 37°C in PBS supplemented with 10% FBS. In one cohort of mice, nonadherent cells were depleted of CD3<sup>+</sup> cells with magnetic beads; 1–3 × 10<sup>5</sup> cells were transferred i.p. into sex-matched B6. Rag1<sup>−/−</sup> mice. Four cohorts of transfers were performed, each with donor Pc cells from B6 and either B6.Sle2c1 or B6.p18<sup>−/−</sup> mice. Within a cohort, the same number of cells was transferred in each recipient. Flow cytometry was performed on the donor Pc cells before transfer and on the recipient Pc and splenic cells 3 wk after transfer. Serum IgM was compared between recipients.

#### Renal pathology

Kidneys of 4–5-mo-old mice were fixed and stained with H&E and periodic acid–Schiff (PAS). Renal lesions were scored as previously described (16). Glomerular lesions were classified as negative, mesangial matrix (PAS<sup>+</sup>) or mesangial cellularia expansion (M), or proliferative glomerulonephritis (glomerular hypercellularity with capillary loop involvement, which was global [P<sub>G</sub>] or segmental). Extent of involvement was graded on a scale of 1–4, Chronic (mononuclear cell) inflammation was classified as perivascular or interstitial (I+) and graded as for glomerular inflammation.

#### Statistical analysis

Data were analyzed with GraphPad Prism 5.0 software with the statistical tests indicated in the text. Nonparametric tests were used when data were not distributed normally. Means, the SEs of the mean (SEM), and the levels of statistical significance for two-tailed tests are shown in the figures.

#### Results

p18 expression is inversely correlated with the size of the B1a cell compartment

The Pc B1a cell compartment of B6.p18<sup>−/−</sup> mice was compared with that of B6.Sle2c1 and B6 mice at 2 mo (Fig. 1A) and 5–7 mo (Fig. 1B) of age. As expected in both cohorts, the number and percentage of B220<sup>+</sup> CD5<sup>+</sup> B1a cells, the ratio of B1a over B220<sup>+</sup> CD5<sup>−</sup> B2 cells (Fig. 1C), and the percentage of IgM<sup>+</sup> CD5<sup>−</sup> B cells (Fig. 1D) were significantly higher in B6.Sle2c1.
than in B6 mice. These values were even higher in B6.p18/− mice, which carried ~5-fold more Pc B1a cells than B6 in both age groups. The number and percentage of Pc B1a cells were also significantly higher in 2-mo-old B6.p18/− than in B6.Sle2c1 mice. Interestingly, the variance was greater in older B6.p18/− mice, suggesting that p18-deficiency is a primary genetic defect affecting the number of Pc B1a cells, a phenotype then subjected to environmental variations as the mice age. The percentage of Sp B1a cells was not affected by p18 expression at 2 mo of age, but was significantly higher in older B6.p18/− mice (Supplemental Fig. 1). As reported previously (15), no difference was observed for Sp B1a cells between B6 and B6.Sle2c1 mice in either of the two cohorts. These data suggest that the size of the B1a cell compartment is directly correlated with p18 expression level. This hypothesis was verified with a cohort of age-matched mice from strains expressing varying levels of p18 (B6, B6.D2/−, B6.p18/−, B6.p18+/−, B6.Sle2, and B6.Sle2c1), and a highly significant negative correlation was obtained between Cdkn2c expression and the percentage of Pc B1a cells (Fig. 1E). However, p18 is haplosufficient for maintaining a normal frequency of Pc B1a cells with similar values obtained for B6 and B6.p18+/− Pc B1a cells (Fig. 1F). Contrary to its effect on cell numbers, p18 deficiency had a minimal effect on the surface marker phenotypes of Pc B1a cells (Supplemental Fig. 1). We have previously noted the presence of IgMhi B1a cells in B6, Sle2c1 mice (14), and this population was also present in most B6.p18/− mice (Supplemental Fig. 1A, IgM histogram). The expression level of B220 was variable in p18-deficient Pc B1a cells, which also expressed lower amounts of CD69 and CD40 than B6. Remarkably, p18 deficiency had no effect on the surface expression of the same markers on Sp B cells (Supplemental Fig. 1B).

p18 deficiency also affected other B cell subsets besides B1a cells (Table I). Overall, p18/− bone marrow (BM) contained less B220+ and IgM+ B cells. However, this reduction was not uniform across B cell developmental stages, with B6.p18/− mice producing more pro-B cells and fewer recirculating B cells (Fr. F) than B6 cells. In the spleen, older B6.p18/− mice showed more immature B cells; in both cohorts of B6.p18/− mice, the percentage of T3 immature B cells was reduced and the percentage of marginal zone (MZ) B cells was expanded. Interestingly, T3 B cells, which have been described as anergic B cells, have been found in lower numbers in autoimmune mice, including the NZM.
p18 deficiency results in the production of ANAs
B1a cells are the major producers of natural autoantibodies (28), and B6.Sle2 mice produce high levels of polyclonal autoreactive IgM (29). However, the individual Sle2 subloci, including Sle2c1, have not been associated with autoantibody production (14). We revisited this topic and found that both Sle2c1 expression and p18 deficiency significantly increased total IgM and IgG levels in older mice (Fig. 2A). Anti-ssDNA IgM and dsDNA IgG were found only in the serum of B6.p18−/− mice, with ~50% and 30% of the mice being positive, respectively. The presence of anti-dsDNA IgG prompted us to further characterize B6.p18−/− sera for the presence of ANAs. We found robust ANA staining by B6.p18−/− and to a lesser extent B6.Sle2c1 sera (Fig. 2B). Among the mice with detectable ANA staining (B6: 4/9, B6.Sle2c1: 4/9, B6.p18−/−: 6/9), the B6.p18−/− and B6.Sle2c1 sera produced a significantly more intense ANA staining and showed more speckled and nuclear staining patterns (Fig. 2C), with the difference reaching statistical significance between B6 and B6.p18−/− (cytoplasmic versus speckled + nuclear; Fisher exact test, p = 0.035). These results showed that p18 deficiency promotes the production of autoantibody with specificities associated with lupus, whereas the residual p18 expression protects B6.Sle2c1 mice from producing high amounts of IgG with nuclear and dsDNA specificities. We investigated the origin of these autoantibodies with ELISPOTs performed with Sp B and PC B1a cells. Although p18 deficiency resulted in a moderate increase in secretion of total IgM by Sp B cells, the increased secretion of anti-ssDNA IgG was mostly limited to p18−/− Pc B1a cells (Fig. 2D). We observed a high secretion of anti-ssDNA IgM by the Sp

Table I. p18 deficiency affects other B cell compartments

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<th>BM</th>
<th>Sp</th>
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<tr>
<td></td>
<td>B220+</td>
<td>IgM+</td>
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<tr>
<td>B6, 2 mo</td>
<td>23.30 ± 1.09</td>
<td>9.60 ± 0.28</td>
</tr>
<tr>
<td>B6.p18−/−, 2 mo</td>
<td>29.20 ± 1.13</td>
<td>12.19 ± 0.49</td>
</tr>
<tr>
<td>B6, 5–7 mo</td>
<td>19.80 ± 0.78</td>
<td>9.42 ± 0.86</td>
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<tr>
<td>B6.p18−/−, 5–7 mo</td>
<td>14.32 ± 1.07</td>
<td>5.35 ± 0.58</td>
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Mean ± standard error; n = 7–15 mice per group. Significance of t tests between age-matched strains is shown as p < 0.05 (underlined), p < 0.01 (bold), p < 0.001 (bold and underlined).
Fr. F B cells, B220+ CD24int IgMint BM cells; MZB, IgMhi CD23+ CD21+ AA4.1+. Pro-B cells, B220+ CD24- IgM+ BM cells; T3, IgMhi CD23+ AA4.1+. Cdkn2c REGULATES B CELLS IN LUPUS MICE

FIGURE 2. p18 deficiency results in the production of autoantibodies. (A) Serum total IgM, anti-ssDNA IgM, total IgG, and anti-dsDNA IgG in 7–12-mo-old B6, B6.Sle2c1, and B6.p18−/− mice. (B) Representative ANA IgG staining of Hep-2 cells incubated with serum from each of the three strains. Original magnification ×20. The B6.Sle2c1 sample shows a speckled pattern, and the B6.p18−/− sample shows a nuclear pattern. (C) ANA pattern distribution and staining intensity in pixels among the mice in each strain that showed a positive ANA stain. (D) ELISPOT analyses of total IgM production by Sp B cells (left) and anti-ssDNA IgM production by Pc (P) and Sp (S) B cells (right). (E) IgM secretion by Pc B1a or Sp B cells purified from B6 and B6. p18−/− mice, cultured for 5 d in medium alone, with LPS or PMA. Each dot represents a single mouse. For (A), (C), and (D), data were compared with Mann-Whitney tests between B6 and B6.Sle2c1 or B6.p18−/−. In (E), t tests were used to compare the two strains for each condition. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. C, Cytoplasmic; N, nuclear; S, speckled.
B cells of one of six B6.p18−/− mice. These results indicate that p18 deficiency not only increases the number of Pc B1a cells, but also skews their repertoire toward more autoreactive specificities. The B6.p18−/− genome derives a significant contribution from 129/Sv, and ANA susceptibility loci have been well documented in the combination of 129/Sv and B6 genomes (30, 31). We determined that these susceptibility loci are of B6 origin in B6.p18−/−, except one SNP in Sle16 that is of 129/Sv origin in both B6.p18−/− and B6.D2−/− mice. Because this locus is ∼1 Mb telomeric to the Fcr and the Slamf gene clusters, and that B6.D2−/− mice do not produce ANA (data not shown), it is unlikely that this locus contributes to ANA production by B6.p18−/− mice.

One of the characteristics of B1a cells is their ability to spontaneously secrete IgM. Pc B1a cells purified from B6.p18−/− mice produced significantly more IgM in medium alone than B6 (Fig. 2E). B6.p18−/− Pc B1a cells also produced more IgM than B6 in response to either LPS or PMA (Fig. 2E). The purity of the B220+/CD5−/IgM+ CD5−/CD5+ cell populations used for these in vitro assays were equivalent between the two strains (data not shown), indicating that p18-deficiency results in a greater intrinsic IgM secretion by B1a cells. LPS stimulation resulted in a similar IgM production by B6.p18−/− and B6 Sp B cells (Fig. 2E). As expected, B6 Sp B cells did not secrete IgM in medium alone or after PMA stimulation, and p18 deficiency did not have any effect in these conditions. These results suggest that p18 deficiency specifically increased the Ab production and autoreactivity by B1a cells, but has less effect on conventional B cells. This finding is consistent with B1a cells differentiating into Ab-secreting cells through a mechanism that does not involve G1 phase arrest.

**p18 deficiency preferentially enhances B1a cell homeostatic expansion**

The preferential expansion of Pc B1a cells relative to other B cell types in B6.p18−/− mice suggested that p18-deficient Pc B1a cells have a greater capacity for homeostatic expansion than other B cells. Intraperitoneal transfers of nonadherent B6.Sle2c1 or B6.p18−−/− Pc cells into B6.Rag1−/− mice resulted in significantly more Pc B1a cells than transfers of B6 cells (Fig. 3A). Both T cells (data not shown) and total IgM+ B cells expanded significantly more from B6.p18−−/− than from B6 donors (Fig. 3B). Among the B cells, the difference was significant only for B1a cells, although the same trend was observed in the other cell types. The expansion of B6.Sle2c1 B1a cells was intermediate between that of B6 and B6.p18−−/− donors (Fig. 3B). Few if any B cells were found in the spleens of the recipient Rag1−/− mice, and no difference were observed between donor strains. The same results were obtained with transfers of nonadherent CD3-depleted Pc cells (data not shown). Finally, the transfer of B6.p18−−/− Pc cells resulted in an increased production of serum IgM compared with B6 donors (Fig. 3C). These results show a preferential expansion of p18-deficient Pc B1a cells in response to homeostatic signals, which suggests that p18 deficiency accelerates the cell cycle of B1a cells, but not of the other B cell subsets.

**Sle2c1-induced expansion of Pc B1a cells is corrected by cyclin D2 deficiency**

p18 regulates the activity of cyclin D2 and cyclin D3 through CDK4 and CDK6. Cyclin D2 is the first cyclin to be activated in PMA-stimulated B1a cells (5). Deficiency in cyclin D3 is compensated by cyclin D2 in B1a cells (4), whereas cyclin D2 deficiency results in a drastic reduction in the number of B1a cells (3). We therefore reasoned that if the B1a cell expansion in B6.Sle2c1 mice is due to an accelerated cell cycle resulting from a decreased p18 expression, the number of B1a cells in B6.Sle2c1 mice should be normalized by cyclin D2 deficiency. The percentage and number of B1a cells and the B1a/B2 ratio were significantly reduced in B6.Sle2c1.D2−/− compared with B6.Sle2c1 mice at either 2 mo (Fig. 4A) or 5–7 mo (Fig. 4B) of age. These results validate the hypothesis that the expansion of Pc B1a cells in B6.Sle2c1 mice are driven by an accelerated cyclin-D2–initiated cell cycle mediated by a low expression of p18.

P18 deficiency synergizes with the lpr mutation to induce T cell hyperplasia and autoimmune pathology

We have previously shown that Sle2c1 greatly enhanced the lymphadenopathy and T cell–driven autoimmune pathology induced by Fas deficiency (16). To test whether this synergy was due to p18 deficiency, we compared B6.p18−−/−.lpr and B6.Sle2c1.lpr mice aged along with B6.lpr controls. Most B6.p18−−/−.lpr and B6.Sle2c1.lpr mice had to be euthanized for animal welfare issues, when their cervical lymph node mass reached an external diameter of ∼1.5 cm. Some mice were also sacrificed when they developed severe dermatitis, which occurred at a similar frequency in B6.p18−−/−.lpr and B6.Sle2c1.lpr mice (5/36 versus 13/84), and only a few mice were found moribund. Given these endpoint parameters, survival of B6.p18−−/−.lpr mice was significantly reduced compared with B6.Sle2c1.lpr mice (Fig. 5A; median survival, 120 versus 146.5 d; log-rank test, p < 0.01). All the B6.lpr mice (n = 22) that were sacrificed as age-matched controls were healthy, and their cervical lymph nodes did not reach the end-point diameter (data not shown). Both B6.p18−−/−.lpr and B6.Sle2c1.lpr mice developed significant lymphadenopathy of the spleen and even more of the lymph nodes (Fig. 5B), which was matched by accordingly increased cell numbers (data not shown). There was no significant

**FIGURE 3.** p18 deficiency results in the homeostatic expansion of Pc B1a cells. (A) Absolute numbers of Pc CD5+ B220− IgM+ B1a cells before (donors [D]) and after (recipients [R]) transfer into B6.Rag1−/− mice according to the donor cells’ strain of origin. Data were compared using paired t tests. (B) Fold expansion of the Pc B cells 3 wk after transfer according to the donor cells’ strain of origin. Expansion was calculated for total IgM+ cells, B1a: CD5+ B220− IgM+ cells, B1b: CD5− B220− IgM+ cells, and B2: CD5− B220− IgM+ cells. (C) Serum IgM in the recipient B6.Rag1−/− mice according to the donor cells’ strain of origin. For (B) and (C), data were compared with t tests between B6 and B6.Sle2c1 or B6.p18−−/−. *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001.

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difference between the two strains, but the B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice were significantly younger, indicating that \textit{p18} deficiency accelerated \textit{lpr}-induced lymphadenopathy as compared with \textit{Sle2c1} expression. As previously shown for B6.\textit{Sle2c1}.\textit{lpr} (16), lymphadenopathy in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice was mostly due to T cells (Fig. 5C). In vivo BrdU incorporation showed high proliferation rates in CD4\textsuperscript{+} and DN T cells, which were significantly higher in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} than in B6.\textit{lpr} mice (Fig. 6A, 6B). Proliferation was lower in B cells, and a difference between strains appeared only after long term BrdU exposure (Fig. 6B). These results suggest that \textit{p18} and Fas deficiency synergize to enhance proliferation, and that this synergy affects T cells more than B cells, which corresponds to the relative increased of T cell numbers in these mice.

The percentage of CD3\textsuperscript{+} T cells was lower in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} than in B6.\textit{Sle2c1}.\textit{lpr} lymph nodes. In the spleen, the percentage of CD3\textsuperscript{+} T cells was not significantly different between B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} and B6.\textit{lpr}, and it was lower than in B6.\textit{Sle2c1}.\textit{lpr} mice (39.91 ± 1.79 versus 46.35 ± 2.14\%; \( p = 0.03 \)).

Both \textit{Sle2c1} expression and \textit{p18} deficiency significantly expanded the number of B220\textsuperscript{+} T cells (Fig. 5D) and DN T cells (Fig. 5E), two subsets that are characteristic of Fas-deficient mice (32). As for the percentage of total CD3\textsuperscript{+} T cells, the B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} values for B220\textsuperscript{+} and DN T cells were intermediate between that of B6.\textit{lpr} and B6.\textit{Sle2c1}.\textit{lpr} mice. This was more pronounced in the spleen, where the percentage of total DN T cells was not significantly different between B6.\textit{lpr} (45.41 ± 1.23\%) and B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} (41.54 ± 1.96\%). \textit{Sle2c1} expression tilts the balance in favor of CD44\textsuperscript{+} memory T cells to the expense of Foxp3\textsuperscript{+} Tregs (16). The same trend was observed in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice, although the difference with B6.\textit{lpr} did not reach significance for Tregs (Fig. 5F). The Foxp3\textsuperscript{+}/CD44\textsuperscript{+} ratio was significantly lower in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice than in B6.\textit{lpr} (0.22 ± 0.2 versus 0.30 ± 0.02; \( p < 0.01 \)).

The overall weaker T cell phenotypes in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice were not due to B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice being younger on average. Indeed, we compared a cohort of 3-mo-old mice and observed similar differences (data not shown).

Overall, these data suggest that \textit{p18} deficiency contributes to, but is not entirely responsible for, the expansion of DN T cells and skewing of Fas-deficient CD4\textsuperscript{+} T cells toward an activated memory phenotype.

We have reported an expansion of the Th1 subset, a known contributor to lupus renal pathology (33), in B6.\textit{Sle2c1}.\textit{lpr} lymphoid organs and kidneys (16). B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice presented significantly more IL-17\textsuperscript{+} CD4\textsuperscript{+} (Fig. 7A, 7B) and IL-17\textsuperscript{+} DN T cells (Fig. 7C) in their spleens than did B6.\textit{lpr} mice. Importantly, a greater percentage of B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} DN T cells produced IL-17 in the kidneys (Fig. 7D), although the number of renal DN T cells was similar between B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} and B6.\textit{lpr} mice (data not shown). As the percentage of CD3\textsuperscript{+} and DN T cells were decreased in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} compared with B6.\textit{Sle2c1}.\textit{lpr} (Fig. 6C, 6D), there was a corresponding decreased in the percentage of IL-17\textsuperscript{+} CD3\textsuperscript{+} splenocytes; however, the percentage of T cells that were IL-17\textsuperscript{+} was equivalent between the two strains (data not shown).

As another biomarker of autoimmune pathology, we compared serum anti-dsDNA IgG, which was found in similar amounts in the three Fas-deficient strains (Fig. 7E). However, whereas all B6.\textit{Sle2c1}.\textit{lpr} mice were producing anti-dsDNA IgG, it was the case for only 60% of the B6.\textit{lpr} and 75% of the B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice (Fig. 7F). This finding corroborated the small effect of \textit{Sle2c1} expression on ANA production that we have reported in older Fas-deficient mice (16). It also indicates that \textit{p18} deficiency–induced ANA (Fig. 3) does not synergize with \textit{lpr}-induced ANA. Finally, renal pathology in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice was also intermediate between B6.\textit{lpr} and B6.\textit{Sle2c1}.\textit{lpr} mice. Glomerulonephritis was largely limited to mesangial expansion in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} mice compared with proliferative glomerulonephritis, which was the predominant presentation of the B6.\textit{Sle2c1}.\textit{lpr} renal pathology (Fig. 7G). Moreover, interstitial and perivascular chronic infiltrates (i.e., ICI) were rarely present in B6.\textit{p18}\textsuperscript{−/−}.\textit{lpr} kidneys, but abundant in B6.\textit{Sle2c1}.\textit{lpr} kidneys (Fig. 7H). Taken together, cellular analysis of lymphoid tissues, anti-dsDNA IgG

\textbf{FIGURE 4.} Cyclin D2 deficiency abrogates \textit{Sle2c1}-induced Pc B1a cell expansion. The absolute number and percentage of B220\textsuperscript{hi} CD5\textsuperscript{−} IgM\textsuperscript{−} B1a cells, the ratio of B220\textsuperscript{hi} CD5\textsuperscript{−} IgM\textsuperscript{−} B1a to B220\textsuperscript{hi} CD5\textsuperscript{−} IgM\textsuperscript{−} B2 cells, and the percentage of IgM\textsuperscript{−} CD5\textsuperscript{−} B220\textsuperscript{hi} cells were compared between 2-mo-old (A) and 5–7-mo-old (B) B6, B6.\textit{Sle2c1}, B6.\textit{Sle2c1}.\textit{D2}\textsuperscript{−/−}, and B6.\textit{D2}\textsuperscript{−/−} mice. Data were compared with the Dunn multiple comparison tests. * \( p \leq 0.05 \)

\( \ast \ast \ast \ast \ast \leq 0.01 \), *** \( p \leq 0.001 \), **** \( p \leq 0.0001 \).
production, and renal pathology indicate that B6.p18\(^{-/-}\).lpr mice have an intermediate phenotype between B6.lpr and B6.Sle2c1.lpr mice.

**Discussion**

The lupus-prone NZM2410 mice present an expanded B1a cell population (9) that we have mapped to the Sle2 locus (29). Expression of Sle2 by itself on a nonautoimmune background is not pathogenic, but its addition to the Sle1/Sle3 lupus susceptibility loci greatly enhances the penetrance and the severity of autoimmune pathology (23). We have shown that the age-dependent accumulation of Pc B1a cells in B6.Sle2 mice was B cell intrinsic and that it was sustained by several mechanisms, including a greater differential proliferation compared with conventional B cells, and a greater output of B1a cells from adult lymphoid organs (13). Congenic recombinant screening mapped the greatest contribution to Pc B1a cell expansion to a 10-Mb NZB-derived interval that we called Sle2c1 (14, 15). Gene expression analysis of Sle2c1 Pc B1a cells identified a differential expression of cell cycle genes, and among them, Cdkn2c, encoding for cyclin-
dependent kinase inhibitor p18^{INK4c}, is located within Sle2c1. A 4-fold lower expression of Cdkn2c in B cells, which corresponds to a higher cell cycle activity, segregated with the expansion of Pc B1a cells in Sle2c1 recombinants (15). A C-to-T transition in the Cdkn2c promoter results in a differential binding for the transcription factor YY-1 to two binding sites in the Sle2c1 allele as compared with the single binding site in the B6 allele, and this accounts for the decreased transcription efficiency of the NZB allele (21). Taken together, these results identified Cdkn2c as the prime candidate to control the size of the B1a cell compartment in B6.

Sle2c1 mice. Although Cdkn2c expression has not been previously associated with B1a cell differentiation or function, it is well documented that cell cycle is regulated differently between B1a and conventional B cells (2). This corresponds to the fact that, contrary to conventional B cells, B1a cells do not proliferate in response to BCR crosslinking, but maintain a stem cell–like ability for self-renewal and proliferation.

To formally prove that a reduced p18 expression was responsible for the Pc B1a cell expansion in B6.

Sle2c1 mice, we compared the B6.

Sle2c1 and B6.p18^{−/−} mice. BrdU incorporation was measured in splenic and lymph node DN, CD4^{+} T cells, or CD3^{−} B220^{+} B cells. Data were compared with t tests. BrdU incorporation in the lymph nodes after 18 h exposure was low and not different between the two strains (data not shown). *p < 0.05, **p < 0.01.

FIGURE 6. p18 deficiency preferentially enhances the in vivo proliferation of lpr T cells. Five-month-old B6.lpr and B6.p18^{−/−}.lpr mice were exposed to BrdU for either 18 h (A) or 7 d (B, C). BrdU incorporation was measured in splenic (A, B) and lymph node (C) DN, CD4^{+} T cells, or CD3^{−} B220^{+} B cells. Data were compared with t tests. BrdU incorporation in the lymph nodes after 18 h exposure was low and not different between the two strains (data not shown). *p < 0.05, **p < 0.01.
conventional B cells. In addition, B6.p18<sup>−/−</sup> mice produced a significant amount of IgM and IgG autoantibodies, including ANAs, which were found at much lower levels in B6.Sle2c1 mice. While the production of ssDNA IgM is likely to be a direct consequence of the expansion of the number of B1a cells, the production of class-switched anti-dsDNA Abs was less expected. B1a cells have the ability to produce anti-dsDNA IgG in diseased NZB/W F1 mice (7). However, the inflammatory conditions encountered in this lupus model are absent in B6.p18<sup>−/−</sup> mice. It is possible that all B1a cells have a small intrinsic probability to produce class-switched autoantibodies, and the mere increase in number of these cells results in the presence of enough anti-dsDNA IgG production. Alternatively, an expanded B1a cell compartment may contribute indirectly to anti-dsDNA IgG production. In this way, B1a cells favor Th17 polarization (10), and Th17 T cells promote the production of autoreactive B cells (36). The fact that T cell–activating CD11b<sup>+</sup> B1 cells, but not spontaneously IgM-secreting CD11b<sup>+</sup> B1 cells, are expanded in SLE patients (12) favors the latter hypothesis. p18 deficiency, however, impairs Ab secretion from conventional B cells (19, 20), which we confirmed in B6.Sle2c1 mice (15). Considering these potentially conflicting contributions, the origin of anti-dsDNA IgG in B6.p18<sup>−/−</sup> mice will have to be addressed directly in future studies.

Sle2c1 expression synergizes with Fas-deficiency to enhance not only lymphoproliferation, but also T cell–mediated immune pathology (16). B6.p18<sup>−/−</sup>.lpr mice showed an even more enhanced lymphoproliferation, which indicates that, in combination with lpr, p18-deficiency affects the proliferation of cells other than B1a cells. Nonapoptotic functions have been reported for FADD-binding death receptor, including proliferation (37). It is possible that the accelerated cell cycle owing to p18 deficiency synergizes with the residual Fas expression found in lpr mice (38) in a novel manner that will have to be elucidated. The extent of T cell activation, however, was not as pronounced in B6.p18<sup>−/−</sup> mice compared with B6.Sle2c1.lpr mice, considering the expansion of DN T cells and memory CD4<sup>+</sup> T cells, the reduced Foxp3<sup>+</sup> Treg compartment, or the expression of activation markers on CD4<sup>+</sup> T cells. B6.p18<sup>−/−</sup>.lpr T cells produced a significant amount of IL-17, consistent with this phenotype being the consequence of an expanded B1a cell component. p18 has been reported to inhibit Ag-induced T cell proliferation (39). Therefore, the T cell activation phenotypes in B6.p18<sup>−/−</sup>.lpr and B6.Sle2c1.lpr mice most likely result from a combination of T cell–intrinsic and T cell–extrinsic B1a cell–dependent factors. The fact that these phenotypes are more severe in B6.Sle2c1.lpr than in B6.p18<sup>−/−</sup>.lpr mice is due either to the existence of a closely linked modifier gene in the Sle2c1 interval or the residual p18 expression, most likely in T cells. We are currently screening Fas-deficient Sle2c1 congenic recombinants to test the former hypothesis. The latter will require a detailed analysis of the cell cycle in p18-deficient and Sle2c1 T cells with and without Fas expression.

These results show that Cdkn2c is a lupus susceptibility gene in the NZM2410 model by regulating the size of and the repertoire of the B1a cell compartment and hence their contribution to autoimmunity. This conclusion is based on results obtained with Cdk2nc-deficient mice. A complete demonstration would require a rescue of the phenotypes by overexpression of the gene. However, to our knowledge this is not feasible because Cdkn2c overexpression induces cell cycle arrest (19, data not shown). In addition, we cannot exclude at this point that the change in B1a cell repertoire in the B6.p18<sup>−/−</sup> mice is due to the expression of 129/Sv loci rather than p18 deficiency, although a careful examination of the 129/Sv genome segregation in that strain makes it unlikely. Other cyclin-dependent kinase inhibitors have been previously associated with lupus. Polymorphisms in human CDKN1A leading to decreased p21<sup>CRP/WAF1</sup> levels have been associated with SLE (40), and p21-deficient mice develop a lupus-like disease through the accumulation of activated and memory
T cells without a major effect on B cells (41). In addition, B cell homeostasis is regulated by the RAPL-mediated translocation of Cdkn1b/p27kip1 to the nucleus, and the forced sequestration of p27 in the cytoplasm leads to a lupus-like phenotype (42). We have not found any association between differences in expression of either p21, p27, or p19ink4d and more B1a cells in mice expressing in the NZB allele of p18 (data not shown), indicating a unique role for p18 in this cell type. These data suggest that the size of lymphocyte subsets is limited by specific cyclin-dependent kinase inhibitors, and that impaired expression of each of them may result in systemic autoimmunity. It should be noted that the analysis of gene-targeted mice has identified mechanisms other than cell cycle that regulate the size of the B1a cell pool, including the strength of the BCR signaling (1). To our knowledge, our results are the first to link a natural polymorphism in a cyclin-dependent kinase inhibitor with a dysregulated cell cycle that expands B1a cells and contributes to autoimmunity. The recent finding of the expansion of B1 cells in lupus patients (12) raises the possibility that an accelerated cell cycle may be also involved in expanding human B1 cells.

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Disclosures
The authors have no financial conflicts of interest.

References

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Cdkn2c REGULATES B CELLS IN LUPUS MICE