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*Published online 16 December 2011*

http://www.jimmunol.org/content/early/2011/12/16/jimmunol.1002362

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/12/16/jimmunol.1002362

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Pre-B Cell Leukemia Homeobox 1 Is Associated with Lupus Susceptibility in Mice and Humans

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Sle1a.1 is part of the Sle1 susceptibility locus, which has the strongest association with lupus nephritis in the NZM2410 mouse model. In this study, we show that Sle1a.1 results in the production of activated and autoreactive CD4+ T cells. Additionally, Sle1a.1 expression reduces the peripheral regulatory T cell pool, as well as induces a defective response of CD4+ T cells to the retinoic acid expansion of TGF-β–induced regulatory T cells. At the molecular level, Sle1a.1 corresponds to an increased expression of a novel splice isoform of Pbx1, Pbx1-d. Pbx1-d overexpression is sufficient to induce an activated/inflammatory phenotype in Jurkat T cells and to decrease their apoptotic response to retinoic acid. PBX1-d is expressed more frequently in the CD4+ T cells from lupus patients than from healthy controls, and its presence correlates with an increased central memory T cell population. These findings indicate that Pbx1 is a novel lupus susceptibility gene that regulates T cell activation and tolerance. The Journal of Immunology, 2012, 188: 000–000.

A large body of literature has demonstrated that CD4+ T cells are actively involved in systemic lupus erythematosus (SLE) pathogenesis. Autoreactive nucleosome-specific T cells provide help to anti-DNA–specific B cells in mice (1) and in SLE patients (2). Lupus-prone mice carry large numbers of activated T cells, and MRL T cells have a lower threshold of activation (3). Signaling defects have been found in SLE CD4+ T cells (4). Additionally, SLE patients and lupus-prone mice present with a reduced regulatory Foxp3+CD4+ T cell (Treg) compartment and/or abnormal Treg function (5). Moreover, a reduction in Treg homeostasis has been directly linked to lupus pathogenesis in the NZB × NZWFl mice (6). Finally, recent studies have identified

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Received for publication July 13, 2010. Accepted for publication November 11, 2011.

This work was supported by grants from the National Institutes of Health and the Alliance for Lupus Research (to L.M.). C.M.C. was partially supported by a National Institutes of Health Training Grant (to Westley H. Reeves).

The microarray data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE22513 and GSE22799 and to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession number HM399110.

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; B6.TC, B6cN/LmJ; EAE, experimental autoimmune encephalomyelitis; eGFP, enhanced GFP; HC, healthy control; iTreg, induced regulatory T cell; LV, lentiviral; MOG, myelin oligodendrocyte glycoprotein; RA, rheumatic acid; RFP, red fluorescent protein; SLE, systemic lupus erythematosus; Treg, regulatory T cell.

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Materials and Methods

Mice

The B6.Slela, B6.Slela.1, B6.Slela.Tcra−/−, and B6cN.LmJ (B6.TC) strains have been previously described (10, 13, 14). C57BL/6J (B6), B6.Tcra−/−, and Th17 T cells as important contributors to lupus nephritis in both humans and mice (7).

Reverse genetics and N-ethyl-N-nitrosourea mutagenesis have demonstrated that deficiency in genes regulating T cell differentiation or activation threshold results in systemic autoimmunity (8). However, the contribution of natural genetic variations to T cell lupus phenotypes remains unknown, except for a mutation in Coronin 1A that alters CD4+ T cell functions and suppresses autoimmunity in the MRL model (9). In the NZM2410 mouse, the Sle1 locus on telomeric chromosome 1 holds the strongest association with lupus nephritis, and its expression results in the production of anti-chromatin autoantibodies (8). Within Sle1, the Slela sublocus induces the production of activated autoreactive CD4+ T cells that help B cells to secrete anti-chromatin IgG (10). Slela also regulates the number and function of Tregs and decreases the effector T cell response to Tregs (11). Congenic recombinants have revealed that the Slela CD4+ T cell phenotypes map to two independent loci, Slela.1 and Slela.2, with each contributing to overlapping but distinct autoimmune alterations (12). This included a reduced peripheral Treg compartment associated with both Slela.1 and Slela.2 loci, which resulted in severely compromised in vitro and in vivo Treg functions when both loci were coexpressed in the B6.Slela mice (12).

This study was conducted to determine the mechanisms by which Slela.1 alters CD4+ T cell functions and to identify the gene responsible for these alterations. We report that Slela.1 impairs T cell homeostasis in response to retinoic acid (RA). Furthermore, we show that Slela.1 corresponds to the differential expression of a novel splice isoform, Pbx1-d, of the Pbx1 gene, which is the only gene located within the Slela.1 congenic interval. Finally, we report that PBX1 is expressed in human CD4+ T cells and that PBX1-d is found at a higher frequency in SLE patients than in healthy controls (HCS).
BrdU incorporation was then measured by flow cytometry. Measured in mice injected i.p. with 2 mg BrdU 18 h prior to sacrifice, and enriched from peripheral blood with the RosetteSep kit (StemCell Technologies). Heatmaps were generated with the Spotfire Decision Site 9 (TIBCO, BeadChips. All data analyses were based on the use of “internal standards” and generalization of the “error model” (17) as presented elsewhere (18). Heatmaps were generated with the Spotfire Decision Site 9 (TIBCO, Palo Alto, CA) with gene subsets created from the list of genes with significant differential expression. Functional analysis of identified genes was performed with Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

Flow cytometry
Flow cytometry on mouse cells was performed as previously described (13). Cells were stained with pretitrated amounts of the following FITC-, PE-, allophycocyanin-, or biotin-conjugated Abs: AA4.1, B220 (RA3-6B2), CD4 (RM4-5), CD19 (1D3), CD21 (G66), CD23 (9D1), CD48 (HM48-1), CD150 (IPO-3), Ly108 (1G3-19D9), or IL-17F (18F10). Live cells were gated using Fixable Viability Dye eFluor 780 (eBioscience). Human CD4+ T cells were enriched from peripheral blood with the RosetteSep kit (StemCell Technologies) and stained with CD3 (SK7), CD4 (RPA-T4), CD45RA (HI100), CD45RO (UCHL1), or isotype controls. mAbs were purchased from either BD Biosciences or eBioscience. In vivo spontaneous cell proliferation was measured in mice injected i.p. with 2 mg BrdU 18 h prior to sacrifice, and BrdU incorporation was then measured by flow cytometry.

Experimental autoimmune encephalomyelitis induction
Experimental autoimmune encephalomyelitis (EAE) was induced with a myelin oligodendrocyte glycoprotein (MOG) peptide and clinical scores were recorded as previously described (16). Briefly, a score of 1 corresponds to a limp tail, score 2 to limp or hindleg weakness, score 3 to hindleg paralysis, and score 4 to fore- and hindleg paralysis. The experiment was performed in 2- to 3-mo-old male mice twice. Mononuclear cells used for flow cytometry were isolated by collagenase treatment of the spinal cord of four B6 and B6.Slela.1 mice on day 20 after induction of EAE. Additionally, cohorts of mice were injected i.p. three times a week starting at day 0 of induction. MOG recall assays were performed as previously described (11), with 2 x 10^5 CFSE-labeled negatively selected B6 CD4+ T cells stimulated with anti-CD3 and anti-CD28 with or without total cells from RA-induced Treg cultures at a 1:1 or 1:2 effector T cell/Treg culture ratio. After 3 d, effector T cell proliferation was assessed by flow cytometry for CFSE dilution in CD4+ live cells. For Th17 reversion, CD4+CD25+ cells were stimulated with anti-CD3 and anti-CD28 in the presence of IL-6 (25 ng/ml, R&D Systems), 2.5 ng/ml TGF-β, and 10 nM RA for 3 d. The Treg induction experiment was performed four times and the Th17 reversion was performed twice, with two to four mice per strain in each experiment.

Pbx1 isoform analysis
Mouse cDNA was synthesized from CD4+ T cells obtained by negative selection using SpinSep reagents (StemCell Technologies) or flow sorting with a FACSArria cytometer from 6-wk-old or 5-mo-old mice. Flow cytometric analysis of the resulting CD4+ T cells consistently showed >90% purity. Th17- and Th2-cytokine-elicited peritoneal macrophages were enriched by adherence before RNA extraction and cDNA synthesis. Human cDNA was synthesized from peripheral blood using the CD4+ T cell enrichment RosetteSep mixture. The following primers were used to detect both murine and human Pbx1 isoforms: 5′-GAA GTG COG CAT CAC AGT CTC-3′ in exon 5, and 5′-CGG CAT CAC GTA GCC GAA TGG AGC-3′ in exon 8. The identification of known or novel isoforms was determined by direct sequencing of the RT-PCR products. Mouse Cd4 was amplified with 5′-GAA CCT GGT GGT GAT GAA AGG AG-3′ and 5′-CAG CCC CCT GCA GAA AAA CCT CT-3′ and mouse Cd11b (Itgam) with 5′-CCC CAA TTA CGT AGC GAA TC-3′ and 5′-TGG TCG GAA CAT CTT TG-3′.

IL-10 production by peritoneal macrophages exposed to apoptotic cells
Th17- and Th2-cytokine-elicited peritoneal macrophages from 5-mo-old mice were cultured with apoptotic B6 thymocytes at a 2:1 ratio for 12 h as previously described (19). IL-10 in the supernatant was detected with an OptEla kit (BD Pharmingen). This experiment was performed four times with two to three mice per strain each. Intracellular IL-10 was measured in splenic CD4+ T cells incubated with leukocyte activation mixture (BD Biosciences) for 5 h.

Statistical analyses
Statistical analyses were performed using the GraphPad Prism 4 software. Unpaired t tests or Mann–Whitney U tests were used depending on whether the data were normally distributed. Multiple-test corrections were made as appropriate. Comparisons of percentages were performed with χ2 tests calculated with the absolute numbers of samples in each category. Statistical significance obtained when p ≤ 0.05 is indicated in the figures.

Results
Slela.1 expression results in the production of autoreactive T cells
Slela.1 corresponds to the 0.3 Mb centromeric segment of Slela (Supplemental Fig. 1) and, as for the entire Slel locus, was de-
rived from the NZW genome (20). *Sle1a.1* expression is necessary for the activated CD4+ T cell phenotypes presented by B6.Sle1a mice (12). In this study, we further characterized the contribution of *Sle1a.1* to T cell autoimmunity. In vitro, *Sle1a.1* T cells produced significantly more IL-2 (Fig. 1A) and IFN-γ (Fig. 1B) than did B6 T cells when stimulated with histone/DNA-loaded B6-irradiated splenocytes used as APCs, but to a lower level than for the full *Sle1a* interval. No difference was obtained with non-loaded APCs, although the medium contains chromatin, suggesting that a higher concentration of histone peptides preloaded on the APCs is necessary to stimulate this polyclonal T cell population. The same result was obtained for IL-4, and no cytokine response was induced by thyroglobulin, a nonnuclear autoantigen control (data not shown). Anti-CD3 stimulation also resulted in the production of more IL-2, IFN-γ (Fig. 1A, 1B), and IL-4 (data not shown) in *Sle1a.1* CD4+ T cells than B6. This indicated that *Sle1a.1* contributes to the production of histone-specific T cells and to the higher response to TCR stimulation that we have observed for *Sle1a* (10). In vivo, *Sle1a.1*-expressing T cells transferred into B6.Sle1.Tcr−/− mice induced the production of anti-chromatin IgG Ab-forming cells (Fig. 1C). Transfers of *Sle1a.1* T cells into B6.Rag2−/− mice showed that cotransferred contaminating *Sle1a.1* B cells were not sufficient to produce significant amounts of anti-chromatin IgG (data not shown). Additionally, *Sle1a.1* T cells increased the percentage of splenic transitional CD21hiCD23lo T1 relative to CD21hiCD23lo follicular B cells (Fig. 1D). Many autoreactive B cells are eliminated in the T1 compartment (21), which is enlarged in the NZM2410 model (22). Thus, *Sle1a.1*-expressing T cells enriched in anti-nuclear specificities are associated with a lower selection threshold for *Sle1*-expressing autoreactive T1 B cells, which have then an increased likelihood to mature into autoantibody-producing cells. Interestingly, contrary to *Sle1* and *Slela* (10), *Sle1a.1* T cell transfers did not induce any autoreactive phenotypes in B6 B cells (data not shown), indicating that expression of other *Sle* genes is necessary for B cells to respond to autoreactive *Sle1a.1* T cells.

As early as 2 mo of age, *Sle1a.1* CD4+ T cells, but not B cells, spontaneously proliferated more than B6 T cells (Fig. 1E, 1F), indicating a T cell-specific effect of *Sle1a.1* expression. This increase was not Ag-specific since naïve B6.Sle1.1.OTII Vβ5+ T cells proliferated significantly more than did B6.OTII T cells, although at a lower level than nontransgenic T cells (Fig. 1G). Overall these in vitro and in vivo results indicate that *Sle1a.1* enhances CD4+ T cell responses to receptor stimulation and induces the production of autoreactive T cells that not only provide help to chromatin-specific B cells but also spontaneously proliferate in vivo.

We have previously reported that *Sle1a.1* expression reduces the size and function of the Treg cell compartment in an age-

![FIGURE 1](http://www.jimmunol.org/)

*Sle1a.1* expression results in the production of autoreactive hyperproliferative T cells. IL-2 (A) and IFN-γ (B) secretion by B6, B6.Sle1a.1, and B6.Sle1a T cells cultured in the presence of histone (○), after anti-CD3 stimulation (□), or in media alone (△). Values between B6 and B6.Sle1a T cells were different at p < 0.01. B6-.Sle1a.1-, or *Sle1a*-expressing T cells were adoptively transferred into B6.Sle1.Tcr−/− mice to assess anti-chromatin IgG Ab-forming cells (AFCs) (C) and the ratio of splenic transitional T1 relative to follicular (Fo) B cells (D) 3 mo after transfer. E-G, CD4+ T cells incorporate more BrdU in B6.Sle1a than in B6 mice after 18 h in vivo exposure. E, Representative FACS plots showing BrdU incorporation in CD4+ gated cells. Quantitation of BrdU incorporation is shown in CD4+ T cells and CD19+ B cells from B6 and B6.Sle1a mice (F) and in Vβ5+CD4+ T cells from B6.OTII and B6.Sle1a.OTII mice (G). *p ≤ 0.05, **p ≤ 0.01.
dependent manner with significant effects detectable at 5–6 mo of age (12). The more sensitive detection afforded by the Foxp3-eGFP reporter construct showed a significantly decreased percentage of Foxp3+CD4+ T cells in B6.Sle1a.1.Foxp3-eGFP spleens as compared with B6.Foxp3-eGFP as early as 3 wk of age, and that difference was enhanced at 3 mo of age (Fig. 2A). In the thymus, the percentage of Tregs was significantly different between the two strains only at 3 mo of age (Fig. 2A). These data show that Sle1a.1 affects the size of the Treg pool early, and they suggest that it does not reduce the generation of thymic Tregs, but rather their maintenance, in the thymus and the periphery. With evidence of an enlarged pool of activated autoreactive T cells, we hypothesized that the B6.Sle1a.1 mice would be more susceptible to EAE than B6. Indeed, B6.Sle1a.1 mice presented with significantly earlier disease onset (clinical score 1, 12.94 ± 0.50 versus 16.5 ± 1.52 d, p = 0.02; or score 2, 14.65 ± 0.35 versus 16.50 ± 0.57 d, p = 0.0037) as well as increased disease severity (Fig. 2B–D). Moreover, CD4+ T cells from MOG-immunized B6.Sle1a.1 mice produced more IFN-γ (Fig. 2E) and IL-17F (Fig. 2F) than B6 in in vitro MOG recall assays. There was also a trend toward greater numbers of CD4+-infiltrating T cells in the lumbar region of the spinal cord of B6.Sle1a.1 mice 20 d postinduction (data not shown). Taken together, this combination of in vitro and in vivo results showed that Sle1a.1 induces the production of inflammatory-activated autoreactive CD4+ T cells and a reduction of the Treg pool.

**Defective responses to RA in Sle1a.1 CD4+ T cells**

B6 and B6.Sle1a.1 CD4+ T cells expressed 390 named genes at a significantly different level, 84% of which had a ≥2-fold dif-

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**FIGURE 2.** Sle1a.1 expression reduces the size of the Treg pool and increases EAE susceptibility. A. Splenic and thymic Treg levels in 3-wk and 3-mo-old B6.Foxp3-eGFP and B6.Sle1a.1.Foxp3-eGFP mice, with representative FACS plots of 3-mo-old spleens. B. EAE average scores in B6.Sle1a.1 (closed symbols) and B6 (open symbols) mice in the days following induction. Squares correspond to RA-treated mice. C. Average day of onset and (D) maximal scores over the 35 d following EAE induction. B–D, Values for 10–15 mice per strain per treatment. MOG recall assays performed with splenocytes collected 10 d after EAE induction in mice (five per strain per treatment) treated with RA or carrier control, measuring IFN-γ in the supernatant (E) and the percentage of IL-17F+CD4+ T cells (F). Experiments shown in B–F were conducted with 2- to 3-mo-old mice. Means ± SEM are shown. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
ference. An Ingenuity Pathway Analysis (Supplemental Fig. 2) revealed signatures in the p16/cell cycle, TCR activation, and cell death pathways (Supplemental Fig. 3A–C). These results validate the increased proliferation and activation observed in Sle1a.1 CD4+ T cells (Ref. 12 and Fig. 1F). The pathway analysis also uncovered a signature of genes involved in RA (Supplemental Fig. 3D), IL-6, and TGF-β signaling (Supplemental Fig. 2). This suggested that Sle1a.1 may affect CD4+ T cells through a defective integration of RA signals during TGF-β-dependent differentiation into induced Tregs (iTregs) or Th17 cells.

To test this hypothesis, B6 and B6.Sle1a.1 CD4+CD25− T cells were stimulated with anti-CD3, anti-CD28, and IL-2 in the presence or absence of TGF-β and RA and then stained for Foxp3. TGF-β alone induced a similar Foxp3 expression in B6 and B6.Sle1a.1 CD4+ T cells (Fig. 3A). As expected, the addition of RA enhanced Foxp3 expression in B6, but significantly

**FIGURE 3.** Sle1a.1 results in defective CD4+ T cell responses to RA. A–C, Foxp3 induction in B6 and B6.Sle1a.1 CD4+CD25− cells by IL-2 and TGF-β in the presence or absence of RA. A, Representative FACS plots showing intracellular Foxp3 expression with TGF-β alone or with RA in B6 and B6.Sle1a.1 CD4+ T cells. Fold induction of Foxp3 by RA obtained with cells from 5- to 6-mo-old mice (10 mice per strain) (B) and 3-mo-old mice (4 mice per strain) (C). B6.TC T cells were included as a Sle1a.1-expressing strain. D and E, Suppression of proliferation by CD4+CD25− cells obtained from 6-wk-old B6 or B6.Sle1a.1 mice and stimulated in the presence of IL-2, TGF-β, and RA. The Treg cultures were added at a 2:1 ratio to CSFE-labeled B6 CD4+ T cells stimulated with anti-CD3 and anti-CD28 for 3 d. D, Representative FACS plot showing proliferation of CSFE+CD4+ T cells with B6 Treg cultures (filled gray), Sle1a.1 Treg cultures (thick line), or no Tregs (thin line). The marker selected for the second round of replication is indicated. E, Comparison of suppression of proliferation by RA-induced Treg cultures from B6 and B6.Sle1a.1 mice. The percentage of suppression was calculated as 100 minus the percentage of the live CD4+ cells that have undergone two rounds of replications with Treg cultures relative to the same proliferation in the absence of Treg cultures. F and G, Foxp3 induction by RA in B6 and B6.Sle1a.1 CD4+CD25− cells stimulated with IL-6 and TGF-β in the presence or absence of RA. F, Representative FACS plots showing intracellular Foxp3 expression with TGF-β and IL-6 with or without RA in B6 and B6.Sle1a.1 CD4+ T cells. G, Fold induction of Foxp3 by RA obtained with cells from 5- to 6-mo-old mice (four mice per strain). In A and F, the values indicate the percentage of CD4+ cells that express Foxp3. Means ± SEM are shown. *p ≤ 0.05, **p ≤ 0.01.
less in B6.Sle1a.1 T cells (Fig. 3A, 3B). A similar defective response to RA was obtained with Sle1a.1-expressing B6.TC mice, a strain with a fully penetrant lupus nephritis (13). The defective Foxp3 induction in response to RA was not a consequence of age-related CD4+ T cell activation in B6.Sle1a.1 and B6.TC mice, as the same results were obtained with 3-mo-old mice (Fig. 3C). These results were confirmed by in vitro suppression assays in which B6 CD4+ T cells were stimulated by anti-CD3 and anti-CD28 Abs in the presence of RA-induced Treg cultures performed with CD4+CD25− T cells obtained from 6-wk-old B6 and B6.Sle1a.1 mice. The B6.Sle1a.1 RA-induced Treg cultures yielded a lower percentage of Foxp3+ T cells than did the B6 cultures (data not shown), leading to a significantly lower suppression of CD4+ T cell proliferation (Fig. 3D, 3E). The alteration of the IL-6 pathway in Sle1a.1 CD4+ T cells (Supplemental Fig. 2) and the competing differentiation fate between Th17 and iTregs (23) suggested that the Th17 compartment may be expanded in B6.Sle1a.1 mice. The number of ex vivo Th17 cells was equivalent in B6 and B6.Sle1a.1 spleens (data not shown). However, the induction of Foxp3 by RA in CD4+CD25− T cells under Th17-polarizing conditions was significantly lower in B6.Sle1a.1 than in B6 mice (Fig. 3F, 3G).

Finally, we examined the effect of RA in vivo using the EAE model. As previously reported (24), RA treatment prevented EAE induction in B6 mice, which showed an average maximal disease score of ~1 (Fig. 2B–D). RA significantly improved the clinical parameters in B6.Sle1a.1 mice, but not to the same extent as in treated B6 mice (Fig. 2B–D). EAE severity in RA-treated B6.Sle1a.1 mice was intermediate between untreated and RA-treated B6 mice, showing that Sle1a.1 expression interferes with RA protection. Furthermore, although RA treatment significantly decreased IL-17F production by both B6 and B6.Sle1a.1 CD4+ T cells, inhibition of IFN-γ production was incomplete in B6.Sle1a.1 T cells (Fig. 2E, 2F). Overall, these data indicate Sle1a.1 results in a defective RA expression of TGF-β–induced Foxp3 expression.

**Sle1a.1 results in the differential expression of Pbx1 splice isoforms**

**Pbx1** is the only gene present in the Sle1a.1 locus (Supplemental Fig. 1). There is no coding sequence polymorphism between the B6 and NZW alleles, except a synonymous single nucleotide polymorphism in exon 4 (rs31432160) and low but similar Pbx1 mRNA expression was found in B6 and Sle1a.1 CD4+ T cells (data not shown). We designed primers in exons 5 and 8 to compare the expression of known isoforms, Pbx1-a and Pbx1-b (Fig. 4A), in B6.Sle1a.1, B6.TC, and B6 CD4+ T cells. Pbx1-a was not detected, but Pbx1-b was expressed by CD4+ T cells from all three strains (Fig. 4B). Interestingly, we uncovered two novel isoforms: Pbx1-c, found mostly in B6.TC, which lacks exon 7 and a portion of exon 6, and Pbx1-d, found in all three strains, which lacks both exons 6 and 7 (Fig. 4A, 4B), corresponding to the DNA binding domain and the Hox binding domain, respectively. Pbx1-d levels were significantly higher in the Sle1a.1-expressing CD4+ T cells as compared with B6 (Fig. 4B, 4C), and we consistently obtained higher expression in B6.TC than in B6.Sle1a.1 T cells. The higher expression of Pbx1-d in Sle1a.1 CD4+ T cells is not secondary to their activation, as the same results were obtained with 6-wk-old mice, in which autoimmunity expression is minimal (Fig. 4D). These data show that Sle1a.1 induces the differential expression of the novel Pbx1-d isoform in CD4+ T cells, and that additional genetic factors in the Sle1, Sle2, or Sle3 loci further enhance this expression.

**Pbx1 is a critical mediator of IL-10 production by macrophages exposed to apoptotic cells** (19). However, apoptotic cells triggered similar secretion of IL-10 in B6 and B6.Sle1a.1 macrophages (Fig. 4E). Both B6 and B6.Sle1a.1 B6 macrophages expressed high levels of Pbx1-b isoform, along with variable and low amounts of Pbx1-c or Pbx1-d in both strains (Fig. 4F). Therefore, there is no strain-specific expression of Pbx1-d in macrophages as is the case for CD4+ T cells. We have previously shown that B6.TC CD4+ T cells produce significantly more IL-10 than B6 (25). To investigate whether this was due to Pbx1NZW, we compared IL-10 production among B6, B6.Sle1a.1, and B6.TC CD4+ T cells. We confirmed a higher IL-10 production in the B6.TC T cells, whereas B6.Sle1a.1 T cells were comparable to B6 (Fig. 4G, 4H). We conclude that Pbx1NZW does not induce Sle1a.1 autoimmune phenotypes by altering IL-10 production.

**Pbx1-d induces T cell activation and decreased apoptosis in response to RA**

Human and mouse Pbx1 exonic structure and amino acid sequence are identical. To test the hypothesis that the Pbx1-d isoform was responsible for the Sle1a.1 phenotypes in CD4+ T cells, we transfected Jurkat T cells with a LV construct expressing Pbx1-d-GFP or control RFP. Jurkat T cells only express Pbx1-a, and LV-Pbx1-d-GFP transfection resulted in the production of the Pbx1-d protein (Supplemental Fig. 4A). The gene expression analysis of GFP+ or RFP+, as well as nontransfected, Jurkat T cells determined that Pbx1-d induced the differential expression of 12 genes (Supplemental Fig. 4B), several of which have been previously associated with T cell survival or activation, or Th17 cell differentiation (Supplemental Table II). A pathway analysis (Supplemental Fig. 4C) predicted a central role for the transcription factor SP1, which is repressed by PBX1 (26), and identified CITED2, which is involved in T cell activation (27), as intermediates between PBX1 and SP1. Finally, for Sle1a.1 CD4+ T cells (Supplemental Fig. 2), the aryl hydrocarbon receptor signaling pathway, which regulates the Th17/Treg differentiation process (28), was differentially expressed in Jurkat T cells overexpressing Pbx1-d.

RA induces apoptosis in Jurkat T cells (29). Pbx1-b overexpression significantly increased the percentage of late apoptotic cells and decreased the percentage of live cells in response to anti-CD3 and anti-CD28 stimulation as compared with Pbx1-d or control-transfected cells (Fig. 5A, 5B). Addition of RA increased the rate of apoptosis but to a lower level in LV-Pbx1-d-transfected cells. Moreover, Pbx1-d–transfected cells accumulated to higher numbers than did control-transfected cells in response to anti-CD3 and anti-CD28 stimulation, whereas cells transfected with Pbx1-b accumulated less than controls (Fig. 5C). These data suggest that overexpression of the two isoforms has an opposite effect, with Pbx1-b promoting apoptosis and Pbx1-d promoting the accumulation of live cells. No difference in proliferation was observed between the two types of transfected cells (data not shown), indicating that the increased cell numbers in Pbx1-d–transfected cells were due to a reduced activation-induced cell death. We also found a reduction of apoptosis in stimulated Sle1a.1-expressing CD4+ T cells in the presence of RA as compared with B6 (Fig. 5D), corroborating the differential expression of genes involved in apoptosis in Sle1a.1 CD4+ T cells (Supplemental Fig. 5C), as well as earlier findings showing apoptosis resistance in Sle1a CD4+ T cells (10). Overall, these data demonstrate that overexpression of Pbx1-d results in an activated/inflammatory phenotype, a decrease in activation-induced cell death, and a defective response to RA in Jurkat T cells and primary mouse CD4+ T cells, strongly suggesting that Pbx1-d is responsible for the Sle1a.1 phenotypes.
PBX1-d is expressed more frequently in the CD4+ T cells from SLE patients than from HCs

We established that human peripheral blood CD4+ T cells express either PBX1-a or PBX1-d or both (Fig. 6A), and we compared the distribution of the PBX1 isoforms in a cohort of SLE patients and HCs (Supplemental Table III). Longitudinal samples obtained from the same individuals showed that the expression of the PBX1 isoforms may vary between samples. Therefore, we treated each sample obtained at each visit independently. The PBX1-d isoform was found significantly more frequently in the SLE patient samples, either considering the three isoform patterns (PBX1-a, PBX1-d, or PBX1-a/d; Fig. 6B) or the presence of the PBX1-d isoform, which occurred either alone or in combination with PBX1-a in 66% of the patients as compared with 43% of the HC samples (p = 0.003). The distribution of the PBX1-a and PBX1-d isoforms was also significantly different between SLE patient and HC individuals (p = 0.005), with 73% of patients versus 49% of HCs ever expressing PBX1-d. PBX1-d expression was similar between white and African Americans, and it was not affected by immunosuppressive drugs or disease activity (Supplemental Table III). However, patients treated with steroids expressed PBX1-d significantly less often than did untreated patients (60.6 versus 77.4%; Fig. 6C). When steroid-treated patients were excluded, the distribution of PBX1 isoforms was even more skewed as compared with HCs (Fig. 6D).

CD4+ T cell leukopenia and increased activation have been consistently reported in SLE patients (30). A significant lower percentage of circulating CD4+ T cells was also found in our cohort of SLE patients (Fig. 6E). Interestingly, expression of PBX1-d was significantly associated with CD4+ T cell leukopenia in the entire cohort of SLE patients and HCs (Fig. 6F). We compared the distribution of memory CD4+ T cell subsets between patients and
HCs based on their CD45RA, CD45RO, and CD62L expression. We found a higher relative level of memory over naive CD4+ T cells in SLE patients (Fig. 6G), and this increase was in the CD45RO+CD62L+ central memory subset (Fig. 6I), whereas there was no difference in the CD45RO+CD62L− effector memory subset (data not shown). The relative levels of memory CD4+ T cells (Fig. 6H) and central memory CD4+ T cells (Fig. 6J) were significantly higher in CD4+ T cells expressing PBX1-d than in CD4+ T cells expressing PBX1-a in the total cohort. CD4+ T cells expressing both isoforms showed an intermediate relative level of memory phenotype. The same results were obtained when SLE samples were analyzed separately (p = 0.02). The HC samples showed the same trend but did not reach significance due to smaller sample size of PBX1-d–expressing samples (data not shown).

Overall, these results show that, as in Slela.1 mice, PBX1-d is expressed in human CD4+ T cells and that this expression is more frequent in SLE patients than in HCs. Furthermore, our data suggest that the expression of this novel isoform is associated with a memory phenotype regardless of disease status.

Discussion

The Sle1 susceptibility locus confers the highest genetic liability in the NZM2410 lupus model (20), and its expression is necessary for full disease development (13, 31). Among the three Sle1 subloci, Slela is associated with a modest production of autoantibody (14), but it increases CD4+ T cell effector functions and reduces the number and function of Tregs, resulting in the production of autoreactive CD4+ T cells (10, 11). In the present study, we showed that Slela.1, which is necessary for the full Slela phenotype (12), induces the production of histone-reactive T cells that provide help to anti-chromatin–specific B cells in vivo. B6.Slela.1 mice present an enhanced susceptibility to EAE, further demonstrating that Slela.1 induces the production of inflammatory autoreactive CD4+ T cells, which was revealed with the experimental colitis model (12). The increased CD4+ T cell

FIGURE 5. Pbx1-d expression decreased activation-induced cell death with and without RA. A. Percentages of late apoptotic (annexin V+7-amino-actinomycin D [7-AAD]+) and live (annexin V−7-AAD−) Jurkat cells transfected with empty vector (EV) control, LV-Pbx1-b, or LV-Pbx1-d and stimulated with anti-CD3 and anti-CD28 with or without RA for 48 h. Representative stains highlighting the percentage of annexin V−7-AAD− late apoptotic cells are shown in B. C. EV control-, LV-Pbx1-b–, or LV-Pbx1-d–transfected Jurkat cell counts over a 72-h period of stimulation with anti-CD3 and anti-CD28. Data shown in A–C were obtained in three independent transfections. D. Fold induction by RA of early apoptosis in mouse CD4+ T cells stimulated with anti-CD3 and anti-CD28 for 24 h. The graph shows the combination of four experiments with three mice per strain in each. Means ± SEM are shown. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 in comparisons with B6 values.
proliferation and the reduced Treg pool occurred at an early age, before B6.Sle1 or B6.TC mice produce autoantibodies, arguing in favor of these phenotypes resulting from a primary genetic defect. The Sle1a.1 CD4+ T cell-specific transcriptional signature included the TGF-β, IL-6, and RA pathways. RA inhibits Th17 differentiation while expanding the TGF-β-mediated differentiation of iTregs and enhancing their suppressive activity in murine (32–35) and human (36, 37) T cells. Therefore, the Sle1a.1 transcriptional signature suggested a defective Th17/Treg homeostasis in response to RA. Indeed, RA-mediated expansion of iTregs and RA-induced Foxp3 expression in Th17-polarizing conditions were significantly reduced in Sle1a.1 T cells. Moreover, the protective effect of RA treatment on EAE induction was less effective in B6.Sle1a.1 than in B6 mice. The Treg pool was depleted in the spleen but not the thymus of 3-wk-old B6.Sle1a.1 mice, suggesting that Sle1a.1 does not affect the output of natural Tregs but limits their maintenance and/or expansion in the periphery. RA expands iTregs directly (38), at least in part by enhancing Foxp3 transcription through TGF-β (24), and indirectly by blocking CD4+CD44hi memory cells from inhibiting iTreg differentiation (39). It is not possible at this point to determine whether an expanded memory T cell subset or a reduced Treg subset is the primary effect of Sle1a.1 expression, and which of these two traits is a consequence of this primary defect. The increased number of memory T cells may also alter the threshold of RA inhibition during Treg induction, but increasing doses of RA did not rescue Sle1a.1 Treg expansion (data not shown), which argues against this hypothesis. The altered TGF-β pathway observed in Sle1a.1 T cells, with notably with the overexpression of TGF-β antagonist Smad7, could be a consequence of a defective response to endogenous RA or it could occur independently. B6 and B6.Sle1a.1 mice presented similar numbers of Th17 cells, and we did not find a Th17 transcriptional signature in Sle1a.1 CD4+ T cells, suggesting that Sle1a.1 affects primarily the RA induction of Tregs.

FIGURE 6. PBX1-d expression is more frequent in lupus patients and correlates with an increased percentage of central memory CD4+ T cells. A, RT-PCR displaying the PBX1-a and PBX1-d isoforms in CD4+ T cells from three representative patients. B, PBX1 isoform distribution in 58 HC and 95 SLE patient samples. The percentage of samples expressing PBX1-a is represented in black, PBX1-d in white, and the coexpression of both by a hatched pattern. C, Comparison of the presence (white) or absence (black) of PBX1-d expression between samples from patients that were treated or not treated with steroids. D, PBX1 isoform distribution in samples from HCs and SLE patients not treated with steroids. Percentage of CD3+CD4+ in HC and SLE patient PBLs (E) and in the total cohort according to the PBX1 isoform expression (F). Relative levels of memory CD4+ T cells—(％CD45RA−CD45RO+CD4+CD3+)／％CD45RA−CD45RO+CD4+CD3+—in HC and SLE patient PBLs (G) and in the total cohort according to the PBX1 isoform expression (H). Naive CD45RO−CD62L+/central memory CD45RO−CD62L+CD4+ T cell ratios in HC and SLE patient PBLs (I) and in the total cohort according to the PBX1 isoform expression (J). B–D, p values correspond to x2 tests. E–J, Medians and interquantile ranges; p values correspond to Mann–Whitney tests.
without a reciprocal effect on the Th17 compartment. RA suppresses inflammatory responses in a variety of autoimmune diseases in animal models, including lupus (40, 41), and a recent study has shown the beneficial effect of RA treatment in a small number of lupus nephritis patients (42). Our results suggest that the Pbx1-d isoform might represent a biomarker for a defective response to RA treatment, a hypothesis that we are currently testing in murine models.

Pbx1, a member of the TALE family of homeodomain proteins, is a transcriptional regulator in a large number of embryonic processes through the modulation of the DNA-binding function of Hox proteins (43), as well as through interactions with Meis and Prep1, two other TALE proteins that regulate chromatin remodeling and coactivator access (44). RA strongly upregulates Pbx1 expression (45, 46) and Pbx1 is required for RA induction of embryonic differentiation (47–49). The analysis of Sle1a.1 CD4+ T cell gene expression linked Pbx1 and RA signaling, suggesting a continuity of this process in adult cells. In the immune system, Pbx1 maintains the quiescence of hematopoietic stem cells, at least in part through TGF-β (50). The function of Pbx1 in adult immune cells has not been described, except for macrophages in which it regulates the production of IL-10 in response to apoptotic cells (19). Pbx1 has been reported to not be expressed in T cells, although the same study showed that transgenic expression of a Pbx1 mutant affects T cell development (51). We showed in this study that Pbx1 is expressed not only in murine but also human CD4+ T cells. Furthermore, we identified a novel splice isoform, Pbx1-d, that is overexpressed by the NZW allele and that is predicted to function as a dominant negative by binding Meis and Prep but not Hox and DNA. A loss-of-function phenotype in B6.Sle1a.1 mice suggests that Pbx1 is required for optimal RA expansion of iTregs or to limit the size of the memory T cell compartment. Pbx1-d transduction in Jurkat T cells was sufficient to induce the expression of genes found in activated T cells or involved in Th17 differentiation, as well as to reduce apoptosis in response to RA. These alterations correlated with the specific transcriptional signatures of Sle1a.1 T cells. We also found that Pbx1-d was expressed at a significantly higher frequency in CD4+ T cells from SSE patients than from HCs. The expression of Pbx1-d was increased in activated/memory CD4+ T cells from murine and human origin, and it was decreased in steroid-treated patients. Overall, in addition to the fact that Pbx1 is the only gene in the critical Sle1a.1 interval, these results strongly suggest a causal relationship between Pbx1-d expression and an autoimmune/activated T cell phenotype. It is likely that activation further enhances Pbx1 expression, since we have consistently observed an increased Pbx1-d expression in B6.TC as compared with B6.Sle1a.1 T cells. This suggests that the level Pbx1-d expression results from a combination of cis-genetic polymorphism(s), represented in this study by the NZW allele, and trans-acting factors associated with T cell activation, which itself can be dependent or independent of Pbx1-d expression. Future studies will test the existence of this amplification loop in both murine and human cells.

It is increasingly recognized that splice variants represent an enormous source of genetic diversity, and that most disease-causing mutations result from alternative splicing (52). In the NOD mouse, type 1 diabetes susceptibility is associated with the differential expression of the lctLTA-4 isoform (53). Moreover, the differential expression of Ly108 splice isoforms between the B6 and NZM2410 alleles regulates B cell (54) and T cell (55) tolerance in the Sle1b locus. The differential expression of Pbx1 splice variants controlling CD4+ T cell tolerance and activation in the Sle1a.1 locus therefore represents the second lupus susceptibility locus attributable to this type of genetic variation. In addition to the synonymous mutation that we confirmed between the B6 and NZW Pbx1 introns. A detailed analysis will be necessary to determine the location of the intronic regulatory motifs responsible for the splicing out of exons 6 and 7 leading to Pbx1-d expression. Human PBX1 maps to 1q23, a region with confirmed linkage to SLE (56).

Future studies will determine to what extent that the increased frequency of Pbx1-d expression in lupus T cells corresponds to genetic polymorphisms in the gene itself.

Acknowledgments

We thank the members of the Morel Laboratory for stimulating discussions, Leilani Zeumer and Xuekun Su for excellent technical help, Neal Benson from the University of Florida Interdisciplinary Center for Biotechnology Research cores for help with flow sorting, Celia Lopez for microarray processing, and Dr. Westley H. Reeves and the staff of the University of Florida lupus clinic for access to patients and HCs. We thank Dr. Vijay Kuchroo (Harvard Medical School) for the gift of B6.Foxp3-eGFP breeders, Dr. Chander Raman (University of Alabama at Birmingham) for advice in the EAE experiment, and Dr. Jane Lian (University of Massachusetts) for the pENTER-TEV-Pbx1b construct.

Disclosures

The authors have no financial conflicts of interest.

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