The Murine Pbx1-d Lupus Susceptibility Allele Accelerates Mesenchymal Stem Cell Differentiation and Impairs Their Immunosuppressive Function

Shun Lu, Leilani Zeumer, Heather Sorensen, Hong Yang, Yunfai Ng, Fahong Yu, Alberto Riva, Byron Croker, Shannon Wallet and Laurence Morel

*J Immunol* 2015; 194:43-55; Prepublished online 21 November 2014;
doi: 10.4049/jimmunol.1401851
http://www.jimmunol.org/content/194/1/43

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/11/21/jimmunol.1401851.DCSupplemental

References

This article cites 50 articles, 7 of which you can access for free at:
http://www.jimmunol.org/content/194/1/43.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Murine Pbx1-d Lupus Susceptibility Allele Accelerates Mesenchymal Stem Cell Differentiation and Impairs Their Immunosuppressive Function

Shun Lu,* Leilani Zeumer,* Heather Sorensen,†‡ Hong Yang,* Yunfai Ng,* Fahong Yu,§ Alberto Riva,§ Byron Croker,* Shannon Wallet,†‡ and Laurence Morel*

Pre-B cell leukemia homeobox 1 (Pbx1)-d is a dominant-negative splice isoform of the gene Pbx1 that corresponds to the NZM2410 lupus susceptibility locus Sle1a1. Pbx1 is required to maintain stem cell self-renewal, including that of mesenchymal stem cells (MSCs). MSCs have immunosuppressive functions that require stem cell maintenance. We tested the hypothesis that the expression of Pbx1-d favors MSC differentiation and impairs their immunosuppressive functions. We demonstrate that Sle1a1 MSCs express high levels of Pbx1-d as compared with congenic C57BL/6J (B6) MSCs. Sle1a1 MSCs grew faster and differentiated significantly more rapidly into osteoblasts than did B6 MSCs. This corresponded to a significant decrease in the expression of genes associated with stemness and an increase in the expression of genes associated with differentiation. Additionally, Sle1a1 MSCs express a gene expression profile associated with an enhanced innate immunity and inflammation. Suppression of Ig production from TLR-activated B6 B cells and IL-2 secretion from activated B6 CD4+ T cells was significantly impaired in Sle1a1 MSCs as compared with B6 MSCs. B6.Sle1a1 MSCs showed intermediate activity in suppressing lupus immunophenotypes in three different mouse models. Taken together, these data suggest that the expression of the lupus susceptibility allele Pbx1-d isoform impairs MSC functions, which may contribute to lupus pathogenesis both through a defective immunosuppression and the promotion of a proinflammatory environment. The Journal of Immunology, 2015, 194: 43–55.

Systemic lupus erythematosus (SLE) is a complex disease with defects in multiple cell types that are mediated through a large number of susceptibility genes whose identity is starting to be revealed (1). Using the NZM2410 lupus-prone mouse, we have identified a number of susceptibility genes and have produced a series of congenic strains that represent a powerful tool to characterize the function of the lupus susceptibility genes singly and in combination (2). This has led to the production of the B6.Sle1.Sle2.Sle3 (TC) strain that coexpresses on a C57BL/6J (B6) background the three Sle susceptibility loci that are necessary and sufficient to develop clinical lupus (3). Among the NZM2410 susceptibility genes, we have identified the pre-B cell leukemia homebox 1 (Pbx1) gene as the gene corresponding to Sle1a1 (4). Sle1a1 is a contributor to the Sle1 global locus, which controls the loss of tolerance to chromatin (5–7), and is necessary for disease initiation (8, 9). NZM2410-derived B6.Sle1a1 (Sle1a1) congenic mice as well as TC mice, which carry Sle1a1, present an elevated number of activated autoreactive CD4+ T cell that provide help to B cells producing anti-chromatin IgG (6, 7).

Pbx1, the only gene located in the Sle1a1 interval, is a well-characterized member of the TALE family of homeodomain proteins that functions as a transcriptional regulator through the modulation of the DNA-binding function of Hox proteins (10). The NZM2410 allele is a splice isoform (Pbx1-d) in which DNA-binding exon 6 and Hox-binding exon 7 are missing and thus induces a dominant-negative function (11). Importantly, the Pbx1 amino acid sequence is identical between mice and humans. Pbx1-d was found more frequently in SLE patients than in healthy controls, and its expression corresponded to a decreased naive/central memory CD4+ T cell ratio, which is characteristic of SLE T cells (4). Prior to this newly discovered role in regulating T cell functions, full-length Pbx1-b was known to be necessary to block lineage-specific differentiation and maintains self-renewal of hematopoietic stem cells (12) as well as mesenchymal stem cells (MSCs) (13). We have shown using an MSC line that the overexpression of Pbx1-d was functionally equivalent to knocking down Pbx1-b, resulting in an accelerated differentiation into osteoblasts (11).

MSCs serve as a reservoir for tissue regeneration through their multipotent differentiation into chondrocytes, osteoblasts, or adipocytes (14). Additionally, MSCs play a role in tissue and immune homeostasis through regulation of apoptosis and immune modulation (15). MSCs suppress T cell proliferation and dendritic cell maturation, and either promote or suppress B cell proliferation and Ab production. Several cases of successful treatment of refractory SLE with allogeneic MSCs have been reported, but treatment of...
two patients with autologous MSCs was not successful (16, 17). Additionally, MSCs from lupus have defective functions, with an impaired differentiation into osteoblasts (18), enhanced senescence and apoptosis, as well as impaired self-renewal (19, 20), providing potential mechanisms for the observed failure of autologous MSC therapies. One study, however, reported that MSCs from autoimmune patients presented immunosuppressive properties similar to those of healthy controls (21). Mixed results have also been obtained with mouse models of lupus (17). MRL/lpr mice have been successfully treated with both allogeneic and syngeneic MSCs in several studies (22–25). In the (NZB × NZW) F1 (BWF1) strain, which has the same

specifically, whole BM was isolated from the femur and tibia, passed by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from
which 1004 were also identified by the previous analysis. We analyzed this list of 1004 genes using the TopoFun program, part of the TopGene suite (https://topgene.cchmc.org/), to identify functional categories showing enrichment for the genes in our list. Note that the RNA-Seq data set can be accessed at http://www.ncbi.nlm.nih.gov/bioproject/?LinkName=sra_bioproject&from_uid=1073997 (accession no. PRJNA264078).

In vitro suppression assays
MSC function was characterized by suppression assays. Following trypsinization, indicated numbers of MSCs were primed with 5 ng/ml IFN-γ (Sigma-Aldrich) overnight, after which primed MSCs were cocultured with either 6 × 10^5 B6 B cells or 6 × 10^5 B6 CD4^+ T cells purified by negative selection with magnetic beads (Miltenyi Biotec). B cells were stimulated with 1 μg/ml LPS or CpG (Invivogen). T cells were stimulated with 6 × 10^5 anti-CD3/CD28 beads (Life Technologies). Cultures were stimulated for 3 d, after which supernatants were probed for either total Ig (B cells) (eBioscience) or IL-2 (T cells) (BD Biosciences) by ELISA. A standard curve and five parameter logistics were used to determine concentrations.

In vivo treatments
We have adapted a previously published protocol (24) to compare the immunosuppressive capacity of Sle1a1 and B6 MSCs to reverse lupus in three different mouse models. Cohorts of age-matched anti-dsDNA IgG^+ mice were injected i.v. with 10^8 BM-derived B6 or Sle1a1 MSCs, or left untreated. We treated TC mice at 25–31 wk of age, BWF1 mice at 21 wk old, and MRL/lpr mice at 8 wk old. Serum was collected before treatment and every 2 wk after treatment for autoantibody measurements. Proteinuria was measured weekly with Albustix strips (Bayer) on a 0–4 scale corresponding to <100, 300, 1000, 3000, and >3000 μg/ml of protein. All mice were sacrificed 8 wk after injection, except for two mice that presented with terminal disease and were sacrificed early in the treatment. Immune cell subset distribution and activation were analyzed by flow cytometry of splenocytes. Renal pathology was scored from H&E and periodic acid–Schiff stains as previously described (31). Briefly, glomerulonephritis (GN) was scored on a semiquantitative 0–4 scale, and qualitied as mesangial or proliferative. Additionally, the samples within each strain were ranked according to increased severity by a pathologist (B.C.) who did not have knowledge of the treatment status. The glomerular deposition of C3 and IgG2a immune complexes was performed on frozen kidney sections and scored as previously described (31). Briefly, glomerulonephritis (GN) was scored on a semiquantitative 0–4 scale, and qualified as mesangial or proliferative. Additionally, the samples within each strain were ranked according to increased severity by a pathologist (B.C.) who did not have knowledge of the treatment status. The glomerular deposition of C3 and IgG2a immune complexes was performed on frozen kidney sections and scored as previously described (31). Glomerular size and the isoform expressed by B6 MSCs was Sle1a1 and B6 MSCs to reverse lupus in three different mouse models. Cohorts of age-matched anti-dsDNA IgG^+ mice were injected i.v. with 10^8 BM-derived B6 or Sle1a1 MSCs, or left untreated. We treated TC mice at 25–31 wk of age, BWF1 mice at 21 wk old, and MRL/lpr mice at 8 wk old. Serum was collected before treatment and every 2 wk after treatment for autoantibody measurements. Proteinuria was measured weekly with Albustix strips (Bayer) on a 0–4 scale corresponding to <100, 300, 1000, 3000, and >3000 μg/ml of protein. All mice were sacrificed 8 wk after injection, except for two mice that presented with terminal disease and were sacrificed early in the treatment. Immune cell subset distribution and activation were analyzed by flow cytometry of splenocytes. Renal pathology was scored from H&E and periodic acid–Schiff stains as previously described (31). Briefly, glomerulonephritis (GN) was scored on a semiquantitative 0–4 scale, and qualitied as mesangial or proliferative. Additionally, the samples within each strain were ranked according to increased severity by a pathologist (B.C.) who did not have knowledge of the treatment status. The glomerular deposition of C3 and IgG2a immune complexes was performed on frozen kidney sections and scored as previously described (31). Glomerular size and the isoform expressed by B6 MSCs was Sle1a1 and B6 MSCs to reverse lupus in three different mouse models. Cohorts of age-matched anti-dsDNA IgG^+ mice were injected i.v. with 10^8 BM-derived B6 or Sle1a1 MSCs, or left untreated. We treated TC mice at 25–31 wk of age, BWF1 mice at 21 wk old, and MRL/lpr mice at 8 wk old. Serum was collected before treatment and every 2 wk after treatment for autoantibody measurements. Proteinuria was measured weekly with Albustix strips (Bayer) on a 0–4 scale corresponding to <100, 300, 1000, 3000, and >3000 μg/ml of protein. All mice were sacrificed 8 wk after injection, except for two mice that presented with terminal disease and were sacrificed early in the treatment. Immune cell subset distribution and activation were analyzed by flow cytometry of splenocytes. Renal pathology was scored from H&E and periodic acid–Schiff stains as previously described (31). Briefly, glomerulonephritis (GN) was scored on a semiquantitative 0–4 scale, and qualitied as mesangial or proliferative. Additionally, the samples within each strain were ranked according to increased severity by a pathologist (B.C.) who did not have knowledge of the treatment status. The glomerular deposition of C3 and IgG2a immune complexes was performed on frozen kidney sections and scored as previously described (31). Glomerular size and the extent of C3 deposition were measured from anti-C3–stained sections averaging four to six glomeruli per sample using the Metamorph 7.5 image analysis software as a percentage of the total glomerular area. Each strain was represented by three lines per strain, first normalized to Gapdh expression and then expressed relative to the B6 average. Relative expression to total Pbx1, Pbx1-a, or Pbx1-b. (F) Pbx1 protein expression in B6 and Sle1a1 MSCs relative to Gapdh. The blots on the left shows Pbx1 expression measured in HEK293 cells transfected with cloned cDNA is shown on the left as reference. (C) Expression of total Pbx1, Pbx1-a, Pbx1-b, and Pbx1-d as measured by SYBR Green incorporation. (D) Pbx1-d expression measured with a TaqMan probe. Results were obtained from three lines per strain, first normalized to Gapdh expression and then expressed relative to the B6 average. Relative expression to total Pbx1, Pbx1-a, or Pbx1-b. (F) Pbx1 protein expression in B6 and Sle1a1 MSCs relative to Gapdh. The blots on the left shows Pbx1 expression measured in HEK293 cells transfected with either Pbx1-b or two different Pbx1-d cDNAs. Results are representative of three experiments. *p < 0.05.

Statistical analysis
Statistical analyses were performed using GraphPad Prism 6.0. Unless specified, the graphs show means and SEM. Comparisons were performed with either two-tailed t tests or Mann–Whitney U tests, depending on the normality of the data distribution. Multiple comparison corrections were used when appropriate. Time-course graphs were compared with two-way ANOVA.

Results
Sle1a1 MSCs express the Pbx1-d isoform at a higher level than do B6 MSCs
We compared Pbx1 isoform expression in MSCs by semiquantitative RT-PCR using the set of primers that amplifies all isoforms (Fig. 1A) that we previously used for CD4^+ T cells (4). The main isoform expressed by B6 MSCs was Pbx1-b, which has been shown by others to be the major isoform expressed in normal MSCs (13), whereas the main isoform expressed by Sle1a1 MSCs was Pbx1-d (Fig. 1B). To quantify this differential expression, we designed isoform-specific qRT-PCR primers, as well as a TaqMan probe for Pbx1-d (Fig. 1A, Supplemental Table I). There was no difference of expression for total Pbx1, Pbx1-a, or Pbx1-b between the two strains, but Sle1a1 MSCs expressed significantly more Pbx1-d than did B6 MSCs (Fig. 1C). This finding was confirmed using a TaqMan probe with six other Pbx1-d–specific primer pairs (Fig. 1D and data not shown). Moreover, the relative ratios of Pbx1-d over total Pbx1, Pbx1-a, or Pbx1-b expression were significantly higher in Sle1a1 than in B6 MSCs (Fig. 1E). Although Western blot analysis of B6 and Sle1a1 MSCs showed a similar amount of total Pbx1 in the two strains, confirming the qRT-PCR results, the isoform results could not be validated at the protein level with currently available Pbx1 Abs, as they bind equally to both isoforms (Fig. 1F). The predicted size of each isoform is 3 aa apart (Pbx1-b, 38 kDa; Pbx1-d, 37 kDa) and thus
cannot be distinguished by electrophoresis as shown by the transfection of HEK293 cells with expression plasmids (Fig. 1F).

We examined Pbx1 expression using an RNA-Seq data set that compared Sle1a1 and B6 MSC RNA expression (Supplemental Table II). The total number of Pbx1 reads was significantly lower in Sle1a1 than in B6 MSCs. The analysis of the individual Pbx1 isoforms detected four isoforms above background, with Pbx1-a being the most abundant and the only one showing a significant difference between strains, with the Sle1a1 MSCs expressing less (Supplemental Table II). Taken together, these data support the fact that Pbx1 transactivates itself, and that Pbx1-d has a dominant-negative function. As shown above, we and others have shown by RT-PCR that Pbx1-b is the most abundant isoform in normal MSCs. Pbx1-d was not found in B6 MSCs, and it was not formally detected above background in Sle1a1 MSCs, which prevented statistical analyses. Isoform detection through RNA-Seq is quite difficult, especially when a gene has several isoforms, and when expression is low, as is this case for Pbx1. We therefore think that our RT-PCR is more reliable, and thus we have demonstrated that Pbx1-d is the major Pbx1 isoform expressed in Sle1a1 MSCs.

Sle1a1 MSCs have a different phenotype than do B6 MSCs
BM-derived cultures from both strains resulted in similar frequency of CD90^+CD73^+ cells; however, the level of expression of surface markers was significantly different (Fig. 2A). Specifically, Sle1a1 MSCs expressed significantly lower levels of markers characteristic of the pluripotent capacity or “stemness” of these cells, such as CD90 and CD105 (Fig. 2B), while expressing higher levels of markers found on differentiated leukocytes, such as MCH class II, CD34, and CD45 (Fig. 2A, 2C). Additionally, Sle1a1 MSCs expanded at a significantly higher rate (Fig. 2D) and differentiated more rapidly into osteoblasts upon induction than did B6 MSCs (Fig. 2E). To this end, protein levels of a marker of osteoblast

**FIGURE 2.** Sle1a1 MSCs differentiate faster than do B6 MSCs. (A) Representative bright field microscopy and flow cytometry comparison of MSCs between the two strains. Original magnification ×60. The number in the upper right corner indicates the percentage of CD90^+CD73^+ cells. The histogram overlays on the right show the expression of three surface makers associated with MSC stemness (CD105 [dotted line], CD90, and CD73) and three markers associated with leukocyte differentiation (MHC class II [blue], CD34 [red], and CD45 [green]). (B and C) Mean fluorescence intensity (MFI) quantitation of the stemness (B) and leukocyte differentiation (C) markers shown in (A). Statistical significance is shown for t tests between strains for each marker (D). Growth rate measured as the number of days necessary to reach each passage. (E) Osteoblast differentiation time course measured as the percentage of Alizarin red^+ cells. For (D) and (E), statistical significance compared the two strains with two-way ANOVA. (F) Bmp4 protein expression in Sle1a1 and B6 MSCs before (day 0) and 6 d after osteogenic differentiation. β-Actin expression is shown as internal control. ***p < 0.001.
differentiation, Bmp4, were significantly higher in Sle1a1 than in B6 MSCs and increased to a much higher level in Sle1a1 MSCs upon osteogenic induction (Fig. 2F). Overall, these results demonstrate that Pbx1-d expression in MSCs is associated with a more differentiated and less stemness phenotype.

Sle1a1 MSCs show a more differentiated gene expression profile than do B6 MSCs

Pbx1 is a transcription factor, and the expression of its dominant-negative allele is predicted to alter gene expression. To test this hypothesis, we used a PCR array that contains MSC-related genes to compare the gene profile of Sle1a1 and B6 MSCs. In accord with our phenotypic data, most genes that were underexpressed in Sle1a1 MSCs, such as Thy1 or Pdgfr, were genes associated with a stem cell phenotype, whereas the genes that were overexpressed in Sle1a1 MSCs were associated with lineage-specific differentiation, such as Gdf7 for chondrogenesis or Bmp4 and Fgf10 for osteogenesis (Fig. 3A). Most of these results were validated by RNA-Seq (Fig. 3B) and qRT-PCR (Fig. 3C) analyses of an independent set of MSC cultures. Additionally, we have validated the increased expression of Bmp4 protein by Sle1a1 MSCs (Fig. 2F).

We identified 12 biological process classes from gene ontology (GO) and three mouse phenotypes (MP) that are either related to stem cells or immune processes (Table I). In particular, the three MP pathways, that is, abnormal innate immunity, increased inflammatory response, and abnormal inflammatory response, showed very strong evidence of enrichment. Examination of the individual genes in the stem cell proliferation pathway (Fig. 3D)

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

**FIGURE 3.** Sle1a1 MSCs express a different gene signature than do B6 MSCs. (A) Expression of MSC-related genes in Sle1a1 MSCs relative to B6 MSCs measured with a PCR array. (B) Expression of the same genes as in (A) detected by RNA-Seq in a new set of MSC lines for each strain with a p value of <0.05. (C) qRT-PCR validation of the same genes as in (A) showing a significant difference between the two strains with a p value of <0.05. Open bars show MSC-specific genes, gray bars show MSC-associated genes, and black bars show genes associated with lineage-specific differentiation. The arrows indicate genes mentioned in the text (n = 3 lines/strain/experiment). Genes in the “GO: stem cell proliferation” (D) and “GO: cytokine production” (E) pathways that were differentially expressed between B6 and Sle1a1 MSCs are shown. The threshold for differential expression was set at −0.5 > log2(fold change) > 0.5 for (D) or −1 > log2(fold change) > 1 for (E), and p < 0.001 for both. (F) qRT-PCR validation of selected genes in the cytokine production pathway. Means ± SEM of n = 3/strain are shown. *p < 0.05 for one-tailed t tests; **p < 0.01, ***p < 0.001 for two-tailed t tests. (G) IL-10 and IL-6 production in MSC supernatant. Means ± SEM of n = 4/strain are shown. *p < 0.05 for two-tailed t tests.
indicated, similar to the gene array and phenotypic analysis, a decreased expression in genes associated with multipotent lineage such as Tbx18, Bdnf, and Zeb31 and, alternatively, an increased expression of genes associated with osteoblast differentiation (Wnt10b, Bmp4), as well as suppression of Wnt signaling by Sfrp2, which leads to decreased self-renewal (32). Examination of the cytokine production pathway indicated an increased expression by Sle1a1 MSCs of cytokines found at higher levels in SLE patients such as IL-10 (33) and IL-33 (34), as well as a very high level of the T cells from SLE patients and that is required for IL-17 production was significantly reduced in Sle1a1 MSCs (Fig. 4A–D). Similarly, B6 MSCs were less suppressive than were B6 MSCs. Specifically, in the presence of IFN-γ, B6 MSCs significantly suppressed Ig production induced by either LPS or CpG up to a 3:1 B cell/MSC ratio, whereas Sle1a1 MSCs were suppressive at only a 1:1 B cell/MSC ratio. Without IFN-γ, B6 MSCs significantly suppressed Ig production induced at a 1:1 B cell/MSC ratio, but no suppression was observed with the Sle1a1 MSCs (Fig. 4A–D). Similarly, B6 MSCs significantly suppressed IL-2 production up to a 3:1 CD4+ T cells/MSC ratio whereas Sle1a1 MSCs were immunosuppressive only at the 1:1 ratio, either in the presence or absence of IFN-γ (Fig. 4E, 4F). Overall, our results demonstrate that Pbx1-d expression in MSCs is associated with a decreased suppressive function.

**Slle1a MSCs have a decreased immunosuppressive capacity in murine models of lupus**

We compared the ability of Sle1a1 MSCs to suppress autoimmune pathology in three models of lupus, that is, BWF1, TC, and MRL/lpr mice. All mice were positive for serum anti-dsDNA IgG at the time of treatment, indicating that disease had been initiated. All mice were sacrificed 8 wk after treatment, except for two TC mice and one MRL/lpr mouse treated with Sle1a1 MSCs that showed terminal clinical disease and were sacrificed at weeks 2, 3, and 6 after treatment, respectively.

We compared the effect of B6-derived MSC and Sle1a1-derived MSC treatments on the production of serum anti-dsDNA IgG as well as ANAs. As previously reported (24), MSC treatments had little effect on the existing production of autoantibodies regardless of the source of MSCs (Fig. 5A–C). A time course analysis of

---

**Table I. Stem cell and immune-related GO pathways differentially expressed between Sle1a1 and B6 MSCs as determined by RNA-Seq**

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>Value</th>
<th>Exact Value</th>
<th>Bonferroni Value</th>
<th>B&amp;H Value</th>
<th>Query List Value</th>
<th>Genome Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO: 0006954</td>
<td>Inflammatory response</td>
<td>0.26</td>
<td>0.0006954</td>
<td>0.0006954</td>
<td>0.0006954</td>
<td>0.0006954</td>
<td>0.0006954</td>
</tr>
<tr>
<td>GO: 0002684</td>
<td>Regulation of immune system process</td>
<td>0.48</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
</tr>
<tr>
<td>GO: 0006955</td>
<td>Immune suppression</td>
<td>0.78</td>
<td>0.0006955</td>
<td>0.0006955</td>
<td>0.0006955</td>
<td>0.0006955</td>
<td>0.0006955</td>
</tr>
<tr>
<td>GO: 0001816</td>
<td>Cytokine production</td>
<td>0.21</td>
<td>0.0001816</td>
<td>0.0001816</td>
<td>0.0001816</td>
<td>0.0001816</td>
<td>0.0001816</td>
</tr>
<tr>
<td>GO: 0002684</td>
<td>Positive regulation of immune system process</td>
<td>0.93</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
<td>0.0002684</td>
</tr>
<tr>
<td>GO: 0002520</td>
<td>Immune system development</td>
<td>0.25</td>
<td>0.0002520</td>
<td>0.0002520</td>
<td>0.0002520</td>
<td>0.0002520</td>
<td>0.0002520</td>
</tr>
<tr>
<td>GO: 0045321</td>
<td>Leukocyte activation</td>
<td>0.19</td>
<td>0.0045321</td>
<td>0.0045321</td>
<td>0.0045321</td>
<td>0.0045321</td>
<td>0.0045321</td>
</tr>
<tr>
<td>GO: 0002764</td>
<td>Immune response-regulating signaling pathway</td>
<td>0.14</td>
<td>0.0002764</td>
<td>0.0002764</td>
<td>0.0002764</td>
<td>0.0002764</td>
<td>0.0002764</td>
</tr>
<tr>
<td>GO: 0002757</td>
<td>Increased inflammatory response</td>
<td>0.23</td>
<td>0.0002757</td>
<td>0.0002757</td>
<td>0.0002757</td>
<td>0.0002757</td>
<td>0.0002757</td>
</tr>
<tr>
<td>GO: 00001846</td>
<td>Abnormal inflammatory response</td>
<td>0.27</td>
<td>0.00001846</td>
<td>0.00001846</td>
<td>0.00001846</td>
<td>0.00001846</td>
<td>0.00001846</td>
</tr>
</tbody>
</table>

*False discovery rate q values were evaluated based on the Bonferroni correction and on the Benjamini–Hochberg (B&H) method.

Number of genes in the pathway that are differentially expressed between the two strains.

Number of genes in the pathway.
biweekly serum anti-dsDNA IgG revealed that both B6 and Sle1a1 MSC treatments of BWF1 mice resulted in the maintenance of anti-dsDNA IgG levels for the first 4 wk (data not shown), an effect that was not sustained. However, at termination of the experiment, untreated BWF1 mice had significantly higher levels of anti-dsDNA IgG than did B6 MSC-treated mice, with an intermediate level of anti-dsDNA IgG observed in mice treated with Sle1a1 MSCs (Fig. 5A). There was a trend for a decrease in anti-dsDNA IgG levels in MSC-treated TC mice (Fig. 5B) and no difference in MRL/lpr mice whether they were treated with MSCs derived from either strain or were left untreated (Fig. 5C). The analysis of terminal ANA serum levels by Hep-2 cell immunofluorescence staining confirmed the results obtained by ELISA for anti-dsDNA IgG. The serum from BWF1 mice treated with B6 MSCs, but not those treated with Sle1a1 MSCs, demonstrated a significant reduction in staining intensity as compared with controls (Fig. 5D, 5E). No consistent difference in ANA reactivity was found in the TC (Fig. 5F, 5G) and MRL/lpr (Fig. 5H, 5I) cohorts. Additionally, the staining pattern, nuclear versus cytoplasmic, was not significantly affected by either MSC treatment in any of the models tested. Taken together, these data suggest that MSC treatments were at best able prevent a further increase of the autoantibody production (anti-dsDNA IgG or ANA) in BWF1 mice, but never to reverse or significantly decrease this process. Importantly, B6 MSCs tended to be more effective than those derived from Sle1a1 mice.

We also compared the renal pathology between treatments within each of the three cohorts using a variety of biomarkers.
Treatment with either B6 or Sle1a1 MSCs tended to reduce the size of glomeruli in both BWF1 and TC mice, but the difference was significant only upon treatment with B6 MSCs (Fig. 6A). The glomerular size of MRL/lpr mice was not affected by either treatment. Glomerular immune complex deposition is a hallmark of lupus nephritis. Treatment with B6 MSCs significantly decreased the amount of C3 found in the glomeruli of BWF1 and MRL/lpr mice, whereas Sle1a1 MSC treatment had a significant effect only on the amount of C3 found in MRL/lpr mice (Fig. 6B, 6C). The MSC treatments had no significant effect on C3 deposits in the kidneys of TC mice. Similar results were obtained for IgG2a glomerular deposits (data not shown). To account for differences of kidney pathology between strains, as well as the different time points in disease development at which the treatment was initiated, we compared the effect of the treatments by ranking blinded samples in order of increasing severity within a cohort. BWF1 mice treated with B6 MSCs showed a significantly reduced pathology as compared with control mice (Fig. 6D). Although there was a trend in the same direction for Sle1a1 MSCs, the difference was not significant. TC mice treated with Sle1a1 MSCs showed a more severe pathology than did mice treated with B6 MSCs and equivalent to that of untreated mice (Fig. 6E). There was no effect of either treatment in MRL/lpr mice (Fig. 6F). Finally, we measured proteinuria on a weekly basis with semiquantitative strips (data not shown) as well as with quantitative microalbuminuria in the terminal samples (Fig. 6G–I). In BWF1 and TC mice, treatment with B6 MSCs significantly decreased proteinuria as compared with controls, whereas Sle1a1 MSCs had an intermediate effect in BWF1 mice and no effect in TC mice (Fig. 6G, 6H). Finally, proteinuria was generally low in our MRL/lpr cohort, with a trend in mice treated with Sle1a1 MSCs presenting higher levels of proteinuria (Fig. 6I).

Body weight loss is an outward biomarker of clinical disease in both BWF1 and TC mice. BWF1 mice treated with B6 MSCs lost significantly less terminal body weight than did controls, whereas there was no difference between mice treated with Sle1a1 MSCs and controls (Fig. 6K). Similarly, TC mice treated with Sle1a1 MSCs lost significantly more terminal body weight than did mice treated with B6 MSCs (Fig. 6K). MRL/lpr mice develop an extensive lymphadenopathy that contributes greatly to an increased body weight. This gain in body weight was significantly reduced by treatment with B6 but not Sle1a1 MSCs (Fig. 6L). Overall, the Sle1a1 MSCs had a reduced ability to reduce clinical pathology in three different models of lupus as compared with B6 MSCs.

Finally, we evaluated the immune phenotypes in the spleens of each cohort 8 wk after treatment. Neither MSC treatment resulted in significantly different numbers or percentages of CD4+ T cells, CD8+ T cells, or B cells (data not shown). No difference was...
observed either for the numbers or percentages of germinal center or class-switched B cells (data not shown). CD4+ T cell activation was, however, affected by MSC treatment. BWF1 mice treated with B6 but not Sle1a1 MSCs showed a significant increase of the CD62L+CD44+ naive CD4+ T cells relative to the CD62L-CD44+ effector memory population (Fig. 7A). This variable was not affected by either MSC treatment in the two other strains. CD25 expression on CD4+ T cells was significantly decreased by B6 but not Sle1a1 MSC treatment in all three cohorts (Fig. 7B). There was no difference, however, in the numbers and percentages of

---

**FIGURE 6.** Effect of MSC treatment on clinical disease. (A) Glomerular size in the BWF1, TC, and MRL/lpr cohorts treated with B6 or Sle1a1 MSCs or untreated controls (none). (B) Glomerular C3 deposition in the same cohorts. The two graphs show means ± SEM, and the statistical significance (*p < 0.05) corresponds to two-tailed t tests with the control group within each cohort. (C) Representative FITC-conjugated anti-C3 IgG staining of kidney sections from BWF1 and MRL/lpr mice treated with B6 or Sle1a1 MSCs or untreated controls. Original magnification ×10. (D–F) Glomerulonephritis severity ranked within each cohort. (G–I) Terminal albuminuria. (J–L) Percentage body change 8 wk after MSC treatment. *p < 0.05, **p < 0.01 for two-tailed t or Mann–Whitney tests.
Foxp3+CD4+ T cells (data not shown). Finally, B6 MSC but not Sle1a1 MSC treatment reduced the percentage of CD4+ and CD8+ T cell blasts in the three cohorts (Fig. 7C, 7D), as well as the percentage of B cell blasts in BWF1 mice (Fig. 7E). Overall, the Sle1a1 MSCs were not as effective in reducing the immunopathology in three different models of lupus as compared with B6 MSCs.

**FIGURE 7.** Effect of MSC treatment on lymphocytes. Splenic T and B cell phenotypes were compared in each cohort 8 wk after treatment. (A) Ratio of naive CD62L+CD44- over effector memory CD62L-CD44+ CD4+ T cells. (B) Percentage of CD4+ T cells expressing CD25. (C) Percentage of CD4+ T cell blasts measured as forward light scatter (FSC)hi. (D) Percentage of CD8+ T cell blasts measured as FSCsh. (E) Percentage of CD19+ B cell blasts measured as FSCsh. *p < 0.05, **p < 0.01 for two-tailed t tests.
Discussion

There are three checkpoints to overcome in lupus pathogenesis (37). Checkpoint 1 involves immune tolerance in the adaptive arm of the immune system, ensuring that autoreactive B and T cells are censored. Checkpoint 2 involves both the adaptive and the innate arms of the immune system to prevent peripheral amplification of the autoimmune response leading to the generation of pathogenic autoantibodies and effector lymphocytes. Checkpoint 3 involves protection of the end organs from pathogenic autoantibodies and effector immune cells. The coordinated activation of disease susceptibility genes at all three checkpoints appears to be necessary for full-blown disease in murine models and humans. The data presented in the present study and in our previous work suggest that Pbx1 is one such susceptibility gene involved in this coordinated activation. We have previously shown that Sle1a1, the locus where Pbx1 is located, regulates the production of autoreactive CD4+ T cells, as well as the homeostasis of the regulatory T cell compartment (4). We now show that Pbx1 regulates MSC immunoregulatory functions.

Pbx1 is an essential gene to maintain stem cell functions, as shown both for hematopoietic stem cells and MSCs (12, 13). MSCs serve as a reservoir for tissue regeneration through their multipotent differentiation (14) as well as a regulator of tissue and immune homeostasis (15). Specifically, MSCs suppress T cell proliferation and dendritic cell maturation, and they have pleiotropic effects on B cells, including either promotion or suppression of B cell proliferation and induction of Ab production. Therefore, MSCs have the potential to regulate checkpoints 1 and 3 in SLE disease progression, making them an attractive therapeutical target.

Based on the dominant-negative function of the NZM2410-derived Pbx1-d lupus susceptibility allele (11), we hypothesized that MSCs expressing Pbx1-d would present a defective maintenance of stem cell functions with an increase in lineage-specific differentiation and thus a poor suppression of inflammation. In this study, we demonstrate that MSCs produced from the congenic B6, Sle1a1 strain that carries the NZM2410 allele of Pbx1 on a B6 background express Pbx1-d as their major isoform, whereas Pbx1-b is the major isoform in B6 MSCs. The RNA-Seq results did not support this conclusion. We think, however, that a number of technical issues, namely the relatively large number of splice isoforms, each of which with a low expression, an insufficient number of sequencing reads, and the short length of the reads, prevented the RNA-Seq to provide informative results on this issue. Please note that we obtained a high level of concordance between RNA-Seq and qRT-PCR results for other genes (Fig. 3 and data not shown). As predicted, Sle1a1 MSC surface marker phenotype, proliferation, osteoblast differentiation, as well as gene expression profiles indicate a decreased stemness and an increased lineage-specific differentiation, including the expression of hematopoietic-derived lineage markers. This was accompanied by a decreased suppressive function.

In vivo, MSCs from aged lupus-prone mice have been reported to not be as effective as those from either aged non-autoimmune mice or young predisease lupus-prone mice, with the suggestion that the inflammatory environment from which they were derived render them less effective (24). Our in vitro and in vivo studies were conducted on MSCs derived from young Sle1a1 mice, whereby this strain does not develop clinical disease (7). This excludes the possibility contribution of a lupus-related inflammatory milieu to the phenotype and altered function of Sle1a1 MSCs. Many soluble factors have been proposed as the mediators of MSC immunosuppression (38). Interestingly, MSCs need to be “licensed” by the inflammatory milieu, largely through IFN-γ signaling (39). SLE is an inflammatory disease in which IFN-γ is found in large quantities (40), and therefore one could assume a robust licensing for MSC immunosuppression. IFN-γ-primed Sle1a1 MSCs provided a level of immunosuppression of B cells equivalent to that of unprimed B6 MSCs, whereas unprimed Sle1a1 MSCs lost their immunosuppressive capacity. It has been shown that TLR ligands can obstruct MSC immunosuppression (41), which may counteract the licensing effect of IFN-γ; therefore, a differential response to TLR ligands could be involved in the reduced immunosuppressive function of Sle1a1 MSCs. However, Sle1a1 MSCs were also defective in suppressing IL-2 production by CD4+ T cells, independent of TLR stimulation. Taken together, this suggests a generalized loss of immunosuppressive functions that may affect multiple pathways as the cells differentiate away from their pluripotent state.

The mechanisms by which MSC treatment ameliorates lupus severity in either patients or mice are not clear, with variable effects reported in different studies and models. Not only strain differences but also differences in the extent of MSC immunosuppressive activity have been observed: MSCs ameliorate renal disease and lymphocyte activation in all lupus models examined, but some studies have reported significant autoantibody suppression, including anti-dsDNA IgG (25), whereas others found no change (24). We compared three lupus-prone mouse models and found the most complete therapeutic effect of B6 MSC treatment in BWF1 mice and NZM2410-derived TC mice, with more variable effects in MRL/lpr mice. BWF1 and TC mice share a common origin, although they differ by several disease mechanisms (42). It is not clear at this time why these two strains were more responsive to MSC treatment than were MRL/lpr mice in our study. The cohort that we used was relatively young (8 wk old), whereas we also treated an older cohort at 14 wk of age, in which disease was too advanced to be reversed or delayed (data not shown).

Note that for all the phenotypes for which B6 MSCs showed a significant effect, Sle1a1 MSCs showed either no or an intermediate effect as compared with untreated mice. B6 but not Sle1a1 MSC treatment decreased CD25 expression on CD4+ T cells and the number of CD4+ blasts in all three models, as well as the ratio of effector memory cells relative to naive CD4+ T cells in the BWF1 mice, whereas treatment did not induce any change in the regulatory T cell populations in any of the three models, consistent with previously reported results (24). An increase in peripheral regulatory T cells has been observed in successful (18) but also failed (43) MSC transplants in SLE patients, which makes the contribution of regulatory T cells to the MSC therapeutic effect in lupus inconclusive, whereas suppression or inhibition of the inflammatory subset of activated CD4+CD25+Foxp3+ cells reported in murine (44) and human (45) lupus is effective in ameliorating some symptoms of disease. Allogeneic BALB/c MSC transfer has been reported to protected MRL/lpr mice from lupus nephritis by inhibiting B cell activation (46). To the contrary, we observed minimal effect of B6 MSC transfer on B cell activation or autoantibody production in our in vivo study, with the exception that B6 and not Sle1a1 MSCs decreased autoantibody production and the formation of B cell blasts in BWF1 mice. Overall, the strongest immunosuppression induced in vivo by B6 MSCs was on CD4+ T cells, whereby the same level of suppression was not achieved by Sle1a1 MSCs.

In this study, we have focused on the defective immunosuppression of Sle1a1 MSCs. Gene expression profiles revealed a pattern of immune-related genes that were overexpressed by Sle1a1 MSCs, predicting an enhanced innate immunity and inflammation in these cells as compared with B6 controls. Some of
these genes have been associated with lupus, such as IL-10 (33), IL-33 (34), and CamK4 (35). High IL-1 receptor levels correlate with lupus severity (47), and polymorphisms in the IL-1A and in the IL-1 receptor antagonist genes are associated with SLE (48).

Additionally, Slen/MSCs secrete a reduced amount of IL-6, a cytokine that has been associated with their immune suppression in multiple studies (49, 50). We also observed that Slen/MSCs express immune surface markers, which suggests that they may also differentiate into innate immune cells. It is therefore possible that MSCs expressing Pbx1-d contribute to lupus pathogenesis not only by a lack of immunosuppression, but also (or possibly mainly) by creating an inflammatory milieu. This hypothesis is supported by our in vivo suppression studies, in which we observed overall worse outcomes, such as death before the study end point, in mice treated with Slen/MSCs.

In conclusion, this study showed that the lupus susceptibility gene Pbx1 not only contributes to autoimmune pathology by impairing T cell tolerance, but also impairs the function of MSCs, which may also contribute to the disease directly or indirectly. Our results also suggest that MSC therapeutic transplants could be less successful with cells collected from donors expressing Pbx1-d, which should be considered as MSC therapies are considered in an increasing number of conditions, including SLE.

Acknowledgments
We thank Dr. Lijun Yang for helpful discussions and Nathalie Kanda for excellent mouse care.

Disclosures
The authors have no financial conflicts of interest.

References


