T cell hyperactivity in lupus as a consequence of hyperstimulatory antigen-presenting cells

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Sle3 is an NZM2410-derived lupus susceptibility locus on murine chromosome 7. Congenic recombination has resulted in a novel mouse strain, B6.Sle3, associated with serum antinuclear autoantibodies (ANAs), T cell hyperactivity, and elevated CD4/CD8 ratios. An OVA-specific TCR transgene was used as a tool to demonstrate that Sle3 facilitated heightened T cell expansion in vitro, and in vivo, following antigen challenge. Indeed, continued T cell expansion was noted even in response to a tolerogenic signal. However, these phenotypes did not appear to be T cell intrinsic but were dictated by hyperstimulatory B6.Sle3 APCs. Importantly, B6.Sle3-derived DCs and macrophages appeared to be significantly more mature/activated, less apoptotic, and more proinflammatory and were better at costimulating T cells in vitro, compared with the B6 counterparts. Finally, the adoptive transfer of B6.Sle3-derived DCs into healthy B6 recipients elicited increased CD4/CD8 ratios and serum ANAs, 2 cardinal Sle3-associated phenotypes. We posit that their heightened expression of various costimulatory molecules, including CD80, CD106, I-Aβ, and CD40, and their elevated production of various cytokines, including IL-12 and IL-1β, may explain why Sle3-bearing DCs may be superior at breached self tolerance. These studies provide mechanistic evidence indicating that intrinsic abnormalities in DCs and possibly other myeloid cells may dictate several of the phenotypes associated with systemic lupus, including ANA formation and T cell hyperactivity.

Introduction

T cells play a central role in driving the development of lupus, both in mice and in humans, as reviewed (1, 2). Earlier studies had indicated that the ablation of CD4 T cells using genetic or experimental approaches had the potential to ameliorate disease (2–7). It is evident that T cells may be promoting disease in a variety of ways, including rendering help to B cells for autoantibody production, as well as facilitating tissue damage in the end organs (8–13). Although it is evident that T cells are essential for disease, the degree to which genetically dictated, intrinsic T cell anomalies contribute to lupus has not been clear.

In this respect, genetic dissection studies in murine models of lupus have contributed some insight. Spontaneously arising lupus in the NZM2410, NZB/NZW, and MRL/lpr strains is polygenic in origin, as reviewed (14–16). Interestingly, these different models share a disease susceptibility locus on mid–chromosome 7 (17–21). In the NZM2410 model, this locus has been termed Sle3. When this locus was introgressed onto the “normal” C57BL/6 (B6) background, it led to low levels of antinuclear autoantibodies (ANAs) and several lymphocyte phenotypes (22–24). Importantly, Sle3-bearing T cells were spontaneously activated and exhibited elevated CD4/CD8 ratios and impaired activation-induced cell death (23). Although each individual locus by itself was not sufficient to engender severe lupus nephritis, epistatic interaction with other lupus susceptibility loci led to full-blown disease (25–27).

A surprise that next surfaced was the apparent cellular origin of the Sle3-associated phenotypes. When BM from allotype-marked B6 and B6.Sle3 congenic mice were cotransferred into B6 hosts, T cells of both origins (i.e., with or without Sle3) exhibited elevated CD4/CD8 ratios and spontaneous T cell activation, phenotypes attributed to Sle3 (28). These studies demonstrated that the Sle3-associated phenotypes may not be encoded in a T cell–intrinsic fashion, although they were BM-transferable. Likewise, the same study also revealed that autoantibody production in the chimeras was also not contingent upon the intrinsic expression of Sle3 within B cells (28).

The present study launches off from the above observations. An OVA-specific, I-Aβ–restricted TCR Tg, “OT-II” (29), was bred onto B6.Sle3 mice. B6.OT-II and B6.Sle3.OT-II mice were then examined to decipher how Sle3 may be impacting T cell activation and tolerance. In addition, the current study also elucidates the cellular players that may be intrinsically responsible for the T cell hyperactivity attributed to Sle3. Importantly, this study draws attention to the potential role of nonlymphocytic myeloid-lineage cells in driving systemic autoimmunity in lupus.

Results

Phenotypes of novel congenic/transgenic mice. As summarized in Table 1, B6.Sle3 mice (without the Sle3 locus) exhibited elevated CD4/CD8 ratios, as well as the serological and pathological phenotypes previously described in B6.Sle3/Sle5 congenics (22, 23). To further study the cellular origins of these phenotypes, the OVA-specific TCR Tg, OT-II (29), was successfully bred onto B6.Sle3 mice. As observed previously in B6.Sle3 congenics (22), B6.Sle3.OT-II mice also possessed low but significant levels of ANAs (Figure 1A), but not glomerulonephritis (data not shown). B6.OT-II and B6.Sle3.OT-II Tg mice exhibited similar numbers of T cells and B cells in their spleens and nodes (data not shown). Importantly, the B6.OT-II and B6.Sle3.OT-II Tg mice expressed both the Vβ5 and the Vα2 TCR transgenes on about 75% of their peripheral CD4+ T cells (Figure 1B). Ex vivo, B6.Sle3.OT-II TCR Tg T cells were not more...
activated (based on CD69 expression), compared with B6.OT-II TCR Tg T cells (Figure 1C).

*Sle3* impacts T cell function: *in vitro* and *in vivo* studies. Although B6.OT-II and B6.6.OT-II mice exhibited similar numbers of Tg T cells, Sle3-bearing Tg T cells were hyperproliferative, as displayed in Figure 2A, with an attendant reduction in activation-induced cell death (data not plotted), consistent with previous findings (23). Previous studies had also shown that the impact of *Sle3* on T cells was not dependent on T cell–intrinsic *Sle3* expression (28). Hence, we designed an in vivo adoptive transfer experiment to confirm this, using the OT-II TCR Tg model. In essence, B6.OT-II Tg T cells were adoptively transferred into B6 or B6.6.OT-II mice, after which the recipient mice were challenged with an immunogenic dose of OVA. In resonance with the earlier report (28), the administration of immunogenic OVA led to a greater degree of T cell expansion in vivo in B6.6.OT-II hosts, with an attendant increase in serum IgG anti-OVA Abs (Figure 2B). After challenge with OVA, T cells in both types of hosts were equally activated (>90% of Tg T cells expressed CD69; data not plotted). Finally, Sle3-bearing T cells appeared relatively recalcitrant to peripheral tolerance induction, as depicted in Figure 2, C and D. Thus, when B6.OT-II mice were first exposed to antigen under a tolerizing regime, their T cells were not able to mount a proliferative response when subsequently challenged with an immunogenic form of OVA. In contrast, the presence of *Sle3* abrogated effective peripheral tolerance in this experimental model (Figure 2D).

*Sle3 encodes aberrant myeloid-lineage cells*. Given that previous allotype-marked BM transfer experiments (28) and the adoptive transfer studies described above (Figure 2B) indicated that the *Sle3*-associated phenotypes may not be T cell intrinsic, we asked whether quantitative or qualitative differences in Sle3-bearing cells of myeloid origin, including various APC subsets, may be responsible for the phenotypic differences noted above. Spleens, lymph nodes, and peripheral blood from 9- to 12-month-old B6 and B6.6.OT-II mice were examined for the numbers and phenotypes of DCs, macrophages, and neutrophils, by flow cytometry, as illustrated in Figure 3, A and B. As noted in Table 2, myeloid-lineage cells from B6.6.OT-II spleens demonstrated several quantitative and qualitative differences. The most consistent difference was the expanded percentages of macrophages in 9- to 12-month-old B6.6.OT-II spleens, which was noted in multiple experiments (Table 2). Since B6 and B6.6.OT-II mice possessed similar numbers of splenocytes, these differences in percentages also translated to differences in the absolute numbers of these cells. Although splenic neutrophils were also expanded in numbers, these differences fell short of statistical significance. There were no significant differences in the numbers of splenic myeloid DCs, though the numbers of lymphoid DCs and plasmacytoid DCs tended to be relatively higher (Table 2, and data not shown).

In addition, DCs, macrophages, and neutrophils isolated from B6.6.OT-II spleens were evidently more activated/mature, based on the expression of several surface markers (Table 2). Thus, for example, although the myeloid DCs were not expanded in numbers in B6.6.OT-II spleens, they exhibited increased surface levels of CD40, CD80, CD86, CD54, CD106 (VCAM-1), and FcR (CD16/32), with similar differences being noted on B6.6.OT-II lymphoid DCs, macrophages, and neutrophils (Table 2). In particular, the surface levels of CD106 were about twice as high on all 4 cell subsets examined, with the corresponding levels in B6-derived cells. Similar changes were also noted in the lymph nodes and peripheral blood of B6.6.OT-II mice (Table 3, and data not shown). Interestingly,
B6.Sle3 lymph node–derived DCs, macrophages, and neutrophils displayed CD40 and I-Aβ levels that were severalfold higher than the respective expression levels on the B6 controls.

As noted above in the spleens and nodes of older B6.Sle3 mice, one could also discern the heightened activation of different myeloid-lineage cells in young (i.e., 2-month-old) B6.Sle3 mice (Figure 3, C and D). Likewise, macrophages cultured from B6.Sle3 BM (using M-CSF) showed similar phenotypic differences, with these differences becoming amplified following LPS stimulation (Figure 3E). This was particularly pronounced with respect to CD80 expression. B6.Sle3-derived BM-cultured DCs revealed similar phenotypic differences, when the DCs were elicited using GM-CSF (Figure 3F). On the other hand, DCs that were cultivated using GM-CSF plus IL-4 yielded mixed results: B6-derived DCs appeared to be more activated in 2 experiments, the inverse pattern was noted in a third experiment, and no differences were noted in a fourth experiment (data not shown). The reason for this variability with DCs elicited by GM-CSF plus IL-4 is not presently clear.

Sle3 myeloid cells exhibit altered cytokine profiles and impaired apoptosis. B6.Sle3-derived macrophages and DCs also exhibited altered cytokine production profiles, compared with the B6 controls, as displayed in Figure 4. Unmanipulated splenic DCs isolated from B6.Sle3 congenics, as well as BM-derived DCs (cultured using GM-CSF), produced more IL-12 but less IL-6 in culture (Figure 4, A and B). In addition, B6.Sle3 DCs also hypersecreted IL-1β and showed a variable difference in TNF-α production (Figure 4B; and see Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI23049DS1). Although IL-12, IL-6, and TNF-α were elevated in B6.Sle3 macrophage supernatant, IL-1β secretion was variably increased (Figure 4, C–E, and Supplemental Figure 1).

Besides the elevated production of proinflammatory cytokines, B6.Sle3-derived myeloid cells also revealed another interesting phenotypic difference — impaired apoptosis (Figure 5, A and B). This difference was demonstrated for both BM-derived DCs and macrophages (Figure 5, A and B). Stimulation with LPS did not alter this phenotypic difference (data not shown). These findings are in line with our recent observation that B6.Sle3 mice also exhibit reduced apoptosis of neutrophils in vitro and in vivo, in an infection challenge model (B. Mehrad and C. Mohan, manuscript submitted for publication). However, Sle3 did not impact the phagocytic po-

Figure 2
Functional responsiveness of B6.Sle3.OT-II T cells. (A) B6.OT-II and B6.Sle3.OT-II splenocytes were cultured with various stimuli and assessed for proliferation. Stimulation index was expressed as cpmantigen/cpmno-antigen. The cpmno-antigen values ranged from 2,000 to 5,000. Each dot represents an independent spleen sample. Horizontal bars indicate the respective group means. The data shown were reproduced in 2 additional studies (Supplemental Table 1 [supplemental material available online with this article; doi:10.1172/JCI23049DS1]). (B) B6.OT-II splenic T cells were transferred i.v. into 2-month-old B6 or B6.Sle3 mice on D0 and challenged with an immunogenic form of OVA on D1, as described in Methods. Seven days after transfer, the numbers of Tg T cells in the recipient spleens (left) and serum IgG anti-OVA levels (right) were assessed. Horizontal bars indicate group means. The displayed data were pooled from 2 independent studies using 5 mice per strain. (C) B6.OT-II and B6.Sle3.OT-II mice (n = 5 per group) were challenged with OVA323-339 in incomplete Freund’s adjuvant on D0 and assessed for their proliferative response to OVA323-339. The vertical bars represent the SEM of triplicate cultures. Data shown are representative of 2 independent studies. Observed differences were not statistically significant. (D) B6.OT-II and B6.Sle3.OT-II mice (n = 5 mice per group) were challenged first with tolerogenic OVA on D0 and then with immunogenic OVA on D10 and examined as described in Methods. The vertical bars represent the SEM of triplicate cultures. In a second confirmatory study (data not shown), the fold difference in cpm between the 2 strain groups was 1.9 (P < 0.04, n = 4 each), at an OVA stimulation dose of 1,000 nM.
in activation, as gauged by the surface expression of CD69 (Figure 5, also demonstrated reduced apoptosis with a concomitant increase of T cell division and proliferation, in a manner dependent on antigen dose and APC number (Figure 6). Similar results were noted when T cell alloreresponsiveness was assayed (data not shown). Although the difference in thymidine incorporation attained statistical significance, the difference in cell division (as assayed by CFSE dilution) failed to attain significance within individual experiments. In both the antigen-specific response experiments and the alloreresponse studies, the use of high numbers of B6-derived DCs tended to dampen the proliferative response of the responding T cells (Figure 6, F and G, and data not shown); in contrast, Sle3-bearing DCs continued to be hyperstimulatory even when high numbers of DCs were used for costimulation.

These differences were most apparent when B6.OT-II TCR Tg T cells were used for the cocultures; B6.Sle3.OT-II Tg T cells behaved fairly similarly irrespective of whether B6 or B6.Sle3 DCs were used for stimulation (Figure 6, E and H). In all experiments, the extent of cell division of the Tg T cells failed to approximate 100%, possibly because of the expression of non-Tg TCR since these studies were executed on a RAG-sufficient background. Finally, T cells cocultured with B6.Sle3 DCs were demonstrated to produce more IFN-γ, compared with T cells cocultured with B6-derived DCs, in some experiments but not others (data not shown), consistent with the elevated IL-12 production by B6.Sle3 DCs.

Sle3 DCs recapitulate the in vivo phenotypes attributed to Sle3. Given that the Sle3 locus facilitated the generation of APCs that were evidently more mature, proinflammatory, and costimulatory, we next asked whether this cellular phenotype might indeed be responsible for the T cell and serological phenotypes noted in B6.Sle3 mice. Importantly, the adoptive transfer of Sle3-bearing DCs (compared with B6-derived DCs) into young B6 hosts, when coupled with LPS coadministration, led to elevated splenic CD4/CD8 ratios (Figure.

Figure 3
Splenic and BM myeloid cell subsets in B6 and B6.Sle3 mice. (A and B) Representative CD11b/CD11c expression profiles observed in 9- to 12-month-old B6 and B6.Sle3 collagenase-treated spleens. The cells in region R2, also expressing CD8, were gated as lymphoid DCs, whereas the cells in region R3 were gated as myeloid DCs. Cells that were in region R4 were gated as macrophages; these cells also exhibited high side scatter and F4/80 expression. Cells in region R5 were gated as neutrophils, and these cells also exhibited high Gr-1 levels. The prevalence of these cells in the spleens of 9- to 12-month-old B6 and B6.Sle3 mice (n = 10–15 each), and the expression levels of CD40, CD80, CD106 (VCAM-1), and FcR on these 4 gated populations, are detailed and statistically compared in Table 2. (C) The respective percentages of the different myeloid cell populations in 2-month-old B6 and B6.Sle3 spleens. (D–F) Expression levels (mean fluorescence intensities, mfi) of various surface markers on 2-month-old splenic CD11b+*, CD11c++ myeloid DCs (D); on BM M-CSF–cultured macrophages, gated on F4/80- cells (E); and on BM GM-CSF–cultured DCs, gated on CD11c- cells (F). For the latter 2 studies, the cells were phenotyped with or without LPS pretreatment (10 ng/ml, 24 hours). For all studies, each dot represents data derived from an individual mouse. Data are representative of 2–3 independent studies each. Depicted P values were computed by comparison of the B6.Sle3 values with the B6 control values, using the Student’s t test. The horizontal bars indicate the respective group means.

tial of macrophages (B. Mehrad and C. Mohan, manuscript submitted for publication). Interestingly, when Sle3-bearing DCs were OVA-pulsed and cocultured with OT-II TCR Tg T cells, the T cells also demonstrated reduced apoptosis with a concomitant increase in activation, as gauged by the surface expression of CD69 (Figure 5, C and D), compared with T cells cocultured with B6 DCs.

Sle3 APCs are superior at costimulating T cells. In keeping with the above findings, the more activated/mature phenotype of B6.Sle3-
7A) and elevated serum autoantibody levels (Figure 7B), 2 cardinal Sle3-associated phenotypes (22, 23). In these adoptive transfer studies, the maximal autoantibody levels were noted on day 40 (D40), i.e., about 4 weeks after the last administration of DCs (see legend to Figure 7). However, this serological outcome was not sufficient for renal disease to ensue (data not shown). The transfer of DCs alone, without any added LPS, or the administration of LPS alone, was insufficient to elicit the above phenotypes (data not shown). These studies demonstrate that Sle3-bearing DCs appear to be sufficient to recreate the Sle3-associated immunophenotypes.

**Discussion**

Sle3 is an NZM2410/NZW-derived locus on proximal chromosome 7 that facilitates spontaneous T cell hyperactivity and low-grade serological autoreactivity, when introgressed onto the normal B6 genetic background (22, 23). The present report adds to the earlier findings in several respects. Firstly, whereas the earlier report had detailed the phenotypic properties of a 40-cM interval on proximal chromosome 7 encompassing Sle3 and Sle5, the present report focuses on mice that bear a 24-cM interval on mid–chromosome 7, encompassing Sle3 but not Sle5 (Table 1 and Figure 1). Secondly, the T cell repertoire is greatly simplified in the current study, owing to the use of an OVA-specific TCR Tg. Finally, and most importantly, the present study directs attention to the potential importance of aberrant myeloid-lineage cells in contributing to the immunological phenotypes associated with this congenic interval. Importantly, the adoptive transfer experiments indicate that the genetic makeup of the APC has the potential to influence the degree of T cell hyperactivity as well as serological autoreactivity, in lupus pathogenesis.

The current findings fortify the conclusions of several previous reports implicating the potential role of enhanced and/or hyperactive APCs in driving autoimmunity. Although the most cited example of elevated mononcytosis in murine lupus is the BXSB strain (39–42), additional forward genetic studies have generated several “engineered” models of disease where systemic autoimmunity and myeloid hyperactivity both co-dominate the clinical phenotype. Thus, for example, Shp-1, lyn CD200/OX2, rel-B, and NfκB2 mice all have the potential to influence both autoimmunity and the myeloid cell compartment (43–47). Although Sle3-bearing APCs resemble APCs from mouse strains exhibiting aberrant expression of the above molecules, Sle3 does not represent an allelic variant of any of the above genes, as they are located on different chromosomes.

The notion that aberrant APCs can breach T cell tolerance is well accepted (48). The underlying mechanism(s) that may explain why Sle3-bearing APCs may be more potent at stimulating T cells in vitro, or in breaching tolerance in vivo, is an important issue to investigate. On the one hand, Sle3-bearing DCs (and other APCs) may be more “costimulatory,” because of their increased surface expression of CD80/B7-1, FcR, CD40, etc. The observation that blockade of B7-CD28 and CD40:CD40L interactions has the potential to ameliorate murine lupus lends support to this thesis (49–51). In addition, their heightened secretion of proinflammatory cytokines may further augment the costimulatory potential of Sle3 APCs. The notion that a proinflammatory milieu could promote the rapid maturation of DCs and impair their T cell–tolerizing potential is well documented (52–55). Clearly, both of the above mechanisms (i.e., increased costimulation and proinflammatory properties) may be synergistic in breaching T cell tolerance, as has been expounded by others (55, 56). Although one might have anticipated a phenotypic difference when Sle3-bearing DCs were adoptively transferred into a B6 host, even without LPS coadministration, this was not the case. Apparently, the coadministration of LPS was essential to uncover the autoimmune potential of B6.Sle3 DCs. It is conceivable that LPS stimulation of the transferred B6.Sle3 DCs may be required for maximally modulating their activation status, cytokine profile, or survival advantage in vivo, drawing from the in vitro observations presented in Figures 3–5.

Based on the in vitro studies in Figure 5, we postulate that the more mature and proinflammatory nature of B6.Sle3 DCs may have conferred a stronger proliferative edge and a better survival advantage to potentially autoreactive CD4 T cells. This could also

### Table 2

Phenotype of DCs, macrophages, and neutrophils in B6 and B6.Sle3 spleens

<table>
<thead>
<tr>
<th></th>
<th>B6</th>
<th>B6.Sle3</th>
<th>P value ^a</th>
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<tr>
<td><strong>Lymphoid DCs</strong></td>
<td></td>
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<tr>
<td>% in spleen ^b</td>
<td>1.8 ± 0.3</td>
<td>2.4 ± 0.5</td>
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<tr>
<td>CD40 mfi ^c</td>
<td>291 ± 57</td>
<td>395 ± 39</td>
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<tr>
<td>CD106 mfi ^c</td>
<td>628 ± 93</td>
<td>1,010 ± 173</td>
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<td>CD80/B7-1 mfi ^c</td>
<td>724 ± 33</td>
<td>1,073 ± 103</td>
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<td>CD54 mfi ^d</td>
<td>451 ± 12</td>
<td>505 ± 23</td>
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<tr>
<td>FcR mfi ^d</td>
<td>743 ± 67</td>
<td>942 ± 67</td>
<td>0.005</td>
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<tr>
<td><strong>Myeloid DCs</strong></td>
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<tr>
<td>% in spleen ^b</td>
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<td>2.7 ± 0.5</td>
<td>NS</td>
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<tr>
<td>CD40 mfi ^c</td>
<td>204 ± 22</td>
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<td>CD106 mfi ^c</td>
<td>455 ± 89</td>
<td>936 ± 249</td>
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<tr>
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<td>CD54 mfi ^d</td>
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<td>339 ± 35</td>
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<td>FcR mfi ^d</td>
<td>525 ± 56</td>
<td>831 ± 104</td>
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<td><strong>Macrophages</strong></td>
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<tr>
<td>% in spleen ^b</td>
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<td>CD40 mfi ^c</td>
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<td>CD80/B7-1 mfi ^c</td>
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<td><strong>Neutrophils</strong></td>
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<td>% in spleen ^b</td>
<td>3.8 ± 1.2</td>
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<td>CD40 mfi ^c</td>
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<tr>
<td>FcR mfi ^d</td>
<td>377 ± 37</td>
<td>449 ± 47</td>
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^a Values pertain to Student’s t test statistical comparisons of B6 versus B6.Sle3 cells isolated from collagenase- and DNase I–treated spleens of 9- to 12-month-old mice. Only surface markers that were differentially expressed on the respective cell populations are listed. ^b Data represent mean ± SEM pertaining to 15 B6 samples and 15 B6.Sle3 samples. Lymphoid DCs were CD11c++ and CD8−, but CD11b− (CD11b−); Myeloid DCs were CD11c++ and CD11b−, but CD11c−, and Gr-1−. Macrophages were F4/80++, CD11b−, and CD11c−. Neutrophils were Gr-1− and CD11b−; but CD11c−, and F4/80−. CData were obtained by examination of 10–15 mice per strain and are representative of at least 2 independent FACS-staining experiments involving 5 B6 mice and 5 B6.Sle3 mice each. Depicted staining intensities were significantly higher than the isotype-control staining, as detailed in Methods. ^c Data pertain to a single FACS-staining experiment involving 5 B6 mice and 5 B6.Sle3 mice but were not reproduced in a second FACS-staining experiment. mfi, mean fluorescence intensity.
account for the increased CD4/CD8 ratios noted in unmanipulated B6.Sle3 mice (23), and in the adoptive transfer experiments (Figure 7). Autoreactive members among the expanded pool of CD4 T cells may in turn have been responsible for the increased serum ANAs observed, both in unmanipulated B6.Sle3 congenics (22, 23) and in the adoptive transfer studies (Figure 7), through T-dependent B cell help. However, a direct impact of Sle3-bearing myeloid cells on B cell function cannot yet be excluded. In addition to their potential impact on systemic T and B cells, it is conceivable that the hyperactivated B6.Sle3 myeloid cells may also be responsible for the enhanced susceptibility to renal disease seen in B6.Sle3 congenics (Y. Fu and C. Mohan, unpublished observations). The notion that neutrophils and macrophages play an essential role in immune-mediated renal disease is well accepted (57, 58), and these cells do appear to play a more prominent role in the renal disease seen in B6.Sle3 mice (Y. Fu and C. Mohan, unpublished observations).

Collectively, it appears likely that the hyperactivated myeloid compartment in B6.Sle3 may be contributing to lupus through multiple mechanisms, both systemically and locally in the end organs. Although the expression of Sle3 within the myeloid cell compartment of these congenics appears to be sufficient to elicit the previously described Sle3-associated phenotypes, one cannot exclude the possibility that Sle3 may also be impacting additional cell types, and these additional cellular mechanisms may also be contributing to lupus pathogenesis. Specifically, one cannot exclude the possibility that T cell–intrinsic expression of Sle3 may be contributing at least in part to the “T cell hyperresponsiveness” noted in these mice. Although the T:DC coculture studies portrayed in Figure 6E might support this thesis, it is clearly possible that T cells isolated from B6.Sle3 congenics may appear to be hyperresponsive simply because they might have already been “primed” by Sle3-bearing APCs in vivo.

The chromosome 7 interval encompassing Sle3 has also been implicated in genetic analyses of other lupus models (18–21, 59). Presently, it is not clear whether the different loci uncovered in the different mouse models represent allelic variants of the same culprit gene(s). Given the repeated mapping of this chromosomal interval in several independent murine lupus studies and models, and the intriguing phenotypes associated with this locus, elucidating the candidate genes within this locus is of paramount importance. Since the phenotypes exhibited by the B6.Sle3-derived macrophages, DCs, neutrophils, and T cells are fundamentally similar (i.e., reduced apoptosis with a concomitant increase in activation status), we hypothesize that a single genetic defect may be responsible for all of these cellular phenotypes. On the other hand, since the studied congenic interval is fairly large, it is certainly possible that 2 or more genes within the disease interval may be contributing to the observed cellular phenotypes. Among the 560 genes/open reading frames located within the Sle3 congenic interval, 1 gene that had potential significance to APC biology was FL (Flt3 ligand). However, sequencing and expression studies of the FL gene in B6 and B6.Sle3 mice have failed to substantiate FL as the culprit gene for Sle3. Progressive narrowing of the recombinant congenic interval and additional candidate gene testing are in progress.

Given that Sle3 is a major disease susceptibility locus in the NZM2410 lupus model, one may infer that the “T cell hyperactivity” in NZM2410 lupus mice may be contingent upon intrinsic APC anomalies, to a significant degree. Lupus T cells have also been phenotyped as being hyperactive in other murine models, as reviewed (1, 2). Based on the present findings, it becomes important to evaluate the extent to which more exuberant APC function may also underlie the T cell hyperactivity noted in other lupus models. Finally, T cell hyperactivity, including several biochemical abnormalities that correlate with T cell hyperresponsiveness, and aberrant DC function have also been documented in human lupus (1, 60–67). The current findings raise the possibility that the T cell phenotypes noted in human SLE may also be influenced by intrinsic differences in DC/myeloid cell function, in addition to possible T-cell–intrinsic anomalies (65–67). Focusing future research efforts on the myeloid compartment may not only shed light on the pathogenic origins of lupus; it may also pave the way for novel therapeutic approaches.

### Methods

**Mice**. B6 and B6.J-Abm12 (used as a source of allogenic T cells) mice were obtained from the Jackson Laboratory, and subsequently bred in our animal colony. B6.Sle3 (i.e., B6.Sle3NZM2410/NZM2410) mice are B6 mice homozygous for a 24-cM congenic interval (D7mit158–D7mit40) encompassing the NZM2410 allele of Sle3. Previous published reports had used a larger chromosome 7 interval encompassing Sle3 (and including Sle5), whereas the present study uses a recombinant offspring that includes Sle3 but excludes Sle5, as illustrated in Table 1. This novel strain retains the cellular phenotypes (i.e.,

### Table 3

<table>
<thead>
<tr>
<th>Phenotype of DCs, macrophages, and neutrophils in B6 and B6.Sle3 lymph nodes</th>
<th>B6</th>
<th>B6.Sle3</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lymphoid DCs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% in lymph node</td>
<td>3.3 ± 0.6</td>
<td>3.2 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>CD40 mfi</td>
<td>1,129 ± 151</td>
<td>1,740 ± 283</td>
<td>0.001</td>
</tr>
<tr>
<td>I-A^d mfi</td>
<td>864 ± 55</td>
<td>1,560 ± 226</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Myeloid DCs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% in lymph node</td>
<td>4.2 ± 2.0</td>
<td>5.2 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>CD40 mfi</td>
<td>720 ± 150</td>
<td>1,325 ± 350</td>
<td>0.006</td>
</tr>
<tr>
<td>CD54 mfi</td>
<td>598 ± 22</td>
<td>734 ± 30</td>
<td>0.008</td>
</tr>
<tr>
<td>FcR mfi</td>
<td>795 ± 156</td>
<td>1,153 ± 241</td>
<td>0.050</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% in lymph node</td>
<td>6.2 ± 1.5</td>
<td>9.7 ± 2.2</td>
<td>NS</td>
</tr>
<tr>
<td>CD40 mfi</td>
<td>382 ± 112</td>
<td>909 ± 311</td>
<td>0.023</td>
</tr>
<tr>
<td>I-A^d mfi</td>
<td>247 ± 55</td>
<td>525 ± 145</td>
<td>0.010</td>
</tr>
<tr>
<td>FcR mfi</td>
<td>102 ± 21</td>
<td>192 ± 26</td>
<td>0.032</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% in lymph node</td>
<td>1.6 ± 0.5</td>
<td>3.2 ± 0.9</td>
<td>0.007</td>
</tr>
<tr>
<td>CD40 mfi</td>
<td>124 ± 32</td>
<td>1,335 ± 3,829</td>
<td>0.034</td>
</tr>
<tr>
<td>CD106 mfi</td>
<td>411 ± 59</td>
<td>1,091 ± 116</td>
<td>0.0002</td>
</tr>
<tr>
<td>I-A^d mfi</td>
<td>182 ± 76</td>
<td>404 ± 62</td>
<td>0.002</td>
</tr>
<tr>
<td>CD80/CD11b mfi</td>
<td>378 ± 88</td>
<td>742 ± 57</td>
<td>0.008</td>
</tr>
<tr>
<td>CD54 mfi</td>
<td>113 ± 16</td>
<td>204 ± 58</td>
<td>0.008</td>
</tr>
<tr>
<td>FcR mfi</td>
<td>459 ± 58</td>
<td>772 ± 118</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*P values pertain to Student’s t test statistical comparisons of B6 cells with B6.Sle3 cells isolated from the inguinal lymph nodes of 9- to 12-month-old mice. Only surface markers that were differentially expressed on the respective cell populations are listed. *Data represent mean ± SEM pertaining to 15 B6 samples and 15 B6.Sle3 samples. Lymphoid DCs were CD11c^int^ and CD11b^+^, but CD11b^+^, Myeloid DCs were CD11c^+^ and CD11b^+^ but CD11b^+^, and Gr-1^+^ Neutrophils were Flt3 ligand^+^ and CD11b^+^, but CD11c^+^ and F4/80^+^ Neutrophils were Flt3 ligand^+^ and CD11b^+^, but CD11c^+^ and F4/80^+^ Data were obtained by examination of 10–15 mice per strain and are representative of at least 2 independent FACs-staining experiments involving 5 B6 mice and 5 B6.Sle3 mice each. *Data pertain to a single FACs-staining experiment involving 5 B6 mice and 5 B6.Sle3 mice but were not reproduced in a second FACs-staining experiment.**
B6.OT-II mice are B6 mice bearing an I-A<sup>b</sup>-restricted, OVA-specific TCR Tg (29), obtained as a kind gift from William Heath (Walter and Eliza Hall Institute, Parkville, Victoria, Australia). B6.OT-II mice and B6.<sup>Sle3</sup> mice were interbred over 2 generations to obtain mice that were homozygous for <sup>Sle3</sup>, and positive for the OT-II TCR Tg; these mice are referred to as B6.<sup>Sle3</sup>.OT-II. The presence of the <sup>Sle3</sup> interval was ascertained by PCR using the microsatellite markers D7mit158, D7mit145, D7mit31, and D7mit40. The presence of the TCR Tg was screened by PCR using a TCR V<sub>β</sub>5-specific primer, and confirmed by FACS, using Abs to TCR V<sub>β</sub>5 and V<sub>α</sub>2. All mice used in this study were bred and housed in a specific pathogen–free colony, and these studies were approved.

Figure 4
Cytokine secretion profiles of B6.<sup>Sle3</sup> DCs and macrophages. (A and B) CD11c magnetic bead–purified splenic DCs from 2- to 3-month-old B6 and B6.<sup>Sle3</sup> mice (n = 4 per group; A), as well as CD11c magnetic bead–purified DCs cultured from B6 or B6.<sup>Sle3</sup> BM (using GM-CSF alone, for 7 days; n = 8–9 mice per group; B), were incubated for 24 hours, with or without 10 ng/ml LPS. Depicted are the IL-12 (p70), IL-6, and IL-1β levels in 24-hour culture supernatant. Each dot represents data derived from splenic or BM DCs of an individual mouse; where the positions of the dots overlap, this has been represented using single dots only, for clarity. Similar findings were noted in a second confirmatory study (Supplemental Figure 1A). (C–E) In addition, 24-hour IL-12, IL-6, and TNF-α secretion by B6- or B6.<sup>Sle3</sup>-derived BM-cultured macrophages in response to LPS was assessed by ELISA. Each dot represents data derived from the BM macrophages of an individual mouse. The horizontal bars represent group means. The data shown were reproduced in 2–3 additional experiments (Supplemental Figure 1B). The depicted P values in A–E were computed by comparison of the B6.<sup>Sle3</sup> values with the B6 control values, using the Student’s t test.

Figure 5
Impaired apoptosis in B6.<sup>Sle3</sup> macrophages and DCs. (A and B) DCs and macrophages were cultured from the BM of B6 and B6.<sup>Sle3</sup> mice, and incubated without any stimuli for 24 hours. Following culture, the extent of apoptosis was gauged by flow cytometry using annexin V and 7-amino-actinomycin D (7-AAD), after gating on CD11c-expressing DCs or F4/80<sup>+</sup> macrophages. Each group and bar represent data gleaned from 6–7 individual mice from each strain. P values were computed by comparison of the B6.<sup>Sle3</sup> values with the B6 control values, using the Student’s t test. The shown horizontal bars indicate the respective group means. (C and D) In addition, DCs cultured from 2-month-old B6 or B6.<sup>Sle3</sup> BM were pulsed with 1,000 nM OVA<sub>323–339</sub>, washed, and cocultured (10,000 per well) with OVA-specific B6.OT-II TCR Tg T cells for varying times, as indicated. After culture, the fraction of T cells that were apoptotic (based on expression of annexin V; C) and the mean fluorescence intensity (mfi) of CD69 on the Tg T cells (D) were assessed by flow cytometry. The respective B6 and B6.<sup>Sle3</sup> values were compared using the Student’s t test (*P < 0.05; **P < 0.001).
by the University of Texas Southwestern Medical Center institutional review board. Both male and female mice were used in equal numbers, since no sex differences in phenotype were observed.

Cell isolation and coculture studies. Spleens and inguinal lymph nodes were cut into small fragments, and then digested with collagenase D (1 mg/ml; Sigma-Aldrich) and DNase I (0.02 mg/ml; Roche Diagnostics Corp.) in RPMI 1640 medium supplemented with 5% FCS, for 20 minutes at 37°C. Digested fragments were washed twice in PBS/2% FCS/5 mM EDTA, and single-cell suspensions were made. For the functional studies, splenic T cells were purified using anti-CD4 magnetic beads (Miltenyi Biotec) and were more than 95% pure.

For the T cell proliferation assays, whole splenocytes or splenic T cells were stimulated with the following I-A<sup>b</sup>-binding peptides:

Figure 6
B6.Sle3 DCs are superior APCs for T cell stimulation. (A–D) DCs cultured from 2-month-old B6 (A and C) or B6.Sle3 (B and D) BM, using GM-CSF plus IL-4 for 7 days, were pulsed with OVA<sub>323–339</sub>, and cocultured with CFSE-labeled, OVA-specific B6.OT-II TCR Tg T cells (A and B) or non-Tg T cells (C and D), and the fraction of T cells that had undergone cell division was assessed by flow cytometry. The histograms pertain to coculture studies that were performed with 10,000 OVA-pulsed DCs, and examined by flow cytometry 96 hours after stimulation. (E) Similar results were obtained using OVA-pulsed CD11c bead–purified splenic DCs from B6 and B6.Sle3 mice cocultured with B6.OT-II or B6.Sle3.OT-II T cells for 96 hours; however, any apparent differences noted failed to attain statistical significance. The data depicted in E are representative of 3 independent experiments. In addition, OVA-specific B6.OT-II TCR Tg T cells were cocultured with varying numbers of unpulsed B6 (white dots) or B6.Sle3 (black dots) BM-cultured DCs and 1,000 nM OVA<sub>323–339</sub> (F), or with 50,000 unpulsed BM-derived DCs and varying doses of OVA<sub>323–339</sub> (G). In both experiments, proliferation was assessed 96 hours after culture, by assaying of <sup>3</sup>H-thymidine incorporation. The data portrayed in F and G are representative of 3 independent experiments; an additional experiment is displayed in H, where the proliferation of B6.OT-II and B6.Sle3.OT-II T cells in response to varying numbers of OVA-pulsed B6 or B6.Sle3 splenic CD11c bead–purified DCs was assessed. Shown P values were computed by comparison of the B6.Sle3 values with the B6 control values, using the Student’s t test.

Figure 7
Adoptively transferred B6.Sle3 DCs recreate Sle3-associated phenotypes. DCs cultured from B6 or B6.Sle3 BM (16–18 mice per strain, pooled from 4 independent experiments) were adoptively transferred into 2-month-old B6 mice on D0, D7, and D14, with coadministration of LPS on D1, D8, and D15. Plotted in A are the terminal splenic CD4/CD8 ratios in the host mice on D20 or D60; since the outcomes were similar at both time points, the results from D20 and D60 experiments have been pooled. Plotted in B are the serum IgG anti–single-stranded DNA Abs (left) and IgG anti–double-stranded DNA Abs (right) at serial time points during the 60-day study period, after the transfer of B6 or B6.Sle3 DCs, with coadministered LPS. All P values shown were computed by comparison of the B6.Sle3 values with the B6 control values, using the Student’s t test. The horizontal bars indicate the respective group means. The data shown were reproduced in 2 additional studies, as portrayed in Supplemental Figure 2.
I-EK37.93 (ASFEAQGALANIAVDKA), histone H476-90 (AKRKTVTAMDV- 
VYAL), and OVA323-339 (ISQVHAAHAINEAG), synthesized at the Uni-
versity of Texas Southwestern protein core facility. For the T:APC coculture 
studies, splenic T cells (5 × 10^6/ml) purified with anti-CD4 beads, and 
splenic or BM-derived DCs (5 × 10^6/ml, or as indicated) irradiated with 30 
Gy and pulsed with OVA (1,000 nM peptide, unless otherwise indicated), 
were cocultured for 24–108 hours, and the T cell response was ascertained 
by measurement of thymidine incorporation, as described previously (23). 
In addition, IFN-γ and IL-4 production was assessed using commercially 
available kits (R&D Systems). In a variation of this assay, the T:DC coculture 
assay was performed using splenic T cells that were labeled with CFSE 
(Invitrogen Corp.), and the degree of cell division was gauged from the serial 
dimination of the CFSE label, as determined by flow cytometry.

Flow cytometric analysis and Abs. Flow cytometric analysis was performed as 
described previously (23, 25, 27). Briefly, cells were first blocked with staining 
medium (PBS, 5% horse serum, 0.05% azide) containing 10% normal rabbit 
serum. Cells were then stained on ice with optimal amounts of FITC-, PE-, 
or biotin-conjugated primary Abs diluted in staining medium for 30 minutes. 
The following dye- or biotin-coupled Abs were obtained from BD Biosciences— 
PharMingen, or CALTAG Laboratories, and were used at pretitrated dilutions: 
CD11b (M1/70), CD11c (HL3), Gr-1 (RB6-8C5), F4/80 (RM2905), Vβ5 
(MR9-4), Vε2 (B20.1), CD40 (3/23), CD54/ICAM-1 (3E2), CD106/VCAAM-1 
(429A), CD16/32 (2.4G2), CD4 (RM4-5), CD5 (53-7.3), CD8 (Ly-2), CD23 
(B3B4), CD25 (7D4), CD43 (S7), CD62L (MEL14), CD69 (H1.2F3), CD80/B7-1 
(16-10A1), and CD86/B7-2 (GL1). Whereas the Abs to CD11b, CD11c, Gr-1, 
CD4, F4/80, and I-A^d were used to gauge the activation/maturation sta-

tus of these cells. Cell staining was analyzed using a FACScan (BD). For the 
data depicted in Tables 2 and 3, and in Figure 3, all isotype-control staining 
were cultured with M-CSF, or IL-4 plus GM-CSF, or GM-CSF alone (10 ng/
liter) purified with anti-CD4 beads, and as indicated. For the in vitro assays, 
BM-derived macrophages or DCs were cultured at 10^6 cells/ml, and 
stimulated with the indicated doses of LPS. In vitro IL-1β, IL-6, TNF-α, and 
IL-12 p70 production was measured using commercial ELISA kits (R&D Sys-
tems). For some experiments, CD11c+ DCs were purified using CD11c mag-
netic beads (either from freshly isolated spleens or from GM-CSF-driven BM 
cultures), following the manufacturer’s instructions (Miltenyi Biotec), before 
use in functional studies. For gauging the degree of apoptosis, BM-cultured 
 macrophages or DCs were cultured with or without LPS (10 ng/ml) for the 
included time periods, after which the cells were stained with annexin V 
(BD Biosciences—PharMingen) and 7-amino-actinomycin D (7-AAD; EMD 
Biosciences) and examined by flow cytometry.

In vivo studies. For the in vivo antigen response experiments, 10 × 10^6 
B6.OT-II splenic TCR Tg T cells were transferred i.v. into 2-month-old 
B6 or B6.Sle3 mice on D0, challenged with OVA (100 µg i.p.) in complete 
Freund’s adjuvant on D1, and examined for serological and splenic cellular 
phenotypes on D7. For the peripheral tolerance studies, 2 sets of experi-
ments were conducted. In 1 experiment, OVA was administered i.p. (100 
µg/mouse) in incomplete Freund’s adjuvant (Figure 2C). A second panel of 
mice were injected with OVA in incomplete Freund’s adjuvant on D0 (100 
µg/mouse), and subsequently with OVA in complete Freund’s adjuvant on 
D10 (100 µg/mouse) (Figure 2D). With both sets of mice, the prolifera-
tive response of splenic T cells to OVA was assessed 5 days after the 
latter injection of OVA. For the adoptive transfer experiments, BM-derived DCs 
cultured using IL-4 plus GM-CSF, as detailed above) from B6 or B6.Sle3 
tissues were infected i.v. into 2-month-old B6 or B6.OT-II recipients (3 
injectons of 10^7 cells per mouse, spaced at weekly intervals). One day after 
each DC transfer, the recipient mice were administered LPS (30 µg i.p.). At the 
ilicated times after transfer, serum autoantibodies were assayed by 
ELISA, and the phenotype of splenocytes was studied by flow cytometry.

Statistics. Where the samples studied were normally distributed, statisti-
cal comparisons were performed using the Student’s t test; otherwise, 
the Mann-Whitney U test was used. These tests were executed using Excel 
(Microsoft Corp.) or SigmaStat (Jandel Scientific).

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