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Defective B cell response to T-dependent immunization in lupus-prone mice

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Abstract

Lupus anti-nuclear Abs show the characteristics of Ag-driven T cell-dependent (TD) humoral responses. If autoAgs elicit the same response as exogenous Ags, lupus should enhance humoral responses to immunization. Blunted responses to various immunizations have, however, been reported in a significant portion of lupus patients. In this study, we show that lupus-prone B6.*Sle1.Sle2.Sle3* (B6.TC) mice produce significantly less Ab in response to TD immunization than congenic controls, while producing significantly more total Ig. This blunted Ab response to TD Ag could be reconstituted with B6.TC B and CD4⁺ T cells. Multiple defects were found in the B6.TC response to NP-KLH as compared to total Ig, including a smaller percentage of B cells participating to the NP-response, a reduced entry into germinal centers, and highly defective production of NP-specific long-lived plasma cells in the bone marrow. B6.TC plasma cells expressed reduced levels of FcγRIIb, which suggests that reduced apoptosis in resident plasma cells prevents the establishment of newly-formed NP-specific plasma cells in bone marrow niches. Overall, these results show that lupus-prone mice responded differently to auto- and exogenous antigens and suggest that low FcγRIIb, hypergammaglobulinemia and high autoantibody production would be predictive of a poor response to immunization in lupus patients.

1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of large amounts of anti-nuclear autoAbs. Numerous studies have shown that both in humans [1] and in murine models [2;3], many of these autoAbs are class-switched and have accumulated somatic hypermutations, which are characteristic of Ag-driven T cell dependent (TD) humoral responses. The kinetics and isotype distribution have suggested that the development of anti-chromatin Abs mimics a normal TD response through autoimmunization [4]. However, this hypothesis has never been formally tested. AM14 transgene-encoded rheumatoid factor autoAbs develop from extra-follicular foci, in spite of the existence of functional germinal centers (GCs) [5;6], suggesting that Abs induced by self and foreign Ags utilize different pathways.

To address whether autoAbs and Abs directed against exogenous TD Ags develop from the same pathways, we have directly compared the TD response between a lupus-prone B6.*Sle1.Sle2.Sle3* (B6.TC), a strain that carries *Sle1*, *Sle2*, and *Sle3*, the three NZM2410 lupus susceptibility loci that are necessary for disease development [7], and its congenic

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control C57BL/6 (B6). NZM2410 and B6.TC mice produce large amounts of class-switched nephrogenic anti-dsDNA Ab [8;9]. If the production of autoAbs mimics normal TD responses, B6.TC mice should display an enhanced humoral response to TD Ags. Here we show that B6.TC mice produce significantly less Ab in response to TD immunization, while producing more total Ig and anti-dsDNA Abs than controls. This reduced humoral response involves multiple factors starting with a diminished production of TD-Ag specific B cells and a reduced entry in GCs, and culminating with the failure to produce long-lived TD-Ag specific plasma cells (PCs) in the bone marrow (BM). This contrasted with the B cells that were not specific for the immunogen, which entered GCs and produced long-live BM PC. These results demonstrate that different pathways are used by foreign and self-Ags in lupus prone mice, and may have implications for the vaccination outcomes in lupus patients.

2 Results

2.1 Lupus-prone mice produced low amounts of Ab to TD immunization

Following primary immunization with NP-KLH in alum, serum anti-NP Abs, total IgG, IgG1 (Fig. 1A left), IgG2a, IgG2b and IgG3 (data not shown), were significantly lower in B6.TC than in control B6 mice during the 25 wks observation period. Anti-NP Abs were not found in pre-immune B6.TC mice at that age. The temporal pattern of anti-NP Ab production was the same between the two strains. However, only B6 mice maintained substantial levels of anti-NP Abs 25 wks post-immunization. The lower TD-response from B6.TC mice was not restricted to NP, as the same results were obtained with anti-KLH (Fig. 1C) and anti-TNP in TNP-CGG immunized mice (Fig. 1A middle). As previously reported [10], total IgG and IgG isotypes were found in significantly higher amounts in B6.TC mice (Fig. 1A right). Although hypergammaglobulinemia predates specific autoAb production in lupus mice, anti-dsDNA IgG was detected in B6.TC mice as early as 11 wks old and greatly increased in mice older than 20 wks of age (Fig. 1D). Anti-dsDNA IgG levels was no significantly different between immunized and non-immunized B6.TC controls (data not shown). The level of anti-NP IgM was also significantly decreased in B6.TC as compared to B6 mice one wk after immunization, and the time required for peak production of anti-NP IgM Ab was delayed until 2 wks after immunization, while total serum IgM was higher in B6.TC than in B6 mice (Fig. 1B). The secondary response to TD-Ags was also defective in B6.TC mice but the difference reached statistical significance only for anti-NP IgG and IgG2a (Fig. 1E). Since the differences between B6 and B6.TC were greater during the primary response, the rest of the paper will show data obtained after primary immunization.

B6.TC mice immunized with TNP-LPS produced titers of anti-TNP Abs similar to those seen in B6 mice, while B6.TC mice immunized with NP-AECM-Ficoll produced significantly more anti-NP IgG Abs than B6 controls (Supplemental Fig. 1). Overall these results show that B6.TC mice produced a significantly reduced amount of Ab in response to TD immunization while producing significantly more total Ig, more Abs to T-independent immunization and more autoAbs.

2.2 Either B6.TC B or T cells are sufficient for a lower Ab response to TD Ag

To address which cellular compartments were responsible for this defect, we transferred individual cell populations from either B6 or B6.TC into B6.Rag mice before immunization. Chimeric mice that received total B6.TC splenocytes showed a significantly impaired anti-NP IgG, IgG1, or IgM (Fig. 2) production as we have reported in intact mice, while total Ig was not different from controls. More interestingly, a combination of purified CD43⁻ B cells and CD4⁺ T cells also reproduced the results obtained with intact animals. Finally, mixing CD43⁻ B cells and CD4⁺ T cells from the two strains indicated that either B6.TC B or T cells were sufficient to reproduce the lower anti-NP humoral response of B6.TC mice. The

combinations of either B6 B cells and B6.TC T cells or B6 T cells and B6.TC B cells resulted in significantly lower amounts of anti-NP IgG, IgG1 or IgM (Fig. 2) than the combination of B6 B and T cells. This clearly showed that the B6.TC CD4⁺ T cell help was sufficient to curtail the anti-NP response from normal B6 B cells, while B6.TC B cells were defective in responding to NP immunization even in the presence of normal CD4⁺ T cell help.

2.3 B6.TC mice produced a reduced B cell response to exogenous TD Ag

The great majority of NP⁺ B cells express the λ_1 light chain [11], which was used as a surrogate marker for NP-specific B cells. The percentage of λ_1 ⁺ or NP-binding B cells or IgM⁺ B cells was significantly decreased in B6.TC spleens as compared to B6 (Fig. 3A). However, the absolute number of λ_1 ⁺ or NP-binding B cells was not different between the two strains (supplemental Fig. 2A and B), since the total number of B cells is significantly higher in B6.TC mice [12]. Interestingly, a significantly lower percentage of proliferating B cells (Fig. 3B) and B cells expressing activated caspase 3 (Fig. 3C) were λ_1 ⁺ or NP-binding in B6.TC than in B6 mice. These results indicate a less active anti-NP B cell response in B6.TC mice than in B6 as compared to the non-NP specific B cell population. It should be noted that in all cellular time-course experiments, each time-point represents a different group of mice, and therefore constitutes an independent replication of the results.

As for total B cells, the percentage of GC GL7⁺ NP-specific B cells or GL7⁺ B cells that were NP⁺ IgM⁺ were decreased in B6.TC spleens 4 wks (Fig. 4A and B) or 1 wk (data not shown) post-immunization, while there was no difference for NP⁻ GL7⁺ B cells. The number of NP⁺ GL7⁺ B cells was however equivalent in both strains due to the elevated lymphocyte number in B6.TC mice (supplemental Fig. 2C and D). The reduced participation of NP-specific B cells in B6.TC GCs was further shown by a significantly reduced affinity of the anti-NP IgG and IgG1 (Fig. 4C). Immunofluorescence staining confirmed the flow cytometry findings. Indeed, large numbers of λ_1 ⁺ IgM⁺ and B220⁺ B cells were found in the B cell zone one wk post-immunization in B6 mice, with foci gathered near and in the GCs (Fig. 5A and B). In contrast, λ_1 ⁺ cells were fewer and scattered all across the B6.TC follicles. Furthermore, the primary extrafollicular foci that are typical of the early TD response were not seen in B6.TC (Fig. 5C). Attempts to perform the same experiment with NP-PE were unsuccessful due to a high background to signal ratio.

2.4 B6.TC mice produce defective NP-specific plasma cells in the BM

The percentage of CD138⁺ splenocytes that were NP-specific B220^{lo/-} plasma cells (PC) was significantly lower in B6.TC spleens (Fig. 6A). As previously reported [13] and seen on the Fig. 6A FACS plots, there was a large accumulation of CD138⁺ cells in B6.TC spleens. Therefore, the total number of NP⁺ CD138⁺ cells was much higher in B6.TC than in B6 spleens (Fig. 6A). Similar results were obtained with PC stained for intracellular λ_1 , from which the NP-specific PC are a subset (Supplemental Fig. 3A). In contrast, B6.TC BM contained significantly fewer NP⁺ PC, assessed either as the percentage of total CD138⁺ cells or the absolute number of NP⁺ B220^{lo/-} CD138⁺ BM cells (Fig. 6B). The percentage of total BM CD138⁺ cells was however equivalent between immunized B6.TC and B6 mice (data not shown). We did not find a difference either with intracellular λ_1 ⁺ PC (Supplemental Fig. 3B), which may be due to the greater propensity of B6.TC λ B cells to expand and differentiate (Morel unpublished). The expression of *Xbp-1*, which is necessary for PC to secrete Abs [14], and to a lesser extent, *Prdm-1*, which encodes for the plasma cell master regulator Blimp-1 [15], mirrored the flow cytometry and ELISPOT results (Supplemental Fig. 4). This indicated that the expression of these two genes was not defective in B6.TC mice. Furthermore, the expression of surface markers on NP⁺ CD138⁺ cells was similar between the two strains both in the spleen and the BM, including IgM,

LFA-1, CXCR4 and CD44 which were differentially expressed between total B6 and B6.TC CD138⁺ cells (Supplemental Fig. 5). Therefore, differential expression of known PC surface markers cannot explain the defective NP response in B6.TC mice.

FcγRIIb has been recently reported to play an essential role in PC apoptosis and turnover [16]. The NZW allele of *Fcgr2b* carried by B6.TC mice is expressed at lower levels than the B6 allele on GC B cells and PC [17]. FcγRIIb was expressed at significantly lower levels on both NP-specific and total PC in B6.TC spleen and BM 4 wks after immunization (Fig. 6C). Interestingly, FcγRIIb was expressed at much higher levels on the NP-specific PC in both strains than on total PC, which is probably related to the fact that on average, the NP-specific PC are of more recent origin than the total PC. The same results were obtained one wk after immunization, although the number of NP-specific PC in the BM was much lower (data not shown). These results suggest that the B6.TC NP-specific PC are, as the total PC population, defective in immune-complex induced apoptosis that is mediated through FcγRIIb.

The numbers of NP-specific and total IgG Ab secreting cells (ASC) were significantly increased in the B6.TC spleens as compared to B6 (Fig. 7A), confirming the flow cytometric analysis (Fig 6A and B). The number of BM NP-specific ASC was not different between the 2 strains (Fig. 7A), but both NP⁺ and NP⁻ ASC BM/spleen ratios were significantly lower in B6.TC mice (data not shown). These results indicate that NP-specific and total PC accumulated in the B6.TC spleen relative to the BM. It is therefore unlikely that the PC splenic accumulation accounts for the lower response to TD Ag in lupus mice.

In spite of the presence of NP-specific ASC in B6.TC BM, the reduced production of anti-NP serum Abs by B6.TC mice (Fig. 1) suggested that they were functionally defective. The normalized amount of anti-NP IgG produced on a per cell basis was similar in the spleen between the two strains, but significantly different in the BM, in which B6.TC ASC barely produced any detectable anti-NP IgG (Fig. 7B). In contrast, total IgG production per cell in either the spleen or BM was similar between the two strains. This result was supported by a size analysis of the ASC spots (Fig. 7C and D). The size of the splenic anti-NP or total IgG ASC spots was not different between the two strains, but BM anti-NP ASC spots were significantly smaller in B6.TC than in B6 mice (% spots $\geq 70 \mu\text{m}$: B6 = 43%, B6.TC = 17%, $\chi^2 = 16.10$, $p < 0.0001$). The size of total BM IgG spots was equivalent between the two strains. These results suggested that the significantly decreased production of anti-NP Ab by B6.TC mice was due to the failure to produce functional NP-specific BM PC, and that the output from B6.TC splenic PC, although increased in number, was not sufficient to compensate for this deficit.

The specific defective function of NP-specific PC in B6.TC BM was further demonstrated by adoptive transfers 30 wks after primary immunization. At this time point, the same pattern of reduced anti-NP IgG and increased total IgG was still found in B6.TC mice (data not shown). B6 or B6.TC splenocytes produced similar anti-NP IgG levels 2 wks after transfer into B6.Rag mice, but only B6 and not B6.TC BM cells produced anti-NP IgG (Fig. 8A). In contrast, B6.TC BM produced significantly higher amounts of total IgG than B6 BM (Fig. 8B). Interestingly, ASC present in the B6.TC spleens, but not BM produced anti-dsDNA and anti-chromatin IgG one week after transfer (Fig. 8C), but not 2 weeks after transfer (data not shown). These latter results suggest that the anti-nuclear Abs are mostly produced by short lived splenic plasmablasts. Overall, these results demonstrate that in spite of abundant total IgG PC in their BM, B6.TC failed to develop long-lived BM anti-NP PC in response to immunization.

3. Discussion

SLE patients [1] and lupus-prone mice [2;3] generate anti-nuclear autoAbs that present the characteristics of Ag-driven TD-immune responses. To formally investigate the response of lupus-prone mice to TD Ags, we have immunized B6.TC and B6 control mice, which share over 95% of their genome, including the Ig heavy chain and light chain loci, the T cell receptor loci, and the entire MHC region, which provide them with identical naïve B and T cell repertoires [7]. These congenic strains therefore represent an ideal experimental system to test whether lupus susceptibility is associated with a diminished humoral response to foreign Ags. Hyper-responsiveness to exogenous TD Ags has been reported in lupus prone mice [18], with the exception of MRL/lpr [19] and PN [20]. This discrepancy may be related to the diverse genetic backgrounds, including between controls and lupus strains, as well as to the fact that the age at which the mice were tested was not consistent. Moreover, these studies did not include a parallel analysis of autoAb production. We have found that B6.TC mice produce a reduced Ab response to various TD Ags as compared to B6, in spite of the fact that they secrete significantly higher amounts of total Ig and autoAbs, including anti-dsDNA Abs. The impairment was greater for the primary than for the secondary response. However, the involvement of memory B and T cells, both of which are abnormal in lupus patients [21;22] but have not yet been characterized in the NZM2410 model, complicates the interpretation of the secondary response findings. These results suggest that the Ab response of lupus mice to TD exogenous Ags is generated through different mechanisms from that of the total Ab pool, which is largely determined in specific-pathogen free conditions by autoAgs and the normal commensal microbial flora. Adoptive transfers demonstrated that either B6.TC B or CD4⁺ T cells were sufficient for the deficient anti-NP humoral response. DCs or stromal cells may play a role in producing these defective B and T cells, but they are not necessary during the anti-NP response. In addition to the transfer experiment, the smaller effect on anti-NP IgM and the increased TI responses in B6.TC mice, strongly suggest that abnormal T cell help may be critical, and additional experiments will be necessary to investigate how B6.TC T cells respond to autoAgs and exogenous TD Ags, and how B cells integrate signals from these T cells.

We have systematically compared the differentiation of the anti-NP B cells to pinpoint the mechanisms responsible for this abortive response. We have confirmed the splenic PC accumulation in B6.TC mice and reduced migration to the BM [13], but it affected both the NP-specific and total PC and this mechanism is therefore not responsible for the specific defect to TD Ag immunization. In contrast, a number of checkpoints in the generation of anti-NP Abs differed between B6.TC and B6 mice. B6.TC B cells showed a reduced participation in the anti-NP response as indicated by a significantly smaller percentage of splenic NP-specific B cells, which proliferated less and underwent less apoptosis than the non-NP specific B cells. The increased proliferation of non-NP specific B6.TC B cells as compared to B6 was consistent with B6.TC expressing *Sle2*, which confers a lower threshold of BCR activation [23]. One might speculate that the reason why NP-specific B6.TC B cells did not have such a proliferative advantage is antigen competition. This well-documented phenomenon in T cells [24] may occur in our model, either directly in B cells or indirectly in T cells, with the majority of the clones actively engaged in autoreactive responses, leaving a reduced pool of cells able to respond to the immunogen.

Flow cytometry, histology and analysis of the Ab affinity showed that proportionally fewer NP-specific B cells entered the GC reactions in B6.TC than in B6 spleens. B6.TC and other lupus-prone mice develop a large number of spontaneous GCs [12], and *Sle1a*-CD4⁺ T cells express a high level of ICOS [25], a co-stimulatory molecule characteristic of follicular helper T cells found in GCs. The specific of the Abs generated through these GCs is currently unknown. However, the association between GC exclusion of NP-specific B cells

and a blunted Ab response suggests that this checkpoint is likely to play a role in the outcome of humoral responses in B6.TC mice. It has been shown recently that the strength of PI3K signaling controls PC differentiation versus class-switch recombination [26]. Multiple pathways including PI3K contribute to an increased BCR signaling strength in our model [27]. The accumulation of splenic PC and reduction of NP-specific cells entering GCs suggest that NP-immunization may induce a stronger BCR signal in B6.TC B cells, which overall results in a decreased Ab response.

The other mechanism that is likely to play a key role in the defective response to TD Ags is the generation of defective NP-specific PC in the B6.TC bone marrow, where normal long-lived non-NP specific PC were produced. B6.TC produced NP-specific PC in the BM, although on a per cell basis, they secreted reduced amounts of anti-NP Abs as compared to B6. Adoptive transfers showed that these B6.TC BM NP-specific PC were short-lived, but although the same mice had increased numbers of long-lived total IgG PC, further demonstrating the defectiveness of B6.TC NP-specific PC residing in the BM. Using the same adoptive transfers, we also found that the antinuclear autoAbs were also mostly produced by short-lived splenic plasmablasts. Using younger non-immunized mice, we and others have shown that NZM2410 and (NZB $\times$ NZW)F1 mice can produce long-lived BM cells [13;28]. These differences warrant further investigation. One can speculate that B6.TC mice have an abundant pre-existing PC population in their BM corresponding to the robust serum Ig levels of unknown specificity found in these mice. According to a recently proposed model [16], the limited BM niches are made available to newly emerging PC through Fc γ RIIb-dependent apoptosis of established secreting PC. The reduced Fc γ RIIb expression on B6.TC PC in the spleen regardless of their specificity predicts a lower apoptosis rate, which may contribute to their accumulation at this early stage of their development. Low Fc γ RIIb expression on the non-NP specific PC, which are in a much higher number than the newly emerged NP-specific PC, would reduce their turn-over and the availability of new niches for the NP-specific PC to develop into highly productive long-lived BM PC. Reduced levels of Fc γ RIIb expression on total PC have also been reported in MRL/lpr and NZB lupus-prone mice [16] and in lupus patients [29]. One could speculate that restoring normal levels of Fc γ RIIb expression on B cells, which has been associated with the elimination of autoimmune pathology in the NZM2410 model [30], may also restore functional long-lived PC specific for exogenous TD Ags in this model.

B6.*Sle1c*, and therefore B6.TC mice carry the *Cr2*^{NZW} allele that is associated with impaired GC formation and reduced humoral response to TD Ag [31]. Some of the phenotypes of B6.*Sle1c* mice are therefore similar in nature to that of *Cr2*-deficient mice [32–35], but not in intensity, most likely because *Cr2*^{NZW} is not a null allele. Moreover, the effect of *Cr2*^{NZW} in isolation is nullified by *Sle1b*, which mediates strong production of anti-chromatin Abs [36] and enhances TD responses [37]. Consequently, B6.*Sle1* mice (which contain both *Sle1b* and *Sle1c*) have a TD-response equivalent to that of B6 (unpublished results). Therefore it is very unlikely that *Cr2*^{NZW} alone is responsible for the phenotypes that we report here for the B6.TC mice. We have also examined the response of B6.*Sle3/5* mice to TD Ags and have shown that this locus was associated with longer IgH CDR3 sequences, increased somatic mutations and receptor editing following NP-SRBC TD immunization [38]. These features normally associated with autoAbs suggest that *Sle3/5* impacts responses to self and autoAg by the same pathways. As we have previously reported for the splenic accumulation of PC [13], the defective response to TD Ag by B6.TC mice is polygenic. We propose that the NZW alleles of *Fc γ RIIb* and *Cr2* may contribute to this defect, but only in interaction with other *Sle* loci, such as *Sle1b* and *Sle3/5*, whose major contribution to the process is to drive the robust autoAb response.

Most lupus patients produce a normal humoral response to vaccination, but a significant minority (20% to 100% depending on the study and the immunogen) do not, despite secreting large amounts of autoAbs [39;40]. This suggests that in some patients, the disease process may impair the response to exogenous Ags. Here we show that the B6.TC mouse may constitute a good model to investigate the mechanisms responsible for poor response to vaccination in lupus patients. Based on our results, we predict that the patients with low Fc γ RIIb expression and/or a high hypergammaglobulinemia or autoAb production may be the most likely to fail to develop protective Ab titers after vaccination due to the failure to make sufficient PC niches available in the BM to produce long-lived Abs against the immunogen.

4 Materials and methods

4.1 Mice

B6.*Sle1/Sle2/Sle3* (B6.TC) [7], C57BL/6J (B6) and B6.129S7-*Rag1^{tm1Mom}/J* (B6.Rag) mice were bred and maintained at University of Florida in specific pathogen free conditions. Unless specified, female mice were used between 2 to 3-months of age at the time of immunization, before any manifestation of clinical disease. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida (protocol D647).

4.2 Spleen and BM chimeras

Splenocytes (30×10^6) or a mixture of CD43⁻ B (20×10^6) and CD4⁺ T (10×10^6) cells were transferred i.v. into B6.Rag mice 1 wk before immunization. The B cell transfers were > 95% B220⁺ and the T cell transfers were > 80% CD4⁺ (supplemental Fig. 6). Long-lived PC were evaluated in B6.Rag chimeras reconstituted with 30×10^6 T cell-depleted (first with pan-T cell magnetic beads from Dynalbiotech, then with anti-CD4 beads from Miltenyi Biotec) splenic or BM cells from B6.TC or B6 mice immunized 30 wks earlier.

4.3 Immunization

The primary response to TD Ags was measured following an i.p. injection with 100 μ g of 4-Hydroxy-3-nitrophenylacetyl-Keyhole Limpet Hemocyanin (NP₁₉-KLH, referred to as NP-KLH) or 2,4,6-Trinitrophenyl-Chicken Gamma Globulin (TNP₁₈-CGG) both from Biosearch Technologies in 100 μ l of Inject alum (Pierce Biotechnology) after various periods of time up to 25 wks. For secondary immunization, mice were boosted with the same dose of NP-KLH in alum 3 wks after the primary immunization, and Ab levels were measured 10 days later. Responses to TI Ags were measured 6 days after i.p. injection with 100 μ g of TNP-LPS or 4-Hydroxy-3-nitrophenylacetic Amino-Ethyl-Carboxy-Methyl-Ficoll (NP₁₂-AECM-Ficoll, Biosearch Technologies) in 200 μ l PBS. In each experiment, 3 to 5 mice per group were used for each time point. Each experiment was repeated at least once.

4.4 Antibody measurements

Anti-dsDNA and anti-chromatin IgG ELISA assays were carried out as previously described [41]. Serum total IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM were detected by sandwich ELISA coated with goat-anti mouse IgG (Accurate Chemical) or IgM (ICN/Cappel) at 1 μ g/ml using Immulon 1B plates in bicarbonate buffer. Serum NP-, TNP-, or KLH- specific IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM were detected by sandwich ELISA coated with NP₃-BSA, NP₁₂-BSA, NP₂₃-BSA, NP₁₆-KLH or TNP₁₈-BSA (Biosearch Technologies) at 10 μ g/ml, or KLH (Sigma-Aldrich) at 50 μ g/ml. After blocking, serial diluted test sera were incubated, and the bound IgG isotypes and IgM were detected by alkaline phosphatase-

conjugated goat anti-mouse IgG (Chemicon), IgG1, IgG2a, IgG2b, IgG3 or IgM (Southern Biotech). All samples were run in duplicate.

4.5 Spleen histology

Immunofluorescence staining was performed as previously described [42]. Frozen sections were stained with the following FITC-, PE-, APC, or biotin-conjugated primary Abs (BD Biosciences): CD138 (281-2), B220 (RA3-6B2), IgM (II/41), Ig λ 1 (R11-153), and GL7. Biotin-conjugated Abs were revealed by Streptavidin-Alexa Fluor 568 or Streptavidin-APC (Invitrogen).

4.6 Flow cytometry

RBC-depleted splenic or BM cells were stained as previously described [36] with the following conjugated mAbs: CD138 (281-2), B220 (RA3-6B2), IgM (II/41), Ig λ 1 (R11-13), Fc γ RIIb (2.4G2) CD44 (IM7), CD184 (2B11/CXCR4), LFA-1 (M17/4), and GL7 (BD Biosciences). NP-specific B cells were detected using NP₁₉-PE (Biosearch Technologies). NP-specific PC were detected by NP₁₉-PE or λ ₁ intracellular staining with Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences). The percentage of NP⁺ CD138⁺ cells in unimmunized mice was very low (Supplemental Fig. 3D), indicating the high specificity of the stain. To measure B cell proliferation, mice were injected i.p. with 100 mg BrdU (BD Biosciences) at 12 h and 24 h prior to sacrifice and BrdU incorporation was then measured with the BrdU Flow kit (BD Biosciences). B cell apoptosis was measured with the active caspase-3 apoptosis kit (BD Biosciences). Stained cells were analyzed on FACSCalibur cytometer (BD Biosciences). Dead cells were excluded based on forward and side scatter characteristics. At least 300,000 events were acquired per sample. Positive staining was determined relative to isotype controls.

4.7 ELISPOT assays

ASC were enumerated by ELISPOT as previously described [13]. Serially diluted splenocytes or BM cells were added to multiscreen filter plates (Millipore) coated with 5 μ g/ml goat anti-mouse IgG, or 50 μ g/ml NP₁₂-BSA. Bound cells were detected with HRP-conjugated-anti-IgG (Southern Biotech), and developed by 3-Amino-9-ethylcarbazole (AEC, Sigma-Aldrich). ASC were then counted and measured using a Bioreader 4000 Pro-x (Bio-Sys GmbH). Measurements of Ab produced by single cells were performed by culturing simultaneously the same number of cells in both filter and 96-well culture plate (CoStar, Corning Life Sciences) for 8 h at 37°C with 5% CO₂. Supernatants were harvested from 96-well plates for ELISA with HRP-conjugated-anti-IgG, and the corresponding numbers of cells per well were enumerated by ELISPOT. The average amount of Ab produced by single cells per hour equals the amount of Abs per well divided by the cell number and by the number of hours in culture.

Prdm-1 and XBP-1 quantitative RT-PCR—Total RNA extracted from B6 and B6.TC spleen or BM with RNeasy Mini Kit (Qiagen) was reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems). PCR was performed using Tagman Gene Expression assay primers and probes and normalized to GAPDH for each assay. The primer and probe sequences for Prdm-1 were as follows: forward, 5'-ACAGAGGCCGAGTTTGAAGAGA-3'; reverse, 5'-AAGGATGCCTCGGCTTGAA-3'; probe, 5'[6-FAM] CCCTGGGATTCCGGCGCTG [TAMRA-6-FAM]-3'. The primers and probe for XBP-1 were the inventoried Applied Biosystems reagents.

4.8 Statistical analysis

Data were analyzed with the GraphPad Prism 4.0 software package with appropriate tests as indicated in the text. Data are shown in figures as mean $\pm$ standard deviation (SD). Significance levels were coded as follows: *: $p < 0.05$, **: $p < 0.01$, and ***: $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard abbreviations

SLE	Systemic lupus erythematosus
B6.TC	B6. <i>Sle1.Sle2.Sle3</i>
B6	C57BL/6
Ab	antibody
TD	T cell dependent
Ag	antigen
BM	bone marrow
GC	germinal center
ASC	antibody secreting cells
PC	plasma cell
PB	plasmablast
TI	T-independent
BCR	B cell receptor
B6.Rag	B6.129S7- <i>Rag1^{tm1Mom}/J</i>
NP-KLH	4-Hydroxy-3-nitrophenylacetyl -Keyhole Limpet Hemocyanin
TNP-CGG	2,4,6- Trinitrophenyl-Chicken Gamma Globulin

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References

1. Lipsky PE. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. *Nat.Immunol.* 2001; 2:764–766. [PubMed: 11526379]
2. Radic MZ, Weigert M. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Ann.Rev.Immunol.* 1994; 12:487–520. [PubMed: 8011289]
3. Craft J, Peng S, Fujii T, Okada M, Fatenejad S. Autoreactive T cells in murine lupus- Origins and roles in autoantibody production. *Immunologic Research.* 1999; 19:245–257. [PubMed: 10493178]
4. Burlingame RW, Rubin RL, Balderas RS, Theofilopoulos AN. Genesis and evolution of antichromatin autoantibodies in murine lupus implicates T-dependent immunization with self antigen. *J.Clin.Invest.* 1993; 91:1687–1696. [PubMed: 8473512]
5. William J, Euler C, Christensen S, Shlomchik MJ. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science.* 2002; 297:2066–2070. [PubMed: 12242446]

6. Herlands RA, William J, Hershberg U, Shlomchik MJ. Anti-chromatin antibodies drive in vivo antigen-specific activation and somatic hypermutation of rheumatoid factor B cells at extrafollicular sites. *Eur.J Immunol.* 2007; 37:3339–3351. [PubMed: 18034429]
7. Morel L, Croker BP, Blenman KR, Mohan C, Huang G, Gilkeson G, Wakeland EK. Genetic reconstitution of systemic lupus erythematosus immunopathology with polycongenic murine strains. *Proc.Natl.Acad.Sci.U.S.A.* 2000; 97:6670–6675. [PubMed: 10841565]
8. Morel L, Rudofsky UH, Longmate JA, Schiffenbauer J, Wakeland EK. Polygenic control of susceptibility to murine systemic lupus erythematosus. *Immunity.* 1994; 1:219–229. [PubMed: 7889410]
9. Liang ZY, Xie C, Chen C, Kreska D, Hsu K, Li LN, Zhou XJ, Mohan C. Pathogenic profiles and molecular signatures of antinuclear autoantibodies rescued from NZM2410 lupus mice. *J.Exp.Med.* 2004; 199:381–398. [PubMed: 14757744]
10. Mohan C, Morel L, Yang P, Watanabe H, Croker B, Gilkeson G, Wakeland EK. Genetic dissection of lupus pathogenesis: a recipe for nephrophilic autoantibodies. *J.Clin.Invest.* 1999; 103:1685–1695. [PubMed: 10377175]
11. Jacob J, Kassir R, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J.Exp.Med.* 1991; 173:1165–1175. [PubMed: 1902502]
12. Xu Z, Duan B, Croker BP, Morel L. STAT4 deficiency reduces autoantibody production and glomerulonephritis in a mouse model of lupus. *Clin.Immunol.* 2006; 120:189–198. [PubMed: 16713741]
13. Erickson LD, Lin LL, Duan B, Morel L, Noelle RJ. A genetic lesion that arrests plasma cell homing to the bone marrow. *Proc.Natl.Acad.Sci.U.S.A.* 2003; 100:12905–12910. [PubMed: 14555759]
14. Shaffer AL, Shapiro-Shelef M, Iwakoshi NN, Lee AH, Qian SB, Zhao H, Yu X, Yang L, Tan BK, Rosenwald A, Hurt EM, Petroulakis E, Sonenberg N, Yewdell JW, Calame K, Glimcher LH, Staudt LM. XBP1, downstream of Blimp-1, expands the secretory apparatus and other organelles, and increases protein synthesis in plasma cell differentiation. *Immunity.* 2004; 21:81–93. [PubMed: 15345222]
15. Shaffer AL, Lin KI, Kuo TC, Yu X, Hurt EM, Rosenwald A, Giltner JM, Yang LM, Zhao H, Calame K, Staudt LM. Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program. *Immunity.* 2002; 17:51–62. [PubMed: 12150891]
16. Xiang Z, Cutler AJ, Brownlie RJ, Fairfax K, Lawlor KE, Severinson E, Walker EU, Manz RA, Tarlinton DM, Smith KGC. FcγRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat.Immunol.* 2007
17. Rahman ZSM, Niu H, Perry D, Wakeland E, Manser T, Morel L. Expression of the autoimmune Fcγr2b NZW allele fails to be upregulated in germinal center B cells and is associated with increased IgG production. *Genes Immun.* 2007; 8:604–612. [PubMed: 17713556]
18. Park CL, Balderas RS, Fieser TM, Slack JH, Prud'homme GJ, Dixon FJ, Theofilopoulos AN. Isotypic profiles and other fine characteristics of immune responses to exogenous thymus-dependent and -independent antigens by mice with lupus syndromes. *J Immunol.* 1983; 130:2161–2167. [PubMed: 6339623]
19. Park CL, Balderas RS, Fieser TM, Slack JH, Prud'homme GJ, Dixon FJ, Theofilopoulos AN. Isotypic profiles and other fine characteristics of immune responses to exogenous thymus-dependent and -independent antigens by mice with lupus syndromes. *J Immunol.* 1983; 130:2161–2167. [PubMed: 6339623]
20. Walker SE. Defective primary and secondary IgG responses to thymic-dependent antigens in autoimmune PN mice. *J Immunol.* 1988; 140:3426–3433. [PubMed: 3283233]
21. Dorner T, Lipsky PE. Correlation of circulating CD27^{high} plasma cells and disease activity in systemic lupus erythematosus. *Lupus.* 2004; 13:283–289. [PubMed: 15230280]
22. Fritsch RD, Shen X, Illei GG, Yarboro CH, Prussin C, Hathcock KS, Hodes RJ, Lipsky PE. Abnormal differentiation of memory T cells in systemic lupus erythematosus. *Arthritis Rheum.* 2006; 54:2184–2197. [PubMed: 16802356]

23. Mohan C, Morel L, Yang P, Wakeland EK. Genetic dissection of systemic lupus erythematosus pathogenesis - Sle2 on murine chromosome 4 leads to B cell hyperactivity. *J.Immunol.* 1997; 159:454–465. [PubMed: 9200486]
24. Kedl RM, Kappler JW, Marrack P. Epitope dominance, competition and T cell affinity maturation. *Current Opinion in Immunology.* 2003; 15:120–127. [PubMed: 12495743]
25. Cuda CM, Wan S, Sobel ES, Croker BP, Morel L. Murine lupus susceptibility locus Sle1a controls regulatory T cell number and function through multiple mechanisms. *J Immunol.* 2007; 179:7439–7447. [PubMed: 18025188]
26. Omori SA, Cato MH, nzelon-Mills A, Puri KD, Shapiro-Shelef M, Calame K, Rickert RC. Regulation of class-switch recombination and plasma cell differentiation by phosphatidylinositol 3-kinase signaling. *Immunity.* 2006; 25:545–557. [PubMed: 17000121]
27. Wu T, Qin X, Kurepa Z, Kumar KR, Liu K, Kanta H, Zhou XJ, Satterthwaite AB, Davis LS, Mohan C. Shared signaling networks active in B cells isolated from genetically distinct mouse models of lupus. *J.Clin.Invest.* 2007; 117:2186–2196. [PubMed: 17641780]
28. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, Radbruch A, Hiepe F, Manz RA. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J Exp Med.* 2004; 199:1577–1584. [PubMed: 15173206]
29. Su K, Yang H, Li X, Li X, Gibson AW, Cafardi JM, Zhou T, Edberg JC, Kimberly RP. Expression profile of FcγRIIb on leukocytes and its dysregulation in systemic lupus erythematosus. *J.Immunol.* 2007; 178:3272–3280. [PubMed: 17312177]
30. McGaha TL, Sorrentino B, Ravetch JV. Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science.* 2005; 307:590–593. [PubMed: 15681388]
31. Boackle SA, Holers VM, Chen XJ, Szakonyi G, Karp DR, Wakeland EK, Morel L. Cr2, a candidate gene in the murine Sle1c lupus susceptibility locus, encodes a dysfunctional protein. *Immunity.* 2001; 15:775–785. [PubMed: 11728339]
32. Chen ZB, Koralov SB, Gendelman M, Carroll MC, Kelsoe G. Humoral immune responses in Cr2(–/–) mice: Enhanced affinity maturation but impaired antibody persistence. *J Immunology.* 2000; 164:4522–4532. [PubMed: 10779753]
33. Fischer MB, Goerg S, Shen LM, Prodeus AP, Goodnow CC, Kelsoe G, Carroll MC. Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science.* 1998; 280:582–585. [PubMed: 9554848]
34. Molina H, Holers VM, Li B, Fang YF, Mariathasan S, Goellner J, StraussSchoenberger J, Karr RW, Chaplin DD. Markedly impaired humoral immune response in mice deficient in complement receptors 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America.* 1996; 93:3357–3361. [PubMed: 8622941]
35. Gatto D, Pfister T, Jegerlehner A, Martin SW, Kopf M, Bachmann MF. Complement receptors regulate differentiation of bone marrow plasma cell precursors expressing transcription factors Blimp-1 and XBP-1. *J Exp.Med.* 2005; 201:993–1005. [PubMed: 15767369]
36. Morel L, Blenman KR, Croker BP, Wakeland EK. The major murine systemic lupus erythematosus susceptibility locus, Sle1, is a cluster of functionally related genes. *Proc.Natl.Acad.Sci.U.S.A.* 2001; 98:1787–1792. [PubMed: 11172029]
37. Croker BP, Gilkeson G, Morel L. Genetic interactions between susceptibility loci reveal epistatic pathogenic networks in murine lupus. *Genes and Immunity.* 2003; 4:575–585. [PubMed: 14647198]
38. Wakui M, Kim J, Butfiloski EJ, Morel L, Sobel ES. Genetic dissection of lupus pathogenesis: Sle3/5 impacts IgH CDR3 sequences, somatic mutations, and receptor editing. *J.Immunol.* 2004; 173:7368–7376. [PubMed: 15585861]
39. Mercado U. Why have rheumatologists been reluctant to vaccinate patients with systemic lupus erythematosus? *J.Rheumatol.* 2006; 33:1469–1471. [PubMed: 16881104]
40. O'Neill SG, Isenberg DA. Immunizing patients with systemic lupus erythematosus: a review of effectiveness and safety. *Lupus.* 2006; 15:778–783. [PubMed: 17153850]
41. Mohan C, Alas E, Morel L, Yang P, Wakeland EK. Genetic dissection of SLE pathogenesis - Sle1 on murine chromosome 1 leads to a selective loss of tolerance to H2A/H2B/DNA subnucleosomes. *J.Clin.Invest.* 1998; 101:1362–1372. [PubMed: 9502778]

42. Duan B, Croker BP, Morel L. Lupus resistance is associated with marginal zone abnormalities in an NZM murine model. *Lab.Invest.* 2007; 87:14–28. [PubMed: 17170739]

Figure 1. Lupus mice produced fewer TD Abs after immunization

(A) Anti-NP IgG and IgG1 levels from NP-KLH immunized mice (left), anti-TNP IgG and IgG1 from TNP-CGG immunized mice (middle), and corresponding total Ig levels (right) in NP-KLH immunized mice. (B) Anti-NP IgM (left), and total IgM (right) from NP-KLH immunized mice. (A, B) Results are shown for 4 mice per strain at the indicated time-points in wks after primary immunization. Sera diluted 1:5,000 for anti-NP and anti-TNP Abs, and 1:50,000 for total Ig. The experiment was performed three times with similar results. (C) Anti-KLH IgG1 at 1, 2 and 4 wks after primary immunization (sera diluted 1:100). Means and SD of 16 mice per strain per time point. (D) Anti-dsDNA IgG levels following primary immunization (sera diluted 1:500). (E) Anti-NP Ab levels 10 days after secondary

immunization (sera diluted 1:10,000). Means and SD of 8 mice per strain. Filled and open symbols represent B6 and B6.TC (indicated as TC) mice, respectively. Asterisks indicate levels of significance of *t* tests between the two strains at a given time point or isotype (*: $p \leq 0.05$; **: $p \leq 0.01$). Stars (*) indicate when statistical significance was reached for every time point in the graph. In (D), the difference between strains was significant either comparing each time point with *t* tests or comparing the slopes of the linear regression lines.

Figure 2. Either B6.TC B or T cells are sufficient for a lower Ab response to TD immunization
 Anti-NP IgG, IgG1, IgM and total IgG, IgG1, IgM levels in B6.Rag mice transferred with splenocytes (SP) or a mixture of splenic B and CD4⁺ T cells, either strain-matched (B6 B + T or TC B + T) or mismatched (B6 B + TC T or B6 T + TC B) 4 wks post-immunization. All sera were diluted 1:5,000. Asterisks indicate levels of significance of Bonferroni's multiple comparison tests (anti-NP-IgG and IgG1), and *t* tests (anti-NP-IgM) (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). Short horizontal lines indicate the mean. TC indicates B6.TC.

Figure 3. NP-KLH-immunized lupus mice produced fewer NP-specific B cells

(A) Frequency of λ_1^+ B220⁺ (left) and λ_1^+ B220⁺ IgM⁺ splenocytes (middle) at the indicated time points after immunization. The FACS plots show representative B220-FITC and NP-PE staining 4 wks after immunization. (B) BrdU incorporation in λ_1^+ (left) and NP⁺ or NP⁻ (middle) B220⁺ B cells 4 wks after immunization. The right FACS plot shows a representative overlay of NP-PE staining on B6 and B6.TC B220⁺ BrdU⁺ using the gating indicated in the left plot. (C) Frequency of λ_1^+ (left) and NP⁺ or NP⁻ (middle) activated caspase 3 B220⁺ B cells 4 wks after immunization. The right FACS plots show a representative overlay of NP-PE staining on B6 and B6.TC B220⁺ caspase 3⁺ cells using the gating indicated in the left plot. (A–C) Means and SD of 3–5 mice per strain per time point. These data are representative results of two independent experiments. Filled and open columns represent B6 and B6.TC (indicated as TC) mice, respectively. Asterisks indicate levels of significance of *t* tests between the two strains at each time point (*: *p* ≤ 0.05; **: *p* ≤ 0.01).

Figure 4. NP-KLH-immunized lupus mice produced fewer NP-specific germinal center B cells
 Percentage of (A) NP⁺ (left axis) or NP⁻ (right axis) GL7⁺ B220⁺ and (B) NP⁺ IgM⁺ GL7⁺ B220⁺ B cells in B6 (filled symbols) and B6.TC (indicated as TC, open symbols) mice 4 wks post-immunization. Representative FACS plots are shown for each graph. (C) Time course of the anti-NP3/anti-NP23 IgG and IgG1 ratio (serum dilution 1:5,000). Means $\pm$ SD of representative results from 2 independent experiments with 3–5 mice per group. Asterisks indicate levels of significance of *t* tests between the two strains (*: $p \leq 0.05$; **: $p \leq 0.01$). Stars (*) indicate when statistical significance ($p \leq 0.05$) was reached for every time point in the graph. Short horizontal lines indicate the mean.

Figure 5. Immunofluorescence staining of B6.TC and B6 spleens after NP-KLH immunization
 The majority of (A) NP-specific λ_1^+ IgM $^+$ cells or (B) λ_1^+ B220 $^+$ cells were located in the B cell zone, concentrated in foci near or in the GL7 $^+$ GC in B6 mice. In contrast, these cells were fewer in number and scattered throughout the follicle in B6.TC (indicated by TC) mice. (C) NP-specific λ_1^+ CD138 $^+$ PB / PC were grouped in extra-follicular foci in B6 mice, but were scattered in B6.TC mice.

Figure 6. B6.TC mice produce proportionally fewer NP-specific plasma cells in the spleen and BM

(A) Upper row: NP⁺ B220^{lo/-} CD138⁺ PC expressed as percentage of CD138⁺ splenocytes and absolute numbers. Lower row: total PC expressed as percentage of splenocytes and absolute numbers. (B) NP⁺ B220^{lo/-} CD138⁺ PC expressed as percentage of CD138⁺ BM cells and absolute numbers. (C) Expression of 2.4G2 on NP⁺ and total (all) B220^{lo/-} CD138⁺ PC in the spleen and BM. Filled and open symbols represent B6 and B6.TC mice (indicated by TC), respectively. Means $\pm$ SD of representative results from 2 independent experiments with 3–5 mice per group, 4 wks after immunization, with representative FACS

plots shown on the right. Asterisks indicate levels of significance of t tests between the two strains (*: $p \leq 0.05$; **: $p \leq 0.01$). Short horizontal lines indicate the mean.

Figure 7. NP-KLH-immunized B6.TC mice produced functionally defective NP-specific ASC (A) Anti-NP IgG⁺ (two left panels) and total IgG ASC (two right panels) in the spleen and BM of B6 and B6.TC (indicated as TC) mice were enumerated by ELISPOT and normalized to 10⁶ cells. Means $\pm$ SD of representative results from 2 independent experiments with 3–5 mice per strain per time point. (B) Normalized anti-NP (two left panels) and total IgG (two right panels) in supernatants from spleen and BM ASC in B6 (filled symbols) and B6.TC (open symbols) mice one wk post-immunization. Asterisks indicate levels of significance of *t* tests (*: *p* $\leq$ 0.05; **: *p* $\leq$ 0.01). (C) ASC diameter distribution calculated on 6 randomly selected wells at the same serial dilution for each strain, with the threshold of detection set at 25 μ M for total IgG and 35 μ M for α NP IgG. (D) Representative ELISPOT wells at the

same serial dilution, showing anti-NP IgG (left) and total IgG (right) ASC in the spleen and BM in B6 and B6.TC mice 4 wks post-immunization illustrating the size difference. The number of spots per well is indicated on the upper right corner. Short horizontal lines indicate the mean.

Figure 8. B6.TC BM did not contain long-lived anti-NP PC

T cell-depleted splenocytes or BM cells obtained from B6 or B6.TC (indicated as TC) mice 30 wks after immunization were transferred into B6.Rag mice. (A) Anti-NP IgG and (B) total IgG levels were measured 2 wks after transfer. (C) Anti-dsDNA and anti-chromatin IgG was measured 1 wk after transfer (left axis) and the levels of these autoAbs in the donors at transfer are shown on the right axis. The experiment was performed twice with identical results. Asterisks indicate levels of significance of *t* tests between the two strains at a given dilution (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$). Short horizontal lines indicate the mean.