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Development of Murine Lupus Involves the Combined Genetic Contribution of the SLAM and FcγR Intervals within the Nba2 Autoimmune Susceptibility Locus

Trine N. Jørgensen,*1,2 Jennifer Alfaro,†,‡,† Hilda L. Enriquez,‡ Chao Jiang,‡ William M. Loo,‡ Stephanie Atencio,* Melanie R. Gubbels Buppp,† Christina M. Mailloux,‡ Troy Metzger,* Shannon Flannery,* Stephen J. Rozzo,* Brian L. Kotzin,*† Mario Rosemblatt,†,‡,‡ Maria Rosa Bono,‡ and Loren D. Erickson‡

Autoantibodies are of central importance in the pathogenesis of Ab-mediated autoimmune disorders. The murine lupus susceptibility locus Nba2 on chromosome 1 and the syntenic human locus are associated with a loss of immune tolerance that leads to antinuclear Ab production. To identify gene intervals within Nba2 that control the development of autoantibody-producing B cells and to determine the cellular components through which Nba2 genes accomplish this, we generated congenic mice expressing various Nba2 intervals where genes for the FcγR, SLAM, and IFN-inducible families are encoded. Analysis of congenic strains demonstrated that the FcγR and SLAM intervals independently controlled the severity of autoantibody production and renal disease, yet are both required for lupus susceptibility. Deregulated homeostasis of terminally differentiated B cells was found to be controlled by the FcγR interval where FcγR1Iib-mediated apoptosis of germinal center B cells and plasma cells was impaired. Increased numbers of activated plasmacytoid dendritic cells that were distinctly CD19+ and promoted plasma cell differentiation via the proinflammatory cytokines IL-10 and IFNα were linked to the SLAM interval. These findings suggest that SLAM and FcγR intervals act cooperatively to influence the clinical course of disease through supporting the differentiation and survival of autoantibody-producing cells. The Journal of Immunology, 2010, 184: 775–786.
the SLAM/CD2 gene cluster (16, 17), and Ifi202 (7). Because of the complicated pattern of disease-associated genes in the Nha2 locus, it is unknown whether the FcγR, SLAM, and Ifi genes contribute to the autoimmune phenotype as a group or as individual gene clusters.

In this study, we directly evaluated the role of Nha2-derived FcγR, SLAM, and Ifi gene clusters in autoantibody production by creating congenic mice that vary in expression of these three intervals. Analysis of congenic strains demonstrated that the severity of ANA and renal disease are linked with the FcγR and SLAM gene clusters with little involvement from the Ifi interval. The most severe autoimmune phenotype occurs in mice carrying both FcγR and SLAM clusters from the parental B6.Nha2 strain. Analyses of immune cell function among the congenic strains revealed that spleen dendritic cells (DCs), including an expanded population of CD19+ plasma-cytoid DCs (pDCs), inappropriately supported PC differentiation in a cytokine-dependent manner that was linked to the SLAM gene cluster. Reduced expression of and apoptosis mediated by FcγRILb were found in B cells that was directly controlled by the FcγR gene interval. Thus, although the FcγR and SLAM gene clusters independently control different immune pathways in murine lupus, together, they contribute to lupus susceptibility by cooperatively controlling autoantibody production.

Materials and Methods

Mice and evaluation of autoimmune phenotype

Congenic B6.Nha2-ABC mice were previously described (7). Congenic strains expressing smaller intervals of the initial Nha2 lupus susceptibility locus were generated by backcrossing 10 generations with B6 mice. These are referred to as B6.Nha2-a (154.7-174.5Mb), B6.Nha2-a’AB (169.1-175.9Mb), B6.Nha2-B (172.6-175.9Mb), B6.Nha2-BC (172.8-194.1Mb), and B6.Nha2-CD (174.3-194.1Mb). Genotyping was performed using a panel of microsatellite markers distributed across the distal chromosome 1 (Supplemental Table I). The positions of markers and various candidate genes with respect to the centromere are given in accordance with the Mouse Chromosome Committee Reports (obtainable at the Mouse Genome Database at www.informatics.jax.org). The PCR products of all D1Mit markers, Crp, and FcγRILb were separated on 10% polyacrylamide gels, whereas real-time PCR reactions for Ifi202 were run on a BioRad iCycler IQ (Bio-Rad, Hercules, CA) to determine whether the marker was of B6 or NZB origin. B6, NZW, NZB, and (NZB x NZW)F1 females (set at 1 unit). Equal amounts of protein were processed for immunoblotting using antisera to Ifi202 (S-19; Santa Cruz Biotechnology, Santa Cruz, CA). DNA samples from sort-purified spleen B cells, conventional DC (cDC), CD19+ pDC, and CD19+ dDC subsets were analyzed for VDJ rearrangement by RT-PCR as previously described (19).

Flow cytometry

Cells were washed and resuspended in 5% bovine calf serum in balanced salt solution and stained with a mixture of mAbs, as noted, for the detection of multiple cell surface and intracellular Ags. Purified rat IgG was used as an isotype control. Non-specific staining was reduced by the addition of rat serum. Stained cells were incubated for 30 min at 4˚C, followed by washing in balanced salt solution. Incubation with streptavidin-PE-cyanin 5.5 was performed for an additional 30 min at 4˚C, followed by washing. DAPI was added to cells at a concentration of 5 µM/ml to discriminate live versus dead cells. After surface staining, samples requiring intracellular staining were labeled for caspase-3 using the Cytofix/Cytoperm reagent (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Cell acquisition was performed with a DakoCytomation CyAn ADP LX (Carpinteria, CA), where a minimum of 100,000 events was collected. Spectral overlap compensation and data analysis were performed using FlowJo software (Tree Star, Ashland, OR). Profiles are presented as 5% probability contours including outliers with gating based on fluorescence-minus-one and isotype controls.

FcγRILb cross-linking

Single-cell suspensions were prepared from spleens of mice that were immunized with the T cell-dependent NP-KLH Ag for 7 d to generate PCs and germinal center (GC) B cells. Cells were depleted of RBC by ammonium chloride-Tris lysis, washed in RPMI 1640, and cultured 4 h in 96-well plates precoated with 10 µg/ml of rat IgG F(ab’)2, or an anti-FcγRILb (2.4G2) MAb ± 20 µg/ml of the caspase-3 inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (ZVAD: MP Biochemicals, Solon, OH). At the end of the culture, cells were harvested and analyzed by flow cytometry to measure apoptosis by intracellular expression of the cleaved active form of caspase-3 from GC B Cells (B220+GL7+) and PCs (B220-/+ CD138+). Cells cultured in 10 µM DMSO served as a positive control for apoptosis.

Isolation of splenic DCs

Spleens were incubated for 45 min at 37˚C in complete RPMI 1640 supplemented with 5% FBS and 1mg/ml collagenase D (Roche, Indianapolis, IN), followed by homogenization. Single-cell suspensions were washed in RPMI 1640 and depleted of RBC by ammonium chloride-Tris lysis. Total CD11c+ DCs were positively selected using anti-CD11c MACS (clone N418; Miltenyi Biotec, Auburn, CA) after the manufacturer’s protocol. As indicated, some experiments used electronically sort-purified DCs using a FACS Vantage SE TurboSort to produce cDC (CD11b+CD11c+), CD19+ pDC (CD11c+CD122+CD19+), and CD19+ dDC (CD11c+IAB20+CD19+) populations of >98% purity.

DC morphology by cytospin

Sort-purified B cells, cDCs, and pDCs were cytospun onto glass slides (5 min at 230g) and fixed in methanol for 5 min at room temperature. Slides were washed and stained with Giemsa (Sigma-Aldrich, St. Louis, MO), followed by digital photography. All images were taken at original magnification x40.

In vitro TLR stimulation

DCs (2 × 105) were cultured in 96-well plates for 24 h in complete RPMI 1640 supplemented with 10% FBS alone or containing 10 µg/ml CpG (ODN 1826; InvivoGen, San Diego, CA), 100 ng/ml LPS (Sigma-Aldrich), or 10µg/ml imiquimod (R837) (InvivoGen). At the end of the culture, supernatants were collected and tested for cytokines by ELISA.

For DC and B cell coculture experiments, electronically sort-purified pDCs and B cells from spleen were cultured separately (1 × 105/well) or together in 96-well round bottom plates for 3 d in complete RPMI 1640 supplemented with 10% FBS alone, 10 µg/ml CpG, or 10 µg/ml nonstimulatory GpC control oligonucleotide for ODN1826 (InvivoGen). Neutralizing mAbs specific for IL-6 (clone MP5-20F3; eBiosciences, San Diego, CA), IL-10 (clone JES52A5; eBiosciences), IL-12 (clone C17.8; eBiosciences), and IFN-gamma (clone XMG1.2; BD Biosciences, San Jose, CA) were added to the culture. The end of the culture, cells were harvested and the number of PCs determined by ELISPOT.
Measurement of cytokines by ELISA

Murine IL-6, IL-10, and IL-12/p70 were measured using the Duo Set kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. IFNγ was determined by sandwich ELISA using the rat anti-mouse IFNγ RMMA-1 mAb for cytokine capture and the rabbit anti-mouse IFNγ polyclonal Ab for cytokine detection (PBL InterferonSource). HRP-conjugated AffiniPure F(ab’)2 fragment donkey anti-rabbit IgG (H+L) was used as a secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Assays were developed using tetramethylbenzidine substrate (BD Biosciences).

Measurement of PCs by ELISPOT

IgM- and IgG-secreting PCs were quantified by ELISPOT assay as previously described (20). Briefly, harvested cells from DC and B cell cultures were reseeded in complete RPMI 1640 and incubated for 6 h on multiscrreen 96-well plates (Millipore, Bedford, MA) precoated with unlabeled anti-mouse IgM and IgG. Serial dilutions of cells were made with an initial cell concentration of 20,000/well. After incubation, plates were washed in 0.05% Tween 20 and incubated with secondary HRP-conjugated Abs to detect mouse IgM and IgG (Southern Biotechnology Associates, Birmingham, AL). ELISPOT assays were developed by FAST 5-bromo-4-chlor-3-indolyl phosphate/NBT chromogen substrate (Sigma-Aldrich). The number of Ig-secreting spots was quantified by direct visual counting using a dual-axis light dissecting microscope.

Statistical analyses

All statistical analyses on autoantibody levels between study groups were performed using the nonparametric, Mann-Whitney U test. A curve comparison test was used to determine statistical differences in the development of proteinuria between study groups. Unpaired comparisons between samples from congenic strains and control mice were analyzed by the Student t test for cell frequencies, mean fluorescence intensities, and cytokine secretion. p values ≤ 0.05 were considered significant and are denoted in figure legends.

Online supplemental material

The PCR primer sequences used for genotyping are shown in Supplemental Table I. A summary of ANA IgG production in 7-mo-old congenic strains and F1 progeny of crosses with NZW mice is shown in Supplemental Table II. A comparison of serum ANA IgG and cytokine (IFNγ, IL-12) levels in 4- and 7-mo-old animals is shown in Supplemental Fig. 1. Measurement of cytokines by ELISA

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Nba2-ABC mice (7), were reduced in congenic strains harboring the NZB-derived Ifi interval, providing further confirmation (Supplemental Fig. 2).

**Inheritance of the NZB FcγR interval from Nba2 results in decreased expression of the FcγRIIb inhibitory receptor during terminal B cell differentiation and is associated with impaired apoptosis**

Signaling via the inhibitory FcγRIIb, which is the only Fcγ receptor expressed on murine B cells (23), can modify B cell responses and mediate apoptosis (24–26). Polymorphisms in the NZB FcγRIIb promoter have been identified within Nba2 that can result in the failure to upregulate FcγRIIb expression on GC B cells and PCs of NZB mice (13–15). Thus, impaired FcγRIIb expression could mediate B cell hyperactivity and survival in congenic B6,Nba2 strains. We therefore determined by flow cytometric analysis whether FcγRIIb membrane expression could be upregulated as B cells differentiated to GC B cells and PCs in vivo. Splenocytes of mice that were immunized for 7 d with the T cell-dependent Ag, NP-KLH, were prepared and labeled with fluorescent-conjugated mAbs specific for the B cell marker B220, the GC marker GL7 (27), the PC marker CD138 (28), and FcγRII/RIII. Because FcγRII is not expressed on murine B cells, this identifies B cells expressing FcγRIIb alone. Results demonstrated that GC B cells and PCs of spleens of control B6 mice express increased FcγRIIb levels compared with mature B cells (Fig. 4A). This pattern of expression was also found on GC B cells and PCs from spleens of control B6 mice as well as on the two main DC subsets, cDC and pDC, in vivo. Analysis of the absolute cell numbers from the various strains revealed an increase in the overall numbers of spleenic B cells among the congenic strains compared with control B6 mice (Fig. 4B). Thus, inheritance of the A, B, or C region of Nba2 influences the frequency of spleenic B cells. Enumeration of total GC B cells within spleens of congenic strains maps this phenotype to the A region because we found significantly increased numbers of GC B cells in congenic ABC, A, and A’B animals compared with B6 mice or congenic lines without the NZB-derived A region (p < 0.05). Finally, total PC numbers were highest in congenic ABC and A’B strains (p < 0.05), suggesting that combined expression of the distal A region and B region within Nba2 control PC survival.

We next determined whether differential FcγRIIb expression among the congenic strains controlled apoptosis. Purified spleen B cells were stimulated with culture grade anti-FcγRII/III Ab or IgG (Fab’2) in vitro and analyzed by flow cytometry for intracellular expression of the cleaved, active form of caspase-3 as an indicator of apoptosis. Results demonstrated that GC B cells and PCs of congenic ABC, A, and A’B mice did not undergo apoptosis in response to FcγRIIb cross-linking compared with cells from control mice and congenic B, BC, and C strains (Fig. 4C). Apoptosis in these latter strains was specific for FcγRIIb because GC B cells and PCs failed to undergo apoptosis when cultured with IgG (Fab’2). Confirmation of FcγRIIb-mediated apoptosis was further provided by analysis of cells from cultures that contained the caspase-3 inhibitor ZVAD. Staining for caspase-3 from all B cells in response to DMSO exposure served as positive controls for all strains. Together, these results indicate that the FcγR interval within Nba2 impairs FcγRIIb expression and function on terminally differentiating B cells, suggesting that this interval influences survival.

**Inheritance of the SLAM interval within Nba2 is associated with heightened numbers of spleen DC subsets that have an altered phenotype**

DCs initiate and regulate humoral immunity by controlling T and B lymphocyte activation through expression of membrane-anchored costimulatory proteins as well as secreted factors (29, 30). Thus, it is believed that DCs play a critical role in maintaining tolerance during Ag recognition by lymphocytes. We therefore determined the frequency of the two main DC subsets, cDC and pDC, in spleens of 4-mo-old female mice based on the level of CD11c and
B220 expression (Fig. 5A). The percentages of cDC and pDC within total spleen cells were used to quantify absolute numbers for each strain. Results demonstrated that total numbers of pDCs were significantly increased in spleens of congenic ABC, A, and B mice compared with control animals. Although modest splenomegaly was observed in the congenic ABC strain, we found no statistically significant differences in the total cellularity of spleens between the subcongenic strains and control mice. In contrast, an increase in the percentage values of total pDCs was determined from ABC (38%), A (34%), and B (29%) strains compared with B6 controls (13%), suggesting that increased numbers of total pDCs is not attributed to a dramatic increase in overall cellularity. Absolute numbers of pDCs were also increased in congenic A and C strains compared with controls, but overall were lower than other strains; these differences were not statistically significant. Total numbers of splenic cDCs among congenic lines were generally higher than in control mice, but these differences were much smaller compared with numbers of pDCs and were not statistically significant. These data suggest that expression of the SLAM interval (B6.Nba2-ABC, -A'B, and -B strains) controls the development and/or life span of pDCs.

We have previously demonstrated that type I IFN (IFNαβ) signaling is involved in the spontaneous development of renal disease in (B6.Nba2-ABC × NZW)F1 mice (21, 31). Within healthy mice, it has been demonstrated that the highest amount of IFNα is produced by a minor subset of pDCs that expresses the marker CD19 (32, 33). We therefore determined the steady-state frequencies of CD19-2 and CD19+ pDCs in spleens of 2- and 4-mo-old mice by flow cytometry (Fig. 5B). Results demonstrated that CD19+ pDCs represented a low percentage of overall pDCs in B6 control mice regardless of age. In contrast, B6.Nba2-ABC mice showed an increased frequency of CD19+ pDCs in an age-dependent manner with CD19+ pDCs representing ∼3% and 25% of total pDCs in 2- and 4-mo-old mice, respectively. Absolute numbers of CD19 pDCs were similar in all strains tested. In contrast, strains expressing the Nba2-derived SLAM interval (ABC, A'B, and B mice) yielded a statistically significant higher number of CD19+ pDCs (Fig. 5B). Siglec-H receptor expression has previously been demonstrated to be restricted to type I IFN-producing pDCs (34, 35). We confirmed that essentially no cDCs (CD11cB220 PDCA1) expressed Siglec-H (Fig. 5C). However, both CD19- and CD19+ pDCs (CD11cB220 PDCA1)
expressing Siglec-H were increased in frequency within congenic ABC, A'B, and B strains compared with control mice. Although a greater percentage of CD19⁻ pDCs expressed Siglec-H relative to CD19⁺ pDCs, quantified numbers of total cells shown in Fig. 5B indicated that the majority of pDCs were CD19⁺. These findings indicate that the central region of \( Nba2 \) inappropriately controls the homeostasis of CD19⁺ pDCs in an age-dependent manner and promotes the expression of Siglec-H on both CD19⁻ and CD19⁺ pDCs, suggesting that these cells may produce IFNα.

Previous studies have demonstrated that \( Nba2 \) does not influence the percentage of marginal zone B cells and B1 B cells in spleen (36). However, because of the dramatic and novel increase in CD19⁺ pDCs within \( Nba2 \) congenic strains, we performed three additional analyses to confirm that this population was not the result of contaminating B cells. First, we electronically sort-purified B cells, cDCs, CD19⁻ pDCs, and CD19⁺ pDCs from spleens of B6.Nba2-ABC mice and analyzed Ig gene rearrangement by PCR using a previously established strategy (19). This approach amplifies DNA corresponding to sequences that are lost on D-J rearrangement (5' \( JH1 \)) and that are lost on V to D-J rearrangement (5' \( DFL16.1 \)). Thus, amplification of \( JH1 \) and \( DFL16.1 \) transcripts will be lost in bona fide B cells. Because it has been demonstrated that low levels of D-J rearrangement can occur in splenic pDCs but not cDCs using other PCR-based strategies (37–39), we chose this strategy as a more unambiguous method to determine whether CD19⁺ pDCs were comprised of B-lineage cells. This measure of Ig gene rearrangement clearly distinguished our cDC, CD19⁻ pDC, and CD19⁺ pDC populations from B cells (Fig. 6A). Second, we morphologically assessed cytospins of sort-purified DCs and B cells by Giemsa staining (Fig. 6B). Results demonstrated that CD19⁺ pDCs had the classical appearance of DCs, including large distinct nuclei, a very small cytoplasm, and dendrite formation (arrows) compared with B cells (40). Finally, we used multiparameter flow cytometry to define cDC and pDC subsets on the basis of cell surface marker expression (41, 42). In this study, we restricted our analyses to congenic ABC, A'B, and B strains as well as control mice because increased pDC numbers were statistically associated with these strains. Results

**FIGURE 3.** Expression levels of \( Ifi202 \) correspond with the \( Nba2 \) genotype of congenic strains. A, Real-time PCR analysis of \( Ifi202 \) expression in spleen cells from 4-mo-old congenic strains and B6 control mice. Results are expressed as the mean ± SEM from six mice. B, Extracts prepared from individual age-matched female mice were analyzed by immunoblotting using Abs specific to the indicated proteins. Densitometry values of protein expression relative to β-actin are shown. Data are representative of two experiments.

**FIGURE 4.** Reduced levels of FcγRIIb expression on PCs and GC B cells are associated with \( Nba2 \) genotype. Spleen cells were prepared from 4-mo-old female mice immunized with 100 μg NP-KLH Ag for 7 d. A, The levels of FcγRIIb membrane expression on DAPI⁵2B220⁺GL7⁺ gated GC B cells (open black histograms), DAPI⁵2B20⁺/⁵2B220⁻/⁵2CD138⁺/⁵2CD1138⁺ gated PCs (solid red histograms), and DAPI⁵2B20⁺/⁵2GL7⁺ gated B cells (solid gray histograms) were determined by flow cytometry. B, Total numbers were quantified for the indicated cell populations from immune animals shown in A. Data shown are expressed as the mean ± SEM for six mice per group. Statistical differences between congenic strains and B6 control mice are indicated as \( p < 0.05 \). C, T cell-depleted spleen cells were cultured 4 h with plate-bound rat IgG (Fab')₂ (gray histograms), anti-FcγRIIb (2.4G2; red histograms) alone, or with the caspase-3 inhibitor ZVAD (blue histograms). Apoptosis was determined by intracellular caspase-3 staining of GC B cells and PCs. Numbers represent the percentage of apoptotic cells. Open black histograms represent isotype-matched control staining. Cells cultured 4 h in the presence of 10 μM DMSO were stained for intracellular caspase-3 (bottom row; red histograms) as a positive control for apoptosis. Solid gray histograms represent isotype-matched Ab staining.
demonstrated that cDCs from all strains exhibited a phenotype consistent with murine cDCs, including high levels of MHC class II (MHCII) expression, negative expression of the pDC marker PDCA1, little CD4 staining, and a bimodal distribution of CD8α that segregates cDCs into lymphoid (CD8αhigh) and myeloid (CD8αlow) DC subsets. CD192 pDCs from control and B6.Nba2-B mice exhibited a phenotype consistent with murine pDCs, including lower levels of MHCII expression, positive staining for PDCA1, and variable levels of CD4 and CD8α staining. CD192 pDCs from congenic ABC and A9B strains demonstrated an equivalent staining profile with the exception of increased MHCII expression. Interestingly, CD19+ pDCs from all strains unimodally expressed high levels of PDCA1, no CD4 staining, and variable but increased levels of CD8α expression compared with their CD192 pDC counterparts. In contrast to CD192 pDCs, CD19+ pDCs from all strains expressed higher levels of MHCII with statistically significant greater expression of MHCII on CD192 pDCs from the ABC and A9B congenic strains compared with control mice and the B congenic strain (Fig. 6C). This was also the case for CD80 expression where CD19+ pDCs expressed higher levels of CD80 compared with CD192 pDCs. However, increased CD86 levels were consistently measured on CD192 pDCs from ABC and A9B strains compared with control mice and the B congenic strain (Fig. 6C). These results suggest that CD19+ pDCs generally exhibit an activated phenotype relative to cDC and CD192 pDCs under steady-state conditions, and that expression of the FcγR and SLAM intervals from Nba2 can augment activation of both CD192 and CD19+ pDCs.

Steady-state expression levels of the costimulatory molecules CD40, CD80, and CD86 were also examined to determine whether pDCs exhibited an altered activation phenotype. Results demonstrated that although CD19+ pDCs overall expressed higher amounts of CD40 compared with their CD192 pDC counterparts, both CD192 and CD19+ pDCs from ABC and A9B congenic strains expressed significantly higher levels of CD40 (Fig. 6C). No significant differences in CD80 expression were measured among the strains albeit CD19+ pDCs generally expressed higher levels of CD80 compared with CD192 pDCs (Supplemental Fig. 4). This is consistent with the expression of CD86 where CD19+ pDCs expressed higher levels of CD86 compared with CD192 pDCs. However, increased CD86 levels were consistently measured on CD192 pDCs from ABC and A9B strains compared with control mice and the B congenic strain (Fig. 6C). These results suggest that CD19+ pDCs generally exhibit an activated phenotype relative to cDC and CD192 pDCs under steady-state conditions, and that expression of the FcγR and SLAM intervals from Nba2 can augment activation of both CD192 and CD19+ pDCs.
measured on CD19+ pDCs, reduced expression levels of CD84 were measured on CD19+ pDCs, and reduced expression of Ly108 was measured on both CD19+ and CD19+ pDCs (Fig. 6C). Expression of SLAM receptor transcript levels in pDCs among congenic strains did not show a strong correlation with protein expression (Supplemental Fig. 5), suggesting that posttranscriptional regulation may contribute to differences in membrane receptor expression on pDCs.

**Combined expression of FcγR and SLAM intervals within Nba2 controls TLR9 sensitivity of pDCs to produce high amounts of cytokines that promote humoral immunity**

Recognition of pathogen-associated molecules by TLRs expressed on DCs triggers their activation that, in turn, results in the production of cytokines IL-6, IL-10, IL-12, and IFNα that promote humoral immunity by inducing growth and differentiation of B cells (44–47). Defective TLR signaling can inappropriately activate DCs, in particular pDCs, to secrete cytokines and has been implicated in facilitating the breakdown of tolerance [reviewed in (48)]. Therefore, we determined the cytokine-producing capacities of freshly isolated DCs in response to TLR signaling. Initial studies analyzed IL-10 and IL-6 secretion from total CD11c+ DCs isolated from spleens of all DCs in response to TLR signaling. Initial studies analyzed IL-10 and cilitating the breakdown of tolerance [reviewed in (48)]. Therefore, particular pDCs, to secrete cytokines and has been implicated in fa-

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control TLR hypersensitivity of DCs. To more thor-
oughly determine cytokine production, we analyzed the capacity of
splenic DCs from control and congenic ABC mice to secrete IL-6,
IL-10, IL-12, and IFNα in response to LPS, CpG, and the TLR7
stimulus R837 (imiquimod). Results demonstrated that although
TLR stimulation induced IL-6 and IL-12 secretion, there were no
differences measured between control and ABC congenic DCs (Fig.
7A). In contrast, DCs from congenic ABC mice produced significantly greater amounts of IL-10 and IFNα compared with control DCs where TLR9 stimulation induced the most cytokine. To determine whether increased levels of IL-10 and IFNα secretion by DCs from congenic ABC mice were the product of CD19+ or CD19+ pDCs, sort-purified pDC subsets were tested for cytokine secretion after TLR stimulation. Results demonstrated that both CD19+ and CD19+ pDCs from B6.Nba2-ABC mice produced significantly higher amounts of IL-10 compared with control pDCs, with the largest amount of IL-10 being produced in response to TLR9 stimulation (Fig. 7B). Similar results were measured for IFNα where both CD19+ and CD19+ pDCs from congenic mice produced the highest amount of cytokine after TLR9 stimulation. These data have at least two important findings. First, Nba2 controls aberrant cytokine production by DCs, yet of the cytokines that influence B cell differentiation is limited to deregulation of IL-10 and IFNα. Second, although Nba2 dramatically increases the frequency of CD19+ pDCs (Fig. 5), the cytokine-producing capacities of both CD19+ and CD19+ pDCs are controlled by Nba2.

TLR9 stimulated pDCs can induce terminal B cell differentiation independent of T cell help and Ag (49, 50). Therefore, we determined the functional capacity of pDCs to license B cells to undergo PC differentiation. Sort-purified pDCs from spleens of congenic ABC, A′B, B, and C strains were cocultured with B6 control B cells in the presence of CpG. After 3 d, the frequency of total Ig-secreting PCs was quantified by ELISPOT. Coculture of
pDCs and B cells from B6 control mice served as a normal control. Results demonstrated that B cells cultured alone, with CpG, or with the control GpC oligonucleotide produced only small numbers of PCs, indicating that by itself TLR9 signaling on B cells minimally supports B cell differentiation (Fig. 8A). Parallel cultures containing only pDCs served as negative controls. Significant numbers of PCs were measured when B cells and pDCs from control and congenic ABC mice were cocultured in the presence of CpG, with the highest number of PCs generated from groups containing congenic ABC pDCs. This response was specific for TLR9 activation because the addition of control GpC did not induce PC differentiation. Furthermore, the ability of pDCs to drive PC differentiation was dependent, in part, by secretion of IL-10 and IFNα as demonstrated by reduced PC numbers when neutralizing mAbs to these cytokines were added to cocultures (Fig. 8B). Significantly high numbers of PCs were generated in cocultures containing pDCs from congenic ABC, A’B, and B strains, suggesting that the Nba2-derived SLAM interval alone is sufficient for promoting heightened Ab production in a TLR9-dependent manner. Analysis of cocultures containing pDCs from congenic C mice, which do not express the SLAM interval from Nba2, produced significantly fewer PCs.

**Discussion**

We have previously shown that when the entire 40 Mb Nba2 locus is expressed on the nonautoimmune B6 background, female congenic animals produce elevated levels of serum ANA, increased B cell proliferation, increased type I IFN, and severe GN when crossed with NZW mice (7, 21). In this study, we analyzed subcongenic strains to determine the gene clusters within Nba2 that contribute to these autoimmune traits. The analysis of five subcongenic strains revealed that combined expression of the FcγR and SLAM intervals (A’B strain), spanning ~6.8 Mb within the central region of Nba2, is sufficient to reproduce the autoimmune phenotype of the parental congenic strain. Further analyses of subcongenic strains that independently express the FcγR interval (A strain) from the SLAM interval (B, BC, and C strains) demonstrated that each of these gene clusters, alone, could not drive autoantibody production. Interestingly, these studies also revealed that genes located proximal to the FcγR interval as well as genes located distal to the SLAM interval do not contribute to ANA production, even if allowed to epistatically interact with susceptibility genes derived from NZW mice (A, C strains × NZW). This suggests that the lupus susceptibility genes located within the Ij family do not mediate a loss in B cell tolerance and autoantibody production. However, genes of the Ij200 family could indirectly contribute to the autoimmune phenotype by inhibiting p53- and E2F1-mediated proapoptotic functions as has previously been suggested (22, 51). Furthermore, data presented in Supplemental Fig. 5 suggests that the SLAM genes, CD48, CD84, and CD229 could be epistatically regulated by genes expressed within the Ij interval.

The contribution of the FcγR interval in mediating autoantibody production appears to be through deregulation of B cell homeostasis by reducing FcγRIIb expression on terminally differentiating B cells, resulting in impaired apoptosis. Human SLE genome screens have consistently identified significant linkage at the 1q23 locus where FcγR genes reside (52–54), suggesting that one or more of the Fc receptor genes contributes to human SLE. This hypothesis is supported in murine experimental systems that have demonstrated a deficiency in the FcγRIIb gene leads to increased ANA IgG (12, 55). Although the most likely candidates in the A’ region of Nba2 are the FcγR genes, it is possible that other genes play a role in B cell differentiation and survival. Fine mapping the 122 genes that span the A’ region by generating additional subcongenic lines that contain narrower intervals will facilitate identifying candidate genes.

How inheritance of the Nba2 B region, containing the SLAM interval, contributes to the disease process is less clear. Genomic characterization of B6.Sle1b congenic mice has demonstrated extensive polymorphisms in exons encoding the extracellular domains of several SLAM receptors, including CD48, CD84, and Ly108 (16). Although it is not understood how these polymorphic variants mediate the Sle1b autoimmune phenotype, differential receptor expression on both T and B lymphocytes may influence cellular activation and survival. Increased levels of the ly108-1 isoform by B cells from B6.Sle1b mice are associated with increased survival and impaired deletion of autoreactive B cells (17). Interestingly, we demonstrated that CD48, CD84, and Ly108 were differentially expressed on DCs from congenic ABC and A’B strains compared with the congenic B strain and control mice.

**FIGURE 7.** The Nba2 locus controls TLR hypersensitivity of DCs to produce proinflammatory cytokines. A, Total CD11c+ DCs were sort-purified from spleens of 4-mo-old B6.Nba2-ABC and B6 control mice, and were cultured 24 h in the absence (no treatment [NT]) or presence of 100 ng/ml LPS, 10 μg/ml CpG, and 10 μg/ml R837. Supernatants were collected and the amount of cytokine was determined by ELISA. Statistical differences between TLR-activated and control cultures (B strain) were indicated by **p < 0.05, ***p < 0.01, and ****p < 0.001. B, Using identical culture conditions, levels of IL-10 and IFNα were determined from purified total pDCs or CD19+ and CD19– pDCs. Data are expressed as the mean ± SEM from 6–9 mice per group. NT, no treatment.
phenotype of pDCs were controlled by both
CD19+ pDCs were similarly elevated in congenic BC and C
strains compared with B6 control mice, further suggesting that
genes within the C region of Nba2 influence the expansion of
CD19+ pDCs. Mellor and coworkers (58) first identified CD19+ DCs as a rare subset of splenic DCs in healthy mice that could suppress T cell proliferation in a manner dependent on expression of the enzyme IDO by CD19+ DCs. Mellor and colleagues (33) subsequently demonstrated that splenic CD19+ DCs acquired potent IDO-dependent T cell suppressor activity after in vivo administration of CpG and that IFNα signaling was essential for IDO expression. Limited phenotypic analysis of these CD19+ DCs makes it unknown whether this population is identical to CD19+ pDCs found in congenic Nba2 strains. However, recent studies have demonstrated that IDO-expressing pDCs directly activated natural T regulatory cells for potent suppressor activity (59), and promoted the generation of inducible T regulatory cells (60). Current studies are underway to determine whether CD19+ pDCs from congenic Nha2 mice express IDO and are able to suppress T cell proliferation.

Consistent with increased numbers of pDCs from congenic ABC and A’B strains, were increased CD40, CD86, and Siglec-E expression levels compared with cDCs, suggesting that combined expression of FcγR and SLAM intervals may trigger heightened steady-state activation levels of pDCs to produce increased proinflammatory cytokines. Analysis of TLR9-stimulated DCs from congenic ABC mice demonstrated that only IL-10 and IFNα levels were significantly increased compared with control DCs, and moreover, were mainly produced by CD19+ and CD19+ pDCs. IL-10 and IFNα have been shown to be associated with proinflammatory properties during an immune response and to be deregulated in autoimmunity. Increased levels of serum IL-10 have been measured in SLE patients and correlate with ANA production and disease activity (61, 62). Furthermore, in both lupus-prone mice (63) and SLE patients (64), administration of a neutralizing IL-10 mAb reduced cutaneous lesions and rheumatologic symptoms, indicating that IL-10 is a major player in autoantibody production and mediating tissue damage. How IL-10 specifically functions in the disease process is unclear, but the capacity of IL-10 to promote B cell proliferation and differentiation to PCs strongly suggests it alters the B cell compartment by increasing the generation of ANA-producing PCs (65, 66). Similarly, IFNα is also believed to be an important proinflammatory cytokine in the generation of autoreactive PCs (44), with pDCs being the primary source of IFNα (67–69). Analysis of pDCs from congenic strains demonstrated that the SLAM interval within Nba2 licenses pDCs to drive PC differentiation in an IL-10- and IFNα-dependent manner.

In conclusion, we have narrowed down the contribution of the Nba2 locus to murine lupus susceptibility to a central region (169.1–175.9 Mb) that contains the FcγR and SLAM intervals. The combined expression of both intervals influences the homeostasis of GC B cells and PCs, and promotes PC differentiation through deregulated activation of pDCs. Importantly, our findings advance our understanding of how susceptibility genes within the telomeric region of chromosome 1 control autoantibody production and indicate that the FcγR and SLAM intervals each control different immune pathways, yet are both required for the development of ANA and renal disease.

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Disclosures
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