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Humoral Autoimmunity to Basement Membrane Antigens Is Regulated in C57BL/6 and MRL/MpJ Mice Transgenic for Anti-Laminin Ig Receptors

Earl H. Rudolph,* Kendra L. Congdon,* Faustina N. A. Sackey,* Muriel M. Fitzsimons,† and Mary H. Foster2*†

Basement membrane proteins are targeted in organ-limited and systemic autoimmune nephritis, yet little is known about the origin or regulation of immunity to these complex extracellular matrices. We used mice transgenic for a nephrotropic systemic lupus erythematosus (SLE) Ig H chain to test the hypothesis that humoral immunity to basement membrane is actively regulated. The LamH-Cμ Ig H chain transgene combines with diverse L chains to produce nephrotropic Ig reactive with murine laminin α1. To determine the fate of transgene-bearing B cells in vivo, transgenic mice were outcrossed onto nonautoimmune B6 and SLE-prone MRL backgrounds and exposed to potent mitogen or Ag in adjuvant. In this work we demonstrate that transgenic auto-antibodies are absent in serum from M6 and M29 lineage transgenic mice and transgenic B cells hypoproliferate and fail to increase Ig production upon exposure to endotoxin or when subjected to B cell receptor cross-linking. Administration of LPS or immunization with autologous or heterologous laminin, maneuvers that induce nonoverlapping endogenous anti-laminin IgG responses, fails to induce a transgenic anti-laminin response. The marked reduction in splenic B cell number suggests that selected LamH-Cμ H chain and endogenous L chain combinations generate autospecificities that lead to B cell deletion. It thus appears that SLE-like anti-laminin B cells have access to and engage a tolerizing self-Ag in vivo. Failure to induce autoimmunity by global perturbations in immune regulation introduced by the MRL autoimmune background and exposure to potent environmental challenge suggests that humoral immunity to nephrotropic basement membrane epitopes targeted in systemic autoimmunity is tightly regulated. The Journal of Immunology, 2002, 168: 5943–5953.

Self-Ag is a primary target of immune effectors in most models of immunologic renal injury, yet our understanding of events that initiate nephritogenic responses is rudimentary. Structurally diverse soluble, matrix, and cell surface proteins and glycolipids distributed throughout the kidney incite injury in animals (reviewed in Ref. 1), whereas basement membrane proteins are the only confirmed Ag targets in human immune nephritis (2–5). Basement membranes are highly organized molecular scaffolds that require proper assembly to correctly present their cell and cytokine binding domains (6). Laminin secretion into the extracellular space and cell receptor-facilitated laminin polymerization are prerequisites for subsequent incorporation and assembly of type IV collagens, nidogens, and proteoglycans into basal lamina lattices. These components interact with adjacent cells via integrins and other receptors to induce cell and tissue differentiation and survival (7). Disruption of these interactions by natural or targeted mutations or by autoantibody binding produces disease, the nature of which depends on the organ distribution of the targeted matrix component (7, 8). Multiple laminin and collagen isoforms exist, each of which has a characteristic tissue restriction. To date, 12 different laminin heterotrimers have been described, each formed by a different combination of the known α-, β-, and γ-chains. Laminin-1 (α1β1γ1) and laminin-5 (α3β1γ2) have been identified as targets of pathogenic autoantibodies in immune nephritis and blistering dermatoses, respectively (9–14). However, the role played by these Ags in inducing or regulating autoimmune responses is unclear, because the accessibility of pathogenic matrix epitopes to B cells and their capacity to crosslink Ig receptors to trigger tolerance or B cell activation is unknown.

It is possible that the manner in which matrix epitopes engage B cell receptors differs substantially from that of freely soluble self-Ag or cell-bound protein capable of capping within the lipid interface of target cell membranes. Considerable work has shown that signals generated by ubiquitous cell membrane-bound or multivalent self-Ag capable of extensive Ig cross-linking are particularly likely to induce editing and clonal deletion, whereas soluble oligovalent self-Ag is more likely to induce a state of functional inactivation termed clonal anergy (15–21). Some self-Ags fail to effectively engage B cells due to subthreshold Ag concentration or molecular or anatomic sequestration, such that the immune system remains ignorant of Ag or tolerance is limited to the T cell compartment (22, 23). Whether signals generated by cell contact with matrix, if it occurs, can commit B cells to similar outcomes is unclear.

We previously reported the generation and initial characterization of an anti-laminin Ig transgenic (Tg) model established by rendering nonautoimmune C57BL/6 (B6) mice Tg for a dominant nephrotropic Ig H chain, termed LamH-Cμ. The H chain V region

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was cloned from an anti-laminin IgG, mAb H50, derived from a systemic lupus erythematosus (SLE)3 MRL/MpJ- Tnfrsf6br (formerly, MRL/MpJ-Fasbr; hereafter, MRL/lpr) mouse with nephritis (24). It binds in vitro to murine laminin-1, including an epitope on the o1 chain expressed in adult glomerulus and renal tubular basement membranes, and forms renal immune deposits in vivo (24). In LamH-Cu Tg lines derived from three founders, two distinct phenotypes were observed (25). Mice of the M7 lineage spontaneously produce nephrotropic anti-laminin Ig but express little B cell membrane IgM, suggesting that M7 B cells are incapable of receiving tolerogenic signals and thus escape normal regulation. Nonetheless, recovery of diverse Tg autantibodies from the M7 lineage reveals that the nonautoimmune mouse has considerable capacity to generate a heterogeneous population of lupus-like anti-matrix B cells. In contrast, progeny of the M29 and M6 Tg lines produced abundant Tg B cells, but anti-laminin mAb were rarely recovered by fusion of LPS-stimulated splenocytes, suggesting that these cells were rendered nonfunctional in vivo (25). Herein we further characterize LamH-Cu Tg B cells in the M29 and M6 lineages and determine whether potent environmental stimulation or the influence of the MRL autoimmune background can induce a Tg anti-laminin response.

Materials and Methods

Animals

Cloning of the anti-laminin LamH-Cu H chain construct and initial characterization of Tg mice are described elsewhere (25, 26). Offspring were genotyped at 2–3 wk by PCR of tail DNA as described (25). Experiments herein were conducted on the seventh through tenth B6 backcross of the M29 and M6 lineages. Tg B6 mice of the M6 lineage were outcrossed with SLE-prone MRL/MpJ breeders for 8–10 generations to reconstitute MRL autoimmune genetic predisposition. Hemizygous Tg and non-Tg mice of either sex reared under conventional conditions were used. B6 and MRL/MpJ breeders and BALB/c (a-allotype) and MRL/MpJ-Tnfrsf6 (j-allotype, MRL autoimmune genetic predisposition. Hemizygous Tg – fl mice are described elsewhere (25, 26). Offspring were genotyped at 2–3 wk by PCR of tail DNA as described (25). Experiments herein were conducted on the seventh through tenth B6 backcross of the M29 and M6 lineages. Tg B6 mice of the M6 lineage were outcrossed with SLE-prone MRL/MpJ breeders for 8–10 generations to reconstitute MRL autoimmune genetic predisposition. Hemizygous Tg and non-Tg mice of either sex reared under conventional conditions were used. B6 and MRL/MpJ breeders and BALB/c (a-allotype) and MRL/MpJ (j-allotype, which serologically cross-reacts with a-allotype) control mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All studies and procedures were approved by the Animal Care and Use Committees of the University of Pennsylvania, Duke University, and the Durham Veteran’s Affairs Medical Center.

Flow cytometry

Single-cell suspensions were prepared and stained as described (21). FACS analysis was performed with a FACSScan (BD Biosciences, San Jose, CA). List mode data were collected with live gating on small lymphocytes (by light scatter) on 10,000–20,000 cellular events and analyzed with CellQuest software (BD Biosciences). To measure cell proliferation, splenocytes were tagged with a nontoxic fluorescent dye, CFSE (Molecular Probes, Eugene, OR), that binds irreversibly to cell proteins. As cells divide, their fluorescence halves sequentially with each generation and provides a marker of proliferation. Single-cell suspensions were labeled with 2.5 μM CFSE and cultured at 1 × 106/ml at 37°C with 5% CO2 for 2.5–3.5 days in proliferation medium with 15% FCS (HyClone Laboratories, Logan, UT), 5–50 μM 2-ME (EM Science, Gibbstown, NJ), 0.3% NaN3 (Fisher Chemical, Pittsburgh, PA), and the following additives obtained from Invitrogen (Carlsbad, CA): 1% MEm nonessential amino acids, 1% sodium pyruvate, 2.5% HEPES, 1% l-glutamine, and 1% penicillin-streptomycin. Mitogens included LPS (Escherichia coli 055:B5; Sigma-Aldrich, St. Louis, MO); anti-IgM-FlaAb3 (Pierce, Rockford, IL) in combination with subnontogenic LPS; PMA (Sigma-Aldrich) in combination with ionomycin (Sigma-Aldrich), or rat anti-mouse recombinant CD40 (Southern Biotechnology Associates, Birmingham, AL) with IL-4 (Sigma-Aldrich). After harvest cells were labeled with PE- or FITC-tagged reagents. Proliferation in B220 lymphocyte-gated cells was measured as the ratio of geometric mean fluorescence intensity (MFI, FL1 for CFSE, logarithmic data) of cells cultured with medium alone divided by geometric MFI of cells cultured with stimulant.

1 Abbreviations used in this paper: SLE, systemic lupus erythematosus; MFI, mean fluorescence intensity; Tg, transgenic; EHS, Engelbreth-Holm-Swarm.

Cell lines and Abs

Reagents for flow cytometry were obtained from BD Pharmingen (San Diego, CA). The origins of cell lines and mAb were described previously: MRL/lpr-derived hybridoma anti-laminin Ig H50 (24) and anti-ssDNA IgM, H130 (27); IgM transfectant LamH/238L (26); Tg anti-laminin mAb 54 and 61 (28); IgM transfectant 238H/3558L, termed BGC (29); and anti-LamH-Id IgG, C10G (30), and anti-DNA IgG H241 (31). Alkaline phosphatase-conjugated anti-isotype reagents and avidin were obtained from Pierce. MOPC 104E and MOPC 141 (Sigma-Aldrich) were used as isotype and allotype standards.

In vitro and in vivo stimulation

Differentiation into Ab-secreting cells was assessed using supernatants of 7– to 10-day cultures of 105 unseparated or T cell-depleted splenocytes plated in 50–75 μg/ml LPS in proliferation medium. Mice were immunized with 0.25 mg/ml emulsified murine Engelbreth-Holm-Swarm (EHS)-laminin (Sigma-Aldrich), human placental laminin (Sigma-Aldrich), or PBS in CFA (Sigma-Aldrich) or RIBI adjuvant (monophosphoryl lipid A + synthetic rethale dicorynomycolate + cell wall skeleton; Sigma-Aldrich). Each mouse was immunized at two sites (i.p. and s.c. interscapular) and 1 wk later a site receiving 100 μl injectant (50 μg laminin in 200 μl adjuvant per mouse). Every 14–21 days serum samples were obtained by tail bleeding and injection protocols repeated (substituting IFA; Sigma-Aldrich) for a total of three for four immunizations per mouse. E. coli LPS (50 or 500 μg; Sigma-Aldrich) dispersed in saline was administered i.p.

ELISA and competition ELISA

Ig concentrations, Ab activity, and allotype-specific binding in serum or culture supernatants were determined by ELISA as described (26, 28). For Ag binding, results are reported as mean OD405 on Ag minus mean OD405 on sham (diluent)-coated wells. For IgM-a anti-laminin assay, positive serum is defined as OD > mean + 3 SD for IgM-b B6 mouse serum. Serum Id expression was determined by ELISA using Immulon II HB plates (Thermo Lab Systems, Franklin, MA) coated overnight at 4°C with culture supernatant containing rat anti-LamH-Id IgG mAb and bound Ig was detected with labeled goat anti-mouse IgG plus IgM.

Expression of LamH Id by laminin-binding Ig was determined by competition ELISA. The dilution of serum that gave 50% of maximal binding to laminin-coated microplates was incubated with varying concentrations of purified rat monoclonal anti-LamH-Id IgG for 1 h at room temperature before plating on laminin-coated microplates. Bound Ig was detected with labeled goat anti-mouse IgG plus IgM. Results are reported as the reciprocal of the concentration of laminin binding: (OD405 with inhibitor/OD405 without inhibitor) × 100. A similar assay substituting purified Tg anti-laminin mAb 61 (28) as inhibitor, detected with goat anti-mouse IgG, was used to determine whether induced endogenous anti-laminin IgBind the same laminin epitopes as do Tg anti-laminin Ig. To determine ssDNA binding by Id serum IgM from MRL mice, dilutions of serum IgM that gave 50% of maximal binding to anti-Id-coated microplates were incubated with soluble ssDNA as inhibitor.

Statistical analyses

All data are shown as mean values ± SD unless otherwise indicated. Comparisons between groups were made using the Student t test. A value of p < 0.05 was considered significant.

Results

Rationale

The autoreactive Ig Tg approach was chosen for this work because the model provides sufficient numbers of B cells bearing the nephritogenic LamH-Cu Ig receptor. This permits study of the receptor under well-defined conditions and within complex immunologic microenvironments in vivo. The type of interaction, if any, between Tg B cells and self-Ag during development and transit in vivo determines cell fate and tolerance phenotype. The model also permits study of the influence on cell fate of different disease-relevant genetic backgrounds and environmental factors and provides insights into mechanisms of regulation of anti-matrix nephritogenic B cells.
Phenotypic characterization of B6 mice expressing the LamH-Cμ Ig H chain transgene

We previously determined that B cells from early generation B6 backcross mice of Tg(B220) M29 and M6 express substantial Tg(B220) surface IgM. FACS analysis of sixth or later generation mice used in the current studies confirms persistence of this phenotype (Fig. 1, A–C; M29 lineage). Surface expression of the H chain transgene was restricted to B220+ spleen cells (i.e., B lymphocytes; Fig. 1B), and endogenous Igh-b allotype was generally excluded on Tg(B220) B cells (Fig. 1E). Dual labeling confirmed transgene LamH Id on B6 IgM-a Tg(B220) B cells from the M29 lineage; endogenous Id is rare in non-Tg littermates (Fig. 2, A and B).

Despite the abundance of Tg(B220) cells, spontaneous anti-laminin autoreactivity attributable to Tg(B220) was rarely detected in sera of unmanipulated B6 Tg(B220) M29 lineage mice (Fig. 3A). Only 1 of 25 mice had detectable anti-laminin IgM-a activity (OD = 0.113). Low to modest levels of Tg(B220) IgM-a were detected in 13 of 25 unmanipulated Tg(B220) M29 lineage mice (Fig. 3B), indicating that a subset of laminin nonreactive Tg(B220) B cells were spontaneously activated in some animals. Any IgM-a detected in non-Tg mice represents nonspecific background staining, as these mice express only the endogenous IgM-b allotype. Low-level Tg(B220) anti-ssDNA activity was detected in serum of 6 of 25 Tg(B220) M29 lineage mice (Fig. 3C). In contrast, Tg(B220) IgM-a, anti-laminin, and anti-DNA activity were readily detected in serum IgM of Tg(B220) mice of the aberrant and presumed unregulated M7 lineage (Fig. 3).

Impaired proliferation and differentiation of LamH-Cμ Tg(B220) B cells

The absence of spontaneous anti-laminin Ig production despite domination of the splenic B cell pool by Tg(B220) B cells indicates that anti-laminin Tg(B220) B cells are not activated by common environmental Ags and suggests the possibility that they are regulated in vivo. To examine this possibility, the ability of B6 Tg(B220) M29 lineage B cells to proliferate in vitro was assessed by flow cytometry using the nontoxic fluorescent dye CFSE. As shown in Fig. 4, B cells from non-Tg littermates proliferate vigorously in response to LPS, as indicated by decreased fluorescence in a significant proportion of B cells, compared with CFSE-stained but unstimulated cells. In contrast, proliferation was dampened among LPS-stimulated B cells from Tg(B220) mice (Fig. 4, B, D, and E). Labeling to detect IgM-a confirmed only limited proliferation among Tg(B220) B cells (Fig. 4F). To examine the possibility that Tg(B220) B cells are functionally competent but selectively nonresponsive to LPS, we assessed proliferation in response to anti-Ig, which mimics cross-linking of surface Ig by multivalent Ag. Anti-Ig induced robust proliferation in non-Tg B cells (Fig. 5, A and B). Proliferation among anti-Ig-stimulated Tg(B220) B cells was markedly diminished (Fig. 5, C–G). In some experiments, there was almost complete absence of a Tg(B220) cell proliferative response to IgM cross-linking. Dampened proliferation was also observed in B6 Tg(B220) B cells stimulated with anti-CD40 and IL-4 (proliferation indices: 1.85 ± 0.06 and 4.52 ± 1.16 for Tg(B220) and non-Tg B220+ cells, respectively; p < 0.05). To determine whether Tg(B220) B cells had a global...
defect in their proliferative capacity, cells were cultured with a combination of protein kinase C agonist PMA (5 ng/ml) and calcium ionophore ionomycin (1 μg/ml). Tg and non-Tg B cells proliferated robustly (Fig. 5H).

To determine whether Ig production by B6 Tg B cells was also dampened, cells were cultured in LPS or anti-IgM-F(ab')2 plus submitogenic LPS for 7–10 days, after which supernatants were assayed for Ab activity. Tg Ig reactivity to laminin was not detected in supernatants from cells subjected to any stimulus (Fig. 6A); sensitivity of the assay was confirmed by ready detection of 5 μg/ml Tg anti-laminin mAb 54 using IgM-a-specific second step (OD 2.40). Increased IgM levels were detected in supernatants from LPS-stimulated non-Tg splenocytes (Fig. 6B), confirming capacity of LPS to induce differentiation. IgM levels increased modestly in a subset of LPS-stimulated Tg supernatants. Neither antilaminin activity nor increased levels of IgM were detected in supernatants of B cells stimulated with PMA and ionomycin (Fig. 6), despite marked B cell proliferation induced by this combination (Fig. 5H).

**B cell receptor density**

Because altered surface expression of IgM and IgD consistent with receptor down-regulation has been described among functionally impaired B cells chronically exposed to soluble self-Ags in vivo, we determined receptor density on Tg and non-Tg B cells. Relative receptor densities were estimated by comparing channel numbers of linear MFI in non-isotype-switched splenic B cells stained with a FITC-conjugated anti-IgM and anti-IgD mixture. In the B6 background, MFI was lower although not statistically different among Tg compared with non-Tg B cells (106.8 ± 54.8 vs 132.6 ± 63.7; value of p not significant).

**B cell number**

Tg mice have smaller spleens and fewer splenic B cells than do their non-Tg littermates (Table I). B cells constituted 12–21% of splenocytes in B6 Tg mice compared with 40–58% in non-Tg mice, and total splenic B cell number was markedly and significantly lower in Tg mice. Consistent with this finding, a greater proportion of Tg splenocytes were T cells (Table I). Although the
FIGURE 5. Blunted in vitro proliferation of B6 LamH-Cμ M29 lineage Tg B cells in response to B cell receptor stimulation. Single-cell suspensions prepared as described in Fig. 4 were cultured with or without 10 μg/ml anti-IgM-F(ab’)_2 plus 5 μg/ml subunitogenic LPS. Cells were harvested and labeled with PE-conjugated reagents for the B cell surface marker B220 before flow cytometric analysis. A–F, Representative dot plots of log fluorescence data gated on lymphocytes on the basis of forward and side scatter for individual mice. G, Proliferation index is calculated as described in Materials and Methods, based on B220+ gated cells. Shown are results from one of three experiments for cells stimulated with anti-IgM-F(ab’)_2. Numbers of mice appear in parentheses. *p < 0.001 vs non-Tg cells. H, Proliferation index for B cells stimulated with a mixture of the protein kinase C agonist PMA (5 ng/ml) and the calcium ionophore ionomycin (1 μg/ml). *p < 0.005.

total number of T cells was reduced in Tg+ spleens, the distribution of CD4+ and CD8+ cells was similar in B6 Tg+ and non-Tg mice.

Characterization of LamH-Cμ Tg MRL/MpJ mice

Because the prototypic nephrotropic anti-laminin IgG H50, donor of the LamH V region gene, originated in an autoimmune mouse, it was of considerable interest to determine whether MRL genetic susceptibility, in the absence of the lpr accelerator gene, influenced expression of LamH-Cμ Tg+ autoreactivity. Multiple backcrosses onto MRL were necessary because disease susceptibility is inherited as a complex polygenic trait involving multiple nonlinked loci. Anti-Id reagents were used to identify Tg+ expression (Fig. 2) (30), because the MRL IgM-j allotype serologically cross-reacts with Tg+ IgM-a.

Similar to their B6 counterparts, the proportion and number of B cells were significantly reduced in MRL Tg+ spleens compared with spleens of non-Tg littermates (Table I). LamH Id and IgM were coexpressed on the surface of Tg+ MRL B cells (Fig. 2, C and D). Endogenous IgD was expressed only at low frequency (<2%) and low intensity (data not shown), confirming efficient allelic exclusion in MRL Tg+ mice. Despite the presence of a substantial population of Tg+ B cells, anti-laminin activity was absent in serum of all but 1 of 19 MRL Tg+ mice (Fig. 7). LamH Id was not detected in serum of the single MRL Tg+ mouse with anti-laminin activity, indicating that the anti-laminin Ig were of endogenous (non-Tg) origin. Anti-ssDNA IgM were detected in serum of all MRL mice (Fig. 7), whereas LamH Id-bearing IgM were detected (defined as OD >0.05, with OD 0.8 for positive control mAb 61 at 20 μg/ml) in only 3 of 19 MRL Tg+ and none of 17 MRL non-Tg mice. Thus, anti-DNA Ig in most MRL Tg+ mice was not Tg in origin. Of the three MRL Tg+ mice producing Id+ IgM, Id binding was not inhibitable with soluble ssDNA for two, whereas ssDNA partially inhibited Id binding (up to 50% inhibition at 500 μg/ml ssDNA) by serum IgM from the third mouse. This suggests that rare combinations of endogenous L chains with the LamH-Cμ transgene or with endogenous Id-related H chains can produce anti-DNA Ig lacking cross-reactivity to laminin.

MRL Tg+ B cells demonstrated impaired proliferation in vitro in response to endotoxin or Ig cross-linking (Fig. 8). Whereas MRL non-Tg B cells proliferate vigorously to LPS or anti-Ig, proliferation of Tg+ B cells was dampened (Fig. 8). Proliferation indices of cells stimulated by 20 μg/ml anti-Ig alone were 1 ± 0.2 (n = 4) and 1.5 ± 0.3 (n = 3) for Tg+ and non-Tg cells, respectively (p < 0.05). Nonetheless, as with B6 mice, both Tg+ and non-Tg MRL B cells proliferated robustly to PMA (5 ng/ml) with ionomycin (1 μg/ml). Anti-laminin IgM were not detected by ELISA in culture supernatants from either Tg+ or non-Tg MRL splenocytes cultured with LPS for 7 days. Low-level anti-ssDNA IgM activity was detected in supernatants from mice of both genotypes (data not shown). MFI of lymphocytes stained with the FITC-conjugated anti-IgM and anti-IgD mixture was significantly lower among MRL Tg+ cells (161.4 ± 24.1 vs 278.7 ± 15.3 for Tg+ and non-Tg cells, respectively, p < 0.001). Taken together, these results indicate functional compromise of Tg+ B cells in MRL mice.

Laminin immunization fails to induce a transgene-encoded response to autologous laminin

To determine whether the nonresponsiveness of LamH-Cμ B cells observed in vitro could be overcome in vivo by potent immune
stimulation and specific Ag immunization, we immunized B6 and MRL LamH-Cμ Tg+ and non-Tg mice with autologous (murine EHS) or heterologous (human placental) laminin in adjuvant and examined the immune response serologically. With one exception, anti-laminin Ig were absent or barely detectable in the preimmune sera of B6 and MRL Tg+/H11001 and non-Tg mice. One MRL Tg+/H11001 preimmune serum had modest spontaneous anti-laminin Ig activity of exclusively IgG isotype, which was therefore not Tg in origin.

Immunization with autologous laminin induced an anti-EHS-laminin Ig response in a subset of Tg+/H11001 and non-Tg B6 and MRL mice (Fig. 9, A and B). Among immunized B6 Tg+ mice, a weak to modest anti-laminin Ig response developed in two of eight, Table I.

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*a, p = 0.0001 vs non-Tg of same background strain.

p < 0.0001 vs non-Tg of same background strain.

FIGURE 6. Ig secretion by mitogen-stimulated B cells. Cells from Tg+ and non-Tg mice were cultured with or without LPS (75 μg/ml), anti-IgM-F(ab')2 (10 μg/ml) plus submitogenic LPS (5 μg/ml), or PMA (5 ng/ml) plus ionomycin (1 μg/ml) for >7 days, after which supernatants were assayed by ELISA. A, Tg anti-laminin IgM-a activity. B, Percentage of change in total IgM, calculated as ((OD with mitogen − OD without mitogen)/OD without mitogen) × 100. Control anti-laminin LamH-Cμ Tg IgM mAb 54 (5 μg/ml) is described in Materials and Methods. Shown are results from one of three experiments.

FIGURE 7. Serum transgene expression in MRL mice. Anti-laminin reactivity (A), transgene Id expression (B), and anti-ssDNA reactivity (C) among serum IgM from unmanipulated LamH-Cμ MRL Tg (n = 19) and non-Tg (n = 13–17) littermate mice. Id expression was measured by capture ELISA using plates coated with rat anti-LamH-Id IgG as described in Materials and Methods, and anti-EHS-laminin and anti-ssDNA reactivity are measured as mean OD405 on Ag-coated minus sham (diluent)-coated wells, using duplicate samples of serum diluted 1/25. Control Ig anti-laminin IgM mAb 54 (5 μg/ml) and mAb 61 (20 μg/ml), anti-laminin IgG, mAb H50 (10 μg/ml), and 238H-Cμ H chain-encoded nonautoactive transfectant IgM, BGC (10 μg/ml) are described in Materials and Methods. Serum from a 6-mo-old MRL/lpr SLE mouse is used at a 1/25 dilution.
whereas six of eight failed to produce anti-EHS-laminin Ig. In the two responding mice, assay for Tg$^+$ allosite revealed little or no IgM-a among serum anti-laminin Ig (OD = 0 and 0.076, respectively, at 1/20 dilution), indicating an endogenous and not Tg origin. This was confirmed by isotype analysis, which revealed exclusively IgG among induced serum anti-laminin Ig. Binding to laminin was not inhibitable with rat anti-LamH-Id IgG, previously determined to recognize and mask the LamH H chain V region Ag binding site (30) (Fig. 9C), indicating that the endogenous B6 response arose from a B cell population that did not share Id with LamH, and ruling out the possibility of isotype switch involving the LamH-C$\mu$ H chain conventional transgene. Anti-EHS-laminin Ig were not detected in serum of any of four B6 Tg$^+$ mice injected with adjuvant alone or in serum of B6 Tg$^+$ mice after i.p. injection of 50 or 500 $\mu$g LPS (data not shown).

Among MRL Tg$^+$ mice, an anti-EHS-laminin response was detected in immune serum from four of six immunized mice (Fig. 9A). As with B6 mice, isotype analysis indicated that IgM did not contribute to the anti-laminin response, consistent with an endogenous origin. This was confirmed by lack of inhibition of Ag binding using anti-LamH-Id (Fig. 9C).

To determine whether immunization-induced endogenous (non-Tg) anti-laminin IgG react with the same epitope(s) on laminin as do nephrotropic Tg$^-$ SLE-like Abs, we attempted to inhibit Ag binding using a Tg$^-$ IgG, termed mAb 61 (28). Whereas laminin binding by SLE-derived mAb H50 is nearly completely inhibited by mAb 61, mAb 61 does not inhibit EHS-laminin binding by evoked B6 or MRL endogenous IgG (Fig. 9D), indicating binding to different laminin epitopes.

A subset of B6 and MRL non-Tg littermate and wild-type B6 mice immunized with EHS-laminin or injected with adjuvant alone also developed weak-to-modest anti-laminin IgG responses (Fig. 9, A and B). Anti-Id did not inhibit laminin binding, similar to the situation with endogenous Ig in Tg$^-$ mice and indicating that the presence of the Tg$^+$ does not alter the endogenous response.
Because the prototypic MRL/pr-derived anti-laminin IgG binds well to both autologous and heterologous laminin (Fig. 10B), we tested whether immunization with human placental Ag in adjuvant superimposed on inherited MRL autoimmune susceptibility would be sufficient to induce a Tg⁺ anti-EHS-laminin response. Binding to heterologous laminin was not detected in preimmune serum with the exception of a low titer in a single MRL Tg mouse. Immunization of ninth generation MRL backcross mice induced an Ig response to heterologous laminin in both Tg⁺ and non-Tg littermates (Fig. 10A). Binding to heterologous laminin was not inhibitable by coinoculation with anti-LamH-Id (Fig. 10B), indicating an endogenous origin. One immunized MRL Tg⁺ mouse developed high-titer Ig reactivity with autologous laminin (Fig. 10C). This anti-EHS-laminin response was predominantly IgG, indicating that it also was endogenous in origin. Low-titer anti-EHS-laminin activity was observed in immune sera from four MRL non-Tg littermates (Fig. 10C).

**Discussion**

We used mice Tg for a murine SLE-derived nephritropic anti-laminin Ig to determine whether pathogenic B cells reactive with basement membrane proteins are regulated in vivo. The LamH-Cμ Ig H chain transgene was previously shown to pair in vitro and in vivo with diverse endogenous L chains to generate anti-laminin specificity (26, 28). We rationalized that mice Tg for LamH-Cμ should be fully capable of generating diverse anti-laminin B cells in their bone marrow. In this work we show that M29 and M6 lineage Tg mice have relatively few B cells in the periphery and fail to produce Tg⁺ autoantibody spontaneously, after mitogen administration, or after Ag immunization. These results suggest that LamH-Cμ Tg⁺ anti-laminin B cells are censored by mechanisms that include clonal deletion and anergy. Functional compromise of surviving Tg⁺ B cells is demonstrated by absence of Tg⁺ IgM-a allotype and anti-laminin Ig in serum of unmanipulated mice; hypoproliferation of Tg⁺ B cells in response to LPS and Ig cross-linking, absence of in vitro LPS-induced differentiation of Tg⁺ B cells, and failure of endotoxin administration or immunization with autologous or heterologous Ag to induce a Tg⁺ immune response.

The reduced ability to differentiate into Ig-secreting cells is a hallmark of B cell anergy as described for anti-ssDNA and anti-HEL B cells (15, 32–34), and is consistent with our previous inability to recover LamH-Cμ Tg⁺ anti-laminin hybridoma from LPS-stimulated splenocytes of M29 or M6 lineage Tg⁺ mice (25). Variability in proliferative responses, which ranged from profound nonresponsiveness to moderate diminution of proliferation of LamH-Cμ Tg⁺ B cells, is reported for other model systems (15, 17, 35–37). This may reflect subtle differences in experimental and environmental conditions or, in Ig H-chain Tg models, expansion in individual Tg⁺ mice of B cells expressing H and L chain combinations with different affinities for Ag. Hypoproliferation could not be attributed to a global defect in Tg⁺ B cell proliferative capacity, because PMA and ionomycin induce robust proliferation and indicate Tg⁺ B cell capacity to activate protein kinase C and support capacitative calcium entry. Cell surface IgM was only minimally down-regulated in our model, 1.2-fold in B6 and 2-fold in MRL Tg⁺ mice, similar to the anergy phenotype described in anti-ssDNA B cells (15) and in contrast to the 10- to 75-fold receptor down-regulation reported in the anti-HEL/HEL silencing model (17, 38). Taken together, the data support the notion that B cell anergy is a heterogeneous phenotype.

The markedly reduced B cell numbers suggest that some anti-laminin LamH-Cμ Tg⁺ B cells meet a fate other than functional silencing. Although a modest decrease in splenic B cells attributed to a shortened half-life of anergic B cells is reported in some energizing models (35, 39, 40), the 8-fold decrease in B cell number observed in LamH-Cμ Tg⁺ mice best mirrors the dramatic hypoproliferality reported in other model systems in which deletion is a dominant mechanism of tolerance (16, 21, 41, 42), and contrasts with the near-normal number of B cells observed in mice Tg for an Ig reactive with foreign Ag (38, 43). This suggests that a large subset of B cells bearing LamH-Cμ H chain/endogenous L chain combinations engage self-Ag in a manner that qualitatively and/or quantitatively is sufficient to promote clonal deletion. High-valency Ag binding capable of promoting extensive B cell receptor cross-linking is postulated to be particularly likely to trigger this outcome (21, 42, 44, 45). Whether these interactions also lead to

**FIGURE 10.** Induction of endogenous anti-laminin Ig responses in MRL Tg⁺ mice by immunization with heterologous laminin. A. Binding to heterologous laminin by serum obtained after the third serial immunization with human placental laminin emulsified in adjuvant for individual Tg⁺ and non-Tg MRL mice. Ag reactivity was measured as the mean OD₄₅₀ on Ag-coated minus sham (diluent)-coated wells using duplicate samples. B. Anti-LamH-Id IgG do not inhibit binding of MRL Tg⁺ immune sera to heterologous laminin. The dilution of immune serum that gave 50% maximal binding to Ag was preincubated with varying concentrations of anti-Id IgG inhibitor before incubation with Ag-coated wells. Results for three individual MRL Tg⁺ mice are shown. Control anti-Id inhibition of heterologous laminin binding by mAb H50 is also indicated (○). C. Binding to autologous (murine EHS) laminin by immune sera induced with heterologous laminin.
receptor editing in LamH-Cμ Tg⁺ B cells is unclear. LamH ιd expression parallels H chain expression, arguing against H chain editing. The proportion of splenic B lymphocytes expressing A L chains is not increased in Tg⁺ mice (our unpublished data), and our inability to recover Tg⁺ hybridoma from LPS-stimulated splenocytes precludes assessment for IA gene bias by this approach. Nonetheless, central tolerizing interactions are known to induce re-expression of recombinate-activating genes 1 and 2 and receptor editing in immature B cells (18, 19). If a proportion of LamH-Cμ Tg⁻ B cells are induced to edit their original L chains, our demonstration of markedly diminished differentiative capacity of surviving Tg⁺ splenocytes suggests that novel Ig generated by editing are themselves autoreactive and regulated in vivo. This is consistent with dominance of the LamH-Cμ H chain in determining autoantigen reactivity (28).

Our inability to induce Tg⁺ anti-laminin Ig by immunization cannot be attributed solely to lack of T cell help, due to a hole in the T cell repertoire or T cell tolerance, because immunization with autologous or heterologous laminin induces an endogenous anti-laminin IgG response in some mice. This suggests that laminin peptides are presented by MHC class II molecules and that functional laminin-reactive T cells are present and primed to provide the necessary help for autoimmunoglobulin production and isotype switch. However, isotype, allotype, and idiotype analyses indicate that in situ help is insufficient to rescue Tg⁺ anti-laminin B cells. This suggests several possibilities with respect to the fate of matrix-reactive LamH-Cμ Tg⁺ B cells. They may express a profound and nonreversible functional block. Alternatively, and as suggested by the relative paucity of splenic B cells, Tg⁺ B cells reactive with laminin may be centrally deleted and thus not present in the periphery to be rescued by T cell help. In this case the Tg⁻ B cells present in the spleen must express specificity for self or foreign Ag other than laminin. It is also possible that laminin epitopes recognized by Tg⁻ B cells are not exposed in the soluble laminin-adjuvant preparations, such that specific receptor engagement, with subsequent Ag processing and presentation, does not occur. In this case our results suggest that bystander T cell help cannot substitute for cognate help in activating Tg⁺ B cells. If there is a role for T cell regulation of transgene expression, and biological properties of laminin isoforms suggest that laminin specific for IgG responses is evident only at the high laminin concentrations (100–200 μg/ml) critical for formation of laminin polymer networks in solution (28). This phenomenon is characteristic of large proteins that aggregate in solution and for which binding to fixed surfaces requires hydrophobic interactions; surface binding leads to exposure of additional epitopes not exposed in the soluble aggregates (51). This high concentration is also necessary to trigger some epithelial cell functions and is consistent with exposure of biologically relevant epitopes only after correct incorporation of the laminin chain within the highly structured basement membrane lattice. Collectively, these observations suggest that soluble laminin may be incapable of effectively engaging and thus tolerizing Tg⁺ laminin-specific Ig receptors in vivo. Soluble laminin clearly does not irreversibly tolerate a subset of endogenous anti-laminin B cells, because an endogenously generated response is readily induced by immunization. However, this endogenous response arises from a nonoverlapping population of Id-negative B cells that recognizes laminin epitopes distinct from those recognized by Tg⁺ and SLE autoantibodies. The relative exposure and reactivity of different epitopes of soluble vs basement membrane-bound laminin are likely to determine their tolerogenic potential. Soluble ssDNA is a potential tolerogen for a subset of Tg⁺ anti-laminin B cells, because several M7-derived Tg⁺ anti-laminin mAb cross-react with ssDNA (28), but DNA cannot account for tolerance in the non-cross-reactive subset.

These studies do not permit us to pinpoint the exact fate of the anti-laminin Tg⁺ B cells. The poor binding to soluble laminin by Tg⁺ Ig precludes use of labeled Ag to probe B cell specificity. Nonetheless, our recovery of 11 different Vk genes from nine different Vk gene families among 17 Tg⁺ anti-laminin mAb from the M7 lineage (28) predicts that similar stochastic mechanisms generate laminin specificity in a large number of newly emerging B cells in the M29 and M6 lineage mice described in this work. An interesting as yet unanswered question is which specificities are deleted and which are permitted to exit the bone marrow. Alternative approaches, including generation of monospecific H and L chain double Ig Tg⁺ mice, will address this.

We found that MRL susceptibility alone is insufficient to permit spontaneous or immunization-induced activation of Tg⁺ anti-laminin B cells. Most studies to date examining tolerance on the MRL background are limited to mice with a superimposed deficiency in Fas/CD95 signaling due to the lpr mutation. Fas deficiency accelerates MRL SLE, in part by exacerbating defects in peripheral tolerance induction (52). Defective tolerance has been attributed primarily to lpr effects on cell death that prolong...
survival of anergic B cells or limit availability of tolerizing apoptosis-related self-Ag (53, 54). However, differential effects due to underlying MRL susceptibility are suggested by failure of MRL/lpr to uniformly break tolerance, as cells reactive with nominal cell membrane-bound and soluble protein Ag are tolerized as efficiently in MRL/lpr mice as in nonautoimmune hosts (55–57) whereas tolerance to DNA is broken (53, 58). Our data suggest that mechanisms that maintain B cell tolerance to laminin epitopes targeted in SLE are preserved in young MRL mice. It remains to be determined what triggers activation of these nephritogenic B cells in disease. Our results are consistent with the view that failure at multiple regulatory checkpoints is necessary to induce full-blown destructive nephritis in systemic autoimmune.

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References


