REVIEW

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Deconstructing B cell tolerance to basement membranes

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Abstract

Basement membrane antigens are frequent targets of autoantibody attack in systemic and organ-restricted autoimmunity. These specialized and highly organized matrices are composed of multiple components with restricted tissue distributions and limited epitope exposure. To dissect mechanisms controlling humoral autoimmunity to nephritogenic basement membrane antigens, we developed autoantibody transgenic models. In mice bearing the LamH Ig transgene encoding B cell receptors specific for laminin, autoreactive B cells are readily generated but actively regulated *in vivo*. In this model, anti-laminin B cells are immunologically censored by mechanisms that include central deletion, κ light-chain editing, and anergy. Tolerance is maintained when the transgene is established in MRL and BXSB genetic backgrounds with inherited autoimmune susceptibility, and despite provocation with potent environmental stimulants. Collectively, these studies indicate that the pathogenic anti-laminin reactivity characteristic of systemic lupus is tightly regulated. A novel anti-collagen transgenic model is used to assess the tolerogenesis of a structurally distinct pathogenic basement membrane epitope and to determine if reactivity to putative cryptic epitopes targeted in organ-restricted disease is regulated. These studies should provide insight into the molecular mechanisms controlling basement membrane autoreactivity and ultimately facilitate the development of novel strategies to inactivate autoreactive cells and treat autoimmune disease.

Key words: B cell tolerance, basement membrane, autoantibody, laminin.

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INTRODUCTION

Autoimmune diseases capable of destroying major organ systems affect up to 22 million people in the United States, and far more worldwide [60] . This ranks autoimmunity and its sequelae third behind cardiovascular disease and cancer as a burden on the U.S. healthcare system [67]. Potent immunosuppressants are often effective in suppressing disease, but these drugs are nonspecific and highly toxic such that adverse effects contribute significantly to morbidity. There is urgent need for novel, less toxic targeted interventions, the design of which will likely depend on mechanistic insight into disease pathogenesis. In this regard, important research has identified and characterized a host of downstream inflammatory cells and mediators that effect local tissue injury. However, considerably less is known about the proximal events that initiate autoimmune responses and which must be interrupted to halt ongoing activation of autoimmune cells. Elucidating these processes is the focus of the research described in this review.

Autoimmunity results when tolerance mechanisms fail. This is a complex and still poorly understood process, involving interactions between multiple genetic and environmental factors acting on a large and diverse population of potentially destructive autoreactive cells. It is estimated that 60% of newly generated B cells bind self antigen [64, 86], as autoreactive cells are constantly generated *in vivo* as the inevitable byproduct of receptor diversity. If these cells are exposed to cognate antigen *in vivo* while in a tolerance-susceptible state and supportive microenvironment, the signals triggered by receptor-antigen engagement prevent cell activation [33]. Cellular responses vary from apoptotic deletion to subtle shifts in activation thresholds. Self antigen itself is an important determinant of cell fate. Antigen structure and other attributes, including valency, accessibility, concentration, and affinity, influence the quality or strength of transmitted signal and thus modify the tolerance phenotype (Fig. 1). This is particularly important for B cells, for which surface Ig receptors recognize native antigen and tolerogen in tertiary conformation.

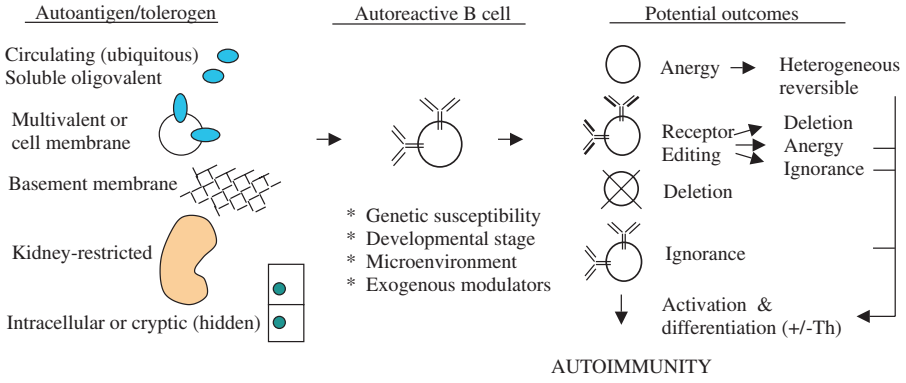

Fig. 1. Potential outcomes for autoreactive B cells after receptor engagement by self antigens with distinct structures and tissue distributions. (Reproduced with permission [41]).

Results from experimental models suggest that ubiquitous multivalent or cell membrane-bound antigens extensively crosslink B cell receptors, generating strong signals that trigger editing or deletion [15, 31, 39, 51, 65, 66, 68, 81, 84]. Soluble oligovalent and low-affinity self antigen crosslink less effectively, generating weaker signals that are more likely to functionally incapacitate the cell [20, 34]. Antigen that fails to effectively engage receptors does not induce tolerance [3, 4, 6, 23, 37, 68]. Antigen sequestration, T cell tolerance, and additional mechanisms provide protection in this setting. An implication of the existence of multiple regulatory mechanisms is that different defects underlie different autoimmune diseases.

These observations prompted us to examine the tolerizing potential of basement membrane components, common targets in autoimmunity (Table 1) and the only confirmed pathogenic targets in human immune nephritis [18, 63, 77]. Basement membranes are specialized extracellular matrices that provide structural support for cell layers and influence cell survival, differentiation, and function. These highly organized protein scaffolds require correct assembly of laminin, collagen, nidogen/entactin, and proteoglycan components to properly expose cell- and cytokine-binding domains [19, 62, 69]. Multiple laminin and collagen isoforms exist, each with a characteristic and, in some cases, highly tissue-restricted distribution. To date, 16 laminin heterotrimers have been described, each formed by the combination of an α, β, and γ polypeptide chain [7]. Laminin-1 (α1, β1, γ1) and laminin-5 (α3A, β3, γ2) have been identified as targets of autoantibody attack in autoimmune nephritis and blistering dermatoses, respectively [1, 5, 8, 12, 13, 22, 25, 36, 45, 47, 52, 59, 61, 75, 79]. Expression of the laminin-1 isoform is limited to the glomerular mesangial matrix in normal adults, but can extend to the basement membrane of capillary loops in diseased kidneys, recapitulating its distribution during embryogenesis [2, 48]. Autoantibodies in lupus mice and patients target a 21-mer peptide within the C-terminus of the α1 laminin chain [5]. This specificity has been exploited therapeutically; peptide administration prevents antibody deposition and disease in MRL/lpr murine lupus [5].

Autoimmunity to collagen isoforms can lead to debilitating damage in joints, skin, lung, and kidney (Table 1) [11, 30, 32, 38, 54, 59, 77, 88, 89]. A well-characterized pathogenic epitope is the NC1 domain of the α3 chain

Table 1. Basement membrane (BM) and matrix targets in immune-mediated diseases

Experimental models	Man
Murine Goodpasture's syndrome	Autoimmune nephritis
α3(IV) collagen NC1 domain	α3(IV) collagen NC1 domain
	tubular BM glycoproteins
Rodent lupus nephritis	α5(IV) collagen NC1 domain
heparin sulfate	
laminin	Systemic lupus erythematosus (SLE)
collagen	laminin
Autoimmune bullous skin disease	Autoimmune bullous skin disease
laminin-5	Cicatricial pemphigoid
Rheumatoid arthritis	laminin-5 (α3 chain)
type II collagen	Epidermolysis bullosa acquisita
type XI collagen	type VII collagen
Pulmonary allograft rejection	Bullous SLE type I
type V collagen	type VII collagen
	Linear IgA disease
	ladinin (LAD-1)

of type IV collagen, the ligand for autoantibody deposition in anti- glomerular basement membrane (GBM) nephritis and Goodpasture's syndrome as well as the target of alloimmune attack in renal allografts of a subset of patients with Alport's nephritis [77, 85, 88]. The native kidneys of Alport's patients lack the Goodpasture epitope, such that this antigen may be attacked as foreign when a normal kidney is introduced at transplantation. Additional tubular basement membrane glycoproteins are pathogenic in certain forms of tubulointerstitial nephritis in man [18, 63].

The origin of anti-matrix autoantibodies is unclear. Organ-restricted distribution and limited accessibility of the basement membrane epitopes raise questions about their capacity to engage or crosslink B cell receptors to trigger tolerance or cell activation. It is unknown if defective tolerance is crucial to disease onset in this setting. To date, tolerance mechanisms have been described primarily in experimental systems based on nominal or ubiquitous antigens, such as neoself antigen hen egg lysozyme, class I major histocompatibility antigen, rheumatoid factor, DNA, Smith antigen, or erythrocyte surface epitopes, several of which have no known association with autoimmune disease. The goal of this review is to summarize our current understanding of B cell regulation by basement membrane tolerogens. It is anticipated that elucidation of these pathways will provide insight into disease pathogenesis and reveal new targets for therapeutic interventions.

REGULATION OF B CELL AUTOREACTIVITY TO LAMININ: THE LamH Ig TRANSGENIC MODEL

To determine if epitopes within basement membrane lattices can tolerize B cells *in vivo*, we constructed unique Ig transgenic mouse models bearing B cells reactive with matrix antigens. The Ig transgenes in these models are reactive with laminin or collagen epitopes known to be targeted in systemic and organ-restricted autoimmune nephritis, respectively. To further determine the influence of autoimmune genetic susceptibility on tolerance and the expression of autoimmunity, the anti-laminin transgene was introduced by breeding onto the non-autoimmune B6 as well as autoimmune-prone MRL, MRL/lpr, BXSB, and NZB backgrounds. Below we describe our LamH anti-laminin Ig transgenic model in detail. This is followed by a brief discussion of the rationale and unique features of the anti-α3(IV) collagen model recently generated and currently under investigation in our laboratory. We used both a genetic approach, as outlined in Table 2, and *in vivo* and *in vitro* B cell stimulation to dissect the regulatory mechanisms and the fate of LamH anti-laminin B cells. An overview and published outcomes from some of these experiments are summarized in Table 3.

An Ig transgenic approach was chosen for this work to provide sufficient B cells of the desired specificity (anti--laminin) with which to unequivocally determine cell fate and tolerance mechanisms at the cellular and molecular levels. Ig transgenic models have proven highly informative in providing insight into the proximal events that initiate and regulate autoimmunity. Mice expressing a transgenic antibody reactive with foreign but not self antigen produce B cells of normal number and function. The fate of B cells expressing an autoantibody transgene varies, depending on the structure, concentration, and anatomic and subcellular distribution of cognate self antigen (reviewed in [41]). We used this powerful tool to enrich for anti-laminin B cells and to examine the outcome of interactions engaging this single specificity within complex *in vivo* networks. If the expression of autoimmunity is suppressed, we can infer that anti-laminin B cells are regulated. Selective breeding of transgenic animals to informative mutant strains permits additional mechanistic insights. Notably, and by design, these models do not assess aspects of disease pathogenesis that depend on isotype switch and related effector functions, including complement activation, Fc receptor engagement, and recruitment of downstream mediators that provoke tissue injury and fibrosis. These latter aspects of disease are readily studied in existing and more suitable experimental models induced by active immunization or passive transfer.

The rearranged VDJ gene (LamH) encoding the heavy-chain variable region of a prototype anti-laminin IgG was cloned and ligated to gene fragments encoding an IgM constant region and Ig regulatory elements that ensure lymphocyte-specific expression [27]. The donor

Table 2. Breeding schemes used to dissect tolerance mechanisms in the LamH anti-laminin Ig transgenic model

Genetic background	LamH Ig heavy chain transgenic lineage	Light chain
B6	M06	Endogenous
	M07	Endogenous
	M29	Endogenous
	M29	V8R (L)
	M29	V8R/V8R (LL)
MRL	M06	Endogenous
MRL/lpr	M06	Endogenous
NZB	M29	Endogenous
BXSB	M29	Endogenous
Rag-KO	M29	V8R (L)
MuMT	M07	Endogenous
	M29	Endogenous

Definitions: V8R is a light-chain gene-targeted transgene generously provided by Dr. Martin Weigert, University of Chicago [71]. Breeders obtained from The Jackson Laboratory (Bar Harbor, ME) include: RagKO, a gene-targeted deletion of recombination activation gene-1 (Rag-1) carried on the B6 background; MuMT, a gene-targeted deletion of membrane-bound IgM carried on the B6 background; B6, C57BL/6 inbred strain; MRL, NZB and BXSB, autoimmune-prone inbred strains. M6, M7 and M29 are three LamH Ig heavy-chain transgenic lineages derived from different founders as previously reported in references [29, 74].

Table 3. Outcomes in anti-laminin Ig B cell receptor transgenic models

Transgene construct	LamH lineage	Genetic background	Model description	Tolerance phenotype	Interpretation
H chain (LamH)	M7	B6	Conventional Ig H chain Tg Polyclonal B cell population	Autoimmunity Very low surface Tg	Defective tolerance signaling permits escape from regulation
H chain	M6, M29	B6	Conventional Ig H chain Tg Polyclonal B cell population ± laminin immunization ± endotoxin administration	Deletion and anergy	Laminin epitopes: – are accessible – engage B cell receptors – trigger tolerogenic signals Tolerance is not readily reversible
H + L chain (double Tg) (LamHxV8R)	M29	B6	Conventional Ig H chain Tg Heterozygous for Vk8Jk5 (κ L chain replacement)	Deletion L chain editing Anergy	Receptor editing permits escape from deletion Edited cells are anergic
H + LL chain (Triple Tg) (LamHxV8R/ /V8R)	M29	B6	Conventional Ig H chain Tg Homozygous for Vk8Jk5 κ editing precluded	Profound central deletion Few IgM+ cells in bone marrow	Tolerogen extensively crosslinks B cell receptors in bone marrow, triggering deletion in absence of opportunity for κ editing
H chain	M29	MRL	Conventional Ig H chain Tg Polyclonal B cell population Autoimmune genetic susceptibility ± laminin immunization ± endotoxin administration	Deletion and anergy	Tolerance is intact despite genetic susceptibility to autoimmunity
H chain	M29	BXSB	Conventional Ig H chain Tg Polyclonal B cell population Autoimmune genetic susceptibility *Yaa* (Y-linked autoimmune accelerator) gene in males	Marked deletion No serum Tg autoantibodies	Tolerance is intact despite genetic susceptibility to severe autoimmunity

Details of the experiments in the B6 and MRL background were previously reported (references [10, 74])

B cell was immortalized by fusion from the unmanipulated spleen of a diseased autoimmune MRL/lpr mouse and was among a group of hydridoma that produced IgG reactive with murine laminin-1. These IgG form glomerular mesangial immune deposits *in vivo* upon passive transfer to naïve recipients and bind the 21-mer α1 laminin peptide identified by Naparstek et al. [5, 28].

Transfection experiments in which the LamH gene was introduced into B cell lines expressing Ig light chains revealed that this Ig heavy chain produced anti-laminin reactivity in combination with multiple different Ig κ light chains [27]. Heavy-chain dominance was confirmed by subsequent analysis of transgenic monoclonal antibodies derived from the M7 line (see below). The LamH heavy chain was thus introduced into the germline of non-autoimmune mice to generate transgenic lines and crossed multiple generations with informative backgrounds to establish inbred lines (Table 2) [29]. The phenotypes described below have been stable over seventeen generations of backcrossing onto C57BL/6 (B6) and MRL.

Initial characterization of the LamH model revealed two distinct phenotypes among three transgenic lines derived from different founders in non-autoimmune B6 mice (Table 3). The aberrant M7 line was unique in that transgene-encoded autoantibodies were spontaneously produced and readily detected in serum and among monoclonal antibody recovered by fusion, whereas transgenic B cells in lines M6 and M29 were tolerized [24, 29]. Fusion of M7 spleens yielded numerous monoclonal antibodies reactive with laminin-1 and variably crossreactive with single-stranded DNA. These antibodies formed renal tubular and glomerular immune deposits with a predominant mesangial pattern when injected into naïve non-autoimmune recipient mice [24]. Flow cytometric analysis revealed very low cell surface expression of the transgene-encoded Ig in M7 B cells, suggesting that defective receptor expression precluded adequate engagement of self antigen or transmission of tolerizing signals, and thus permitted B cells to escape regulation. B cell development and survival in M7 mice likely depends on heavy-chain allelic inclusion with the

Table 4. Properties supporting anergy in M29 lineage LamH Ig transgenic B cells

Absence of transgenic autoreactivity in serum
Failure to recover autoreactive transgenic monoclonal antibodies by fusion of mitogen-stimulated B cells
Impaired *in vitro* proliferation in response to Ig cross-linking, endotoxin and CD40 plus IL-4
Impaired *in vitro* production of autoantibodies by mitogen-stimulated B cells
Shortened life span as determined by BrdU labeling
Altered surface expression of activation and maturation markers

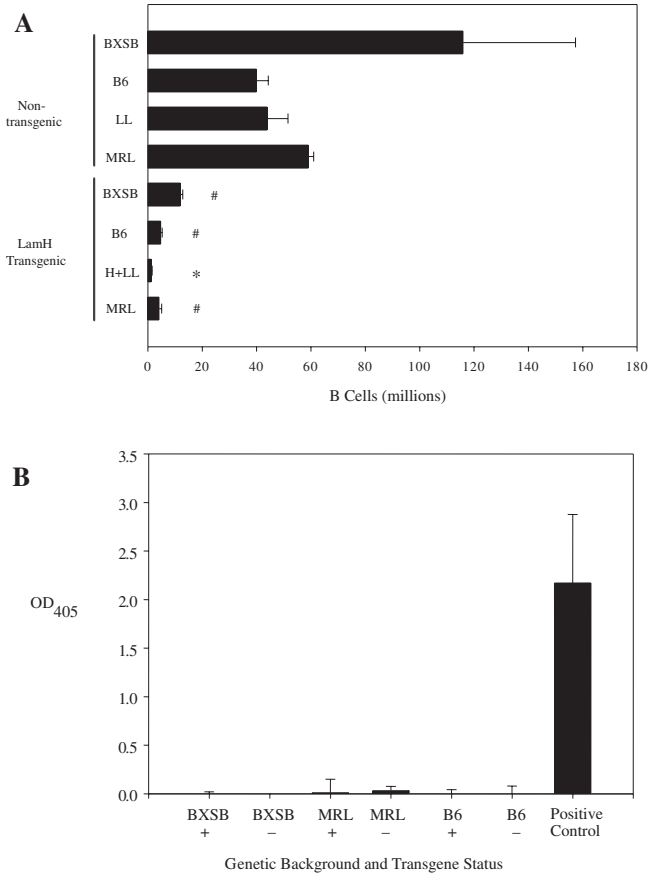

Fig. 2. Deletion of transgenic anti-laminin B cells and absence of serum transgene-encoded autoantibody. **A** – the number of B220+ B cells in spleen was calculated using flow cytometry and the total number of nucleated cells recovered from each spleen. Values are mean ±SD. Genetic backgrounds are indicated: B6, non-autoimmune C57BL/6; MRL and BXSB, autoimmune-prone inbred strains; H+LL, triple-mutant for the LamH heavy-chain transgene and homozygous for the V8R gene-targeted light chain on the B6 background; LL, homozygous for V8R gene-targeted light chain only on the B6 background. Transgenic refers to the LamH Ig heavy-chain transgene. *$p<0.005$, # $p<0.0001$, comparing transgenic vs. non-transgenic mice within same background. **B** – serum transgene anti-laminin reactivity, measured as the mean OD405 on antigen-coated minus sham (diluent)-coated wells, using duplicate serum samples diluted 1/20 or 1/25. Mean OD405 for positive control anti-laminin IgM monoclonal antibodies (5 μg/ml) is also shown. A subset of this data was previously reported [10, 41, 74] .

expression of endogenous IgM, a hypothesis currently under investigation in our lab using genetic approaches that eliminate the expression of endogenous Ig heavy chains. Nonetheless, based on the M7 phenotype, we conclude that normal mice have the capacity to produce a large and diverse repertoire of anti-basement membrane B cells in their bone marrow. Insight into the mechanisms by which these cells are normally regulated was provided by subsequent analysis of the M6 and M29 lines.

Detailed analysis of M6 and M29 lineage mice revealed that LamH transgenic anti-laminin B cells are regulated by central deletion, κ light-chain editing, and anergy [10, 74]. Anti-laminin antibodies were not detected in serum or among monoclonal antibodies despite *in vitro* and *in vivo* strategies to activate B cells (Tables 3 and 4). Moreover, B cell numbers were markedly decreased in M6 and M29 spleens. M29 mice had 7- to 10-fold fewer B cells than did their non-transgenic littermates (Fig. 2), consistent with the marked deletion of transgene-bearing B cells. Examination of bone marrow revealed that deletion occurred centrally; there were few IgM^+ $B220^+$ B cells in transgenic mouse marrow compared with abundant surface Ig-positive B cells in the marrow of non-transgenic littermates.

Most B cells that escaped deletion in M29 mice did so by editing their κ light-chain receptors [10]. Receptor editing is an efficient and common salvage process by which an Ig receptor is replaced by a different receptor through continued Ig gene rearrangements. During normal B cell development, antibody genes are assembled by DNA recombination in a process catalyzed by products of the V(D)J recombinase-activating genes (RAG-1 and -2). Expression of a functional non-autoreactive Ig chain then suppresses further RAG gene expression. Editing occurs when the RAG genes remain active or become reactivated in B cells that have been developmentally arrested at the pre-B to immature B cell stage, either by autoantigen-triggered signaling or inadequate signaling through poorly expressed non-autoreactive receptors [16, 17, 31, 50, 57, 70, 72, 73, 81, 82]. This permits secondary Ig gene recombination and the production of a new Ig chain that replaces the original chain and alters B cell specificity. Light-chain editing most frequently occurs at the active κ allele; this entails recombination of an upstream Vk gene segment with a downstream Jk gene to replace the existing Vk-Jk rearrangement. Editing on the second κ allele, either λ allele, or the second heavy-chain locus, as well as heavy-chain VH replacement, are also reported. Signals capable of inducing deletion or anergy can trigger editing, and restoration of adult spleen B cell populations to near normal numbers is reported.

We used Ig κ-chain site-directed, or "knocked-in", V8R transgenic mice [71], generously provided by Dr. Martin Weigert, University of Chicago, to determine the role of editing in LamH transgenic mice. In V8R mice, the rearranged Vk8Jk5 light-chain transgene is

targeted into the genomic Ig light-chain locus [71]. The V8R light chain generates anti-DNA Ig when combined with the lupus 3H9 heavy chain [14, 72] and is similar to light chains expressed by a subset of anti-laminin monoclonal antibodies derived from M7 mice [24]. Transfection of a cell line producing the V8R light chain with the LamH heavy chain yields an antibody strongly cross-reactive with laminin and ssDNA [10], a specificity observed among a subset of LamH M7 transgenic antibodies [24].

M29 LamH transgenic mice were bred with V8R mice to generate H+L and H+LL double- and triple--transgenic mice in which one or both light-chain loci were replaced with the V8R light-chain. Because V8R uses Jk5, the most downstream Jk gene, further editing at the targeted κ allele is thus prevented. Characterization of the new double-mutant mice revealed a key role for κ editing in rescuing LamH heavy-chain transgenic B cells [10]. Neither anti-DNA nor anti-laminin transgenic Ig were recovered from serum, nor were autoantibodies recovered among the few monoclonal antibodies derived from endotoxin-stimulated splenocytes of H+L mice. These antibodies bore an unmutated LamH transgenic heavy chain, as determined by cDNA sequencing, suggesting that the altered specificity arose by replacement or mutation of the V8R light chain. This was confirmed by molecular analysis, which revealed productive secondary gene rearrangements on the non-targeted κ allele. PCR amplification of hybridoma genomic DNA derived from all four LamH transgene-encoded monoclonal antibodies obtained from H+L mice yielded products consistent with a Vk rearrangement to Jk1. Additionally, PCR amplification using promiscuous Vk primers revealed extensive κ rearrangements in a H+L spleen cDNA library. Over half of 36 light-chain sequences were endogenous (non--transgenic) in origin, and at least 13 independent κ rearrangements were represented. Strikingly, when κ editing was prevented by replacing both endogenous alleles with V8R in H+LL triple-transgenic mice, massive B cell deletion resulted (Fig. 2). IgM-expressing B cells were absent from H+LL Tg mouse bone marrow. Thus receptor editing was crucial for rescue from deletion of anti-laminin B cells encoded by LamH and V8R.

Analysis further suggested that most of the B cells generated by editing were autoreactive. The small population of transgene-bearing B cells that reach the spleen in heavy-chain transgenic or H+L double-transgenic mice are anergic (Tables 3 and 4). Transgenic B cells secrete only low levels of Ig despite co-culture with endotoxin or Ig crosslinking, and few monoclonal antibodies are recovered from transgenic spleens despite LPS stimulation. Transgenic B cells hypoproliferate in response to LPS, Ig crosslinking, and anti-CD40+IL-4 stimulation, and in some experiments receptor crosslinking fails to induce B cell proliferation completely. The transgenic B cell receptor is capable of signaling, as revealed by prompt calcium flux [41], protein tyrosine phosphorylation, and NF-κB nuclear translocation [Clark et al., in press] in response to Ig cross-linking. H+L B cells have shortened life spans as determined by BrdU labeling and express surface markers consistent with prior antigen engagement, these being features of anergy.

Collectively, our observations suggest that LamH anti-laminin B cells have access to and engage a tolerizing self antigen in C57BL/6 mice. Tolerogen engagement in the bone marrow appears to provide a window of opportunity for editing, during which secondary Ig gene rearrangements at the κ loci replace the original autoreactive light chains with chains of different specificity. This κ editing does not abrogate autoreactivity altogether, however, but rather generates new autospecificities, based on the anergic phenotype in residual B cells. The new specificities may include reactivity to ssDNA; this nucleic acid is recognized by a subset of dual-specific LamH monoclonal antibodies. Alternative autospecificities could also arise from initial H+L chain pairings or via receptor revision or somatic mutation in the periphery. Thus more than one tolerogen may contribute to the complex tolerance phenotype in M6 and M29 Tg mice. In keeping with this possibility, laminin α1 is not expressed in mouse bone marrow and is expressed in the periphery only in selected epithelial basement membranes [21, 35]. Whether this represents promiscuity in LamH B cell receptor binding to different laminin chains, as suggested for integrin receptors and discussed in reference [74], or a second as yet undefined specificity awaits clarification.

ANTI-LAMININ B CELLS ARE REGULATED IN AUTOIMMUNE MICE

The studies described above reveal that B cells reactive with laminin epitopes are tolerized in non-autoimmune mice. These same laminin epitopes are subject to autoantibody attack in mice and patients with systemic lupus erythematosus, suggesting that reactive B cells escape regulation in this setting. To determine the effect of host modifier genes on the fate of nephritogenic anti--laminin B cells, we established the tolerance-susceptible LamH transgene on backgrounds prone to autoimmune nephritis. This takes advantage of unique Ig transgenic mice bearing disease-relevant specificities and a defined and highly reproducible tolerance phenotype, and genetically homogeneous disease strains with uniform disease phenotypes similar to human nephritis.

Mice of the LamH M29 line were crossed 7 or more generations with recombinant strains MRL and BXSB. Each inbred lupus strain carries a unique constellation of susceptibility genes and immune alterations and spontaneously develops disease resembling human SLE including severe nephritis [80]. MRL susceptibility derives from a composite genome of LG/J (75%), AKR/J, B6, and C3H/Di and includes two powerful renal disease-modifying loci on chromosomes 7 and 12, a glomerulonephritis-linked locus on chromosome 10,

and chromosome 5 and 7 loci linked to anti-dsDNA Ig independent of nephritis [83, 87]. Markedly accelerated disease occurs in MRL/lpr mice homozygous for the *lpr* mutation in the gene encoding Fas/CD95 apoptosis receptor; however, *lpr* alone fails to induce disease in non-autoimmune strains, indicating the need for preexisting MRL defects [46, 90]. The BXSB strain is derived from B6 and SB/Le stock, with male-specific acceleration contributed by the Y-chromosome-linked autoimmune accelerator, or *Yaa*, gene [40, 42, 55]. Mortality is 50% at 6 months in male BXSB compared with 20 months for females. The as yet unidentified *Yaa* defect is functionally expressed in B but not in T lymphocytes [26, 58]. *Yaa*+B cells can receive help for autoantibody production from T cells from non-autoimmune mice, but non-autoimmune B6 and CBA/J mice bearing *Yaa* do not develop lupus.

Immune phenotyping revealed that tolerance was not overcome by MRL or BXSB inherited autoimmune susceptibility (Table 3). Like their B6 counterparts, MRL LamH transgenic mice do not produce transgene-encoded anti-laminin autoantibodies [74]. MRL transgenic mice have markedly fewer splenic B cells than do non-transgenic littermates, consistent with the deletion of anti-laminin B cells (Fig. 2). The residual MRL transgenic B cells hypoproliferate to endotoxin and Ig crosslinking, and cells cultured in LPS produce little autoantibody, consistent with an anergic phenotype. Similarly, transgene-encoded autoantibodies are not detected in serum of BXSB transgenic mice, and BXSB transgenic mice have almost tenfold fewer peripheral B cells than do non-transgenic littermates (Fig. 2). These differences are present in older, 10- to 12-month BXSB transgenic mice, indicating that receptor editing is inadequate to replenish peripheral B cell repertoires. This likely reflects a shortened life span of residual anergic cells, although this has not yet been formally tested in BXSB transgenic lineages. Notably, there are no significant differences in cell number between female and male BXSB transgenic mice (not shown); thus, the *Yaa* gene has no detectable effect on central deletion or regulation of anti-laminin B cells in this model.

Notably, tolerance was not overcome by exposure to potent environmental challenge, even when superimposed on autoimmune susceptibility. Transgenic anti-laminin antibodies were not induced in B6 or MRL LamH transgenic mice administered endotoxin or immunized with homologous or heterologous laminin in adjuvant [74]. Immunization did induce endogenous anti-laminin IgG that recognized non-overlapping epitopes on laminin, suggesting that T cell help was solicited. This points to a fundamental difference in origin and regulation of lupus-like versus endogenous anti-laminin B cells.

Our inability to recover anti-laminin Ig from B6, MRL, and BXSB transgenic mice despite genetic susceptibility and/or *in vivo* stimulation suggests that lupus-like B cells with specificity for laminin are either deleted from the repertoire or subject to profound functional inactivation. Central deletion was unimpaired in multiple backgrounds, including mice with aggressive lupus. This differs from the more glaring breakdown in tolerance reported for anergic anti-ssDNA and anti-Sm B cells in MRL/lpr mice [56, 76] and likely reflects differences in the mechanisms maintaining tolerance. This clearly begs the question: from where do the anti-laminin Ig found in disease arise, and how do they escape tolerance? In this regard we have detected rare transgene-encoded anti-laminin Ig in Tg MRL/lpr mice with superimposed Fas/CD95 deficiency and accelerated disease, suggesting that escape from regulation can occur when multiple regulatory mechanisms fail [Clark et al., manuscript in preparation].

BASEMENT MEMBRANE ANTIGEN IN SYSTEMIC VS. RENAL-RESTRICTED AUTOIMMUNITY

Basement membrane antigens are targeted in both systemic and organ-restricted autoimmunity. Lupus nephritis develops in the setting of systemic autoimmunity dependent on interactions of multiple susceptibility genes and associated with numerous immune alterations and lymphocyte signaling aberrations. In contrast, kidney-restricted autoimmunity can arise in otherwise healthy individuals. This suggests a fundamental difference in the regulation of B cells that promote nephritis in these two settings. We propose that this difference revolves around tolerogenesis of the nephritogenic antigens. In this scenario, renal-limited disease depends primarily on local injury that permits immune recognition of normally hidden organ-restricted antigen. Loss of tolerance is not essential, since autoreactive cells never "see" sequestered antigen and thus are neither activated nor tolerized; they remain immunologically "ignorant". In this regard, there is substantial evidence that potentially pathogenic autoreactive B cells exist as non-tolerized ignorant cells within the healthy immune repertoire [4, 37, 44, 53, 78]. Occasionally, factors converge to both activate cells and permit antibody access to antigen [4, 49, 53].

The prototype organ-restricted autoimmune disease targeting basement membrane in man is Goodpasture's syndrome and its kidney-restricted counterpart is anti-GBM disease. Both can lead to rapid loss of kidney function. Goodpasture's syndrome is further complicated by lung hemorrhage. Disease results from anti-GBM autoantibodies that target the NC1 domain of the α3 chain of type IV collagen, or α3(IV) [77, 88]. Goodpasture's syndrome arises spontaneously in man and can be induced experimentally by passive transfer of anti-GBM antibodies or by active immunization. The clinical manifestations are attributed to the highly organ- and tissue-restricted distribution of α3(IV) collagen, which is expressed primarily in glomerular and alveolar basement membranes [9, 43]. The pathogenic epitopes have been localized to the EA and EB regions of the NC1

domain, normally buried in the native NC1 hexamer. The antibody epitopes are conformational and inaccessible to antibody binding unless the NC1 hexamer becomes dissociated [9]. Thus the epitopes are cryptic and presumably immunologically privileged, predicting that reactive B cells do not engage cognate antigen during development and are not tolerized. This further implies that primary regulation in this setting resides at a different level, such as within the T cell repertoire. We are currently testing this hypothesis in a novel autoantibody transgenic model targeting α3(IV) collagen epitopes.

SUMMARY AND FUTURE DIRECTIONS

Basement membrane components are targeted in diverse autoimmune diseases, yet the origin of this destructive autoreactivity remains unclear. Ig transgenic models provide an opportunity to track these autoreactive cells within the complex immunologic microenvironment of the whole animal and to glean insight into their regulation *in vivo*. Using the LamH Ig transgenic model we have characterized tolerance mechanisms controlling humoral anti-laminin reactivity associated with lupus nephritis. Immune characterization reveals a highly reproducible tolerance phenotype that is surprisingly preserved within autoimmune-prone strains carrying different constellations of disease susceptibility genes, including a strain prone to aggressive disease. Most anti-laminin B cells are deleted centrally, whereas a small residual population of cells are rendered anergic, many of which escaped deletion by editing their κ chains. The complex phenotype and evidence of dual specificity raise the possibility that multiple or crossreactive tolerogens are involved in regulation, a situation mirroring the antigen crossreactivity that is a hallmark of lupus. Less clear is the mechanism by which these tightly regulated lymphocytes ultimately escape tolerance in disease. Characterization of a unique model that tracks anti-collagen B cells specific for cryptic epitopes associated with organ-restricted disease should better define the spectrum of regulatory mechanisms engaged in controlling autoreactivity against structurally diverse matrix antigens. Unresolved areas await further investigation, including the contribution of immunological ignorance and sequestered antigens to autoimmune disease, the minimal requirements for effective B cell tolerance, the regulatory checkpoints most susceptible to host modifier gene influences, and the cause of failure of regulation. Dissection of these factors will permit development of individualized immunosuppressive strategies.

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