

Deficiency in CD22, a B Cell-specific Inhibitory Receptor, Is Sufficient to Predispose to Development of High Affinity Autoantibodies

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Summary

CD22 is a B cell-specific transmembrane glycoprotein that acts to dampen signals generated through the B cell antigen receptor (BCR): B cells from CD22-deficient mice give increased Ca^{2+} fluxes on BCR ligation. Here we show that this B cell hyperresponsiveness correlates with the development of autoantibodies. After the age of eight months, CD22-deficient mice developed high titers of serum IgG directed against double-stranded DNA; these antibodies were of multiclonal origin, somatically mutated, and high affinity. Increased titers of antibodies to cardiolipin and myeloperoxidase were also noted. The results demonstrate that a single gene defect exclusive to B lymphocytes is, without additional contrivance, sufficient to trigger autoantibody development in a large proportion of aging animals. Thus, CD22 might have evolved specifically to regulate B cell triggering thresholds for the avoidance of autoimmunity.

Key words: B lymphocyte • autoimmunity • threshold • CD22 • inhibitory receptor

Several autoimmune diseases are characterized by the presence in serum of high affinity antibodies to self antigens, with the B cells producing them having undergone heavy chain class switching and somatic hypermutation. This suggests that T cell help has been available, at least under these pathological conditions, to facilitate maturation of an anti-self response. The defect is, however, unlikely to lie simply in the inappropriate provision of T cell help: several models reveal that multiple loci can contribute to predisposition to autoimmune disease (1–4), with intrinsic defects in the B cell lineage able to play an important role (5, 6).

Although defects in B cell apoptosis can certainly accelerate autoimmune disease (7), several lines of evidence suggest that an intrinsic hyperresponsiveness of B cells to antigen encounter could also be a contributory cause of autoimmunity. Thus, genetic dissection of the contributing loci in a mouse model of systemic lupus erythematosus reveals that one of the loci (*Sle2*) leads to B cell hyperactivity (8). Furthermore, the response to self antigen by B cells that express an autoreactive immunoglobulin transgene can be significantly affected by mutations that affect B cell antigen receptor (BCR)¹ signaling (9, 10).

We were interested in determining whether mutations affecting B cell signaling would be sufficient to predispose

autoantibody development in an otherwise normal mouse. Is a hyperresponsiveness that is restricted to the B cell compartment nevertheless sufficient to so perturb the immune system that the necessary help is recruited to allow development of high affinity antiself antibodies?

To this end, we made use of CD22-deficient mice, which exhibit a relatively mild B cell hyperresponsiveness (11–14). CD22 is a B cell-specific transmembrane glycoprotein that associates with BCR and possesses an extracellular domain that binds α -2,6-sialylated glycoconjugates (15–18). It acts as a negative regulator of antigen receptor signaling, with levels of BCR cross-linking that are too low to generate a detectable signal in B cells from control mice, nevertheless giving rise to a calcium flux with CD22-deficient B cells (11–14). Indeed, even halving the abundance of CD22 on the cell surface leads to enhanced BCR signaling (19). In our initial characterization of CD22-deficient mice, we noted a small (approximately twofold) increase in total serum IgM (but not IgG) together with a corresponding increase in total Ig anti-DNA titers in 5-mo-old animals that might be ascribable to an expanded B1 cell population (11). However, here we show that as the CD22-deficient mice age, they have a dramatically increased likelihood of producing somatically mutated, high affinity autoreactive IgG. Thus, it appears that there is tight regulation of BCR signaling and that the perturbations caused by CD22 deficiency can trigger the development of autoimmunity.

¹Abbreviations used in this paper: BCR, B cell antigen receptor; ds, double-stranded; ES, embryonic stem cell.

Materials and Methods

Mice. Mice were generated from chimeras established using a previously described embryonic stem cell (ES) clone (11) containing a targeted integration of a *tk-neo* cassette into *Cd22*. The chimeras (created using C57BL/6 blastocysts) were bred against both C57BL/6 and BALB/c mice, and mice from the F2 generation were maintained for up to 20 mo with tail bleeds taken every 4–6 wk. A cohort of control (129 × C57BL/6)F2 mice (that do not carry any targeted gene alteration) was established analogously. Animals were either bred in our own conventional facility or in a specific pathogen-free (barrier) unit following delivery by Caesarian section and fostering onto C57BL/6 × CBA females in isolators.

Analysis of Autoantibodies. Serum titers of IgG anti-double-stranded (ds) DNA were measured as described elsewhere (20) using alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., Ltd.). Sera from four MRL/lpr mice were always titrated in parallel, with one of these sera assigned a titer of 5 U/ml. The assay was calibrated using a high affinity IgG2a monoclonal anti-dsDNA antibody (S22) from mouse 9612 (see below); 1 U/ml in the ELISA was given by 24 μg/ml of S22. Titers of other IgG autoantibodies were similarly determined using plates that had been coated with either cardiolipin (Sigma Chemical Co.; 100 μg/ml in ethanol) or myeloperoxidase (Calbiochem Corp.; 250 ng/ml in sodium bicarbonate, pH 9.2). Antibody isotypes were determined using reagents from PharMingen.

Hybridomas were established from unimmunized mice by fusion with NS0 cells and autoantibodies in the supernatants monitored by ELISA developing with biotinylated goat anti-mouse κ (Southern Biotech) and alkaline phosphatase-conjugated streptavidin (Dako). The binding of monoclonal anti-DNA antibodies at 20°C to a 5'-biotinylated ds 48mer oligonucleotide that had been immobilized on a streptavidin-coated chip (SA-Biacore chip; Pharmacia) was monitored by surface plasmon resonance as previously described (21).

Sequencing of Expressed V_H Segments. Oligo-dT-primed cDNA prepared from RNA extracted from the hybridomas was PCR amplified using a consensus V_H oligonucleotide for forward priming (5'-CGGGATCCTGAGGTGCAGCTGGAGGAGTC [22])

in conjunction with either 5'-CGGAATTCGGGGCCAGTGG-ATAGAC or 5'-CGGAATTCGGGACCAAGGGATAGAC for priming back from the C_H1 domain of $C\gamma1$, $\gamma2a$, and γb or of $C\gamma3$, respectively. PCR products were sequenced directly as well as ligated into pUC18 with multiple DNA clones sequenced from each hybridoma.

Results

We have previously described (11) an ES line (derived from 129 mice) that carries a targeted integration of a neomycin resistance gene into the *CD22* gene; this cell line was used to establish chimeric mice by injection into C57BL/6 blastocysts and germline transmission of the targeted allele (yielding $CD22^{+/-}$ heterozygotes) obtained following breeding with both C57BL/6 and BALB/c females. Cohorts of animals from the F2 generations of both sets of breedings were followed with time for the development of IgG anti-dsDNA antibody. On both backgrounds, high titers of anti-DNA antibodies developed with age (particularly after 8 mo) in many of the $CD22^{-/-}$ animals but not in the $CD22^{+/+}$ litter-matched controls. That the development of these autoantibodies was due to the targeted integration into the *CD22* gene is confirmed by the fact that IgG anti-dsDNA was not detected in the sera of control (129 × C57BL/6)F2 mice (Fig. 1).

The titer of anti-DNA antibody in the *CD22*-deficient mice is, in many cases, of a comparable order to that found in 12-mo-old MRL/lpr mice. By 18 mo of age, over 70% of the *CD22*-deficient mice have at some time shown evidence of IgG anti-dsDNA antibody in their sera at concentrations >1.5 U/ml; none of the 42 control mice revealed titers of this magnitude (Fig. 2). We have also followed a limited number of $CD22^{+/-}$ heterozygotes and found that

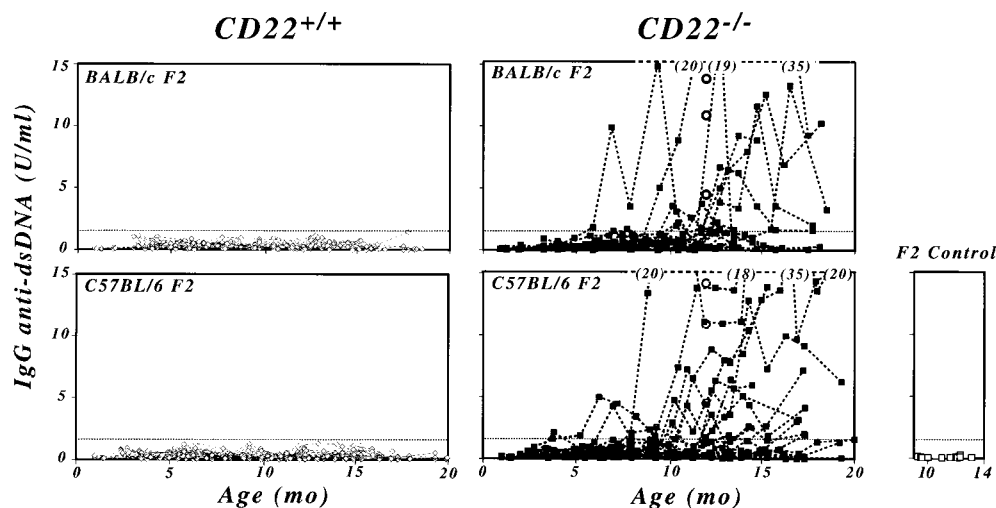


Figure 1. IgG anti-dsDNA antibodies in the sera of *CD22*-deficient and control mice. Serum IgG anti-dsDNA was measured by ELISA and compared in $CD22^{-/-}$ (center panels; ■) and $CD22^{+/+}$ littermates (left panels; ◇). These mice derive from the F2 generation of conventionally housed, *CD22*-targeted ES chimeric mice bred against BALB/c (top panels) or C57BL/6 (bottom panels). These cohorts initially comprised 16 *CD22*-deficient and 17 $CD22^{+/+}$ mice from the BALB/c breedings and 21 $CD22^{+/+}$ mice from the C57BL/6 breedings. Elevated titers of IgG anti-dsDNA were not detected in sera of aged C57BL/6 or 129 control mice. In addition, the ti-

ters of IgG anti-dsDNA were monitored in the sera of 18 control (129 × C57BL/6)F2 mice that did not carry a targeted gene modification (bottom right panel) to exclude the possibility that a *Cd22*-linked polymorphism might result in autoantibody development in the context of a (129 × C57BL/6)F2 background. The titers of IgG anti-DNA in sera of four MRL/lpr mice are indicated (○). Definition of units and calibration of the assay is described in Materials and Methods. Autoantibody titers did not differ significantly between males and females.

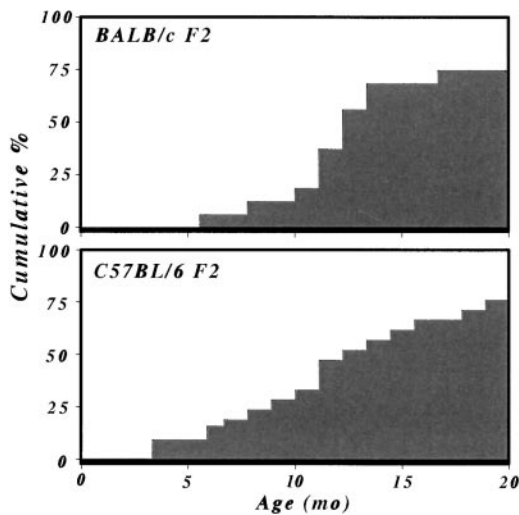


Figure 2. The percentage of CD22-deficient mice that have exhibited serum IgG anti-DNA titers >1.5 U/ml at some stage during their lives, plotted as a function of age. None of the control mice exhibited titers of this magnitude.

3/11 had developed IgG anti-dsDNA by 12 mo of age (not shown).

Life expectancy among CD22-deficient mice was decreased (10/43 weaned CD22-deficient mice having died by 15 mo of age compared with 1/43 CD22^{+/+} controls), with at least 4 of the deaths due to infection. However, all but one of these deaths occurred after 7 mo of age. Furthermore, we did not detect proteinuria or antibody deposition in glomeruli in the mice harboring autoantibodies. This lack of pathology may well correlate with the fact that anti-DNA titers do not simply rise with age but, in individual animals, often rise, regress, and rise again.

dsDNA is not the sole target of autoantibody development. Mice were also monitored for the development of antibodies to cardiolipin and myeloperoxidase; a clear distinction between the CD22-deficient and control siblings was found here as well (Table I and Fig. 3 A). The largest cohort of animals was followed under conditions of conventional housing, but we also compared autoantibody de-

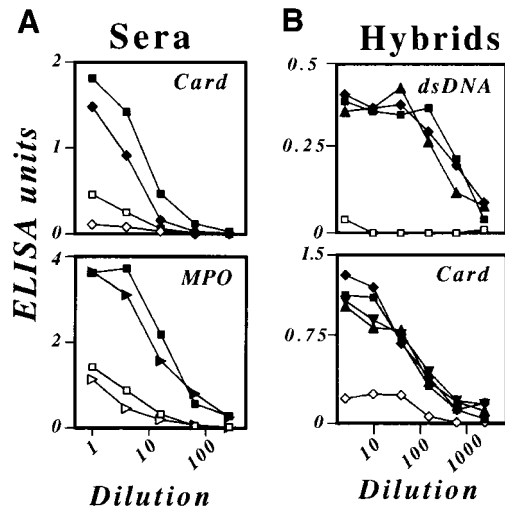


Figure 3. ELISA titration of autoantibodies in CD22-deficient mice. (A) Titers of serum IgG antibodies to cardiolipin (Card) and myeloperoxidase (MPO) in the sera of 18-mo-old mice. Filled symbols, CD22-deficient mice from the C57BL/6 breedings; mouse 9449 (■) and 9714 (◆) were conventionally housed, whereas 748 (▲) was from the SPF facility. Open symbols, litter-matched CD22^{+/+} controls. (B) Titers of autoantibody in supernatants of hybridomas obtained from fusions performed on CD22-deficient mice. Anti-DNA ELISA: S20 (■), S35 (◆), and S48 (▲) with the cardiolipin-specific hybrid S19 (□) providing a negative control. Anticardiolipin ELISA: S14 (▲), S19 (■), S28 (▼), and S72 (◆) with hybrid S8 (◇) providing a negative control. These hybrids all expressed IgG antibodies (subclass and V_H sequences are given in Fig. 5), but S14 and S28 were Card-specific IgMs with unassigned V_Hs.

velopment in CD22-deficient and control mice housed in a barrier unit. The results (Table I) reveal that autoantibodies also develop under these cleaner conditions.

Subclass typing of serum autoantibodies revealed that, in both the C57BL/6 and BALB/c breedings, IgG2a anti-DNA was found in ~80% of the autoimmune animals. However, >50% of the autoimmune mice contained anti-DNA antibodies of multiple IgG subclasses. To obtain more detail about the nature of these antibodies, hybridomas were established from two unimmunized, 18-mo-old, CD22-deficient females (mouse 9612 from the BALB/c breedings and

Table I. Autoantibodies in the Sera of CD22-deficient and Control Mice

Antigen	Conventionally housed				Barrier housed	
	12-mo-old		18-mo-old		14-mo-old	
	CD22 ^{+/+}	CD22 ^{-/-}	CD22 ^{+/+}	CD22 ^{-/-}	CD22 ^{+/+}	CD22 ^{-/-}
Cardiolipin	0/19*	1/16	0/17	3/14	0/5	2/7
Myeloperoxidase	0/19	3/16	0/17	3/14	0/5	6/7
dsDNA	0/25	10/19	0/25	10/15	0/5	4/7

Serum IgG autoantibodies were monitored at the ages indicated. All mice are from the C57BL/6 breedings. Two of the conventionally housed 18-mo-old as well as two of the barrier-housed 14-mo-old CD22-deficient mice simultaneously harbored antibodies against cardiolipin, myeloperoxidase, and DNA.

*Mice harboring autoantibodies/total mice.

9449 from the C57BL/6 breedings), as well as two CD22^{+/+} litter-matched controls. No anti-dsDNA IgG was detected in the supernatants from 198 wells from the control fusions; strong titers, however, were detected in 20/302 wells from the CD22-deficient mice (18 of these from mouse 9612; 2 from mouse 9449; Fig. 3). Similarly, whereas no cardiolipin-specific hybrids were detected in the control fusions, 19 positives were obtained from the CD22-deficient mice (10 from mouse 9612; 9 from mouse 9449). The majority of these cardiolipin-specific antibodies were IgMs, although mouse 9612 gave two IgA and mouse 9449 gave two IgG3 anticardiolipin antibodies.

The hybridomas were then expanded for further characterization. Analysis of the anti-DNA antibodies by surface plasmon resonance using a biotinylated oligonucleotide as antigen revealed that several bound DNA very tightly, with dissociation half-lives in the range of 8–500 min (Fig. 4). To ascertain whether the B cells producing these antibodies were clonally related and whether they had undergone somatic hypermutation, the V_H sequences were determined from several of the anti-DNA antibodies from mouse 9612. The results demonstrate that the anti-DNA antibodies within a single CD22-deficient mouse derive from multiple, clonally expanded B cell progenitors that have undergone class switching and somatic hypermutation. Thus, for example, hybridoma S48 appears to be derived from S30, as they carry the same V_H36–60/J_H2 rearrangement but with S48 harboring multiple additional somatic mutations (several to arginine), which could account for its increased affinity (Fig. 5 A). Similarly, S31 (IgG1) and S35, S66, S11, and S15 (all IgG2a) all express the same (V_H558 family member)/J_H2 rearrangement with an arginine-rich CDR3 (characteristic of many anti-DNA antibodies [23–25]); the individual antibodies differ, however, in the extent of accumulated somatic mutations (Fig. 5 B). In contrast, S20 and S22 carry distinct rearrangements of the same V_H7183 family member (Fig. 5 C). Thus, paralleling observations

previously made with other autoimmune mice (25), multiple independent B cells appear to have seeded an ongoing anti-DNA response. Similarly, in respect of the two cardiolipin-specific IgG3s, analysis of their V_H sequences revealed them to be clonally related (Fig. 5 D).

Discussion

The development of autoantibodies in CD22-deficient mice reveals that a single gene defect exclusive to B cells is sufficient to trigger autoimmunity in a large proportion of mice. This presumably means that the restriction of the provision of T cell help to foreign antigens is intrinsically imperfect: T cell help for an autoantibody response can be elicited by a hyperreactive B cell compartment.

The CD22-deficient animals do not, however, go on to develop autoimmune disease. This is consistent with genetic analyses of predisposition to systemic autoimmune disease in lupus-prone mouse strains, which reveal a role for multiple genetic loci (1–4, 26). Indeed, one of the loci contributing to autoimmunity in NZM mice (*Sle3*) has been mapped to a region of chromosome 7 in the vicinity of *Cd22* and, when bred into C57BL/6 mice, causes production of IgG anti-dsDNA antibodies as well as lupus nephritis (27). It will be interesting to ascertain whether this, at least in part, reflects a functionally relevant *Cd22* polymorphism. By analogy with studies in the MRL mouse (28), it will also be interesting to ascertain whether mutations in Fas or its ligand exacerbate autoimmunity in CD22-deficient mice.

The precise mechanism by which CD22 deficiency predisposes to autoimmunity remains to be definitively identified, but we believe the hyperresponsiveness of CD22-deficient B cells to BCR ligation is likely to be of central importance. Phosphorylation of CD22 on its cytoplasmic tyrosines following BCR ligation is mediated by the Lyn kinase and leads to the recruitment of the phosphatase SHP1 (29–34). It is therefore notable that deficiencies in either Lyn or SHP1 both lead to autoimmunity (35–38). However, this autoimmunity is more severe than that in CD22-deficient animals and is most unlikely to simply reflect defects in CD22-mediated regulation of BCR. Indeed, the increased severity probably correlates with both Lyn and SHP-1 being implicated in signal transduction through multiple cell-surface receptors, with their functions not being limited to the B cell lineage.

Thus, the significance of the autoantibody development in CD22-deficient mice lies in the fact that these autoantibodies arise as a consequence of a relatively mild perturbation that is exclusive to B lymphocytes and that affects the BCR signaling threshold. Experiments performed using transgenic mice that have been engineered to express high affinity autoreactive specificities on a substantial proportion of their B cells have revealed that the fate of such B cells is sensitive to modifications in CD22, Lyn, and SHP-1 as well as other genes that affect BCR signaling (9, 10). Our findings are entirely consistent with these earlier studies but reveal that CD22 deficiency alone, without additional con-

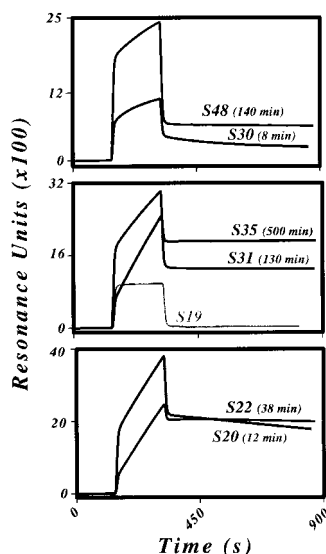


Figure 4. Binding kinetics of the monoclonal anti-DNA antibodies as measured by surface plasmon resonance. Antibody binding to a biotinylated ds oligodeoxyribonucleotide immobilized on streptavidin bound to the chip is depicted in resonance units and was monitored as a function of time. The dissociation half-life (min) calculated for each antibody is indicated in parentheses. The S19 (anticardiolipin) antibody (center) was included as a negative control.

A

	1	20	CDR1	40	----CDR2----	70	----D----	----JH2----->	
S30	<u>EV</u> OLEESRPSLVRPFSVLTCSVTGDSIT		SGYWN	WVRKFFGKLEFMGFMS	YSGNTYYNPSLKS	RISITRDTSKNHYFLHLNSVTTEDSATYYCA	KSTMITTPF	FDYWGQGTTLTVSS	IgG1
S48	-----A-----		I-----R-----	VYI-----	T-R-----	R-Q--Y-Q-----	T-----R	-----	IgG1

B

S47	-----L-----	--W--	--R-----	N-Y-GSGR-N-D-----S	-----V-T--T-----QL-----	V--T	RKAYYRHG	WFAWYWGQGLTVTUSA	IgG2a
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	1	20	CDR1	40	----CDR2----	70	----D----	----JH3----->	
G/L	<u>EV</u> OLEESGPPELVKPGASVVKMSCKASGYTFT		SYVMH	WVKQKPGQGLEWIG	YINPYNDGTYKNEKFKG	KATLTSDKSSSTAYMEFSSLTSEDSALYYCA	RRRASYGNSFV	YWGQGLTVTUSA	
S31	-----N-----		-----I-----	-----A-----	-----S-----	-----F-----	-----Y-----	-----	IgG1
S35	-----T-K-----		-----I-----	-----ST-N-----D-----	-----S-----	-----F-----	-----Y-----	-----	IgG2a
S11	-----K-----		-----I-----	-----ST-N-----D-----	-----S-----	-----F-----	-----Y-----	-----	IgG2a
S15	-----I-----		-----L-----	-----ST-N-----D-----T-----	-----S-----	-----F-----	-----Y-----	-----	IgG2a
S66	-----T-----		-----I-----	-----S--S--D-----	-----S-----	-----F-----	-----Y-----	-----	IgG2a

C

	1	20	CDR1	40	----CDR2----	70	----D----	----JH2/3----	
G/L	<u>EV</u> OLEESGGGLVQPGGSRKLSCAASGFTFS		SFGMH	WVRQAFPEKLEWVA	YISSGSSTIYYADTVKG	RFTISRDNPKNTLFLQMTSLRSEDATAMYYCAA	---D---	FDYWGQGTTLTVSS	IgG3
S20	-----G-----		-----I-----	-----R-----	-----S-----	-----F-----	-----Y-----	-----	IgG1
S22	-----G-----		-----R-----	-----R-----	-----S-----	-----F-----	-----Y-----	-----	IgG1

D

	1	20	CDR1	40	----CDR2----	70	----D----	----JH2-----	
S19	<u>EV</u> OLEESGAELVKPGTSSVKMSCKTSGYTFT		NVWIT	WVTRPFGQGLEWIG	DIFPLNGGTNSNEKFKN	MATLTVDTSSNTAYMQLSLLTSDDSAVYYCC	LGNS	FDNWGQGTTLTVSS	IgG3
S72	-----A-----		-----R-----	-----S-----	-----Y-----	-----A-----	-----A	-----Y-----	IgG3

Figure 5. V_H sequences of autoantibodies. Amino acid numbering is according to Kabat. The identity of the underlined amino acids is determined by the forward-priming oligonucleotide. (A–C) Sequences of anti-DNA antibodies, derived from a single fusion performed on an 18-mo-old female CD22-deficient mouse (9612) from the BALB/c breedings. (A) Sequences of the clonally related anti-DNA antibodies S30 and S48, which use a V_H36-60/J_H2 rearrangement. The parental germline V_H could not be unambiguously assigned, but, based on V_H36-60 sequences in the database, S30 is likely to prove much closer to the germline sequence than S48. (B) V_H amino acid sequences compared with a family of clonally related hybrids (S31, S35, S11, S15, and S66). G/L indicates the presumed sequence of the parental germline V_H (J558 family member) from which these sequences derive, deduced on the basis of intracloal sequence comparison as well as the published database of V_H sequences. Hybridoma S47 from the same fusion uses a distinct but related V_H rearrangement. (C) Sequences of S20 and S22, which use different rearrangements of V_H61-1P (a V_H7183 family member [43]) as their germline progenitor. (D) Sequences of clonally related anticardiolipin IgG3s (obtained from mouse 9449), which use a V_HJ558/J_H2 rearrangement. Dashes, identities; periods, silent nucleotide substitutions.

trivance, is sufficient to predispose autoimmunity in normal animals.

It is attractive to speculate from our results that the major physiological function served by CD22 in normal mice is to mediate the avoidance of autoimmunity. In light of the diminished level of CD22 expression in immature B cells (39), we previously suggested (11) that CD22 plays a role in raising the threshold of sensitivity to antigen that accompanies differentiation of an immature B cell (sensitive to tolerization/deletion by low affinity antigen) into a mature B cell that awaits triggering by exogenous antigen (40). Such a proposal could well explain the autoimmunity in CD22-deficient mice. However, a role for CD22 should also take into account the specificity of its extracellular domain for

α -2,6-sialoglycoconjugates (18). Intriguingly, the sialylated moieties present on eukaryotic membranes enhance the interaction between complement components C3b and factor H, thereby leading to inhibition of the alternative complement pathway; this serves to bias activation of the innate immune system toward microbial infection and away from autoreactivity (41, 42). Maybe CD22 recognition of the sialoglycoconjugates expressed on mammalian cells serves an analogous role in the adaptive immune system, dampening the BCR signaling that might otherwise be triggered by low affinity autoantigens. It will be interesting to ascertain whether making mutations in the CD22 extracellular domain that abolish recognition of sialoglycoconjugates will be sufficient to predispose autoimmunity.

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