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Modulation of Autoimmunity by TLR9 in the Chronic Graft-vs-Host Model of Systemic Lupus Erythematosus

Zhongjie Ma,* Fangqi Chen,* Michael P. Madaio,2† Philip L. Cohen,*‡ and Robert A. Eisenberg3*

Chronic graft-vs-host (cGVH) disease is induced in nonautoimmune mice by the transfer of alloreactive T cells that recognize foreign MHC class II. It closely resembles systemic lupus erythematosus, with antinuclear Abs and immune-mediated nephritis. Recent work has implicated TLRs, particularly TLR9, in the recognition of certain autoantigens in vitro and in vivo. To explore further the role of TLR9 in systemic autoimmunity, we induced cGVH disease in C57BL/6 (B6) mice lacking TLR9, including B6 mice expressing the anti-DNA-encoding IgH transgenes 3H9 or 56R (B6.3H9.TLR9−/−, B6.56R.TLR9−/−). We found that cGVH disease caused breakdown of B cell tolerance to chromatin and DNA in TLR9−/− recipients of alloreactive cells, yet that nephritis was less severe and that some autoantibody titers were lower compared with B6-cGVH controls. Spleen lymphocyte analysis showed that cGVH disease strikingly depleted marginal zone B cells in B6 mice, but did not influence T cell subsets in either B6 or B6-TLR9−/− hosts. B6.56R.TLR9−/− mice had less spontaneous production of autoantibodies than B6.56R mice, but there were no significant differences between B6.56R and B6.56R.TLR9−/− postinduction of cGVH disease. Taken together, these results suggested that TLR9 may worsen some aspects of systemic autoimmunity while alleviating others. The Journal of Immunology, 2006, 177: 7444–7450.

Systemic lupus erythematosus (SLE)4 is characterized by the production of autoantibodies against chromatin, Sm, DNA, and other nuclear Ags, as well as the deposition of Ig in various organs, including the kidneys and skin. These immunologic events are accompanied by the development of glomerulonephritis, although the underlying mechanisms are not fully understood (1). Previous work showed that the murine chronic graft-vs-host (cGVH) syndrome is a useful system to probe the mechanisms of tolerance loss in SLE B cells (2). In this model, SLE is induced by transferring alloreactive splenic cells from non-autoimmune bm12 mice into congenic, nonautoimmune B6 recipients (3). The donor and recipient cells differ by three amino acids of their MHC class II molecules, and this difference is sufficient to make the two strains fully alloreactive. In this cGVH model, the allo-helper T cells of the donor react against incompatible I-A structures of the host and generate abnormal help, which activates a subpopulation of B cells to become self-reactive (1, 4). Although evidence indicates that cGVH disease results in breakdown of B cell tolerance, the biochemical mechanisms that mediate this process are poorly understood.

Adaptive immunity is mediated by T and B lymphocytes, which are clonally distributed and show specificity and memory. In contrast, innate immunity is characterized by a lesser degree of specificity, and is mediated by the action of NK cells, dendritic cells, macrophages, and neutrophils. Accumulating evidence has demonstrated that certain aspects of innate immune systems are shared between insects and vertebrates and play crucial roles in host protection (5, 6). Furthermore, the innate and adaptive immune systems interact in critical ways. Recent reports indicate in fact that the innate immune pathways may contribute to the triggering of B cells to produce autoantibodies (7). Specifically, the TLR9, which has been thought to recognize bacterial DNA, appears to mediate the recognition of autologous DNA by B cells in vitro (8). In addition, the production of anti-DNA Abs in vivo in the MRL/lpr mouse model appeared to be dependent on TLR9 in one study, but not another (9, 10). In the present work, we have tested further whether TLR9 might be implicated in systemic autoimmunity in vivo using the cGVH reaction. This model permits the ready use of IgH transgenic strains on a C57BL/6 background. In addition, because the cGVH reaction induces autoimmunity in a normal mouse, it allows the examination of the effect of the transgenes on both tolerance and autoimmunity. We found that certain features of experimental SLE, such as the production of anti-DNA and antichromatin Abs, are variably dependent on TLR9. Other mechanisms, perhaps involving other innate immunity receptors, may also be of importance (11).

Materials and Methods

Mice

C57BL/6 (B6) mice, and B6.C-H-2bm12/KhEg(bm12) mice, were originally obtained from The Jackson Laboratory, and TLR9 knockout (TLR9−/−) mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan). The generation of B6.3H9 and B6.56R site-directed transgenic mice has been previously described (12). All mice were maintained in our mouse colony at the University of Pennsylvania Medical Center (Philadelphia, PA). Recipient and donor mice were sex- and age-matched within each experiment. The genotype of the TLR9−/− mice was determined by PCR of tail DNA.
cGVH disease was induced as previously described (3). Briefly, recipient mice (8–10 wk old) were injected (i.p.) with single-cell suspensions of 1 × 10^8 donor splenocytes, prepared by pressing donor spleens through a wire mesh screen in HBSS. Blood samples were obtained from experimental mice before the induction of cGVH disease and at 2, 4, 7, 10, and 13 wk thereafter. Sera were stored at -20°C for later analysis.

**Measurement of total IgG**

Total IgG was assessed by ELISA. Goat anti-mouse Fab at 3 μg/ml for total IgG (Jackson ImmunoResearch Laboratories) was coated onto plates as the first step of the ELISA. The biotinylated Abs were goat anti-mouse pFc’ for total IgG. Mouse IgG (clone HB63) was used as a standard in these assays.

**Detection of autoantibodies**

Autoantibodies were assessed by ELISA, as described previously (13, 14). The autoantigens were as follows: 1) chromatin, purified from chicken erythrocyte nuclei, and used at 5 μg/ml; 2) dsDNA, from calf thymus (Sigma-Aldrich), used at 3 μg/ml; and 3) IgG2b (clone CBPC101), used at 3 μg/ml for IgM rheumatoid factor (RF). Autoantibody results from individual ELISA were standardized against a reference serum (15).

**Isotype-specific anti-chromatin and anti-dsDNA**

The isotypes of anti-chromatin and anti-dsDNA were assessed by ELISA. Plates were coated with chromatin (5 μg/ml) and dsDNA (3 μg/ml). The IgGl, IgG2a, IgG2b, and IgG3 isotypes were detected with affinity-purified, biotinylated goat anti-rabbit IgGl, IgG2a, IgG2b, and IgG3 (pFc’-specific) on different plates (Southern Biotechnology Associates), followed by avin-alkaline phosphatase and substrate (Sigma-Aldrich).

**Evaluation of nephritis**

Urine protein concentrations were detected using Uristix reagent strips (Miles Laboratories). Serum creatinine was measured at 13 wk using a kit (Cayman Chemicals). Mice were sacrificed at that time, and the severity of nephritis was determined by light microscopy, as described previously (16). Briefly, for light microscopy, the severity of glomerular, interstitial, and vascular lesions was determined independently by blinded grading by one study author (M. P. Madaio, Temple University, Philadelphia, PA) on a scale of 0 to 4+. Multiple sections at a minimum of two different levels were observed. Each section typically involved evaluation of over 50 glomeruli and more than 20 blood vessels, and the interstitium contained within two to three longitudinal sections of kidney.

**Immunofluorescence staining of cell suspensions**

The following conjugated Abs were purchased from BD Pharmingen: anti-CD19 (clone 1D3), anti-CD21 (clone 7G6), and anti-CD23 (clone B3B4). A total of 1.5 × 10^6 cells was incubated with directly labeled Abs for 30 min and washed. Cells were analyzed on a BD Biosciences FACS Calibur using FlowJo software.

**Statistical and data analyses**

Statistical significance was determined using an unpaired nonparametric Mann-Whitney U test for proteinuria and kidney disease. Other data were compared by the Student t test. Each experiment used n = 5–8 mice per group. Protocols with the nontransgenic (Ig H chain) recipients were repeated once with results comparable to those presented. Data are shown as the mean ± SD.

**Results**

**Induction of cGVH autoimmune disease**

cGVH disease was established in unirradiated recipient mice by i.p. injection of a single dose of 1 × 10^8 donor spleen cells. Mice were followed for survival and for periodic determination of urinary protein and collection of serum. As shown in Fig. 1, all mice in the B6 → B6, B6 → TLR9^-/-, and bm12 → TLR9^-/- groups survived to 13 wk after the induction of cGVH disease. In contrast, two of eight mice in the bm12 → B6 group had died of their induced lupus by the time the experiment was terminated at 13 wk. However, no B6.3H9 or B6.56R mice died after induction of cGVH disease (data not shown).

**Total IgG increased in all recipients of alloreactive cells**

Total IgG increased in all cGVH groups (bm12 donors) compared with the corresponding controls (B6 donors) (Fig. 2). Levels of IgG in the bm12 → TLR9^-/- group were significantly lower than in the bm12 → B6 group only at 4 and 10 wk (p < 0.05) (Fig. 2A). The presence or absence of TLR9 did not influence the increased IgG levels induced by cGVH disease in either 3H9 or 56R mice. The 3H9 and 56R strains spontaneously develop high levels of IgG. Interestingly, the lack of TLR9 lowered their IgG levels, in contrast to what was seen in B6 mice without an anti-DNA transgene (Fig. 2, B and C).

**Anti-dsDNA and anti-chromatin**

As expected, the bm12 → B6 positive control group showed substantial elevations of anti-dsDNA titers. Interestingly, the TLR9^-/- mice had significant levels of anti-DNA before the induction of cGVH disease, and these did not increase further after challenge with bm12. However, because the anti-DNA levels actually fell somewhat in their control group, the levels in the bm12 → TLR9^-/- group anti-dsDNA were significantly higher than in B6 → TLR9^-/- only at 4 wk, but lower than in bm12 → B6 at 2 and 4 wk (Fig. 3A). Anti-dsDNA in all the 3H9 and 56R cGVH groups was higher than in the corresponding controls. Interestingly, in these transgenic recipients, TLR9 made no difference. The levels of anti-dsDNA in the bm12 → 3H9.TLR9^-/- were actually higher than in B6 → 3H9, whereas the reverse pattern was true for 56R, but the differences never reached statistical significance. The 56R strain develops spontaneous levels of anti-dsDNA Abs. Interestingly, the lack of TLR9 lowered their levels, in contradistinction to what was seen in B6 mice without an anti-DNA transgene (Fig. 3, B and C). The patterns of the anti-chromatin responses in all groups were similar to those directed at dsDNA (Fig. 4).

**IgM RF**

All cGVH groups showed increased production of RF IgM anti-IgG2b. Strikingly, the presence of TLR9 played no significant role in any of the three B6 recipient substrains (Fig. 5).

**The subclasses of anti-dsDNA and anti-chromatin**

The subclasses of anti-dsDNA and anti-chromatin were measured at 4 wk postinduction of cGVH disease. Anti-dsDNA IgG1, IgG2b, and IgG3 were significantly lower in bm12 → TLR9^-/- than in bm12 → B6 mice (Fig. 6A). Anti-chromatin IgG1, IgG2a,
and IgG2b were also significantly lower in bm12 B6 mice than in bm12 B6 mice (p < 0.05) (Fig. 6B).

Renal disease

Proteinuria was measured at 2, 4, 7, 10, and 13 wk to assess renal involvement during cGVH disease. As shown in Fig. 7A, both bm12 → TLR9−/− and bm12 → B6 mice showed significant elevation of urine protein excretion, compared with the B6 → TLR9−/− and B6 → B6 groups, respectively. However, proteinuria in the bm12 → TLR9−/− mice was significantly lower than in bm12 → B6 mice (p < 0.05). The 3H9 and 56R cGVH models also showed significant proteinuria, with again no effect of TLR9 (Fig. 7, B and C). Serum creatinine was measured at 13 wk. All cGVH groups showed significant elevation of serum creatinine compared with non-GVH groups. However, creatinine in the bm12 → TLR9−/− mice was significantly lower than in bm12 → B6 mice (p < 0.05). The bm12 → B6 positive control group showed substantial elevations of anti-dsDNA titers. Anti-dsDNA in all the 3H9 and 56R cGVH groups was higher than in the corresponding controls. *p ≤ 0.05, comparing cGVH and control recipients; +p ≤ 0.05, comparing cGVH groups with and without TLR9; #, p ≤ 0.05, comparing control groups with and without TLR9.
significantly higher than those of B6 → B6 and B6 → TLR9−/− mice; however, the pathology in bm12 → TLR9−/− mice was less severe compared with bm12 → B6 mice (Fig. 9).

**B cell and T cell subpopulations**

Spleen B cell subpopulations were examined by FACS at the time of sacrifice 13 wk postinduction of GVH. The only significant difference between the TLR9−/− and B6 recipients of bm12 cells (cGVH groups) was a less pronounced depletion of marginal zone B cells in the knockouts (data not shown).

**Discussion**

Increasing evidence has linked the innate and adaptive immune systems, in response to both foreign immunogens and autoantigens (17, 18). A recent study has shown that RF-positive B cells could be activated much more efficiently in vitro when the Ig bound by the BCR was complexed with chromatin (7). This activation was dependent on TLR9-mediated recognition of DNA released by the cultured murine cells themselves. These data led to the novel hypothesis that the activation of B cells specific for nuclear Ags may depend on TLR recognition. Further work from the same group has provided additional in vitro data to support this idea (19).
In vivo studies have shown a complex relationship between TLR9 and B cell tolerance. Recently, two published papers have investigated the role of TLR9 in the MRL/lpr model of SLE, with seemingly contradictory results. In one study based on the F2 generation of a cross of MRL/lpr with the knockout strain, the TLR9/H11002/H11002/H11002 mice had lower levels of autoantibodies against DNA-containing Ags, whereas another study, using a more fully backcrossed MRL/lpr-TLR9/H11002/H11002/H11002 strain, found increased anti-DNA titers (9, 10). Subsequent publications have been similarly paradoxical. In C57BL/6-lpr mice, the TLR9/H11002/H11002/H11002 locus decreased the antinucleosome response, but increased anti-DNA titers (20). In a spontaneous SLE model based on an induced germline mutation in phospholipase C/H9253, the TLR9/H11002/H11002/H11002 locus resulted in increased levels of autoantibodies, whereas in the FcγRIIB/H11569 model, TLR9/H11002/H11002/H11002 locus caused decreased anti-DNA Abs (21, 22).

TLR9 regulates Abs in cGVH mice, but not those with IgH anti-DNA transgenes

In the current study, we have used the cGVH model to explore further the role of TLR9 in the loss of B cell tolerance in SLE. The transfer of spleen cells from MHC class II-disparate donors (bm12) into B6 mice provides allo-help to the recipient B cells and results in a spectrum of autoantibodies comparable to what is seen in spontaneous SLE (23). Thus, the loss of tolerance by B cells in this model parallels that seen in spontaneous disease, even though the nature of the T cell help, through allore cognition, is presumably different. Our present findings with this system have confirmed a role for TLR9 in the development of autoimmunity. However, the nature of the effect was complex, as previous work has suggested.

Anti-dsDNA and anti-chromatin autoantibodies were significantly lower in bm12 → TLR9/H11002/H11002/H11002 than in bm12 → B6 mice. Levels in subclasses shown were significantly lower in bm12 → TLR9/H11002/H11002/H11002 than in bm12 → B6 mice. Symbols represent each individual animal. Horizontal bar, Mean value for each group. *p ≤ 0.05, comparing cGVH and control recipients; +, p ≤ 0.05, comparing cGVH groups with and without TLR9.

FIGURE 6. IgG subclasses of anti-dsDNA and anti-chromatin autoantibodies at 4 wk postinduction of cGVH. A, anti-ds DNA; B, anti-chromatin; C, 56R recipients with and without TLR9. Levels in subclasses shown were significantly lower in bm12 → TLR9/H11002/H11002/H11002 than in bm12 → B6 mice. Levels in subclasses shown were significantly lower in bm12 → TLR9/H11002/H11002/H11002 than in bm12 → B6 mice. Symbols represent each individual animal. Horizontal bar, Mean value for each group. *p ≤ 0.05, comparing cGVH and control recipients; +, p ≤ 0.05, comparing cGVH groups with and without TLR9.

FIGURE 7. Proteinuria in cGVH mice. A, B6 recipients with and without TLR9. B, 3H9 recipients with and without TLR9. C, 56R recipients with and without TLR9. Significant differences in urine protein excretion, serum creatinine, and proteinuria were found in all groups as described in Results. *p ≤ 0.05, comparing cGVH and control recipients; +, p ≤ 0.05, comparing cGVH groups with and without TLR9.
the λ1+ L chain are stimulated to proliferate by a TLR9 ligand, whereas λ1− 3H9 B cells have their spontaneous proliferation inhibited by a TLR9 antagonist (24). These seemingly divergent results further emphasize the complexity of the role of TLR9 in modifying the response of B cells to DNA.

Interestingly, the B6-TLR9−/− mice had significant levels of anti-DNA Abs before the induction of cGVH, which were not seen in B6 mice of the same age. In contrast, the lack of TLR9 downregulated the spontaneous loss of tolerance to DNA in B6.56R mice. TLR9 has been shown to be important in shifting the immune response to a Th1-like response (8), and thus to have an effect on the IgG2a and IgG2b isotypes (21). In our data in B6 cGVH mice, however, the isotype distribution of anti-dsDNA antibodies was not influenced by TLR9, which suggests that the role of TLR9 signaling in the anti-DNA response goes beyond a shift in cytokines. In all three sets of recipients, IgM RF was unaffected by TLR9. We also found a modest reduction in renal disease in TLR9−/− cGVH mice. The B cells in TLR9−/− mice responded to cGVH with the expected phenotypic changes we have previously reported, except for less depletion of the marginal zone B cell subset in bm12 → TLR9−/− compared with bm12 → B6 mice (2, 25).

**TLR9 has complex effects on different autoantibodies**

Overall, then, our results indicate that the lack of TLR9 does not interfere with the basic alloreactivity of the cGVH model, in that B cells are activated and some autoantibodies (RF) are produced at expected levels. However, TLR9 deficiency does have variable effects on the production of autoantibodies against DNA or DNA-containing Ags (chromatin). In the wild-type B6 recipients, TLR9 deficiency may cause some failure of normal tolerance to DNA. However, under the stimulus of cGVH disease, TLR9 deficiency rather seems to protect against the loss of tolerance. Similarly, in the B6.56R mouse, which spontaneously loses tolerance to DNA (our unpublished data), TLR9 deficiency opposes autoreactivity, whereas cGVH disease in either B6.56R or B6.3H9 mice is unaffected. These latter results may reflect the strong forces favoring autoimmunity resulting from the combination of the autoantibody transgene and alloreactive T cell help. Alternatively, the rearranged Ig H chain transgene may bypass a TRL9-dependent step in B cell development.

The failure of the presence of TLR9 to affect RF titers despite decreased levels of autoantibodies to dsDNA and chromatin in the
B6-TLR9$^{-/-}$ cGVH mice is curious, particularly given the in vitro results with RF specific B cells from Marshak-Rothstein and colleagues (7, 19). It suggests that in vivo, at least in the cGVH model, the production of RF is not driven by DNA-containing immune complexes.

The in vivo recognition of DNA by B cells (and perhaps other cells, such as dendritic cells) therefore depends in part on the TLR9 receptor, but the consequences of this recognition may differ dramatically in different contexts, and thus may be hard to predict. In some situations, the induction of tolerance may be facilitated by the activation of TLR9 by DNA, in others it may be the loss of tolerance that is affected, and in still others, it may be irrelevant. This complexity is not really surprising because in general the induction of B cell tolerance and the breakdown of tolerance require Ag recognition by the same BCR. It is the context of the recognition, that is, the presence of other signals and the stage of maturation of the B cell, that determines the outcome. In a sense, tolerance and autoantibody production, at least for the anti-dsDNA specificity, must share some common pathways, including the involvement of TLR9$^{-/-}$. Thus, any targeting of TLR9 for potential therapeutic interventions in SLE will need to be done carefully.

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Disclosures

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