Immunologically Active Autoantigens: The Role of Toll-Like Receptors in the Development of Chronic Inflammatory Disease

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Key Words
systemic autoimmune disease, Toll-like receptor 9, Toll-like receptor 7, autoantibodies

Abstract
Pattern recognition receptors (PRRs), expressed on cells of both the innate and adaptive immune systems, serve as sentinels, waiting to alert the host to the first signs of microbial infection and to activate the initial line of immune defense. Research has increasingly demonstrated that many of the same PRRs also recognize self-epitopes that either are released from dying or damaged cells or are present at the surface of apoptotic cells or apoptotic bodies. In this context, PRRs play a critical role in tissue repair and the clearance of cellular debris. However, failure to appropriately regulate self-responses triggered by certain PRRs can have serious pathological consequences. The Toll-like receptor (TLR) gene family represents a case in point. TLR7, 8, and 9 were originally identified as receptors specific for bacterial and viral RNA and DNA, but more recent in vitro and in vivo studies have now linked these receptors to the detection of host RNA, DNA, and RNA- or DNA-associated proteins. In this context, they likely play a key role in the development of systemic autoimmune diseases.
INTRODUCTION

Autoimmune diseases occur when the adaptive immune response is aberrantly directed to host tissues. Systemic lupus erythematosus (SLE) and other related chronic inflammatory conditions are routinely associated with high titers of IgG autoantibodies reactive with ubiquitously expressed intracellular autoantigens. These autoantibodies and immune complexes containing these autoantibodies accumulate in the joints, kidneys, and other tissues, where they provoke inflammatory responses leading to tissue damage and destruction. Several environmental and genetic factors contribute to the development of systemic autoimmune diseases, and thus the cause and clinical course of these conditions are highly variable. This diversity is reflected in the myriad of genetic lesions that give rise to animal models for SLE, including defects in apoptotic pathways, improperly regulated B and T cell signaling cascades, and inefficient clearance of cell debris. Remarkably, in all these models, as well as in many patients afflicted with SLE, autoantibodies are directed against a relatively limited set of nucleoprotein particles (1, 2). A clear understanding of the processes that allow these particular epitopes to become immunogenic should provide important insights for the design of safer and more specific therapeutics.

Another possible link between infection and autoimmunity involves the cross-reactivity between infectious agents and particular self-antigens. Examples of such molecular mimicry include B cells that specifically recognize a particular region of both the Epstein Barr virus EBNA-1 protein and the Ro autoantigen (5). According to this model, B cells that recognize any component of a subcellular particle would be expected to preferentially bind, internalize, and process proteins associated with the complex and then to present elevated levels of these particular peptides (in the context of MHC) to potentially self-reactive T cells. This initial amplification loop could lead to epitope spreading—that is, to the production of autoantibodies against additional components of the particle. As part of the apoptotic process, many of the common autoantigens are redirected from the nucleus and/or cytoplasm to apoptotic blebs on the cell surface (6). Autoantibodies specific for one kind of particle may bind to apoptotic bodies that incorporate additional particles, thereby facilitating the FcγR-mediated uptake of this material by activated APCs and leading to a second phase of amplification in which the autoantibody response spreads to additional subcellular particles. Although clearly an attractive hypothesis, molecular mimicry cannot explain why autoantibodies reactive to the same restricted set of autoantigens are found in both SLE patient populations and in spontaneous noninfectious
animal models of SLE, and even in germ-free autoimmune-prone mice (7).

The cytotoxic activity of antiviral effector cells provides a third potential connection between infection and autoimmunity. Many of the common autoantigens are cleaved by either granzymes or caspases (8), proteases that mediate the cytopathic events initiated by T or natural killer (NK) cell cytotoxic populations. Autoantigen cleavage can theoretically create neoepitopes, either because the resulting fragments form novel conformational determinants or because realignment of the protein sequences provided to the antigen processing machinery leads to the presentation of previously cryptic peptides. Additional examples of autoantigen modification, such as phosphorylation, dephosphorylation, oxidation, citrullination, isoaspartylation, and transglutamination, may provide additional forms of neoepitopes. Many of these modifications occur during the process of apoptotic cell death or result from tissue injury and/or inflammation (9–11).

Although apoptotic cell death is generally considered silent or tolerogenic, the release of modified self-antigens in the context of inflammation, whether infectious or noninfectious, may suffice to activate both T cells and B cells not yet tolerized to the newly formed determinants. This neoepitope model implies a relatively passive role for autoantigens: T cell or B cell recognition in the appropriate microenvironment leads to immune activation. Although a tuned-up immune system, molecular mimicry, and the release of modified self-antigens may all contribute to a loss of tolerance, can these factors alone account for the target specificity of the autoantibody repertoire? Insights garnered over the past decade suggest not. Rather, evidence increasingly suggests that autoantigens may play a much more proactive role in promoting and sustaining systemic autoimmune diseases than was previously appreciated. As outlined in this review, certain self-components may in fact be autoantigens because they are inherently biologically active. That is, they have the capacity to engage effectively a PRR and thereby provoke an inflammatory response that then leads to the activation of autoantigen-specific T cells and B cells and the production of autoantibodies.

**TOLL-LIKE RECEPTORS 9 AND 7**

**TLR9 and TLR7 Ligands**

The PRRs so far most closely associated with autoantibody production are Toll-like receptor 9 (TLR9) and TLR7, which, together with TLR3 and TLR8, form a TLR subfamily predominantly located in cytoplasmic compartments of the endoplasmic reticulum, endosome, and lysosome lineage. By contrast, the other TLR family members are mainly expressed on the plasma membrane. Because of their location, TLR9- and TLR7-initiated signaling cascades are inhibited by drugs such as chloroquine and bafilomycin that interfere with endosome acidification and/or maturation (12). Both bacterial and viral DNA can effectively activate TLR9 (13, 14). TLR9 is also the receptor responsible for the adjuvant activity of oligodeoxynucleotides (ODNs) containing hypomethylated CpG motifs that are currently involved in clinical trials as both cancer and viral vaccine adjuvants (15, 16).

TLR7 recognizes viral ssRNA and at least some mammalian ssRNA sequences (17–19). Although the recognition unit for TLR7 remains poorly defined, both UG-rich RNA and ssRNAs containing unmodified bases appear to be preferentially recognized (19–21). TLR9 and TLR7 are constitutively expressed in B cells and plasmacytoid DCs (pDCs), and both of these cell types are closely tied to disease pathogenesis in SLE and related systemic diseases through the production of autoantibodies and IFN-α, respectively (22, 23). TLR8 also recognizes ssRNA, but TLR8 does not appear to be expressed in mice. In humans, TLR8 has a very different cell distribution...
than TLR7 and is found mainly in cells of the monocyte/myeloid lineage, where TLR8 engagement elicits a distinctly different set of cytokines than TLR9/7 engagement of pDCs (24). Whether TLR8 serves a proinflammatory or protective role in SLE remains to be determined.

**BCR/TLR9 and BCR/TLR7 Synergy in the In Vitro Response of B Cells to SLE-Associated Autoantigens**

A functional link between TLR9 expression and autoreactive B cell activation was first revealed in studies involving B cell receptor (BCR) transgenic murine B cells. B cells expressing transgene-encoded autoreactive receptors have been valuable experimental tools for exploring mechanisms of B cell tolerance. They have also played a critical role in the evaluation of autoantigen biological activity. The AM14 transgenic line, originally developed by Weigert and colleagues (25–27), expresses a receptor specificity commonly found in the IgG autoantibody repertoire of Fas-deficient MRL autoimmune-prone mice (25). These rheumatoid factor antibodies recognize IgG2a (26), and B cells expressing this receptor escape the tolerance-inducing mechanisms that eliminate more avid self-reactive cells (27). Although mature AM14 B cells on a nonautoimmune-prone background remain relatively quiescent or undergo abortive activation, on an autoimmune-prone background they readily convert to autoantibody-producing plasmablasts (28, 29). This conversion was recapitulated in vitro by stimulating AM14 B cells with autoantigen-containing immune complexes (ICs). Importantly, AM14 B cells did not proliferate in response to foreign protein–containing ICs or to heat-aggregated IgG2a (30). These experiments pointed to a critical property of autoantigen ICs that could not be provided by nonautoantigen ICs, namely the ability to engage a PRR.

The relevant PRR depends on the nature of the autoantigen. One type of stimulatory IC forms when anti-DNA or anti-nucleosome IgG2a monoclonal antibodies (mAbs) are added to primary AM14 B lymphocyte cultures; these antibodies bind to material presumably released from cells damaged during the cell preparation procedure or cells that fail to survive in culture. The IC-activated AM14 B cells rapidly enter the cell cycle, as detected by 3H-thymidine incorporation. The addition of DNase results in degradation of the antigen and eliminates the proliferative response. This DNA-associated IC response is dependent on expression of both the AM14 BCR and TLR9 (31, 32).

A second type of stimulatory IC forms when IgG2a mAbs reactive with RNA, or RNA-associated proteins, are added to primary B cell cultures. The addition of purified ribonucleoprotein (RNP) particles significantly enhances the potency of these antibodies, presumably by increasing the concentration of or valency of the ICs. Stimulation by the RNA ICs is sensitive to RNase, dependent on TLR7, and dramatically enhanced by the addition of type 1 IFN (33), which serves to upregulate TLR7 (34). Both DNA and RNA IC stimulation of AM14 B cells are inhibited by ODNs that block TLR9 and TLR7 signaling and also by chloroquine or bafilomycin. Overall, the data are consistent with a model in which the AM14 BCR binds to a DNA-/RNA-containing IC and then directs the IC to an internal compartment where the complexed DNA/RNA triggers TLR9 or TLR7; both the BCR and the TLR are required for B cell activation.

Although the BCR/TLR paradigm was originally identified by IC activation of IgG2a-reactive B cells, additional in vitro studies indicate that TLR engagement is also involved in the activation of B cells that directly bind DNA or other autoantigens and may therefore play an important role in the early stages of autoantibody production.
FcγR/TLR Synergy Contributes to the Activation of Dendritic Cells and Other Cytokine-/Chemokine-Producing Cells

DNA- and/or RNA-containing ICs can also bind to low-affinity FcγRs on various types of APCs. Sera obtained from patients with SLE or other systemic autoimmune disorders can induce pDCs to produce high amounts of the type 1 IFN, IFN-α (35–37). This activity can be recapitulated by mixing IgG antibodies purified from patient sera with either apoptotic or necrotic cell debris, or by mixing the same antibodies with CpG-rich DNA or purified SmRNP (20, 38–41). Blocking antibodies specific for FcγRIIa (CD32) inhibit IFN-α production (36, 42). Experiments with TLR-deficient murine pDCs as well as inhibitory ODNs and inhibitors of endosome acidification have further linked this response to TLR7 and TLR9 (20, 41, 43) (summarized in Table 1). Type 1 IFNs are thought to play a critical role in the etiology of SLE through a variety of mechanisms that include direct

### Table 1  In vitro evidence for TLR involvement in autoantigen-mediated activation of B cells, DCs, or other APCs

<table>
<thead>
<tr>
<th>Responder cells</th>
<th>Relevant cell surface receptor</th>
<th>Autoantigen</th>
<th>Relevant TLR</th>
<th>Criteria for TLR involvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine B cells</td>
<td>BCR anti-IgG2a AM14</td>
<td>IgG2a antihistone + spent culture fluid</td>
<td>TLR9 &gt; TLR7</td>
<td>MyD88 knockout, TLR9 knockout, Unc-93B knockout, Inh-ODN, Inh-Endo/H+</td>
<td>31, 32, 113</td>
</tr>
<tr>
<td>Murine B cells</td>
<td>BCR anti-IgG2a AM14</td>
<td>IgG2a anti-RNA + spent culture fluid, IgG2a anti-SmD + SmRNP</td>
<td>TLR7</td>
<td>TLR7 knockout, Inh-ODN, Inh-Endo/H+</td>
<td>33</td>
</tr>
<tr>
<td>Murine B cells</td>
<td>BCR anti-DNA VH3H9</td>
<td>Spent culture fluid</td>
<td>TLR9 (TLR7?)</td>
<td>Inh-ODN</td>
<td>114</td>
</tr>
<tr>
<td>Murine mDC</td>
<td>FcγRIIa</td>
<td>IgG2a antihistone + spent culture fluid or nucleosomes</td>
<td>Partially TLR9</td>
<td>TLR9 knockout, Inh-ODN</td>
<td>51</td>
</tr>
<tr>
<td>Murine pDC</td>
<td>U1 snRNP + DOTAP</td>
<td>TLR7</td>
<td>TLR7 knockout</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Murine pDC</td>
<td>IgG3 anti-SmB/D mAb + U1 snRNP</td>
<td>TLR7</td>
<td>TLR7 knockout</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Human pDC</td>
<td>FcγRIIa (CD32)</td>
<td>SLE IgG anti-DNA</td>
<td>TLR9</td>
<td>TLR9/CD12-transfected HEK cells, Inh-ODN, Inh-Endo/H+</td>
<td>50</td>
</tr>
<tr>
<td>Human PBMCs</td>
<td>U1 snRNP + DOTAP, SLE sera + U1 snRNP</td>
<td>TLR7</td>
<td>RNase-sensitive Inh-ODN, Inh-Endo/H+</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Human pDC</td>
<td>SLE anti-DNA sera + apoptotic cell debris</td>
<td>TLR9</td>
<td>TLR9-specific Inh-ODN</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Human pDC</td>
<td>SLE IgG anti-RNP + apoptotic cell debris</td>
<td>TLR7</td>
<td>TLR7-specific Inh-ODN</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Human pDC</td>
<td>FcγRIIa (CD32)</td>
<td>SLE IgG + U1 snRNP</td>
<td>TLR7</td>
<td>RNase-sensitive Inh-ODN, Inh-Endo/H+</td>
<td>40</td>
</tr>
</tbody>
</table>

*aDC, myeloid dendritic cells; pDCs, plasmacytoid dendritic cells.

bAM14, rheumatoid factor BCR transgenic line; VH3H9, anti-DNA BCR transgenic line.

cInh-ODN, inhibitory oligodeoxynucleotide; Inh-Endo/H+, inhibitor of endosome acidification.

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Table 2  Examples of cell types that coexpress FcγRs together with TLR7, TLR8, and/or TLR9

<table>
<thead>
<tr>
<th>Cell typea</th>
<th>TLR7</th>
<th>TLR8</th>
<th>TLR9</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDCs (H, M)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>115–119</td>
</tr>
<tr>
<td>CD8+ (lymphoid) cDCs (M)</td>
<td>−</td>
<td>+</td>
<td></td>
<td>119</td>
</tr>
<tr>
<td>CD8− (myeloid) cDCs (M)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>119, 120</td>
</tr>
<tr>
<td>Skin-derived mast cells (M)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>121</td>
</tr>
<tr>
<td>Langerhans cells</td>
<td></td>
<td></td>
<td>+</td>
<td>122</td>
</tr>
<tr>
<td>Monocytes DC (H)</td>
<td>+ or −</td>
<td>+</td>
<td></td>
<td>117, 123</td>
</tr>
<tr>
<td>Myeloid DCs (H)</td>
<td>+ or −</td>
<td>+</td>
<td></td>
<td>123</td>
</tr>
<tr>
<td>Activated neutrophils (H)</td>
<td>+</td>
<td>+</td>
<td></td>
<td>124</td>
</tr>
<tr>
<td>Platelets (M, H)</td>
<td></td>
<td></td>
<td>+</td>
<td>125</td>
</tr>
</tbody>
</table>

aH, human; M, mouse.

PBMC: peripheral blood mononuclear cell

- effects on DCs, cytotoxic effector cells, and B cells (44, 45). SLE patients often develop an IFN signature associated with the peripheral blood mononuclear cell (PBMC) expression of numerous IFN-inducible genes (46, 47). Moreover, genetic polymorphisms associated with IFN regulatory factor 5 (IRF5), a proinflammatory cytokine-associated transcription factor downstream of TLR9 and TLR7, are strong risk factors for the development of SLE (48, 49). However, despite the notoriety of IFN-α in the SLE literature, one must keep in mind that SLE ICs also induce pDCs to produce proinflammatory cytokines and chemokines other than IFN-α (50).

- pDCs are not the only cell type that can express FcγRs together with TLR9 and/or TLR7 (for other examples, see Table 2). For example, DNA/nucleosome-containing ICs, and not protein ICs, induce murine bone marrow–derived myeloid DCs to secrete TNF-α through a mechanism that is dependent on FcγRIII and at least partially dependent on TLR9 (51). In addition, GM-CSF-activated neutrophils produce remarkably high levels of IL-8 in response to SLE ICs (50), an intriguing observation considering the neutrophil signature apparent in a subpopulation of SLE patients (47). Whether TLR9- and/or TLR7-expressing NK cells, skin-derived mast cells, or Langerhans cells respond to SLE ICs remains to be determined. DNA- or RNA-containing IC activation of these populations could potentially contribute to the pathogenesis of SLE or other autoimmune diseases through a variety of mechanisms.

An important issue to be considered in such future studies (as well as in some of the published SLE IC reports) is whether a role for TLR7 or TLR9 is confirmed or merely implied. ICs (that do not contain DNA and/or RNA) have been reported to effectively stimulate cells through FcγR engagement (52–54), and therefore the stimulatory activity of verified control ICs will need to be directly compared with that of SLE ICs. dsRNA can also activate the innate immune system through non–TLR-associated cytoplasmic receptors such as the helicases RIG-1 (retinoic acid–inducible gene 1) and MDA-5 (melanoma differentiation–associated gene 5) (55, 56). IPS-1 (IFN-β promoter stimulator protein 1), an adapter protein downstream of RIG-1 and MDA-5, is also involved in the detection of cytosolic dsDNA (57). Although it is unclear whether RIG-1, MDA, or other potential cytoplasmic receptors are accessible to RNA or DNA internalized through FcγR, that possibility cannot be ruled out at this point.
Effect of TLR9 Deficiency on Murine Models of Autoimmunity

Although the in vitro analysis implicates both DNA- and RNA-associated autoantigens in the TLR-dependent activation of transgenic B cells and DCs, the relevance of these observations to actual disease pathogenesis can only be confirmed by determining the effect of aberrant TLR expression on in vivo models of systemic autoimmune disease. The initial analyses of TLR-deficient autoimmune-prone mice have provided intriguing results and indicate that the various models of spontaneous lupus may be differentially regulated by TLR9 and/or TLR7 (Table 3).

B6 mice lacking the inhibitory FcγRIIB receptor produce IgG autoantibodies and develop glomerulonephritis (58). If these FcγRIIB-deficient mice inherit the high-affinity 56R heavy chain anti-DNA transgene, they develop accelerated disease. Remarkably, MyD88-deficient FcγRIIB-deficient mice produce dramatically lower amounts of pathogenic IgG2a and IgG2b autoantibodies than do MyD88-sufficient FcγRIIB-deficient mice, and as a result they have reduced renal disease and prolonged survival. TLR9 is implicated in this model, as TLR9-deficient FcγRIIB-deficient mice also show decreased IgG2a and IgG2b autoantibody titers (59).

The impact of TLR9 on disease parameters in Fas-deficient (lpr) or Ali5 autoimmune-prone mice appears to be more complex and is complicated by somewhat conflicting published data (60–64). In studies from three out of four laboratories, TLR9-deficient autoimmune mice have a significantly different autoantibody repertoire than their TLR9-sufficient littermates, as best visualized by HEp-2 immunofluorescent staining patterns. Sera from TLR9-deficient lpr mice usually give a homogeneous nuclear pattern, indicating the presence of autoantibodies specific for Sm-RNP, and Sm-binding activity has been confirmed by Western blot analysis. Consistent with the phenotype of the FcγRIIB-deficient TLR9-deficient mice, these TLR9-deficient MRL/lpr sera fail to show homogeneous nuclear or metaphase chromosome staining activity. However, many of the TLR9-deficient sera give a speckled nuclear pattern, consistent with antibodies reactive with snRNPs, and the majority of these sera also stain the cytoplasm, a reactivity not apparent in TLR9-sufficient lpr mice and Ali5 mice, with an apparent shift to a nucleolar staining pattern. Unexpectedly, despite the absence of circulating anti-dsDNA or anti-chromatin antibodies, all studies agree that the clinical disease of TLR9-deficient lpr mice is significantly exacerbated when compared with TLR9-sufficient lpr mice. The TLR9−/− mice have higher IgG titers, more extensive lymphadenopathy and splenomegaly, and more severe nephritis when compared with their TLR-sufficient littermates, and on an MRL background the median survival decreases from 25 weeks to 16 weeks.

Effect of TLR7 Deficiency on Murine Models of Autoimmunity

In contrast to TLR9-deficient lpr mice, TLR7-deficient lpr mice still produce anti-dsDNA/chromatin autoantibodies but not anti-Sm/RNP autoantibodies. The TLR7-deficient lpr mice also have lower IgG2a and IgG3 titers than their TLR-sufficient littermates, as well as reduced numbers of activated T cells and pDCs. Nevertheless, renal disease is decreased only modestly (61). Notably, lpr/lpr mice, deficient in the TLR/IL-1
<table>
<thead>
<tr>
<th>Animal model</th>
<th>TLR defect</th>
<th>Genetic background</th>
<th>DNA-reactive autoantibodies</th>
<th>RNP-reactive autoantibodies</th>
<th>Other disease parameters</th>
<th>Survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>lpr/lpr</td>
<td>MyD88-deficient</td>
<td>mixed</td>
<td>ANA: loss of homogeneous nuclear stain</td>
<td>ANA: loss of speckled nuclear stain WB: no anti-Sm</td>
<td>ND</td>
<td>ND</td>
<td>33</td>
</tr>
<tr>
<td>lpr/lpr</td>
<td>TLR9-deficient</td>
<td>mixed</td>
<td>ANA: loss of homogeneous nuclear and mitotic plate stain Crithidia: decreased binding</td>
<td>ANA: retention of speckled nuclear stain (acquire cytoplasmic stain) Sm WB: increased frequency Sm ELISA: increased titer</td>
<td>Increased lymphadenopathy and T cell activation, renal disease unchanged</td>
<td>ND</td>
<td>60</td>
</tr>
<tr>
<td>lpr/lpr</td>
<td>TLR3-deficient</td>
<td>mixed</td>
<td>ANA: no change</td>
<td>ANA: no change Sm WB: no change Sm ELISA: no change</td>
<td>Cell activation unchanged, renal disease unchanged</td>
<td>ND</td>
<td>60</td>
</tr>
<tr>
<td>lpr/lpr</td>
<td>TLR9-deficient</td>
<td>MRL</td>
<td>ANA: no change dsDNA ELISA: no change</td>
<td>ND</td>
<td>Increased lymphadenopathy and IgG2a and IgG3 titers, exacerbated renal disease and salivary gland involvement</td>
<td>ND</td>
<td>62</td>
</tr>
<tr>
<td>lpr/lpr</td>
<td>TLR9-deficient</td>
<td>B6</td>
<td>ANA: loss of homogeneous nuclear staining pattern Crithidia: unchanged Nucleosome ELISA: decreased binding dsDNA ELISA: unchanged</td>
<td>ANA: acquisition of speckled nucleolar staining pattern</td>
<td>Increased splenomegaly and lymphadenopathy, increased proteinuria</td>
<td>No change at 24 wks</td>
<td>63</td>
</tr>
<tr>
<td>Ali5</td>
<td>TLR9-deficient</td>
<td>B6</td>
<td>ANA: reduction in homogeneous nuclear staining pattern Nucleosome ELISA: decreased binding dsDNA ELISA: unchanged</td>
<td>ANA: acquisition of speckled nucleolar staining pattern</td>
<td>More severe renal disease</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>Lpr/Lpr</td>
<td>TLR9-deficient</td>
<td>MRL</td>
<td>ANA: loss of homogeneous nuclear and mitotic plate stain Crithidia; decreased binding dsDNA ELISA: no change</td>
<td>ANA: retention of speckled nuclear stain (acquire cytoplasmic stain) Sm ELISA: increased titer</td>
<td>Increased circulating IFN-α and pDC and T cell activation, increased lymphadenopathy and IgG2a and IgG3 titers, more severe skin disease, exacerbated nephritis</td>
<td>Median survival reduced from 25 to 16 weeks</td>
<td>61</td>
</tr>
<tr>
<td>Lpr/Lpr</td>
<td>TLR7-deficient</td>
<td>MRL (3–7 BC)</td>
<td>ANA: retention of homogeneous nuclear and mitotic plate stain dsDNA ELISA: no change</td>
<td>ANA: reduced frequency of speckled nuclear stain Sm or Sm/RNP ELISA: loss of binding</td>
<td>Decreased pDC and T cell activation, decreased lymphadenopathy and IgG2a and IgG3 titers, less severe skin disease, slightly less severe nephritis</td>
<td>ND</td>
<td>61</td>
</tr>
<tr>
<td>FcyRII−/−</td>
<td>MyD88-deficient</td>
<td>B6</td>
<td>dsDNA ELISA: decreased IgG2a and IgG2b titers</td>
<td>ND</td>
<td>Reduced renal disease</td>
<td>Prolonged</td>
<td>59</td>
</tr>
<tr>
<td>56R</td>
<td>FcyRII−/−</td>
<td>MyD88-deficient</td>
<td>B6</td>
<td>dsDNA ELISA: decreased IgG2a and IgG2b titers</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>56R</td>
<td>FcyRII−/−</td>
<td>TLR9-deficient</td>
<td>B6</td>
<td>dsDNA ELISA: decreased IgG2a and IgG2b titers</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>564 Igi</td>
<td>TLR7-deficient</td>
<td>B6</td>
<td>ND</td>
<td>Idiotype ELISA: decreased spontaneous secretion of 564 autoAb</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>FcyRII−/−</td>
<td>TLR7-duplication</td>
<td>B6</td>
<td>ANA: loss of homogeneous nuclear stain</td>
<td>ANA: nucleolar staining pattern</td>
<td>Increased splenomegaly and proteinuria</td>
<td>Median survival reduced from 9 to 4.5 mons.</td>
<td>67, 69</td>
</tr>
<tr>
<td>Skl Yaa</td>
<td>TLR7-duplication</td>
<td>B6</td>
<td>ND</td>
<td>Protein array: increased IgG titers for RNA-associated autoantigens</td>
<td>Increased splenomegaly and T and B cell activation</td>
<td>Decreased</td>
<td>68</td>
</tr>
</tbody>
</table>

*564 Igi and 56R refer to BCR-targeted genes encoding autoantibodies reactive with RNA-associated or DNA-associated autoantigens, respectively; Ali5, gain of function mutation in PLCγ2.

*ANA, immunofluorescent staining of HEp-2 cells, Crithidia, immunofluorescent staining of Crithidia kinetoplast as a measure of anti-dsDNA antibody; WB, Western blot.

*ND, not determined.*
adapter protein MyD88, and therefore unable to signal through either TLR9 or TLR7, failed to produce both DNA- and Sm-reactive antibodies, but the mice were on a mixed genetic background, and clinical disease was not evaluated (33). TLR7 also appears to play a role in the activation of B cells that express the transgene-encoded 564 Igi receptor. 564 Igi B cells spontaneously produce antibodies that give a cytoplasmic HEp-2 staining pattern and form ICs that deposit in the kidney. TLR7-deficient 564 Igi B cells no longer spontaneously produce 564 Igi antibodies (65).

Overall, the data demonstrate that TLR9 is involved in the in vivo production of anti-DNA/chromatin antibodies, whereas TLR7 is involved in the production of antibodies reactive with RNA-associated autoantigens. Remarkably, TLR9 deficiency appears to have a beneficial effect in FcγRIIB-deficient mice, but not in lpr or Ali5 mice. One interpretation of these data is that in the FcγRIIB model the autoantibody repertoire is more skewed toward DNA-associated autoantigens than it is in lpr mice and is therefore more sensitive to TLR9 perturbations. However, in lpr and Ali5 mice, in which TLR9 deficiency is associated with more severe autoimmune disease, TLR9 appears to serve a protective function, perhaps by contributing to the production of IgM anti-DNA/chromatin antibodies involved in the clearance of apoptotic debris. Alternatively, IC engagement of the TLR9 signaling cascade (or stimulation of cells that preferentially express TLR9) may, in some cases, lead to the production of immunosuppressive cytokines not elicited by the TLR7 pathway. Another possibility is that both anti-DNA/chromatin and anti-RNP autoantibodies form pathogenic ICs but that the TLR7-elicted component is more proinflammatory, perhaps through type 1 IFN cross-regulation. In the absence of anti-DNA-producing cells, the microenvironmental niche for the RNP-reactive plasmablasts may expand, thereby allowing for the increased production of the more pathogenic autoantibodies. Resolution of this conundrum awaits the analysis of TLR9/TLR7-double deficient lpr mice as well as additional autoimmune-prone TLR9/TLR7-double deficient strains.

Effect of TLR7 Overexpression on Murine Models of SLE

The Yaa mutation was first described as an autoimmune accelerator locus responsible for the exacerbated SLE-like disease exhibited by male BXSB mice (66). The genetic basis for this phenotype is the duplication of a 4 Mb region of the pseudoautosomal region of the X chromosome and translocation of this region to the Y chromosome. TLR7 is included in this interval, and Yaa mice express double the normal level of TLR7, both at the RNA and protein level, and respond twice as well as normal mice to synthetic TLR7 ligands (67, 68).

Although B6.Yaa mice do not develop clinical symptoms of SLE, the Yaa locus greatly exacerbates disease progression of the B6.FcγRIIB-deficient and B6.Sle1 strains (69, 70). Importantly, there is a dramatic shift in the autoantibody repertoire of the FcγRII−/−.Yaa mice. As the HEp2 staining pattern shows, the FcγRII−/−.Yaa sera produce a speckled nuclear/cytoplasmic pattern, more commonly associated with autoantibodies reactive with nucleoli or other RNA/protein particles, rather than producing the antichromatin antibodies characteristic of FcγRII−/− mice (69). Allotype analysis of sera obtained from allotype-disparate FcγRII−/−.Yaa + FcγRII−/− mixed bone marrow radiation chimeras demonstrate that the propensity to target RNA-associated antigens is an inherent property of the FcγRII−/−.Yaa B cells (67). The Yaa mutation had similar effects on the range of autoantibodies produced by B6.Sle1 mice. B6.Sle1 mice normally produce nonpathogenic IgG chromatin-reactive autoantibodies. However, as determined by protein arrays, B6.Sle1.Yaa mice develop high IgG autoantibody titers against an extensive...
panel of RNA-associated autoantigens (68). The B6.Sle1.Yaa mice also develop pronounced splenomegaly, increased numbers of activated T cells, and aberrant expansion of most immune cell lineages, especially monocytes and neutrophils. They also develop renal disease, with a mortality rate of greater than 50% by 8 months.

Together, the data suggest that a twofold increase in the level of TLR7 expression can have a profound effect on disease outcome by promoting the production of autoantibodies reactive with RNA-associated autoantigens (similar to the autoantibodies present in the TLR9-deficient lpr mice) and support a model in which RNA-associated autoantigens exacerbate clinical disease (Figure 1). Nevertheless, the Yaa translocation includes 15–20 other reading frames, and one or more of these gene products may also contribute to the onset and/or persistence of autoimmune disease in these mice. In this context, one must consider the effect of the Yaa mutation on mice that express transgene-encoded autoreactive BCRs. 4C8 mice express an IgM transgene specific for red blood cells that was originally derived from NZB mice. 4C8.Yaa mice develop a dramatically accelerated lethal anemia, with 100% mortality by 8 weeks of age, compared with 0% mortality of the non-Yaa littermates. By contrast, the Yaa mutation does not appear to change the phenotype of mice that inherit Sp6, a transgenic receptor specific for DNA (71). The Sp6.Yaa outcome is consistent with the link between Yaa and TLR7, and not TLR9. However, there is no obvious connection between TLR7 and red blood cell–reactive antibodies, other than the fact that autoimmune disease in NZB mice is associated with elevated IFN-α production, and NZB mice lacking the type I IFN receptor have reduced titers of red blood cell– and DNA-reactive autoantibodies and less severe renal disease (72, 73). Whether 4C8 autoantibodies, or even the Yaa RNA-associated autoantibodies, depend on TLR7 will need to be determined by evaluating the appropriate TLR7-deficient Yaa strains.

**OTHER EXAMPLES OF IMMUNOLOGICALLY ACTIVE AUTOANTIGENS**

Despite the preponderance of autoantibody targets in SLE associated with DNA- and/or RNA-containing macromolecular particles, not all autoantigens fall into this category. A number of these non-DNA/RNA autoantigens also have inherent proinflammatory activities that, under normal conditions, most likely promote appropriate self-limiting responses to cell death or tissue injury. Nevertheless, in the context of certain environmental or genetic factors, these molecules may also contribute to the inappropriate activation of the innate immune system and in some cases become prominent autoantibody targets. Although many of these molecules have been found to engage either TLR2 and/or TLR4, other receptor families are also used in the detection of these molecules. A complete description of all biologically active autoantigens is beyond the scope of the current review, but we briefly describe representative examples here.

**Heat Shock Proteins**

Heat shock proteins (Hsps) constitute a group of molecules that function inside the cell and mediate the folding and unfolding of other proteins in response to cellular stress. However, Hsps can be expressed on the cell surface, released from dead or dying cells, or even actively secreted, and they have been detected in the serum of patients with atherosclerosis or other inflammatory conditions (74). Hsp60, Hsp70, and gp96 have been reported to activate macrophages and DCs through either TLR2 or TLR4, even under conditions in which their effect is unlikely because of microbial contaminants (75, 76), and autoantibodies to Hsp60 are present in SLE patients.
Discrepant outcomes of TLR9 and TLR7 deficiency in autoimmune-prone lpr/lpr mice. DNA or RNA released from dying or damaged cells can be recognized by autoreactive B cells and then transported by the BCR to an internal endosomal/lysosomal compartment, where TLR9 is effectively engaged by DNA (left) or TLR7 is effectively engaged by RNA (right). The activated B cells secrete autoantibodies that, together with additional autoantigen, form immune complexes (ICs) that bind FcγRs expressed by pDCs (and other antigen-presenting cells). The FcγRs transport the ICs to an internal endosomal/lysosomal compartment, where autoantigens again engage TLR9 or TLR7. Prior viral exposure may result in the production of IFN-α that enhances the response to RNA or RNA-associated autoantigens by upregulating TLR7 expression. The activated pDCs upregulate costimulatory molecules and produce cytokines, such as IFN-α, that promote further autoantibody secretion and also drive other proinflammatory aspects of the immune response. Possible explanations that might account for the more severe clinical phenotype of TLR9-deficient autoimmune prone mice may include absence of TLR9-enhanced clearance of apoptotic debris or TLR9-enhanced activation of a cell that produces inhibitory cytokines or that induces Treg activity. Alternatively, increased activation through a TLR7-dependent pathway may lead to increased levels of pro-inflammatory cytokine production.
with antiphospholipid syndrome, as well as in patients with atherosclerosis (77). Hsp90 is a common autoantibody target in SLE (78, 79) and is recognized by the α2-macroglobulin receptor, CD91 (80).

High-Mobility Group Box 1
High-mobility group box 1 (HMGB1) is a DNA-binding protein, present in almost all cell types, that serves as a transcriptional regulator. It is also passively released from necrotic cells or inducibly secreted by activated monocytes and macrophages (81). Extracellular HMGB1 functions as a late mediator of inflammation and has a variety of proinflammatory activities, including macrophage activation, DC maturation, induction of cytokine production, and promotion of T cell and neutrophil survival (82). These effects are thought to be mediated through the receptor for advanced glycation end products (RAGE) as well as through TLR2 and TLR4 (83, 84). Anti-HMGB1 antibodies are frequently detected in patients with rheumatoid arthritis, SLE, Sjögren’s syndrome, and scleroderma (82).

Glucosaminoglycans
Glucosaminoglycans (GAGs) are linear polysaccharides made up of repeating uronic acid/hexosamine disaccharide units, diversified through modifications such as sulfation and deacetylation. GAGs are normally associated with tissue-specific core proteins on the cell membrane or in the extracellular matrix but are released as soluble molecules as a result of cell injury (85). Heparan sulfate (HS) and hyaluronic acid (HA) represent two of the four major GAG families. HS-derivatized molecules serve a wide range of functions that include the regulation of coagulation as well as cytokine production. The proinflammatory capacity of HS is further revealed by its capacity to trigger a systemic inflammatory response syndrome through interactions with TLR4 (86). HS autoantibodies have been detected in patients with SLE, primary antiphospholipid syndrome, and scleroderma (87). High molecular weight HA is broken down during injury, and the resulting low molecular weight HA fragments mediate a variety of activities through interactions with CD44, the receptor for HA-mediated motility (RHAMM), TLR4, or TLR2. These include T cell activation, DC maturation, and the release of proinflammatory cytokines (85, 88, 89). Intriguingly, in a lung injury model, high molecular weight HA serves a protective function and limits the injury response (89). HA-reactive autoantibodies have been detected in patients with endocrine disorders (90). HA may also exacerbate disease in rheumatoid arthritis patients by stabilizing RF-containing ICs (91) or by activating cells through the CD44 receptor (92).

Apoptotic Cells and Oxidized Lipids
Intact cells and native unmodified low-density lipoproteins (LDL) are poorly immunogenic. In contrast, oxidation-induced neoepitopes on lipoproteins and apoptotic cells play important roles in both health and disease. Clearance of apoptotic cells by phagocytes is an essential homeostatic mechanism that serves to remove from the body damaged cells or cells that have completed their physiological function. Apoptotic cell clearance is promoted by molecules released by or expressed on the surface of apoptotic cells such as translocated phosphatidylserine and oxidized lipids, including lysophosphatidylcholine (LPC). LPC serves as both a chemotactic factor (93) and a prominent target molecule for phagocytosis-promoting natural IgM (94) and, along with other oxidized lipids, can be recognized by scavenger receptors such as CD36 (95).

The uptake of apoptotic debris is generally associated with the induction of an anti-inflammatory and tolerogenic response (96).
However, impaired clearance of apoptotic cells or apoptotic debris can lead to inflammation and lupus-like autoimmunity, as illustrated by mice that fail to express receptors or bridging molecules involved in the clearance process (97–101). The mechanisms whereby a normally anti-inflammatory and tolerogenic stimulus is converted to a productive immune response are poorly understood, although the demonstration that oxidized lipoproteins on apoptotic cells can be immunogenic and proinflammatory offers a possible explanation (102). In atherosclerosis, there is convincing evidence that oxLDLs are directly proinflammatory and that innate and adaptive immune responses to oxLDL are major contributors to disease pathogenesis (103). Certain forms of oxLDL directly engage TLR2 and TLR4 (104, 105), and oxLDL uptake via scavenger receptors, although not directly proinflammatory, may lead to effective antigen presentation to T cells. Remarkably, mAbs reactive with proinflammatory oxidation-induced epitopes on oxLDL also bind to apoptotic cells (106), and uptake of oxLDL and apoptotic debris is mediated by overlapping scavenger receptors (107). A potential functional correlation between oxLDL and apoptotic debris may explain the elevated incidence of atherosclerosis in patients with SLE (108) and/or the exacerbated SLE-associated clinical features of autoimmune-prone gld mice that are also ApoE-deficient and as a consequence have a profound defect in apoptotic cell clearance (109). IgG autoantibodies reactive with oxLDL are found in patients with SLE, particularly in those patients with cardiovascular disease (110).

**Chemotactic Autoantigens**

Autoantibodies specific for particular aminoacyl-tRNA synthetases are common in patients afflicted with forms of polymyositis or dermatomyositis. There is no evidence so far that these molecules, or associated RNA fragments, can engage TLR7. However, histidyl-tRNA synthetase, more commonly known to clinicians as Jo-1, reportedly has chemotactic activity for T cells, activated macrophages, immature DCs, and CCR3-transfected HEK293 cells. By the same criteria, another autoantigen, asparaginyl-tRNA synthetase, has chemotactic activity mediated through CCR3, whereas nonautoantigenic tRNA synthetases are not demonstrably chemotactic (111).

**CONCLUDING REMARKS**

In vitro and in vivo data now provide clear evidence that TLR9 and TLR7 contribute to the activation of autoreactive B cells and to the production of autoantibodies reactive with nucleic acids and DNA- and RNA-associated autoantigens. In addition, ICs containing immunostimulatory DNA and/or RNA fragments are potent ligands for pDCs and other FcγR+ TLR7/9+ APCs. Nevertheless, the overall effect of combined TLR9/TLR7 deficiency on systemic autoimmune diseases such as SLE remains to be determined. Unexpectedly, the initial phenotypes of the TLR9- and TLR7-deficient mice together with the identification of the Yaa mutation as a genetic translocation resulting in the duplication of TLR7 suggest that TLR7 may be the more relevant therapeutic target. Ultimately, investigators will need to determine the effect of TLR7, TLR8, and/or TLR9 blockade on systemic diseases in human patients, where differences in cell type expression of the individual TLR family members may influence therapeutic responses. Moreover, the functional association between TLR7, 8, and 9 and systemic autoimmune disease may only be the tip of the iceberg; it is increasingly apparent that a variety of other autoantigens may also serve as endogenous ligands for additional TLR and non-TLR PRRs. Whether these interactions can actually initiate and/or promote autoimmune diseases or other chronic inflammatory conditions is an important topic for future investigations.
SUMMARY POINTS

1. DNA- and RNA-associated autoantigens activate autoreactive B cells in vitro through a mechanism that depends on the BCR and TLR9 and TLR7, respectively. The response to RNA autoantigens is enhanced by type I IFN, presumably through upregulation of TLR7.

2. DNA- and RNA-containing ICs activate DCs though a mechanism that depends on uptake through FcγRs and TLR9 and/or TLR7. IC-activated pDCs produce high levels of IFN-α that can promote the activity of many of the effector mechanisms associated with SLE and other systemic autoimmune diseases.

3. TLR9 deficiency in vivo leads to decreased anti-dsDNA autoantibody production, but effects on end organ disease and survival are variable. TLR9−/− 56R FcγRII−/− mice do not make pathogenic IgG2a and IgG2b anti-DNA autoantibodies; TLR9−/− lpr and Ali5 mice develop increased autoantibody titers for RNA-associated autoantigens and develop more severe clinical features of SLE.

4. TLR7 deficiency in vivo leads to decreased titers of RNA-reactive autoantibodies. TLR7−/− lpr mice develop clinical features of SLE that are slightly less severe than the TLR7+/+ control group.

5. The Yaa mutation results from duplication of a 4 Kb segment of the X chromosome that includes TLR7. FcγR−/− Yaa mice and Sle1.Yaa mice make elevated titers of IgG autoantibodies reactive with RNA autoantigens and develop more severe features of SLE than their non-Yaa littermates.

FUTURE ISSUES

1. How will the combined effects of TLR7 and TLR9 deficiency influence disease manifestations in both lpr and non-lpr models of SLE and what will be the effect of TLR7/9 blockade on human disease?

2. Do gene products other than TLR7 contribute to the Yaa phenotypes?

3. How do DNA-/RNA-containing ICs affect FcγR+ TLR7+ and/or TLR9+ cell populations other than pDCs? Will RNA-containing ICs activate FcγR/TLR8-expressing cells and what will be the consequences of this activation?

4. What other PRRs are used by autoantigens to elicit immune system activation?

DISCLOSURE STATEMENT

U.S. patent application 10/487,885 entitled Method and Composition for Treating Immune Complex-Associated Disorders and corresponding foreign applications have been licensed and provide royalty income.

ACKNOWLEDGMENTS

We apologize in advance to all the investigators whose research could not be appropriately cited owing to space limitations. We extend a special thanks to our many collaborators for thoughtful...
discussions pertaining to the topic of this review, in particular Drs. A. Krieg, M. Shlomchik, G. Viglianti, and R. Corley, and to the agencies that have supported our own research studies, NIAMS, National Kidney Foundation, Lupus Research Institute, and the Alliance for Lupus Research.

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