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Epstein-Barr virus and molecular mimicry in systemic lupus erythematosus

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Abstract
Systemic lupus erythematosus (SLE or lupus) is a complex disease with a multifactoral etiology, with genetic, hormonal, and environmental influences. Molecular mimicry as a result of viral infection may contribute to the development of lupus. The pattern of autoantibody development in lupus is consistent with initiation through molecular mimicry, as the initial autoantigenic epitopes that have been observed are limited and cross-reactive with viral proteins. Autoantibody specificity may then later diversify to other autoantigens through B-cell epitope spreading. Epstein-Barr virus (EBV) is an excellent candidate to be involved in molecular mimicry in lupus. EBV infection has been associated with lupus through serological and DNA studies. Infection with EBV results in the production of the viral protein Epstein-Barr virus nuclear antigen-1 (EBNA-1), antibodies against which cross-react with lupus-associated autoantigens, including Ro, Sm B/B′, and Sm D1, in lupus patients. The immune response against EBV, and EBNA-1 in particular, differs among lupus patients and healthy controls, with controls maintaining a limited humoral response and failing to produce long-standing cross-reactive antibodies. We hypothesize that the humoral immune response to EBNA-1 in susceptible individuals leads to the generation of cross-reactive antibodies. Through the process of epitope spreading, these cross-reactive antibodies target additional, non-cross reactive autoepitopes, spread to additional autoantigens, and become pathogenic, leading eventually to clinical lupus. This paper reviews some of the current literature supporting roles for EBV exposure and epitope spreading in SLE.

Keywords: *Systemic lupus erythematosus, Epstein-Barr virus, cross-reactivity, autoimmunity, epitope spreading, autoantibody*

Introduction

Systemic lupus erythematosus (SLE or lupus) is a complex systemic autoimmune disease. Clinical presentation of lupus varies among patients, including a wide range of possible organ system damage [1]. Autoantibodies serve as the most common and unifying characteristic of lupus. These antibodies are directed against a large but limited collection of autoantigenic targets, including DNA chromatin and its component histones, components of the spliceosome, the Ro/La complex, among others [2,3]. This complexity has made the complete understanding of lupus etiology and pathogenesis difficult. No factor, either genetic or environmental, appears to be solely causal for most of the patients. Therefore, most investigators strongly suspect that lupus has a multifactoral etiology.

The available immunochemical evidence is consistent with a mechanism of lupus autoantibody generation through molecular mimicry. A molecular mimicry mechanism is possible when the antibodies formed, either spontaneously or as a consequence of a heteroimmune response, cross-react. That is, these antibodies bind two different antigens. Immunochemically some structural feature of each of the two antigens is sufficiently similar to one another to imitate or be a mimic. This antibody then plays the

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initiating role in the generation of the humoral immune response against the second antigen.

In the case of systemic lupus the data are consistent with at least three scenarios of autoimmunity being generated from the heteroimmune response against EBNA-1: PPPGRRP of EBNA-1 cross-reacting with PPPGMRPP of Sm B/B′ [4], amino acids (aa) 35–58 of EBNA-1(GPAGPRGGGRGRGRGRGR-GHNDGG) and aa 95–119 of Sm D1 (RRPGGR-GRGRGRGRGRGRGRGA) [5], and aa 58–72 (GGSGSGPRHRDGVRR) of EBNA-1 cross-reacting with aa 169–180 (TKYKQRNSWSHK) of 60 kDa Ro [6].

Cross-reactions may also occur without there being any pathological significance. Of all the known and postulated cross-reactions (Table I) in only a few is a pathogenic role possible. The HIV infection rate is too low to be considered seriously as a primordial immune response from which lupus autoimmunity could emerge. Though not directly tested, association with anti-70K nRNP autoantibodies and cytomegalovirus is not associated with the general lupus phenotype [12–14].

Although, the initial cross-reactions are expected and observed to be very limited, usually to a single structure, autoantibody binding can diversify over time through the process of B cell epitope spreading. Human B cell epitope spreading was first described in the anti-Sm system in human SLE [15,16]. These processes have been implicated in lupus by analysis of pre-diagnosis serum samples in human SLE that documents the considerable diversification of antibody specificity over time. Furthermore, the pattern of autoantigens recognized in SLE is consistent with what has been observed in animal models of epitope spreading.

Much of our current evidence suggests that Epstein-Barr virus (EBV) is likely to be involved in the initiation of lupus. EBV has many characteristics that are ideal for involvement in the development of autoimmunity, which will be discussed below. EBV infection has been associated with lupus through several different lines of evidence, including serological association of the presence of anti-EBV-viral capsid antigen (VCA) with lupus, association of EBV DNA, with lupus, cross-reactivity of EBV proteins with SLE-associated autoantigens, and a number of other lupus-specific immune differences [12–14,18,19]. The humoral immune response against two lupus-associated autoantigens, Sm and 60 kDa Ro demonstrates both substantial epitope spreading and cross-reactivity with EBNA-1. This review will focus on how EBV infection may initiate early events in autoimmunity, and propose mechanisms through which such early events could lead to lupus (Figure 1).

Table I. Cross-reactivity of viral proteins with lupus-associated autoantigens.

Virus	Viral antigen	Self-antigen	Reference
Epstein-Barr Virus	EBNA-1	Sm B/B′	[4]
		SM D	[5,6]
		Ro	[7]
	EBNA-2	Sm D	[8]
Cytomegalovirus	gB	U1 70K	[9]
HIV	P24	Sm B/B′	[10]
HRES-1	P28	U1 70K	[11]

Patterns of autoantibody appearance

Recent work from our group examined the prevalence of autoantibody specificies in SLE before disease occurrence. The Department of Defense Serum Repository contains samples from more than 5 million Active Duty Military Service men and women. We investigated seven serum autoantibodies before SLE diagnosis from 130 patients [20]. A pre-diagnosis sample was positive for a lupus autoantibody in 88% of all patients with a mean appearance of 3.3 years prior to diagnosis [20].

These samples make tracking the order and appearance of autoantibodies possible. Antinuclear antibodies (ANA) (by HEp-2 immunofluorescence screening), Ro/SSA, La/SSB and antiphospholipid antibodies appeared first, at a mean of 3.4 years prior to diagnosis. These were followed by anti-dsDNA antibodies with a mean of 2.2 years prior to diagnosis, which in turn are followed by anti-Sm and anti-nRNP antibodies with a mean time of appearance of 1.2 years prior to diagnosis [20]. These are a minimum time estimate of the actual time from the first appearance of the antibody to diagnosis, since samples from patients with autoantibodies already present in the first available sample were not considered in this analysis. Therefore, the actual length of time between the initial development of antibodies and the observation of clinical manifestations is likely much longer.

The number and type of antibody specificities in individual patients also increased over time. At the appearance of the first autoantibody specificity, antibodies from individual sera recognized an average of 1.5 out of 7 antigens evaluated. At the time of diagnosis, the average number of autoantigens recognized was 3.0, a change that occurred progressively in time and clearly demonstrates the broadening of autoimmunity over time [20]. This progressive accrual of autoantibodies is consistent with autoimmunity resulting from molecular mimicry, which may begin by cross-reactivity with a very small number of autoepitopes and subsequent development of additional specificies through epitope spreading.

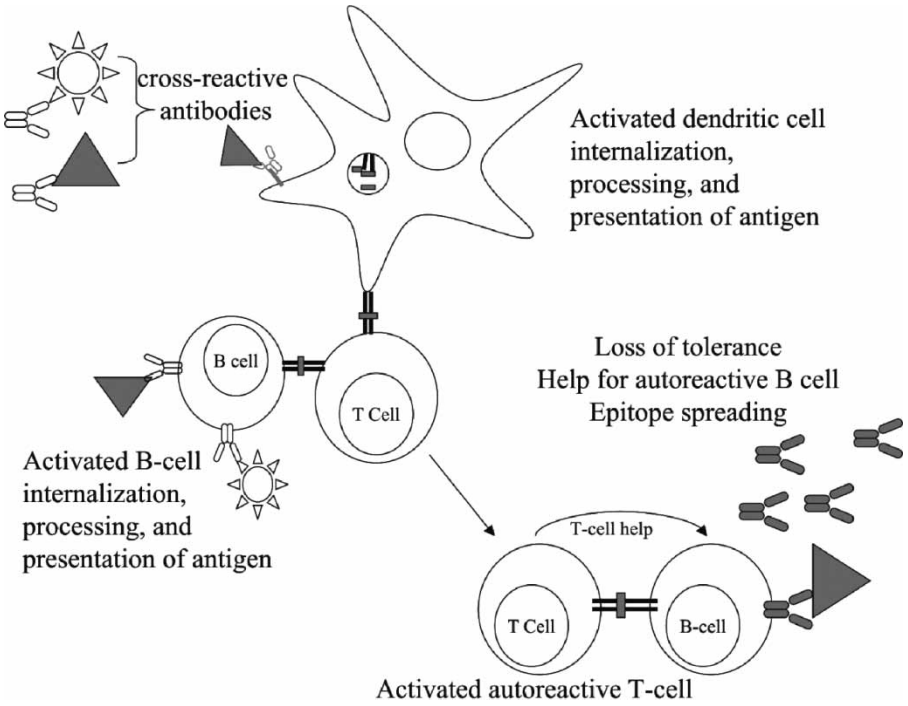

Figure 1. Molecular mimicry resulting in autoimmunity. Cross-reactive anti-pathogen antibodies bind self proteins. The antibodies could be either in soluble form or as part of the B cell antigen receptor. Antibody-bound self protein (grey) is internalized and processed by APC, and peptides presented to T cells, allowing loss of self- tolerance. Genetic predisposition or flaws in T-cell tolerance may be necessary for this process to occur. B cell receptor bound self protein is internalized by B cells, processed, and presented to T cells, breaking tolerance. Autoreactive T cells provide help for autoreactive B cells, leading to maturation and diversification of the autoantibody response.

Epitope spreading in lupus

When autoimmunity first initiates with the first cross-reactive epitope, tolerance is lost. Autoimmunity may spread to other epitopes on the same antigen, other molecules in the same complex, or even other complexes [21]. B cell epitope spreading is thought to occur when T cells with specificity for one epitope are able to provide help to B cells specific for another. B cells recognize epitopes in the context of intact antigens while T cells recognize processed peptides presented in the context of MHC. B cells capable of binding one of several epitopes on a particular protein could bind, internalize, and present antigen from that protein to T cells in the context of MHC class II. Similarly, B cells may be capable of binding to and internalizing one component of a multimolecular complex. Peptides derived from proteins in the complex could then be presented to T cells [22,23]. T cells would then provide help to the B cell in the form of cytokines and ligation of CD 40. Since the peptide presented to the T cell is not necessarily the antigen bound by the B cell, one antigen-specific T cell could activate a variety of B cells with different epitope specificities [24] (Figure 2).

Examination of serial serum samples from patients with reactivity to Sm revealed that temporal epitope spreading occurs in spliceosomal autoimmunity. After recognition of the initial epitope, PPPGMRPP, antibody recognition spreads to neighboring epitopes on the protein. PPPGMRGP (aa 193–200) was the next epitope bound by Sm autoantibodies, and the autoantibody specificity spread from there to

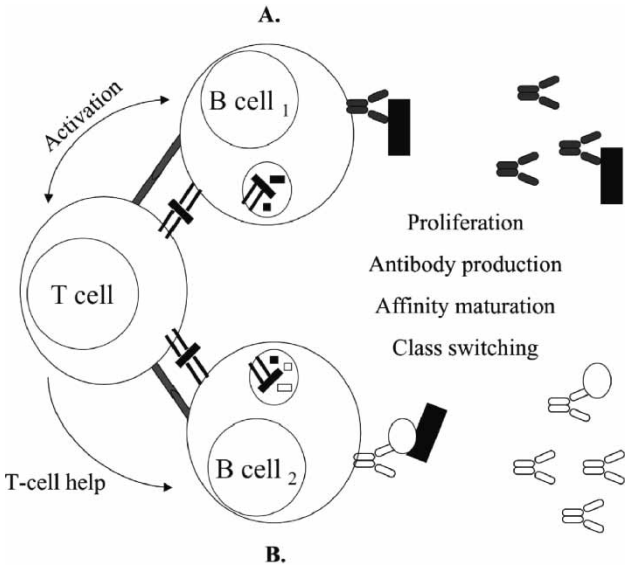

Figure 2. Process of epitope spreading. A: B cells specific for an initial antigen (designated by the black rectangle) can present peptides derived from that antigen to T-cells. B: B cells specific for a different epitope or antigen (open circle) that forms a complex with the initial antigen is able to internalize the entire complex, and present peptides from the original antigen to the T-cell, which then provides help for a mature antibody response.

include multiple epitopes [16]. Epitope spreading in the case of Sm autoimmunity is not limited to the Sm B/B′ protein. Patients who develop Sm autoimmunity will almost invariably develop anti-RNP [25], demonstrating the ability of lupus autoimmunity to spread between proteins. The human anti-Ro response develops in a similar fashion as the anti-Sm response, with initial recognition of one epitope, followed by spreading to multiple sites [7].

Epitope spreading also occurs in models of spontaneous SLE. In a non-immunized mouse model of lupus, longitudinal analysis of MRL-*lpr*/*lpr* mice showed that the antibody response to hnRNP A2/B1 diversified from peptides aa 35–55 to aa 50–70 [26,27]. Anti-chromatin antibodies are also generated spontaneously in these mice. On average, the first antibodies specific for chromatin to arise in this model recognize discontinuous, native epitopes, followed by antibodies to dsDNA or histone proteins [28]. These findings demonstrate epitope spreading in a model that was not driven by immunization, but by naturally arising autoimmunity.

EBV and lupus

The available evidence strongly suggests that EBV infection is involved in the development of SLE. Two studies examining the prevalence of EBV antibodies in pediatric lupus patients detected a significant increase in EBV infection in lupus patients (OR $>$ 49, OR $>$ 14) compared to matched controls [12,13]. EBV DNA was also found significantly more frequently in these patients compared to controls [12,13]. Indeed, EBV DNA was recovered from all of the patients studied. Significant increases in EBV seroconversion were also found in adult lupus patients (OR = 9.4, CI 95% [1.5-infinity] corrected for familial control issues) [14,18,19].

EBV proteins are cross-reactive with a number of SLE autoantigens. The lifelong, periodically reactivating nature of EBV infection insures that there will be multiple opportunities for these cross-reactive proteins to encounter the immune system. In addition, the natural host cells of EBV are B cells, which are responsible for the production of the autoantibodies that are central to SLE. Infection with EBV activates and/or immortalizes B-cells, and interferes with the normal regulatory mechanisms that control antibody production. A protein expressed during viral latency, LMP-1, mimics the B-cell stimulatory signal from CD40 [29]. EBV produces a Bcl-2 homolog that inhibits apoptosis in infected cells [30], and a viral IL-10 homologue which also can likely modify the host immune system [31].

EBV and molecular mimicry in lupus

60 kDa Ro system

Additional supportive evidence for a role for EBV in SLE comes from the human 60 kDa Ro system. Anti-Ro is among the first autoantibody specificities developed in patients who will eventually be diagnosed with lupus [20]. In our recent published work, serial serum samples were analyzed in which Ro autoantibodies developed after the first sample was collected [7]. By solid-phase peptide epitope mapping, nine of 29 patients had monospecific antibodies to the same single epitope, TKYKQRNGWSHK, amino acids 169–180. Seventeen of the remaining twenty patients also recognized this peptide in addition to other specificities. Sera from later time points were available for the nine patients who had originally generated antibodies only against aa 169–180, and the antibodies present in these samples demonstrated spreading over time to multiple Ro epitopes [7]. Other work has shown this Ro169 peptide to be a key 60 kDa Ro humoral SLE epitope [32–34]

When these anti-Ro antibodies from patients in the earliest stages of Ro autoimmunity were purified, they were found to cross-react with EBNA-1 at amino acids 58–72 [7]. Immunity to EBNA-1 was always present in these patients before Ro autoimmunity could be detected, indicating that EBV infection came before the development of autoimmunity. In several cases, the progression of antibody reactivity from EBV-VCA, to EBNA-1, to Ro could be observed in serial serum samples [7].

Similarly, immunization of rabbits or mice with peptides derived from the Ro autoantigen led to generation of antibodies against not only the immunizing peptide, but also multiple epitopes of Ro, and against several other autoantigens. Immunization of mice with 52 kDa Ro lead to antibodies against 48 kDa Ro and 60 kDa Ro, and immunization with 60 kDa Ro similarly resulted in anti-48 and 52 kD Ro immunity [35]. Several studies investigating the immunization of rabbits or mice with peptides derived from the 60 kDa Ro protein found that immunity developed against many different proteins, including anti-La, Sm B′ SmD1, nRNP A and nRNP C antibodies. Time course analysis revealed that autoantibodies appeared first against Ro, then SmB/B′, followed by nRNP A and C. Antibodies to La were among the last to appear [36–41].

Spliceosomal Sm system

Early epitopes of Sm autoantibodies cross-react with EBNA-1, the major latent protein of EBV infection. In four patients who developed anti-Sm reactivity while under observation, the first anti-Sm B/B′ response was mapped to few epitopes, with the repeated peptide sequence PPPGMRPP always being

recognized by the first Sm-positive sample. Not only is PPPGMRPP the first antigen bound by the anti-Sm B response, antibodies targeted against this sequence account for an overwhelming proportion of the anti-Sm antibody population, highlighting its critical role in anti-Sm autoimmunity [16]. In one experiment, 98% of Sm-binding sera recognized PPPGMRPP [42]. Purified rabbit antibodies against the EBNA-1-derived peptide PPPGRRP from strongly cross-react with the Sm epitope PPPGMRPP [4], and purified anti-PPPGMRPP from human lupus patient sera cross-react with PPPGRRP[43].

Immunization of rabbits with the SmB/B′-derived peptides PPPGMRPP or PPPGIRGP on a branched lysine support lead to the production of antibodies against up to 96 distinct sites dispersed throughout the spliceosome, demonstrating that antibody recognition had spread from the immunizing peptide to novel epitopes. Immunized rabbits also developed antibody reactivity against Sm D (12 of 13 rabbits), nRNP 70K (12/13 rabbits), Sm C (13/13), RNP A (11/13), double stranded DNA (dsDNA), and ANA, as well as features of lupus such as thrombocytopenia, seizures and proteinuria [4,44].

Immunization of mice with the peptide PPPG-MRPP led to strain-specific epitope spreading. Some strains (129/J, A/J, AKR/J, Balb/c, PL/J and SJL/J) produced antibodies that bound multiple epitopes of SmB/B′ and nRNP upon immunization, while other strains (C3H/HeJ, C57Bl/6J, C57Bl/10J, C57BL/J, DBA/2J and NZB/Binj) produced antibodies only against the immunizing peptide [45]. These immunization experiments confirmed the data obtained using the rabbit model, and also indicated that genetic background is an important component of epitope spreading. Repetition of this experiment by an independent group confirmed the development of ANA and epitope spreading to additional determinants of Sm B/B′ and the development of autoantibodies against RNP A and Ro [46]. A third attempt to demonstrate epitope spreading to the native forms of Sm and U1RNP in response to PPPGMRPP immunization using a different immunogenic backbone showed lupus-like autoimmune-mediated disease, including kidney damage, but no evidence of epitope spreading in the three rabbits tested [47].

EBNA-1 antibodies from lupus patients also cross-react with Sm D1. EBNA-1 contains a large glycine–arginine (GR) repeat that is homologous with a region on the C-terminal end of Sm D1, a lupus-associated autoantigen and component of the spliceosome [5,6]. Approximately 30% of tested sera from lupus patients bound to Sm-derived peptides containing the GR region, while sera from patients with rheumatoid arthritis, systemic sclerosis, or Sjögren's syndrome did not. Interestingly, the only non-lupus sera that bound were from patients with infectious mononucleosis [6]. When the Sm D1 GR-specific antibodies were purified, it was found that they also bound to EBNA-1 [6]. Intriguingly, although some normal EBV-infected individuals as well as lupus patients make antibodies against the EBNA-1 GR repeat, only antibodies from the lupus patients will cross-react with the homologous sequence derived from Sm D1 [48]. Furthermore, epitope analysis revealed that 8/9 sera from Sm D-reactive lupus patients bound to the GR repeat region of Sm D1, as did sera from lupus patients without Sm specific antibodies by precipitin. Normal controls did not bind any Sm D-derived epitopes [49]. Symmetrical dimethylation of Sm D1 and Sm D3 peptides may render them more antigenic [50].

Animal models confirmed the importance of the GR-repeat in Sm autoimmunity. Immunization of rabbits with a peptide containing the Sm D1 GR repeat region led to anti-Sm D1 immunity, as well as some epitope spreading to Sm D3 [51]. Immunization of mice with the cross-reactive EBNA-1 peptide is sufficient to cause Sm D1 autoimmunity, illustrating the potential for cross-reactive antibodies to EBNA-1 to participate in molecular mimicry [6]. Antibodies from MRL *lpr*/*lpr* mice, which spontaneously develop a lupus-like syndrome, also bind to the GR-repeat region of Sm D1 [50].

Animal models demonstrate that a cross-reactive anti-EBNA-1 immune response can lead to lupus-like disease. Immunization of rabbits with the cross-reactive epitopes derived from either Ro or EBNA-1 led to antibody recognition of EBNA-1 and of other lupus autoantigens, including Sm B′, nRNP, and dsDNA. These rabbits also developed clinical features, such as leukopenia, thrombocytopenia, and increases in serum creatinine [7]. Peptide immunization, however, is not the natural route through which an organism would encounter EBNA-1. We expect that the human cases of lupus developed anti-EBNA-1 first and then, with maturity, the cross-reactive specificities. To see if a more physiologic method of introducing EBNA-1 would still produce autoimmunity, Sundar injected mice with EBNA-1 DNA expression vectors and found that expression of EBNA-1 led to the production of anti-Sm and anti-DNA antibodies [52]. These experiments show that full-length, endogenous expression or peptide immunization EBNA-1 can lead to broad autoimmunity.

SLE unique EBV responses

The ubiquitous nature of EBV infection naturally raises the question of why these cross-reactive epitopes do not lead to autoimmunity in all infected people. Only three likely possibilities exist. The difference is between those who develop lupus because of the host response to EBV, because of differences in the EBV infection, or because the penetrence of lupus is low and random. This last possibility seems to be eliminated

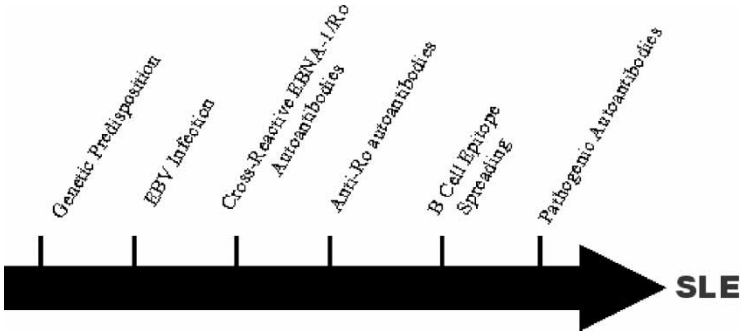

Figure 3. Development of SLE. SLE is initiated when a genetically susceptible individual is infected with EBV. Cross-reactive antibodies are generated as a result of infection. These antibodies allow the targeting by the immune system of proteins containing the cross-reactive epitope or epitopes. Epitope spreading then generates a range of autoantibodies. When these autoantibodies attain the required specificity or concentration, they become pathogenic, and SLE ensues.

by the strong genetic component in SLE [53,54], which is also consistent with the host response being the important variable. To this point there is no evidence that lupus risk varies with viral strain.

The answer may be found in the lupus-specific differences in the humoral immune response to EBNA-1. In a study of EBV-positive pediatric lupus patients and controls, patients were more likely to have anti-EBNA-1 than controls, with 36/36 patients compared to 25/36 controls having antibody recognizing EBNA-1 ($p < 0.005$) [17]. Lupus through anti-Sm B/B′ or anti-Ro may be impossible in normal individuals who do not develop anti-EBNA-1 antibodies.

Epitope mapping of the EBNA-1 antibody specificity in these pediatric SLE patients revealed that lupus patients recognize a much broader range of epitopes than controls. Control sera primarily recognized two epitopes, both of which are part of a large glycine–alanine repeat. Sera from lupus patients, however, bound to multiple epitopes found throughout the protein [17]. Increased diversity of EBNA-1 epitope recognition by lupus patients may result in production of cross-reactive antibodies, which participate in molecular mimicry to initiate autoimmunity.

The reasons for these lupus-specific alterations in EBNA-1 immunity are not completely known. However, cross-reactive antibodies, including those against PPPGMRPP, are produced during infectious mononucleosis. In people who do not develop lupus, these antibodies are generally cleared by 4 months after the resolution of mononucleosis [55].

SLE patients carry increased EBV viral loads, and cellular immunity against EBV is altered in SLE patients [17,56,57]. EBV viral load was found to be 40-fold higher in lupus patients than controls, resulting from increased numbers of latently infected cells in the periphery [56,57]. Such an increase in viral load may provide a large increase in cross-reactive antigen. The cellular immune response to EBV differs in lupus, with a decreased cytotoxic T-cell response and heightened CD $4+$T cell reactivity [57]. Such differences in infection or T-cell immunity as well as genetic or other factors may prevent lupus patients from eliminating these cross-reactive antibody specificities or, alternatively, allow them to return, allowing molecular mimicry to develop.

From EBV infection to lupus

Cross-reactivity of anti-pathogen antibodies with self-proteins is a compelling hypothesis to explain the initial loss of tolerance seen in SLE. Under this hypothesis, cross-reactive anti-pathogen antibodies are formed in response to viral infection, and bind self-proteins. For example, as a result of EBV infections, anti-EBNA-1 antibodies that also bind to 60 kD. Ro are formed and EBNA-specific B cells that can bind Ro with their antigen receptor are activated. These antibodies could be either immunoglobulins in soluble form, or as B cell antigen receptors, since either dendritic cells or B cells are capable of presenting antigen to T-cells. Dendritic cells are efficient at initiating T-cell immunity, whereas B cells present peptides from their specific antigen with extremely high efficiency [22,58]. Antibody-bound self-protein would be internalized and processed by these antigen-presenting cells, and self-peptides presented to T cells, potentially resulting in the loss of tolerance to these self-antigens [59]. Autoreactive T cells could then provide help for autoreactive B cells, leading to maturation and diversification of the autoantibody response (Figure 2). Recognition of self antigens in an inflammatory milieu of cytokines, such as would result from viral infection, could also predispose antigen presenting cells (APCs) to present self-antigen in a stimulatory, as opposed to tolerogenic, manner.

Development of lupus autoimmunity may begin with molecular mimicry involving EBV infection and

cross-reactivity to cellular autoantigens. Cross-reactivity between EBNA-1 and multiple lupus antigens has been observed in lupus patients, and the ability of EBNA-1 to induce lupus-like autoimmunity has been demonstrated in multiple animal models. The progression from EBV infection to the diverse autoimmune response seen in SLE may be easily imagined (Figure 3). EBV infects a lupus-susceptible person, resulting in molecular mimicry due to the production of cross-reactive anti-EBNA-1 antibodies. These autoantibodies react with self-proteins such as Ro, Sm B/B′, or Sm D1. Epitope spreading increases the autoimmune response to novel epitopes on the protein. Cross-reactivity and physical association allow the immune response to spread beyond the initial protein, and develop sufficiently to cause clinical lupus.

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