Curr Rheumatol Rep (2016) 18: 1
DOI 10.1007/s11926-015-0552-x

SCLERODERMA (J VARGA, SECTION EDITOR)

Fresh Insights into Disease Etiology and the Role of Microbial Pathogens

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Published online: 23 December 2015

Abstract Pathogens have been implicated in the initiation and/or promotion of systemic sclerosis (scleroderma, SSc); however, no evidence was found to substantiate the direct contribution to this disease in past years. Recently, significant advances have been made in understanding the role of the innate immune system in SSc pathogenesis, supporting the idea that pathogens might interact with host innate immune-regulatory responses in SSc. In light of these findings, we review the studies that identified the presence of pathogens in SSc, along with studies on pathogens implicated in driving the innate immune dysregulation in SSc. The goal of this review is to illustrate how these pathogens, specifically viruses, may play important role both as triggers of the innate immune system, and critical players in the development of SSc disease.

Keywords Scleroderma · Systemic sclerosis · EBV · CMV · Innate immunity · Fibrosis

This article is part of the Topical Collection on *Scleroderma*

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Introduction

The immune system walks a fine line to distinguish self from harmful non-self to preserve the integrity of the host. However, certain kind of infections can result in the breakdown of self-tolerance, leading to immunopathology and autoimmunity. A potential role for microbial involvement in the pathogenesis of systemic sclerosis (scleroderma, SSc) was recognized, and viral infections have been implicated in the initiation and/or promotion of most autoimmune diseases, including SSc. In the past years, this hypothesis was primarily supported by serological, molecular, and epidemiological studies on several different environmental microbes that were found associated with SSc. However, no evidence has demonstrated a clear and direct contribution of these factors to the disease.

Recently, a number of studies highlighted the potential driving role that the innate immune response can play in activation of cells responsible for the central pathological triad of SSc: vasculopathy, inflammation, and fibrosis. The evolutionary older innate immune system is classically viewed as first line of host defense, and it represents the sole mode of protection against pathogens in non-immune cells, although it is an intricate system in which immune and non-immune cells play a role. Toll-like receptors (TLRs) have a particularly instructive role in innate immune responses against microbial pathogens, as well as in the subsequent induction of adaptive immune responses [1]. Intriguingly, several molecular innate immune mediators were found to underlie inflammation in SSc lesional tissues [2, 3]. Among these mediators, the interferons (IFNs) have captured the most attention due to their central role in regulating a multitude of biological functions in the innate immune system, facilitating their primarily antiviral activity [4, 5]. Microarray and proteome-wide studies largely documented the presence of "IFN signatures" as a common

theme in most of the organs involved in SSc, such as skin, lungs, and peripheral blood mononuclear cells (PBMCs) [2, 3, 6–11]. In vitro studies showed that activation of the innate immune response in immune and non-immune cells, including fibroblasts and endothelial cells (ECs), by specific TLR ligands, induced high expression of several IFN- and TGFβ-responsive genes also seen upregulated in SSc skin, such as CXCL9, OAS2, PAI-1, and ET-1 [12–16]. Based on these findings, it appears that dysregulation of the innate immune system might contribute to the activation of key effector cells such as fibroblasts and ECs, leading to production of genes implicated in SSc pathogenesis. Further genetic studies that strongly associate polymorphisms in TLRs and IFN pathways (IRF5, IRF7,IRF8 STAT4) with SSc confirm the correlation of innate immune dysregulation and IFNs with the risk of developing SSc [17, 18].

The rationale for studying the role of pathogens is very attractive since the etiology of autoimmunity, as well as the origin innate immune dysregulation is largely unexplained in SSc. Herein, we discuss the evidence that the pathogens associated with SSc have a mechanistic role in influencing disease development.

Herpesviruses

Herpesviruses are a large family of DNA viruses and can be subdivided into α−, β− (cytomegalovirus (CMV)) or γ− (Epstein-Barr virus (EBV)) subfamilies based on their biological functions and sequence similarities. Common to all herpesviruses is the ability to cause lytic infections, to replicate in permissive cells, and to establish latency in distinct specialized cells, such as lymphocytes (γ) and/or myeloid (β), as occurs with EBV and CMV, respectively. Importantly, while the immunological control of herpesviruses is achieved by both the innate and adaptive immune systems, the innate antiviral immune response (interferon (IFNs)) and NK cells have key roles in monitoring the containment of herpesvirus lytic infection (replication phase) [19, 20•]. In this regard, it is important to know how innate immune system detection translates into anti-herpesvirus host defense, and how the viruses seek to evade this innate detection to establish persistent infection.

For a long time, EBV and CMV have been associated with SSc; however, most of the evidence was based only on the serological patterns often detected in SSc patients. Numerous studies reported elevated levels of antibodies against EBV and CMV antigens in most SSc patients, indicating the presence of a recent and active infection [21, 22•, 23]. Moreover, the cross-reactivity between several EBV and CMV antigens with cellular autoantigens raised multiple theories including epitope spreading in generating a host antiviral response that is reactive toward self-antigens [24]. Thus, EBV and CMV have been considered the best candidates in priming autoreactive immune responses and in initiating the immune abnormalities in SSc.

Over the past decades, advances have been made in the innate immunity field to clarify how herpesviruses are initially recognized during cellular infections. It has been proposed that distinct viral genes trigger host innate immune responses in the infected cells. While the role of EBV and CMV as disruptors of acquired immunity was largely studied in SSc, their ability to interfere with the host innate immune system is less known and almost unexplored in most autoimmune diseases, including SSc. Understanding the interaction between herpesviruses and the host innate immune system might clarify the mechanisms used by viruses to influence the development of SSc disease.

EBV and Autoimmunity in SSc

Multiple serological, molecular, and epidemiological studies support the theory of EBV playing an important role in triggering autoimmunity in SSc. A persistent widespread infection and frequent EBV reactivation was found in patients with SSc [18, 21, 25–29]. While the hypothesis of molecular mimicry between several EBV antigens and self-antigens clearly links EBV infection to autoimmunity in SSc, there are mechanisms related to the viral pathology which make EBV unique in its contribution to the induction of autoimmunity [30]. For instance, it is well established that EBV initiates a transformative, latent infection in selected B-lymphocytes, leading to their proliferative expansion as lymphoblastoid cell lines (LCL) and immortalization in vitro [31, 32]. This process requires the expression of distinct EBV transforming proteins and expression of oncogenic genes which can be seen in EBV-associated tumors [31, 33]. Early studies reporting induction of recombination-activating genes (RAGs) by EBV in both lytic infected and in immortalized B-cells indicate that EBV dysregulates the process of receptor editing and revision, processes used to rescue B-cells with autoreactive or missing primary antigen receptor specificity, through RAG reexpression and secondary immunoglobulin rearrangement [34–37]. Autoreactive antibodies, like all other antibodies are produced in developing B-lymphocytes by variable diversity and joint gene segment (V(D)J) recombination regulated by RAG proteins [38]. It was shown that EBV generates transient levels of autoantibodies in patients with infectious mononucleosis (IM), suggesting that secondary immunoglobulin gene rearrangements by EBV-induced RAGs could be the mechanism employed by the virus in the production of the autoantibodies in IM [34]. Translating this concept to SSc, it was reported that B-lymphocytes from healthy donors upon EBV transformation were able to produce anti-topoisomerase I antibody (Scl-70), suggesting that the production of these specific autoantibodies might be directly related to EBV

infection in SSc, since anti-topoisomerase I antibody is considered a specific marker for SSc [39].

Additional findings of lytic EBV dysregulation further link EBV infection to SSc disease. Specifically, SSc patients more often exhibited high titers of EBV antibodies including increased titers of anti-viral capsid antigen (VCA)-Ig(VCA)-IgG antibodies that suggest that a recent EBV infection or reactivation occurred in SSc [25, 31, 40]. Moreover, a recent study showed that antibodies to distinct EBV antigens were detected more frequently than specific SSc and systemic lupus erythematosus (SLE) autoantibodies in these patients. Specifically, SSc and SLE patients, who tested negative for anti-dsDNA and Scl-70/anti-centromere autoantibodies, were positive for at least one of the antibodies against EBV antigens, suggesting that both of these diseases may be associated with a defective immune response to EBV infection, predisposing EBV to persistent activation in these patients [21].

In light of this new observation the following questions could be raised: does EBV trigger autoimmunity, and if so, is the innate antiviral immune response to EBV a critical mediator?

EBV, Innate Immunity, and Fibrosis in SSc

Evidence suggests that EBV is ubiquitous in human populations and establishes benign lifelong silent infections of B-lymphocytes occasionally interrupted by viral reactivation and virion production. This reflects the fine balance struck between host immune control over virus infection and the ability of the virus to evade this control. In healthy hosts, the immune system forces EBV to enter the "true latency" state, where the virus hides inside the nucleus of lymphocytes asymptomatically. EBV in true latency is neither pathogenic nor visible to the host immune systems due to the lack of any lytic or latent viral protein production [31].

While the acquired immune system tightly controls distinct viral gene expression programs and enables EBV to persist in immunocompetent hosts without doing much harm, the host innate immune system plays a critical role in blocking the initiation of EBV lytic infection [41, 42]. In the early stage of lytic infection, the host mounts an innate immune response represented by activation of several signaling pathways, including IFN production mediated by TLRs [43]. One of the most potent immune-stimulating components of EBV is the unmethylated form of genomic DNA. Based on studies using synthetic DNA, the current paradigm is that TLR9 recognizes CpG-motif-containing DNA, which is abundantly present in the EBV genome [31]. During the replication phase, the linear double-stranded DNA remains free of CpG methylation to facilitate the amplification of EBV genomes. At this point, the unmethylated EBV genome becomes visible to the innate immune system and can be detected by TLR9 [44, 45]. The hypomethylated genomes disappear in the true latency phase after the inhibition of productive viral replication, and EBV circularizes its DNA to an episome which then gets heavily methylated, strongly impairing its ability to activate TLR9 or other TLRs [45, 46]. Treatment with EBV DNA activates cultured plasmacytoid dendritic cells (pDCs), B-lymphocytes, and monocytes through the TLR9 signaling pathway. Under specific viral programs, EBV is also recognized by other TLRs, such as TLR7 and its downstream signaling mediators, IRF5 and IRF7 in B-lymphocytes [47, 48]. Moreover, the Epstein-Barr-virus-encoded small RNAs (EBERs) are also implicated as mediators of innate immune activation since they can interact with both the RIG-I and TLR3 sensors of adjacent cells when released by infected cells [49, 50]. Together, these results suggest that activation of the innate immune response by EBV is dependent on the viral programs carried in the infected cells.

Activation of non-immune cells in SSc skin induced by viral products has also been investigated over the past few years since EBV/LMP1/latency-antigen mRNA was found in SSc skin [29]. However, the cellular source of EBV viral products was never identified. Recently, our group reported the expression of EBV viral mRNA and lytic proteins in the majority of fibroblasts, mainly myofibroblasts, as well as endothelial cells in the skin of SSc patients, indicating that EBV replication is ongoing in the skin, and also that SSc fibroblasts/myofibroblasts and ECs might represent a target of EBV infection in SSc skin [22•]. Supported by the notion that lytic EBV activates the host innate immune system, expression of TLR7/9, IRF5/7, and the EBV-BZLF1 lytic gene was found increased in EBV-infected SSc fibroblasts in vitro, suggesting that EBV mediates the fibroblast antiviral response via the TLR7/9-MyD88 activation pathway. Because EBV induces a large spectrum of genes in infected B-lymphocytes and epithelial cells, it was of particular interest to investigate whether EBV could also alter the fibroblast pro-fibrotic response in infected cells. Intriguingly, expression of TGFβ1, and several TGFβ-regulated genes, including α-SMA and collagen-1, were also found upregulated in infected fibroblasts, suggesting that the activation of SSc fibroblasts might be related to EBV infection in these cells [22•]. These results lead to the idea that EBV lytic infection might be dysregulated in SSc and the lytic viral program might regulate both the inflammatory and the fibrotic processes in SSc skin, but the driving role of TLR in inducing pro-fibrotic genes in infected fibroblasts remains to be substantiated.

Among several EBV lytic genes, one has been reported to upregulate TGFβ expression in Burkitt's lymphoma (BL) cell lines and in epithelial cells. BZLF1/Zta, which was found abundantly expressed in SSc skin fibroblasts, is known to regulate several host cellular functions including innate immunity and cellular apoptosis, although its primary role is to disrupt viral latency and transactivate the expression of EBV/early-lytic genes [51–53]. Among its host cellular

targets, activation of the TGFβ pathway is one of the strategies employed by the EBV/Zta gene to modulate the host immune response in infected B-lymphocytes and epithelial cells [42, 54, 55]. Moreover, BZLF1/Zta was shown to upregulate TGFβ1 mRNA levels and biologically active protein and to interact with numerous key cellular transcriptional regulatory factors including EGR1 in cells expressing this gene [55–58]. Given the fact that TGFβ is required by the virus during the lytic stage of the infection, it is possible that TGFβ might represent one of the mechanisms employed by EBV to dampen the host innate immune response. Supporting this observation, it was recently shown that TGFβ inhibits CpG DNA-induced type I IFN production transcriptionally via ubiquitination of TRAF6 [59].

Collectively, these results suggest that EBV targets the innate immune system during the lytic phase of viral infection. Moreover, in order to enhance the efficiency of lytic viral replication, EBV employs several strategies to evade the innate immune response, and activation of TGFβ might represent one of them.

EBV and Fibroblasts

While there is ample evidence that EBV hides in latent form in memory B-lymphocytes in the majority of the world healthy population, there is limited evidence that EBV infects fibroblasts. Fibroblasts are not considered a typical target of EBV infection since they are negative for the CD21-EBV receptor [20•, 60] used by EBV for cell entry. Early studies showed Zebra protein expression in synovial fibroblasts from one patient with rheumatoid arthritis, and EBNA1 protein expression in lung fibroblasts, suggesting that EBV might infect fibroblasts even if, like epithelial cells, they are negative for CD21 [61–66]. Recently, our group reported that EBV is able to infect human dermal fibroblasts using monocytes or dendritic cells as a vehicle for infection in vitro. This finding suggests that EBV uses alternative strategies to infect fibroblasts that bypass the absence of CD21, resembling the described transmission of EBV to human epithelial cells by cell-to-cell contact [22•, 60, 67]. While the mechanism by which EBV infects fibroblasts in vivo and in vitro is still uncertain, there is growing interest in exosomes. Those specialized membranous vesicles derived from the endocytic compartment can carry and deliver functional mRNA, miRNAs as well as proteins to various cells [68–70]. Given the fact that thousands of EBV-miRNA copy numbers have been detected in exosomes from LCL-infected cells, it would be conceivable that EBV-containing exosomes may be continuously secreted and transferred from the infected cells to uninfected neighboring cells, including fibroblasts [70, 71, 72•]. In light of this observation, further research will be warranted to evaluate the presence of EBV-miRNA/protein-containing exosomes in SSc skin, as well as clarify whether EBV-infected immune cells might transfer functional EBV-RNA and protein to fibroblasts through exosomes.

Cytomegalovirus and Autoimmunity

Serological and epidemiological studies link infection of human cytomegalovirus (HCMV) to the onset of SSc, suggesting that HCMV is a possible trigger for autoimmunity in SSc. Specifically, a higher prevalence of IgA anti-CMV antibodies was found in patients with SSc. The presence of antibodies against multiple CMV proteins cross-reacting with endothelial cell and fibroblast surface proteins implicates CMV role in triggering both endothelial cell apoptosis and fibroblast activation in SSc [23, 40, 73, 74]. Moreover, monoclonal antibodies against topoisomerase I recognized peptide of an autoantigen sharing homology with the HCMV-derived UL70 protein, support the theory that CMV might utilize the molecular mimicry mechanism to trigger SSc [75]. Therefore, due to its capacity to induce powerful polyclonal immune responses, CMV mimicry has been considered as a potential initiator of humoral SSc autoimmunity.

Cytomegalovirus, Innate Immunity, Vasculopathies, and Fibrosis in SSc

While the association between HMCV serology and SSc is generally recognized, whether HCMV plays a direct role in activating the innate immune system in SSc, at this stage, is still unknown. The fact that multiple members of TLR and non-TLR signaling pathways were found downregulated during HCMV active infection suggests that CMV might use a strategy similar to EBV to avoid the innate immune system [76]. This would seem likely because other studies reported that CMV structural components directly activate the innate immune system in infected cell lines. For instance, TLR2 was been found to functionally recognize HCMV through direct interaction with the envelope tegumental glycoproteins, gB and gH, suggesting that the innate immune response activated by HCMV might be primarily dependent on viral envelope proteins and independent of viral replication in permissive fibroblasts [76]. Moreover, there was a reported interaction of CMV with TLR7 and TLR9 in human plasmacytoid dendritic cells (pDC), suggesting that TLR involvement might also be related to the cell types in which the virus establishes the infection [77]. The downregulation of TLR signaling pathways may help CMV infect pDCs and transfer to endothelial cells and fibroblast during early infection, while activation of TLRs in these cells may increase during viral reactivation at a later stage contributing to SSc disease.

The contribution of the CMV lytic gene in promoting fibrosis was considered in SSc. Earlier studies showed detection of CMV immediate early lytic gene IE1 in SSc skin [29]. Supported by this observation and based on CMV's tropism

to infect endothelial cells as well as a broad range of cells within its host, including fibroblasts, the effect of the CMV IE1 gene was tested in human fibroblasts. It was found that fibroblasts expressing the CMV-IE1 gene were capable of transiently inducing high levels of CTGF, TIMP-1 and Collagen-1 mRNAs, partially mediated by TGFβ, suggesting that HCMV lytic infection can produce several pro-fibrotic cytokines implicated in SSc skin disease [78]. The association of viral active infection with fibrosis was further confirmed by studies in animal models. Murine CMV (MCMV) infection induced vascular lesions similar to that seen in SSc tissues in mice carrying an IFNγR deficiency [79]. These results were particularly intriguing, since they were the first to suggest that chronic CMV infection might be directly involved in inducing endothelial cell injury by a mechanism independent of molecular mimicry/autoimmunity, and that MCMV chronic infection, in the presence of aberrant IFNγ signaling recapitulated SSc vascular/fibrotic features. Given the importance of IFNγ-producing cells in protection against CMV disease and in controlling viral lytic reactivation, these results suggest that the cell activation, vascular proliferation and fibroblast pro-fibrotic responses induced by CMV viral products might only contribute to SSc in conditions of a predisposing aberrant immune response [80].

Parvovirus B19 and SSc

Parvovirus B19 was speculated to be a causative agent in SSc since serum parvovirus viremia was more often detected in SSc patients than healthy subjects, and the presence of parvovirus B19 was more frequently detected in SSc bone marrow biopsy specimens, suggesting that the bone marrow may represent a reservoir from which the parvovirus spreads to SSc tissue [81, 82]. Endothelial injury in patients with parvovirus was suggested to reflect a combination of direct cytotoxicity and humoral immunity. It was described that parvovirus can persistently infect SSc fibroblasts and that this might be responsible for marked cell alteration [40]. However, how parvovirus B19 impacts innate immunity and the mechanism by which it might cause fibrosis await further validation in SSc.

Conclusions

Infections are known to be involved in the initiation and promotion of SSc [21, 22•, 29, 40]. Viral infections such as EBV and CMV appear to be the main candidates to trigger SSc due to their multifaceted and multidirectional relationships with the development of this disease. The studies highlighted in this review suggest that EBV and CMV have the capacity to elicit a strong immune activation as well as to contribute to the pro-fibrotic process in SSc. In the case of EBV, the finding of anti-topoisomerase antibody that could be induced by the oncogenic genes expressed in immortalized B-lymphocytes supports the hypothesis that EBV modulates the production of specific autoantibodies in SSc. The presence of viral proteins in the skin strongly indicates that EBV could be the source of tissue injury in SSc skin. Moreover, activation of the innate immune response by lytic-genes could represent one of the mechanisms employed by EBV to drive inflammation and pro-fibrotic responses in infected SSc fibroblasts. On the other hand, CMV also seems to be involved in the development of SSc, although its contribution to the disease appears to be different than EBV. Specifically, CMV might promote vascular injury in SSc, based on its tropism for endothelial cells, and the capacity to induce anti-CMV antibodies cross-reacting with endothelial cell and fibroblast surface proteins.

Modulation of the host innate immune responses is a key component in herpesviruses life. In fact, herpesviruses are very common pathogens and the majority of healthy adult populations show serological evidence of past EBV and CMV infection. However, evidence of viral reactivation is rarely reported in the healthy population carrying a viral genome [42]. While the host innate antiviral responses mediated by IFNs promptly monitor and limit those infections, the reason why the innate immune system of SSc patients fails to limit EBV and CMV infection remains to be understood. The finding of active EBV and CMV infections, as well as the evidence of higher susceptibility of SSc patients to specific pathogens, including fungi (*Rhodotorula glutinis*) [83], strongly support the idea that dysregulation of SSc antiviral immune response could facilitate pathogen persistent infections in the disease. Furthermore, one animal study confirmed this observation by showing the development of vascular lesions similar to those seen in SSc only in mice infected with MCMV, in the condition of an impaired IFNγ response [79]. Although the reason for this has not yet been taken into account, one explanation could be that SSc genetic backgrounds might affect important key regulators of the host defense, predisposing the host immune response to an uncontrolled EBV and CMV infection. If this is the case, SSc patients might poorly handle certain kinds of infection, facilitating viruses such as EBV to display their pathogenic potential in infecting atypical target cells, such as fibroblasts, or, even worse, in transforming these cells. In addition, due to EBV's oncogenic potential that etiologically links this virus to a remarkably wide range of tumors, of B-cell and non-B-cell origin, it would be interesting to determine whether aberrant immune responses might also increase the oncogenic capacity of this virus, or other oncogenic viruses, to induce cancer in SSc patients [20•, 84•].

Overall, further research is needed to better understand the interaction of pathogens with genes that confer susceptibility

to autoimmunity in SSc. Perhaps understanding the dynamic interplay among genetics, immune responses, and pathogens might shed a light on the origin of SSc pathogenesis.

Acknowledgments We wish to thank Paul Haines for the critical proofreading of the manuscript.

Compliance with Ethical Standards

Conflict of Interest Antonella Farina and G. Alessandra Farina declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

Funding This study was supported by the NIH-NIAMS grant 1R03AR062721-01 and Scleroderma Foundation Established investigator (G.A.F).

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