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Epstein–Barr Virus Infection Induces Aberrant TLR Activation Pathway and Fibroblast–Myofibroblast Conversion in Scleroderma

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Scleroderma (SSc) is a complex and heterogeneous connective tissue disease mainly characterized by autoimmunity, vascular damage, and fibrosis that mostly involve the skin and lungs. Epstein–Barr virus (EBV) is a lymphotropic γ-herpesvirus that has co-evolved with human species, infecting >95% of the adult population worldwide, and has been a leading candidate in triggering several autoimmune diseases. Here we show that EBV establishes infection in the majority of fibroblasts and endothelial cells in the skin of SSc patients, characterized by the expression of the EBV noncoding small RNAs (EBERs) and the increased expression of immediate-early lytic and latency mRNAs and proteins. We report that EBV is able to persistently infect human SSc fibroblasts *in vitro*, inducing an aberrant innate immune response in infected cells. EBV–Toll-like receptor (TLR) aberrant activation induces the expression of selected IFN-regulatory factors (IRFs), IFN-stimulated genes (ISGs), transforming growth factor-β1 (TGFβ1), and several markers of fibroblast activation, such as smooth muscle actin and Endothelin-1, and all of these genes play a key role in determining the profibrotic phenotype in SSc fibroblasts. These findings imply that EBV infection occurring in mesenchymal, endothelial, and immune cells of SSc patients may underlie the main pathological features of SSc including autoimmunity, vasculopathy, and fibrosis, and provide a unified disease mechanism represented by EBV reactivation.

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INTRODUCTION

Fibrosis is a pathologic scarring process that has been considered the hallmark of systemic sclerosis (scleroderma (SSc)), a connective tissue disease characterized by autoimmunity, inflammation, and vasculopathy, leading to progressive fibrosis mostly of skin and lungs. Fibroblasts, mainly myofibroblasts, clearly have a necessary and fundamental role in promoting fibrosis (Varga and Abraham, 2007). Evidence suggests that innate immune activation of IFNs by Toll-like receptors (TLRs) may play a role in the pathogenesis of inflammation in many autoimmune diseases, initially assessed in systemic lupus erythematous and more recently in SSc, implying that dysregulation of the innate immune response underlies the overactive immune system of individuals who are susceptible to autoimmune disease (Theofilopoulos, 2012).

Recently, it has been shown by our group and by other independent studies that IFN signature gene expression is increased in peripheral blood mononuclear cells (PBMCs) and in the skin of SSc patients (York *et al.*, 2007; Farina *et al.*, 2010a). Furthermore, we showed that double-stranded RNA/polyinosinic–polycytidylic acid, a TLR3 ligand, stimulates IFN production, inflammation, and markers of vascular activation in SSc skin (Farina *et al.*, 2010b, 2011).

Although our studies strongly supported the potential key role for double-stranded RNA/polyinosinic–polycytidylic acid to contribute to innate immune activation in SSc, the origin of the innate immune dysregulation in SSc is unknown, and to date there is no obvious evidence addressing any potential exogenous/endogenous source of RNA that might represent a TLR3 ligand in SSc skin. In order to address this question, we asked whether viral RNAs could be detected in the skin and serve as innate immune ligands in SSc. Previous studies in animal models have shown that murine cytomegalovirus infection in immunocompromised mice resembles the pathological processes seen in autoimmune diseases, particularly in SSc, suggesting a link between herpesvirus infection and the development of fibrosis (Pandey and LeRoy, 1998). However, no direct evidence of cytomegalovirus infection, such as the presence of viral protein or production of viral progeny, has yet been found in SSc. As herpesvirus family has been linked to

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Abbreviations: DC, dendritic cell; EBER, EBV-encoded RNA; EBV, Epstein–Barr virus; HD, healthy donor; IRF, IFN-regulatory factor; ISG, IFN-stimulated gene; LdSSc, lesional diffuse SSc skin; MO, monocyte; NLdSSc, nonlesional diffuse SSc skin; PBMC, peripheral blood mononuclear cell; SMA, smooth muscle actin; SSc, scleroderma; TGF, transforming growth factor; TLR, Toll-like receptor

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the development of fibrosis, we focused our attention on Epstein–Barr virus (EBV), a γ-herpesvirus that has been a leading candidate in triggering several autoimmune diseases (Ebrahimi *et al.*, 2001; Niller *et al.*, 2008; Dreyfus, 2011; Stoolman *et al.*, 2011). This virus is a biologically plausible source for endogenous innate immune activation as it is ubiquitous in nature, establishes a life-long silent infection with continuous virus production because of reactivation, and most importantly, modulates the human immune system, evolving immune evasion strategies to the host antiviral response (Young and Rickinson, 2004; Munz *et al.*, 2009; Martorelli *et al.*, 2012).

We show that EBV is able to infect human dermal fibroblasts, and modulate the innate immune response in infected fibroblasts, inducing fibroblast–myofibroblast conversion typical of a profibrotic phenotype. We further demonstrate the presence of EBV viral transcripts and proteins in the majority of fibroblasts and endothelial cells in the skin of SSc patients, supporting a crucial role of EBV in SSc pathogenesis. Viral infection of nonimmune cells might provide a persistent source of tissue injury and induce chronic inflammation and fibrosis in SSc skin.

RESULTS

EBV transcripts are present in skin and in PBMCs from SSc patients

The EBV noncoding RNA, EBV small RNA (EBER) is the most abundant viral transcript in latently infected cells, and can activate TLR3 and retinoic acid-inducible gene 1 (RIG-I) (Samanta *et al.*, 2008; Iwakiri *et al.*, 2009). We investigated EBER (EBER1/EBER2) expression in SSc skin by "*in situ*" hybridization in sections from representative SSc patients (demographics and clinical characteristics are specified in Supplementary Tables S1 and S2 online). We found a striking accumulation of EBER-positive cells (EBER+ cells) in the deep dermis of lesional/forearm (LdSSc) and nonlesional/back (NLdSSc) SSc skin (Figure 1a and c). EBER+ cells were distributed among the bundles of the extracellular matrix in the deep dermis, showing spindle morphology and identified as fibroblasts by the shape and location. The EBER signal was mostly detected in cell nuclei, although certain nuclei of those cells appear mostly destroyed and disintegrated (Figure 1a). No EBER+ cells were detected in healthy donor (HD) skin (Figure 1d, Supplementary Table S2 online). As myofibroblasts play a pivotal role in fibrotic SSc skin, we asked whether EBER+ cells may express smooth muscle actin (SMA), a marker for myofibroblasts. Immunohistochemical staining of serial skin biopsies showed that most, but not all SMA-stained cells, colocalized with EBER+ cells (Figure 1e and f), Interestingly EBER+ cells were also detected around blood vessels, identified as smooth muscle cells by SMA immunohistochemical staining (Figure 1f, lower panels). We also observed EBER+ cells in the myoepithelial layer of the cutaneous glands (Figure 1g), as well as in most of the endothelial cells in dermal ectatic vessels (Figure 1c). No EBER staining was detected in smooth muscle cells and myoepithelial or endothelial cells from HDs (Supplementary Figure S1 online). EBER+ fibroblasts were also found in patients with limited SSc (data not shown).

It is known that in B cells, detection of EBV DNA and EBER or lytic RNAs/proteins identifies latent or active infection, respectively (Okano *et al.*, 2005). Thus, we asked whether EBV infection is associated with expression of lytic or latency genes in SSc skin. We found mRNA expression of both BZLF1, the viral transactivator that drives EBV reactivation, switching from latency to the lytic replication, and EBNA1, one of the nine latency genes, present in LdSSc skin (Supplementary Figure S2a online). Complementary DNA sequencing confirmed BZLF1 and EBNA1 specificity of the reverse transcriptase–PCR products (data not shown). BZLF1 was also detected in NLdSSc and in none of the control skin (Supplementary Figure S2b online and Supplementary Table S2 online).

As EBV persists in B cells, we also investigated the state of EBV infection in SSc PBMCs. We found that BZLF1 gene and lytic and latency proteins were significantly increased in PBMCs from SSc patients (Supplementary Figure S2c online and Supplementary Tables S2 and S7 online). Although all the patients and HDs were seropositive for EBV, a striking increased production of antibodies against EBV viral capsid antigen, the marker of acute EBV infection, was detected in SSc sera (Supplementary Tables S1 and S3 online).

EBV DNA is detected in the skin of SSc patients

To further confirm the presence of EBV infection in SSc skin, we measured EBV genome DNA. We found EBV EBER1 in LdSSc (Supplementary Figure S2d online) and NLdSSc skin but rarely in HD skin (Supplementary Table S2 online). DNA sequencing confirmed that amplified DNA was indeed EBER1 (data not shown). Increased copies of EBV DNA were found in LdSSc compared with HD skin (Supplementary Figure S2e online). As it has been shown that most of the immunosuppressive medications used to treat organ transplant recipients elevate EBV load and reactivate lytic/latency genes as has also been shown in patients with systemic lupus erythematous or other autoimmune disease, we selected a group of SSc patients naive for any treatment compared with SSc patients who received immunosuppressive therapy (Green *et al.*, 2000; Larsen *et al.*, 2011). We found no statistical differences in EBV DNA viral load (EBER1) and EBER expression in the skin of the treatment-naive SSc patients compared with the treated group (Supplementary Figures S2f and S4a–c online and Supplementary Tables S4 and S5 online). Undetectable levels of EBV DNA were measured in the skin from foreskin samples as well as in 293 cells as negative control.

Assessment of viral proteins in SSc skin

We next investigated whether EBV transcripts might produce viral proteins in SSc skin. Comparable to data obtained with EBV transcripts, we found that the majority of the SSc patients positive for EBV mRNA showed expression of lytic as well as latent proteins in the skin (Supplementary Figure S3a–f online and Supplementary Tables S6 and S7 online). It is noteworthy that no lymphocyte infiltration was observed in areas where ZEBRA-positive cells were detected. Nuclei of endothelial cells and smooth muscle cells were also positive for BFLF2 and ZEBRA (Supplementary Figure S3c online). No difference in ZEBRA protein skin expression was observed between

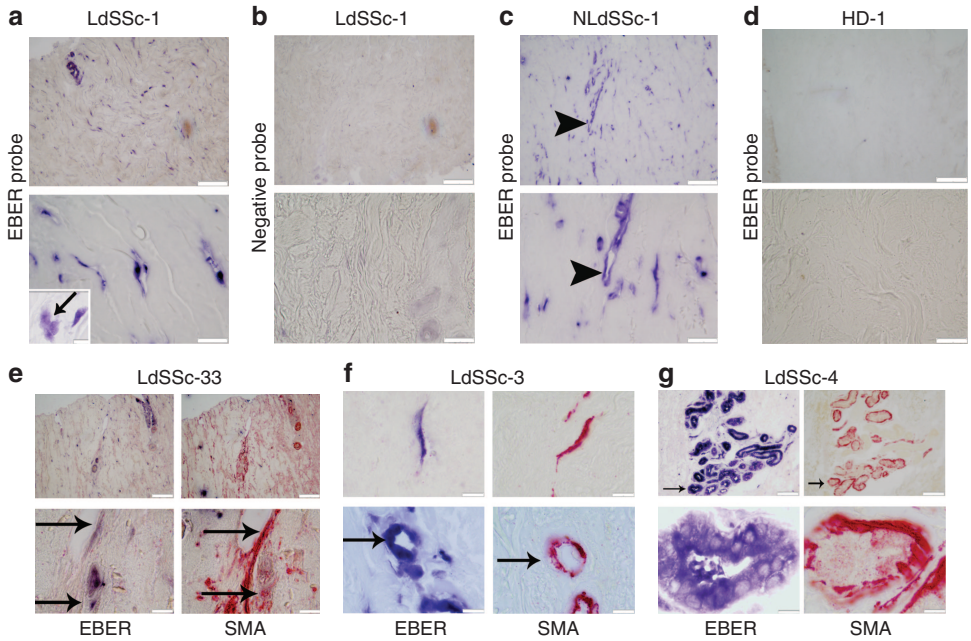

Figure 1. The Epstein–Barr virus (EBV)–encoded RNA (EBER)–infected cells in scleroderma (SSc) skin. (**a**–**c**) Representative images of *in situ* hybridization (ISH) in lesional (LdSSc-1) and in nonlesional diffuse skin (NLdSSc-1) (**a**) showing EBER+ spindle cells in the deep dermis; (**b**) EBER staining in LdSSc-1 skin with probe control; (**c**) EBER staining in endothelial cells of ectatic vessels in the skin (arrowhead); (**d**) EBER staining of healthy donor (HD) skin; (upper panels bar = 100 μm, lower panels 50 μm). (**e**, **f**, upper panel) Colocalization of EBER+ cells detected by ISH (blue) with α-smooth muscle actin (αSMA) cells (red) detected by immunohistochemical staining (IHC) in serial LdSSc skin sections (bar = 100 μm and 50 μm); (**f,** lower panel) EBER+ staining in blood vessels smooth muscle cells identified by αSMA staining by IHC in serial skin section (arrows) (lower panel, bar = 50 μm). (**g**) EBER+ cells detected in myoepithelial glands surrounded by SMA fibers in skin (bar = 50 μm).

biopsies from patient's naive to immunosuppressed treatment and biopsies from patients treated with immunosuppression (Supplementary Figure S4d–f online). Notably, we did not find expression of the late/EBV protein gp350/220 (Supplementary Figure S5 online), or detection of mature virus production by electron microscopy in SSc skin (data not shown), suggesting that the virus lytic cycle is abortive (Martel-Renoir *et al.*, 1995). None of the HD skin sections showed specific staining for ZEBRA and BFLF2 (Supplementary Figure S5 online).

EBV infects human SSc fibroblasts *in vitro*

EBV mainly infects B lymphocytes through CD21 receptor, although it is generally accepted that it also infects epithelial cells, even though they are CD21 negative. Thus, its presence in fibroblasts was unexpected. No expression of EBV DNA, mRNA, and/or protein was detected in primary fibroblast cell lines. The first attempt of using cell-free EBV or recombinant EBV p2089 (EBV-p2089) failed to infect any fibroblast cell lines and it prompted us to explore how the virus gains access into fibroblasts. As monocytes (MOs) have been found to be increased in the perivascular areas of SSc skin, we used purified MOs or dendritic cells (DCs) from HDs bound with EBV-p2089 to perform transfer infection, as indicated by previous reports using resting B cells as a transfer vehicle for EBV infection of epithelial cells (Shannon-Lowe *et al.*, 2006; Feederle *et al.*, 2007; York *et al.*, 2007). MO or DC virus-binding efficiency was detected by indirect immunofluorescence assay using EBV anti-capsid antibody (10–20% of MOs or DCs were able to bind the virus; Figure 2a). No EBV infection was detected in MO/DCs recovered after fibroblast transfer infection. After the transfer infection, 10% cell/field of SSc fibroblasts showed nuclear p2089–green fluorescent protein fluorescence at 5 days and 4 weeks after infection, respectively (Figure 2b and c and Supplementary Figure S6a online). As fibroblasts do not represent the natural target of EBV, we analyzed the expression of latency as well as lytic proteins in order to identify the viral strategy in this EBV-infected cell type. EBV lytic proteins, BFLF2/BFRF1, and latent antigen/EBNA2 were expressed at 4 weeks after infection in the nuclei of infected SSc cells defined as fibroblasts by collagen-1 and collagen-11 staining and by shape (Figure 2d–f and data not shown). We did not find the expression of late lytic gene/BLLF1, suggesting that abortive EBV replication occurs in EBV-infected SSc fibroblasts (data not shown). We did not find any cleavage of poly (ADP-ribose) polymerase protein in EBV-p2089 compared with mock-infected SSc fibroblasts, confirming that EBV does not induce apoptosis in infected SSc cells, as well as in infected B cells (Supplementary Figure S6b online).

MO/DCs derived from different HDs consistently infected all SSc fibroblast cell lines included in this study. Infected SSc fibroblast cultures were monitored by indirect immunofluorescence assay. Fibroblasts from NLdSSc and from HDs were also occasionally infected (ratio of 1 of 4). EBV-p2089 DNA persisted up to 6 months in infected LdSSc fibroblasts, whereas EBV-p2089-infected NLdSSc fibroblasts and EBV-p2089-infected HD fibroblasts died after 20 days after infection.

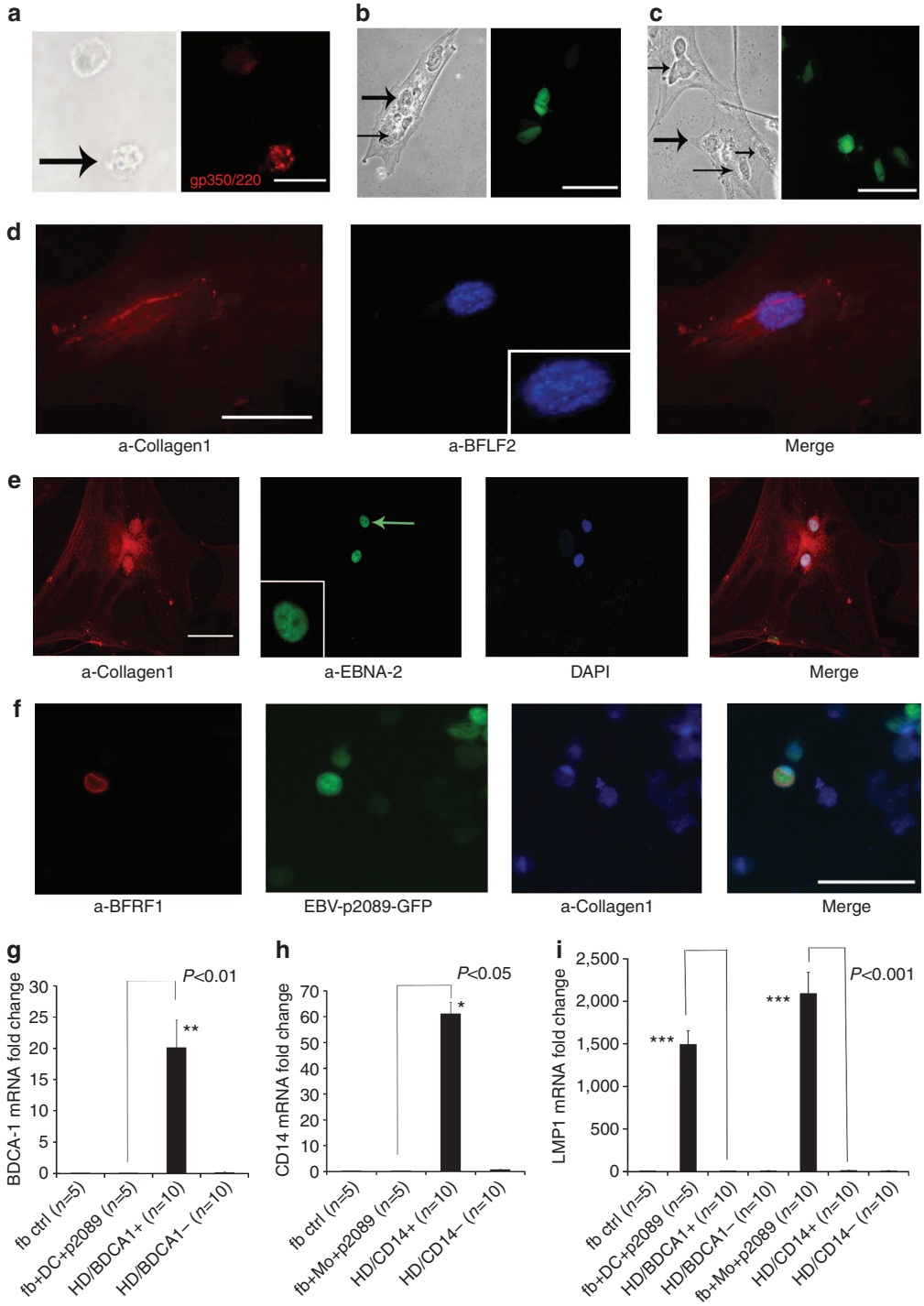

Figure 2. *In vitro* infection of scleroderma (SSc) fibroblast. Monocytes (MOs) and dendritic cells (DCs) from healthy donors (HDs) previously exposed to Epstein–Barr virus (EBV)-p2089 were co-cultured with SSc fibroblasts as described in the Materials and Methods. (**a**) Indirect immunofluorescence (IF) staining for EBV gp350/220 antigen of MOs bound to EBV-p2089 (left panel: phase-contrast light microcopy, bar = 20 μm). (**b**, **c**) Detection of EBV-p2089 green fluorescent protein (GFP) in fibroblasts at 5 days (**b**) and 4 weeks (**c**) after infection (left panel: phase-contrast light microcopy, bar = 20 μm). (**d**, **e**) Double IF of adherent or in suspension fibroblasts (**f**) costained with the indicated antibodies at 4 weeks after infection (square insert, bar = 10 μm). Diaminidino-2-phenylindole (DAPI) was used as counterstaining for the nuclei. (**g–i**) mRNA expression of indicated genes in p2089 fibroblasts transfer-infected cultures, CD14 + /CD14 − and BDCA1 + /BDCA1 − population from HDs by semi-quantitative PCR. ctrl, control; fb, fibroblast; LMP1, latent membrane protein 1. Data are expressed as the fold change normalized to mRNA expression in a single sample from HD. Bars represent mean ± SEM $*P<0.05$; $**P<0.01$; $***P<0.001$.

To exclude immune cell contamination in the fibroblast population, markers of MOs and DCs were evaluated by semi-quantitative PCR. CD14 and BDCA1 mRNA expression were undetectable in MOs, or DC transfer-infected or mock-infected fibroblasts, although those markers were expressed in MOs and DCs used as shuttle infection in

fibroblast cultures (Figure 2g and h). Interestingly, latent membrane protein 1 (LMP1) mRNA expression was found significantly abundant in EBV-infected SSc cells, whereas no LMP1 expression was detected in uninfected-, mock infected, or MO/DCs from HDs, confirming that EBV is infecting SSc fibroblasts (Figure 2i). Indirect immunofluorescence assay staining for CD14/BDCA1 was absent in infected SSc fibroblasts (data not shown).

EBV activates TLR pathway in infected SSc fibroblasts

To explore the interaction between EBV and fibroblast innate immune responses, we examined the expression of TLR-activated genes in infected cells at 4 weeks after infection. Expression of TLR7 and TLR9 mRNA was significantly induced in EBV/p2089-infected SSc fibroblasts (Figure 3a), as well as the mRNA levels of IFN-regulatory factor 7 (IRF7), IRF5, and IRF4, and selected IFN-stimulated genes (ISGs) (Figure 3a–c). Remarkably, tumor necrosis factor-α, a gene found modulated by EBV in B lymphocytes, was also robustly increased in EBV/p2089-infected SSc fibroblasts. No increased expression of TLRs, IRFs, or ISGs was observed in mock-infected or in parallel uninfected fibroblast cultures.

EBV induces a profibrotic phenotype in infected SSc fibroblasts

Next we asked whether viral interaction with SSc fibroblasts might also promote proliferation and expression of activation markers as it does in B cells (Miller *et al.*, 1972). Specifically, we evaluated expression of genes and protein known to be related to SSc profibrotic phenotype (Varga and Abraham, 2007). We found coexpression of EBV lytic protein BFRF1 and SMA antigen in SSc fibroblasts, and increased expression of genes implicated in fibroblast–myofibroblast conversion such as transforming growth factor-β1 (TGFβ1), endothelin 1 (EDN1), and SMA, and several TGFβ-regulated genes (EGR1, PAI1, COMP, and COLIV) in EBV/p2089-infected SSc fibroblasts at 4 weeks after infection (Figure 4a, b, and e and Supplementary Figure S7 online). A persistent activation of phospho-SMAD2, a critical mediator of TGFβ-induced collagen secretion, was also detected in the cell lysates of SSc fibroblasts infected either by EBV/p2089 or EBV (Figure 4c).

TLR7 and TLR9 agonists stimulate IFN-regulated genes in fibroblasts from healthy control skin

To evaluate whether activation of TLR7/TLR9 mimics the innate immune modulation induced by EBV in infected SSc fibroblasts, parallel fibroblast cultures generated from the same SSc patients and HDs not exposed to the virus were stimulated with TLR ligands, namely R837/imiquimod (for TLR7) and CpGODN2006 oligonucleotide (for TLR9) (Schoenemeyer *et al.*, 2005; Paun *et al.*, 2008).

We found that activation of the TLR pathway by CpGODN2006, or a combination of R837 and CpGODN2006 ligands, significantly induced MX2, OAS2, and CXCL9 expression in HD fibroblasts 24 hours after treatment (Supplementary Figure S8a online); chronic activation of TLR pathway induced CXCL9 expression in HD fibroblasts treated for 4 weeks (Supplementary Figure S9a online). No expression of TGFβ-regulated genes and/or increase of collagen secretion were detected in acute/chronic TLR-stimulated HD fibroblasts (Supplementary Figure S8c online and data not shown). In contrast, we did not find activation of IFN-inducible gene responses, as well as any increase of collagen secretion upon acute/chronic stimulation by TLR7/9 ligand agonists in SSc fibroblasts (Supplementary Figures S8b, d and S9b online).

DISCUSSION

We show here that EBV DNA, mRNAs, and proteins are present in SSc skin, and the majority of the cells expressing EBER RNA are fibroblasts. To investigate the consequences of EBV infection in stromal cells, we developed an innovative methodology that requires MOs and DCs as a transfer vehicle for infection of fibroblasts. Here, we report that EBV is able to infect human fibroblasts "*in vitro*" and activate a fibroblast innate immune response. Importantly, EBV infection of SSc fibroblasts promotes the SSc profibrotic phenotype, inducing an aberrant TLR activation pathway that is responsible for expression of IRFs, selected ISGs, TGFβ1, and several markers of fibroblast activation, such as SMA and Endothelin-1, in infected SSc cells. Our study provides mechanistic insight into how EBV infection of stromal cells affects fibroblast–myofibroblast conversion, which is consistent with the phenotype seen in SSc skin.

EBV infection in LdSSc and in NLdSSc skin

We detected expression of the EBV transactivator BZLF1 gene, latency genes, and early lytic protein *in vivo* in PBMCs, as well as in the skin, indicating that EBV infection is systemic and ongoing in SSc. Intriguingly, EBV expression of EBER, BZLF1, and EBNA1, and EBV proteins for ZEBRA, BFRF1, and BFLF2, were expressed in LdSSc and prominently in NLdSSc, suggesting that a similar "EBV footprint" was found in areas not associated with fibrosis. Previous studies have shown almost identical disease-specific patterns of gene expression from LdSSc, NLdSSc skin biopsies, and from fibroblast cultures, revealing that both LdSSc and NLdSSc shared the same gene expression (Whitfield *et al.*, 2003; Fuzii *et al.*, 2008); our findings of EBV transcriptional programs in SSc skin are in line with these data. As the mechanism of how the virus persists in certain infected cells and not in others is still not clear, a possible explanation would be that the innate immune system of infected cells does not allow EBV to survive in the context of NLdSSc skin.

Moreover, in SSc skin biopsies we found that smooth muscle cells, myoepithelial cells, and most of the endothelial cells expressed EBER RNA, suggesting that EBV tropism *in vivo* could be broader than initially thought and extended to other cell types as permissive targets in SSc.

EBV and innate immunity

EBV has long been proposed as a common associated factor for autoimmune diseases: systemic lupus erythematous, rheumatoid arthritis, and multiple sclerosis, as EBV latent antigens and high titers of EBV antibodies have been detected more often in these patients; moreover, high titers of IgM viral capsid antigen antibodies were found in SSc, implying a recent EBV infection in this disease (Lunemann and Munz, 2007; Niller *et al.*, 2008; Arnson *et al.*, 2009). In support of

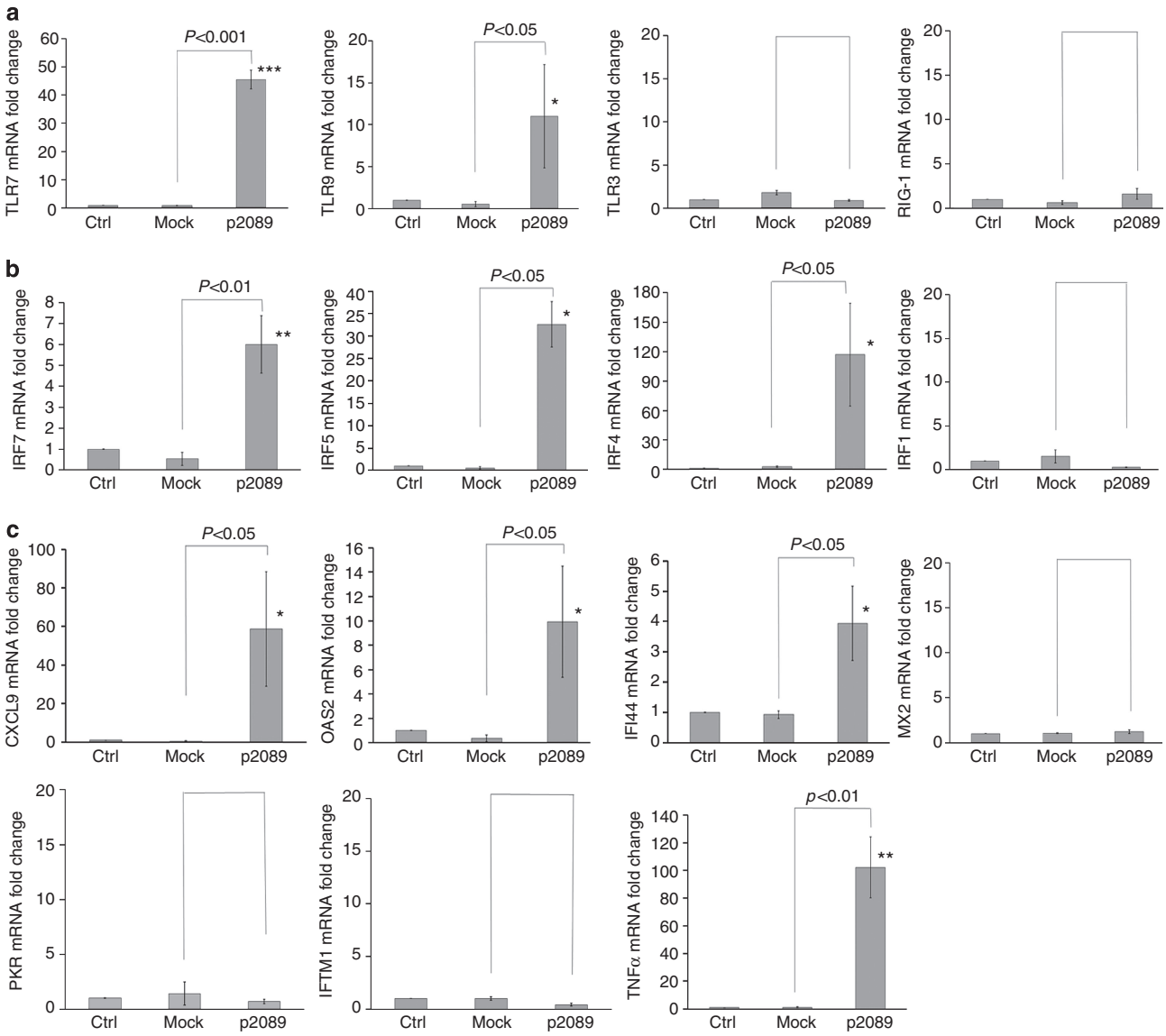

Figure 3. Epstein–Barr virus (EBV) activates innate antiviral response in infected scleroderma (SSc) fibroblasts. Monocytes (MOs) and dendritic cells (DCs) from healthy donors (HDs) bound to EBV-p2089 were co-cultured with dermal fibroblasts from SSc patients. MOs and DCs not exposed to EBV-p2089 were co-cultured with SSc fibroblasts as mock infection; fibroblasts left untreated were used as control (Ctrl). After 72 hours, MO, DCs, and EBV-p2089-free virus were removed from fibroblasts cultures and total RNA extracted at 4 weeks after infection. (**a**–**c**) mRNA expression of Toll-like receptors (TLRs), IFN-regulatory factors (IRFs), IFN-stimulated genes (ISGs), and tumor necrosis factor (TNF) in EBVp2089-infected, mock-infected, and control fibroblasts from SSc patients evaluated by semi-quantitative PCR. Fold changes shown on the graph are normalized to mRNA expression by each corresponding untreated cell line. Bars represent mean ± SEM from five separate SSc fibroblast lines. *P*-values were calculated using two-tailed *t*-test $*P<0.05$; $**P<0.01$; $***P<0.001$.

this association, LMP1 antigen mRNA was detected in SSc skin; however; the cellular source of the EBV product was not identified (Vaughan *et al.*, 2000). We show here that EBV early lytic infection occurs in fibroblasts of SSc patients, suggesting that fibroblasts might represent the crucial target of EBV infection in SSc skin.

Several mechanisms have been described to explain how EBV triggers autoimmune disease, such as antigen cross-reactivity with self-nuclei protein and/or bystander activation of autoreactive cells (Langland *et al.*, 2006; Samanta *et al.*, 2008; Iwakiri *et al.*, 2009; Munz *et al.*, 2009). In this study we reveal an unreported feature employed by EBV that involves viral RNA and DNA triggering the innate immune system of nonimmune cells. Specifically, we show that EBV triggers the innate immune system activating TLR-like antiviral responses. The contribution of EBV infection to the innate immune system is unexplored in fibroblasts. EBV activates TLR7 in B cells although additional mechanisms have recently been proposed to explain how EBV might activate TLRs or different pattern recognition receptors in other cellular systems (Lindhout *et al.*, 1994; Martin *et al.*, 2007; Samanta *et al.*, 2008). We found that EBV increases mRNA levels of IRF5/IRF7 and TLR7/9, suggesting that EBV might signal through the TLR7/9-pathway in infected fibroblasts; however, further experiments are required to address this issue. Several studies showed that IRF5 and IRF7 are critical mediators of

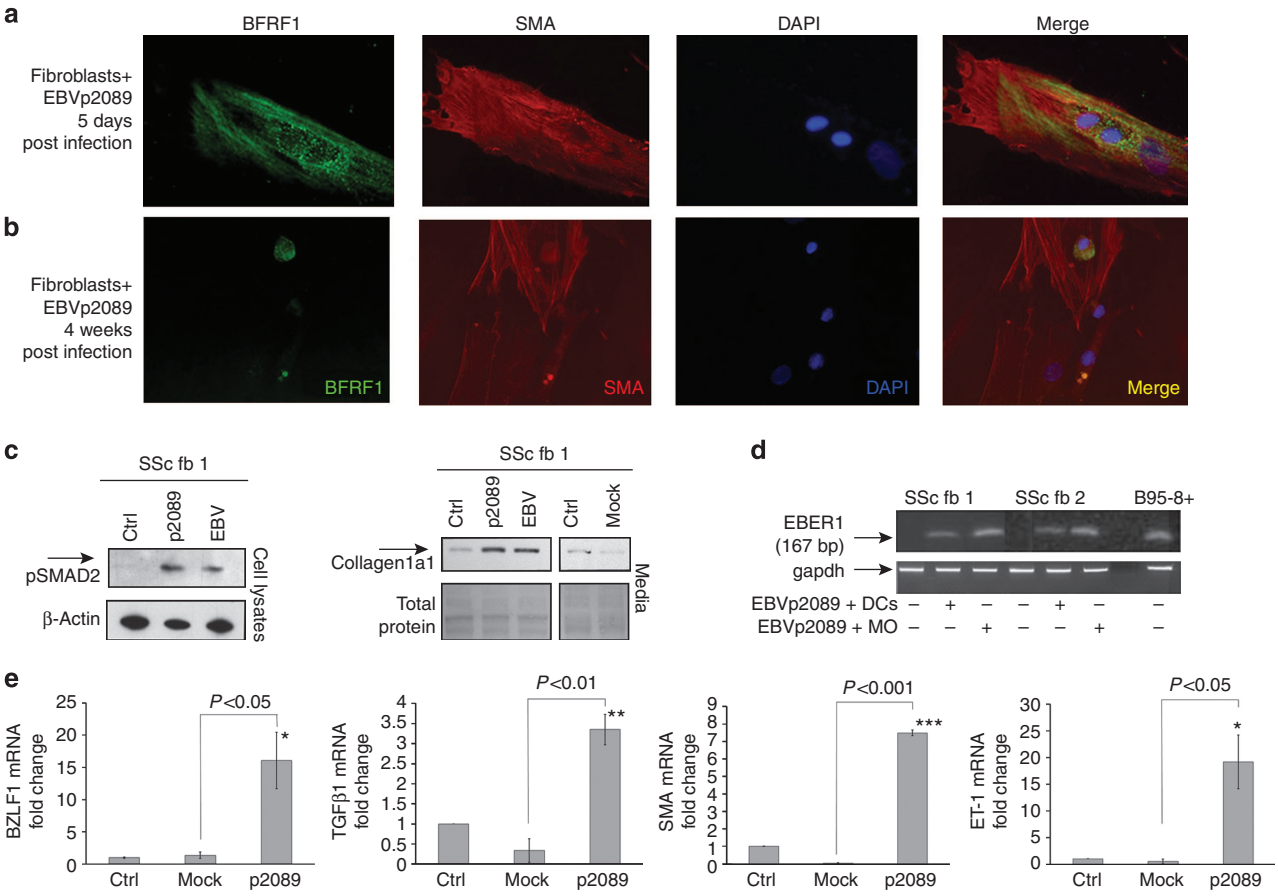

Figure 4. Epstein–Barr virus (EBV) induces myofibroblasts activation markers in infected scleroderma (SSc) fibroblasts. (**a, b**) Double immunofluorescence (IF) staining of adherent fibroblasts stained with EBV early/lytic -BFRF1 and α-smooth muscle actin (αSMA) antigen (red) as indicated. Diaminidino-2-phenylindole (DAPI) was used as counterstaining for the nuclei (scale bar = 50 μm). (**c**) Western blot was performed to determine pSMAD2 and type I collagen secretion in cell lysates and in the media of indicated fibroblast (fb) cultures at 4 weeks after infection. Total protein loading was determined by Ponceau-S staining. (**d**) Representative PCR products of EBV DNA in two SSc fibroblast cell lines infected with monocyte (MO)-EBV-p2089 and dendritic cell (DC)-EBV-p2089 at 4 weeks after infection; DNA from B95-8-EBV-positive cells was used as positive control. (**e**) mRNA expression of indicated genes in EBV-p2089-infected and in mock-infected fibroblasts at 4 weeks after infection evaluated by semi-quantitative PCR. TGFβ1, transforming growth factor-β1. Bars represent mean ± SEM from five separate SSc fibroblast lines. *P*-values were calculated using two-tailed *t*-test **P*<0.05; ***P*<0.01; ****P*<0.001.

TLR7/TLR9 signaling in response to single-stranded RNA and CpG DNA in several cell types including fibroblasts (Schoenemeyer *et al.*, 2005; Tamura *et al.*, 2008). Moreover, IRF5 and IRF7 seem to be equally required by the host and the virus, as both are crucial for the host in activating the IFN system and for the virus in regulating several EBV proteins necessary for viral B-cell transformation in infected cells (Ning *et al.*, 2003; Ning *et al.*, 2005; Paun and Pitha, 2007; Savitsky *et al.*, 2010; Barton *et al.*, 2011).

ISGs, TGFβ, and EBV

We found that activation of TLR signaling by EBV promotes fibroblast antiviral response that culminate with the induction of selected ISGs and TGFβ-regulated genes that have been found to be increased in PBMCs (tumor necrosis factor-α, CXCL9, OAS2) and in the skin of patients with SSc (CXCL9, OAS2, SMA, COMP, and EGR1) (Farina *et al.*, 2010b; Radstake *et al.*, 2010). In addition, we also found increased SMAD2 phosphorylation and collagen proteins in EBV-infected SSc fibroblasts, suggesting that EBV directly activates the TGFβ transduction pathway. As expected, we found that activation of TLR7/TLR9 by R837 and CpG-ODN ligands significantly induced ISGs in HD fibroblasts (MX2, IFI44, OAS2, CXCL9); nevertheless, none of these ligands induced TGFβ-regulated genes and collagen in HD fibroblasts, suggesting that TLR activation in the absence of chronic EBV infection is unable to stimulate a TGFβ response. Intriguingly, selected ISGs such as PKR, MX2, and TLR3, which are transcriptionally regulated by type I IFN, were not induced by the virus, suggesting that EBV upregulates a distinct ''IFN signature'' incompetent to fully stimulate the protective IFN response in infected SSc fibroblasts. It is well accepted that EBV is a poor IFN inducer permitting efficient lytic replication in B cells, although the likelihood that it might happen in fibroblasts is unexplored (Kikuta, 1986; Spender *et al.*, 2001). Specifically, EBV has evolved multiple strategies to evade the immune system specifically suppressing and/or blocking several pathways of the IFN response, mainly by inhibiting IRF7 activity (Elia *et al.*, 1996; Hahn *et al.*, 2005;

Langland *et al.*, 2006; Wang *et al.*, 2009; Wu *et al.*, 2009; Bentz *et al.*, 2010; Michaud *et al.*, 2010; Samanta and Takada, 2010; Wang *et al.*, 2011). Our data show that EBV induces expression of IRF4 in infected fibroblasts, suggesting that it might serve as a repressor of IFNα/β in fibroblasts as it is known in immune cells (Hrdlickova *et al.*, 2001; Negishi *et al.*, 2005; Paun and Pitha, 2007; Wang *et al.*, 2011).

EBV and fibroblast infection

Although fibroblasts are negative for the EBV receptor CD21, spontaneous EBV fibroblast infection was detected in a primary cell line from rheumatoid arthritis synovial tissue (Koide *et al.*, 1997). We did not detect EBV DNA in SSc fibroblast cell lines (Figure 4d). Here we report a system that successfully infects fibroblasts ''*in vitro*,'' providing evidence that EBV is able to infect human dermal fibroblasts using MOs or DCs as a vehicle for infection (Lindhout *et al.*, 1994; Savard *et al.*, 2000). It is likely that EBV uses alternative strategies to infect fibroblasts that bypass the absence of CD21, similar to the described transmission of EBV to human epithelial cells by cell-to-cell contact (Janz *et al.*, 2000). There is a growing interest in exosomes, the specialized membranous vesicles derived from the endocytic compartment that can carry and deliver functional mRNA, miRNAs, and proteins to various cells (Valadi *et al.*, 2007; Pegtel *et al.*, 2010; Zomer *et al.*, 2010). Thousands of EBV miRNA copy numbers have been detected in exosomes from lymphoblastoid cell line–infected cells, suggesting that EBV-containing exosomes may be continuously secreted and transferred from the infected cells to uninfected neighboring cells (Pegtel *et al.*, 2010; Wurdinger *et al.*, 2012). Possibly, EBV-infected immune cells might transfer functional EBV RNA and protein to fibroblasts through exosomes.

Our data show that EBV persists in fibroblasts exploiting both lytic and latent cycles. We did not detect any late viral product gp350/220 and/or mature virions in SSc biopsies, suggesting that EBV replication is not complete in infected SSc fibroblasts and possibly occurs in an abortive cycle, whereas viral DNA might be conserved in growing fibroblast cultures. Previous studies showed that the EBV lytic cycle is abortive in several EBV-associated diseases and in specific infected cells (Bibeau *et al.*, 1994; Martel-Renoir *et al.*, 1995; Al Tabaa *et al.*, 2009; Al Tabaa *et al.*, 2011). Perhaps, fibroblasts do not provide the necessary environment for productive infection and hence the virus is not able to perform the normal replication program.

EBV/p2089-recombinant and EBV/B95-8-cell-derived virus show similar cellular tropism in infecting LdSSc, NLdSSc, and HD fibroblasts ''*in vitro*,'' suggesting that EBV infection can occur in mesenchymal cells. Intriguingly, we noticed that LdSSc and to a lesser degree NLdSSc as well as HD fibroblast lines could all be infected by EBV; however, only fibroblasts from LdSSc and occasionally from NLdSSc skin were able to support sustained viral presence for up to 6 months. These results suggest that the LdSSc fibroblast phenotype might predispose to EBV chronic infection, promoting EBV survival in the cells, whereas fibroblasts from HDs appear to be able to control EBV infection, clearing the virus. Possibly, EBV infection spontaneously resolves in the context of presumably immunocompetent condition. In our case infected cells from HD might still have a competent immune system able to induce a full IFN response controlling the EBV infection. Characterization of ''EBV-IFN-signature'' deserves further investigation specifically in these cells.

EBV and SSc profibrotic phenotype

Activated fibroblasts are considered the principal mediators of fibrogenesis in SSc. It is known that SSc fibroblasts show a profibrotic phenotype with sustained TGFβ activation, increased collagen production, and increased number of myofibroblasts (Varga and Abraham, 2007). We found that the EBV lytic cascade is associated with upregulation of TGFβ1, several TGFβ-regulated genes, including SMA, and increased collagen by infected fibroblasts. Intriguingly, the EBV transactivator BZLF1 gene has been linked to development of fibrosis in some other EBV-associated diseases, although its primary role is to disrupt viral latency and transactivate the expression of early lytic genes (Grogan *et al.*, 1987; Guenther *et al.*, 2010). Specifically, BZLF1 was shown to interact with numerous key cellular transcriptional regulatory factors including TGFβ1/3 and EGR1 in infected epithelial cells (Cayrol and Flemington, 1995; Adamson and Kenney, 1999; Chang *et al.*, 2006; Tsai *et al.*, 2009). Thus, BZLF1 interaction with one of these factors might also be responsible for SMAD2 phosphorylation and TGFβ1 increased expression in EBV-infected SSc fibroblasts.

Previous studies have suggested a role for EBV infection as in the pathogenesis of smooth muscle tumors in patients with clinical immunosuppression. Specifically, EBER was found expressed in smooth muscle tumor cells and in myofibroblasts from sclerosing nodular transformation of the spleen, suggesting that EBV-infected myofibroblasts could be a common pathway of a fibrosclerotic process occurring in splenic inflammatory tumor-like lesion, although the origin of EBER+ myofibroblasts was not fully understood (Lee *et al.*, 1995; Weinreb *et al.*, 2007; Kashiwagi *et al.*, 2008). Our *in vitro* data showed that EBV induces a myofibroblast phenotype in infected SSc fibroblasts.

Our results show that SSc fibroblasts have greatly diminished IFN-inducible gene response upon TLR7/9 agonist stimulation, possibly one of the reasons that EBV is able to infect and persist in SSc fibroblasts. Further experiments are needed to understand whether EBV infection might induce epigenetic changes in infected fibroblasts, as it does in B cells and lymphoblastoid and epithelial cells, blunting the IFN response in ''transformed'' cells (Gregorovic *et al.*, 2011; Banerjee *et al.*, 2013; Queen *et al.*, 2013).

Overall, our study provides compelling evidence that EBV infection could contribute to fibrosis in SSc skin through multiple factors and a combination of subsequent pathological events such as virus host cellular interactions that may lead to aberrant activation of TLR pathway in infected SSc fibroblasts. This pathway activates selected ISGs and cytokines and influences fibroblast profibrotic phenotype driving

myofibroblast conversion. These results indicate that EBV infection might cause the patho-immunogenetic alterations seen in SSc fibroblasts, and those abnormalities are related to the viral strategies that subvert the host innate immune response in infected cells.

Concurrent EBV infection occurring in mesenchymal, endothelial, and immune cells of SSc patients may underlay the main pathological features of SSc including autoimmunity, vasculopathy, and fibrosis, and provide a unified disease mechanism represented by EBV reactivation.

MATERIALS AND METHODS

Study subjects

All study subjects met the criteria for SSc as defined previously (LeRoy *et al.*, 1988). The study was conducted under a protocol in adherence to the Declaration of Helsinki Principles and approved by the Boston University Medical Center, Institutional Review Board, and all subjects gave written informed consent. Skin biopsies were performed as previously described (Farina *et al.*, 2010a,b)

EBER "*in situ*" hybridization

In situ hybridization for EBV-encoded RNA (EBER) was carried out using FITC (FITC-l-labeled peptide nucleic acid probed for EBER and peptide nucleic acid *in situ* hybridization detection kit (DAKO, Carpinteria, CA)) on paraffin-embedded skin sections according to the manufacturer's protocol.

Virus preparation

EBV was obtained from producing-B95.8 cell line and from p2089 cell line as previously described (Delecluse *et al.*, 1998; Farina *et al.*, 2000).

Isolation of MOs and monocyte-derived DCs from HDs

PBMCs from 10 HDs were isolated by Ficoll Paque gradient centrifugation (Pharmacia, Uppsala, Sweden). CD14+/monocytes were positively selected using anti-CD14 mAb-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA) confirmed by flow cytometry (Cirone *et al.*, 2007). Each group of infection ($n=6$) was carried out using fibroblasts from SSc and HDs, and MOs/DCs from a single HD at the time. MOs/DCs isolated from different HDs were used to infect the same SSc fibroblast cell lines. CD14+/− and BDCA1+/− selection markers were also evaluated by semi-quantitative PCR.

Transfer infection

Donor cells (MOs and DCs) obtained from 10 HDs were exposed to EBV and/or p2089 at known multiplicities of infection for 3 hours at 4 °C; after extensive washing, 10^4/cells were added to confluent fibroblast culture in 8-well chamber slides (Feederle *et al.*, 2007). At 72 hours after co-cultivation with human primary fibroblast, supernatants (MOs/DCs) were removed and cultured separately from the infected fibroblast. After 2 days, total RNA was extracted from MOs and DCs. Transfer infection in fibroblasts cultures was assayed 72 hours after the initiation of co-culture by counting the percentage of green fluorescent protein–positive cells in trypsinized acceptor cell suspensions. The p2089-infected, mock-infected, and control fibroblasts were cultured in DMEM (10% fetal calf serum) and monitored by indirect immunofluorescence assay or PCR up to 6 months after infection. Total RNA was extracted from p2089-infected-, mock-infected, and control SSc fibroblasts at 4 weeks after infection.

Statistical analyses

All data are expressed as mean ± SE. The means between two groups were analyzed by Student's *t*-test, Wilcoxon test, and Fisher's exact test. Significance was taken at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Author contributions

AF and GAF designed experiments; AF, GAF, MC, MY, SL, CP, and SM performed experiments; GAF, AFag, and RL provided reagents; AF, AFag, MT, RL, and GAF prepared the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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