Supplementary Materials

Supplemental Methods

Nucleic Acid Extraction.

DNA was extracted from skin using the Qiagen Extraction Kit (Qiagen, Valencia, CA) according to the manufacturer`s protocol. DNA was detected using PCR as previously described (Iwakiri *et al.*, 2009). EBV viral load was quantified using StepOnePlus Sequence Detector (Applied-biosystems, Life Technologies, Grand Island, NY). The standard curve was created automatically with AB program using EBV-B95-8 quantitated DNA PCR control (Advanced Biotechonologies INC) (Jebbink *et al.*, 2003). Different sets of EBER1 primers were used for PCR (Iwakiri *et al.*, 2009) and qPCR (Strowig *et al.*, 2008).

Fibroblast culture

Primary human dermal fibroblast explant cultures from diffuse SSc lesional (LdSSc) and non lesional (NLdSSc) and healthy donors (HDs) were established as described previously (Jelaska *et al.*, 1996). LdSSc and HD fibroblasts were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin, utilized at passage 3–6. Fibroblasts were incubated in serum-free DMEM for 24h prior to the addition of TLR agonists: R837 imiquimod, sspolyU/ (1mg/ml) (Invitrogen, Grand Island, NY), CpG-ODN-2006 (5μM) (Invitrogen, Grand Island, NY), TGFβ (5ng/ml) (R&D, Minneapolis, MN).

RNA preparation and real-time polymerase chain reaction (q-PCR).

Human tissue and fibroblasts were processed as describe before (Farina *et al.*, 2010b). The synthesized cDNAs were used as templates for quantitative real-time PCR and primed used as described before (Farina *et al.*, 2010a; Farina *et al.*, 2010b). All real

time-PCR was carried out using StepOnePlus Sequence Detector (Applied Biosystems, Life Technologies, Grand Island, NY) and TaqMan primers and probes were purchased from AppliedBiosystem (Life Technologies, Grand Island, NY) and used as recommended by the supplier. The change in the relative expression of each gene was calculated using $\Delta\Delta$Ct formula choosing a healthy human subject (Livak and Schmittgen, 2001). Target and control reactions were run on separate wells of the same q-PCR plate (Farina *et al.*, 2010a).

Nested RT-PCR.

Human Skin and PBMCs were processed as described before (Farina *et al.*, 2010a; Pendergrass *et al.*, 2010). The synthesized cDNAs were used as templates for nested RT- PCR. BZLF1 and EBNA-1 were used at condition as described before (Gonnella *et al.*, 1997).

Primers.

Primers used to detect viral lytic and latent genes were designed using Primer Express software (Applied Biosystems, Life Technologies, Grand Island, NY) and synthesized by Integrated DNA Technologies. Expression of mRNA for BZLF1, LMP1, and BFLLF1 was detected using SYBRGreen chemistry and TaqMan endogenous control amplification (Applied Biosystems, Life technologies, Grand Island, NY). To assure the specificity of primer set, amplicons generated from PCR reaction were analyzed for specific melting temperatures by using the melting curve software. Primers sets sequences are summarized in Table S8.

Protein extraction

Proteins were extracted from SSc and HD skin biopsies using Trizol methods described by the manufacturer (Invitrogen, Grand Island, NY), and suspended in 1% SDS. Samples (30µg) were heat denatured with reducing agent and loaded onto 12% SDS-PAGE gels.

Western Blot analysis.

Cellular extracts were prepared by sonication of cells in 10 % NU-PAGE (Invitrogen, Grand Island, NY), in reducing condition. Blotted protein were probed with each primary monoclonal antibody respectively for EBNA2 (DAKO, Carpinteria, CA), BFLF2 (Gonnella *et al.*, 2005) BFRF1 (Farina *et al.*, 2005; Farina *et al.*, 2000), Rta (Argene, bioMérieux, Inc. Durham, NC), Zta (Argene, bioMérieux, Inc. Durham, NC), a-collagen1a1-antibody (SouthernBiotech, Birmingham, Alabama), anti-b-actin-antibody (Sigma, St. Louis, MO), or polyclonal antibodies for BFRF1 (Farina *et al.*, 2005), Rta (kindly provided by Prof. G.Miller), and then probed with secondary antibody and visualized using super signal chemiluminescence kit (Thermo Scientific, Pittsburg, PA).

Immunohistochemisrty.

Tissue sections were deparaffinised as described before using primary antibodies described above in western-blotting and immunocytostaining (Farina *et al.*, 2009). Hematoxylin (Thermo Scientific, Pittsburg, PA) was used to counterstain the cells.

Immunocytochemistry.

Infected cell were washed with PBS, trypsinized and spotted on to coverslip as previously described (Farina *et al.*, 2004; Farina *et al.*, 2000; Gonnella *et al.*, 2005). Slides were stained with mouse monoclonal antibodies against EBNA2 (PE2), BZLF1 (Argene), BFLF2(Gonnella *et al.*, 2005), CD20 (DAKO, Carpinteria, CA), CD21 (DAKO, Carpinteria, CA), BDCA-1 (MiltenyiBiotec, Auburn,

CA), BFRF1 (Farina *et al.*, 2005), polyclonal antibodies against Collagen1 (SouthernBiotech, Birmingham, Alabama), and BFRF1-R319 (Farina *et al.*, 2000) and secondary-antibodies-Cy3-conjugated (Jackson IR, West Grove, PA), Alexafluor-350 goat-anti mouse antibody (Invitrogen, Grand Island, NY), or 488-labeling (Zenon kit; Invitrogen, Grand Island, NY). Cell nuclei were counterstained with DAPI (Sigma, St. Louis, MO).

EBV-VCA-IgG/EBNA1-IgG bio-assay quantification. EBV- EBNA1-IgG ELISA was conducted in sera from 34 HDs and 78 SSc patients according to the supplied protocol (NovaGnost, Siemens Victoria, Australia); EBV-VCA-IgG ELISA ELISA was conducted in sera from 34 HDs and 78 SSc patients according to the supplied protocol (Novagnost SIEMENS, Victoria, Australia).

Supplementary Figures

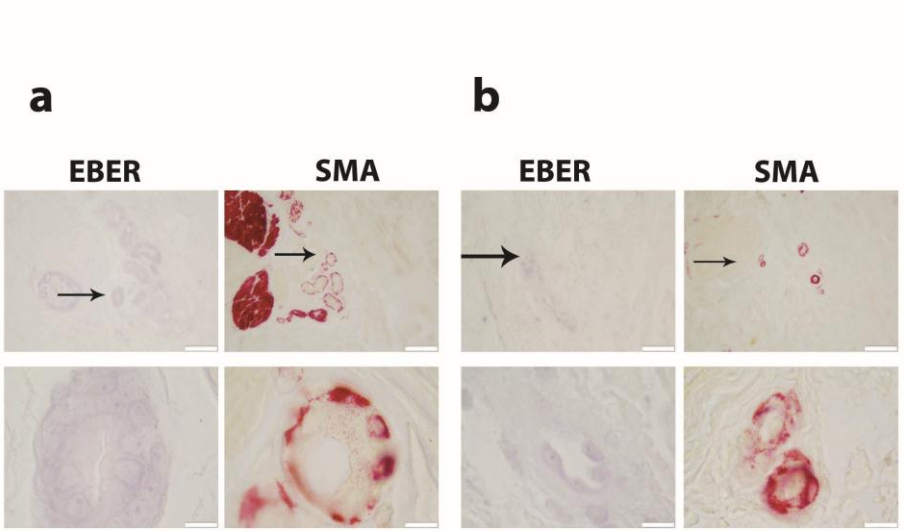

Fig. S1. **EBER probe in the skin of healthy donors**.
(a and b) Representative images of EBER in situ hybridization (ISH) in the skin from 2 healthy donors (HDs) (left panels); α-smooth muscle actin (αSMA) staining by IHC in serial sections from skin of 2 HDs (right-panels) (scale/bars upper-panels, 100µm, lower-panel 50µm).

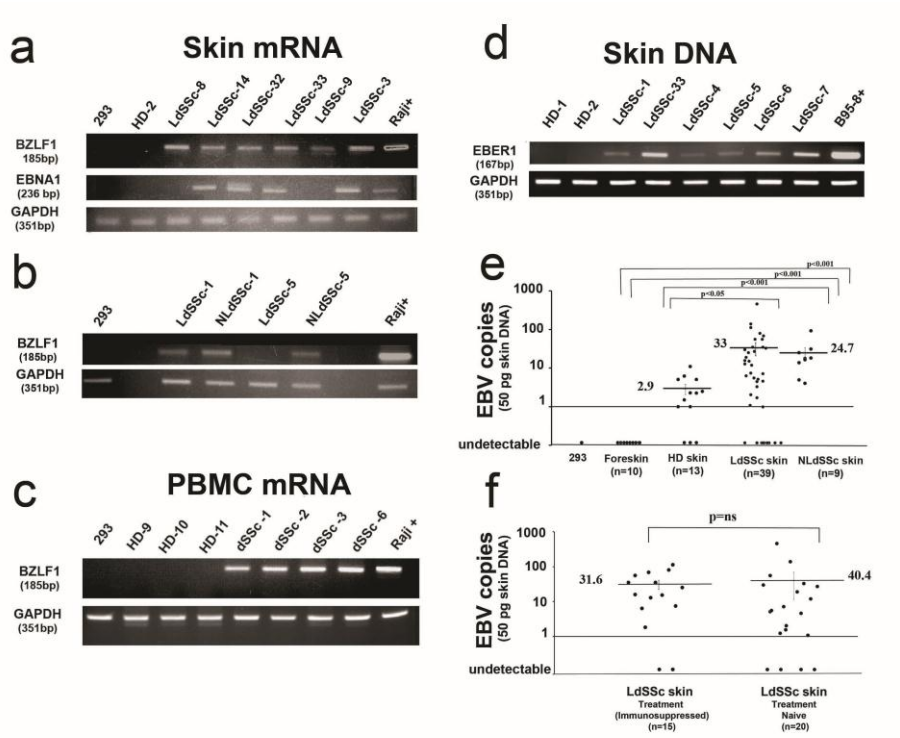

Fig. S2. **Expression of EBV transcripts in skin and PBMCs of SSc patients.**

(a) Representative gel electrophoresis of EBV-lytic/latency genes (BZLF1/EBNA1) RT-PCR products from 6 lesional diffuse SSc (LdSSc) and one representative normal skin (HD); 293 and Raji cells were use as negative and positive control respectively. (b) RT-PCR products of EBV-lytic-gene BZLF1 in (LdSSc) and non/lesional (NLdSSc) from 2 representative patients. (c) RT-PCR products of EBV-lytic-gene BZLF1 in PBMCs from dSSc and 2 representative HDs subjects. (d) PCR products of EBV DNA (EBER1) in LdSSc and in 2 HDs representative skin; DNA from B95-8-EBV-positive cells were used as positive control, GAPDH used as internal control; incidence of EBV DNA and BZLF1/EBNA1 transcripts in screened skin section are summarized in Supplemental Table 2. (e-f) Detection of EBV-load by q-PCR; each sample was tested in duplicates and normalized by endogenous internal control. Shown here are copies of viral nucleic acid in the skin calculated by standard curve. 293 cells and Foreskin dermal skin were used as negative control. The average of copies number is represented by horizontal line ± SE. p-values calculated using Wilcoxon two samples test.

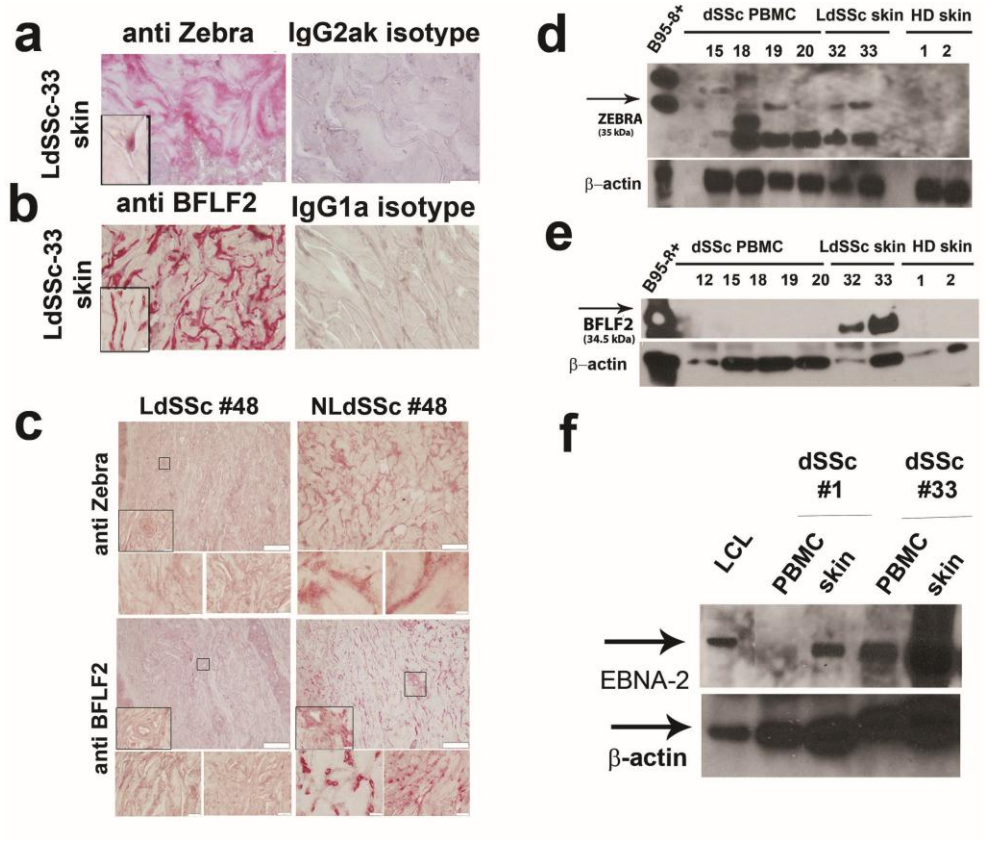

Fig. S3. **Expression of EBV proteins in the skin of SSc patients.**

(a-c) Immuno-histochemistry (IHC) in serial tissue sections from lesional (LdSSc) and non-lesional (NLdSSc) skin samples. Nuclear localization of EBV-lytic-protein/Zebra in scattered fibroblasts (square insert) and in the matrix, and expression of the early lytic BFLF2 protein in LdSSc deep dermis obtained from the same patient (a-b). Expression of Zebra and early-lytic-BFLF2 proteins in LdSSc and NLdSSc skin from the same patient (c). Crude lysate from skin and PBMCs of representative SSc patients and HDs (d and e), skin and PBMCs samples obtained from the same patients (f), were separated on SDS-PAGE, blotted on PVDF and probed with the indicated antibodies; lysate from B95-8 EBV infected cells were used as positive control; β-actin was used as loading control. Numbers represent distinct patients enrolled in the study. (red-staining bar scale 100µm (upper-panels) and 10µm (lower and squares panels).

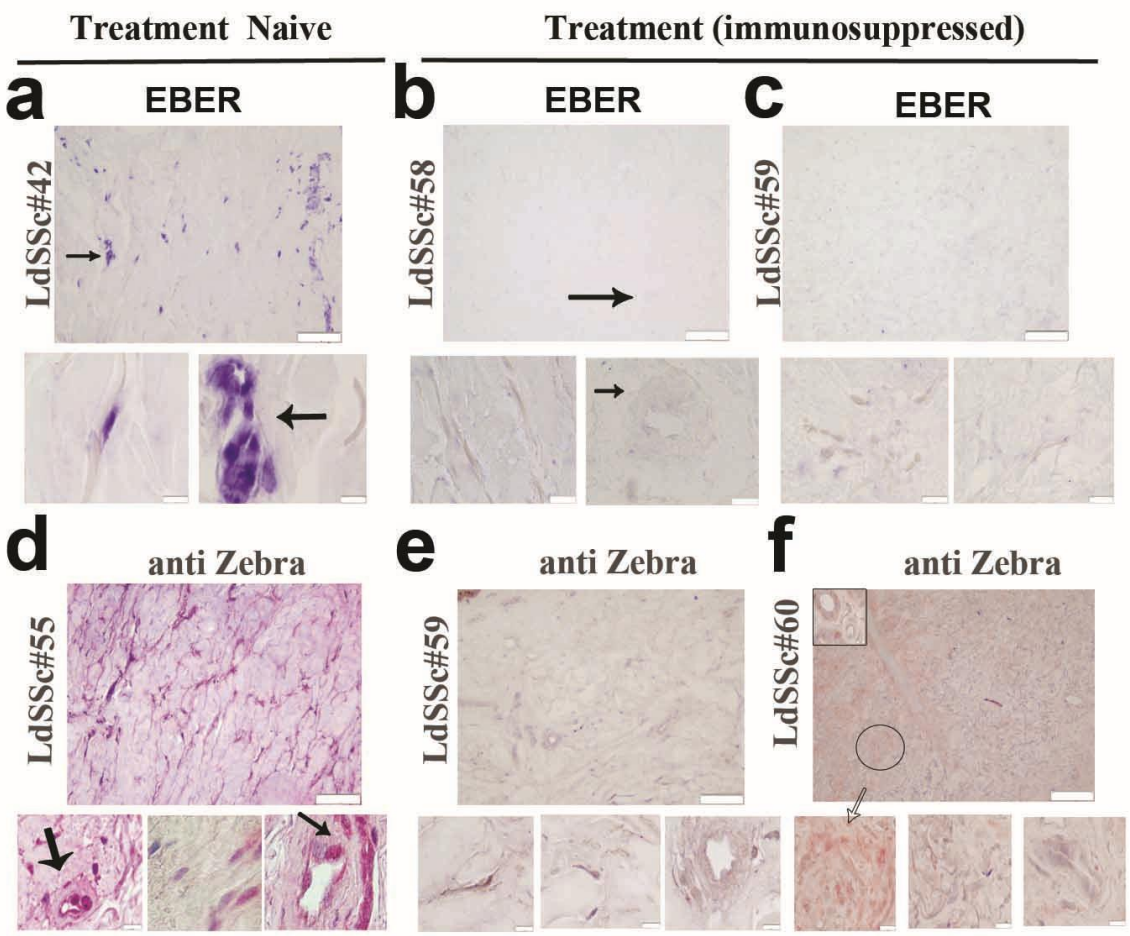

Fig. S4. **EBV RNAs and antigens in the skin of SSc patients**.
(a-c) Representative images of EBERs in situ hybridization (ISH), and (d-f) immuno-histochemistry (IHC) of EBV-lytic-protein Zebra in serial tissue sections from lesional skin (LdSSc) of two SSc cohort patients naïve vs immunosuppressed treatment (arrows indicate vessels positive or negative for EBERs staining). Numbers on the side represent distinct patient enrolled in the study, whose clinical characteristics are summarized in Table S5. Bar scale 100µm (upper panels) and 10µm (lower and squares panels)

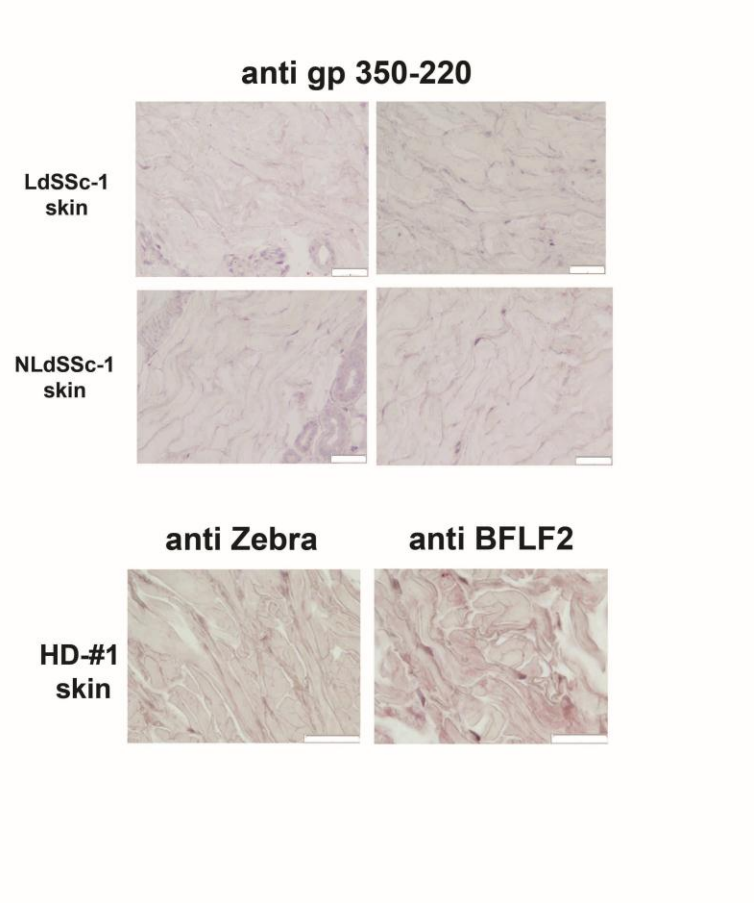

Fig. S5. **EBV antigens expression in the skin.**
Immuno-histochemistry (IHC) in serial skin tissue sections from lesional (LdSSc), non-lesional (NLdSSc) and healthy-donor (HD) skin sample of indicated EBV-proteins (red-staining bar scale 50µm). Numbers on the side represent distinct patient enrolled in the study.

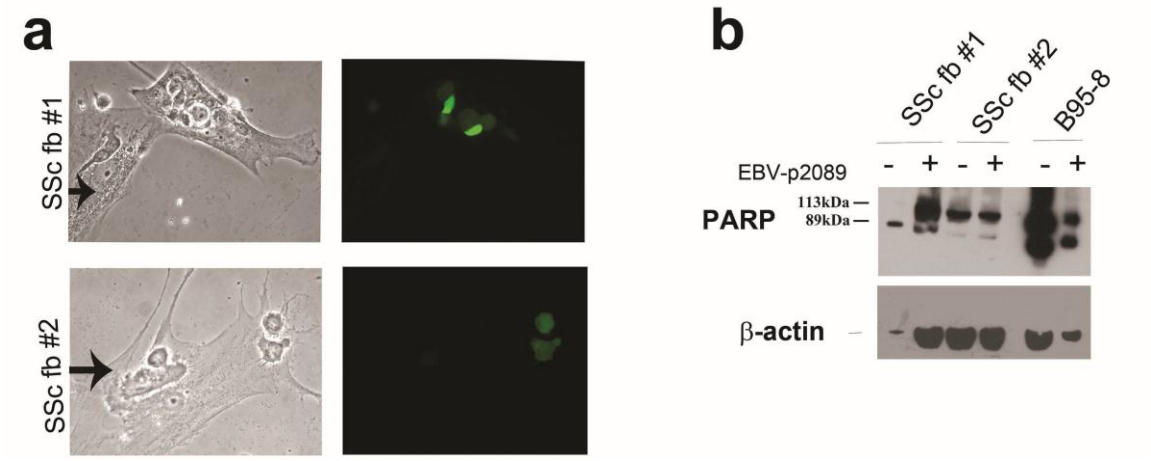

Fig. S6. **EBV-p2089-recombinant-virus infects human SSc-fibroblasts "*in vitro*"**.
(a) Inverted microscope image of 2 EBV-p2089-infected-SSc-fibroblast 4 week/post infection (PI). GFP expression indicates recombinant ebv- infected cells. (left panel: phase-contrast-light-microcopy; bar scale 20 mm). (b) Western-blot analysis of Poly (ADP-ribose) polymerase (PARP) protein in cell lysates from EBV-p2089 and mock-infected-SSc-fibroblast-cultures at 4-week-PI. B95-8 EBV-activated was used as positive control.

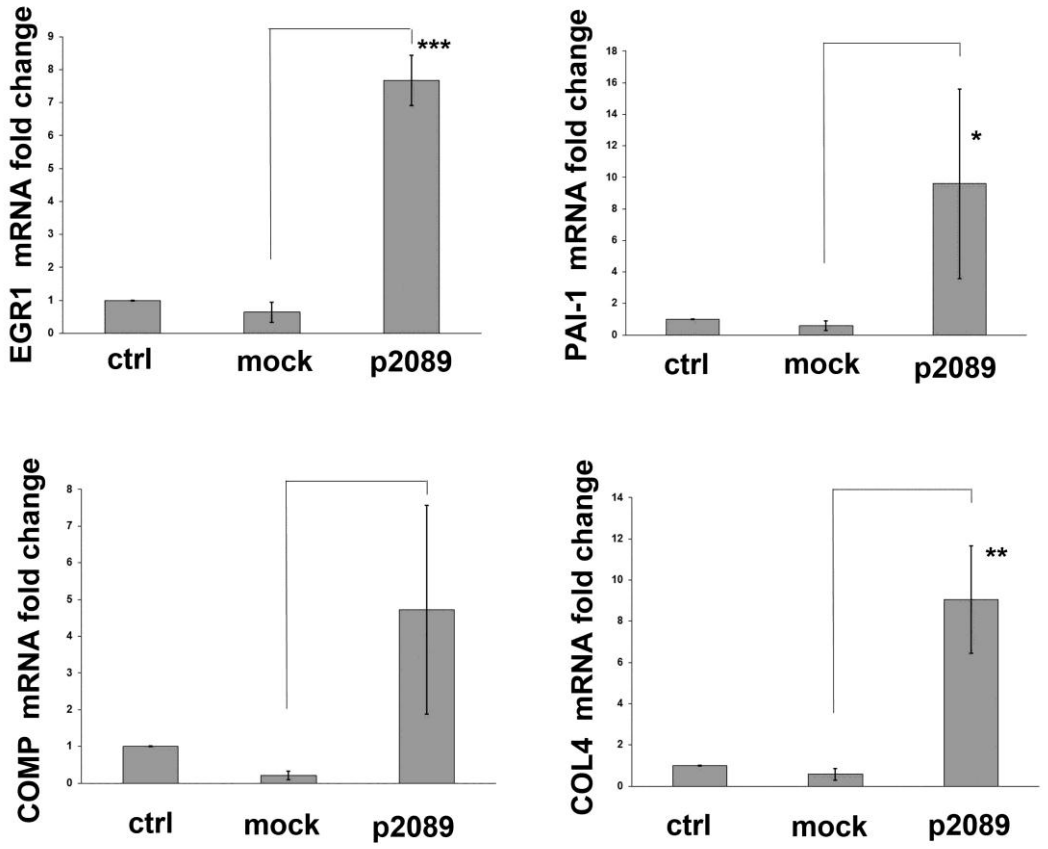

Fig. S7. **Expression of TGFβ-responsive genes in fibroblasts infected with EBV.**
mRNA expression of indicated genes in EBVp2089-infected, mock-infected and control fibroblasts from SSc patients after 4/week post infection, evaluated by qPCR. Fold-changes shown on the graph are normalized to mRNA expression by each corresponding untreated cell lines. Bars represent mean ± S.E.M. from 3 separate experiments from different SSc-fibroblast-cell-lines. p-values calculated using two-tailed T-test. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$

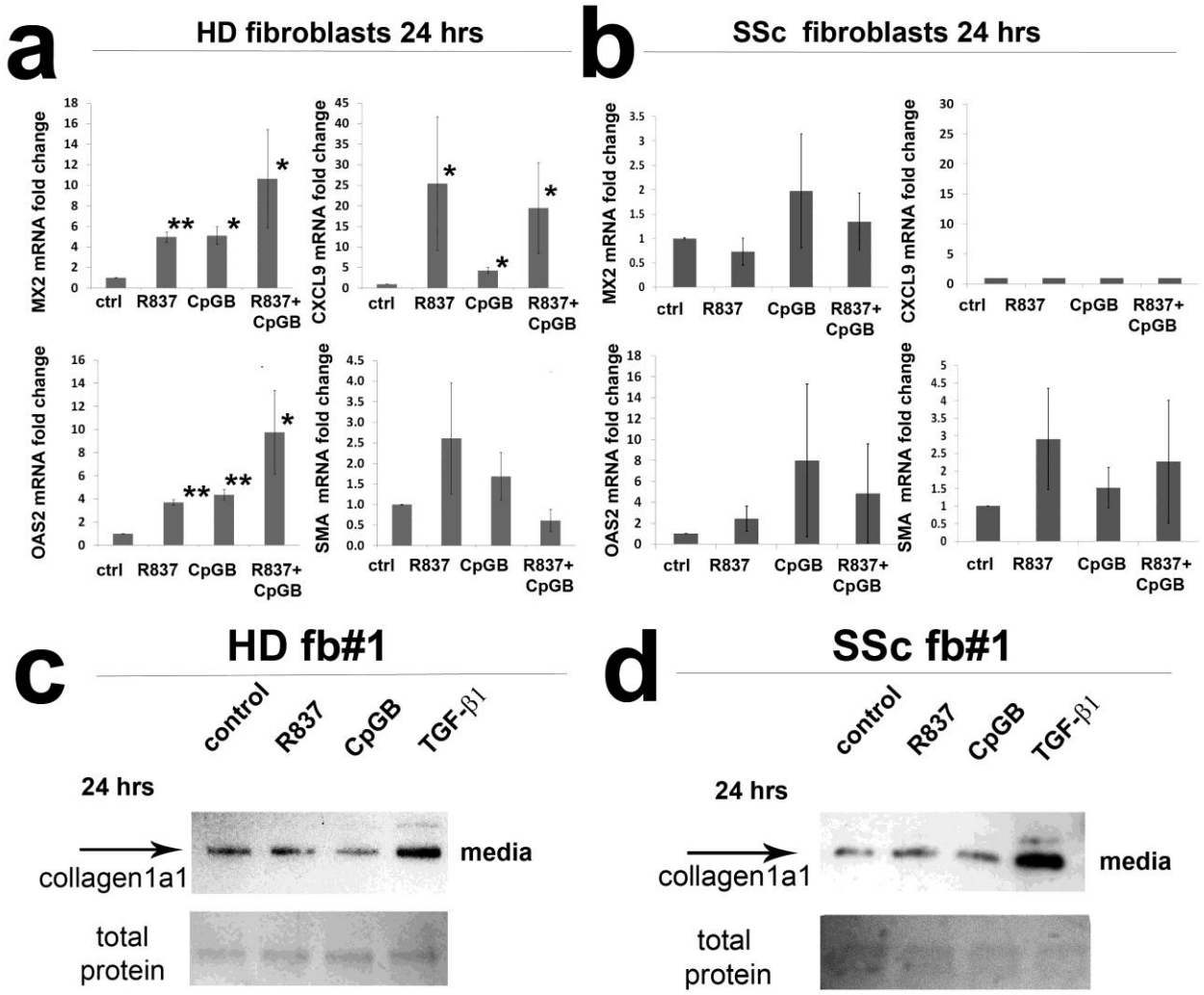

Fig. S8. **Expression of Interferon-stimulated-genes (ISGs) in human dermal fibroblasts by TLRs stimulation.**
Fibroblasts explanted from healthy donors (HDs) (a) and from lesional skin of patients with dSSc (b), were starved o/n and incubated with TLR-agonist-ligands as indicated for 24hrs. mRNA was harvested and analyzed by qPCR. Fold-changes shown on the graph are normalized to mRNA expression by each corresponding untreated cell lines. Bars represent mean ± S.E.M. from 3 separate experiments using 3 different cell lines. p-values calculated using two-tailed T-test. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$. (c-d) Western blot analysis was performed to determine type I collagen secretion in the media of indicated fibroblasts cultures after indicated treatment. Total protein loading was determined by Ponceau-S staining of the filter after western transfer (bottom panel).

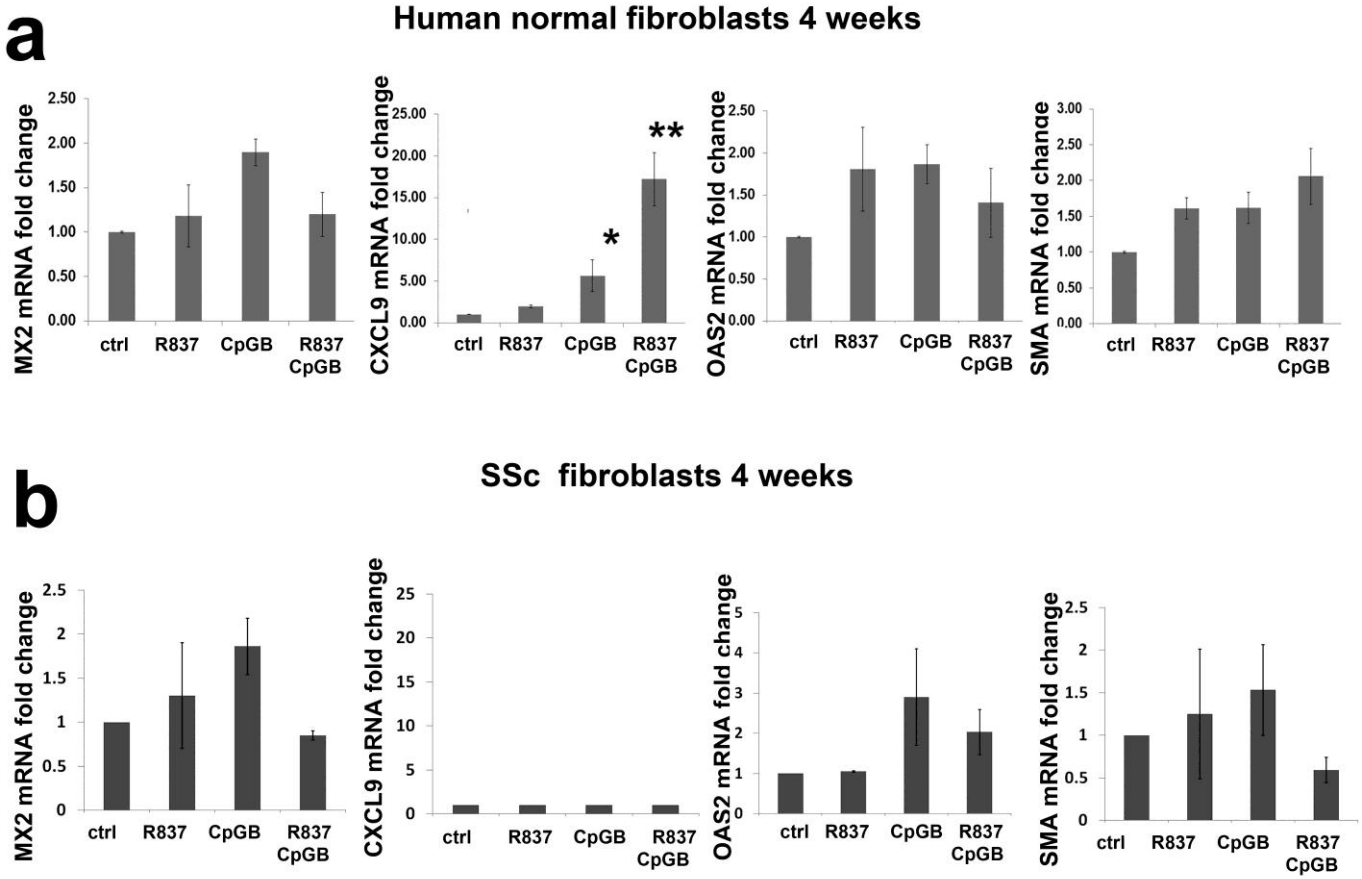

Fig. S9.**Expression of Interferon-stimulated-genes (ISGs) in human dermal fibroblasts by TLRs chronic stimulation.** Fibroblasts explanted from healthy donors (HDs) (a) and from lesional skin of patients with dSSc (b), were incubated with TLR-agonist-ligands as indicated and treated for 3 times/week for 4 weeks in presence of FBS 10%. mRNA was harvested and analyzed by qPCR. Fold-changes shown on the graph are normalized to mRNA expression by each corresponding untreated cell lines. Bars represent mean ± S.E.M. from 3 separate experiments using 3 different cell lines. p-values calculated using two-tailed T-test. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$.

Table S1. Demographics and Clinical characteristics of SSc patients and Healthy Donor (HD) subjects

		SSc	**HD**
Subjects (n)		89	36
Age mean ± SE (years)		47.9 ± 2.1	42 ± 2.8
Sex (F/M)		72/17	27/9
Race %	**Caucasian** **African-American** **others**	86.6 6.7 6.6	93.4 - 6.6
diffuse SSc (dSSc) (n)		80	-
limited SSc (lSSc) (n)		9	-
EBV seropositive (anti-EBNA1)		100% (78/78)	100% (34/34)

Table S2. Detection of Epstein-Barr virus genomes and their products in PBMCs and skin tissues of patients with SSc and HD subjects

	PBMC RT-PCR	**Skin** RT-PCR		RNA in situ	**DNA**[1]
	BZLF1	**BZLF1**	**EBNA1**	**EBER**	**EBER1**
dSSc (n=80)	10/21 (47%)	22/59 (37%)			
LdSSc (n=50)	-	17/50 (34%)	23/64 (36%)	11/23 (47%)	20/22 (90%)
NLdSSc (n=9)	-	5/9 (55%)	3/9 (33%)	2/4 (50%)	3/3 (100%)
lSSc (n=9)	-	1/7 (14%)	3/9 (33%)	2/5 (40%)	6/6 (100%)
HD (n=36)	2/20 (10%)	0/16 -	0/16 -	0/15 -	2/7 (26%)
p value LdSSc vs HD	p<0.01	p<0.05	p<0.05	p<0.0001	p<0.05

[1]: DNA detected by PCR

Table S3: serological profile to EBV in patients with diffuse (dSSc) and limited (lSSc) SSc disease.

	Age	d.d. (years)	ANA (auto-ab)	IgG-VCA* ELISA	Immunosuppressant Therapy
dSSc#67	35			300	none
dSSc#68	26	3	n/a**	243	mycophenolate
dSSc#69	36	n/a	n/a	282	none
dSSc#70	67	n/a	n/a	261	none
dSSc#71	52	4	Scl-70	181	gleevec
dSSc#72	71	4	n/a	175	none
dSSc#73	61	6	n/a	175	methrotrexate
dSSc#74	29	n/a	n/a	170	none
dSSc#75	55	n/a	Scl-70	158	none
dSSc#76	53	5	+	163	prednisone
dSSc#77	38	10	+	113	cyclophosphamide
dSSc#78	67	6	+	44	cyclophosphamide
dSSc#79	53	4	n/a	12	gleevec
dSSc#80	42	8	Scl-70	12	prednisone
dSSc#81	54	7	Scl-70	11	none
dSSc#82	55	7	Scl-70	9	none
lSSc#19	66	12	+	241	none
lSSc#20	71	8	+	191	none
lSSc#21	84	15	+	66	none
lSSc#22	63	6	n/a	57	methrotrexate
lSSc#23	63	5	n/a	48	mycophenolate
lSSc#24	45	10	n/a	41	prednisone
lSSc#25	48	n/a	+	28	prednisone
lSSc#26	41	7	n/a	8	none
lSSc#27	67	4	+	6	none

Anti-VCA* = antibody to the viral capsid antigen, positive >1.1 U/mL; negative < 0.37**;**

n/a=** not available

Table S4. Demographics and clinic characteristics of two cohorts of SSc patients Treatment Naïve vs Treatment

	Age	EBER "*in situ*" RNA	d.d. (mo)*	MRSS	Medications
LdSSc #42	64	**+++**	5	31	naive
LdSSc #43	62	**++**	36	12	naive
LdSSc #44	37	**+++**	9	21	naive
LdSSc #45	39	**++++**	192	29	naive
LdSSc #46	61	**++**	12	26	naive
LdSSc #47	53	**+++**	8	42	naive
LdSSc #48	61	**++**	12	26	naive
LdSSc #49	42	neg	24	27	naive
LdSSc #50	65	neg	9	50	naive
LdSSc #51	62	neg	252	20	naive
LdSSc #52	53	neg	18	42	naive
LdSSc #53	61	**++**	14	26	naive
LdSSc #54	58	**++**	18	31	naive
LdSSc #55	41	**+**	6	30	naive
LdSSc #56	57	**+++**	3	42	naive
LdSSc #57	53	neg	38	28	naive
LdSSc #58	55	neg	30	5	mycophenolate
LdSSc #59	47	neg	50	2	mycophenolate
LdSSc #60	38	neg	21	24	methotrexate
LdSSc #61	54	**+**	44	27	mycophenolate
LdSSc #62	49	**++**	6	10	cellcept
LdSSc #63	53	**++**	53	31	cytoxan
LdSSc #64	53	**+++**	18	23	cytoxan
LdSSc #65	51	**+++**	35	16	prednisone
LdSSc #66	54	neg	55	31	Ab-interferon type I

LdSSc = Lesional diffuse SSc
***d.d. mo=** duration of disease in months; MRSS: Modified Rodnan skin score

Table S5. Comparison of EBER “in situ” RNA expression in SSc patients Treatment-Naïve vs Treatment-Immunosuppressed

	Treatment -naïve (TN)	**Treatment-immunosuppressed (T)**
LdSSc (n total)	**16**	**9**
EBERs positive (n) (%)	**11 (68%)**	**5 (55%)**
EBERs negative (n) (%)	**5 (32%)**	**4 (44%)**
TN vs T (Fisher exact test)	**P=0.6**	

Table S6. (part I): EBV expression pattern in lesional skin (LdSSc) and auto-antibodies profile in patients with SSc diffuse disease.

LdSSc	**Lytic/ gene**	**Latency/genes**		**Lytic/proteins**	**auto-antibodies**
	Zta	**EBERs**	**EBNA1**	**(Zebra/Rta/ BFLF2)**	
#1	+	+	+	+	
#3	+	+	+		
#4		+			
#5	negative	negative		+	negative
#6	+		negative	+	Scl-70
#7	+		negative		positive
#8	+		negative	+	Scl-70
#9	+		negative	negative	positive
#10	negative		negative	negative	Scl-70
#11	negative		negative		positive
#12	+		negative	+	Scl-70
#13			+		positive
#14	+		+	+	Scl-70
#15	negative		negative	negative	
#17	negative		negative		positive
#18	negative		negative		Scl-70
#19			negative		negative
#20			negative		negative
#21		negative	negative		Scl-70
#22		+	+	+	negative
#23		+	negative		
#24			+		
#25		negative	+		positive
#26	negative			+	positive
#28					positive
#29	+		negative		positive
#30	negative		negative		negative
#32	+		+	+	Scl-70
#33	+	+	+	+	negative

Table S6. (part II): EBV expression pattern in lesional skin (lSSc) and auto-antibodies profile in patients with limited SSc disease.

lSSc	**Lytic/gene**	**Latency/genes**		**Lytic-proteins**	**auto-antibodies**
	Zta	**EBER**	**EBNA1**	**(Zebra/Rta/ BFLF2)**	
#27	negative		negative		
#31	negative		+		+
#34	+		neg	+	ACA
#36	negative		neg	+	negative
#37	negative	+	neg	+	+
#38		negative	neg	negative	+
#39	negative	negative	neg	+	+
#40		+	+	+	+
#41		negative	+		+

Table S7. Detection of Epstein-Barr virus products in PBMCs and skin tissues of patients with SSc and HDs

	Zebra*	RTA*	BFRF1*	BFLF2*	EBNA2*
LdSSc skin (n=50)	11/50 (22%)	10/50 (20%)	13/50 (26%)	4/6 (66%)	24/50 (48%)
HD skin (n=10)	0/10	1/20 (3.3%)	1/20 (3.3%)	0/4	1/20 (3.3%)
SSc PBMC (n=21)	11/21 (52%)	11/21 (52%)	11/21 (52%)	6/21 (28%)	10/21 (47.6%)
HD PBMC (n=20)	3/20 (15%)	1/20 (5%)-	1/20 (5%)		1/20 (5%)

*= evaluated by Western-blot

Table S8. Real-time PCR primers used for detection of viral cDNAs.

GENE	Forward (5'-3')	Reverse (5'-3')
qPCR		
BZLF1	-TCGCATTCCTCCAGCGAT-	-AACCTGGAGACAATTCTACTGTTCAA-
LMP1	-CAGTCAGGCAAGCCTATGA-	-CTGGTTCCGGTGGAGATGA-
BLLF1	-CATTGGTAGCCGTTCGTGTGATAAT-	-GCGAGCAATCGGACATTTGACAT-

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