Effects of Parvovirus B19 In Vitro Infection on Monocytes from Patients with Systemic Sclerosis: Enhanced Inflammatory Pathways by Caspase-1 Activation and Cytokine Production

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Parvovirus B19 (B19V) has been proposed as a triggering agent for some autoimmune diseases including systemic sclerosis (SSc). In this study, we investigated whether B19V infection in vitro differently activates inflammatory pathways, including those dependent on caspase-1 activation, in monocytes from patients with SSc and healthy controls. We showed that B19V can infect both THP-1 cells and primary monocytes but is not able to replicate in these cells. B19V infection increases the production of tumor necrosis factor-α and induces NLRP3-mediated caspase-1 activation in both THP-1 cells differentiated with phorbol 12-myristate 13-acetate and in monocytes from patients with SSc but not from healthy controls. B19V infection was sufficient for THP-1 to produce mature IL-1β. Monocytes from patients with SSc required an additional stimulus, here represented by lipopolysaccharides, to activate cytokine genes. Following B19V infection, however, lipopolysaccharide-activated monocytes from patients with SSc strongly increased the production of IL-1β and tumor necrosis factor-α. Altogether, these data suggest that viral components might potentiate the response to endogenous and/or exogenous toll-like receptor 4 ligands in monocytes from patients with SSc. The B19V-mediated activation of inflammatory pathways in monocytes might contribute to the disease progression and/or development of specific clinical phenotypes.

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INTRODUCTION

Systemic sclerosis (SSc) is a complex autoimmune disease characterized by vascular and immunological abnormalities with progressive fibrosis involving the skin and internal organs. The main hallmarks of this disease are injury to the endothelial cells, overproduction of extracellular matrix proteins, and aberrant activation of both immune and nonimmune effector cells (Le Roy, 1992). Innate immune response plays a relevant role in the pathogenesis of SSc (O’Reilly, 2014).

Higher levels of cytokines and molecules involved in inflammation and fibrosis are produced by circulating mononuclear cells from patients with scleroderma compared with healthy subjects (Dantas et al., 2018; Duan et al., 2008). In addition, recent evidence has placed the chronic activation of the inflammasome and the production of the “inflammasome proteins” as central drivers of fibrosis (Artlett et al., 2011). Although the nature of triggers inducing the activation of innate immunity in SSc remains unclear, environmental factors, including infectious agents, seem to have a crucial role in the onset and/or progression of disease. The “infection hypothesis” has been studied extensively and a possible role of some viruses, such as cytomegalovirus (Arcangeletti et al., 2018; Lunardi et al., 2006), Epstein-Barr virus (EBV) (Farina et al., 2014, 2017), and parvovirus B19 (B19V) (Ferri et al., 1999; Ohtsuka and Yamazaki, 2004) has been suggested in the pathogenesis of SSc.

B19V is a small nonenveloped single-stranded DNA virus with an icosahedral capsid composed of two structural proteins, VP1 and VP2, differing from each other for a sequence of 227 amino acids at the amino-terminal of the VP1 protein, called the VP1-unique region (Agbandje et al., 1994). The major nonstructural viral protein, NS1, is involved in viral replication and in the pathogenesis of some B19V-associated diseases (Kerr, 2016).

B19V infection is associated with a wide range of clinical manifestations, including erythema infectiosum, arthralgia and arthritis, transient aplastic crisis, and chronic bone marrow failure (Young and Brown, 2004). After primary infection, B19V DNA persists lifelong in several tissues, mainly and in particular in bone marrow, heart, liver,
synovia, and skin (Norja et al., 2006). The functional consequences of such persistence are still unknown. A pathogenic role of chronic B19V infection in some autoimmune diseases has been suggested by the presence of typical clinical features and/or autoantibodies in patients with an ongoing B19V infection. Consequently, it was supposed that the persistence of the virus might be responsible for the induction of autoimmunity, mainly through a molecular mimicry mechanism (Lunardi et al., 1998).

The hypothesis that B19V can contribute to the onset and/or evolution of SSc is further supported by a number of studies reporting that B19V DNA persists more frequently in the bone marrow and skin of patients with SSc compared with control subjects (Ferri et al., 1999; Ohtsuka and Yamazaki, 2004) and that anti-B19V NS1 antibodies, markers of persistent infection, are more frequently detected in patients with SSc compared with controls (Ferri et al., 1999). Moreover, the presence of mRNA for B19V and for tumor necrosis factor-α (TNF-α) was demonstrated in endothelia, fibroblasts, and perivascular inflammatory cells (Magro et al., 2004), and a limited B19V replication was reported in the monocytic U937 cell line. (Munakata et al., 2006). In vitro studies have demonstrated that B19V is able to infect normal human fibroblasts and endothelial cells (Zakrzewska et al., 2005), and B19V DNA can persist in SSc fibroblasts (Ferri et al., 2002a). Although these data suggest the possible involvement of B19V in SSc, the mechanism by which the virus interferes in the disease’s pathogenesis remains unclear.

In this study, we investigated whether B19V is able to induce the activation of caspase-1-dependent inflammatory pathways in human monocytes and whether these pathways are differently activated in patients with SSc compared with healthy controls.

RESULTS

Patients and controls

The main characteristics of patients with SSc and controls are reported in Table 1.

B19V infection of primary monocyte cultures and monocytic THP-1 cells

As a first step, we investigated whether primary cultured CD14⁺ monocytes are susceptible to B19V infection. CD14⁺ cells, isolated from theuffy coats of normal donors, were infected with B19V and analyzed at different time points post-infection for the presence of viral DNA and mRNAs. Parallel infections were performed using the THP-1 cell line.

Neither CD14⁺ nor THP-1 cells were able to sustain the replication of the viral genome or its transcription, as the levels of B19V DNA at the end of the absorption/penetration period (T0) did not increase at any time (see Supplementary Figure S1 online), and no viral mRNAs were observed.

B19V activates NLRP3 inflammasome in THP-1 cells

To provide evidence that B19V activates inflammasome pathways, we used THP-1 cells differentiated with phorbol 12-myristate 13-acetate as a consolidated model for inflammasome studies (Chanput et al., 2014). The cells were infected with B19V, and the mRNA expression of inflammasomes frequently involved in viral infections, such as NLRP3, AIM2, and IFI16 (Chen et al., 2018; Torii et al., 2017), was evaluated by reverse transcriptase–PCR. After 6 hours post-infection, the NLRP3 mRNA was significantly increased compared with mock-infected cells, whereas AIM2 and IFI16 mRNA were not upregulated (Figure 1a).

To investigate whether B19V is able to activate caspase-1 in the context of the NLRP3 inflammasome, we infected the cells in the absence or presence of glybenclamide, a specific NLRP3 inhibitor (Chen et al., 2018). Western blot analysis revealed that B19V induces the cleavage of a caspase-1 precursor that was completely inhibited by glybenclamide (Figure 1b). Comparable data were obtained by measuring caspase-1 activity using the Caspase-Glo 1 Inflammasome Assay (Promega, Madison, WI) (Figure 1c).

Moreover, B19V infection of THP-1 cells induces caspase-1-dependent cleavage of the pro–IL-1β precursor in mature IL-1β; this phenomenon was inhibited by glybenclamide and by the specific caspase-1 inhibitor YVAD-CHO (Figure 1d).

### Table 1. Characteristics of Patients and Controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with SSc</th>
<th>Healthy Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>Age, years (median [range])</td>
<td>55 [38–60]</td>
<td>49 [38–61]</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3 (16)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Female</td>
<td>16 (84)</td>
<td>6 (75)</td>
</tr>
<tr>
<td>B19 antibody status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG⁺ IgM⁺</td>
<td>13 (76)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>IgG⁺ IgM⁻</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IgG⁻ IgM⁺</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>IgG⁻ IgM⁻</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Unviable</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Disease duration, years (median [range])</td>
<td>6[1–19]</td>
<td></td>
</tr>
<tr>
<td>Clinical subgroups, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse cutaneous</td>
<td>5 (26)</td>
<td></td>
</tr>
<tr>
<td>Limited cutaneous</td>
<td>14 (74)</td>
<td></td>
</tr>
<tr>
<td>Clinical manifestations, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raynaud phenomenon</td>
<td>19 (100)</td>
<td></td>
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<tr>
<td>Digital ulcers</td>
<td>9 (47)</td>
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<tr>
<td>Puffy fingers</td>
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<td>Telangiectasia</td>
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<tr>
<td>Intestinal lung disease</td>
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<tr>
<td>Esophageal dysfunction</td>
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<tr>
<td>Arthralgia</td>
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<tr>
<td>Sicca syndrome</td>
<td>14 (74)</td>
<td></td>
</tr>
<tr>
<td>Modified Rodnan skin score (median [range])</td>
<td>8 [0–33]</td>
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</tr>
<tr>
<td>Autoantibodies, n (%)</td>
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<tr>
<td>Positive ANA</td>
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</tr>
<tr>
<td>Positive ACA</td>
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<td></td>
</tr>
<tr>
<td>Positive anti-SC170</td>
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<td></td>
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<tr>
<td>Treatment, n (%)</td>
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<tr>
<td>Steroids (low dosage)</td>
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<td></td>
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<tr>
<td>Prostanoids</td>
<td>16 (84)</td>
<td></td>
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<tr>
<td>Bosentan</td>
<td>5 (26)</td>
<td></td>
</tr>
<tr>
<td>Calcium antagonist</td>
<td>13 (68)</td>
<td></td>
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</tbody>
</table>

Abbreviations: ACA, anti-centromere autoantibodies; ANA, anti-nuclear autoantibodies.
B19V induces caspase-1 activation in cultured monocytes from patients with SSc

Next, we investigated whether B19V activates caspase-1 in CD14⁺ cells from patients with SSc or healthy controls. At 6 hours post-infection, a quantitative determination of viral DNA and mRNAs was carried out by PCR and reverse transcriptase–PCR, respectively, and levels of caspase-1 activity were measured through a functional Caspase-Glo 1 Inflammasome Assay.

The amount of viral DNA detected in infected monocytes ranged from 1.98E⁰⁵ to 5.25E⁰⁸ (median, 1.42E⁰⁷). There was no difference in the viral load between patients and controls (median, 1.86E⁰⁷ and 1.07E⁰⁷, respectively). Moreover, no B19V mRNAs were detected in the infected cells. The NLRP3 mRNA showed a significant increase at 6 hours post-infection compared with mock-infected cells; in contrast, no upregulated expression of AIM2 and IFI16 was observed (Figure 2a).

B19V infection of CD14⁺ cells from patients with SSc induced an increase in caspase-1 activation, which was completely inhibited in the presence of YVAD, a specific caspase-1 inhibitor (Figure 2b). No significant differences were detected in monocytes from healthy controls compared with mock-infected cultures.

Cytokine production is induced by B19V infection in monocytes from patients with SSc and controls

Because B19V infection induces caspase-1 activation, we investigated whether the virus can also induce the production of mature IL-1β in CD14⁺ monocytes from patients with SSc or from healthy donors. For this purpose, we collected the supernatants of mock-infected or B19V-infected cultures and measured the concentrations of mature IL-1β by Immunoplex array. The concentrations of other pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-8, whose production is often activated by mature IL-1β (Stylianou and Saklatvala, 1998), were also measured.

No increase in IL-1β production was observed in B19V-infected CD14⁺ monocytes either from patients with SSc or from healthy donors (Figure 3a). The production of TNF-α was significantly induced by B19V infection in monocytes from patients with SSc. In contrast, only two of eight of cultures from healthy controls showed increased production of TNF-α, and statistical analysis did not reveal significant differences compared with mock-infected cells (Figure 3b). The B19V-induced production of TNF-α positively correlated with the amount of viral DNA detected at the end of incubation time (Figure 3c and d), suggesting a role for viral DNA in cytokine production, likely through the activation of toll-like receptor (TLR) 9. In fact, using the HEK-293XL cell line expressing human TLR9 and viral DNA–bearing vector, we were able to demonstrate the activation of the TLR9 signal (see Supplementary Figure S2 online).

The production of IL-6 and IL-8 was induced by virus infection in monocytes from patients with SSc, but statistical analysis did not reveal significant differences compared with control cultures (data not shown).

B19V infection potentiates lipopolysaccharide-induced inflammatory response

Emerging evidence has suggested that, in vivo, monocytes of patients with SSc may be activated by factors released from damaged endothelial cells (O’Reilly, 2018). These danger-
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Figure 2. Inflammasome-related gene expression and caspase-1 activity of B19V-infected CD14+ cells from patients with SSc or healthy controls. (a) The bar graph shows fold changes of mRNA expression (NLRP3, AIM2, and IFI16 genes) compared with that of control cells of three different experiments (mean ± SE; mRNA expression was quantified by RT-PCR at 6 hours p.i. (b). Caspase-1 activity was determined by Caspase-Glo 1 Inflammasome Assay. Results are expressed as RLUs. The boxes extend from range IQR*1.5, with a horizontal line at the median and an asterisk indicating the mean value. Statistical analysis was performed by Wilcoxon rank test for paired samples and Friedman test (Bonferroni correction); *P < 0.05 was considered significant. B19V, parvovirus B19; IQR, interquartile range; p.i., post-infection; RLU, relative luminescence unit; RT-PCR, reverse transcriptase–PCR; SE, standard error; SSc, systemic sclerosis.

DISCUSSION
Different studies have demonstrated the role of the inflammasome in the development of fibrosis (Gasse et al., 2007; Pan et al., 2019; Zhang et al., 2019). The upregulation of at least 40 genes involved in the inflammasome pathway and NLRP3-mediated secretion of IL-1β and IL-18 were detected in scleroderma fibroblasts (Artlett et al., 2011).

Many viruses can activate inflammasomes (Delaloye et al., 2009; Ichinohe et al., 2010; Muruve et al., 2008), and it has been hypothesized that persistent infection and chronic inflammasome activation is involved in the pathogenesis of SSc (Artlett et al., 2011).

B19V can infect the SSc target cells (fibroblasts and endothelial cells) (Zakrzewska et al., 2005) and frequently persists in the bone marrow and skin of patients with SSc (Ferri et al., 1999; Ohtsuka and Yamazaki, 2004).

In this study, we demonstrated that B19V is able to induce NLRP3 gene expression, NLRP3-dependent caspase-1 activation, and caspase-1–mediated IL-1β secretion in THP-1 cells differentiated with phorbol 12-myristate 13-acetate. Monocytes from patients with SSc highly express the NLRP3 gene and activate caspase-1 following B19V infection. Compared with THP-1 cells, however, they need additional stimuli to increase their IL-1β production, suggesting that B19V infection is not sufficient per se to activate the expression of the IL-1β gene. A significant increase in IL-1β production in B19V-infected monocytes from patients with SSc occurred only after LPS stimulation of infected cells.

Based on these data, we suggest that additional stimuli (in our experimental model, LPS) are required to induce the expression of some cytokine genes. Supporting this

associated molecular patterns can trigger TLR4 and initiate pro-inflammatory signals (Chen and Nuñez, 2010). To reproduce in vitro the stimulation conditions that are likely active in vivo, we used suboptimal doses of lipopolysaccharide (LPS), the main TLR4 ligand, to activate monocytes at the same time as infection with B19V.

In the presence of LPS, B19V infection induced a significant increase in IL-1β production in CD14+ monocytes from 17 of 19 patients with SSc compared with cultures stimulated with LPS alone. LPS-stimulated monocytes from four of eight normal donors also increased their IL-1β production following infection, but the analysis of the whole data did not reveal significant differences (Figure 4a). In addition, LPS-stimulated monocytes also increased TNF-α production following infection with B19V, and this increase reached statistical significance in monocytes from patients with SSc but not in monocytes from controls (Figure 4b).

Cytokine production by monocytes from patients with SSc and correlation with clinical parameters
Because the cytokine profile of peripheral blood mononuclear cells (PBMCs) from patients with SSc may be associated with selected clinical manifestations (Dantas et al., 2018), we evaluated whether caspase-1 activation and cytokine production by monocytes from patients with SSc correlated with the main clinical manifestations of the disease. We found a positive correlation between the levels of caspase-1 activity induced by B19V infection in LPS-stimulated monocytes and the disease’s duration, particularly in anti-B19V IgG-positive patients (Figure 5a–c). Moreover, the caspase-1 activation following virus infection in LPS-stimulated monocytes was much higher in patients with a disease duration of more than 3 years compared with recently diagnosed patients (Figure 5d). Finally, the caspase-1 activation following virus infection in LPS-stimulated monocytes was much higher in patients with sicca syndrome (Figure 5e).
hypothesis, LPS stimulation of B19V-infected SSc monocytes also amplified TNF-α production. Recently, De Nardo and colleagues (2009) reported that signaling crosstalk between TLR4 and TLR9 amplifies the inflammatory response of macrophages. In particular, LPS pretreatment primes the inflammatory response (e.g., TNF-α production) of mouse macrophages to the CpG DNA, the main TLR9 ligand. In vivo, monocytes of patients with SSc may be activated by factors released from damaged endothelial cells that have become apoptotic but have not been cleared appropriately (O’Reilly, 2018). These danger-associated molecular patterns bind TLR4 and initiate pro-inflammatory signals (Chen and Nunez, 2010), eventually potentiated by viral infections.

Thus, we can speculate that B19V activates molecular pathways, including the TLR9 pathway, which might synergize with those activated by LPS or other endogenous or exogenous TLR4 ligands.

It should be emphasized that markers of infection with other viruses such as EBV and cytomegalovirus are frequently detected in patients with SSc (Farina et al., 2014; Fattal et al., 2014). Recently, it has been reported that 50% of scleroderma patients have an active EBV infection and that EBV DNA is present in almost all scleroderma monocytes (Farina et al., 2017). It has been also demonstrated that EBV activates the AIM2 inflammasome in human monocytes (Torii et al., 2017).

The ability of B19V to activate the NLRP3 inflammasome discloses the autoinflammatory side of SSc, which might contribute to its multistep, multifactorial pathogenesis on an autoimmune basis (De Santis and Selmi, 2015; Ferri et al., 2018). In this regard, it is not surprising that the caspase-1 activation indexes are more marked in patients with a long-term disease, in which the combined action of danger-associated molecular patterns and other triggers including viral infections places the circulating monocytes in a state of preactivation, able to amplify the levels and even the duration of the inflammatory response. Finally, it is important to note that caspase-1 activation induced by B19V is more evident in patients with sicca syndrome.
These findings might suggest that different triggers and/or pathogenetic factors may be responsible for each SSc symptom and/or for different disease phenotypes (Ferri et al., 2018). Further studies on a greater number of patients are needed, however, to support this hypothesis and maybe to reveal novel correlations.

One unanswered question is which viral component is responsible for the monocyte activation observed in our study? In a recent study, B19V NS1 stimulation of PBMCs from adult-onset Still disease induced significant upregulation of mRNA levels of NLRP3, caspase-1, and IL-1β, and the secretion of respective proteins, compared with PBMCs from healthy controls. (Chen et al., 2018).

Because no B19V gene expression was observed in THP-1 cells and monocytes, we hypothesize that viral structural components, such as capsid VP1/VP2 proteins and/or viral DNA, may be involved in this phenomenon.

It has been demonstrated that the B19V VP1-unique region domain, located on the outside of the capsid (Kawase et al., 1995), presents phospholipase A2 activity that is necessary for viral infectivity (Zadori et al., 2001) and is involved in inflammatory reactions (Canaan et al., 2004; Dorsch et al., 2002; Lu et al., 2006).

An in silico study (Lanini et al., 2011) reported that B19V’s CpG index is very high compared with other DNA viruses, suggesting that B19V can strongly stimulate TLR9-dependent innate immunity. Moreover, using the HEK-293XL cell line expressing human TLR9, we were able to demonstrate the activation of the TLR9 signal induced by B19V DNA (see Supplementary Figure S2).

Therefore, we can speculate that the monocyte activation observed in this study might be due to the stimulation effect of the VP1-unique region protein and/or viral DNA sequences, a hypothesis needing further in-depth studies.

As mentioned previously, the pathogenesis of SSc may recognize a multifactorial (genetic, infectious, and/or environmental factors) and multistep process, which may include the B19V infection. The virus might be involved at different pathogenetic steps of the disease, including the impaired bone marrow production of endothelial cell precursors, as well as the endothelial and fibroblast activity. The B19V-mediated activation of inflammatory pathways in monocytes, demonstrated in this study, might strongly contribute to the disease progression and/or development of specific clinical phenotypes.

**MATERIALS AND METHODS**

**Patients and controls**

This study included 19 unselected patients with SSc (16 women and 3 men, ranging from 38 to 60 years, median age: 55 years) consecutively referred to the Rheumatology Unit, University-Hospital Policlinico of Modena, Italy. All patients fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism criteria for SSc and were classified according to the extent of skin involvement as limited SSc or diffuse SSc (van den Hoogen et al., 2013). Clinical, epidemiological, and serological investigations, including the modified Rodnan skin score to evaluate the extent of skin fibrosis (Clements et al., 1995; Khanna et al., 2017), the main visceral organ involvement, and SSc-associated autoantibodies, were carried out according to standardized methodologies (Ferri et al., 2002, 2014); clinical and serological features referred to at the time of this study were thoroughly reviewed from patients’ medical records.

Eight age- and sex-matched healthy subjects were enrolled as controls. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Institutional Review Board and Area Vasta Emilia Nord Ethical Committee (project identification code: 2742016). Participants gave their written informed consent.

**Laboratory investigations**

Peripheral blood samples (12 ml) were collected from each patient and control. The DNA was extracted from 100 μl of sample using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Milan, Italy), and detection of B19V DNA was performed by real-time PCR using the Parvovirus B19 ELiTe MGB Kit (EliTechGroup, Puteaux, France). The detection of serum anti-B19V antibodies was carried out using the Novagnost Parvovirus B19 IgG/IgM ELISA kit BEP III System (Siemens Healthcare GmbH, Erlangen, Germany).

**Figure 4. B19V potentiates IL-1β and TNF-α production in cultured monocytes stimulated with LPS from patients with SSc. (a) IL-1β and (b) TNF-α production was determined by Immunoplex array in supernatants of LPS-stimulated and B19V-infected (LPS + B19V) or LPS-stimulated and uninfected (LPS) monocytes from patients or controls at 6 hours p.i. The boxes extend from range IQR*1.5, with a horizontal line at the median and an asterisk indicating the mean value. Statistical analysis was performed by Wilcoxon rank test for paired samples; P < 0.05 was considered significant. B19V, parvovirus B19; IQR, interquartile range; LPS, lipopolysaccharide; p.i., post-infection; SSc, systemic sclerosis; TNF-α, tumor necrosis factor-α.
Cell cultures
PBMCs were obtained by Ficoll-Hypaque density gradients of blood samples from patients and controls and from buffy coats provided by AOU Careggi as previously described (Clemente et al., 2011). CD14⁺ cells were isolated from PBMCs using anti-CD14⁺ conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Clemente et al., 2013). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotic mix (penicillin, 10,000 U/ml; streptomycin, 10 mg/ml), here referred to as complete medium (CM) at 37°C with CO₂.

THP-1 cells were obtained from ATCC (Manassas, VA) and cultured in CM. Cells were treated with phorbol 12-myristate 13-acetate (300 ng/ml) for 30 minutes before their use.

B19V infection
For in vitro infection, viremic plasma samples from healthy donors containing a high titer of B19V (1E⁺11 to 1E⁺12 genome equivalents/ml), kindly donated by Professor Modrow (Germany) and by Kedrion S.p.A. (Italy), were used as a source of B19V. CD14⁺ or THP-1 cells were cultured in CM at a density of 2 x 10⁶ cells/well and incubated with B19V viremic plasma at a multiplicity of infection of 1,000 genome equivalents/cell, with or without LPS (500 ng/ml). As controls, cells treated with only LPS or with B19V-negative plasma were used. Following the adsorption/penetration period (T₀), the cells were washed with phosphate buffered saline and incubated in CM. At T₀ and at different time points, the cells were lysed for protein, DNA, or RNA extraction.

Nucleic acid extraction and amplification
The B19V DNA extracted from cell cultures using E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Norcross, GA) was quantified by real-time quantitative PCR (Toppinen et al., 2015). For quantification, dilutions of pHRT-1 plasmid, kindly donated by Professor Gallinella (University of Bologna), were used. To check DNA extraction and amplification, all samples were analyzed by real-time PCR for the Adaptor Related Protein Complex-3 Subunit Beta-1 sequence (Bio-Rad, Hercules, CA) (Arvia et al., 2017).

The RNA extracted from cultured cells using the E.Z.N.A. Total RNA Kit 1 (Omega Bio-tek, Norcross, GA) was retro-transcribed using the PrimeScript RT reagent kit with gDNA Eraser (Takara Clontech, St-Germain-en-Laye, France) and analyzed for the presence of spliced transcripts of B19V VP1 protein by real-time PCR modified from Nguyen et al. (2002). To detect and quantify B19V NS1 mRNA, cDNA was amplified by real-time PCR as described previously. To quantify mRNA for 18S, NLRP3, AIM2, and IFI16, reverse transcriptase–PCRs were carried out using PrimePCR SYBR Green Assay (Bio-Rad, Hercules, CA).

Cytokine production and caspase-1 activity studies
CD14⁺ cells (2 x 10⁶ cells) from patients with SSc or controls were infected with B19V in the absence or presence of LPS (500 ng/ml). Cells were washed with phosphate buffered saline and incubated with CM for an additional 4 hours. Supernatants were collected, and cytokine concentration was determined using a Milliplex kit (Merck KGaA, Darmstadt, Germany) and Bio-Plex (Bio-Rad, Hercules, CA) apparatus. Caspase-1 activity was measured in cell cultures in the presence or absence of 60 μM YVAD using the Caspase-Glo 1 Inflamasome Assay (Bio-Rad, Hercules, CA).

Western blot
B19V-infected or control THP-1 cells differentiated with phorbol 12-myristate 13-acetate (2 x 10⁶ cells) were cultured with or without LPS (500 ng/ml), 100 μM glybenclamide, 200 μM YVAD-CHO, or DMSO (Sigma-Aldrich, St. Louis, MO) for 6 hours, lysed with...
radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA) in the presence of phosphatase and protease inhibitor cocktails (Sigma-Aldrich, Saint Louis, MO) and centrifuged at 12,000 g. Proteins (40 μg) were loaded onto precast polyacrylamide TGX Stain-Free gel (Bio-Rad, Hercules, CA), blotted onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), and stained with rabbit anti-caspase-1 (Cell Signaling Technology, Danvers, MA), rabbit anti-β-actin or anti-β-tubulin (Santa Cruz Biotechnology, Dallas, TX); anti-rabbit IgG (Bio-Rad, Hercules, CA) was used as a secondary antibody at a 1:3,000 final dilution. The densitometric analysis was performed using Image Lab software (Bio-Rad, Hercules, CA).

Statistical analysis

Statistical analyses were performed using Fisher exact test for the analysis of frequency. Numerical data were expressed as mean ± standard error if they were in normal distribution, or median and interquartile range if they were not in Gaussian distribution. Mann-Whitney U test (exact test) and Wilcoxon rank sum test for two-independent samples were used to compare differences in cytokine production. Statistical significance was defined as a P ≤ 0.05. Spearman rank correlation coefficient was used to examine the relationship between two continuous variables. Analysis of variance and paired t-test were used to analyze densitometric values of western blot. A probability value of P ≤ 0.05 was considered significant. Statistical analysis was performed using R software version 3.5.1.

Data availability statement

Datasets related to this article can be found at https://www.researchgate.net (name of repository: Maria Gabriella Torcia).

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CONFICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: MT, KG, TZ; Formal Analysis: MT; Funding Acquisition: CF, KZ; Investigation: RA, AMC, GC; Methodology: RA; Resources: DG, GS; Supervision: CF; Writing - Original Draft Preparation: RA, MT, MGT, KZ; Writing - Review and Editing: CF, MGT, KZ

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.03.1144.

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Supplementary Figure S1. Time course of B19V infection of THP-1 cultures and cultured CD14^+ from normal donors. The cells were infected at a multiplicity of infection of 1,000 geq/cell. Quantitative evaluation of viral DNA was done at 2 hours (T0), 6 hours, 24 hours, 48 hours, and 72 hours, and at 6 days and 8 days p.i. Bars represent mean ± SE values from three different experiments. B19V, parvovirus B19; geq, genome equivalents; p.i., post-infection; SE, standard error.

Supplementary Figure S2. B19V DNA activates TLR9. The HEK-293XL cell line expressing human TLR9 was stably transfected with a plasmid encoding a luciferase reporter gene regulated by NF-κB regulatory promoter (pNifty, Invitrogen, Carlsbad, CA). Cells were plated in triplicate (70,000 cells/well) in the absence or presence of 0.5 μM ODN 2006 (Invitrogen). Viral DNA-bearing pGEM vector or empty vector was diluted in 0.5 μl of lipofectamine 2000 and added to the culture at the final concentrations of 1, 0.5, and 0.25 μg/ml. Cells were cultured for 5 hours at 37°C. TLR9-mediated NF-κB activation was measured as luciferase activity using the Steady-Glow Luciferase Assay System (Promega, Madison, WI) as directed by the manufacturer. Absorbance and luminescence were evaluated using a Victor (PerkinElmer, Waltham, MA) microplate spectrophotometer. Results are expressed as the fold change (%) in luciferase units relative to control not stimulated cultures. B19V, parvovirus B19; TLR, toll like receptor.