Antibodies against human cytomegalovirus late protein UL94 in the pathogenesis of scleroderma-like skin lesions in chronic graft-versus-host disease

Rocco Pastano1, Chiara Dell’Agnola2, Caterina Bason3, Federica Gigli1, Cristina Rabascio1, Antonio Puccetti4, Elisa Tinazzi3, Gianluigi Cetto2, Fedro Peccatori1, Giovanni Martinelli1 and Claudio Lunardi3

1Hematology Division, Bone Marrow Transplant Unit, European Institute of Oncology, Milan, Italy
2Department of Medicine, Section of Clinical Oncology, University of Verona, Verona, Italy
3Department of Medicine, Section of Internal Medicine, University of Verona, Verona, Italy
4Institute G. Gaslini and University of Genoa, Genoa, Italy

Correspondence to: Claudio Lunardi; E-mail: claudio.lunardi@univr.it

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Abstract

Human cytomegalovirus (hCMV) infection and its reactivation correlate both with the increased risk and with the worsening of graft-versus-host disease (GVHD). Because scleroderma-like skin lesions can occur in chronic GVHD (cGVHD) in allogeneic stem-cell transplant (HCT) patients and hCMV is relevant in the pathogenesis of systemic sclerosis (SSc), we evaluated the possible pathogenetic link between hCMV and skin cGVHD. Plasma from 18 HCT patients was tested for anti-UL94 and/or anti-NAG-2 antibodies, identified in SSc patients, by direct ELISA assays. Both donors and recipients were anti-hCMV IgG positive, without autoimmune diseases. Patients’ purified anti-UL94 and anti-NAG-2 IgG binding to human umbilical endothelial cells (HUVECs) and fibroblasts was performed by FACS analysis and ELISA test. HUVECs apoptosis and fibroblasts proliferation induced by patients’ anti-NAG-2 antibodies were measured by DNA fragmentation and cell viability, respectively.

About 11/18 patients developed cGVHD and all of them showed skin involvement, ranging from diffuse SSc-like lesions to limited erythema. Eight of eleven cGVHD patients were positive for anti-UL94 and/or anti-NAG-2 antibodies. Remarkably, 4/5 patients who developed diffuse or limited SSc-like lesions had antibodies directed against both UL94 and NAG-2; their anti-NAG-2 IgG-bound HUVECs and fibroblasts induce both endothelial cell apoptosis and fibroblasts proliferation, similar to that induced by purified anti-UL94 and anti-NAG-2 antibodies obtained from SSc patients.

In conclusion, our data suggest a pathogenetic link between hCMV infection and scleroderma-like skin cGVHD in HCT patients through a mechanism of molecular mimicry between UL94 viral protein and NAG-2 molecule, as observed in patients with SSc.

Keywords: endothelial cell apoptosis, fibroblast proliferation, tetraspanin NAG-2

Introduction

Graft-versus-host disease (GVHD) represents a major complication in allogeneic stem-cell transplant (HCT) patients (1–3). Chronic GVHD (cGVHD) shows clinical features similar to those of several autoimmune diseases; in particular, skin lesions may resemble those observed in patients with systemic sclerosis (SSc), an autoimmune disease in which autoantibodies directed against cell-surface antigens may induce endothelial cell damage, apoptosis, and fibroblasts activation within the skin and visceral organs (4–6). Moreover, anti-topoisomerase I [Scl-70 (7)] autoantibodies present in diffuse cutaneous SSc are also detected in a percentage of skin cGVHD patients (8–11).

Latent human cytomegalovirus (hCMV) infection has been suggested to be involved in the pathogenesis of SSc (12–16). High levels of IgG antibodies to the polyglycine motifs of hCMV detected in SSc patients represented the first indirect evidence of a link between the hCMV and the disease. Moreover, the cross-reaction of anti-Scl-70 autoantibodies with a peptide sequence of the UL70 protein of hCMV suggested that molecular mimicry may play a role in initiating the autoimmune response. More recently, some of us have provided direct evidence of a pathogenic link between hCMV infection and SSc by identifying a subset of anti-hCMV antibodies directed against the UL94 viral protein in patients with SSc, but not in the general...
population. Such viral protein shows homology with the NAG-2 molecule, a tetraspanin associated with integrins, expressed on both normal endothelial cell surface and normal fibroblasts. Anti-UL94 antibodies cross-reacted with cell-surface NAG-2-integrin complex inducing apoptosis of endothelial cells and fibroblasts proliferation, key features of SSc (17–19). hCMV infection is associated also with an increased risk of cGVHD, and hCMV reactivation correlates with the clinical worsening of GVHD (20, 21). However, a direct pathogenetic link between hCMV infection and cGVHD is still lacking. In a recent view of cGVHD as an autoimmune disease, CD13 represented a key molecule in hCMV infection, and anti CD13 autoantibodies were detected in hCMV-positive HCT patients (22). However, no evidence of a correlation between this host-target antigen, hCMV infection, and tissue damage was found.

The aim of this work was to evaluate whether the anti-UL94 and anti-NAG-2 cross-reactive antibodies, found to be pathogenic in SSc, are present also in the plasma of HCT patients with cGVHD and scleroderma-like skin lesions and may be correlated to the onset of scleroderma skin features in these patients.

Patients and methods

Patients

Plasma samples available from 18 patients undergone HCT for hematological malignancies between 2003 and 2006, at Bone Marrow Transplant Unit of Division of Hematooncology, European Institute of Oncology, Milan, Italy, were retrospectively analyzed for the presence of anti-UL94 and anti-NAG-2 antibodies. The characteristics and the outcome of the patients considered in the study are summarized in Table 1. All of them had HLA-identical sibling donors, and the majority received a preparative nonmyeloablative regimen, mainly based on Fluorouracil combined with Total Body Irradiation (TBI). The median follow-up was 7 months (range: 1–83). None of the patients or the donors was suffering from autoimmune diseases. Five recipients were hCMV IgG negative, but their donors were IgG positive. Ten patients reactivated hCMV (Table 2) evaluated by pp65 and PCR, often requiring a multiline preemptive therapy with Gancyclovir, Valgancyclovir, or Foscavir. 2) evaluated by pp65 and PCR, often requiring a multiline preemptive therapy with Gancyclovir, Valgancyclovir, or Foscavir.

Peptide synthesis

The peptides UL94 (VTLGGAGIWLLP), NAG-2 (CGVLGVGIL), and the irrelevant control (VTLPKSDVELP) were manually synthesized using the standard method of solid-phase peptide synthesis, which follows the 9-fluorenylmethoxycarbonyl strategy with minor modifications (17), then purified by reverse-phase high-performance chromatography, and their molecular weights were confirmed by electrospray mass spectrometry.

Enzyme-linked immunosorbent assay

The direct ELISA for antibody binding to the UL94 and NAG-2 peptides has been previously described (17, 18) and has been used with some modifications.

Briefly, the synthetic peptides were both used at the concentration of 20 µg ml–1 in PBS to coat polystyrene plates (Immunon II, Dynax, Ashford, UK). Plasma of all the patients was diluted 1:200 in 1% BSA and 0.05% Tween-20 in PBS. Plasma from normal age and sex-matched subjects were used as control group.

After 1 h blocking with 3% BSA in PBS, diluted plasma was added to the plates and incubated overnight (O/N) at 4°C. After washing, alkaline phosphatase–conjugated antiserum against human IgG (Sigma-Aldrich) was added in diluting buffer to the plates, and then incubated up to 3 h at room temperature. After washing, we measured the bound enzymatic activity with p-nitrophenyl phosphate (Sigma-Aldrich). Optical density (OD) values higher than the mean plus 3 SD of each plasma dilution of the control group were judged as positive.

To test the antibody levels, a plasma dilution from 1:200 to 1:1,600 has been performed, and the ELISA has been carried out as previously described.

Cell culture

To test the binding ability of patients’ anti-hCMV antibodies to cells, we isolated human umbilical endothelial cells (HUVECs) and cultured them in standard conditions. Human dermal fibroblasts and their growth medium were purchased from Promocell Bioscience Alive (Heidelberg, Germany). HUVECs were used between passages 2 and 5 and dermal fibroblasts between passages 3 and 6. The assay for the antibodies binding to HUVECs and fibroblasts has been previously described (17, 18).

Briefly, monolayer of HUVECs fixed with 0.1% glutaraldehyde or fibroblasts was incubated at 37°C for 2 h with patients’ plasma diluted 1:50 in their growth medium, containing 1% BSA. IgG binding was detected with a peroxidase-conjugated anti-human IgG (Amersham, UK) after 1 h of incubation. The enzymatic reaction was read within 30 min at 405 nm. Results were expressed in OD. Plasma of SSc patients and normal donors was used as positive and negative control respectively.

Total IgG extraction and affinity purification of anti-UL94 and anti-NAG-2 antibodies

Total IgG and affinity-purified anti-UL94 and anti-NAG-2 IgG antibodies were obtained from patients’ plasma. Plasma samples were applied to protein A-sepharose (Sigma), O/N, at 4°C. The columns were washed with PBS. Bound IgG were eluted with 0.1 M glycine (pH 2.5) and dialyzed against PBS. The purity of the preparation was assessed by SDS–PAGE followed by silver staining. To affinity purify IgG antibodies directed against UL94, NAG-2, or the irrelevant peptide, each peptide (5 mg of peptide per gram of dried sepharose powder) was coupled with sepharose 4B (Pharmacia, Uppsala, Sweden), according to the manufacturer’s instructions. Aliquots of the total IgG diluted in PBS were applied to each column separately. The columns were washed with PBS. Bound anti-UL94 and anti-NAG-2 peptide-specific IgG were eluted with 0.1 M glycine (pH 2.5) and dialyzed against PBS. Preparation purity was assessed as previously described. The anti-peptide antibodies obtained and the total IgG were used in functional experiments of cell proliferation and apoptosis.
HUVECs and fibroblasts were incubated with antibodies affinity purified against UL94 or NAG-2 from HCT and SSc patients or with antibodies affinity purified against an irrelevant control peptide. Antibody binding was revealed with Cy-5 conjugated antihuman IgG antibodies (Jackson Immunoresearch). Data were acquired on a FACScalibur and analyzed using FlowJo 8.8.2 software. Results are expressed as mean fluorescence intensity (MFI).

HUVECs and fibroblasts were incubated also with 10 µl of patients’ plasma for 30 min in ice. As positive control, we used plasma from a patient with SSc who was known to be positive for anti-UL94 and anti-NAG-2 antibodies, and as negative control we used plasma from a HCT patient without skin involvement who had tested negative for the presence of anti-UL94 and anti-NAG-2 antibodies. Antibody binding was revealed with Cy-5-conjugated antihuman IgG antibodies. Samples were run on a FACScalibur flow cytometer and analyzed with CellQuest program.

Measurement of apoptosis
The extent of internucleosomal DNA fragmentation was quantified using a commercially available kit (Roche Biochemical, Indianapolis, Indiana) according to the manufacturer’s instructions. Cells plated at 10⁵ cells ml⁻¹ were cultured for 16 h in microtiter plates with the presence or absence of the following apoptotic stimuli: anti-UL94, anti-NAG-2, and total IgG purified antibodies (20 µg ml⁻¹) from HCT and SSc patients, or TNF-alpha as positive control (50 ng ml⁻¹). The test allows the detection of mono and oligonucleosomes released into the cytoplasm by dividing the absorbance value obtained in the cells exposed to the antibody treatment with the absorbance value of the untreated cells. The enrichment factor was used as apoptosis index. An increase in the enrichment factor of 1.0 corresponded approximately to 8–12% of apoptotic cells.

Proliferation assay
In further experiments, anti-NAG-2 affinity-purified antibodies were tested for their ability to induce fibroblasts proliferation. Fibroblasts, plated at 5000 cells per well, were cultured for 24 h in microtiter plates with anti-NAG-2 affinity-purified antibodies (15 µg ml⁻¹) from HCT and SSc patients (positive control) and with antibodies against the irrelevant peptide (negative control). Cell viability was measured using a commercially available kit (Alexis Biochemicals, San Diego, California, USA). Results were expressed in OD.

Statistical analysis
Calculations were performed with the SPSS 19 statistical package. Comparison of OD levels in patients with or without cGVHD and in patients with or without skin involvement has been carried out using t-test for independent samples. The correlation between the presence of anti-UL94 and anti-NAG-2 antibodies in patients with skin involvement versus patients without skin involvement was performed with a nonparametric test [analysis of variance (ANOVA)]. Finally, the correlation between the presence of anti-UL94 and anti-NAG-2 antibodies in patients with cGVHD vs patients without it has been performed by a nonparametric test (Kruskall–Wallis).

### Table 1. Characteristics of HCT patients considered in this study

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/gender</th>
<th>Diagnosis</th>
<th>Therapy lines pre-HCT</th>
<th>Conditioning regimen</th>
<th>GVHD prophylaxis</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24/M</td>
<td>HL</td>
<td>9 (with auto HSCT, splenectomy)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 1055</td>
</tr>
<tr>
<td>2</td>
<td>53/F</td>
<td>CLL</td>
<td>2</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Alive, day 1755</td>
</tr>
<tr>
<td>3</td>
<td>61/M</td>
<td>AML (M4)</td>
<td>8 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Alive, day 1839</td>
</tr>
<tr>
<td>4</td>
<td>61/F</td>
<td>MM</td>
<td>3 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Alive, day 1047</td>
</tr>
<tr>
<td>5</td>
<td>59/M</td>
<td>AML (M2)</td>
<td>4</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Alive, day 1977</td>
</tr>
<tr>
<td>6</td>
<td>45/M</td>
<td>HL</td>
<td>6 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 166</td>
</tr>
<tr>
<td>7</td>
<td>51/M</td>
<td>NHL (mantle)</td>
<td>9 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 471</td>
</tr>
<tr>
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<td>48/F</td>
<td>CLL</td>
<td>5 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 168</td>
</tr>
<tr>
<td>9</td>
<td>51/F</td>
<td>CLL</td>
<td>2</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 212</td>
</tr>
<tr>
<td>10</td>
<td>63/M</td>
<td>MM</td>
<td>9 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Alive, day 979</td>
</tr>
<tr>
<td>11</td>
<td>47/M</td>
<td>CML (accelerate phase)</td>
<td>1</td>
<td>Myelablative</td>
<td>MTX CSA</td>
<td>Dead, day 203</td>
</tr>
<tr>
<td>12</td>
<td>67/M</td>
<td>MM</td>
<td>5</td>
<td>Myelablative</td>
<td>MMF CSA/FK</td>
<td>Dead, day 184</td>
</tr>
<tr>
<td>13</td>
<td>20/M</td>
<td>T-cell lymphoblastic lymphoma</td>
<td>7 (with auto HSCT)</td>
<td>Myelablative</td>
<td>MTX CSA</td>
<td>Dead, day 405</td>
</tr>
<tr>
<td>14</td>
<td>53/M</td>
<td>Peripheral T-cell lymphoma</td>
<td>5 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 229</td>
</tr>
<tr>
<td>15</td>
<td>61/M</td>
<td>T-cell NHL</td>
<td>4 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 73</td>
</tr>
<tr>
<td>16</td>
<td>25/M</td>
<td>T-cell lymphoblastic lymphoma</td>
<td>8 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 41</td>
</tr>
<tr>
<td>17</td>
<td>36/M</td>
<td>DLBC lymphoma</td>
<td>5 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 169</td>
</tr>
<tr>
<td>18</td>
<td>27/M</td>
<td>DLBC lymphoma</td>
<td>9 (with auto HSCT)</td>
<td>Nonmyelablative</td>
<td>MMF CSA</td>
<td>Dead, day 36</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; DBLC, diffuse large B-cell lymphoma; HL, Hodgkin’s lymphoma; HSCT, autologous hematopoietic stem cell transplant; MM, multiple myeloma; NHL, non-Hodgkin’s lymphoma.
Results

Detection of anti-UL94 and anti-NAG-2 antibodies and clinical correlation in HCT patients

To evaluate the presence of anti-UL94 and anti-NAG-2 antibodies in HCT patients, the synthesized UL94 and NAG-2 peptides (17) were incubated with patients’ plasma, obtained after HCT (Fig. 1A and B). The plasma concentration of anti-UL94 and anti-NAG-2 antibodies was very high because they were still detectable at a plasma dilution of 1:1600 in direct binding assay (Fig. 1C and D).

About 15/18 patients survived after day 100 post HCT and were suitable for GVHD evaluation; focusing on 11 patients who developed cGVHD (Table 2), 7 of them did reactivate hCMV often requiring multiline treatments. Two patients suffered from acute GVHD. All GVHD patients showed skin involvement, ranging from diffuse SSc-like lesions, as observed in patients 1–3 to limited SSc-like lesions (patients 4 and 5) to a diffuse erythema or limited erythema (Table 2). Using t-test for independent samples, a statistical correlation was observed between the cGVHD and the anti-UL94 and anti-NAG-2 antibodies’ levels [absorbance (mean ± SD): 0.252 ± 0.110 and 0.358 ± 0.176, respectively] compared with patients who did not develop cGVHD (absorbance [mean ± SD]: 0.152 ± 0.033 and 0.226 ± 0.060) with a P = 0.01 and 0.03, respectively (Fig. 2A and B). Moreover, this statistical significance is maintained when we compare the presence or absence of anti-UL94 and anti-NAG-2 antibodies in patients with cGVHD with those patients who do not develop the disease (Table 2; P = 0.04). When we consider patients with and without skin involvement, the presence of anti-UL94 and anti-NAG-2 antibodies is again statistically different using the nonparametric test, ANOVA (P = 0.017).

In particular, the three patients with diffuse SSc-like lesions (Table 2) were positive for both anti-UL94 and anti-NAG-2 antibodies (Fig. 1A and B).

Considering the two patients with limited scleroderma, patient 4 resulted negative for anti-UL94 and anti-NAG-2 antibodies; he had a very limited cutaneous SSc-like skin lesions. In addition, during GVHD immunoprophylaxis, he re-activated hCMV twice and was treated with Valcyte; on the contrary, patient 5 was positive for anti-UL94 and anti-NAG-2 antibodies and his cutaneous SSc-like skin lesions were more extended than the previous one.

Five patients with cGVHD (Table 2) showed a diffuse skin erythema, without evidence of scleroderma-like lesions and two of them were negative for both anti-UL94 and anti-NAG-2 antibodies.

Patient 13 was positive for anti-UL94 antibodies, but negative for anti-NAG-2 antibodies, and he showed a skin erythema limited to the head and mouth due to lichen after brain radiotherapy; he died because of disease progression.

Remarkably, the five patients who did not develop acute or cGVHD and any skin involvement resulted negative for anti-UL94 and anti-NAG-2 antibodies unrespectable to hCMV IgG antibodies status (Table 2).

Purified anti-UL94 and anti-NAG-2 antibodies from HCT patients with diffuse scleroderma-like skin lesions are functionally active

We subsequently tested the ability of purified anti-UL94 and anti-NAG-2 antibodies to bind endothelial cells and fibroblasts both by FACS analysis (Fig. 3A–D) and ELISA test (data not shown): antibodies obtained from HCT and SSc patients behaved similarly. Moreover, the binding of antibodies to HUVECs and fibroblasts was present also when patients’ plasma was used (Fig. 4A and B).

Finally, to evaluate whether purified anti-UL94 and anti-NAG-2 antibodies were functionally active, affinity-purified antibodies from the three patients with cGVHD and skin lesions resembling diffuse scleroderma were incubated with HUVECs to assess their ability to induce cell apoptosis. Indeed, HUVECs underwent internucleosomal DNA fragmentation in the same manner as observed with the antibodies purified from SSc patients (Fig. 5A). This effect was
comparable to 70% of the level of DNA fragmentation seen after the exposure of HUVECs to 50 ng ml–1 of TNF-alpha. Similar data were obtained following HUVEC’s exposure to patients’ purified total IgG (Fig. 5B). Finally, purified anti-NAG-2 antibodies from the same HCT patients were able to induce fibroblasts proliferation as observed with SSc patients’
derived antibodies (Fig. 5C). Similar results were obtained with plasma-derived total IgG (data not shown).

Discussion

In this retrospective study, we included patients who underwent HCT after a multiline treatment comprising autologous stem-cell transplant (Table 1), and this can explain the high mortality rate observed in our patient group.

GVHD is a substantial cause of morbidity and mortality and the major determinant of long-term outcome and quality of life in HCT patients, with those with an extensive cGVHD characterized by an unfavorable natural history.

The extent of cGVHD, that is, limited versus extended is more important than the histological severity (23). Skin cGVHD lesions, particularly, those resembling diffuse SSc could be a second and invalidating disease caused by HCT, explaining the great effort provided for a better management of this disorder and therefore for limiting patients’ exposure to corticosteroids. Although skin cGVHD is mainly explained by the attack of engrafted allo-hematopoietic cells and their progeny against host cutaneous tissues resulting in lichenoid or sclerodermatous changes (2, 3), many additional mechanisms can contribute to the development of this syndrome. Indeed, as observed in SSc, scleroderma-like skin cGVHD is characterized also by vascular tissue damage and fibrosis. Moreover, autoantibodies are present in the two disease conditions, making the two entities even more similar (23, 24). Autoantibodies directed against the PDGF receptor (PDGFR) that is able to activate fibroblasts have been found not only in SSc patients but also in HCT patients who developed skin cGVHD (25, 26).

HCMV remains one of the most common infections after HCT, resulting in significant morbidity and occasional mortality. Moreover, clinical observations suggest that hCMV infection could play a critical role in cGVHD. Indeed hCMV persists in a latent status following primary infection and causes high morbidity in immunosuppressed individuals, such as HCT patients (27). cGVHD is more common among patients who experienced reactivation of hCMV infection than among those without signs of active viral infection (28), defining hCMV viral load monitoring as the best opportunity for risk stratification in these patients, thus allowing the use of pre-emptive treatment and the monitoring of the response to therapy (29).

Majority of our patients with GVHD experienced hCMV reactivation (9 patients out of 13), and in particular, 7 patients out of 11 who developed cGVHD with skin involvement had hCMV reactivation. Moreover, seven patients with cGVHD showed the presence of antibodies directed against hCMV-derived protein UL94, a subtype of anti-hCMV antibodies found in SSc patients. Patient 14, the only one who experienced hCMV reactivation without developing GVHD, was refractory to any treatment, included HCT, and was heavily immunosuppressed.

The pathogenetic link between the hCMV infection and the development of skin cGVHD scleroderma is still unknown. Latent hCMV infection has been shown to be involved in the pathogenesis and progression of SSc through a mechanism of molecular mimicry because anti-UL94 viral protein
antibodies cross-react with the cell-surface tetraspanin NAG-2 and are functionally active by inducing apoptosis of endothelial cells and fibroblasts, proliferation, key features of SSc (17–19). Similarly, antibodies directed against UL94 and NAG-2 were detected in HCT patients with cGVHD and skin involvement and behaved in the same manner as those obtained from SSC subjects because they bound endothelial cells and fibroblasts and were functionally active, suggesting a mechanism of molecular mimicry as described in scleroderma (17).

Remarkably, the three patients with cGVHD, who developed a diffuse SSC-like skin involvement, had also antibodies directed against NAG-2 molecule. Based on these results, the pathogenetic mechanism underlying hCMV-related tissue damage in hCMV-positive HCT patients with SSC-like skin lesions seems to be similar to the one occurring in SSC patients, potentially linking the viral infection with the skin tissue damage following HCT.

In this regard, the vast majority of HCT patients (8/11) who developed cGVHD were positive for anti-UL94 and

Fig. 3. Affinity-purified anti-UL94 and anti-NAG-2 IgG antibodies bind endothelial cells and fibroblasts. (A) Binding to HUVECs of antibodies directed against UL94 affinity purified from an SSc patient (blue: MFI ± SD = 16 ± 3.5) and from HCT patient 1 (red: MFI ± SD = 16.5 ± 2.9). (B) Binding to HUVECs of antibodies directed against NAG-2 affinity purified from an SSc patient (blue: MFI ± SD = 16.8 ± 3.5) and from HCT patient 1 (red: MFI ± SD = 16.4 ± 3.1). Negative control: (gray: MFI ± SD = 5.3) HUVECs incubated with anti-irrelevant peptide antibodies. Representative example of three independently performed experiments that generated the same FACS profiles. (C) Binding to fibroblasts of antibodies directed against UL94 affinity purified from an SSc patient (blue: MFI ± SD = 22 ± 2) and from HCT patient 1 (blue: MFI ± SD = 16.2 ± 2.8). (D) Binding to fibroblasts of antibodies directed against NAG-2 affinity purified from an SSc patient (blue: MFI ± SD = 25.3 ± 2.2) and from HCT patient 1 (red: MFI ± SD = 23.8 ± 2). Negative control: (gray: MFI ± SD = 3.2) fibroblasts incubated with anti-irrelevant peptide antibodies. Representative example of three independently performed experiments that generated the same FACS profiles. MFI: mean fluorescence intensity.
**Fig. 4.** FACS analysis of HUVECs and fibroblasts incubated with HCT patients’ plasma. (A) HUVECs and (B) fibroblasts were incubated with medium alone (a), with plasma obtained from SSc patients used as positive controls (b,c), with plasma from HCT patients 1–3 with diffuse-like SSc lesions (d,e,f), and with plasma obtained from patient 4 (g), patient 11 (h), and patient 16 (i). Representative example of three independently performed experiments that generated the same FACS profiles. X-axis: FL4-H, fluorescence intensity; y-axis: counts.

**Fig. 5.** Anti-UL94 and anti-NAG-2 antibodies purified from patients with SSc-like skin cGVHD are functionally active. (A) Apoptosis of endothelial cells induced by anti-UL94 (dark gray bars) and anti-NAG-2 antibodies (light gray bars): antibodies affinity purified from an SSc patient used as positive control (bar 2) and from HCT patients 1, 2 and 3 who developed a diffuse skin SSc-like disease (bars 3, 4, and 5, respectively). Bar 1 (black bar): negative control = cells incubated with antibodies purified against an irrelevant peptide gave an apoptotic index = 1. The bars indicate the mean ±SD of experiments performed in triplicates. (B) Apoptosis of endothelial cells induced by total IgG antibodies obtained from an SSc patient used as positive control (bar 2) and from HCT patients 2, 5, 11, 16 (bars 3–6). Bar 1 (black bar): negative control = cells incubated with total IgG obtained from a normal donor gave an apoptotic index = 1. The bars indicate the mean ±SD of experiments performed in triplicates. (C) Proliferation (gray bars) of fibroblasts induced by anti-NAG-2 antibodies affinity purified from an SSc patient (positive control, bar 2) and from HCT patients 1, 2, and 3 (bars 3, 4, and 5, respectively). Bar 1 (black bar): negative control = cells incubated with antibodies purified against an irrelevant peptide. Data are expressed as cell viability (OD) (y-axis). The bars indicate the mean ±SD of experiments performed in triplicates.
anti-NAG-2 antibodies; three patients survived and developed diffuse and invalidating skin lesions similar to those observed in SSc. Unfortunately, four of them died prematurely after cGVHD development and had only limited skin lesions. We can hypothesize that a longer survival could have allowed the onset of a diffuse skin involvement. In this view, measurement of anti-UL94 and anti-NAG-2 antibodies after HCT could be useful for risk stratification of development of SSc lesions. We, therefore, may suggest that testing the patients for the presence of anti-UL94 viral protein antibodies may help in achieving a better management of HCT patients with cGVHD in preventing the possible onset of scleroderma-like skin lesions.

Even more interestingly, the identification of NAG-2 as a key molecule involved in the tissue damage process observed in skin cGVHD, particularly in the ones with SSc-like phenotype, opens new perspectives in terms of both an immunosuppressive treatment and a target therapy tailored on this specific molecule (30).

In conclusion, although prospective studies with a larger number of patients are needed, our data suggest a link between HCMV infection and the pathogenesis of skin cGVHD resembling scleroderma.

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**Conflict of interest**

The authors have no conflicting financial interest.

**References**