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Arthritis & Rheumatology
Vol. 0, No. 0, Month 2024, pp 1–11
DOI 10.1002/art.42746
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Adeno-Associated Virus Type 5 Infection via PDGFRα Is Associated With Interstitial Lung Disease in Systemic Sclerosis and Generates Composite Peptides and Epitopes Recognized by the Agonistic Immunoglobulins Present in Patients With Systemic Sclerosis

Gianluca Moroncini,[1] Silvia Svegliati,[2] Antonella Grieco,[2] Massimiliano Cuccioloni,[3] Matteo Mozzicafreddo,[2] Chiara Paolini,[2] Silvia Agarbati,[2] Tatiana Spadoni,[2] Angela Amoresano,[4] Gabriella Pinto,[4] Qingxin Chen,[5] Devis Benfaremo,[1] Cecilia Tonnini,[2] Martina Senzacqua,[2] Silvia Alizzi,[6] Karen Nieto,[5] Doreen Finke,[2] Nadia Viola,[7] Donatella Amico,[8] Mario Galgani,[4] Stefano Gasparini,[1] Lina Zuccatosta,[7] Stefano Menzo,[1] Martin Müller,[5] Jürgen Kleinschmidt,[5] Ada Funaro,[9] Antonio Giordano,[2] Antonio La Cava,[4,10] Peter Dorfmüller,[11] Antonio Amoroso,[6] Piero Pucci,[4] Antonio Pezone,[4] Enrico V. Avvedimento,[4] and Armando Gabrielli[12]

Objective. The etiopathogenesis of systemic sclerosis (SSc) is unknown. Platelet-derived growth factor receptors (PDGFRs) are overexpressed in patients with SSc. Because PDGFRα is targeted by the adeno-associated virus type 5 (AAV5), we investigated whether AAV5 forms a complex with PDGFRα exposing epitopes that may induce the immune responses to the virus–PDGFRα complex.

Methods. The binding of monomeric human PDGFRα to the AAV5 capsid was analyzed by in silico molecular docking, surface plasmon resonance (SPR), and genome editing of the PDGFRα locus. AAV5 was detected in SSc lungs by in situ hybridization, immunohistochemistry, confocal microscopy, and molecular analysis of bronchoalveolar lavage (BAL) fluid. Immune responses to AAV5 and PDGFRα were evaluated by SPR using SSc monoclonal anti-PDGFRα antibodies and immunoaffinity-purified anti-PDGFRα antibodies from sera of patients with SSc.

Results. AAV5 was detected in the BAL fluid of 41 of 66 patients with SSc with interstitial lung disease (62.1%) and in 17 of 66 controls (25.75%) ($P < 0.001$). In SSc lungs, AAV5 localized in type II pneumocytes and in interstitial cells. A molecular complex formed of spatially contiguous epitopes of the AAV5 capsid and of PDGFRα was identified and characterized. In silico molecular docking analysis and binding to the agonistic anti-PDGFRα antibodies identified spatially contiguous epitopes derived from PDGFRα and AAV5 that interacted with SSc agonistic antibodies to PDGFRα. These peptides were also able to bind total IgG isolated from patients with SSc, not from healthy controls.

Conclusion. These data link AVV5 with the immune reactivity to endogenous antigens in SSc and provide a novel element in the pathogenesis of SSc.

Supported by grants from Fondazione di Medicina Molecolare e Terapia Cellulare, Associazione Italiana lotta alla Sclerodermia, Gruppo Italiano per la Lotta alla Sclerodermia, and Drs. Andrea and Diego Della Valle.

[1]Gianluca Moroncini, MD, PhD, Devis Benfaremo, MD, Stefano Gasparini, MD, Stefano Menzo, MD: Università Politecnica delle Marche and Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy; [2]Silvia Svegliati, PhD, Antonella Grieco, PhD, Matteo Mozzicafreddo, PhD, Chiara Paolini, PhD, Silvia Agarbati, PhD, Tatiana Spadoni, PhD, Cecilia Tonnini, BS, Martina Senzacqua, PhD, Doreen Finke, PhD, Antonio Giordano, MD: Università Politecnica delle Marche, Ancona, Italy; [3]Massimiliano Cuccioloni, PhD: Università di Camerino, Camerino, Italy; [4]Angela Amoresano, PhD, Gabriella Pinto, PhD, Mario Galgani, PhD, Antonio La Cava, MD, PhD, Piero Pucci, PhD, Antonio Pezone, PhD, Enrico V. Avvedimento, MD, PhD: Federico II University, Napoli, Italy; [5]Qingxin Chen, PhD, Karen Nieto, PhD, Martin Müller, PhD, Jürgen Kleinschmidt, PhD: German Cancer Research Center (DKFZ), Heidelberg, Germany; [6]Silvia Alizzi, MD, Antonio Amoroso, MD: Università Politecnica delle Marche, Ancona, Italy, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy, and Heinrich Heine University, Düsseldorf, Germany; [7]Nadia Viola, BS, Lina Zuccatosta, MD: Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy; [8]Donatella Amico, MD, PhD: Ospedali Riuniti Marche Nord, Pesaro, Italy; [9]Ada Funaro, PhD: Università di Torino, Torino, Italy; [10]Antonio La Cava, MD, PhD: University of California, Los Angeles, CA; [11]Peter Dorfmüller, MD, PhD: University of Giessen and Marburg Lung Center, Giessen, Germany; [12]Armando Gabrielli, MD: Università di Torino and Azienda Ospedaliera Universitaria Città della Salute e della Scienza, di Torino, Torino, Italy.

Drs. Moroncini, Svegliati, and Grieco are co-first authors and contributed equally to this work.

Additional supplementary information cited in this article can be found online in the Supporting Information section (http://onlinelibrary.wiley.com/doi/10.1002/art.42746).

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Address correspondence via email to Armando Gabrielli, MD, at a.gabrielli@staff.univpm.it; or to Enrico V. Avvedimento, MD, PhD, at avvedim@unina.it.

Submitted for publication February 6, 2023; accepted in revised form November 1, 2023.

INTRODUCTION

Systemic sclerosis (SSc; scleroderma) is an autoimmune, multisystem disorder of unknown etiology and unclear pathogenesis. It is characterized by microvasculature damage, circulating autoantibodies, and fibroblast activation that lead to fibrosis of the skin and visceral organs.[1,2] SSc is a major cause of disability and carries high morbidity and mortality associated with the fibrotic and microvascular alterations.[3,4]

It has been suggested that SSc can be triggered by viruses,[5] and the interferon type I gene expression signature is frequently observed in patients with SSc.[6–8] However, the search for putative disease-promoting viruses in SSc has so far been inconclusive.

Platelet-derived growth factor receptor (PDGFR) signaling, directly or indirectly, is involved in SSc.[9] PDGFRs are the targets of several viruses[10–13] and are typically overexpressed in SSc.[14] We also reported the presence of stimulatory anti-PDGFRα antibodies in the serum of patients with SSc,[15] and we cloned agonistic anti-PDGFRα autoantibodies from patients with SSc.[16]

Anti-PDGFRα autoantibodies are of particular interest for the following reasons: (1) they induced an SSc-like phenotype in normal human fibroblasts,[15] (2) they induced fibrosis ex vivo,[17] (3) they stimulated the proliferation and migration of human pulmonary artery smooth muscle cells (suggesting a role in the formation of the neointima, which is the cardinal feature of the vascular abnormalities in SSc),[18] and (4) they triggered a well-characterized signaling pathway leading to fibrosis.[19] Furthermore, these antibodies selectively recognized specific domains in the receptor. For example, antibodies targeting extracellular domain I of the receptor were biologically inactive, whereas those recognizing the extracellular domain II were stimulatory in inducing fibrosis and redox stress.[16]

It has to be noted that the detection of these antibodies is not trivial because they cannot be detected by nonfunctional bioassays,[16,20,21] underscoring their functional role. Moreover, it has to be considered that these antibodies can have conformational epitopes that are distant in the primary sequence but contiguous in the tertiary structure of the receptor on the cell surface.[16] Interestingly, adeno-associated virus type 5 (AAV5) is known to enter several cell types through PDGFRα,[22–24] making it a candidate worth studying in the induction of anti-PDGFRα in SSc.

AAV5 is a nonenveloped, single-stranded DNA adeno-associated virus that belongs to the genus *Dependoparvovirus* within the Parvoviridae family[25]; it requires a helper virus, such as adenovirus or herpes simplex virus, or cellular stress, to replicate.[26] To date, 13 serotypes of AAV have been identified.[27] AAVs gain access to specific cell surface receptors after attaching to glycans or glycoconjugates on the cell membrane.[28] The current knowledge is that AAVs do not cause any human diseases and have been used for in vivo therapeutic gene delivery.[29] Building on these considerations, we hypothesized that AAV5 may recognize and bind specific domains in the receptor and may target the immune response to the receptor and the viral capsid.

MATERIALS AND METHODS

Patients. Sixty-six consecutive Caucasian patients with interstitial lung disease (ILD)-SSc were studied. All patients with SSc fulfilled the EULAR/American College of Rheumatology preliminary criteria for the classification of SSc.[30] The patients were classified into subgroups according to whether they had diffuse cutaneous scleroderma (dcSSc) or limited cutaneous scleroderma (lcSSc)[31] and then were divided into those with early (<3 years for dcSSc and <5 years for lcSSc) or late (>6 years for dcSSc and >10 years for lcSSc) disease from the first non–Raynaud phenomenon symptom.[32] All patients had not received any immunosuppressive treatment during the previous 6 weeks. The presence of ILD was confirmed by x-ray and/or high resolution computed tomography (HRCT). The control group included 66 patients who underwent bronchoalveolar lavage (BAL) for lung involvement other than SSc ILD and, in selected in vitro experiments, healthy controls. The protocol with patients' information and consent forms was approved by the Ethics Committee of the Università Politecnica delle Marche (number 2017-518). The study was conducted in accordance with the Declaration of Helsinki, 5th edition (2000). Written informed consent was obtained from all patients.

Molecular docking analysis. The three-dimensional structure of the AAV5 envelope subunit[33] was obtained from the Protein Data Bank. Human PDGFRα and anti-PDGFRα V_HPAM-$V_κ$16F4, a stimulatory human anti-PDGFRα monoclonal autoantibody cloned from B cells of a patient with SSc, were homology modeled as previously reported.[16]

The binding partners were uploaded on the ClusPro server,[34] and the amino acids involved in the interaction between AAV5 and monomeric PDGFRα and between the predicted AAV5–PDGFRα complex and V_HPAM-$V_κ$16F4 were identified using PyMol software (PyMOL Molecular Graphics System, version 2.1). Energy-based docking between trans-peptides, minimized using GROMACS (version 2021.5), and V_HPAM-$V_κ$16F4 was performed using SwissDock server.[35] Other methods are reported in the supplementary methods section.

Statistical analyses. The two-sample test for equality of proportions was used to analyze the results of the BAL study, presented as percentages of patients who tested positive for AAV5 DNA, and for the analysis of patients positive for both anti-PDGFRα and anti-AAV5 antibodies. The significance of the differences in AAV5 transduction in wt and PDGFRα-knockout A549 cell line was determined by Student's *t*-test and Mann-Whitney test for the analysis of the quantitative Polymerase Chain Reaction

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results. Data are expressed as mean ± SD or as median value and a range. Data were analyzed using Prism software (Graph-Pad). All reported *P* values are two-sided. A *P* value of <0.05 was considered statistically significant. Access to primary datasets (generated during the study) and referenced datasets (datasets analyzed in the study) are available.

RESULTS

AAV5 in the lungs of patients with SSc with ILD. Because the lung is the main disease target in SSc and is responsible for high morbidity and mortality,[36,37] we investigated the possibility that it might be affected by the presence of AAV5-infected cells. Immunohistochemistry was performed on frozen lung tissues from five patients with SSc and six control lung specimens (from lobectomy or pneumonectomy for localized lung cancer), and the results were confirmed by chromogenic in situ hybridization on paraffin-embedded lung sections from two patients and two controls (Supplementary Table 1). A representative experiment is shown in Figure 1. All samples were analyzed in a blinded manner by two independent investigators (Antonio Giordano and MS). In SSc lungs of five patients with SSc, but not in controls, AAV5 was detected mainly in cells lining the alveolar space—many of them with the cytologic appearance of type II pneumocytes (Figure 1A and B)—and, to a lesser extent, in interstitial macrophage-like cells. In lung tissue of patients with lung cancer used as controls, AAV5 immunoreactivity was only found in interstitial cells. Positivity was found in two other controls, one affected by primary arterial hypertension with fibrotic remodeling and pneumocytic hyperplasia and the other by nonspecific interstitial pneumonia with extensive interstitial fibrosis (data not shown). Using prosurfactant protein C (pro-SP-C) and thyroid transcription factor-1 (TTF-1) as markers of type II pneumocytes,[38,39] we found by double staining and confocal microscopy that a consistent number of type II pneumocytes positive for pro-SP-C or TTF-1 were variably positive for AAV5 (mean ± SD 17% ± 6.2% and 11% ± 4.5%, respectively; Figure 1C and Supplementary Figure 1). Of note, AAV5-positive type II pneumocytes coexpressed PDGFRα, mainly at the apical segment of the cells, and facing the alveolar space (Figure 1D and Supplementary Figure 2). The specificity of the signals was suggested by the strong positivity for PDGFRα—and not for AAV5—of fibroblast-like cells in the interstitial space (Figure 1D). Extensive quantification of multiple confocal microscopy images of SSc lung sections (n = 14), as described in the supplementary methods section, indicated positivity for AAV5 capsid (mean ± SD 16% ± 5.1%) and PDGFRα (mean ± SD 22% ± 5.5%), with an overlay mean ± SD percentage of 10.69% ± 4.4% (Supplementary Figure 3A and B, Supplementary Table 2). Interestingly, a mean ± SD of 63.77% ± 10% of the AAV5 signal overlapped with the PDGFRα signal, whereas a mean ± SD of only 46.49% ± 9.8% of the PDGFRα signal overlapped with the AAV5 signal (Supplementary Figure 3C). A strong linear correlation between the two signals was observed (R^2 = 0.97, Supplementary Figure 3D).

Of note, colocalization of AAV5 and PDGFRα was also observed by confocal microscopy in peripheral blood mononuclear cells (PBMCs) from patients with SSc (Supplementary Figure 4). Quantification of multiple confocal microscopy images of SSc PBMCs (n = 11) showed an AAV5 capsid mean ± SD positivity of 11.85% ± 3.4% and a PDGFRα mean ± SD positivity of 21.5% ± 2.67%, with an overlay mean ± SD percentage of 6.05% ± 2% (Supplementary Figure 5A and B, Supplementary Table 3). Notably, a mean ± SD of 52.2% ± 10.9% of the AAV5 signal overlapped with the PDGFRα signal, whereas a mean ± SD of only 28.4% ± 9.1% of the PDGFRα signal overlapped with the AAV5 signal (Supplementary Figure 5C). Also in this case, the two signals had a strong linear correlation (R^2 = 0.93, Supplementary Figure 5D).

Taken together, the aforementioned data indicate that (1) immunostaining for the AAV5 capsid correlates with PDGFRα expression in both lung sections and PBMCs of patients with SSc, (2) type II pneumocytes co-express PDGFRα and AAV5 positivity, and (3) not all PDGFRα-expressing cells are also positive for the AAV5 capsid.

AAV5 in BAL from patients with SSc. The presence of AAV5 DNA was investigated in the BAL fluid from 66 patients with SSc (53 female and 13 male; mean ± SD age 56.9 ± 15.2 years) with ILD and 66 control patients with lung disease other than SSc (38 female and 28 male; mean ± SD age 62.3 ± 15.1 years). Thirty-seven patients with SSc (56%) had the limited cutaneous form of SSc, and 29 (44%) had the diffuse cutaneous form. The lung disorders different from SSc in controls are reported in Supplementary Table 4. The results showed positivity for AAV5 DNA in the BAL fluid from 41 patients with SSc (62.1%; 95% confidence interval [95% CI] 49.3%–73.8%) and 17 controls (25.75%; 95% CI 15.8%–38.0%) (P < 0.001) (Figure 2A). It is worth mentioning that seven additional patients with SSc lacking viral DNA in the BAL fluid had positivity for AAV5 DNA in their PBMCs (Supplementary Figure 4), for a totality of 48 positive patients (72.7%). The only feature distinguishing patients with SSc with AAV5-positive BAL fluid from those with AAV5-negative BAL fluid was disease duration (SSc AAV5 positive: mean ± SD 5.3 ± 0.9 years vs SSc AAV5 negative: mean ± SD 2.4 ± 0.8 years; P < 0.05) (Supplementary Table 5). Quantitatively, the AAV5 DNA content was comparable between the BAL fluid from patients with SSc and the controls who tested positive for AAV5 (P = 0.191) (Figure 2B). To rule out the possibility that a mutated AAV5 could have accounted for the virus detection in the SSc lungs, the complete nucleotide sequence of the viral *cap* gene in the BAL fluid of three patients and three controls was verified. Sequences were identical to the prototypical European AAV5 prototype (account number Y18065.1) in both groups.

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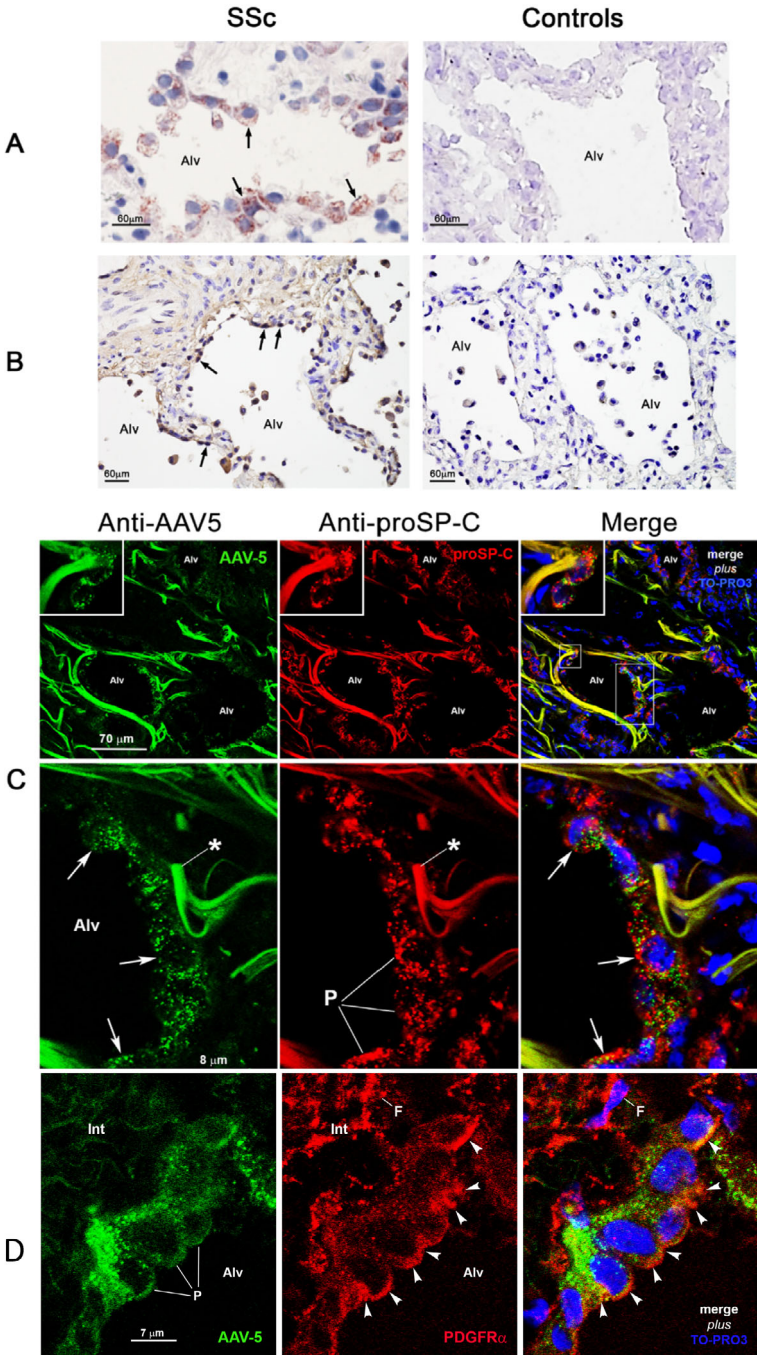

Figure 1. AAV5 and PDGFRα expression in lung tissue of patients with SSc. A representative experiment from one patient with SSc is shown. (A, left) Immunohistochemical staining of the lung of one patient with SSc shows AAV5 in cells lining the alveolar space resembling the cytologic appearance of type II pneumocytes (arrows). (A, right) No staining was detected with an isotype control antibody. (B, left) Representative microscopic images of chromogenic in situ hybridization for AAV5 was applied to paraffin-embedded lung sections from two patients with SSc and two controls. A labeled DNA probe was used to hybridize to AAV5 DNA sequence. Strong signals for AAV5 set specifically in pneumocytes (arrows). (B, right) Antisense probe was used as control. (C) A lung section from one patient with SSc is shown at low magnification. AAV5 capsid–specific staining (left panels, green) is present in several epithelial cells lining the alveolar cavities that are also positive for the type II pneumocyte marker pro-SP-C (middle panels, red). The larger area framed in the right upper panel is enlarged in the lower panels, while the smaller area is shown as insets of the upper panels. They both show at higher magnification pneumocytes positive for pro-SP-C infected by AAV5 (arrows). Asterisks (*) indicate bulk of collagen and other extracellular matrix components that exhibit autofluorescence. Semiquantitative analysis showed that 17% ± 6.2% of pro-SP-C + type II pneumocytes expressed AAV5. (D) AAV5-positive pneumocytes (left panel, green) are positive for PDGFRα (middle panel, red) that is mainly detectable on their cellular luminal side (arrowheads), indicating colocalization of the two (right panel). In the interstitial space, a fibroblast-like cell is positive for PDGFRα. AAV5, adeno-associated virus type 5; Alv, alveolar cavity; F, fibroblast-like cell; Int, interstitial space; P, pneumocytes; PDGFRα, platelet-derived growth factor receptor alpha; pro-SP-C, prosurfactant protein C; SSc, systemic sclerosis.

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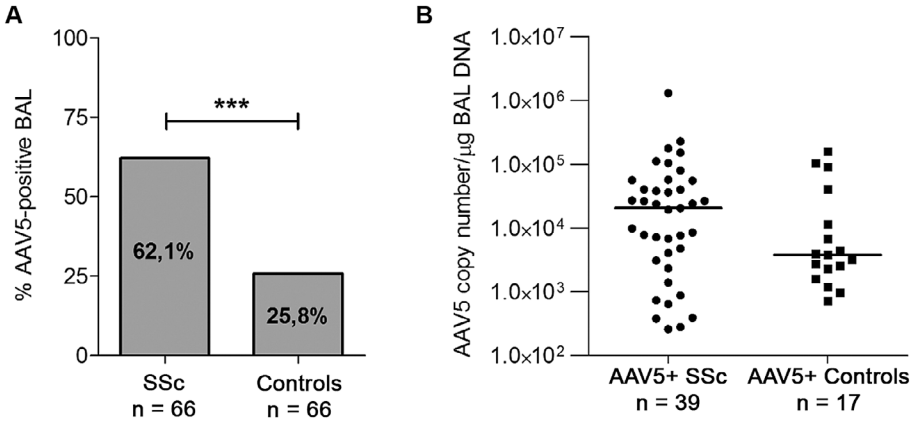

Figure 2. AAV5 genomic sequences in bronchoalveolar lavage (BAL) fluid from patients with SSc and controls. (A) Percentage of patients with SSc (n = 66) and controls with lung disease other than SSc (n = 66) positive for AAV5 DNA in cells recovered from BAL fluid. (B) AAV5 DNA copy number in cells recovered from BAL fluid of patients with SSc (n = 41) and controls (n = 17) who tested positive for AAV5. Data are expressed as a median value and a range (patients with SSc: median 20,595, range 260 to 1.3 × 10^6; controls: median 3,800, range 720 to 16 × 10^4). ***$P < 0.001$. AAV5, adeno-associated virus type 5; SSc, systemic sclerosis.

The AAV5 capsid binds human PDGFRα. The interaction between the AAV5 capsid and PDGFRα was studied through in silico molecular docking using the homology modeled three-dimensional structures of human monomeric PDGFRα[16] and the crystal structure of the AAV5 capsid monomeric subunit. It was found that the predominant binding at the external surface of the capsid with the receptor occurred at a region spanning the second and third extracellular immunoglobulin-like domains of PDGFRα (Figure 3A). These regions of the receptor discriminate agonistic from nonfunctional anti-PDGFRα antibodies that bind to the receptor's domain I of the receptor.[16] The AAV5-PDGFRα interacting sequences are shown in Supplementary Figure 6A and B.

To validate the in silico model, we analyzed the binding of PDGFRα to purified AAV5 capsid protein by surface plasmon resonance (SPR) using histidine-tagged recombinant human monomeric PDGFRα (rhPDGFRα-His) immobilized and folded into a native-like conformation.[16] The capsid protein of AAV2, a close serotype belonging to the same viral family, was tested in parallel as a control. The rhPDGFRα was found to bind AAV5 with a 370-fold higher affinity as compared to AAV2, with a faster recognition phase (higher value of association constant, k_{ass}) and a greater stability of the complex (lower value of dissociation constant, k_{diss}) (Figure 3B).

Next, the functional role of human PDGFRα in AAV5 transduction was evaluated with a CRISPR/Cas9 PDGFRα knockout variant in the A549 cell line (a type II pulmonary epithelial cell line) and related control. Transduction efficiency of the AAV5 virus expressing luciferase (AAV5-FF-Luc) in A549-PDGFRα knockout cells was significantly reduced as compared to that of A549 control or cells cotransduced with AAV2-FF-Luc (Figure 3C and D).

The type I transmembrane protein KIAA0319L Is a cellular receptor (AAVR) involved in the cellular entry of several AAV serotypes, including AAV5.[23] The comparison of binding of the predicted sites of AAV5 to AAVR[40] and PDGFRα showed that the AAV5 capsid binding site for AAVR was distinct from that used to engage rhPDGFRα (Supplementary Figure 6A and B). Furthermore, when AAVR was immobilized onto the SPR device and tested for binding to the AAV5 capsid, the binding to AAV5 of AAVR (Supplementary Figure 6C) displayed an equilibrium dissociation constant in the sub-micromolar range, similar to that reported previously for the AAV2–AAVR interaction.[23] On the other hand, the interaction between AAV5 and rhPDGFRα displayed a 100-fold higher affinity, associated with a faster kinetic association phase (Figure 3B). Taken together, these data (1) demonstrate that AAV5 binds with high-affinity PDGFRα in human cells, (2) identify the PDGFRα domains bound to the AAV5 capsid, and (3) demonstrate that human PDGFRα can be the cell entry receptor for AAV5 as indicated by colocalization studies.

Immune responses to the AAV5–PDGFRα complex. Next, we investigated whether the PDGFRα–AAV5 complex (Figure 3A) associated with anti-PDGFRα immune responses. To this aim, we took advantage of the V_HPAM-$V_κ$16F4 human monoclonal stimulatory anti-PDGFRα antibody, whose epitopes have been experimentally identified and validated.[16] V_HPAM-$V_κ$16F4 antibody, immobilized onto the SPR device to test binding to AAV5, formed a high-affinity complex with AAV5 (Figure 4A [left]), with an equilibrium dissociation constant in the sub-micromolar range and with about four-fold higher affinity than the control V_HPAM-$V_κ$16F4–AAV2 complex (Figure 4A [right]). This indicates that the stimulatory

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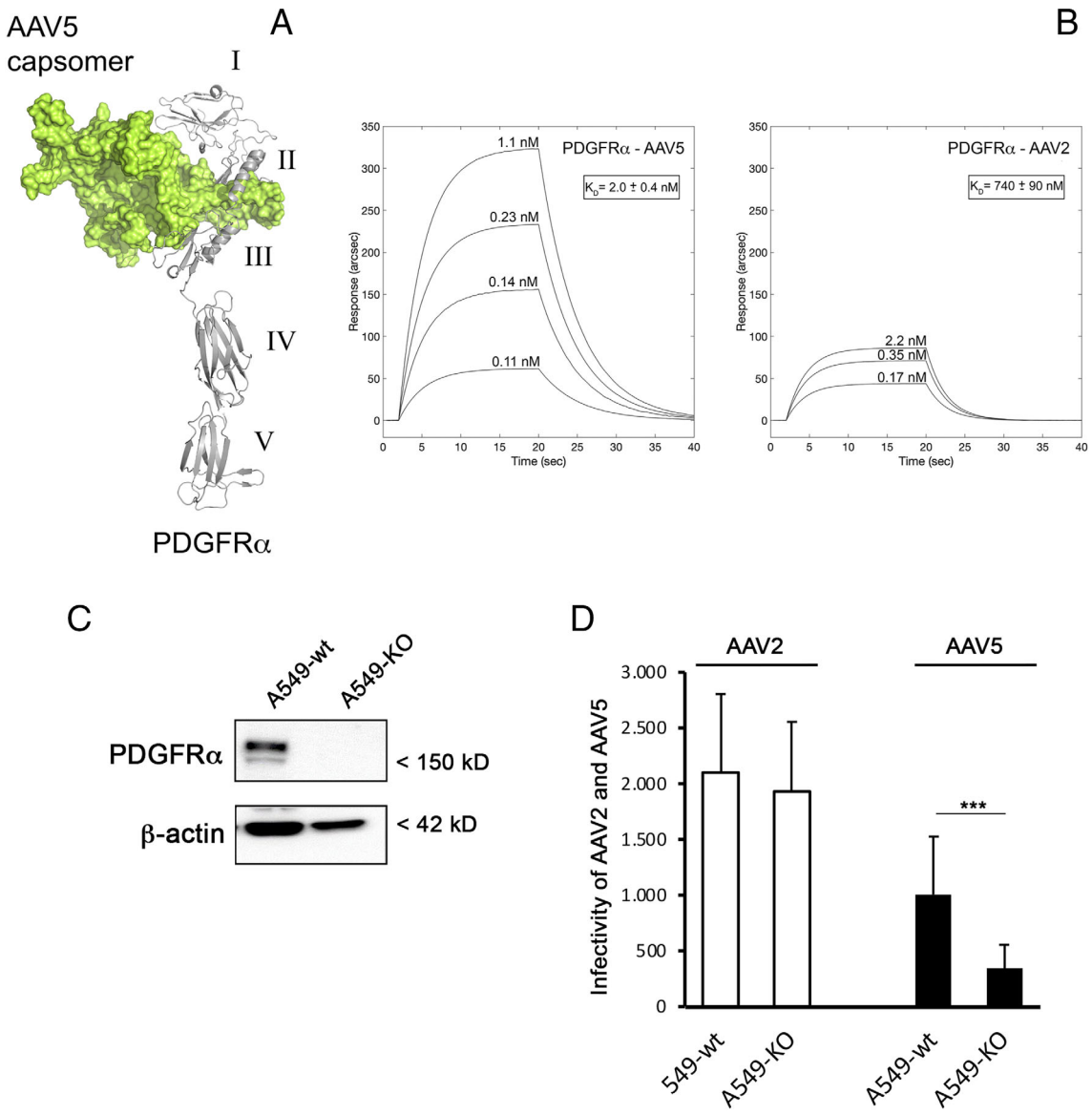

Figure 3. Binding of AAV5 to human PDGFRα. (A) Molecular docking model predicting the binding of AAV5 capsid monomeric subunit (green) to the extracellular region of monomeric PDGFRα (gray). AAV5 capsomer binding site lies between the second and third PDGFRα Ig-like extracellular domains (indicated in roman numerals from V to I). (B) Binding curves (measured in arc/seconds [arcsec] over time) of histidine-tagged monomeric recombinant human PDGFRα immobilized on the biosensor chip, after saturation with different concentrations (expressed in nanomoles [nM]) of AAV5 capsid monomeric subunits. AAV2 capsid monomeric subunits were tested as control. Dissociation constants (Kd) are indicated in the boxes. (C) The immunoblot of A549 cells before (wild-type [wt]) and after PDGFRα knockout (KO) by CRISPR/Cas9 technology. (D) AAV5 and AAV2 transduction in wt and PDGFRα KO A549 cells (A549-KO) is shown as a mean ± SD of three independent experiments. ***$P < 0.001$. AAV5, adeno-associated virus type 5; PDGFRα, platelet-derived growth factor receptor alpha.

antibody to PDGFRα $V_HPAM\text{-}V_κ16F4$ recognizes also the AAV5 capsid.

Identification of spatially contiguous epitopes of AAV5 and PDGFRα recognized by SSc autoantibodies. In silico molecular docking was optimized by restricting the AAV5 capsid–PDGFRα interaction to the extracellular domain II recognized by the agonistic antibody $V_HPAM\text{-}V_κ16F4$—and not by the control antibody $V_HPAM\text{-}V_κ13B8$. To identify the epitopes of the AAV5 capsid and PDGFRα epitopes, jointly recognized by the $V_HPAM\text{-}V_κ16F4$ antibody, we first evaluated in silico the interaction between $V_HPAM\text{-}V_κ16F4$, the AAV5 capsid, and PDGFRα focusing on the variable region (Fab) of the monoclonal autoantibody and the AAV5–PDGFRα complex (Figure 4B). This analysis showed that (1) the PDGFRα epitope recognized by $V_HPAM\text{-}V_κ16F4$ was not included in the PDGFRα domain that interacts with AAV5 and (2) the $V_HPAM\text{-}V_κ16F4$ anti-PDGFRα antibody recognized two spatially contiguous peptides formed by the

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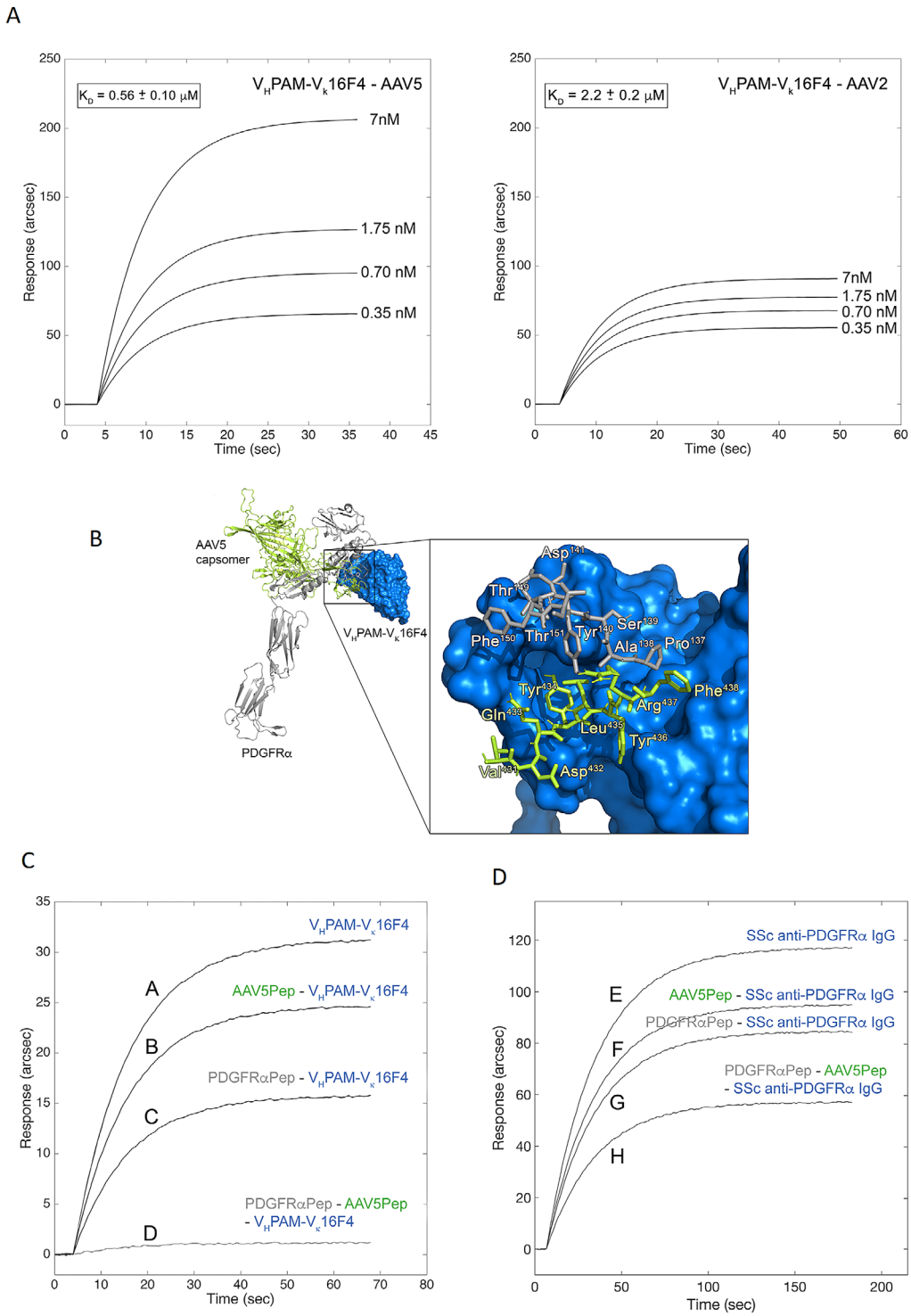

Figure 4. Immune responses against AAV5-PDGFRα spatially contiguous epitopes. (A) Binding curves (measured in arc/seconds [arcsec] over time) of $V_HPAM\text{-}V_κ16F4$ immobilized on the biosensor chip after saturation with different concentrations (expressed in nanomoles [nM]) of AAV5 (left panel) and AAV2 (right panel). Dissociation constants (Kd) are indicated in the boxes. (B) Molecular docking model predicting binding of $V_HPAM\text{-}V_κ16F4$ to the complex formed by AAV5 capsid monomeric subunit and the extracellular region of monomeric PDGFRα. The predicted epitopes of $V_HPAM\text{-}V_κ16F4$ are indicated as PDGFRα-pep (gray) and AAV5-pep (green). (C) Binding curves (measured in arc/seconds [arcsec] over time) of $V_HPAM\text{-}V_κ16F4$ antibody probing rhPDGFRα-His immobilized on the biosensor chip and saturated with AAV5 capsid monomeric subunits are shown. $V_HPAM\text{-}V_κ16F4$ binding to this complex was measured before (curve A) and after preincubation with an AAV5-peptide (curve B) or PDGFRα-peptide (curve C) or both mixed (curve D). (D) Experiment performed as in panel D but $V_HPAM\text{-}V_κ16F4$ was replaced by immunoaffinity purified SSc anti-PDGFRα antibodies. AAV5, adeno-associated virus type 5; PDGFRα, platelet-derived growth factor receptor alpha; rhPDGFRα, histidine-tagged recombinant human monomeric PDGFRα.

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amino acid sequences LYRF (derived from the AAV5 capsid) and PASY (derived from PDGFRα) with high affinity (K_d of 5.182×10^{-8} M). In addition, the V_HPAM-$V_κ$16F4 anti-PDGFRα antibody recognizes other composite peptides formed by the amino acid sequence FTVG (derived from PDGFRα), SARN ($K_d = 6.103 \times 10^{-9}$ M), and SVSA ($K_d = 3.124 \times 10^{-9}$ M) peptides derived from the AAV5 capsid (Supplementary Figure 7). In all, the same PDGFRα epitopes that bound in silico the AAV5 capsid were recognized as well by the agonistic anti-PDGFRα antibodies using different techniques (SPR, conformational PDGFRα peptide library, and single amino acid mutagenesis).[16]

To confirm the in silico predictions, the rhPDGFRα-coated surface of the biosensor was saturated with AAV5 before addition of the monoclonal antibody V_HPAM-$V_κ$16F4. The results showed the formation of a receptor–capsid ternary complex (Figure 4C, curve A). To rule out that V_HPAM-$V_κ$16F4 binding was limited to PDGFRα, peptide sequences corresponding to the predicted AAV5 (VDQYLYRF) and PDGFR (PASYDTFT) binding domains of V_HPAM-$V_κ$16F4 were synthesized and preincubate—alternatively or in combination—with V_HPAM-$V_κ$16F4 prior to the addition of the rhPDGFRα–AAV5 complex. Notably, both peptides, independently, partially inhibited V_HPAM-$V_κ$16F4 binding (Figure 4C, curves B and C) and completely abrogated the binding of the antibody when combined (Figure 4C, curve D). Similar results were obtained when V_HPAM-$V_κ$16F4 was replaced by the immunoaffinity-purified anti-PDGFRα serum antibodies from patients with SSc (the polyclonal nature of these antibodies explains the partial inhibition of the binding to the peptides) (Figure 4D). Together, these data indicate that anti-PDGFRα autoantibodies purified from the sera of patients with SSc as well the cloned V_HPAM-$V_κ$16F4 PDGFRα autoantibody react to both human PDGFRα and the AAV5 capsomere.

DISCUSSION

This study shows that (1) PDGFRα allows AAV5 internalization in cells (Figure 1 and 3C and D); (2) a high percentage of patients (62.1%) with SSc with ILD harbor AAV5 in the lung (Figure 2); and (3) a spatially contiguous epitope composed of a stretch of amino acids of PDGFRα and the AAV5 capsid is recognized by antibodies in vivo that can target PDGFRα (Figure 4).

The presence of AAV5 in BAL fluid of 25.75% of the controls with different lung diseases confirms the notion that AAV5 is endemic in the human population, as was also indicated by the presence of anti-AAV5 neutralizing antibodies and circulating INF-γ + T cells that react to AAV5 in 30% and 24% of healthy donors, respectively.[41] AAV5 is a nonpathogenic and poorly immunogenic virus. Although it does not directly participate in the pathogenesis of SSc, our data suggest that it can contribute to the generation of neoantigens, some of which can target the immune reactivity to PDGFRα.

After binding to a primary receptor, the capsid protein of AAVs interacts with a co-receptor which leads to virus internalization via endocytosis. Primary cell surface receptors for AAV5 include heparin sulfate proteoglycans, N-terminal galactose, and N- or O-linked sialic acid moieties. Secondary receptors for AAV5 are PDGFRα and AAVR, as also shown here.[23,42] Our data confirm previous findings[23] that demonstrate that AAVR (the KIAA0319L transmembrane protein) is a receptor for multiple AAV serotypes, including AAV5. Although in our experiments KIAA0319L displayed a lower affinity to AAV5 capsid compared to human PDGFRα (Supplementary Figure 6C), a single nucleotide polymorphism in the *KIAA0319L* locus, rs2275247, was strongly associated with systemic lupus erythematosus, scleroderma, and higher expression of the receptor.[43]

The fact that the KIAA0319L sequence of amino acids recognizing the AAVR capsid is distinct from the peptide bound to human PDGFRα (Supplementary Figure 6B) suggests that the capsid can bridge a connection between PDGFRα and the KIAA0319L receptor. This trimeric complex sustained by the viral capsid may be recognized as an exogenous protein and thus targeted by immune cells.

The association between viral (exogenous) and endogenous (PDGFRα) antigens has also been implicated in other human infections. For example, cytomegalovirus (CMV), a ubiquitous human herpesvirus, requires PDGFRα to induce a robust inflammatory response,[10] and it has also been linked to SSc.[44,45] It remains to be solved whether the heterogeneous phenotypes of SSc depend directly or indirectly on the virus involved—CMV or AAV5 or other AAV serotypes—or whether CMV is only one of the helper viruses necessary for a productive AAV5 replication.

The finding that 28.3% of our cohort of patients with SSc did not have AAV5 sequences could be explained by an on-and-off viral replication and by the consideration that only patients with evidence of ILD underwent BAL for ethical reasons. These aspects do not allow us at present to formally establish whether the viral infection would occur before or after clinical evidence of the disease.

Another finding that deserves an explanation is the longer duration of disease in patients with AAV5-positive BAL fluid. We believe that the protracted systemic disease, characterized by increased secretion of disease-related cytokines and growth factors,[46] stimulated the expression of PDGFRα in the lungs, increasing chances of local infection or reactivation of AAV5. Incidentally, the virus might also be present in other locations, driving as well the production of antibodies in patients with AAV5-negative BAL fluid as shown by seven patients with SSc who did not have AAV5 in the BAL fluid but tested positive for the virus in the peripheral blood.

We propose that high expression of PDGFRα may facilitate AAV5 infection and (re)activation, possibly with helper virus co-infections, and this (wherever occurring) can induce, in

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genetically susceptible individuals, self-reactive immune responses to PDGFRα epitopes physically associated with the virus capsid and AAVR (as indicated by the co-presence of anti-PDGFRα and anti-AAV5 antibodies; Supplementary Figure 8). Of note, the anti-PDGFRα antibodies recognizing the II domain of the receptor (the activation loop physically linked to the capsid)[16] are biologically active, present at high levels in patients with early disease,[15] and capable of inducing the fibrotic phenotype in patients with SSc.[15-19] However, we cannot rule out that AAV5 can contribute to the development of fibrosis by directly activating macrophage-like interstitial cells (Figure 1).

The remarkable finding here is also the identification of nonlinear epitopes spanning the sequences of two cell receptors, PDGFRα, KIAA0319L, and the AVV5 capsid. The spatial organization of PDGFRα sequences, exposing its II activating domain in this trimeric complex, explains the generation of stimulating autoantibodies of PDGFRα. These antibodies isolated from patients with SSc have been cloned and functionally tested in vivo and in vitro. They induce reactive oxigen species and fibrosis in vivo[17] and in vitro[19] through PDGFRα activation.[16] Our data imply that SSc antibodies recognize a neoantigen composed of two peptides derived from two different proteins, the AAV5 capsid and PDGFRα (Figure 4A, C, and D). The structure of the complex is better defined by in silico modeling (Figure 4B), which shows that the AAV5 peptide interacts with the second extracellular domain of PDGFRα in proximity to PDGFRα epitopes targeted by anti-PDGFRα autoantibodies (Figure 4B).

We acknowledge two limitations of this study: (1) the limited number of controls and patients, which prevents a conclusive subgroup analysis; and (2) the impossibility, for ethical reasons, of performing BAL in patients without HRCT evidence of ILD. While our results do not indicate that AAV5 is the cause of scleroderma, they suggest that AAV5 favors the formation and presentation of a peculiar and composite peptide complex containing epitopes from different proteins, PDGFRα, AAVR, and the viral capsid, which stabilizes this complex. Due to the physical proximity of the capsid sequences to cell proteins, PDGFRα or KIAA0319L, it is likely that immune cells primarily recognize the abundant capsid segment and inefficiently the endogenous cellular proteins (PDGFRα or KIAA0319L) associated with the capsid, probably due to high levels of the capsid. Reduction of the capsid peptide levels with time would increase the visibility of the trimeric complex (PDGFRα–capsid–KIAA0319L), which is recognized as a viral antigen by immune cells and consequently enhances the immune response. The appearance of agonistic anti-PDGFRα antibodies in genetically susceptible individuals is the result of the trimeric complex, which exposes the second domain of the receptor (Figure 4B) that is necessary for its activation.[16] This scenario also could explain why AAV5-positive BAL fluid is associated with longer disease duration.

This model excludes molecular mimicry of viral and endogenous proteins as an underlying mechanism inducing autoimmunity. Instead, as shown in Figure 4B, the formation of a single unique complex formed by viral and endogenous protein epitopes would facilitate the emergence of reactive epitopes. Spatial association of different and noncontiguous epitopes maintained by viral protein(s) has been suggested by other autoimmune diseases. For example, it has recently been reported that Epstein-Barr virus (EBV) infection dramatically increases the odds of developing multiple sclerosis,[47] and while this has been attributed to molecular mimicry between EBV and the glial cell adhesion molecule (GlialCAM) that is the target of autoantibodies,[48,49] it can also be speculated that the GlialCAM peptide(s) might be able to form a complex with viral proteins and the entry receptor (Eph and Eph-receptor binding proteins, ephrins).[50]

In conclusion, the results from the study presented here point to a new understanding of some aspects of the pathogenesis of SSc and autoimmune diseases that could have diagnostic and therapeutic implications to assess with subsequent work.

ACKNOWLEDGMENTS

We thank Professor Rosaria Gesuita (Università Politecnica delle Marche, Ancona, Italy) for statistical analysis; Professor Michael S. Chapman and Dr. O. Davulcu (Oregon Health & Science University, Portland) for the bacterially expressed fusion protein of polycystic kidney disease domains 1–5 of AAVR described by Pillay et al[17]; Dr. Lorena Zentilin (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) for AAV5 and AAV2 capsids; and Dr. Gianni Di Maio for his help in the preparation of the manuscript.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gabrielli had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Moroncini, Svegliati, La Cava, Avvedimento, Gabrielli.

Acquisition of data. Grieco, Paolini, Agarbati, Spadoni, Pinto, Chen, Benfaremo, Tonnini, Senzacqua, Alizzi, Viola, Amico, Galgani, Gasparini, Zuccatosta, Menzo, Müller, Leinschmidt, Giordano, Amoroso.

Analysis and interpretation of data. Moroncini, Svegliati, Cuccioloni, Mozzicafreddo, Amoresano, Nieto, Finke, Funaro, Giordano, La Cava, Dorfmüller, Pucci, Pezone, Avvedimento, Gabrielli.

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