MATRIX PATHOBIOLOGY

Ex Vivo Pathogenicity of Anti–Laminin γ 1 Autoantibodies

Florina Florea,^{*} Claudia Bernards,[†] Marzia Caproni,[‡] Jessika Kleindienst,^{*} Takashi Hashimoto,[§] Manuel Koch,[†] and Cassian Sitaru^{*†}

From the Department of Dermatology,^{*} University Medical Center Freiburg, and the Center for Biological Signaling Studies (BIOSS),[¶] University of Freiburg, Freiburg, Germany; the Institute for Dental Research and Oral Musculoskeletal Biology,[†] Center for Biochemistry, and Center for Molecular Medicine Cologne (CMMC), Medical Center, University of Cologne, Cologne, Germany; the Section of Dermatology,[‡] Department of Surgery and Translational Medicine, University of Florence, Florence, Italy; and the Department of Dermatology,[§] Kurume University School of Medicine, Kurume, Japan

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Address correspondence to
Cassian Sitaru, Ph.D., M.D.,
Universitäts-Hautklinik,
Hauptstr. 7, 79104 Freiburg,
Germany. E-mail: cassian@mail.sitaru.eu

Autoimmunity against laminins has been described in several autoimmune diseases (including mucous membrane pemphigoid, anti–laminin γ 1 pemphigoid, and connective tissue diseases), in pregnancy loss, and in infections such as Chagas disease. Except for anti–laminin-332 mucous membrane pemphigoid, adequate evidence has been lacking for the tissue injury potential of laminin-specific antibodies and the pathogenic epitopes. We evaluated the pathogenic potential of antibodies targeting laminin γ 1, a major constituent of basement membranes and the main antigen in anti–laminin γ 1 pemphigoid. Rabbit antibodies were generated against fragments of the N-terminus and C-terminus of murine laminin γ 1, and their ability to disrupt ligand interactions and/or to activate complement and granulocytes was assessed using previously established *ex vivo* assays. Our findings document a pathogenic potential of antibodies targeting the laminin γ 1 N-terminus. These antibodies interfere with the binding of nidogen to laminin and can activate granulocytes and the complement cascade. We detected antibodies with different degrees of reactivity with laminin γ 1 N-terminus in patients with anti–laminin γ 1 pemphigoid, cutaneous lupus erythematosus, and scleroderma. Our results provide mechanistic insights into the tissue damage associated with laminin autoimmunity and could facilitate development of appropriate diagnostic tools and therapeutic strategies. (*Am J Pathol* 2014, 184: 494–506; <http://dx.doi.org/10.1016/j.ajpath.2013.10.019>)

Autoimmunity is characterized by a failure or breakdown of self-tolerance mechanisms, leading to a B-cell and/or T-cell–mediated immune response directed against the self that can induce tissue damage and result in autoimmune disease. Among the immunological players involved in autoimmune disease development, the inappropriate production of autoantibodies that mediate tissue injury plays a role in several of the more than 80 described autoimmune diseases.¹ The interplay between antibodies and self-antigen, either circulating or tissue-bound, may induce damage by several effector mechanisms, including inflammatory and noninflammatory complement-mediated and Fc-mediated reactions, as well as Fc-independent noninflammatory pathways.

Antibodies bound to self-antigens from cell surfaces can trigger tissue damage by Fc-mediated or complement-mediated mechanisms, including induction of cell phagocytosis, cell lysis, and recruitment and activation of inflammatory cells, as is described in autoimmune diseases such as autoimmune

hemolytic anemia, rheumatoid arthritis, myasthenia gravis, and bullous pemphigoid. Antibodies may, however, exert direct pathogenic effector functions in an Fc-independent manner, including stimulation of the bound receptor (as in Graves' disease) or disruption of protein–protein interactions through steric hindrance (as in pemphigus).

The *in vivo* pathogenicity of autoantibodies in several autoimmune disorders has been demonstrated by the passive transfer of antigen-specific IgG from patients or immunized animals.¹ *Ex vivo* models have also proved useful in confirming the tissue damage-inducing potential of antibodies in autoimmune diseases, including skin blistering disorders, multiple sclerosis, neuromyelitis optica, and antiphospholipid syndrome.^{2–5}

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Autoantibodies against laminins have been reported in several human diseases, including anti-laminin-332 mucous membrane pemphigoid,² anti-laminin γ 1 pemphigoid (previously known as anti-p200 pemphigoid),⁶ lupus erythematosus,^{7,8} systemic sclerosis and Raynaud phenomena,⁹ cardiomyopathy and myocarditis,¹⁰ endometriosis and pregnancy loss,¹¹ and Chagas disease.¹² Laminin γ 1 is an extracellular protein present in all basement membranes as a constitutive chain of almost all laminin isoforms. It has a critical role in embryogenesis; its absence in mice results in lack of basement membrane formation and death at the peri-implantation stage of embryonic development.¹³ Through its short arm located at the N-terminus, laminin γ 1 binds nidogen, which cross-links the two meshworks of laminins and collagen IV within the basement membrane.¹⁴ Laminin γ 1 and γ 2 chains are critically involved in laminin recognition by integrins through their C-terminal regions.¹⁵ Integrins mediate the adhesive interactions of laminin with cells, which are fundamental for tissue development, differentiation, and function.

In the skin, laminin γ 1 is present as a constituent of laminin-311, -321, and -511, which can bind to integrins α 3 β 1, α 6 β 4, and α 6 β 1.¹⁶ It has been recently shown that serum of anti-laminin γ 1 pemphigoid patients reacts mainly with the C-terminus of laminin γ 1, but epitopes located outside this fragment may also be targeted.^{6,17,18} Anti-laminin γ 1 pemphigoid is an autoimmune subepidermal blistering disease characterized by circulating and tissue-bound antibodies and a neutrophil-rich inflammatory infiltrate.⁶ Interestingly, despite the widespread distribution of the autoantigen, the pathology remains organ-specific. The pathogenic potential of antibodies targeting laminin-111 is still poorly understood. Immunization with the autoantigen and passive transfer of specific antibodies in animal models had little effect or (depending on the experimental setting) induced limited renal disease, myositis, abortion, or reproductive failure.^{19–23}

Because a skin-specific effect had not yet been demonstrated for autoantibodies against laminin γ 1,¹⁸ in the present study we addressed the *ex vivo* pathogenic potential of laminin γ 1-specific antibodies. For this purpose, rabbit antibodies were generated against N- and C-terminal fragments of mouse laminin γ 1. Subsequently, their direct effect on

nidogen and integrin interactions was investigated in a cell adhesion assay and a solid-phase ligand inhibition and disruption assay. Furthermore, the ability of the newly generated antibodies to activate the complement system and/or inflammatory cells was assessed in a complement fixation test and antibody-induced granulocyte-dependent dermal–epidermal separation assay or by measurement of reactive oxygen species (ROS) production. Our findings show that anti-laminin γ 1 antibodies, especially those targeting the N-terminus, have pathogenic potential either through direct inhibition and/or disruption of the nidogen 1 binding or by Fc-mediated activation of granulocytes and complement cascade. Interestingly, autoantibodies against laminin γ 1 N-terminus were detected not only in cases of anti-laminin γ 1 pemphigoid, but also in cutaneous lupus erythematosus (CLE) and scleroderma.

Materials and Methods

Proteins

Recombinant human laminin-511 was purchased from Bio-Lamina (Stockholm, Sweden), natural mouse laminin-111 from Life Technologies–Invitrogen (Darmstadt, Germany; Carlsbad, CA), and recombinant human α 6 β 4 from R&D Systems (Minneapolis, MN). Human laminin γ 1 N-terminus was expressed in a mammalian system as described previously.²⁴ The murine laminin γ 1 fragments and nidogen 1 were recombinantly expressed in a mammalian or a bacterial system, as described below.

Heterologous Expression of Recombinant Forms of Murine Laminin γ 1 and Nidogen 1

The DNA sequence data for murine laminin γ 1 was retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/nuccore>, accession number NM_010683) using the accession number NM_010683. The cDNA sequence encoding mouse laminin γ 1 N-terminal fragment [mLn γ 1-Nterm, amino acids (aa) 30–988] was synthesized from total RNA (SuperScript III kit; Life Technologies–Invitrogen) using primers as presented in Table 1 and cloned via NheI/BamHI restriction sites into a modified pCEP-Pu expression vector containing a thrombin

Table 1 Primer Sequences for PCR Amplification of cDNA Fragments of Murine Laminin γ 1 and Nidogen 1

Fragment	Restriction site	Primer sequence
mLn γ 1-Nterm	NheI BamHI	Forward: 5′-CACGCTAGCAGCCATGGACGAGTGCGCGGAT-3′ Reverse: 5′-TAGGATTCCCGACGTGTGGGCCTAGGAAC-3′
mLn γ 1-Cterm-1	BamHI SalI	Forward: 5′-GATCGGATCCTTCTACAATCGGTCCTGGCCTG-3′ Reverse: 5′-GATCGTCGACTCAAATCTCCAGCGCAGTTTGA-3′
mLn γ 1-Cterm-2	BamHI SalI	Forward 5′-GATCGGATCCGCAGGAGAAAAATCAAACCTGCGC-3′ Reverse: 5′-GATCGTCGACTCAGGCGTCAGCGGCAGCATTTG-3′
mLn γ 1-Cterm-3	BamHI SalI	Forward: 5′-GATCGGATCCAACCGGACCATAGCTGAAGCC-3′ Reverse: 5′-GATCGTCGACTCAGGGCTTCTCGATGGACGG-3′
Nidogen 1	NheI NotI	Forward: 5′-AAAGCTAGCCTGAATCGCCAGGAGCTCTTCCC-3′ Reverse: 5′-CTGACGTAACCTTGCTTTACTCGCCGGCGTTAT-3′

cleavage site next to a 2×StrepII/FLAG tag at the C-terminal end of the protein sequence.²⁵ 293-EBNA cells were transfected with the expression vector, and the supernatant was collected and supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (Sigma-Aldrich, Munich, Germany; St. Louis, MO). After filtration, the supernatant was passed through a Strep-Tactin Sepharose column (IBA, Göttingen, Germany), and the recombinant protein was eluted with buffer [100 mmol/L Tris, 150 mmol/L NaCl (pH 7.4)] containing 2.5 mmol/L D-desthiobiotin (Sigma-Aldrich). For the removal of the 2×StrepII/FLAG tag, the purified glycosylated protein (Supplemental Figure S1) was incubated with 1 U bovine thrombin (Sigma-Aldrich) per milligram protein overnight, at room temperature [(in Tris-buffered saline with 5 mmol/L CaCl₂ (pH 8.2))]. Afterward, the protein was applied on a Strep-Tactin Sepharose column and the digested protein was collected in the flow-through.

The cDNA sequences coding for the three overlapping fragments of murine laminin γ 1 C-terminus (aa 1018–1607) were cloned into prokaryotic vectors and expressed in *Escherichia coli* as glutathione *S*-transferase (GST)–fusion proteins according to protocols described previously.²⁶ Synthesis of the primers used in PCR (Table 1), as well as cloning of the cDNA encoding the fragments into pUC57 vector, was performed at GenScript (Piscataway, NJ). The cDNA sequences were subcloned into the linearized pGEX-6P-1 vector (Amersham; GE Healthcare, Freiburg, Germany; Little Chalfont, UK) using the BamHI/SalI cutting site, resulting in the recombinant vectors pGEX-LAMC1-Cterm-1, pGEX-LAMC1-Cterm-2, and pGEX-LAMC1-Cterm-3. The correct ligation and in-frame insertion of various DNA fragments were confirmed by DNA sequence analysis performed by GATC Biotech (Konstanz, Germany). Recombinant GST-fusion proteins were expressed in *E. coli* strain BL21 and purified by glutathione agarose affinity chromatography, as described previously.²⁶

The DNA sequence data for murine nidogen 1 was retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>, accession number NP_035047.2). The full-length murine nidogen 1 cDNA sequence obtained by reverse transcription from total RNA (SuperScript III kit, Life Technologies–Invitrogen) was cloned into a modified pCEP-Pu vector containing a thrombin cleavage site next to a 2×StrepII/FLAG tag at the N-terminal end of the protein sequence and expressed in 293-EBNA cells. Primer sequences are presented in Table 1. Protein concentrations were determined by spectrophotometry at 280 nm (NanoDrop 1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA).

Immunoblot Analysis

Recombinant 2×StrepII/FLAG–mLn γ 1-Nterm, GST-tagged C-terminal fragments (GST-mLn γ 1-Cterm-1, GST-mLn γ 1-Cterm-2, GST-mLn γ 1-Cterm-3), and 2×StrepII/FLAG–nidogen 1 were fractionated by SDS-PAGE, transferred onto nitrocellulose, and analyzed by immunoblotting.^{25,26} For

detection of the GST-tagged fragments, after blocking, the membrane was incubated for 2 hours at room temperature with goat polyclonal antibody specific to GST (GE Healthcare) diluted 3000-fold. Subsequently, the bound goat IgG was detected with 5000-fold diluted horseradish peroxidase (HRP)–labeled antibody (Promega, Mannheim, Germany; Madison, WI) and diaminobenzidine. GST alone was used as control. For detection of recombinant 2×StrepII/FLAG–nidogen 1, the membrane was incubated with murine monoclonal antibody specific to StrepII tag (IBA) diluted 2000-fold, for 1 hour at room temperature. Next, the murine antibody was detected with a 3000-fold diluted HRP-coupled antibody (Dako, Hamburg, Germany; Carpinteria, CA), and the signal was visualized with chemiluminescence substrate. The recombinant 2×StrepII/FLAG–fusion mLn γ 1-Nterm and the tag-free mLn γ 1-Nterm were detected using the 1000-fold diluted serum of rabbits immunized with mLn γ 1-Nterm, for 1 hour at room temperature, followed by incubation with the 3000-fold diluted HRP-coupled antibody specific to rabbit IgG (Dako) and then signal detection by chemiluminescence.

Antibodies

Rabbit polyclonal antibodies were produced against the recombinant mLn γ 1-Nterm fragment and the GST-tagged mLn γ 1-Cterm-1, -2, and -3 fragments according to established protocols.²⁷ Rabbit polyclonal antibodies were also generated against the recombinant GST-fusion protein containing sequences of murine collagen VII (GST-mCVII-1, aa 1-300 and GST-mCVII-2, aa 281-594)²⁶ and used as controls in several experiments, as specified.

Affinity Purification of IgG

IgG from rabbit serum was purified by protein G Sepharose 4 fast flow affinity column chromatography (GE Healthcare), as described previously.²⁷ The eluted fractions were concentrated by ultrafiltration using Amicon Ultra-15 (15 mL, 30 kDa) centrifugal filter units (EMD Millipore, Billerica, MA) in a centrifuge at 3600 $\times$ g and 4°C, 45 minutes per cycle. Protein concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Human Sera

Serum samples were obtained from patients with anti-laminin γ 1 pemphigoid ($n = 7$), CLE (discoid CLE, $n = 13$; subacute CLE, $n = 2$; acute CLE, $n = 4$), dermatomyositis ($n = 9$), systemic sclerosis, ($n = 10$); and localized scleroderma, ($n = 14$). Anti-laminin γ 1 pemphigoid patients were characterized by i) blisters on the skin; ii) linear deposits of IgG at the dermal–epidermal junction (DEJ), as observed under direct immunofluorescence (IF) microscopy; iii) circulating IgG binding the dermal side of the 1 mol/L NaCl-split skin, as observed under indirect IF microscopy; and iv) immunoblot reactivity with the

200-kDa protein from dermal extracts. Normal control serum samples were obtained from healthy donors ($n = 20$). Informed consent was obtained from patients whose material was used in the study, in adherence with Helsinki principles.

Complement Fixation Test

The ability of the antibodies to activate the complement cascade was assessed *in vitro* by a complement fixation test, according to described protocols.²⁶ In brief, cryosections (6 μ m thick) of normal mouse skin were incubated with normal rabbit serum, with rabbit serum raised against mLn γ 1-Nterm (diluted 1:10) or against GST-mLn γ 1-Cterm-1, -2, or -3 (diluted 1:2), or with purified rabbit IgG antibodies targeting the C-terminal fragments of laminin γ 1 (diluted 1:10), followed by incubation with serum from a healthy human donor fivefold diluted in gelatin Veronal buffer (Sigma-Aldrich) as a source of complement. Rabbit polyclonal antibody targeting GST-mCVII-2 (diluted 1:10) was used as control. Bound human complement C3 was subsequently detected with 100-fold diluted fluorescein isothiocyanate-labeled antibody (Cappel; MP Biomedicals, Eschwege, Germany; Santa Ana, CA). Sections were counterstained with 1000-fold diluted DAPI (Sigma-Aldrich).

Ex Vivo Cryosection Assay

The blister-inducing potential of antibodies targeting different fragments of murine laminin γ 1 was determined by an *ex vivo* assay of antibody-induced granulocyte-dependent dermal–epidermal separation, as described previously.²⁶ In brief, cryosections of murine skin were incubated with rabbit serum against mLn γ 1-Nterm (diluted 1:5), GST-mLn γ 1-Cterm-1, -2, or -3 (diluted 1:2) or with purified rabbit IgG specific to mLn γ 1-Nterm (diluted 1:10), GST-mLn γ 1-Cterm-1, -2, or -3 (diluted 1:5). Rabbit polyclonal antibody specific to GST-mCVII-1 (diluted 1:5) was used as control. Leukocytes isolated from healthy human donors were added to cryosections and incubated at 37°C for 3 hours. The extent of dermal–epidermal separation was assessed under a microscope and quantified as the percentage of the split area at the DEJ relative to the total length of the DEJ.

Solid-Phase Binding Assay

Recombinant mLn γ 1-Nterm [5 μ g/mL in 0.1 mol/L carbonate buffer (pH 9.3)] was coated onto 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) overnight, at 4°C. The wells were then blocked with 1% bovine serum albumin (BSA) (fraction V; Sigma-Aldrich) in PBS and subsequently incubated with serial dilutions of recombinant mouse 2 $\times$ StrepII/FLAG-fusion nidogen 1. The amount of bound nidogen was detected with 5000-fold diluted HRP-tagged mouse antibody against StrepII tag (AbD Serotec, Kidlington, UK; Raleigh, NC).

For enzymatic reaction, 50 μ L of orthophenylenediamine (Sigma-Aldrich) solution in water and 0.1% (v/v) H₂O₂ was added to each well. The reaction was stopped after 10 minutes with 50 μ L/well of 0.5 mol/L H₂SO₄, and absorbance at 490 nm was read using a microplate reader (Labsystems Multiskan Multisoft; Thermo Fisher Scientific).

For the inhibition assay, mLn γ 1-Nterm-coated wells were first incubated with serial dilutions of rabbit serum reactive with mLn γ 1-Nterm, patient's serum, or normal rabbit or human serum for 1 hour at room temperature. Subsequently the recombinant 2 $\times$ StrepII/FLAG-tagged nidogen 1, 3.5 nmol/L in blocking buffer was added to the wells and the plate was incubated for 2 hours at room temperature. After washing, the bound nidogen was detected with 5000-fold diluted HRP-tagged antibody against StrepII tag (AbD Serotec).

For the disruption of protein binding assay, wells were first incubated with nidogen 1, followed by incubation with serial dilutions of rabbit serum against mLn γ 1-Nterm or normal rabbit serum; subsequently, the nidogen 1 was detected. Inhibition and disruption were calculated as percentages, relative to uninhibited controls.

For integrin binding inhibition assay, published protocols were followed, with minor modification.²⁸ In brief, 10 nmol/L recombinant human laminin-511 was coated onto 96-well microtiter plates in 0.1 mol/L carbonate buffer (pH 9.3) overnight, at 4°C. Wells were washed with PBS, blocked with 1% BSA in PBS, and then incubated with a 25-fold diluted mix of rabbit serum specific to GST-mLn γ 1-Cterm-1, -2, or -3 or with normal rabbit serum in blocking buffer. After washing with PBS containing either 1 mmol/L MnCl₂ and 2 mmol/L MgCl₂ or 10 mmol/L EDTA, 80 nmol/L recombinant human integrin α 6 β 4 (R&D Systems) in blocking buffer was added in the presence of 2 mmol/L MgCl₂ and 1 mmol/L MnCl₂ or of 10 mmol/L EDTA and incubated for 2 hours at room temperature. After washing, the bound integrin α 6 β 4 was detected by a 3000-fold diluted HRP-labeled rabbit polyclonal anti-His antibody (Novus Biologicals, Littleton, CO). Readings obtained in the presence of 10 mmol/L EDTA were subtracted. The binding of integrin was calculated as percentage of the value obtained in the absence of rabbit serum.

Cell Culture and Cell Adhesion Assay

HaCaT immortalized human keratinocytes (Dr. Petra Boucamp, DKFZ, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (Biochrom; Merck Millipore, Billerica, MA). Cell adhesion assay was performed according to published protocols, with minor modification.²⁹ In brief, 1 μ g/well of recombinant human laminin-511 or BSA was coated onto 96-well, non-tissue-treated microtiter plates (Nalge Nunc International, Roskilde, Denmark; Penfield, NY). After blocking for 2 hours at 4°C with 1% BSA in PBS, HaCaT

cells were added to the wells as a single-cell suspension at a density of 1×10^5 cells per well in serum-free medium supplemented with 2 mmol/L MgCl_2 and 1 mmol/L MnCl_2 , followed by incubation for 60 minutes at 37°C in the presence of 5% CO_2 . For inhibition assays, a 25-fold diluted mix of rabbit sera raised against the three GST-mLn γ 1-Cterm fragments, or normal rabbit serum, or the monoclonal antibody 4C7 (Merck Millipore) (50- or 100-fold diluted) was incubated with laminin-511-coated wells for 60 minutes at room temperature before incubation with cells. The 4C7 antibody can interfere with cell attachment by interacting with the laminin $\alpha 5$ LG domain.³⁰ After washing, cells were fixed with 3% formalin solution and stained with 0.1% crystal violet. Adhesion was quantified by lysing the adherent cells with 2% SDS solution and determining the amount of released dye by reading the absorbance at 550 nm with a microtiter plate reader. Readings obtained for nonspecific cell adhesion in the presence of BSA were subtracted. Cell attachment was expressed as percentage of the adhesion in the absence of rabbit serum.

Binding of Anti-Laminin γ 1 Antibodies in Murine Skin

Organ culture of skin explants was performed according to published protocols, with minor modification.³¹ In brief, punch biopsies (0.5 cm $\times$ 0.5 cm) of mouse tail were incubated for 12 hours at room temperature or at 37°C with 300 μL of rabbit serum specific to mLn γ 1-Nterm or normal rabbit serum diluted 1:10 in culture medium. The culture medium consisted of Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% (v/v) fetal bovine serum (FBS), 4 mmol/L L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all from Biochrom). After a washing, the samples were frozen and stained for the detection of rabbit IgG deposition by IF microscopy.

For antibody injection into mice, 6- to 8-week-old BALB/c female mice with a body weight of approximately 17 g were obtained from Charles River Laboratories International (Wilmington, MA). All injections were performed on mice narcotized by inhalation of isoflurane or subcutaneous administration of a mixture of 100 $\mu\text{g}/\text{g}$ ketamine and 15 $\mu\text{g}/\text{g}$ xylazine. The experiments were approved by the local Animal Care and Use Committee (G-02/2012) and were performed by certified personnel. Mice were injected subcutaneously twice with 15 mg of purified rabbit IgG specific to mLn γ 1-Nterm ($n = 5$) or with normal rabbit IgG ($n = 3$). Skin samples were frozen in Tissue-Tek OCT optimal cutting temperature compound (Sakura Finetek Europe, Zoeterwoude, Netherlands) and analyzed by direct IF microscopy for IgG deposition.

Enzymatic Deglycosylation of Recombinant Mouse Laminin γ 1 N-Terminus

Analysis of post-translational glycosylation of the recombinant murine 2 $\times$ StrepII/FLAG-laminin γ 1 N-terminus expressed in a eukaryotic system was performed by enzymatic deglycosylation in 200 mmol/L ammonium hydrogen phosphate buffer.

Protein (10 μg) was incubated with 500 units PNGase F (New England Biolabs, Ipswich, MA) overnight, at 37°C . The deglycosylated protein was analyzed on 12% SDS-PAGE gel under reducing conditions and was detected by immunoblotting with polyclonal rabbit antibodies specific to mLn γ 1-Nterm.

Indirect IF Microscopy

Detection of rabbit antibodies by indirect IF microscopy was performed according to established protocols, with minor modification.^{27,32} In brief, cryosections of normal mouse or human skin were incubated first with 100-fold diluted rabbit serum or 10-fold diluted Biotin-tagged rat anti-laminin γ 1 monoclonal antibody (Thermo Fisher Scientific), followed by incubation with 100-fold diluted Alexa Fluor 488-labeled goat antibody against rabbit IgG or Alexa Fluor 488-labeled streptavidin (both from Life Technologies—Invitrogen). For unmasking cryptic epitopes, cryosections were treated with 6 mol/L urea in 0.1 mol/L glycine (pH 2.2), for 5 minutes on ice, before incubation with rabbit serum, according to published protocols.³² Images were acquired using a Nikon Eclipse 80i microscope equipped with a Nikon DS-Fi1 camera and Nikon NIS-Elements D software (F 4.00.06).

Measurement of Circulating Serum Autoantibody Reactivity by ELISA

Reactivity of circulating antibodies from rabbit serum with laminin-111 and -511 was tested by enzyme-linked immunosorbent assay (ELISA) assay according to published protocols, with minor modification.²⁶ In brief, 96-well plates (Greiner Bio-One) were coated overnight with 500 ng/well of native or heat-denatured (30 minutes, at 80°C) mouse laminin-111 (Life Technologies—Invitrogen) or recombinant human laminin-511 (Biolamina, Sundbyberg, Sweden) in 0.05 mol/L carbonate-bicarbonate buffer (pH 9.3). After blocking, the wells were incubated with 100-fold or serially diluted rabbit serum and bound IgG antibodies were subsequently detected with a 5000-fold diluted HRP-labeled chicken antibody (Novus Biologicals).

For detection of circulating antibodies from patient's serum, ELISA plates were coated with 500 ng/well of recombinant human or mouse laminin γ 1 N-terminus and subsequently incubated with 50-fold diluted human serum. Bound antibodies were detected with a 5000-fold diluted HRP-labeled goat antibody (Abcam, Cambridge, UK). For enzymatic reaction, 50 μL of orthophenylenediamine (Sigma-Aldrich) solution in water and 0.1% (v/v) H_2O_2 was added to each well. The reaction was stopped after 10 minutes with 50 $\mu\text{L}/\text{well}$ of 0.5 mol/L H_2SO_4 . Absorbance at 490 nm was read using a microplate reader (Labsystems Multiskan Multisoft; Thermo Fisher Scientific).

ROS Measurement by Luminol Chemiluminescence

Measurement of ROS production was performed in a luminol assay according to published protocols.³¹ Plate-bound rabbit

immune complexes were formed using the recombinant mLn γ 1-Nterm or natural mouse laminin-111 and serum from rabbits immunized against the laminin γ 1 fragments. In brief, 96-well microtiter plates were coated with 10 μ g of mouse laminin-111 (Life Technologies—Invitrogen) or 1 μ g of recombinant mouse mLn γ 1-Nterm in 0.1 mol/L carbonate buffer (pH 9.3). The wells were washed three times with PBS (pH 7.2) containing 0.05% Tween 20, after each step. After blocking for 1 hour with PBS–Tween 20 containing 1% BSA (fraction V; Sigma-Aldrich), wells were incubated for 2 hours with several dilutions of rabbit serum against mLn γ 1-Nterm (100-, 200-, 400-fold diluted) or a mix of rabbit sera targeting GST-mLn γ 1-Cterm-1 and GST-mLn γ 1-Cterm-3 (25-, 50-, and 100-fold diluted). Human leukocytes were isolated from the peripheral blood of healthy donors. After 3% dextran sedimentation, the erythrocytes were lysed using a hypotonic solution of 0.2% NaCl. The leukocytes were further washed in Dulbecco's modified Eagle's medium (Lonza) without supplements and were resuspended at a final density of 3×10^6 cells/mL. Cell viability was tested with Trypan Blue, and only preparations with viability greater than 95% were used. ROS production was measured using luminol chemiluminescence. The leukocytes were preincubated for 5 minutes at 37°C with 150 μ mol/L luminol (Sigma-Aldrich), and then 100 μ L of the cell suspension was added to each well. All procedures were performed in the dark. Chemiluminescence intensity was recorded continuously for 1 hour. Kinetic measurement of ROS production in leukocytes stimulated with 500 nmol/L phorbol 12-myristate 13-acetate (Sigma-Aldrich) was performed as control.

In Silico Analysis

Amino acid sequences of murine and human laminin γ 1 were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide>, accession numbers NP_034813.2 and NM_002284.3), and sequence alignment was performed using an algorithm developed by Smith and Waterman.³³ *In silico* prediction of linear B-cell epitopes was performed using publicly available software (<http://tools.immuneepitope.org/tools/bcell>, last accessed June 26, 2012) that computes an antigenic score based on a prediction algorithm developed by Kolaskar and Tongaonkar,³⁴ and CBTOPE³⁵ (<http://www.imtech.res.in/raghava/cbtope>, last accessed October 22, 2012) was used for prediction of conformational B-cell epitopes of a protein from its amino acid sequence. The sequence corresponding to the short arm is longer than the one for the α -helical C-terminus domain, and therefore the number of epitopes was calculated per 100 amino acid residues.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 6 (GraphPad Software, San Diego, CA). Statistical differences were calculated using two-tailed, paired Student's *t*-test. Data were considered significantly different if $P < 0.05$.

Results

Expression of Recombinant Laminin γ 1 and Nidogen 1 Forms

The mLn γ 1-Nterm fragment (Figure 1 and Supplemental Figure S1) and nidogen 1 (Supplemental Figure S2) were recombinantly expressed in 293-EBNA cells, using a modified eukaryotic expression vector pCEP-Pu (Table 1). The 2 $\times$ StrepII/FLAG-fusion recombinant proteins were purified by Strep-Tactin Sepharose affinity chromatography. The tag of the recombinant mLn γ 1-Nterm fragment was subsequently removed by thrombin digestion. The cDNA sequences coding for the three C-terminal fragments of murine laminin γ 1 were cloned into prokaryotic expression vector pGEX-6P-1 and expressed in *E. coli* (Table 1 and Figure 1A). The GST-fusion mLn γ 1-Cterm-1, -2, and -3 recombinant fragments were purified by glutathione-affinity chromatography. With SDS-PAGE separation, all proteins

Figure 1 Characterization of recombinant murine laminin γ 1 fragments. **A:** Laminin γ 1 consists of a short arm at its N-terminus, containing globular structures (IV, VI) alternating with rod-like domains composed of epidermal growth factor-like repeats (III, V), and an α -helical domain at its C-terminus (I, II) that forms the coiled-coil domain by binding other laminin chains. The sequence corresponding to mLn γ 1-Nterm fragment was cloned into a modified pCEP-Pu vector and expressed in 293-EBNA cells; the fragments of the C-terminus corresponding to the α -helical domain were cloned into pGEX-6P-1 and expressed in *E. coli*. Amino acid residue numbers are indicated above the fragments. **B:** When separated by 10% SDS-PAGE, recombinant GST-tagged mLn γ 1-Cterm-1, -2, and -3 fragments (lanes 2, 3, and 4, respectively) and recombinant mLn γ 1-Nterm (lane 5) migrated consistently to their calculated molecular masses. Migration positions of the molecular weight marker (lane 1) are indicated on the left. **C:** Recombinant mLn γ 1 fragments were electrophoretically transferred to nitrocellulose. GST-tagged recombinant mLn γ 1-Cterm-1, -2, and -3 fragments (lanes 1, 2, and 3, respectively) were detected with goat antibody specific to GST. GST (lane 4) was used as control. The 2 $\times$ StrepII/FLAG-fusion mLn γ 1-Nterm fragment (lane 5) and the tag-free mLn γ 1-Nterm fragment (lane 6) were detected with rabbit antibodies specific to mLn γ 1-Nterm. Migration positions of molecular weight markers are indicated on the left.

migrated consistently with their calculated molecular masses (Figure 1B and Supplemental Figure S2B). With immunoblotting, the GST-tagged recombinant forms of laminin γ 1 C-terminus were stained by an anti-GST antibody, similar to the GST used as control (Figure 1C). The 2 $\times$ StrepII/FLAG-fusion nidogen 1 (Supplemental Figure S2C) was detected by an anti-StrepII antibody, and the generated polyclonal rabbit antibodies were used for detection of the 2 $\times$ StrepII/FLAG-fusion mLn γ 1-Nterm and tag-free mLn γ 1-Nterm (Figure 1C).

In Silico Analysis of Antigenicity of Murine Laminin γ 1

For the murine and human forms of laminin γ 1 (with a length of 1607 and 1609 amino acids, respectively), alignment analysis using the Smith–Waterman algorithm indicated an identity of 92.9% and positivity of 96.7%. The epitopes targeted by antibodies from anti-laminin γ 1 pemphigoid patients have been suggested to be located mainly within the C-terminus of the protein.^{6,17} By *in silico* prediction analysis, B-cell epitopes are distributed throughout the entire mouse laminin γ 1, with an average antigenic score slightly higher for the short arm, compared with the C-terminus forming the coiled-coil domain (Supplemental Table S1 and Supplemental Figure S3). Kolaskar and Tongaonkar antigenicity analysis predicted 42 linear epitopes for the N-terminus sequence (aa 1–1028) and 18 for the C-terminus (aa 1029–1607).³⁴ With CBTOPE used to predict conformational epitopes, 67 probable epitope residues were found for the N-terminus and 19 for the C-terminus.³⁵ The sequence for the nidogen binding site (domain III 3–5, aa 769–932) contains six linear antigenic determinants and five probable conformational epitopes.

Characterization of Reactivity and Specificity of Antibodies Generated against Laminin γ 1

The specificity of antibodies from immune rabbit serum was determined by indirect IF microscopy on normal mouse skin and ELISA using native and heat-denatured mouse laminin-111. Serum from rabbits immunized with the recombinant mLn γ 1-Nterm exhibited linear binding at the DEJ and also staining of dermal blood vessels (Figure 2A), similar to the monoclonal rat anti-laminin γ 1 antibody used as control (Figure 2E). Circulating antibodies from rabbits immunized against the three fragments of C-terminus laminin γ 1 chain (GST-mLn γ 1-Cterm-1, -2, and -3) weakly stained the DEJ and dermal blood vessels (Figure 2, B–D). By ELISA, rabbit serum raised against C- or N-terminal fragments of laminin γ 1 exhibited different degrees of reactivity against mouse laminin-111 (Figure 2F).

Murine and human laminin γ 1 are highly homologous and share predicted B-cell epitopes to a large extent (Supplemental Table S2). We used indirect IF to further test the cross-reactivity of the newly generated antibodies against murine laminin γ 1 with the human protein on cryosections of normal human skin. With one exception (rabbit serum raised

Figure 2 Characterization of rabbit antibodies specific to laminin γ 1 fragments by indirect IF microscopy and ELISA. **A–E:** Cryosections of murine skin were incubated with serum from rabbits immunized with mLn γ 1-Nterm (**A**), GST-mLn γ 1-Cterm-1 (**B**), GST-mLn γ 1-Cterm-2 (**C**), or GST-mLn γ 1-Cterm-3 (**D**), followed by staining with anti-rabbit IgG Alexa Fluor 488-labeled secondary antibody (**E**). Skin basement membrane and dermal capillaries are indicated by arrows. **F:** Serial dilutions of rabbit serum were tested by ELISA using murine laminin-111 as a substrate. Rabbit serum raised against C- or N-terminal fragments of laminin γ 1 differed in degree of reactivity against mouse laminin-111. Data are expressed as means $\pm$ SD. Experiments were conducted in duplicate. Original magnification, $\times$ 400.

against mLn γ 1-Cterm-2), all the sera exhibited linear binding in the human epidermal basement membrane and also in the walls of dermal blood vessels (Figure 3, A–D).

Laminin γ 1 C-Terminus-Specific Antibodies React with Cryptic Epitopes

The C-terminus of laminin γ 1 consists of an α -helix that trimerizes into a coiled coil, and we therefore assessed the availability of the epitopes within the C-terminus for rabbit antibodies by comparing reactivity with the native and denatured forms of laminin. Indirect IF microscopy of murine skin cryosections revealed that the serum raised against laminin γ 1 C-terminal fragments stained stronger the skin treated with acid urea for unmasking epitopes than the native skin (Supplemental Figure S4, A and B). Similarly, ELISA revealed a higher reactivity with heat-denatured laminin-111 or -511 than with the native proteins (Supplemental Figure S4, C and D).

Laminin γ 1 N-terminus-Specific Antibodies Bind in Mouse Skin

A prerequisite for developing a mouse model for antibody-induced skin disease is binding of antibodies in the skin. We

Figure 3 Cross-reactivity of rabbit antibodies specific to murine laminin $\gamma 1$ fragments with the human protein by indirect IF microscopy. **A–D:** Cryosections of human skin were incubated with serum from rabbits immunized with mLn $\gamma 1$ -Nterm (**A**), GST-mLn $\gamma 1$ -Cterm-1 (**B**), GST-mLn $\gamma 1$ -Cterm-2 (**C**), or GST-mLn $\gamma 1$ -Cterm-3 (**D**). After a PBS washing, the respective sections were incubated with anti-rabbit IgG Alexa Fluor 488–labeled secondary antibody. Skin basement membrane and dermal capillaries are indicated by **arrows**. Original magnification, $\times 400$.

therefore assessed whether the laminin $\gamma 1$ N-terminus–specific antibodies are able to bind in the mouse skin both in an *ex vivo* model of skin organ culture and *in vivo*. Incubation of normal mouse skin explants with anti–laminin $\gamma 1$ N-terminus–specific rabbit serum led to the deposition of rabbit IgG at the DEJ and in the walls of dermal blood vessels (**Figure 4A**). The same binding pattern was observed after injection of purified rabbit antibodies into normal mice (**Figure 4C**), but no binding was observed for normal rabbit IgG (**Figure 4, B and D**).

Laminin $\gamma 1$ N-Terminus–Specific Antibodies Interfere With the Ligand Function of Nidogen 1 Binding of Laminin $\gamma 1$

We further asked whether antibodies against laminin $\gamma 1$ influence its binding to nidogen 1, a well-described ligand for laminins containing the $\gamma 1$ chain. The interaction site is located within a single epidermal growth factor–like domain from the N-terminal part of laminin $\gamma 1$. Indeed, the recombinant purified mLn $\gamma 1$ -Nterm fragment bound with high-affinity nidogen 1 in our solid-phase assay (**Figure 5A**). In this system, antibodies generated against mLn $\gamma 1$ -Nterm inhibited the binding of nidogen 1 to mLn $\gamma 1$ -Nterm in a concentration-dependent manner (**Figure 5B**). These antibodies also exhibited a concentration-dependent ability to disrupt binding of nidogen to mLn $\gamma 1$ -Nterm, although to a lesser extent than observed in the inhibition assay (**Figure 5C**).

Antibodies Specific to Laminin $\gamma 1$ C-Terminus Do Not Interfere With Integrin-Mediated Adhesion to Laminin

The binding of laminin to integrins through the LG domains, which plays a major role in cell adhesion, is

modulated by the C-terminal fragment of laminin $\gamma 1$ chain.¹⁵ We therefore addressed the question of whether the antibodies targeting the C-terminus of laminin $\gamma 1$ inhibit laminin–integrin interaction. In a solid-phase assay, a mix of the antibodies targeting laminin $\gamma 1$ C-terminal fragments did not influence the binding of integrin $\alpha 6 \beta 4$ to laminin-511 (**Figure 6A**). Furthermore, we observed no significant inhibition of HaCaT cell adhesion on recombinant human laminin-511 in the presence of the same antibodies (**Figure 6B**).

Laminin $\gamma 1$ –Specific Antibodies Activate Complement *ex Vivo*

To test the Fc-dependent pathogenic potential of antibodies against laminin $\gamma 1$, we assessed the ability of skin-bound antibodies to activate complement, using an *ex vivo* complement fixation assay. Ln $\gamma 1$ -Nterm–specific rabbit antibodies bound to normal mouse skin were able to activate complement in the walls of dermal blood vessels and, to a lesser extent, at the DEJ (**Figure 7A**). In contrast, no complement activation ability was detected for the antibodies specific to any of the three GST-mLn $\gamma 1$ -Cterm fragments (**Figure 7, B–D**). Antibodies raised against collagen VII, used as a positive control, exhibited linear fixation of complement at the epidermal basement membrane (**Figure 7E**). No complement activation was observed in samples incubated with normal rabbit serum (**Figure 7F**).

Laminin $\gamma 1$ –Antibody Complexes Elicit ROS Production in Granulocytes

In a further set of experiments, we tested whether laminin $\gamma 1$ –specific antibodies from immune complexes are able to

Figure 4 Laminin $\gamma 1$ -Nterm–specific antibodies bind in murine skin explants and *in vivo*. **A and C:** After incubation of murine skin samples with anti-mLn $\gamma 1$ -Nterm rabbit serum, the antibodies stained the DEJ and dermal vessel walls (**A**). The same antibodies bound in a similar pattern in the skin after injection in mice (**C**). **B and D:** Normal rabbit serum (NRS) exhibited no binding in the skin samples (**B**) or *in vivo* (**D**). Skin basement membrane and dermal capillaries are indicated by **arrows**. Original magnification, $\times 400$.

Figure 5 Binding of nidogen 1 to mouse laminin γ 1 N-terminus and interference of specific antibodies with the binding. **A:** Serial dilutions of recombinant 2 $\times$ StrepII/FLAG-fusion nidogen 1 were incubated with 500 ng/well of laminin γ 1-Nterm or BSA coated onto 96-well microtiter plates. Binding was detected by an ELISA-based solid-phase assay with an antibody against StrepII tag. **B:** Recombinant mLn γ 1-Nterm (500 ng/well) coated onto 96-well microtiter plates was incubated first with serial dilutions of rabbit serum anti-mLn γ 1-Nterm (RSA-mLn γ 1-Nterm) or the same dilutions of NRS, followed by incubation with 3.5 nmol/L mouse nidogen 1–2 $\times$ StrepII/FLAG. The bound nidogen was detected with an anti-StrepII tag antibody. **C:** Recombinant mLn γ 1-Nterm (500 ng/well) coated onto 96-well microtiter plates was incubated first with 3.5 nmol/L mouse nidogen 1–2 $\times$ StrepII/FLAG. Subsequently, serial dilutions of rabbit serum anti-mLn γ 1-Nterm or the same dilutions of NRS were added. Mouse nidogen 1 was detected with an anti-StrepII tag antibody. Data are expressed as means $\pm$ SD. Experiments were conducted in triplicate. OD, optical density.

elicit ROS production in granulocytes isolated from healthy human donors. Immobilized immune complexes formed by antibodies specific to mLn γ 1-Nterm bound to mouse laminin-111 or recombinant mLn γ 1-Nterm coated onto ELISA plates were able to activate human granulocytes, thereby inducing the release of ROS, as detected by chemiluminescence assay. In contrast, rabbit sera raised against the GST-mLn γ 1-Cterm fragments failed to induce ROS production in the same assay (Figure 8).

Laminin γ 1-Specific Antibodies Induce Granulocyte-Dependent Dermal–Epidermal Separation

Clinical and laboratory findings in patients suggest that, to fully unfold their pathogenic potential, autoantibodies against laminin γ 1 may need to recruit and activate inflammatory cells, in addition to their complement-fixing capacity. We therefore investigated the ability of laminin γ 1-specific antibodies to induce granulocyte-dependent dermal–epidermal separation in an *ex vivo* assay, using sections of normal skin incubated with antibodies and normal leukocytes. When coincubated with leukocytes purified from healthy donors, antibodies specific to the N-terminus of laminin γ 1 bound at the murine DEJ induced subepidermal splits (Figure 9A), but to a lesser extent than the antibodies against collagen VII used as control (Figure 9E). In contrast, no blister formation was observed in sections incubated with antibodies against the C-terminal fragments (Figure 9, B–D).

Figure 6 Interference of laminin γ 1-specific antibodies on laminin–integrin interaction and cell adhesion. **A:** ELISA wells coated with 10 nmol/L laminin-511 were first incubated with a 25-fold diluted mix of rabbit antibodies against GST-mLn γ 1-Cterm-1, -2, and -3 (RSA-mLn γ 1-Cterm) or with NRS; subsequently, recombinant integrin α 6 β 4 was added in the presence of 2 mmol/L MgCl₂ and 1 mmol/L MnCl₂, or 10 mmol/L EDTA. The bound integrin was detected by incubation with an HRP-labeled rabbit polyclonal anti-His antibody. OD readings obtained in the presence of EDTA were subtracted as background. Binding was expressed as a percentage relative to results obtained in the absence of rabbit antibodies. **B:** Recombinant laminin-511 or BSA (1 μ g/well) was immobilized on microtiter plates and further incubated with a 25-fold diluted mix of rabbit antibodies against GST-mLn γ 1-Cterm-1, -2, and -3 (RSA-mLn γ 1-Cterm), with NRS, or with 50-fold diluted monoclonal antibody 4C7. HaCaT cells were then allowed to adhere to the wells for 60 minutes at 37°C in the presence of 2 mmol/L MgCl₂ and 1 mmol/L MnCl₂, followed by staining with crystal violet. Readings obtained for the nonspecific cell adhesion in the presence of BSA were subtracted. Cell adhesion was expressed as a percentage, relative to results obtained in the absence of rabbit antibodies. Data are expressed as means $\pm$ SD. Experiments were conducted in duplicate (A) or triplicate (B).

Figure 7 Complement fixation ability of laminin γ 1-specific rabbit serum. Cryosections of normal mouse skin were incubated with rabbit serum; fresh normal human serum was added, as a source of complement. Human C3 was detected with a fluorescein isothiocyanate-labeled antibody. The sections were counterstained with DAPI. **A:** Antibodies against mLn γ 1-Nterm were able to activate the human complement in dermal blood vessels and, to a lesser extent, linearly along the DEJ. **B–D:** In contrast, no complement activation ability was detected for antibodies specific to GST-mLn γ 1-Cterm-1, -2, or -3 fragments. **E:** Serum from a rabbit immunized against GST-mCVII-2 strongly fixed the complement linearly along the DEJ. **F:** No complement activation was observed on the cryosections incubated with NRS. Skin basement membrane and dermal capillaries are indicated by arrows. Original magnification, $\times 400$.

Laminin γ 1 N-Terminus-Reactive Antibodies in Patients with Anti-Laminin γ 1 Pemphigoid and Collagenoses

Although most of the antibodies in anti-laminin γ 1 pemphigoid patients react with the C-terminus of laminin γ 1, reactivity with epitopes within the N-terminus is also reported.¹⁸ We identified serum from one patient with significant reactivity in ELISA to both human (Supplemental Figure S5) and mouse recombinant laminin γ 1 N-terminus. In the laminin–nidogen binding assay, the serum did not interfere with the binding of nidogen to laminin (data not shown). Circulating antibodies targeting the N-terminus of laminin γ 1 were detected by ELISA in 3/19 (16%) patients with cutaneous lupus erythematosus (2 discoid CLE; 1 subacute CLE) and in 2/14 (8%) patients with scleroderma (1 systemic sclerosis; 1 localized scleroderma) (Supplemental Figure S5).

Discussion

Laminin γ 1 has been recently described as the autoantigen in anti-laminin γ 1 pemphigoid, a rare autoimmune bullous

disease of the skin. In addition, autoantibodies against laminin-111 with unclear pathogenic relevance have been reported in patients with lupus erythematosus,^{7,8} systemic sclerosis, and Raynaud phenomena,⁹ cardiomyopathy and myocarditis,¹⁰ endometriosis,¹¹ and Chagas disease.¹² Anti-laminin γ 1 pemphigoid is characterized by subepidermal blistering, a neutrophil-dominated inflammatory infiltrate in the upper dermis, and tissue-bound and circulating serum antibodies binding the skin basement membrane. Interestingly, indirect IF microscopy revealed that the antibodies label only the DEJ; no staining of the vessel basement membrane was observed. Furthermore, despite the widespread distribution of laminin γ 1, the lesions are restricted to skin and mucous membranes.⁶ In skin, laminin γ 1 is incorporated in laminin-321/311 and laminin-511; the latter is well expressed in hair follicles, but also in dermal vessels, in addition to the DEJ. It has been proposed that laminin γ 1-specific antibodies mediate blistering by interfering with laminin–integrin interaction.^{6,17,18} Because their pathogenic potential had not yet been demonstrated, in the present study we investigated the pathogenic potential of laminin γ 1-specific antibodies.

Wet-laboratory epitope mapping studies indicate a distribution of antigenic determinants throughout the entire laminin γ 1. Although epitopes recognized by antibodies from anti-laminin γ 1 pemphigoid patients cluster within the C-terminus of laminin γ 1 chain, few epitopes were shown to reside within the N-terminal domain.^{6,17,18} Immunochemical analysis of laminin-111 identified important antigenic activity for fragments located outside the C-terminus.^{36,37} Our *in silico* analysis indicated that linear and conformational antigenic determinants are distributed throughout the entire

Figure 8 Antibody-elicited production of ROS measured by chemiluminescence. Immobilized immune complexes were formed by rabbit antibodies with mouse laminin-111 or mLn γ 1-Nterm coated onto ELISA plates. Granulocytes isolated from healthy human donors were preincubated with luminol and subsequently added to the wells. Chemiluminescence intensity was recorded continuously for 1 hour. Immune complexes formed by rabbit antibodies specific to mLn γ 1-Nterm and recombinant mLn γ 1-Nterm (mLn γ 1-Nterm+RSa-mLn γ 1-Nterm), or with laminin-111 (In111+RSa-mLn γ 1-Nterm), elicited ROS production, whereas no release of ROS was detected in the laminin-111-coated wells incubated with a mix of rabbit antibodies targeting GST-mLn γ 1-Cterm-1 and -3 (In111+RSa-mLn γ 1-Cterm). No activation of granulocytes was observed in wells incubated with NRS (mLn γ 1-Nterm+NRS, In111+NRS). Data are expressed as means $\pm$ SD. Experiments were conducted in duplicate. RLU, relative luminescence units.

Figure 9 Granulocyte activation ability of laminin $\gamma 1$ -specific rabbit serum. Frozen sections of mouse skin were incubated with rabbit serum and, after washing, leukocytes isolated from healthy human donors were added. **A–D:** Rabbit antibodies specific to mLny1-Nterm bound to normal mouse skin recruited and activated granulocytes, inducing subepidermal splits (**A**). In contrast, no blister formation was observed on sections incubated with rabbit serum against GST-mLny1-Cterm-1 (**B**), GST-mLny1-Cterm-2 (**C**), or GST-mLny1-Cterm-3 (**D**). **E:** Percentage of dermal-epidermal separation (DES). Data are expressed as means $\pm$ SEM. Data represent multiple experiments [cryosections incubated with rabbit serum raised against GST-mCVII-1 (RSa-mCVII-1, $n = 18$); rabbit antibody against mLny1-Nterm (RIGa-mLny1-Nterm, $n = 18$), GST-mLny1-Cterm-1 (RIGa-mLny1-Cterm-1, $n = 16$), GST-mLny1-Cterm-2 (RIGa-mLny1-Cterm-2, $n = 16$), or GST-mLny1-Cterm-3 (RIGa-mLny1-Cterm-3, $n = 16$); and NRS ($n = 15$).] $^{**}P < 0.01$.

murine laminin $\gamma 1$, with the short arm harboring more epitopes, compared with the C-terminus forming the coiled-coil domain. We observed reactivity with the N-terminus of laminin $\gamma 1$ in 1/7 (14%) patients with anti-laminin $\gamma 1$ pemphigoid and in 5/52 (10%) patients with connective tissue diseases (CLE, systemic sclerosis, and localized scleroderma).

Because the relevant contribution to tissue damage of the antigenic determinants within the N-terminal laminin $\gamma 1$ had not been investigated previously, we addressed the pathogenic potential of antibodies directed against epitopes located within both the N- and the C-terminus of laminin $\gamma 1$. The newly generated laminin $\gamma 1$ -specific antibodies exhibited different degrees of reactivity with the skin basement membrane and dermal blood vessels (as indicated by indirect IF microscopy) and with laminin-111 (as indicated by ELISA). A weaker binding in the skin was

observed for the antibodies raised against the C-terminal domain of laminin $\gamma 1$, perhaps because of lower recognition of the native conformation of laminins (detected also by ELISA). As suggested by our *in silico* antigenicity analysis of human and murine laminin $\gamma 1$, the antibodies cross-reacted with the human antigen, as indicated by both indirect IF microscopy and ELISA. Subsequently, the laminin $\gamma 1$ fragment-specific antibodies were characterized with regard to their ability to mediate tissue damage through Fc-dependent and Fc-independent pathways.

By interacting with other components of the basement membrane, laminins play both an architectural and a functional role. We therefore addressed the ability of the antibodies to directly interfere with the ligand function of laminin. Nidogen 1 and 2 have been shown to connect the laminin network with the collagen IV meshwork in basement membranes, along with perlecan. The high-affinity binding site is located within the domain III 3–5 of the short arm of laminin $\gamma 1$,¹⁴ and its important developmental role has been demonstrated in mice, in which ablation of this site led to renal and lung basement membrane defects.³⁸ Additionally, Breitzkreutz et al³⁹ reported inhibition of basement membrane formation by a nidogen-binding recombinant $\gamma 1$ chain fragment in skin organotypic cocultures. After treatment of the coculture with this fragment, they observed an increased tendency for splitting at the tissue interface. Furthermore, early treatment with the recombinant fragment diminished or abolished deposition of nidogen and laminin $\gamma 1$, whereas a delayed treatment (after the basement membrane was formed) led to an almost complete, but reversible, loss of nidogen and laminin $\gamma 1$. Consistent with such report, in the present study the antibodies raised against the N-terminus of laminin $\gamma 1$ were able to inhibit and disrupt the interaction of nidogen with laminin. Taken together, the findings suggest that this Fc-independent potential of anti-laminin $\gamma 1$ short-arm antibodies to induce tissue damage might unfold during basement membrane remodeling in both physiological and pathological processes. In our small cohort of patients with anti-laminin $\gamma 1$ pemphigoid, we found only one serum with significant reactivity with human and murine laminin $\gamma 1$ N-terminus. This serum did not interfere with the binding of nidogen to laminin. A possible explanation is the low reactivity with epitopes located within the nidogen binding site.

Laminins have a crucial functional role through cell receptor-mediated mechanisms. By interacting with skin integrins such as $\alpha 6\beta 4$ and $\alpha 3\beta 1$, laminin-511 is a potent adhesive ligand for keratinocytes.²⁸ The interaction site maps to the C-terminal globular domain of laminin α chain, but binding of the integrin requires heterotrimerization with β and γ chains. Furthermore, the C-terminal region of laminin $\gamma 1$ is important in modulating integrin-laminin-511 interaction through the third glutamic acid residue, although not directly binding to integrin.¹⁵ In one study most antibodies from anti-laminin $\gamma 1$ pemphigoid patients reacted with the 246-amino-acid C-terminus of laminin $\gamma 1$

chain, and it has been speculated that the mechanism of blister formation might rely on their interference with laminin–integrin binding.⁶ In the present study, however, the laminin γ 1 C-terminus–specific antibodies inhibited neither keratinocyte attachment to laminin-511–coated wells, nor the laminin-511–integrin α 6 β 4 interaction.

A well-described mechanism of tissue damage by antibodies is their ability to trigger activation of the innate immune system, including the complement cascade and inflammatory cells. In the present study, direct IF microscopy on perilesional skin of anti–laminin γ 1 pemphigoid patients revealed deposition of C3 at the DEJ. Furthermore, human and animal anti–laminin-111 antibodies are able to fix complement *in vitro*.^{40,41} Our present findings show that antibodies specific to laminin γ 1 are able to activate complement *ex vivo*, to different extents depending on their molecular fine specificity. In the complement fixation test, the complement was activated mainly in dermal blood vessels, and to a lesser extent at the DEJ of mouse skin, suggesting a possible involvement in vascular pathology. Of note, anti–laminin-111 antibodies have been reported in vasculitis patients.⁴² Both the lack of antibody deposition in blood vessels from most anti–laminin γ 1 pemphigoid patients and the skin-specific pathology remain puzzling. Although Dainichi et al⁶ reported one patient with evident reactivity with laminin from the skin blood vessels, most of the sera reacted with dermal rather than blood-vessel laminin. As Dainichi et al⁶ speculated, tissue-specific differences in post-translational modifications of the γ 1 chain, such as N-glycosylation or laminin trimer conformation, might account for differing antibody reactivity in these patients. Although binding of laminin γ 1 N-terminus–specific antibodies in blood vessels might not be relevant for pathogenesis of anti–laminin γ 1 pemphigoid, it might have relevance for other autoimmune disorders, including vasculitis and connective tissue diseases, and its contribution to tissue damage should be addressed in further studies.

Inflammatory infiltrates dominated by neutrophils are a common finding in anti–laminin γ 1 pemphigoid patients, and we therefore assessed the ability of the laminin γ 1–specific antibodies to activate leukocytes. Immune complexes formed by N-terminus–specific antibodies with the targeted antigen elicited ROS production of granulocytes, as shown by luminol assay. In addition, skin-bound antibodies were to some extent able to induce dermal–epidermal separation by activating human leukocytes. The tissue injury might therefore be triggered by antibody-mediated activation of leukocytes and subsequent release of proteases and/or NADPH oxidase, as shown in several inflammatory skin blistering diseases.² Consistent with recently published findings,¹⁸ antibodies targeting laminin γ 1 C-terminal fragments were not able to induce ROS production, nor did they induce split formation in skin cryosections.

Both the antibody-dependent dermal–epidermal separation assay and the complement fixation test require an initial binding step of antibodies in the skin cryosections. The

differences in the Fc-dependent pathogenic potential of N- and C-terminus–specific antibodies in both the cryosection assay and the complement binding test may rely on their different affinity for the native skin laminins. However, two recent studies showed that purified laminin γ 1 C-terminus–specific patient or rabbit IgG could not induce dermal–epidermal separation *ex vivo*, nor complement activation and blistering *in vivo*, although it bound in the skin basement membrane.^{18,43} Taken together, these results suggest that other factors, in addition to poor binding, might account for the lack of pathogenicity of these antibodies.

Human anti–laminin γ 1 antibodies have low availability and are therefore not well suited for use in animal models, which normally require injections of high amounts of specific antibodies over a long period of time. Antibodies of rabbit origin are an appropriate alternative for animal model studies. Laminin γ 1 N-terminus–specific antibodies have pathogenic potential *ex vivo* and, because they bind at the DEJ and in dermal blood vessels in murine skin, they can be used to establish an animal model of laminin autoimmunity.

In conclusion, the present results demonstrate a pathogenic potential of anti–laminin γ 1 antibodies *ex vivo*. The epitopes relevant to tissue damage are located within the N-terminal part of the γ 1 chain, and both Fc-mediated and Fc-independent pathways appear to be involved. Thus, antibodies targeting laminin γ 1 N-terminus strongly bind in murine skin and are able to inhibit and disrupt the binding of nidogen 1, and also to activate leukocytes and the complement. These results extend our understanding of the pathogenesis of diseases associated with laminin autoimmunity and should greatly facilitate the development of appropriate diagnostic tools and therapeutic strategies.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.10.019>.

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