From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

blood

Prepublished online November 9, 2007;
doi:10.1182/blood-2007-05-092825

Autoantibodies to the C-terminal subunit of RLIP76 induce oxidative stress and endothelial cell apoptosis in immune-mediated vascular diseases and atherosclerosis

Paola Margutti, Paola Matarrese, Fabrizio Conti, Tania Colasanti, Federica Delunardo, Antonella Capozzi, Tina Garofalo, Elisabetta Profumo, Rachele Rigano, Alessandra Siracusano, Cristiano Alessandri, Bruno Salvati, Guido Valesini, Walter Malorni, Maurizio Sorice and Elena Ortona

Articles on similar topics can be found in the following Blood collections
Hemostasis, Thrombosis, and Vascular Biology (2497 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://bloodjournal.hematologylibrary.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
Copyright 2011 by The American Society of Hematology; all rights reserved.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.
Blood First Edition Paper, prepublished online November 9, 2007; DOI 10.1182/blood-2007-05-092825

AUTOANTIBODIES TO THE C-TERMINAL SUBUNIT OF RLIP76 INDUCE OXIDATIVE STRESS AND ENDOTHELIAL CELL APOPTOSIS IN IMMUNE-MEDIATED VASCULAR DISEASES AND ATHEROSCLEROSIS

[1]Paola Margutti, [2]Paola Matarrese, [3]Fabrizio Conti, [1]Tania Colasanti, [1]Federica Delunardo, [4]Antonella Capozzi, [4]Tina Garofalo, [1]Elisabetta Profumo, [1]Rachele Riganò, [1]Alessandra Siracusano, [3]Cristiano Alessandri, [5]Bruno Salvati, [3]Guido Valesini, [2]Walter Malorni, [4]Maurizio Sorice, and [1]Elena Ortona

[1]Dipartimento di Malattie Infettive, Parassitarie e Immunomediate, Istituto Superiore di Sanità, Rome, Italy; [2] Dipartimento del Farmaco, Istituto Superiore di Sanità, Rome, Italy; [3]Dipartimento di Clinica e Terapia Medica, Cattedra di Reumatologia, Università "Sapienza", Rome, Italy; [4]Dipartimento di Medicina Sperimentale, Università "Sapienza", Rome, Italy; [5]Dipartimento di Scienze Chirurgiche, Università "Sapienza", Rome, Italy

Address correspondence to: Elena Ortona, Department of Infectious, Parasitic and Immune-Mediated Diseases; Section of Immune-Mediated Diseases; Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy. Phone: +39.06.49902760; Fax.+39.06.49382886; E-mail elena.ortona@iss.it

Copyright © 2007 American Society of Hematology

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Abstract

Although detection of autoantibodies in the peripheral blood from patients with immune-mediated endothelial dysfunctions has so far failed to provide tools of diagnostic or pathogenetic value, putative bioindicators include anti-endothelial cell antibodies, a heterogeneous family of antibodies that react with autoantigens expressed by endothelial cells. In this study, to identify endothelial autoantigens involved in the autoimmune processes causing endothelial damage, we screened a human microvascular endothelial cell cDNA library with sera from patients with Behçet's disease. We identified antibodies to the C-terminus of Ral binding protein1 (RLIP76), a protein that catalyzes the ATP-dependent transport of glutathione (GSH) conjugates including GSH-4-hydroxy-*t*-2,3-nonenal, in the serum of a significant percentage of patients with various diseases characterized by immune-mediated endothelial dysfunction, including Behçet's disease, systemic sclerosis, systemic lupus erythematosus and carotid atherosclerosis. These autoantibodies increased intracellular levels of 4-hydroxy-*t*-2,3-nonenal, decreased levels of GSH and activated C-Jun NH2 Kinase signaling (JNK), thus inducing oxidative stress-mediated endothelial cell apoptosis. The dietary antioxidant alpha-tocopherol counteracted endothelial cell demise. These findings suggest that autoantibodies to RLIP76 play a pathogenetic role in immune-mediated vascular diseases and represent a valuable peripheral blood bioindicator of atherosclerosis and immune-mediated vascular diseases.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Introduction

Autoantibodies directed against normal host antigens are a common feature of many autoimmune diseases. Some of the markers are pathogenic, whereas others they may be merely an epiphenomenon due to tissue damage and serve as markers for organ involvement or outcomes.[1] Pathogenic autoantibodies may act directly on target organs by immune-complex deposition, complement activation or by binding distinct soluble or membrane proteins blocking or activating their biological activity.[2] Anti-endothelial cell antibodies (AECA) are a heterogeneous group of antibodies detected in autoimmune vasculitis, vasculopathies, atherosclerosis and other diseases caused by vessel-wall damage.[3] AECA induce an endothelial perturbation *in vitro*, increasing adhesion molecule expression and secretion of pro-inflammatory cytokines and chemokines. Some evidence suggests that AECA favour ischemic events by inducing apoptosis.[4-7] The pathogenetic role of AECA in ischemia receives support from their frequent association with disease activity in several autoimmune vasculitis and vasculopathies including Behçet's disease (BD), systemic lupus erythematosus (SLE) and systemic sclerosis (SS).[3, 8, 9] Until recently, few published data were available on the endothelial cell autoantigens recognized by AECA.[10] Identifying endothelial autoantigens involved in the immune-mediated processes during endothelial dysfunctions could help to explain how chronic inflammation of the vascular wall initiates and progresses. Screening a cDNA expression library is a powerful technique that identifies previously uncharacterized antigens from patients' sera containing antibodies.

In this study, designed to identify new antigenic targets of AECA, we screened a human microvascular endothelial cell (HMVEC) cDNA expression library with sera from patients with BD, a systemic form of primary vasculitis and identified a strongly reactive clone encoding Ral binding protein1 (RLIP76/RALBP1). RLIP76 is a Ral effector, GTPase-activating protein[11] expressed in several malignant cell lines and, in smaller amounts, in non-

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

malignant human cell lineages of endothelial, epithelial and aortic smooth muscle origin, as well as in erythrocytes.[12, 13] Like ABC protein, it catalyzes ATP-dependent transport and extrusion from the cell of anionic, e.g. glutathione (GSH) conjugates, such as GSH-4-hydroxy-*t*-2,3-nonenal (GS-HNE), leukotrienes and weakly cationic compounds, i.e. anthracyclins. 4-hydroxy-*t*-2,3-nonenal (4-HNE) is an end product of lipid peroxidation that induces oxidative stress, causes apoptosis, activates several signaling pathways and is conjugated with GSH.[14-21] By lowering GS-HNE, RLIP76 helps to maintain cell homeostasis.[22] Cells subjected to mild, transient oxidative stress redistribute RLIP76 on the membrane surface thus expelling 4-HNE at a higher rate.[23] In experiments to identify the immunoreactive region of RLIP76 we cloned and expressed the N- and C- terminal regions and by enzyme-linked immunosorbent assay (ELISA) we measured IgG specific to RLIP76 in patients with immune-mediated endothelial dysfunction**,** BD, SLE, SS, and carotid atherosclerosis. Secondly, we analyzed the endothelial RLIP76 expression, localized the protein on endothelial cells in physiological conditions and after mild oxidative stress, and investigated the pathogenic effects of the specific autoantibodies. In particular, we studied the intracellular levels of 4-HNE and GSH and anti-RLIP76 antibody induced C-Jun NH2 Kinase signaling activation (JNK) activation and apoptosis. The data we present here provide evidence that sera from patients with various diseases characterized by immune-mediated endothelial dysfunctions contain autoantibodies specific to the C-terminal region of RLIP76. These autoantibodies may have a pathogenetic role inducing oxidative stress-mediated apoptosis in endothelial cells.

Materials and Methods

Patients

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

We studied thirty-seven unselected out-patients with BD (10 women, 27 men; mean age 42.2 years, range 27-58 years; mean disease duration 7.9 years, range 0-24 years), 40 consecutive patients with SLE (35 women, 5 men; mean age 40.1 years, range 19-71 years; mean disease duration 8.2 years, range 0.4-24 years), 65 consecutive patients with SS (60 women, 5 men; mean age 57 years, range 20-77 years; mean disease duration 6.2 years, range 0.9-30 years) attending the Rheumatology Division of "Sapienza" University of Rome. All patients with BD fulfilled the diagnostic criteria of the International Study Group for BD.[24] Glucocorticoids were used in 46.1% of patients with BD, immunosuppressive drugs (cyclosporine A, methotrexate, azathioprine, chlorambucil) in 56.4%, infliximab in 5.1%, interferon α in 5.1%, and 10.2% of the patients with BD were not treated. Patients who had two of the seven findings (oral and genital ulcerations, skin lesions, eye involvement, positive pathergy test, thrombophlebitis and arthritis), or multiple erythema nodosum with severe inflammation and with an elevated erythrocyte sedimentation rate and positive C-reactive protein were assumed to have active disease. According to these criteria, 44.4 % of patients had active disease. The frequency of the HLAB51 allele was 68.7%. SLE was diagnosed in accordance with the American College of Rheumatology revised criteria.[25] Glucocorticoids were used in 74% of patients with SLE, hydroxychloroquine in 48.1%, immunosuppressive drugs (azathioprine, cyclophosphamide, cyclosporine A, methotrexate, mycophenolate mofetil) in 37%, and 11.1% were not treated. SS was diagnosed in accordance with the criteria of the American Rheumatism Association.[26] Of the 65 patients with SS 15 were receiving low doses of glucocorticoids (<10 mg prednisone daily) and 9 patients were also undergoing immunosuppressive therapy (cyclosporine A or cyclophosphamide). As controls, we also enrolled 43 patients with infectious mononucleosis and 46 healthy subjects (27 women, 19 men; mean age 45 years, range 34-53 years).

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

We also enrolled 66 consecutive patients with carotid atherosclerosis undergoing carotid endarterectomy at the Department of Surgical Sciences of University of Rome, "Sapienza". The indications for surgery, based upon the recommendations published by the Asymptomatic Carotid Atherosclerosis Study and the North American Symptomatic Carotid Endarterectomy Trial were clinically asymptomatic, severe or pre-occlusive carotid-artery stenosis equal to or more than 70%, clinically asymptomatic stenosis with ipsilateral signs of cerebral ischemia on computed tomographic scan and clinically symptomatic stenosis.[27, 28] To correct cardiovascular risk factors, all patients received 150 mg of aspirin for 4 weeks before endarterectomy. Exclusion criteria were recent infection (< 1 month), autoimmune disease, malignancy and inflammatory diseases. We also excluded patients receiving statins. Venous peripheral blood was drawn from patients before endarterectomy and from 25 sex- and age-matched healthy subjects with no ultrasonographically evident carotid atherosclerotic disease recruited as controls.

Informed consent was obtained from each patient and the Istituto Superiore di Sanita Institutional Review Board approved the study.

Immunoscreening of the cDNA expression library

A commercially available HMVEC cDNA library (Stratagene, Cambridge, UK) was screened with the serum from 2 of the 37 patients with BD, essentially as previously described.[29] The two patients'sera were selected on the basis of their AECA positive immune reaction and disease activity. Positive plaques were re-screened with the same pool of sera to obtain the clonality and phages were recovered as pBluescript by single-stranded rescue using the helper phage (Stratagene) according to the manufacturer's instructions and used to transform SolR XL1 cells.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Identification, amplification and cloning in expression vector of the cDNA subunits

The nucleotide sequence of the cloned cDNA insertions was sequenced with automated sequencer ABI Prism 310 collection (Applied Biosystems, Foster City, CA, USA) and the sequence compared with the GenBank sequence database using the Blast program revealed 100% identity with RLIP76 (NM 006788). The cDNA insertion was amplified by PCR to obtain the N- and C-terminal regions using as primers the oligonucleotides with restriction sites. For the N-terminal region we used: forward (BamHI restriction site underlined) 5′ GCATGGATCCATGACTGAGTGCTTCCTG 3′, reverse (Hind III restriction site underlined) 5′ GCATAAGCTTAGTTCCTTTGCAATGACATG 3′; for the C-terminal subunit we used: forward (BamHI restriction site underlined) 5′ GCATGGATCCCCAGAATGTAACTATCTTCTG 3′, reverse (HindIII restriction site underlined) 5′ GCATAAGCTTTCAGATGGACGTCTCCTT 3′. The amplified fragments were run in 2% agarose gel, purified by Qiaex kit following the manufacturer's instruction (Qiagen GmbH, Hilden, Germany) and after digestion with the restriction enzymes (Promega Corporation, Madison, Wisconsin, USA) cloned in pQE (Qiagen) expression vector.

Expression and purification of the recombinant antigens

The fusion proteins were expressed in *Escherichia coli* SG130009 cells, purified by affinity of NI-NTA resin for the six-histidine tail and eluted under denaturing conditions according to the manufacturer's instruction (Qiagen) using a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA).

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

SDS-PAGE and immunoblotting

After 10% SDS-PAGE under reducing conditions, immunoblotting was performed as previously described.[30] In brief, the antigen was loaded at concentrations of 3 μg/lane and was revealed by human sera diluted 1:100 and by a monoclonal antibody to six-histidine tail (Qiagen). Peroxidase-conjugated goat anti-human IgG and anti-mouse IgG sera (Biorad, Richmond, CA, USA) were used as second antibodies. Strips were developed with 3-3' diaminobenzidine (Sigma-Aldrich).

ELISA

ELISA was developed essentially as previously described.[31] In brief, polystyrene plates (Maxisorp, Nunc, Rochester, NY) were coated with the antigen (0.1 μg/well) in 0.05 M $NaHCO_3$ buffer, pH 9.5, and incubated overnight at 4°C. Plates were blocked with 100 μl/well of PBS with 0.05% Tween20 (PBS-Tween) containing 3% milk, for 1 hour at 37 °C. Optimal serum dilution was established in preliminary experiments (1:10-1:500). For the RLIP76 C-terminal subunit serum reactivity peaked at a dilution of 1:100 and remained unchanged at higher concentrations whereas for the RLIP76 N-terminal subunit it remained under detectable values at each serum dilution tested (data not shown). After blocking with 3% milk, plates were therefore incubated with human sera diluted 1:100 in PBS-Tween and 1% milk. Peroxidase conjugated goat anti-human IgG (Biorad) diluted 1:3000 in PBS-Tween containing 1% milk was incubated 1 hour at room temperature. O-phenylenediamine dihydrochloride (Sigma-Aldrich) was used as substrate and the optical density (OD) was measured at 490 nm. Means + 2 standard deviations of the OD reading of the healthy controls were considered as the cut-off level for positive reactions. All assays were performed in

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

quadruplicate. Data were presented as the mean OD corrected for background (wells without coated antigen). The results of unknown samples on the plate were accepted if internal controls (two serum samples, one positive and one negative) had an absorbance reading within mean ± 10% of previous readings. To inhibit specific IgG, the sera from two patients with BD were incubated overnight at room temperature with 10 μg/ml of the same antigen used to coat ELISA plates according to the method reported by Huang and colleagues.[32] As negative controls for the inhibition analysis, the sera were pre-incubated with 10 μg/ml of an unrelated recombinant antigen or 40 μg/ml of bovine serum albumin.

Cultures of human umbilical-vein endothelial cells (HUVEC) at the third to fourth passage were used to detect AECA (IgG), using a cell-surface ELISA on living cells, as previously reported.[33]

Antibodies specific to RLIP76

Antibodies from patients' sera were purified as previously described.[29] In brief, antigen (50 μg) was spotted onto a nitrocellulose filter and incubated with the sera from patients with BD used for the immunoscreening. The bound antibodies were eluted with glycine 100 mM, pH 2.5, mixed for 10 minutes and neutralized with Tris-HCl 1 M, pH 8. Antibodies from a preparation of intravenous immunoglobulin (IVIG) precipitated by saturated ammonium sulfate solution (SAS) were used as control. Endotoxin contamination of antibodies, as determined by the quantitative chromogenic *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD) was <0.03 EU/μg of protein.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Mouse polyclonal antibodies to RLIP76 C-ter obtained by a standard immunization protocol and mouse monoclonal antibody to six-histidine (Qiagen) were used as positive controls.

Culture conditions of endothelial cells

The primary cultures HMVEC-L (Provitro GmbH, Berlin, Germany) or the immortalized hybridoma cell line EAhy926 or HUVEC isolated by collagenase perfusion from normal-term umbilical cord veins were used as endothelial cells. Cells were grown to 60-70% confluence and seeded at 5 x 10^6 well on glass cover slips. To induce mild oxidative stress, cells were treated with 30 µM H_2O_2 for 30 min. After this time cells were incubated for an additional 30 min, 6 and 24 hours in H_2O_2-free medium or with human anti-RLIP76 C-ter antibodies at a concentration of 40 µg/ml, reported by Singhal *et al.* as the optimal concentration for inducing apoptosis.[12] As a control we used the same concentration of human IgG in the medium. To rule out endotoxin contamination the same experiments were run in the presence of polymyxin B (10 µg/ml) (Sigma-Aldrich). In some experiments, cells were also pre-incubated for 2 hours with 30 µM pan-caspase inhibitor zVAD (Alexis, San Diego, CA, USA) or for 24 hours with 30 µM alpha-tocopherol (α-TCPH, Sigma-Aldrich).

Cellular localization of RLIP76

An indirect immunofluorescence assay was developed on endothelial cells, as previously described.[34] Cells were permeabilized with acetone/methanol 1/1 (vol/vol) for 10 min at 4°C, soaked in balanced salt solution (Sigma) for 30 min at 25°C and then were incubated for 30 min at 25°C in the blocking buffer (2% BSA in PBS, containing 5% glycerol and 0.2% Tween-20). After washing three times with PBS, cells were incubated for 1 hour at 4°C with

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

human anti-RLIP76 antibodies and with control human IgG (0.1 μg/μl) in PBS containing 1% BSA. Fluorescein isothiocyanate-conjugated anti-human IgG (γ-chain specific, Sigma-Aldrich) were then added and incubated at 4°C for 30 min. After washing with PBS, fluorescence was analysed with an Olympus U RFL microscope (Olympus, Hamburg, Germany) or by a flow cytometer.

RLIP76 immunoprecipitation

Cell-free lysates from EAhy926 were immunoprecipitated with mouse polyclonal anti-RLIP76 C-ter antibodies. In brief, cells were lysed in lysis buffer (20 mM HEPES, pH 7.2, 1% Nonidet P-40, 10% glycerol, 50 mM NaF, including protease inhibitors). To preclear non-specific binding, cell free lysates were mixed with protein A-acrylic beads (Bio-Rad) and stirred in a rotary shaker for 1 hour at 4°C. After centrifugation (500 x g for 1 min), the supernatant was immunoprecipitated with mouse polyclonal anti-RLIP76 C-ter antibodies (3 μg) plus protein A-acrylic beads. The immunoprecipitates were subjected to 7.5% SDS-PAGE and immunoblotting with human anti-RLIP76 antibodies. Immunoreactivity was assessed by the chemiluminescence reaction using the enhanced chemoluminescence (ECL) Western blotting system (Amersham).

Immunohistochemistry

The superior thyroid artery, obtained from a patient after thyroidectomy, was immediately frozen. Cryostat sections of the artery were incubated with mouse anti-RLIP76 serum and with a biotinylated anti-mouse antibody and peroxidase-labeled streptavidin. Specimens were reincubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) and nuclei were counterstained with Mayer's haematoxylin. Controls included isotype-matched IgG and elimination of the primary antibody step.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

4-Hydroxynonenal quantification

To evaluate the formation of 4-HNE adducts with histidine cells fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) were stained with specific monoclonal antibody against 4-HNE (10 µg/ml, R&D Systems, Inc. Minneapolis, USA) for 1hour at 4°C. After washing cells were incubated with an anti-mouse antiserum conjugated with Alexa-488 (Molecular Probes, Eugene, OR, USA). After 30 min at 37° C, cells were washed twice and then analyzed on a cytometer. For fluorescence microscopy observations, cells were also counterstained with Hoechst before analyses by a Nikon Microphot equipped with intensified video microscopy (IVM) by a CCD camera (Carl Zeiss, Germany).

Staining for intracellular GSH

Intracellular GSH was detected by monochlorobimane (Molecular Probes) staining as previously described.[35] Samples were analyzed with an LRS II cytometer (Becton & Dickinson, San Jose', CA, USA) equipped with a UVB laser. Data obtained were analyzed by DIVA software (Becton & Dickinson).

Annexin V assay

Apoptosis was quantitatively evaluated by flow cytometry with the annexin-V-fluorescein isothiocyanate apoptosis detection kit (Eppendorf, Milan, Italy) which distinguishes early apoptotic (single annexin V positive), late apoptotic (double annexin V/propidium iodide positive) and necrotic cells (single propidium iodide positive).

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Activation of caspase-3

The activation state of caspase-3 was evaluated with the CaspGLOW fluorescein active caspase staining Kit (MBL, Woburn, MA, USA). Control and treated cells were incubated with FITC-conjugated caspase-3 inhibitor (DEVD-FMK) for 1 hour at 37°C, following the manufacturer's instructions. Samples were thereafter washed three times and immediately analyzed on a cytometer equipped with an FL-1 channel.

Activation of JNK

To evaluate the activation state of JNK by flow cytometry, we used a rabbit anti-JNK polyclonal antibody (BD/Pharmingen Oxford, UK) able to recognize human JNK1 phosphorylated at T183 and Y185. Cells were fixed with paraformaldehyde (4% in PBS), permeabilized with Triton X-100 (0.05% in PBS) and then stained with anti-JNK (pT183/pY185) followed by addition of FITC-conjugated anti-rabbit for 45 minutes at 4°C. After washings, cells were resuspended in PBS and analyzed on a cytometer.

Statistical analysis

For the analysis of the associations between the clinical characteristics of patients and anti-RLIP76 antibodies chi-square test was used to evaluate differences between percentages and the Mann-Whitney unpaired test was used to compare quantitative variables. Linear regression analysis (r correlation coefficient) was used to identify significant correlations. For the flow cytometry studies, at least 20,000 events were acquired. Data were recorded and statistically analyzed with a Macintosh computer using CellQuest Software (Becton & Dickinson). Student's *t* test was used for statistical analysis of mean values of the biologic variants analyzed in endothelial cells under the different treatments. Statistical significance of

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

flow cytometry studies was calculated with the parametric Kolmogorov-Smirnov (K/S) test. Unless otherwise indicated**,** *P* values of less than 0.01 were considered significant.

Results

Identification of RLIP76 by immunoscreening of the HMVEC expression library and its characterization

To identify genes encoding putative endothelial antigens we immunoscreened a HMVEC expression library with IgG from the serum of two patients with BD. Besides clones with cDNA insertion of Sip1, a known BD autoantigen;[29] we identified, other strongly reactive clones with a 1968 base-pair open reading frame and a predicted amino acid sequence 655 residues long that had 100% identity with the glutathione conjugate transporter RLIP76. To identify the immunoreactive region of RLIP76 we cloned and expressed two distinct overlapping subunits corresponding to the N- and the C-terminal fragments (Figure 1A). These fragments showed the expected molecular sizes of 41.1 kDa for the C-terminal region and 39.2 kDa for the N-terminal region by 10% SDS-PAGE. In immunoblotting analysis the patients' serum IgG used for immunoscreening the library recognized only the C-terminal fragment of RLIP76 (RLIP76 C-ter Figure 1B).

Serum IgG immunoreactivity to RLIP76

We analyzed serum IgG immmunoreactivity to the N- and C- terminal regions of RLIP76**.** When we investigated the prevalence of serum anti-RLIP76 C-ter antibodies in patients with diseases characterized by endothelial dysfunction and controls, ELISA detected IgG specific to RLIP76 C-ter in sera from all the groups of patients studied (11/37 (30%) patients with BD,

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

11/65 (17%) patients with SS and 10/40 (25%) patients with SLE**)** but in no sera from controls **(**patients with mononucleosis or age- and sex-matched healthy subjects) (Figure 2A). ELISA also detected serum anti-RLIP76 C-ter antibodies in 27 of the 66 patients with carotid atherosclerosis (41%) but in no age- and sex-matched healthy subjects (Figure 2B). Pre-absorption with RLIP76 C-ter itself of the sera from two patients with BD completely inhibited the antibody immunoreactivity thus confirming the specificity of ELISA (data not shown). No tested patients' or controls' sera reacted with RLIP76 N-ter ($OD_{490} < 0.05$). To assess the association of the clinical features in each disease with anti-RLIP76 C-ter antibody reactivity we then subgrouped the patients according to the presence of serum anti-RLIP76 C-ter antibodies. For BD we considered ocular, genital, skin or vascular involvement (Table 1); for SLE, SLEDAI, skin or kidney involvement, neuropsychiatric manifestations and serological markers (triglycerides, cholesterol, HDL, LDL) (Table 2); for SS, lung fibrosis and skin score (Table 3); and for carotid atherosclerosis, diabetes, hypertension, cardiovascular diseases in relatives and hypercholesterolemia (Table 4). Although no significant difference was found between the presence of serum anti-RLIP76 C-ter antibodies and clinical variables, in the SS group, sera from patients with lung fibrosis more frequently contained RLIP76 C-ter antibodies than sera from patients without (6/11, 54% *vs* 18/54, 33%). Considering as indicators of disease activity SLEDAI for patients with SLE, skin score and lung fibrosis for patients with SS, and the criteria defined in Material and Methods for patients with BD, we found no significant association between the presence of serum anti-RLIP76 antibodies and disease activity. No association was found between the presence of serum anti-RLIP76 antibodies and serum AECA or therapeutic regimen in the various subgroups (data not shown). Overall these data suggest that anti-RLIP76 antibodies are a new immunological marker shared by patients with various immune-mediated endothelial diseases.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Localization of RLIP76 in EAhy926 cells under physiological conditions and after oxidative stress

We analyzed RLIP76 expression in EAhy926 endothelial cells and in vascular tissue. Immunoprecipitation analysis showed that RLIP76 antibodies can immunoprecipitate the protein from EAhy92 endothelial cells *in vitro* (Figure 3A). Immunohistochemistry provided evidence that this protein is expressed on vascular endothelium *in vivo* (Figure 3B). To find out whether oxidative stress induces a redistribution of RLIP76 from the intracellular compartment into the membrane surface of endothelial cells, and to identify the extracellular region we analyzed qualitatively (by fluorescence microscopy) and quantitatively (by flow cytometry) RLIP76 C-ter in EAhy926 cells under physiological conditions and under mild oxidative stress. [23, 36] Immunofluorescence analysis with human purified antibodies specific to RLIP76 C-ter disclosed a redistribution of RLIP76 C-ter on the surface membrane of oxidative-stressed cells (Figure 3C). Accordingly, semi-quantitative analysis of surface expression clearly showed that under physiological conditions**,** RLIP76 C-ter was weakly expressed in the membrane. Under mild oxidative stress, membrane expression rapidly increased at 30 min, peaked at 6 hours and diminished at 24 hours (Figures 3D,E).

Effects of anti-RLIP76C-ter antibodies on intracellular 4-HNE and GSH levels in EAhy926 endothelial cells

To find out more about the role of RLIP76 in the cell response to oxidative stress we studied the effects of anti-RLIP76 C-ter antibodies on EAhy926 endothelial cells under physiological conditions and after mild oxidative stress.

First, we analyzed the formation of 4-HNE adducts with histidine (Figures 4A-C). As expected, when endothelial cells were exposed to H_2O_2, 4-HNE formation increased (Figures

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

4A,C). When cells were allowed to recover for 24 hours in fresh culture medium, 4-HNE levels returned to baseline. Adding anti-RLIP76 C-ter antibodies to the medium completely prevented endothelial cell recovery (Figures 4B,C). A time-course analysis in H_2O_2-treated cells clearly confirmed that the time-dependent recovery of 4-HNE to baseline levels was abolished by adding anti-RLIP76 C-ter antibodies to the medium (Figure 4D). 4-HNE levels were significantly higher in cells incubated for 6 h and 24 h with anti-RLIP76 C-ter antibodies than in cells cultivated without. In untreated endothelial cells, anti-RLIP76 C-ter antibodies also induced *per se* a time-dependent increase in 4-HNE (Figure 4D).

Because intracellular 4-HNE detoxification involves the most abundant cellular thiol-containing peptide, GSH, we analyzed the time courses of endothelial intracellular GSH content.[37] At 30 min after cells had been exposed to H_2O_2, intracellular levels of GSH decreased significantly and at 6 h and 24 h recovered to baseline levels. The time-dependent recovery of GSH levels was abolished by adding anti-RLIP76 C-ter antibodies to the medium. In untreated cells incubated for 30 min, 6 h and 24 h with anti-RLIP76 C-ter antibodies GSH levels significantly decreased (Figure 4E). Linear regression analysis showed a strong negative correlation between intracellular 4-HNE and GSH ($r = -0.94$, $P < 10^{-4}$). Control purified total IgG from healthy subjects left 4-HNE and GSH levels appreciably unchanged (data not shown). Collectively, these data suggest that anti-RLIP76 C-ter antibodies not only lower physiological cellular defenses against oxidative stress, but also directly *per se* increase the formation of oxidative by-products.

Effects of anti-RLIP76C-ter on induction of cellular apoptosis

The well-known relationship among apoptosis, 4-HNE and GSH prompted us next to analyze the possible role of anti-RLIP76 C-ter antibodies as an apoptotic inducer in endothelial cells.[38] Anti-RLIP76 C-ter antibodies induced apoptosis in a significant percentage of H_2O_2-treated

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

and untreated cells ($P < 0.01$ after 30 min, 6 and 24 hours of antibody incubation) (Figures 5A,C). Under all experimental conditions**,** apoptosis correlated positively with 4-HNE levels ($r = 0.8$, $P < 10^{-4}$) and negatively with GSH ($r = -0.75$, $P < 10^{-4}$). In experiments incubating cells with purified total IgG from healthy subjects endothelial cell apoptosis remained unchanged (data not shown).

Because the foregoing results suggested that apoptosis induced by anti-RLIP76 C-ter antibodies could be mediated at least in part by 4-HNE (and possibly by other oxidized lipids) we analyzed the same time course in cells pre-treated with alpha-tocopherol (α-TCPH), the most active form of vitamin E in humans, known to prevent lipid oxidation. As expected, under all the experimental conditions, cell pre-treatment with α-TCPH completely prevented apoptosis induced by anti-RLIP76 C-ter antibodies (Figures 5B,D). We also found that apoptosis induced by anti-RLIP76 C-ter antibodies was caspase-dependent as evidenced by detection of caspase-3 enzymatic activity (Figures 6A,B). The pan-caspase inhibitor zVAD significantly prevented anti-RLIP76 C-ter antibody-induced caspase-3 activation (Figure 6C) and apoptosis (data not shown).

To find out whether anti-RLIP76 -ter antibodies activate the typical oxidative signaling pathway, we investigated JNK phosphorylation. Treatment with anti-RLIP76 C-ter antibodies, either alone or in combination with H_2O_2, led to the phosphorylation of JNK in a large percentage of cells. JNK activation started after 30 minutes, peaked after 6 hours and decreased 24 hours after exposure to anti-RLIP76 C-ter antibodies (Figures 6D,E).

Effects of anti RLIP76 C-ter antibodies on microvascular cells

Besides using the EAhy926 line, a model of macrovascular endothelium**,** we analyzed the biological effects of anti-RLIP76 C-ter antibodies on a primary microvascular cell line (HMVEC-L). When these cells were subjected to mild H_2O_2-induced oxidative stress,

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

RLIP76 expression at the cell surface increased (Figure 7A). In H_2O_2-treated and in untreated HMVEC-L, anti-RLIP76 C-ter antibody-exposure for 24 hours induced 4-HNE adduct formation (Figure 7B), GSH depletion (Figure 7C) and apoptosis (Figures 7D,E). Both α-TCPH and the caspase inhibitor zVAD completely prevented RLIP76 C-ter antibody-induced apoptosis in HMVEC-L cells (Figure 7F). JNK-mediated signaling started 24 hours after cells were exposed to anti-RLIP76 C-ter antibodies (Figure 7G).

Discussion

In this study, using a molecular cloning strategy, we identified a new antigenic target of AECA involved in the autoimmune processes causing endothelial damage. The main finding in this study is that the C-terminal subunit of RLIP76 we cloned is a novel autoantigen in various immune-mediated diseases characterized by endothelial dysfunction, BD, SLE, SS and carotid atherosclerosis. Our findings strongly suggest that autoantibodies specific to the RLIP76 C-terminus exert pathogenetic effects on the endothelium by inducing oxidative stress-mediated apoptosis.

Our results extend current knowledge about the glutathione conjugate transporter RLIP76. We provide evidence that the C-terminal immunoreactive region of RLIP76 may be accessible to serum antibodies on the surface of both macrovascular and microvascular endothelium. Confirming published data,[23] we also found that after mild, transient oxidative stress, RLIP76 redistributes from the intracellular compartment to the plasma cell membrane. More important, we provide new evidence showing anti-RLIP76 C-ter antibodies in sera from patients with immune-mediated vascular diseases. In our *in vitro* experiments, by blocking the physiological function of RLIP76 to throw out GS-HNE, these autoantibodies depleted cellular antioxidant defenses, thereby causing oxidative imbalance increasing 4-HNE intracellular levels, decreasing GSH

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

levels and ultimately inducing apoptosis in endothelial cells under physiological conditions and after mild oxidative stress. These findings are consistent with previous studies suggesting that inhibition of RLIP76 with a rabbit specific IgG induced apoptosis.[39, 40] In our experiments, the antioxidant α-TCPH, the main component of vitamin E, counteracted the effects of anti-RLIP76 C-ter antibodies on cell apoptosis confirming that these autoantibodies induce oxidative stress-mediated apoptosis.

Oxidative stress, expressed by increased 4-HNE level, was found in several degenerative and immune-mediated diseases, confirming the idea that it could have a pathogenetic role in such diseases.[41-46] Our findings suggest that 4-HNE could induce apoptosis also through immune-mediated mechanisms. Although the precise mechanism of 4-HNE-induced apoptosis is unclear, several studies show that 4-HNE reduces cellular GSH.[38] GSH is an endogenous thiol that plays an important role as antioxidant in regulating cellular redox status.[47] In particular, GSH has a pivotal role in protecting macromolecules, such as DNA, from cyclic adduction by 4-HNE, suppressing the oxidation of lipids and, consequently, reducing the formation of 4-HNE. Hence, depletion of GSH in tissues finally leads to an increased formation of oxidized products.[48] Decreased GSH levels have been found in numerous diseases such as cancer, viral infections and immune dysfunctions.[49]

As expected, cytofluorimetric analysis showed that anti-RLIP76 C-ter antibodies activate JNK, thus inducing the signaling pathway typical of oxidative damage.[23, 39] Oxidative stress-mediated JNK activation is a critical component that decides cell fate in response to various stress stimuli.[50]

Our study provides new evidence suggesting that the various diseases related to endothelial dysfunction may arise through a common pathogenetic mechanism, possibly involving anti-RLIP76 C-ter antibodies. We found anti-RLIP76 C-ter antibodies not

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

only in the sera from patients with autoimmune vasculitis and vasculopathies, but also in the sera of a high percentage (41%) of patients with carotid atherosclerosis. The presence of pathogenetic serum autoantibodies specific to the RLIP76 C-terminus in patients with atherosclerosis supports the recently emerging role of the immune system in the development and progression of atherosclerosis. Atherosclerosis arises as a vascular wall response to endothelial injury. Endothelial cell apoptosis may be an important mechanism of vascular injury leading to the disruption of the endothelial barrier with vascular leak, extravasation of plasma proteins and exposure of the prothrombotic sub-endothelial matrix.[51, 52] Accumulating evidence suggests that endothelial cell apoptosis could play a critical role as an initial pathogenic event in atherosclerosis, SS and SLE.[53-56]

In this study we found that anti-RLIP76 antibodies bound RLIP76 on endothelial cells. We cannot exclude the possibility that anti-RLIP76 antibodies may induce oxidative damage or complement-mediated cytoxicity, or both, in other cellular types besides endothelial cells. In fact RLIP76 protein is expressed by various types of cells including endothelial cells even though contrasting data have been reported on its expression on endothelium in brain.[57, 58]

The potent activity of anti-RLIP76 antibodies we observed *in vitro* clearly disagrees with their lack of correlation with clinical disease activity. One explanation might be that anti-RLIP76 antibodies *in vivo* failed to induce oxidant mediated-damage because this protein is poorly expressed on the cell surface. Our experiments showed RLIP76 expression and anti-RLIP76 antibody induced cellular damage in *unperturbed* endothelial cells in culture. Cells in culture can nevertheless spontaneously undergo oxidative stress thus increasing surface expression of RLIP76. Hence, we consider it unlikely that *in vivo* anti-RLIP76 antibodies stimulate cellular damage without a pre-

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

existing oxidant-mediated damage caused for instance by inflammation or infections and further studies will be necessary to clarify whether the presence of anti-RLIP76 C-ter antibodies in the serum could be a risk factor for the onset of vascular diseases. A combination of several antibodies rather than a single autoantibody alone could be used as biological markers to monitor disease manifestations and progression. In single patients, an antibody panel that mirrors the various stages of disease might be needed to optimize therapy and prevent disability. Studies are in progress to clarify the effective role of anti-RLIP76 antibodies *in vivo* and to provide new insights into their potential pathogenicity. An important question our study leaves unanswered is whether a diet rich in anti-oxidant elements, in particular vitamin E, counteract the pathogenic effect of anti-RLIP76 C-ter antibodies.

How the RLIP76 C-terminus becomes autoantigenic remains unclear. Our study leaves open the possibility that RLIP76 becomes antigenic through mechanisms of molecular mimicry. Further investigations will clarify the possible cross-reaction with molecules from microorganisms associated with immune-mediated vascular diseases and RLIP76.

Overall, our study suggests that future research should be aimed at evaluating the potential therapeutic effectiveness of antioxidant agents in patients with endothelial dysfunction who have serum anti-RLIP76C-ter antibodies. A valuable therapeutic approach in these patients, could be to immunomodulate or block the anti-RLIP76 immune response. Besides, natural products having an anti-oxidant activity, such as vitamin E, could represent an optional complementary therapeutic tool. Finally, autoantibodies specific to the RLIP76 C-terminus might in the long run become a marker of prognostic value in these human diseases.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Acknowledgments

We wish to thank Dr Angela Tagliani for help in immunohistochemical analysis. This work was supported by a research grant from the Italian Ministry of Health (project no. 6ACF/1) to EO, grant ISS/NIH to WM and Fondazione Umberto Di Mario, ONLUS to GV

Conflict of interest: The authors have declared that no conflict of interest exists.

Author contributions: PMar and EO conceived the idea. PMat conducted the cytofluorimetric experiments on endothelial cell lines and participated in the design of the study and analysis of data. PMar and TC screened the library, conducted the ELISA experiments, participated in the design of the study and analysis of the data. FD cloned and sequenced cDNA, purified the recombinant protein and helped to interpret the data. FC and CA participated in the design of the study and in the analysis of data, helped to draft the manuscript and were responsible for the autoimmune patients selection. RR and EP participated in the design and revision of the study. AS participated in the analysis and interpretation of data and helped to draft the manuscript. BS was responsible for the selection of patients with atherosclerosis. GV participated in the design of the study and in the revision of the manuscript. AC and TG conducted the immunofluorescence analysis on endothelial cell lines and participated in the design of the study. PMar, EO, MS and WM coordinated the study and drafted the manuscript. All authors read and approved the final manuscript.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

References

1. Lee SJ, Kavanaugh A. 4. Autoimmunity, vasculitis, and autoantibodies. J Allergy Clin Immunol. 2006 Feb;117:S445-50.
2. Martin F, Chan AC. Pathogenic roles of B cells in human autoimmunity; insights from the clinic. Immunity. 2004 May;20(5):517-27.
3. Meroni P, Ronda N, Raschi E and Borghi MO. Humoral autoimmunity against endothelium: theory or reality? Trends Immunol. 2005;26:275-81.
4. Belizna CC, Duijvestijn A, Hamidou M and Cohen Tervaert JW. Antiendothelial cell antibodies in vasculitis and connective tissue disease. Ann Rheum Dis. 2006;65:1545-50.
5. Youinou P, Le Dantec C, Bendaoud B, Renaudineau Y, Pers JO and Jamin C. Endothelium, a target for immune-mediated assault in connective tissue disease. Autoimmun Rev. 2006;5:222-8.
6. Jamin C, Dugue C, Alard JE, Jousse S, Saraux A, Guillevin L, Piette JC and Youinou P. Induction of endothelial cell apoptosis by the binding of anti-endothelial cell antibodies to Hsp60 in vasculitis-associated systemic autoimmune diseases. Arthritis Rheum. 2005;52:4028-38.
7. Chauhan SK, Tripathy NK and Nityanand S. Antigenic targets and pathogenicity of anti-aortic endothelial cell antibodies in Takayasu arteritis. Arthritis Rheum. 2006;54:2326-33.
8. Navarro M, Cervera R, Font J, Reverter JC, Monteagudo J, Escolar G, Lopez-Soto A, Ordinas A and Ingelmo M. Anti-endothelial cell antibodies in systemic autoimmune diseases: prevalence and clinical significance. Lupus. 1997;6:521-6.
9. Oelzner P, Deliyska B, Funfstuck R, Hein G, Herrmann D and Stein G. Anti-C1q antibodies and antiendothelial cell antibodies in systemic lupus erythematosus -

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

relationship with disease activity and renal involvement. Clin Rheumatol. 2003;22:271-8.

10. Youinou P. New target antigens for anti-endothelial cell antibodies. Immunobiology. 2005;210:789-97.
11. Jullien-Flores V, Dorseuil O, Romero F, Letourneur F, Saragosti S, Berger R, Tavitian A, Gacon G, Camonis JH. Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. J Biol Chem. 1995;270:22473-7.
12. Singhal SS, Awasthi YC and Awasthi S. Regression of melanoma in a murine model by RLIP76 depletion. Cancer Res. 2006;66:2354-60.
13. Sharma A, Zimniak P, Awasthi S and Awasthi YC. RLIP76 is the major ATP-dependent transporter of glutathione-conjugates and doxorubicin in human erythrocytes. Arch Biochem Biophys. 2001;391:171-9.
14. Awasthi S, Cheng J, Singhal SS, Saini MK, Pandya U, Pikula S, Bandorowicz-Pikula J, Singh SV, Zimniak P and Awasthi YC. Novel function of human RLIP76: ATP-dependent transport of glutathione conjugates and doxorubicin. Biochemistry. 2000;39:9327-34.
15. Awasthi S, Sharma R, Yang Y, Singhal SS, Pikula S, Bandorowicz-Pikula J, Singh SV, Zimniak P and Awasthi YC. Transport functions and physiological significance of 76 kDa Ral-binding GTPase activating protein (RLIP76). Acta Biochim Pol. 2002;49:855-67.
16. Awasthi S, Singhal SS, Sharma R, Zimniak P and Awasthi YC. Transport of glutathione conjugates and chemotherapeutic drugs by RLIP76 (RALBP1): a novel link between G-protein and tyrosine kinase signaling and drug resistance. Int J Cancer. 2003;106:635-46.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

17. Ramana KV, Bhatnagar A, Srivastava S, Yadav UC, Awasthi S, Awasthi YC and Srivastava SK. Mitogenic responses of vascular smooth muscle cells to lipid peroxidation-derived aldehyde 4-hydroxy-trans-2-nonenal (HNE): role of aldose reductase-catalyzed reduction of the HNE-glutathione conjugates in regulating cell growth. J Biol Chem 2006;281:17652-60.
18. Awasthi YC, Sharma R, Cheng JZ, Yang Y, Sharma A, Singhal SS and Awasthi S. Role of 4-hydroxynonenal in stress-mediated apoptosis signaling. Mol Aspects Med. 2003;24:219-30.
19. Usatyuk PV and Natarajan V. Role of mitogen-activated protein kinases in 4-hydroxy-2-nonenal-induced actin remodeling and barrier function in endothelial cells. J Biol Chem. 2004;279:11789-97.
20. Yang Y, Sharma R, Sharma A, Awasthi S and Awasthi YC. Lipid peroxidation and cell cycle signaling: 4-hydroxynonenal, a key molecule in stress mediated signaling. Acta Biochim Pol. 2003;50:319-36.
21. Minekura H, Kumagai T, Kawamoto Y, Nara F and Uchida K. 4-Hydroxy-2-nonenal is a powerful endogenous inhibitor of endothelial response. Biochem Biophys Res Commun. 2001;282:557-61.
22. Awasthi S, Singhal SS, Yadav S, Singhal J, Drake K, Nadkar A, Zajac E, Wickramarachchi D, Rowe N and Yacoub A, Boor P, Dwivedi S, Dent P, Jarman WE, John B and Awasthi YC. RLIP76 is a major determinant of radiation sensitivity. Cancer Res. 2005;65:6022-8.
23. Cheng JZ, Sharma R, Yang Y, Singhal SS, Sharma A, Saini MK, Singh SV, Zimniak P, Awasthi S and Awasthi YC. Accelerated metabolism and exclusion of 4-hydroxynonenal through induction of RLIP76 and hGST5.8 is an early adaptive response of cells to heat and oxidative stress. J Biol Chem. 2001;276:41213-23.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

24. International Study Group for Behçet's Disease. Criteria for diagnosis of Behçet's Disease. Lancet. 1990;335:1078-1080.

25. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997;40:1725.

26. Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum. 1980;23:581-90.

27. Barnett HJ, Taylor DW, Eliasziw M, Fox AJ, Ferguson GG, Haynes RB, Rankin RN, Clagett GP, Hachinski VC, Sackett DL, Thorpe KE, Meldrum HE and Spence JD. Benefit of carotid endarterectomy in patients with symptomatic moderate or severe stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med. 1998;339:1415-25.

28. Executive Committee for the Asymptomatic Carotid Atherosclerosis Study, Endarterectomy for asymptomatic carotid artery stenosis. JAMA. 1995;273:1421–28.

29. Delunardo F, Conti F, Margutti P, Alessandri C, Priori R, Siracusano A, Riganò R, Profumo E, Valesini G, Sorice M and Ortona E. Identification and characterization of the carboxy-terminal region of Sip-1, a novel autoantigen in Behcet's disease. Arthritis Res Ther. 2006;8:R71.

30. Margutti P, Ortona E, Vaccari S, Barca S, Riganò R, Teggi A, Muhschlegel F, Frosch M and Siracusano A. Cloning and expression of a cDNA encoding an elongation factor 1 β/δ protein from *Echinococcus granulosus* with immunogenic activity. Parasite Immunol. 1999;21:485-92.

31. Margutti P, Delunardo F, Sorice M, Valesini G, Alessandri C, Capoano R, Profumo E, Siracusano A, Salvati B, Riganò R and Ortona E. Screening of a HUAEC cDNA

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

library identifies actin as a candidate autoantigen associated with carotid atherosclerosis. Clin Exp Immunol. 2004;137:209-15.

32. Huang X, Johansson SGO, Zargari A, Zargari A and Nordvall SL. Allergen cross-reactivity between *Pityrosporum orbiculare* and *Candida albicans*. Allergy. 1995;50:648-56.
33. Conti F, Alessandri C, Bompane D, Bombardieri M, Spinelli FR, Rusconi AC, Valesini G. Autoantibody profile in systemic lupus erythematosus with psychiatric manifestations: a role for anti-endothelial-cell antibodies. Arthritis Res Ther. 2004;6:R366-72.
34. Margutti P, Sorice M, Conti F, Delunardo F, Racaniello M, Alessandri C, Siracusano A, Riganò R, Profumo E, Valesini G, Ortona E. Screening of an endothelial cDNA library identifies the C-terminal region of Nedd5 as a novel autoantigen in systemic lupus erythematosus with psychiatric manifestations. Arthritis Res Ther. 2005;7:R896-R903.
35. Sahaf B, Heydari K, Herzenberg LA and Herzenberg LA. Lymphocyte surface thiol levels. Proc Natl Acad USA. 2003;100:4001-5.
36. Yadav S, Singhal SS, Singhal J, Wickramarachchi D, Knutson E, Albrecht TB, Awasthi YC and Awasthi S. Identification of membrane-anchoring domains of RLIP76 using deletion mutant analyses. Biochemistry. 2004;43:16243-53.
37. Falletti O, Cadet J, Favier A and Douki T. Trapping of 4-hydroxynonenal by glutathione efficiently prevents formation of DNA adducts in human cells. Free Radic Biol Med. 2007;42:1258-69.
38. Raza H and John A. 4-hydroxynonenal induces mitochondrial oxidative stress, apoptosis and expression of glutathione S-transferase A4-4 and cytochrome P450 2E1 in PC12 cells. Toxicol Appl Pharmacol. 2006;216:309-18.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

39. Yang Y, Sharma A, Sharma R, Patrick B, Singhal SS, Zimniak P, Awasthi S and Awasthi, YC. Cells preconditioned with mild, transient UVA irradiation acquire resistance to oxidative stress and UVA-induced apoptosis: role of 4-hydroxynonenal in UVA-mediated signaling for apoptosis. J Biol Chem. 2003;278:41380-8.

40. Awasthi S, Singhal SS, Singhal J, Yang Y, Zimniak P and Awasthi YC. Role of RLIP76 in lung cancer doxorubicin resistance: III. Anti-RLIP76 antibodies trigger apoptosis in lung cancer cells and synergistically increase doxorubicin cytotoxicity. Int J Oncol. 2003;22:721-32.

41. Awasthi YC, Sharma R, Cheng JZ, Yang Y, Sharma A, Singhal SS and Awasthi S. Role of 4-hydroxynonenal in stress-mediated apoptosis signaling. Mol Aspects Med. 2003;24:219-30.

42. Rittner HL, Hafner V, Klimiuk PA, Szweda LI, Goronzy JJ and Weyand CM. Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. J Clin Invest. 1999;103:1007-13.

43. Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER and Mizuno Y. Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. Proc Natl Acad Sci. 1996;93:2696-701.

44. Montine KS, Reich E, Neely MD, Sidell KR, Olson SJ, Markesbery WR and Montine TJ. Distribution of reducible 4-hydroxynonenal adduct immunoreactivity in Alzheimer disease is associated with APOE genotype. J Neuropathol Exp Neurol. 1998;57:415-25.

45. Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W and De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2002;166:490-5.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

46. Uchida K. 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. Prog Lipid Res. 2003;42:318-43.

47. Dalton TP, Chen Y, Schneider SN, Nebert DW and Shertzer HG. Genetically altered mice to evaluate glutathione homeostasis in health and disease. Free Radic Biol Med. 2004;37:1511-26.

48. Sarangarajan R, Apte SP and Ugwu SO. Hypoxia-targeted bioreductive tyrosine kinase inhibitors with glutathione-depleting function. Anticancer Drugs. 2006;17:21-4.

49. Townsend DM, Tew KD and Tapiero H. The importance of glutathione in human disease. Biomed Pharmacother. 2003;57:145-55.

50. Kutuk O, Basaga H. Apoptosis signaling by 4-hydroxynonenal: a role for JNK-c-Jun/AP-1 pathway. Redox Rep. 2007;12(1):30-4

51. Winn RK and Harlan JM. The role of endothelial cell apoptosis in inflammatory and immune diseases. J Thromb Haemost. 2005;3:1815-24.

52. Mallat Z and Tedgui A. Apoptosis in the vasculature: mechanisms and functional importance. Br J Pharmacol. 2000;130:947-62.

53. Rajagopalan S, Somers EC, Brook RD, Kehrer C, Pfenninger D, Lewis E, Chakrabarti A, Richardson BC, Shelden E, McCune WJ and Kaplan MJ. Endothelial cell apoptosis in systemic lupus erythematosus: a common pathway for abnormal vascular function and thrombosis propensity. Blood. 2004;103:3677-83.

54. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G. Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. Arthritis Rheum. 2000;43:2550-62.

55. Jun JB, Kuechle M, Harlan JM and Elkon KB. Fibroblast and endothelial apoptosis in systemic sclerosis. Curr Opin Rheumatol. 2003;15:756-60.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

56. Stoneman VE and Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond). 2004;107:343-54.

57. Soranzo N, Kelly L, Martinian L, Burley MW, Thom M, Sali A, Kroetz DL, Goldstein DB, Sisodiya SM. Lack of support for a role for RLIP76 (RALBP1) in response to treatment or predisposition to epilepsy. Epilepsia. 2007;48:674-83.

58. Awasthi S, Hallene KL, Fazio V, Singhal SS, Cucullo L, Awasthi YC, Dini G, Janigro D. RLIP76, a non-ABC transporter, and drug resistance in epilepsy. BMC Neurosci. 2005;6:61.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Table 1. Clinic characteristics of patients with Behçet Disease divided according to the presence of serum anti-RLIP76 IgG immunoreactivity

Characteristics	Patients with serum anti-RLIP76 IgG (n = 11)	Patients without serum anti-RLIP76 IgG (n = 26)	*P* value
Age (years, median; range)	33 (28-54)	43.5 (27-58)	> 0.05
Sex (males/females)	8/3	19/7	> 0.05
Disease duration (years, median; range)	4.8 (0-14)	6 (0-24)	> 0.05
C-reactive protein mg/L median; range)	3.2 (3.2-15.2)	4.4 (0-129)	> 0.05
Genital ulcerations %	45.4	38.5	> 0.05
Skin lesions %	54.5	61.5	> 0.05
Eye involvement %	54.5	69.2	> 0.05
Vascular manifestations %	36.4	20.8	> 0.05
Arthrites %	9	23.1	> 0.05
HLA B51 %	75	66.6	> 0.05
Active disease %	45.4	42.3	> 0.05

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Table 2. Clinic characteristics of patients with Systemic Lupus Erythematosus divided according to the presence of serum anti-RLIP76 IgG immunoreactivity

Characteristics	Patients with serum anti-RLIP76 IgG (n = 10)	Patients without anti-RLIP76 IgG (n= 30)	*P* value
Age (years, median, range)	36 (19-44)	41 (26-71)	0.03
Sex (males/females)	0/10	5/25	> 0.05
Disease duration (years, median, range)	4 (0.5-24)	7 (0.4-22)	> 0.05
SLEDAI (median, range)	0 (0-18)	2 (0-25)	> 0.05
Skin lesions %	60	46.6	> 0.05
Arthrites %	80	66.6	> 0.05
Neuropsychiatric manifestations %	30	26.6	> 0.05
Kidney involvement %	20	30.0	> 0.05
Cytopenia %	70	73.3	> 0.05
Serositis %	20	36.7	> 0.05
Anti-phospholipid syndrome %	20	36.7	> 0.05

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Table 3. Clinical characteristics of patients with Systemic Sclerosis divided according to the presence of serum anti-RLIP76 IgG immunoreactivity

Characteristics	Patients with anti-RLIP76 IgG (n = 11)	Patients without anti-RLIP76 IgG (n = 54)	*P* value
Age (years, median; range)	57 (47-65)	52 (20-77)	> 0.05
Sex (males/females)	1/10	4/50	> 0.05
Duration disease (years, median; range)	6.5 (1-19)	6 (0.9-30)	> 0.05
Erythrocyte sedimentation rate (median; range)	16 (10-30)	16 (2-64)	> 0.05
C-reactive protein, mg/L (median; range)	3 (0-72)	3 (0-48)	> 0.05
C3 (median; range)	144 (72-179)	113 (69-286)	> 0.05
C4 (median; range)	22 (11-51)	22 (14-46)	> 0.05
Lung fibrosis %	54.5	33.3	> 0.05
Skin score (median; range)[a]	13 (4-30)	8 (4-27)	> 0.05

[a]Skin score is the assessment of skin thickening.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Table 4. Clinical characteristics of patients with Carotid Atherosclerosis divided according to the presence of serum anti-RLIP76 IgG immunoreactivity

Characteristics	Patients with anti-RLIP76 IgG (n = 27)	Patients without anti-RLIP76 IgG (n = 39)	*P* value
Age (years, median; range)	76 (68-82)	71.5 (65-80)	> 0.05
Sex (males/females)	19/8	28/11	> 0.05
Diabetes[a] %	23	28	> 0.05
Smoking[b] %	70	62.5	> 0.05
Hypertension[c] %	67	87	> 0.05
Cardiovascular diseases in relatives %	57	40	> 0.05
Hypercholesterolemia[d] %	14	14	> 0.05

[a]Diabetes is defined as fasting glucose levels ≥ 140 mg/dl or need for antidiabetic medications. [b]Smoking is defined as current smokers. [c]Hypertension is defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥ 90 mm Hg, or need for hypertensive medication. [d]Hypercholesterolemia is defined as total cholesterol >200 mg/dL or need for lipid-lowering therapy.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Figure legends

Figure 1. The amino acid sequence and the immunochemical characterization of the N- and C-terminal regions of RLIP76. (A) The nucleotide sequence of the cloned cDNA (GenBank accession number NM 006788) was divided in two subunits by PCR with specific primers. The cDNA subunits were cloned in an expression vector and the N- and C-terminal regions of the protein were expressed and purified. The overlapped amino acids were squared. (B) The molecular size and the purity of the expressed proteins were confirmed by 10% SDS-PAGE stained by Coomassie blue (lane 1, N-terminal region; lane 2, C-terminal region) and serum immunoreactivity was analyzed by immunoblotting (lanes 3-6, C-terminal region; lanes 7-10, N-terminal region). Lanes 3, 7: monoclonal antibody specific to six-histidine tail; lanes 4, 8: serum pool from the two patients with BD used in screening the library; lane 5, 9: representative serum from a healthy subject; lanes 6, 10: control without serum.

Figure 2. Anti- RLIP76 C-ter antibodies in patients and healthy controls.

(A) Box-whisker plot of anti-RLIP76 C-ter IgG in patients with Behçet's disease (BD), systemic lupus erythematosus (SLE), systemic sclerosis (SS), infectious mononucleosis and from sex and age-matched healthy donors (NHS). (B) Box-whisker plot of anti-RLIP76 C-ter IgG in patients with carotid atherosclerosis and from sex and age-matched healthy donors (NHS). Median, quartiles, range, and possibly extreme values are indicated. The broken line represents the cut-off (mean + 2 SD for the healthy controls). Outliers are represented as crosses.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

Figure 3. RLIP76 expression and localization.

(A) EAhy926 were immunoprecipitated with mouse polyclonal anti-RLIP76 C-ter antibodies. The immunoprecipitates were analyzed by Western blotting, using anti-human RLIP76 C-ter antibodies. Bound antibodies were visualized with HRP-conjugated anti-human IgG and immunoreactivity was assessed by ECL. Virtually no reactivity was found with immunoprecipitates obtained using non RLIP76-specific IgG (irrelevant). (B) RLIP76 expression in vascular tissue was detected by immunohistochemistry on tissue arrays with histological sections from normal vascular human tissue incubated with mouse anti-RLIP76 C-ter antibodies. Intense immunoreactivity was observed in vascular endothelium. (C) Immunofluorescence analysis of RLIP76 distribution in EAhy926 untreated cells (left panel) or treated with 30 μM H_2O_2 30 min (right panel). (D, E) Flow cytometry analysis after surface staining of EAhy926 cells with antibody to the RLIP76 C-terminus. (D) Results obtained in a representative experiment. Full light gray histogram: untreated control cells; red histogram: H_2O_2-treated cells incubated for 30 min with fresh medium; black histogram: H_2O_2-treated cells incubated for 6 hours with fresh medium; blue histogram: H_2O_2-treated cells incubated for 24 hours with fresh medium. (E) Time-course evaluation of RLIP76 expression in untreated cells and in cells treated with H_2O_2 and then incubated 30 min, 6 and 24 hours in fresh medium (mean ± SD of the results obtained from three different experiments). (*) Represents $P<0.01$ by Student's t-test.

Figure 4. Intracellular 4-HNE and GSH levels in EAhy926 endothelial cells.

(A, B) Immunofluorescence analysis of the formation of 4-HNE adducts with histidine in EAhy926 cells stained with a 4-HNE-specific antibody and counterstained with Hoechst dye to reveal nuclei. (A, left panel) untreated cells; (A, right panel) H_2O_2-treated cells; (B, left

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

panel) H_2O_2-treated cells incubated for 24 hours with fresh medium; (B, right panel) H_2O_2-treated cells incubated for 24 hours with medium containing anti-RLIP76 C-ter antibodies.

(C) Quantitative analysis of 4-HNE adducts by flow cytometry in a representative experiment. Gray histogram: untreated control cells; red histogram: H_2O_2-treated cells; black histogram: H_2O_2-treated cells incubated for 24 hours with fresh medium; blue histogram: H_2O_2-treated cells incubated for 24 hours with medium containing anti-RLIP76 C-ter antibodies. Numbers represent median values of fluorescence intensity.

(D) Quantitative time course evaluation of 4-HNE intracellular content in untreated control cells, in cells incubated 30 min, 6 h and 24 h with anti-RLIP76 C-ter antibodies, in cells treated with H_2O_2 and in cells treated with H_2O_2 and then incubated for an additional 30 min, 6 and 24 hours in fresh medium or in medium containing anti-RLIP76 C-ter antibodies. Statistical analysis performed by Student's *t*-test indicated $P<0.01$ for : 1. control untreated cells *vs* cells incubated with anti-RLIP76 C-ter antibodies for 30 min, 6 h and 24 h; 2. untreated cells *vs* H_2O_2 treated cells incubated for 30 min and 6 h in fresh medium; 3. H_2O_2 treated cells incubated in fresh medium *vs* H_2O_2 treated cells incubated for 6 h and 24 h with medium containing anti-RLIP76 C-ter antibodies.

(E) Quantitative time-course evaluation of GSH intracellular content. Statistical analysis performed by Student's *t*-test indicated $P<0.01$ for: 1. untreated cells *vs* H_2O_2 treated cells incubated for 30 min in fresh medium; 2. untreated cells *vs* cells incubated for 30 min, 6 h and 24 h with anti-RLIP76 C-ter antibodies; 3. H_2O_2 treated cells incubated in fresh medium *vs* H_2O_2 treated cells incubated for 6 h and 24 h with medium containing anti-RLIP76 C-ter antibodies. Data reported in D and E are the mean ± SD of the results obtained from three different experiments.

Figure 5. Induction of apoptosis by anti-RLIP76 antibodies in EAhy926 endothelial cells.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

(A, C) Flow cytometry analysis after double staining with annexin V/propidium iodide of untreated control cells; cells incubated for 30 min, 6 h and 24 h with anti-RLIP76 C-ter antibodies and in cells treated with H_2O_2 and then incubated additional 30 min, 6 h and 24 h in fresh medium or with medium containing anti-RLIP76 C-ter antibodies. (B, D) Cells were also pre-treated with α-TCPH.

(A, B) Results obtained from three independent experiments are reported as mean ± SD. (C, D) Dot plots from a representative experiment. Numbers represent the percentage of annexin V single positive (early apoptosis, bottom right quadrant) or annexin V/PI double positive cells (late apoptosis, lower right quadrant). Statistical analysis performed by Student's *t*-test indicated $P<0.01$ for: 1. control untreated cells *vs* H_2O_2-treated cells; 2. control untreated cells *vs* cells incubated for 30 min, 6 h and 24 h with anti-RLIP76 C-ter antibodies and 3. cells treated with H_2O_2 and then incubated in fresh medium *vs* cells treated with H_2O_2 and then incubated for 6 h and 24 h with medium containing anti-RLIP76 C-ter antibodies. $P<0.01$ for any treatment (A) *vs* the same treatment performed after α-TCPH pre-incubation (B).

Figure 6. Apoptotic pathway induced by anti-RLIP76C-ter in EAhy926 cell line.

(A) Flow cytometry data were obtained in the absence (first row) or in the presence (second row) of 30 μM pan-caspase inhibitor zVAD. The numbers in each panel refer to the percentage of cells containing caspase 3 in its active form. Results obtained in a representative experiment are reported. (B, C) Graphs showing the mean ± SD of the percentages of cells with the active form of caspase 3 obtained from three different experiments done without (B) or with (C) zVAD. Statistical analyses indicate a significant ($P<0.01$) decrease in caspase 3 activity in cells pre-treated with zVAD before anti-RLIP76 C-ter antibody exposure. (D) Quantitative flow cytometry analysis investigating the JNK activation state 6 hours after the various treatments with a polyclonal antibody able to identify JNK (pT183/pY185). The

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

numbers in each panel refer to the percentage of cells containing JNK in its active form. Results obtained in a representative experiment are reported. (E) Time course analysis of the activation state of JNK. Statistical analyses of the results obtained from three independent experiments (reported as mean ± SD) indicate a significant difference ($P<0.01$) between cells treated with RLIP76 C-ter antibody, alone or in combination with H_2O_2, and both untreated and H_2O_2-treated cells at any time point considered.

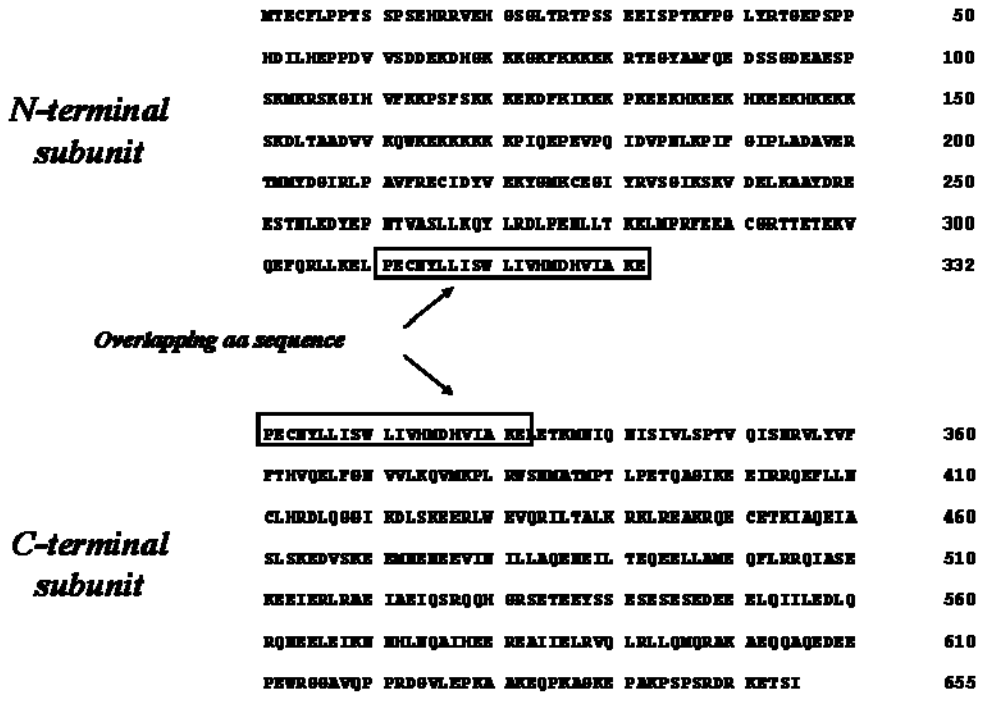
Figure 7. Effects of anti-RLIP76 antibodies on human microvascular primary cells. (A) Flow cytometry analysis of HMVEC-L cells, either untreated or H_2O_2-treated cells, after surface staining with antibody to RLIP76. Mean ± SD of the results obtained from three different experiments. (*) Represents $P<0.01$ by Student's *t*-test. (B, C) Quantitative flow cytometry analysis of 4-HNE adducts and GSH intracellular content in: untreated control cells, cells treated with H_2O_2 30 min, cells incubated with anti-RLIP76 C-ter antibodies for 48 hours and cells treated with H_2O_2 30 min and then incubated for an additional 48 hours in medium containing anti-RLIP76 C-ter antibodies. Data reported in (B, C) are the mean ± SD of the results obtained from three different experiments. Student's *t*-test indicated $P< 0.01$ for: control untreated cells *vs* H_2O_2 treated cells; untreated cells *vs* cells incubated with anti-RLIP76 C-ter antibodies for 48 hours, and untreated cells *vs* H_2O_2 treated cells incubated for 48 hours with medium containing anti-RLIP76 C-ter antibodies. (D) Dot plots from a representative experiment performed 48 hours after different treatments. Numbers represent the percentage of annexin V single positive (early apoptosis, bottom right quadrant) or annexin V/PI double positive cells (late apoptosis, lower right quadrant). (D) Flow cytometry analysis of apoptosis after double staining with annexin V/propidium iodide of untreated control cells; cells treated with H_2O_2 and then incubated at different time points with fresh medium or with medium containing anti-RLIP76 C-ter antibodies. Cells were also treated for

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

the same times with anti RLIP76 C-ter antibodies given alone. Results obtained from three independent experiments are reported as mean ± SD. Student's *t*-test indicated $P<0.01$ for: 1. control untreated cells *vs* cells incubated for 24 and 48 hours with anti-RLIP76 C-ter antibodies and 2. cells treated with H_2O_2 and then incubated in fresh medium *vs* cells treated with H_2O_2 and then incubated for 24 and 48 hours with medium containing anti RLIP76 C-ter antibodies. (F) Quantitative flow cytometry analysis of apoptosis in cells treated with anti-RLIP76 C-ter antibodies for 24h and 48h pre-treated or not with zVAD or α-TCPH as indicated in Materials and Methods section. As control, cells were also treated at the same time points with zVAD or α-TCPH given alone. Results obtained from three independent experiments are reported as mean ± SD. (G) Quantitative flow cytometry analysis of the JNK activation state obtained with a polyclonal antibody specific for active form of JNK (pT183/pY185) in cells treated with anti-RLIP76 in the presence or absence of α-TCPH. (*) represents $P<0.01$ by Student's *t*-test.

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

A



B

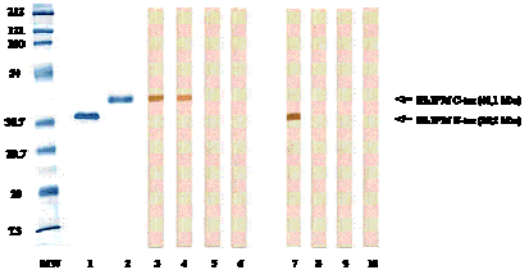


Figure 1

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

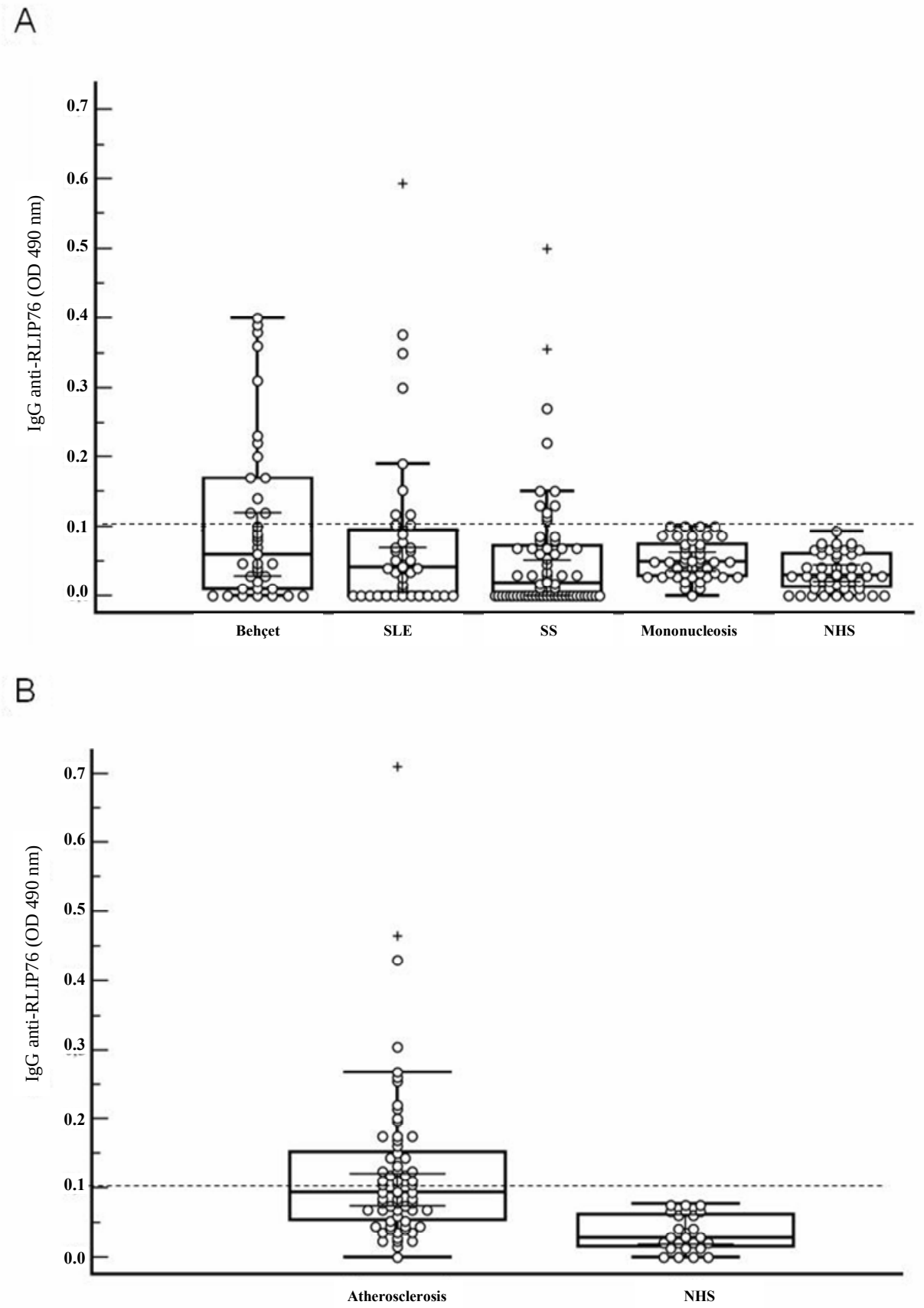


Figure 2

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

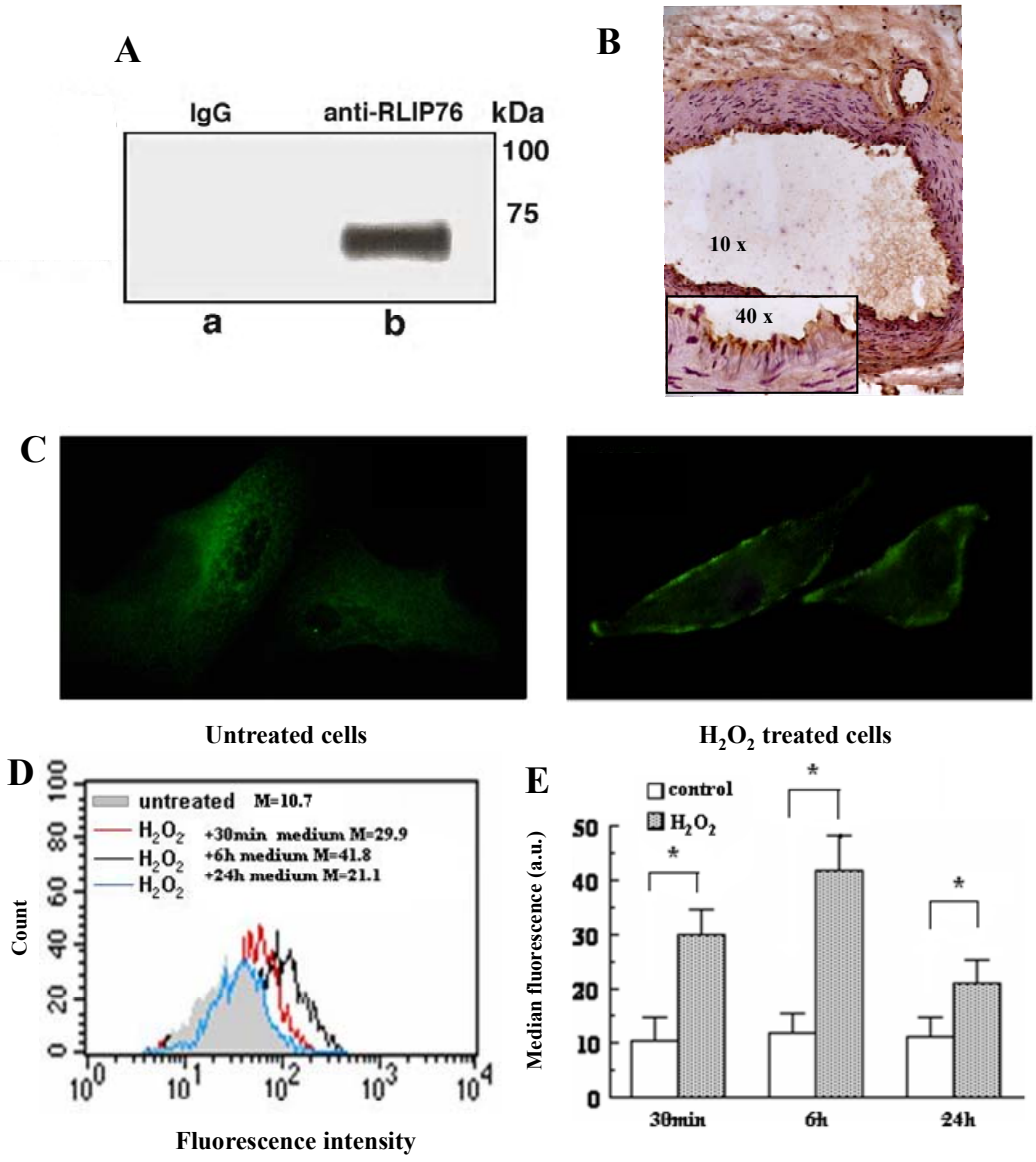


Figure 3

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

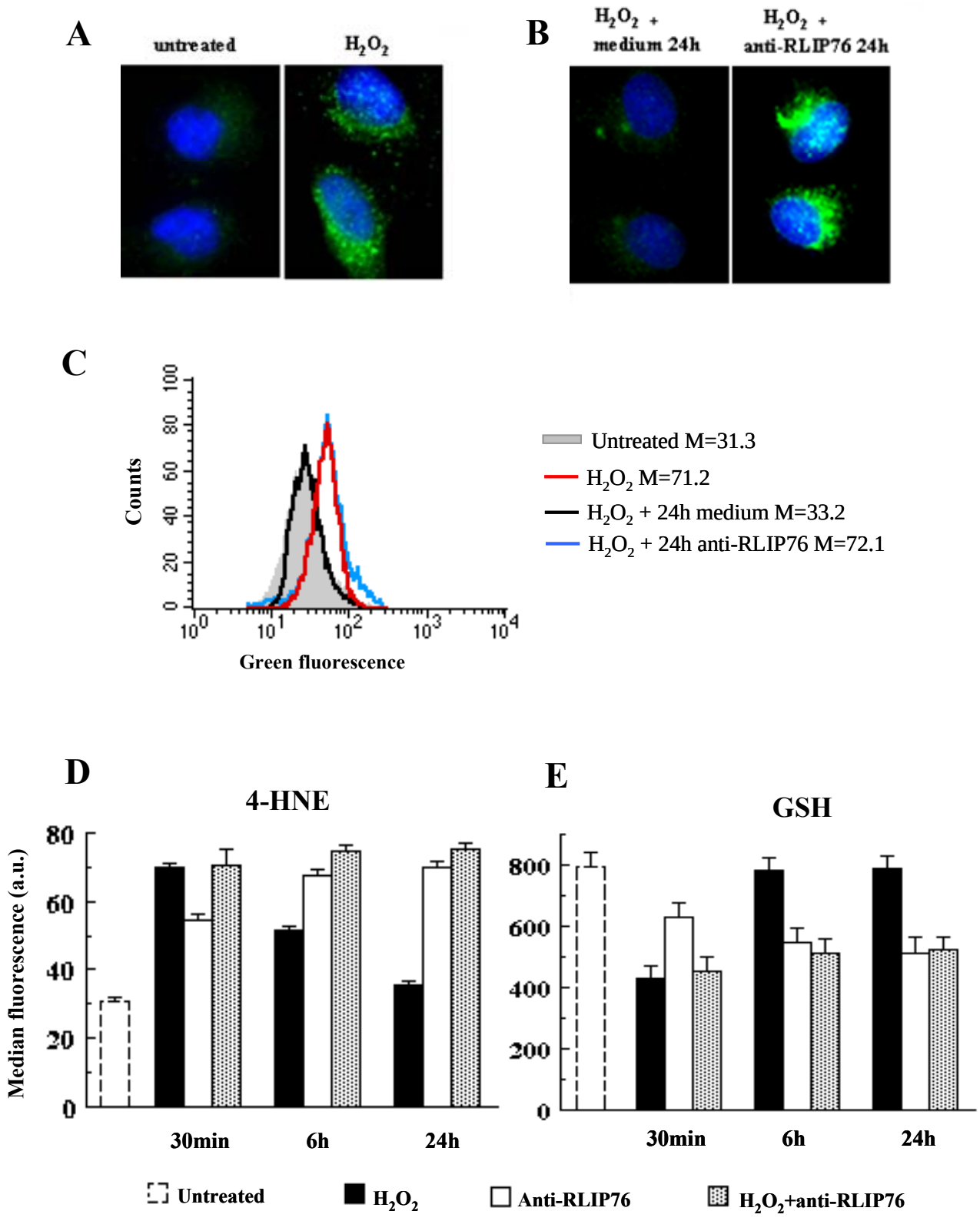


Figure 4

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

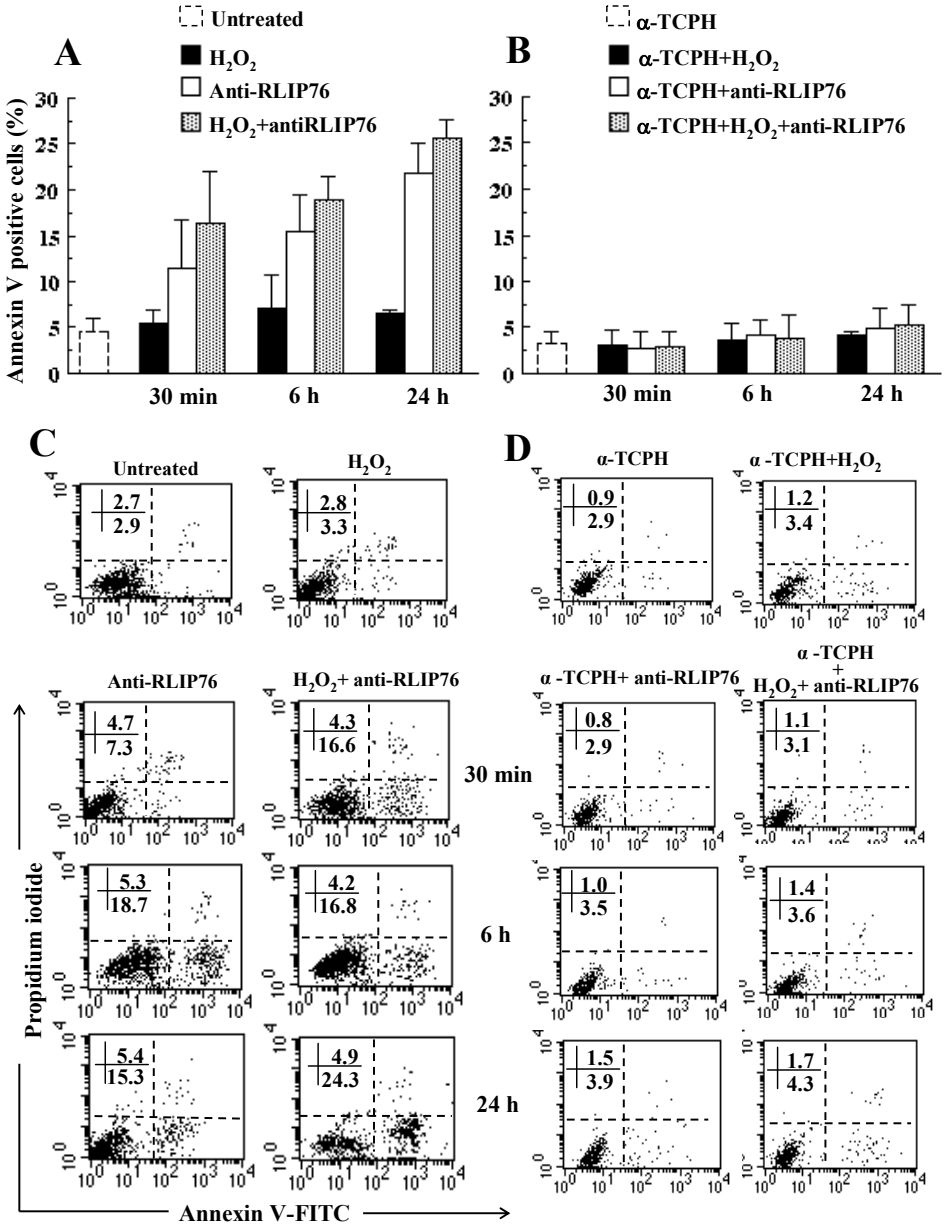


Figure 5

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

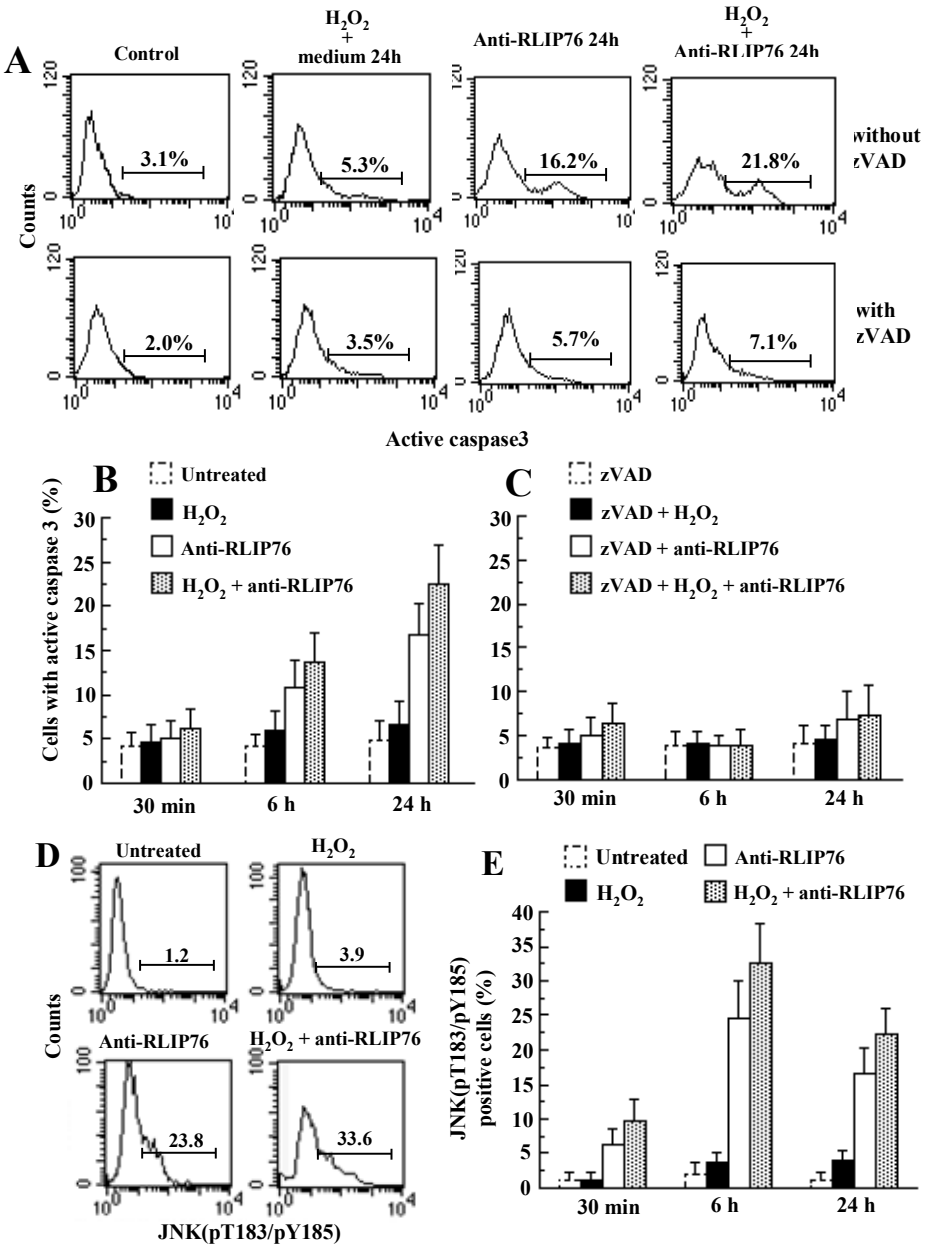


Figure 6

From bloodjournal.hematologylibrary.org by guest on June 6, 2013. For personal use only.

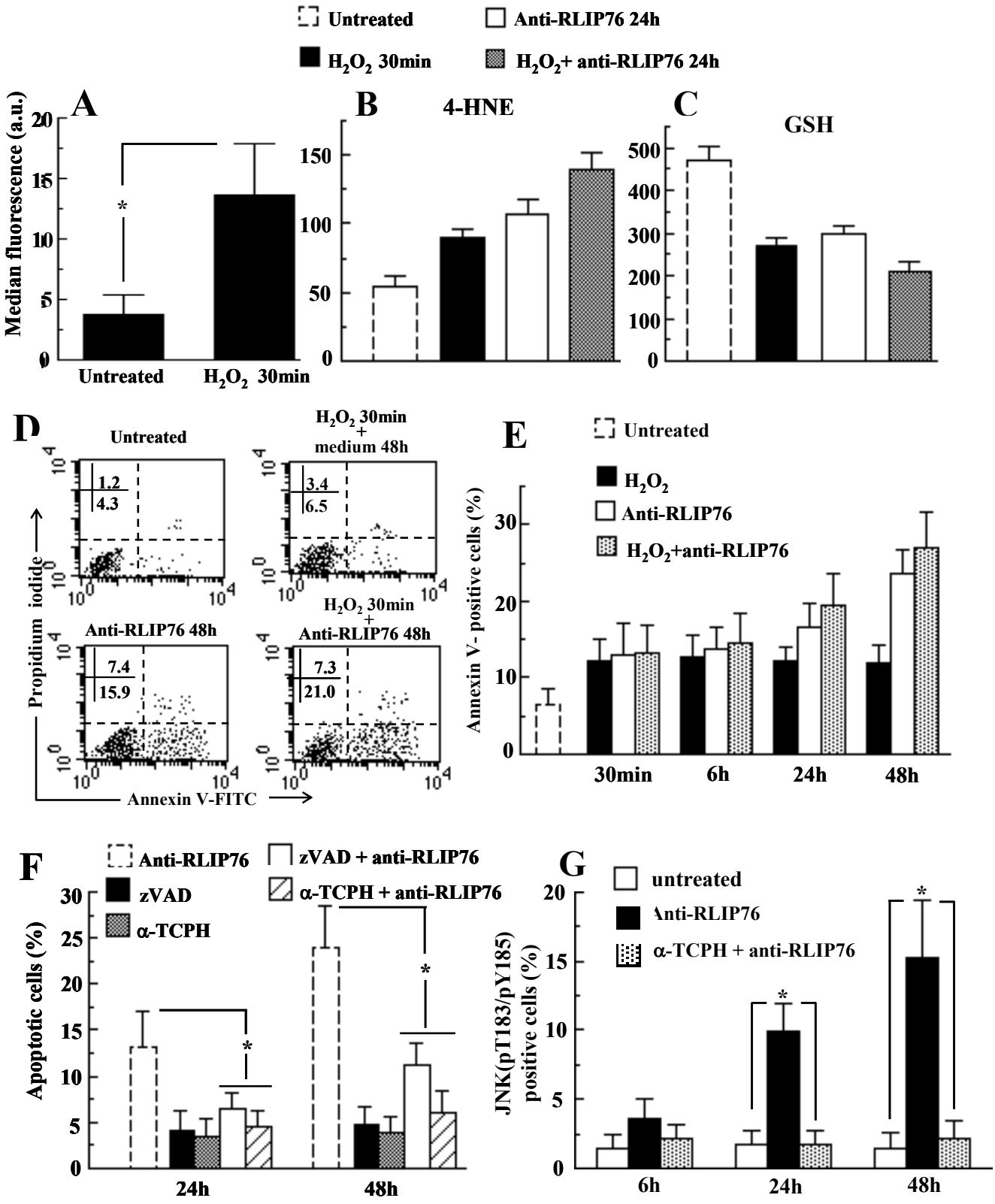


Figure 7