Role of tissue inhibitor of metalloproteinase-1 in development of autoimmunity lymphoproliferation

by Elena Boggio, Manuela Indelicato, Elisabetta Orilieri, Riccardo Mesturini, Maria Clorinda Mazzarino, Maria Francesca Campagnoli, Ugo Ramenghi, Umberto Dianzani, and Annalisa Chiocchetti

Haematologica 2010 [Epub ahead of print]

Citation: Boggio E, Indelicato M, Orilieri E, Mesturini R, Mazzarino MC, Campagnoli MF, Ramenghi U, Dianzani U, and Chiocchetti A. Role of tissue inhibitor of metalloproteinase-1 in development of autoimmunity lymphoproliferation. Haematologica. 2010; 95:xxx
doi:10.3324/haematol.2010.023085

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.

Haematologica (pISSN: 0390-6078, eISSN: 1592-8721, NLM ID: 0417435, www.haematologica.org) publishes peer-reviewed papers across all areas of experimental and clinical hematology. The journal is owned by the Ferrata Storti Foundation, a non-profit organization, and serves the scientific community with strict adherence to the principles of open access publishing (www.doaj.org). In addition, the journal makes every paper published immediately available in PubMed Central (PMC), the US National Institutes of Health (NIH) free digital archive of biomedical and life sciences journal literature.

Support Haematologica and Open Access Publishing by becoming a member of the European Hematology Association (EHA) and enjoying the benefits of this membership, which include free participation in the online CME program.
Role of tissue inhibitor of metalloproteinase-1 in development of autoimmunity lymphoproliferation

Running title: TIMP-1 in autoimmunity and lymphoproliferation

Elena Boggio,1 Manuela Indelicato,2 Elisabetta Orilieri,1 Riccardo Mesturini,1 Maria Clorinda Mazzarino,3 Maria Francesca Campagnoli,4 Ugo Ramenghi,4 Umberto Dianzani,1 and Annalisa Chiocchetti1

1Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Sciences, “A. Avogadro” University of Eastern Piedmont, Novara, Italy; 2Department of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome, Italy; 3Department of Biomedical Sciences, University of Catania, Catany, Italy, and 4Department of Pediatrics, University of Turin, Turin, Italy

Correspondence

Umberto Dianzani Department of Medical Science University of Eastern Piedmont “A. Avogadro” via Solaroli 17, I-28100 Novara, Italy. Phone: international +39.0321.660658. Fax: international +39.0321.620421. E-Mail: chiocche@med.unipmn.it

Funding

This work was supported by grants from Fondazione Cariplo Ricerca (Milan), AIRC (Milano), Compagnia di San Paolo (Torino), Fondazione Amici di Jean (Torino), FISM Fondazione Italiana Sclerosi Multipla-Cod. 2008/R/11 (Genova), Fondazione Cassa di Risparmio di Torino-Alfieri Project (Turin), to UD and Regione Piemonte (Ricerca Sanitaria Finalizzata Project and Regione Piemonte "Piattaforme Innovative Project") to UD and AC.
**Background.** Inherited defects decreasing function of the Fas death receptor cause the Autoimmune Lymphoproliferative Syndrome and its variant Dianzani Autoimmune Lymphoproliferative Disease; analysis of the lymphocyte transcriptome from a patient detected striking overexpression of Osteopontin and Tissue Inhibitor of Metalloproteinases-1. Since previous work on Osteopontin had detected increased serum levels in these patients, associated with variations of its gene, the aim of this work was to extend the analysis to Tissue Inhibitor of Metalloproteinases-1.

**Design and Methods.** Tissue Inhibitor of Metalloproteinases-1 levels were evaluated in sera and culture supernatants from patients and controls by enzyme-linked immunosorbent assay. Activation- and Fas-induced cell death were induced, in vitro, using anti-CD3 and anti-Fas antibodies respectively.

**Results.** Tissue Inhibitor of Metalloproteinases-1 levels were higher in sera from 32 patients (11 with Autoimmune Lymphoproliferative Syndrome and 21 with Dianzani Autoimmune Lymphoproliferative Disease) than in 50 healthy controls (p<0.0001), unassociated with variations of the Tissue Inhibitor of Metalloproteinases-1 gene. Both patients’ groups displayed increased serum levels of Osteopontin too. In vitro experiments showed that Osteopontin increased Tissue Inhibitor of Metalloproteinases-1 secretion by peripheral blood monocytes. Moreover, Tissue Inhibitor of Metalloproteinases-1 significantly inhibited both Fas- and activation-induced cell death of lymphocytes.

**Conclusions.** These data suggest that high Osteopontin levels may support high Tissue Inhibitor of Metalloproteinases-1 levels in Autoimmune Lymphoproliferative Syndrome and Dianzani Autoimmune Lymphoproliferative Disease, and hence worsen the apoptotic defect in these diseases.

**Key words:** osteopontin, tissue Inhibitor of metalloproteinases-1, lymphoproliferation, apoptosis, autoimmunity.
Introduction

Fas/Apo-1 (CD95) is a ubiquitous death receptor and cells expressing it undergo apoptosis upon interaction with Fas ligand (FasL)\(^1\)\(^-\)\(^3\). In lymphocytes, Fas triggering does not induce apoptosis in resting and recently activated T-cells, but the apoptosis-inducing pathway is connected to Fas several days after cell activation. This Fas/FasL interaction is involved in shutting-off immune responses, lymphocyte lifespan regulation, and maintenance of peripheral tolerance.

Inherited defects decreasing Fas function causes the autoimmune lymphoproliferative syndrome (ALPS) characterized by polyclonal accumulation of lymphocytes in the spleen and lymph nodes with lymphoadenomegaly and/or splenomegaly and development of autoimmune manifestations that predominantly involve blood cells.\(^1\) Typically, ALPS patients display expansion of TCR\(\alpha\)\(\beta\) positive, CD4/CD8 double-negative (DN) T cells in the peripheral blood and lymphoid tissues, and defective function of Fas. Moreover, ALPS patients are predisposed to develop lymphomas in adulthood. Most ALPS cases are ascribed to deleterious mutations of the Fas gene (ALPS type Ia), though a few patients carry mutations of the FasL gene (ALPS type Ib) or the caspase 10 gene (ALPS type II). However, a substantial proportion of ALPS patients displays defective Fas-induced cell death (FICD) but the mutated gene is not known (ALPS type III).\(^4\)\(^,\)\(^5\)

We described a picture that fulfils the first three criteria, but lacks expansion of DN T cells and mutations of the Fas, FasL or caspase-10 genes.\(^6\)\(^,\)\(^7\) Lack of DN cells has diagnostic relevance since search for these cells is a first level analysis to diagnose ALPS, but it may also mark immunopathologic differences since DN T cells might play a direct role in ALPS development. Since the complete paradigm of ALPS could not be demonstrated, this disease has been provisionally named Dianzani Autoimmune Lymphoproliferative Disease (DALD) by Mckusick (OMIM reference #605233; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM).\(^6\) A further point is that an ALPS-
like disease may even be caused by apoptotic defects unrelated to Fas function since Olivera et al have described a patient carrying a mutation in the n-Ras gene, who displayed normal FICD, but defective non receptor-mediated mitochondrial apoptosis.

In addition to causal mutations, ALPS development may be influenced by the genetic background. This may explain the incomplete penetrance of mild mutations. It has been shown for the mouse model of ALPS, i.e. MRL/lpr/lpr and MRL/gld/gld mice carrying mutations of the Fas and FasL genes respectively, since these mutations cause a much milder clinical picture in strains other than MRL one. In humans, a role may be played by variations of the osteopontin (OPN) gene since some of them result in a 8 fold increase in the risk of developing DALD, possibly because they increase production of OPN through mRNA stabilization. OPN is a phosphorylated glycoprotein containing an arginine-glycine-aspartate motif and displays both adhesive and cytokine functions. It is secreted by activated macrophages and activated T cells, and is present in extracellular fluids, at sites of inflammation, and in the extracellular matrix of mineralized tissues. It interacts with a variety of cell surface receptors, including several integrins and CD44. Binding of OPN to these cell surface receptors stimulates cell adhesion/migration and triggers several signaling pathways. OPN in vivo mainly acts as a pro-inflammatory cytokine by chemoattracting monocytes/macrophages and stimulating T helper 1 differentiation. DALD patients and MRL/lpr/lpr mice display increased serum levels of OPN, that may favour disease development by inhibiting activation-induced cell death (AICD), which is a further mechanism to switch off the immune response. AICD is induced by lymphocyte reactivation through the antigen receptor, it is partly independent from Fas function, and may functionally compensate the Fas-function defect in ALPS patients.

Our attention to OPN has been prompted by a cDNA array analysis comparing expression of genes involved in lymphocyte apoptosis and proliferation in a DALD patient and her healthy brother. Apart from OPN, we detected a second transcript clearly hyperexpressed in the
patient, namely that of Tissue Inhibitor of Metalloproteinase 1 (TIMP-1), which belongs to a family of proteins functioning as specific inhibitors of matrix metalloproteinases (MMPs).\textsuperscript{15} This observation was intriguing, since TIMP-1 also acts as an autocrine and paracrine factor that influences several functions of immune cells, including apoptosis. For example, it inhibits AICD in Hodgkin lymphoma cells and upregulates the anti-apoptotic protein BclX\textsubscript{L} in Burkitt lymphoma cells. Moreover, human recombinant TIMP-1 (rTIMP-1) inhibits the cell-mediated cytotoxicity that may play a role in lymphocyte AICD.\textsuperscript{16-18} These observations prompted the present investigation of the role of TIMP-1 in ALPS and DALD development. We found that TIMP-1 serum levels were higher in both ALPS and DALD patients than in healthy controls, but sequencing of the TIMP-1 gene showed that this was not ascribable to its variations. By contrast, we found that recombinant OPN (rOPN) stimulated TIMP-1 expression by monocytes in vitro. Moreover, we showed that TIMP-1 inhibits both FICD and AICD of lymphocytes in vitro, and these data suggest that it may play a role in ALPS and DALD development by worsening the apoptotic defect.
Design and Methods

Patients

We analyzed 11 ALPS (6 type I, 5 type III) and 21 DALD patients followed at the Pediatric Department, University of Turin, Italy and 50 age-matched healthy controls. ALPS was diagnosed from the presence of all the following criteria: 1) autoimmune manifestations; 2) chronic non-malignant lymphadenopathy (two or more enlarged lymph nodes over 2 cm in diameter) and/or splenomegaly; 3) defective Fas-induced apoptosis in vitro, 4) mutations in the Fas, FasL, or caspase-10 genes and/or expansion of DN T cells in the peripheral blood. The Fas, FasL, caspase-10, and OPN genes were sequenced from genomic DNA, as previously reported by Chiocchetti et al10. DALD was diagnosed from the presence of the first three criteria only.

Common infectious causing lymphadenopathy (Epstein Barr Virus, Cytomegalovirus, Rubella and/or Toxoplasma) were ruled out by serological testing; histology of the lymph nodes was evaluated in patients with bulky adenopathies indicative of malignancy. Patients were screened for serum autoantibodies (anti-phospholipid, anti-nuclear, anti-native-DNA, anti-mitochondrion and anti-smooth-muscle antibodies); anti-blood-cell autoantibodies were sought if peripheral cytopenia was present. Serum immunoglobulin (Ig) levels were determined and lymphocyte immunophenotype was investigated. DN T cell levels were determined by immunofluorescence and flow cytometry, and used to differentiate ALPS and DALD; DN T cell expansion was defined as DN T cells higher than 2% of total TCR__+ circulating lymphocytes. Written informed consent was obtained from patients and controls. The study was planned according to the guidelines of the local ethical committee.
Cells
PBMC were separated by the Ficoll-Hypaque (Lympholyte-H, Cedarlane Laboratories, Netherlands) density-gradient centrifugation. Cultures were performed in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin (Invitrogen, Burlington, ON, Canada).

CD4$^+$ or CD8$^+$ T cells, and monocytes were negatively purified from PBMC using magnetic bead kits (Miltenyi-Biotec GmbH, Germany). CD19$^+$ B cells were purified from tonsils of children undergoing routine tonsillectomies and further fractionated by discontinuous Percoll (Pharmacia Biotech, Uppsala, Sweden) density gradients as previously described by Dono et al$^{19}$. Cell purity was checked by immunophenotypic analyses and was higher than 95%.

In some experiments, cells were treated with recombinant OPN (rOPN) derived from the murine myeloma cell line NS0, anti-OPN neutralizing polyclonal antibody, recombinant TIMP-1 (rTIMP-1), anti-TIMP-1 neutralizing polyclonal antibody; all these reagents were from R&D system (Minneanapolis, USA).

Spontaneous cell death was evaluated by the Trypan blue exclusion test and assessment of lactate dehydrogenase (LDH) release in the culture supernatants with the CytoTox-One™ Homogeneous Membrane Integrity Kit (Promega, Madison, USA).

Array analysis
Panorama Human Cytokine Gene Arrays (PRCK0002) and Panorama Human Apoptosis Array (PRAP0002) were purchased from Sigma-Genosys (London, United Kingdom) as previously described by Chiocchetti et al$^{10}$. 
ELISA

Concentrations of OPN and TIMP-1 in sera and culture supernatants and rOPN and rTIMP-1 were measured by ELISA according to the manufacturers (IBL, Germany respectively and GE Healthcare, Piscataway, NY, USA). Absorbance was detected with a microplate reader (Bio-Rad, Hercules, CA, USA) and the I-smart program was used to calculate the standard curve.

Real-Time RT-PCR

Total RNA was isolated from PBMC cultures, treated or not with rOPN (500 ng/ml), using the Nucleospin RNAII kit (Machery-Nagel, Germany). RNA (500ng) was retrotranscribed by the ThermoScript™ RT PCR System (Invitrogen, Burlington, ON, Canada). TIMP-1 and OPN expression were evaluated with a gene expression assay (Assay-on Demand: TIMP-1, Assay No. Hs99999139_m1; Assay-on Demand: OPN, Assay No. Hs00167093_m1 Applied Biosystem, Foster City, CA, USA). The Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH Assay No. Hs99999905_m1) was used to normalize for cDNA amounts. Real time PCR was performed using the 7000 Sequence Detection System (Applied Biosystem) in duplicate for each samples, in a 20 µL final volume containing 0.5 µL diluted cDNA, 10 µL TaqMan universal PCR master mix (Applied Biosystem), and 1 µL Assay-on Demand mix. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. The results were analyzed with a standard curve model.
**Intracellular staining of TIMP-1**

Intracellular staining of TIMP-1 was performed on cells permeabilized using the FIX&PERM kit (Caltag, Burlingame, CA). Monocytes were treated with or without 500 ng/mL rOPN for 6h, in the presence of 10 µg/mL Brefeldin A (Sigma Aldrich St. Louis MO) and then stained with a FITC-conjugated anti-CD14 antibody (Caltag), fixed, permeabilized, and stained with a PE-conjugated anti-TIMP-1 antibody (R&D system) prior to analysis with a FACSCalibur cytofluorimeter (BD Biosciences).

**Cell death assays**

AICD and FICD were evaluated, as previously reported by Chiocchetti et al.¹⁰, on T cell lines obtained by activating PBMC with PHA at days 0 (1 µg/mL) and 12 (0.1 µg/mL) and cultured in RPMI 1640 medium + 10% FBS + IL-2 (2-10-100 U/mL) (Sigma-Aldrich). AICD was assessed at day 6, FICD at day 18. In the AICD assay, cells were cultured in wells coated with anti-CD3 mAb (OKT3, 10 µg/mL), whereas in the FICD assay they were cultured in the presence of a soluble anti-Fas mAb (CH11, UPSTATE 1 µg/mL). In these assays, cells (5 x 10⁴/well) were cultured for 16 hours in the presence or absence of titrated amounts (0.60, 0.125 and 0.25 µg/mL) of rTIMP-1 or alkylated-rTIMP-1 (0.250 µg/mL), or in the presence of an anti-TIMP-1 neutralizing antibody (3 µg/mL). Live cells were then counted in each well using the trypan blue exclusion test. Assays were performed in triplicate and results were expressed as relative cell survival % calculated as follows: (total live cell count in the assay well / total live cell count in the respective control well) X 100.
Statistical analysis

The Mann-Whitney U-test was used to compare unpaired data of different groups and Wilcoxon’s signed rank test for analysis of paired data in a group. TIMP-1 and OPN values were expressed as median and 25th-75th percentile (interquartile range, IQR). Correlations were tested by Pearson analysis. All p values were 2-tailed and the significance cut-off was p<0.05. Statistical analysis was performed with the GraphPad Instat software (GraphPad Software, San Diego, California, USA) software.
Results

TIMP-1 expression is increased in ALPS and DALD patients

The transcriptome of PHA-activated PBMC derived from a DALD patient and her healthy brother was evaluated by cDNA macroarrays containing 573 genes involved in cell apoptosis or proliferation. Analysis showed altered expression of several genes in the patient. The most striking differences were increased expression of OPN, previously reported, and TIMP-1 (Figure 1A). To confirm increased expression of both TIMP-1 and OPN mRNA, we assessed their level by real time PCR, and found that PHA-activated PBMC from the patient displayed 1.6- and 8-fold higher levels of TIMP-1 and OPN, respectively, than those from the healthy brother (Figure 1B).

To move this observation to the protein level and assess its generality, we measured TIMP-1 in the sera of 11 ALPS (6 ALPS-I, 5 ALPS-III) and 21 DALD patients and 50 healthy age-matched controls by ELISA. Results showed that TIMP-1 levels were about 1.5 fold higher in both ALPS (median=132 ng/mL, IQR 117-171) and DALD (median=131 ng/mL, IQR 122-141) patients than in the controls (median=83 ng/mL; IQR 56-110; p<0.0001 vs both groups) (Figure 2). Separate analysis of ALPS-I and ALPS-III patients showed that TIMP-1 levels were higher in both groups of patients than in the controls (ALPS-I: median=167 ng/mL, IQR 140-171, p<0.001 ALPS-III: median=117 ng/mL, IQR 115-119, p<0.05) without significant differences between the two groups of patients. Figure 2 also shows the serum levels of OPN, which were higher in both ALPS (median= 274 ng/mL, IQR 125-339, p<0.05) and DALD (median=254 ng/mL, IQR 191-302, P<0.001) patients than in the controls (median=142 ng/mL, IQR 96-194).

The TIMP-1 gene is located in the X chromosome. Some of us have previously shown that the +372C variant of the TIMP-1 gene is associated to Italian male patients affected from Systemic Sclerosis, while no association was observed in females. To determine whether the
TIMP-1 increase was associated with variants of the TIMP-1 gene, we sequenced its exons and intron boundaries in all patients and controls. Results detected 5 single nucleotide polymorphisms (http://snpper.chip.org; rs5953060, rs4898, rs6609533, rs2070584, rs6609534), but their allelic and genotypic frequencies were not different in patients and controls, even after selection for gender, and not associated to TIMP-1 levels (data not shown). Moreover, TIMP-1 levels were not significantly different in males and females in both the control and patient groups, which shows that gender does not influence levels.

**OPN induces TIMP-1 expression in vitro**

Since patients displayed increased serum levels of both TIMP-1 and OPN, we investigated the functional interplay between OPN and TIMP-1 which are both involved in the inflammatory response. To this aim, we evaluated the effect of rOPN on TIMP-1 expression in vitro. PBMC from healthy controls were cultured in the presence and absence of rOPN, and TIMP-1 expression was evaluated at different times both at the mRNA and protein level. Real-time PCR evaluation of the TIMP-1 mRNA showed that rOPN induced TIMP-1 upregulation that peaked after 6h, and then decreased during the following 16h and 24h (Figure 3A). ELISA evaluation of protein secretion showed that untreated PBMC displayed substantial secretion of TIMP-1 after 24h of culture that declined at 72h, whereas rOPN increased TIMP-1 secretion by about 50% during the first 24h (p<0.05) and levels did not decline during the 72h culture (p<0.01) (Figure 3B). The OPN effect on TIMP-1 secretion was not ascribable to its capacity to protect cells from spontaneous apoptosis since live cell counts were not different in OPN-treated and -untreated wells at any culture time (12-24-48-72h). Moreover, the rOPN effect was not due to the small amount of endotoxin possibly contaminating the rOPN preparation (< 1 EU/µg rOPN), since TIMP-1 secretion was not induced by a 10 fold higher dose of LPS in control experiments (data not shown).
Furthermore, proportions of CD14⁺, CD4⁺, CD8⁺ and CD19⁺ cells, detected by immunofluorescence and flow cytometry, were similar in OPN-treated and -untreated wells at any culture time (data not shown). Further experiments evaluated the effect of titrated amounts of rOPN (0.05-2.5 µg/mL) on TIMP-1 secretion by PBMC after 48h of culture and showed that the effect was dose-dependent; the 2.5 µg/mL dose of rOPN increased TIMP-1 secretion by about 2 fold (Figure 3C).

To detect which cell type was secreting TIMP-1 in our assay, we evaluated its secretion by purified CD4⁺ or CD8⁺ T cells, CD19⁺ B cells, and CD14⁺ monocytes (0.5 X 10⁶/mL each) cultured in the presence and absence of rOPN (0.5 µg/mL). ELISA evaluation of TIMP-1 in the supernatants collected after 48h showed that both the basal and the OPN-induced secretion of TIMP-1 were mainly detectable in monocytes, whereas the other cell types secreted minimal amounts (Figure 4A). The specificity of the rOPN effect was assessed in parallel experiments in which monocytes were treated with rOPN (0.5 µg/mL) in the presence of an anti-OPN neutralizing mAb (10 µg/mL). Results showed that OPN neutralization abrogated the TIMP-1 secretion induced by rOPN (Figure 4A).

To confirm the effect of rOPN on TIMP-1 production by monocytes, we analyzed TIMP-1 expression at the single-cell level by intracytoplasmic immunofluorescence to detect TIMP-1 before secretion. Purified monocytes cultured with or without rOPN (0.5 µg/mL) for 6h were surface-stained with a FITC-conjugated anti-CD14 mAb, permeabilized, and then intracellular-stained with a PE-conjugated anti-TIMP-1 mAb. Intracellular staining confirmed that TIMP-1 was basally produced by monocytes, and it was substantially upregulated by rOPN (Figure 4B). This effect was not ascribable to OPN-mediated modulation of cell death in culture, since similar cell death levels were detected in OPN-treated and -untreated cells by assessment of LDH release and by the Trypan blue exclusion test (data not shown).
TIMP-1 inhibits T cell apoptosis

Since ALPS and DALD are ascribed to defective lymphocyte apoptosis and we had already found that OPN may contribute to the defect by inhibiting AICD\textsuperscript{10}, we investigated the role of TIMP-1 in AICD or FICD in PHA-derived T cell lines obtained from healthy controls. In the presence of rTIMP-1 (0.25 µg/mL), cells were treated with anti-CD3 or anti-Fas mAb to induce AICD and FICD respectively and cell survival was evaluated after 16h. Results showed that rTIMP-1 significantly inhibited both FICD (p<0.05), and, at higher levels, AICD (p<0.001) (Figure 5). Titration experiments showed that the effect was dose-dependent in the 0.06-0.25 µg/mL range, which overlaps with the ranges detected in our patients’ sera (data not shown). To assess its specificity, we evaluated the effect of rTIMP-1 (0.25 µg/mL) on AICD and FICD in the presence and absence of an anti-TIMP-1 neutralizing antibody (3 µg/mL). Results showed that the anti-TIMP-1 antibody abrogated the rTIMP-1 effect on both AICD and FICD, supporting its specificity. To assess relationship with the MMP inhibitory activity of TIMP-1, we compared the effect of rTIMP-1 and its alkylated form, which loses the MMP-inhibitory activity, on FICD and AICD\textsuperscript{17,21}. Results showed that rTIMP-1 alkylation didn’t abrogate the rTIMP-1 effect on both types of cell death. These experiments were performed using T cell lines cultured in the presence of low doses of IL-2. Since the IL-2 level can influence T cell sensitivity to FICD and AICD, we repeated these experiments using T cells cultured in the presence of high doses of IL-2 (10 and 100 U/mL) to rule out the possibility that the TIMP-1 effect was due to downmodulation of T cell activation. Results showed that TIMP-1 inhibited FICD and AICD also in T cells cultured in high IL-2 levels (Supplemental Figure 1).
Discussion

This work shows that TIMP-1 levels are increased in ALPS and DALD patients, and suggests that they worsen the lymphocyte apoptotic defect causing these diseases. This possibility is supported by our in vitro data showing that TIMP-1 inhibits both FICD and AICD of activated T cells, just as other workers have shown that it inhibits B cell apoptosis induced by both Fas-dependent and -independent pathways, and apoptosis of several other cell types.17 The increased TIMP-1 levels were not associated with variants of the TIMP-1 gene and may thus be secondary to other stimuli. IL-6, IL-1α, b-FGF, EGF, and PDGF induce TIMP-1 secretion22, but their involvement in our patients is unlikely since only a few of them displayed increased levels of these cytokines (data not shown). By contrast, an effective stimulus might be OPN since its levels are significantly increased in both ALPS and DALD patients. In line with this possibility, our in vitro experiments showed that OPN substantially induced TIMP-1 secretion in monocytes at concentrations comparable to those detectable in vivo.

The effect of TIMP-1 on lymphocyte apoptosis was not dependent on its inhibition of MMPs, since it was preserved in alkylated TIMP-1 which loses the MMP-inhibitory activity. This is in line with several works showing that TIMP-1 exerts anti-apoptotic activity in several cell lines independently of its MMP-inhibitory activity 23,24.

One possibility is that the effect on FICD and AICD is mediated by TIMP-1 interaction with its receptor CD63, a tetraspanin that is often used as a marker of late endosomes, but is also expressed on the surface of several cell types including activated T cells. In line with this possibility, CD63 has been shown to deliver costimulatory signals to T cells25 and exert an anti-apoptotic effect in other cell types.26 The TIMP-1 interaction with CD63 may also be involved in the TIMP-1 capacity to inhibit cell-mediated cytotoxicity, reported by other authors27, since CD63 marks the cytolytic granules of cytotoxic cells and its surface expression is increased in active cytotoxic cells. This inhibitory effect may play a role in
ALPS and DALD development since cell-mediated cytotoxicity may partly compensate the apoptosis defect in these diseases.\textsuperscript{14,27}

An alternative possibility is that the anti-apoptotic effect of TIMP-1 it is mediated by triggering of CD44, that has been shown to form a ternary complex with proMMP-9 and TIMP-1, inhibiting apoptosis of the UT-7 erytroid cell line.\textsuperscript{28} This effect is independent from the MMP-inhibitory activity. This molecular interplay is intriguing since OPN is known to upmodulate expression of CD44, which in turn is an OPN receptor\textsuperscript{29,30}. In this scenario, OPN, TIMP-1, and CD44 may build up an anti-apoptotic network where OPN upmodulates expression of both CD44 and TIMP-1, and CD44 triggers survival signals by interacting with both OPN and, through pro-MMP-9, TIMP-1. It is noteworthy that the anti-apoptotic effect of OPN that we previously described in cultured T cells was probably independent from TIMP-1 since, differently from TIMP-1, OPN inhibited AICD but not on FICD; moreover, we found that OPN induced TIMP-1 secretion in PBMC and monocytes, but not in T cells.

In addition to its effect on lymphocyte apoptosis, TIMP-1 may theoretically exert other effects in ALPS development. For instance, ALPS patients often display hypergammaglobulinemia and other authors have reported increased IL-10 levels.\textsuperscript{31} Both of these features may be influenced by TIMP-1, since it has been shown to regulate B cell differentiation and induce expression of IL-10\textsuperscript{16,32}. Moreover, TIMP-1 serum levels have been correlated with hypergammaglobulinaemia in patients with eosinophilic fasciitis.\textsuperscript{33} However, we did not detect any significant correlation between TIMP-1 and IL-10 levels or hypergammaglobulinemia in our patients (\textit{data not shown}).

In conclusion, these data suggest that the high OPN serum levels of ALPS patients may worsen the apoptotic defect causing ALPS not only by direct inhibition of AICD, but also by induction of high TIMP-1 levels inhibiting both AICD and FICD. This may open the way to novel approaches to ALPS/DALD therapy aimed at inhibiting the effects of these concurring factors.
Authorship and Disclosures

AC and UD drafted the manuscript, all authors contributed to the revision. EB, MI, EO and RM performed the experiments. MFC and UR diagnosed and characterized patients. EB, MI and AC performed statistical analyses. UR, MCM and UD critically revised the paper. All authors edited and approved the written manuscript.

The authors reported no potential conflicts of interests.
References


Figure 1. TIMP-1 is hyper-expressed in PHA-activated lymphocytes from a DALD patient. (A) Macroarray membranes hybridized with cDNA from a DALD patient and her healthy brother. Black arrows: OPN, white arrows: TIMP-1. (B) mRNA levels of TIMP-1 (left panel) and OPN (right panel) in lymphocytes evaluated by real-time PCR. GAPDH was used to normalize cDNA amounts. Results are expressed as fold-increase relative to the brother's levels.
Figure 2. ALPS and DALD patients display increased TIMP-1 and OPN serum levels. Serum concentrations of TIMP-1 (A) and of OPN (B) in 11 ALPS (ALPS-I: white squares, 5 ALPS-III: black squares) and 21 DALD patients (black triangles), and 50 healthy controls (black diamonds). The horizontal bars indicate the median values for each group and boxes represent IQR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney U-test).
Figure 3. rOPN induces TIMP-1 expression in vitro. (A) TIMP-1 mRNA evaluated in cell lysates by real-time PCR. (B) TIMP-1 protein evaluated in the culture supernatants by ELISA. (C) TIMP-1 protein secreted after 48h treatment with titrated rOPN. Means±SE from 6 experiments; *p<0.05, **p<0.01 (Wilcoxon’s signed rank test).
Figure 4. Monocytes are the main cell type secreting TIMP-1 in response to rOPN. (A) ELISA evaluation of TIMP-1 secreted by CD4⁺ or CD8⁺ T cells, CD19⁺ B cells, CD14⁺ monocytes after 48h culture with/without rOPN and anti-OPN antibody. Means±SE from 5 experiments *p<0.05 (Mann-Whitney U test). (B) Intracellular TIMP-1 staining of CD14⁺ monocytes cultured for 6h with/without rOPN.
Figure 5. rTIMP-1 inhibits AICD and FICD of T cells. AICD (A) and FICD (B) were induced in PHA-derived T cell lines from healthy controls in the presence/absence of rTIMP-1 (0.25 µg/mL), alkylated rTIMP-1 (0.25 µg/mL), and anti-TIMP-1 antibody (3 µg/ml). Results are expressed as relative cell survival % and are the mean±SE of 5 experiments. * p<0.05; ** p<0.01; ***p<0.001 (Mann-Whitney U-test).
Online Supplementary Figure 1. rTIMP-1 inhibits AICD and FICD of T cells cultured in high dose IL-2. AICD (A) and FICD (B) were induced in PHA-derived T cell lines from healthy controls, cultured in the presence of high levels (10–100U/mL) of IL-2 in the presence/absence of rTIMP-1 (0.25 μg/mL). Results are expressed as relative cell survival % and are the mean±SE of 6 experiments. * p<0.05; ** p<0.01 (Mann-Whitney U-test).