Role of tissue inhibitor of metalloproteinases-1 in the development of autoimmune lymphoproliferation

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ABSTRACT

Background
Inherited defects decreasing function of the Fas death receptor cause autoimmune lymphoproliferative syndrome and its variant Dianzani's autoimmune lymphoproliferative disease. Analysis of the lymphocyte transcriptome from a patient with this latter condition detected striking over-expression 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's autoimmune lymphoproliferative disease) than in 50 healthy controls ($P<0.0001$), unassociated with variations of the tissue inhibitor of metalloproteinases-1 gene. Both groups of patients also had increased serum levels of osteopontin. *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's autoimmune lymphoproliferative disease, and hence worsen the apoptotic defect in these diseases.

Key words: osteopontin, tissue inhibitor of metalloproteinases-1, lymphoproliferation, apoptosis, autoimmunity.

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Introduction

Fas/Apo-1 (CD95) is a ubiquitous death receptor and cells expressing it undergo apoptosis upon interaction with Fas ligand.[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/Fas ligand interaction is involved in shutting off immune responses, lymphocyte lifespan regulation, and maintenance of peripheral tolerance.

Inherited defects decreasing Fas function cause autoimmune lymphoproliferative syndrome (ALPS) which is 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, patients with ALPS have expansion of TCRαβ positive, CD4/CD8 double-negative 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 cases of ALPS are ascribed to deleterious mutations of the Fas gene (*FAS*) (ALPS type Ia), although a few patients carry mutations of the Fas ligand gene (*FASL*) (ALPS type Ib) or the caspase 10 gene (*CASP10*) (ALPS type II). However, a substantial proportion of ALPS patients have 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 double negative T cells and mutations of *FAS*, *FASL* or *CASP10*.[6,7] Lack of double-negative T cells has diagnostic relevance since a search for these cells is a first level analysis to diagnose ALPS, but it may also mark immunopathological differences since double-negative T cells might play a direct role in the development of ALPS. Since the complete paradigm of ALPS could not be demonstrated, this disease was provisionally named Dianzani's 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.*[8] have described a patient carrying a mutation in the *NRAS* gene, who displayed normal FICD, but defective non receptor-mediated mitochondrial apoptosis.

In addition to causal mutations, the development of ALPS may be influenced by the genetic background. This could explain the incomplete penetrance of mild mutations. This 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 the MRL one.[9] In humans, variations of the osteopontin gene (*OPN*) may play a role since some of them result in an 8-fold increase in the risk of developing DALD, possibly because they increase production of osteopontin through mRNA stabilization.[10] Osteopontin is a phosphorylated glycoprotein containing an arginine-glycine-aspartate motif and has 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.[11] It interacts with a variety of cell surface receptors, including several integrins and CD44. Binding of osteopontin to these cell surface receptors stimulates cell adhesion/migration and triggers several signaling pathways.[12] *In vivo* osteopontin mainly acts as a pro-inflammatory cytokine through its chemo-attraction of monocytes/macrophages and stimulation of T helper 1 differentiation.[13] DALD patients and MRL*lpr/lpr* mice have increased serum levels of osteopontin, which may favor disease development by inhibiting activation-induced cell death (AICD),[10] this being a further mechanism of switching 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.[14]

Our attention to osteopontin was 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 osteopontin, we detected a second transcript clearly hyper-expressed in the patient, namely that of tissue inhibitor of metalloproteinases 1 (TIMP-1), which belongs to a family of proteins functioning as specific inhibitors of matrix metalloproteinases (MMP).[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's lymphoma cells and up-regulates the anti-apoptotic protein BclX-L in Burkitt's lymphoma cells. Moreover, human recombinant TIMP-1 inhibits the cell-mediated cytotoxicity that may play a role in lymphocyte AICD.[16-18] These observations prompted the present investigation of the role of TIMP-1 in the development of ALPS and DALD.

Design and Methods

Patients

We analyzed 11 patients with ALPS (6 type I, 5 type III) and 21 with DALD 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: (i) autoimmune manifestations; (ii) chronic non-malignant lymphadenopathy (two or more enlarged lymph nodes over 2 cm in diameter) and/or splenomegaly; (iii) defective Fas-induced apoptosis *in vitro*; (iv) mutations in the *FAS*, *FASL*, or *CASP10* genes and/or expansion of double-negative T cells in the peripheral blood. The *FAS*, *FASL*, *CASP10* and *OPN* genes were sequenced from genomic DNA, as previously reported by Chiocchetti *et al.*[10] DALD was diagnosed when only the first three criteria were fulfilled.

Common infections causing lymphadenopathy (Epstein-Barr virus, cytomegalovirus, rubella and 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 a peripheral cytopenia was present. Serum immunoglobulin (Ig) levels were determined and lymphocyte immunophenotype was investigated. Double-negative T-cell levels were determined by immunofluorescence and flow cytometry, and used to differentiate ALPS and DALD; double-negative T-cell expansion was defined as the proportion of dou-

ble-negative T cells being more than 2% of the total TCRαβ+ circulating lymphocytes. Written informed consent to inclusion in this study was obtained from patients and controls. The study was planned according to the guidelines of the local ethical committee.

Cells

Peripheral blood mononuclear cells were separated by Ficoll-Hypaque (Limpholyte-H, Cedarlane Laboratories, the Netherlands) density-gradient centrifugation. Cultures were performed in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (Invitrogen, Burlington, ON, Canada).

$CD4^+$ or $CD8^+$ T cells, and monocytes were negatively purified from peripheral blood mononuclear cells 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 osteopontin derived from the murine myeloma cell line NS0, anti-osteopontin neutralizing polyclonal antibody, recombinant TIMP-1, and 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 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, UK) and were used as described by Chiocchetti *et al.*[10]

Enzyme-linked immunosorbent assays

Concentrations of osteopontin and TIMP-1 in sera and culture supernatants and recombinant osteopontin and recombinant TIMP-1 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' recommendations (IBL, Germany and GE Healthcare, Piscataway, NY, USA, respectively). 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 reverse transcriptase polymerase chain reaction

Total RNA was isolated from peripheral blood mononuclear cell cultures, treated or not with recombinant osteopontin (500 ng/mL), using the Nucleospin RNAII kit (Machery-Nagel, Germany). RNA (500ng) was reverse transcribed by the ThermoScript™ reverse transcriptase polymerase chain reaction (RT PCR) System (Invitrogen, Burlington, ON, Canada). *TIMP1* 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 tissue inhibitor of metalloproteinases-1

Intracellular staining of TIMP-1 was performed on cells permeabilized using the Fix&Perm kit (Caltag, Burlingame, CA, USA). Monocytes were treated with or without 500 ng/mL recombinant osteopontin for 6 h, in the presence of 10 μg/mL brefeldin A (Sigma Aldrich, St. Louis, MO, USA) and then stained with a fluorescein isothiocyanate-conjugated anti-CD14 antibody (Caltag), fixed, permeabilizated, and stained with a phycoerythrin-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 peripheral blood mononuclear cells with phytohemagglutinin at days 0 (1 μg/mL) and 12 (0.1 μg/mL) and cultured in RPMI 1640 medium + 10% fetal bovine serum + interleukin-2 (2, 10 and 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 monoclonal antibody (OKT3, 10 μg/mL), whereas in the FICD assay they were cultured in the presence of a soluble anti-Fas monoclonal antibody (CH11, UPSTATE 1 μg/mL). In these assays, cells (5×10^4/well) were cultured for 16 h in the presence or absence of titrated amounts (0.60, 0.125 and 0.25 μg/mL) of recombinant TIMP-1 or alkylated recombinant TIMP-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 are expressed as percentage 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 osteopontin values are expressed as medians and 25^{th}-75^{th} percentiles (interquartile range, IQR). Correlations were tested by Pearson's analysis. All *P* values are two-tailed and values less than 0.05 are considered to indicate a statistically significant result. Statistical analyses were performed with GraphPad Instat software (GraphPad Software, San Diego, California, USA).

Results

Expression of TIMP1 is increased in patients with autoimmune lymphoproliferative syndrome and Dianzani's autoimmune lymphoproliferative disease

The transcriptome of phytohemagglutinin-activated peripheral blood mononuclear cells derived from a patient with DALD and her healthy brother was evaluated by cDNA macroarrays containing 573 genes involved in cell apoptosis or proliferation. This analysis showed altered expression of several genes in the patient. The most striking differences were increased expression of *OPN,* previously reported,[10] and *TIMP1* (Figure 1A). To confirm increased expression of both *TIMP1* and *OPN* mRNA, we

assessed their level by real-time PCR, and found that phytohemagglutinin-activated peripheral blood mononuclear cells from the patient displayed 1.6- and 8-fold higher levels of *TIMP1* 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 from 11 patients with ALPS (6 ALPS-I, 5 ALPS-III) and 21 with DALD and from 50 healthy age-matched controls by ELISA. The 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; $P<0.0001$ *versus* both groups) (Figure 2). Separate analyses 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 osteopontin, 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 *TIMP1* gene is located in the X chromosome. Some of us have previously shown that the +372C variant of the *TIMP1* gene is associated with Italian male patients affected by systemic sclerosis, while no association was observed in females.[20] To determine whether the increase in TIMP-1 was associated with variants of the *TIMP1* gene, we sequenced the exons and intron boundaries of this gene in all patients and controls. Five single nucleotide polymorphisms (htp//snpper.chip.org; rs5953060, rs4898, rs6609533, rs2070584, rs6609534) were found, but their allelic and genotypic frequencies were not different between patients and controls, even after selection for gender, and they were not associated with TIMP-1 levels (*data not shown*). Moreover, TIMP-1 levels were not significantly different between males and females in either the control group or the group of patients, which shows that gender does not influence the levels of this protein.

Osteopontin induces tissue inhibitor of metalloproteinases-1 expression in vitro

Since patients displayed increased serum levels of both TIMP-1 and osteopontin, we investigated the functional interplay between osteopontin and TIMP-1 which are both involved in the inflammatory response. To this aim, we evaluated the effect of recombinant osteopontin on TIMP-1 expression *in vitro*. Peripheral blood mononuclear cells from healthy controls were cultured in the presence and absence of recombinant osteopontin, and TIMP-1 expression was evaluated at different times both at the mRNA and protein levels. Real-time PCR evaluation of the *TIMP1* mRNA showed that recombinant osteopontin induced *TIMP1* up-regulation, which peaked after 6 h and

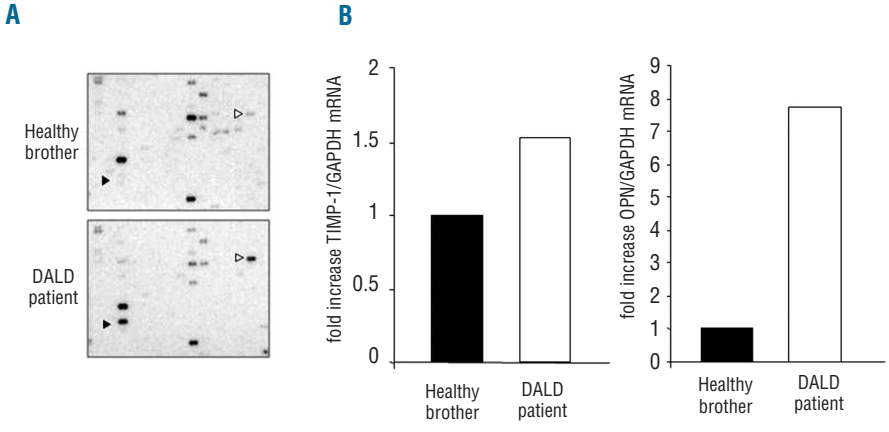

Figure 1. TIMP-1 is hyper-expressed in phytohemagglutinin-activated lymphocytes from a DALD patient. (A) Macroarray membranes hybridized with cDNA from a DALD patient and her healthy brother. Black arrows: osteopontin, white arrows: *TIMP-1*. (B) mRNA levels of TIMP-1 (left panel) and *OPN* (right panel) 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.

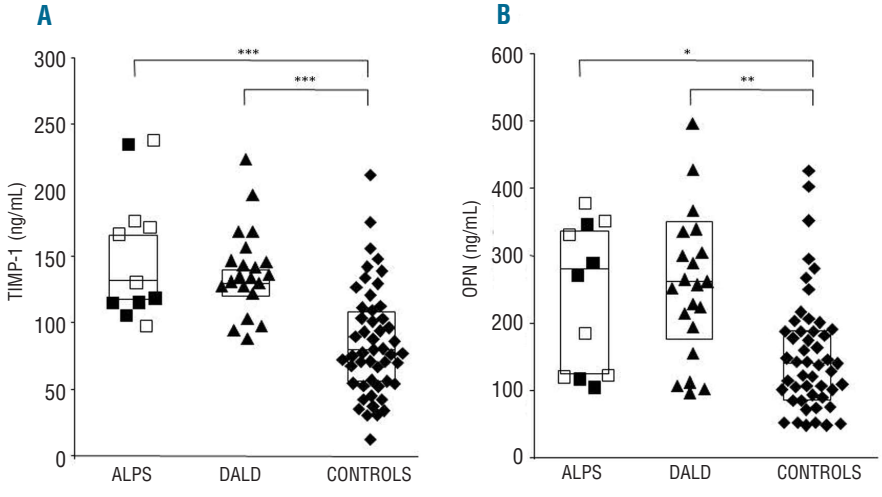

Figure 2. ALPS and DALD patients have increased serum levels of TIMP-1 and osteopontin (OPN). Serum concentrations of TIMP-1 (A) and of OPN (B) in 11 ALPS (6 ALPS-I: white squares, 5 ALPS-III: black squares) and 21 DALD patients (black triangles), and 50 healthy controls (black diamonds). The horizontal 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).

then decreased over the following 16 h and 24 h (Figure 3A). ELISA evaluation of protein secretion showed that untreated peripheral blood mononuclear cells secreted substantial amounts of TIMP-1 after 24 h of culture and that the secretion declined at 72 h, whereas recombinant osteopontin increased TIMP-1 secretion by about 50% during the first 24 h ($P<0.05$) and levels did not decline during the 72 h culture ($P<0.01$) (Figure 3B). The effect of osteopontin on TIMP-1 secretion was not ascribable to its capacity to protect cells from spontaneous apoptosis[18] since live cell counts were not different in osteopontin-treated and -untreated wells at any culture time (12, 24, 48 or 72 h). Moreover, the effect of osteopontin was not due to the small amount of endotoxin possibly contaminating the recombinant preparation (<1 EU/μg recombinant osteopontin), since TIMP-1 secretion was not induced by a 10-fold higher dose of lipopolysaccharide in control experiments (*data not shown*).

Furthermore, proportions of $CD14^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells, detected by immunofluorescence and flow cytometry, were similar in osteopontin-treated and -untreated wells at any culture time (*data not shown*). Further experiments evaluated the effect of titrated amounts of recombinant osteopontin (0.05-2.5 μg/mL) on TIMP-1 secretion by peripheral blood mononuclear cells after 48 h of culture and showed that the effect was dose-dependent; the 2.5 μg/mL dose of recombinant osteopontin increased TIMP-1 secretion by about 2-fold (Figure 3C).

To detect which type of cell was secreting TIMP-1 in our assay, we evaluated its secretion by purified $CD4^+$ or $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes (0.5×10^6/mL each) cultured in the presence and absence of recombinant osteopontin (0.5 μg/mL). ELISA evaluation of TIMP-1 in the supernatants collected after 48 h showed that both basal and osteopontin-induced secretion of TIMP-1 were mainly detectable in monocytes, whereas the other cell types secreted minimal amounts (Figure 4A). The specificity of the effect of osteopontin was assessed in parallel experiments in which monocytes were treated with recombinant osteopontin (0.5 μg/mL) in the presence of an anti-osteopontin neutralizing monoclonal antibody (10 μg/mL). Results showed that osteopontin neutralization abrogated the TIMP-1 secretion induced by recombinant osteopontin (Figure 4A).

To confirm the effect of recombinant osteopontin 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 recombinant osteopontin (0.5 μg/mL) for 6 h were surface-stained with a fluorescein isothiocyanate-conjugated anti-CD14 monoclonal antibody, permeabilized, and then intracellularly stained with a phycoerythrin-conjugated anti-TIMP-1 monoclonal antibody. Intracellular staining confirmed that TIMP-1 was basally produced by monocytes, and that it was substantially up-regulated by recombinant osteopontin (Figure 4B). This effect was not ascribable to osteopontin-mediated modulation of cell death in culture, since similar levels of cell death were detected in osteopontin-treated and -untreated cells by assessment of lactate dehydrogenase release and by the trypan blue exclusion test (*data not shown*).

Tissue inhibitor metalloproteinases-1 inhibits T-cell apoptosis

Since ALPS and DALD are ascribed to defective lymphocyte apoptosis and we had already found that osteopontin may contribute to the defect by inhibiting AICD,[10] we investigated the role of TIMP-1 in AICD or FICD in phytohemagglutinin-derived T-cell lines obtained from healthy controls. In the presence of recombinant TIMP-1 (0.25 μg/mL), cells were treated with anti-CD3 or anti-Fas monoclonal antibody to induce AICD and FICD, respectively, and cell survival was evaluated after 16 h. The results showed that recombinant TIMP-1 significantly inhibited both AICD ($P<0.01$) and, at higher levels, FICD ($P<0.001$) (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 recombinant TIMP-1 (0.25 μg/mL) on AICD and FICD in the presence and absence of an

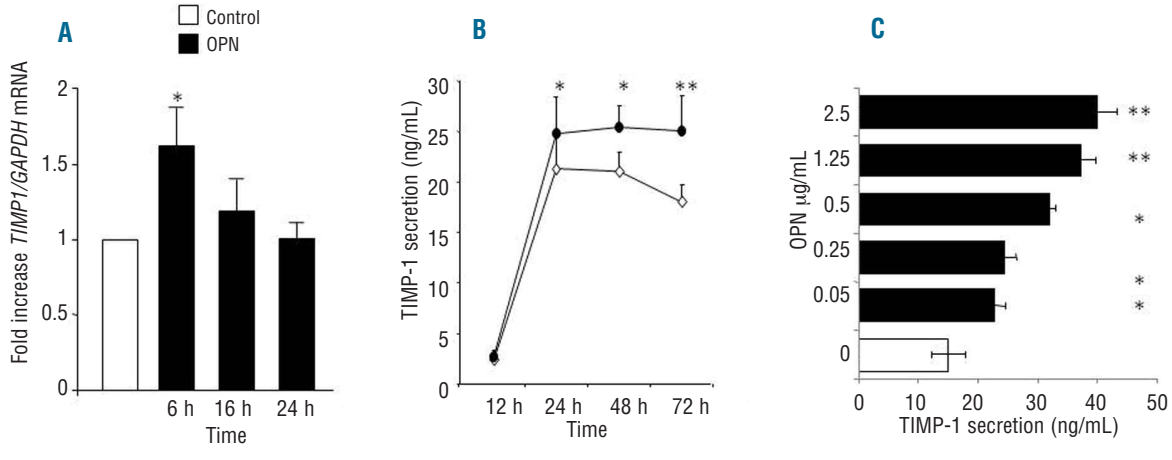

Figure 3. Recombinant osteopontin (OPN) induces TIMP-1 expression *in vitro*. (A) *TIMP-1* mRNA evaluated in cell lysates by real-time PCR. (B) TIMP-1 protein evaluated in the culture supernatants by ELISA. (C) TIMP-1 protein secreted after 48 h treatment with titrated recombinant OPN. Means±SE from six experiments; **P*<0.05, ***P*<0.01 (Wilcoxon's signed rank test).

anti-TIMP-1 neutralizing antibody (3 μg/mL). The anti-TIMP-1 antibody abrogated the effect of recombinant TIMP-1 on both AICD and FICD, supporting its specificity. To assess the relationship with the matrix metalloproteinase inhibitory activity of TIMP-1, we compared the effect of recombinant TIMP-1 and its alkylated form, which loses matrix metalloproteinase-inhibitory activity, on FIDC and AICD.[17,21] The results showed that alkylation of recombinant TIMP-1 did not abrogate the effect of recombinant TIMP-1 on either type of cell death. These experiments were performed using T-cell lines cultured in the presence of low doses of interleukin-2. Since the level of interleukin-2 can influence T-cell sensitivity to FIDC and AIDC, we repeated these experiments using T cells cultured in the presence of high doses of interleukin-2 (10 and 100 U/mL) to rule out the possibility that the effect of TIMP-1 was due to down-modulation of T-cell activation. We found that TIMP-1 inhibited FIDC and AIDC also in T cells cultured with high levels of interleukin-2 (*Figure 6*).

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 researchers have shown that it inhibits B-cell apoptosis induced by both Fas-dependent and Fas-independent pathways, and apoptosis of several other cell types.[17]

The increased levels of TIMP-1 were not associated with variants of the *TIMP1* gene and may thus be secondary to other stimuli. Interleukin-6, interleukin-1α, basic fibroblast growth factor, epidermal growth factor, and platelet-derived growth factor induce TIMP-1 secretion,[22] but their involvement in our patients is unlikely since only a few of them had increased levels of these cytokines (*data not shown*). By contrast, an effective stimulus might be osteopontin since its levels are significantly increased in both ALPS and DALD patients. In line with this possibility, our *in vitro* experiments showed that osteopontin 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 matrix metalloproteinases, since it was preserved in alkylated TIMP-1 which loses matrix metalloproteinase-inibitory activity. This is in line with several studies showing that TIMP-1 exerts anti-apoptotic activity in several cell lines independently of its matrix metalloproteinase-inhibitory activity.[23,24]

One possibility is that the effect on FIDC and AICD is mediated by the interaction of TIMP-1 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 co-stimulatory signals to T cells[25] and exert an anti-apoptotic effect on other cell types.[26] The interaction between TIMP-1 and

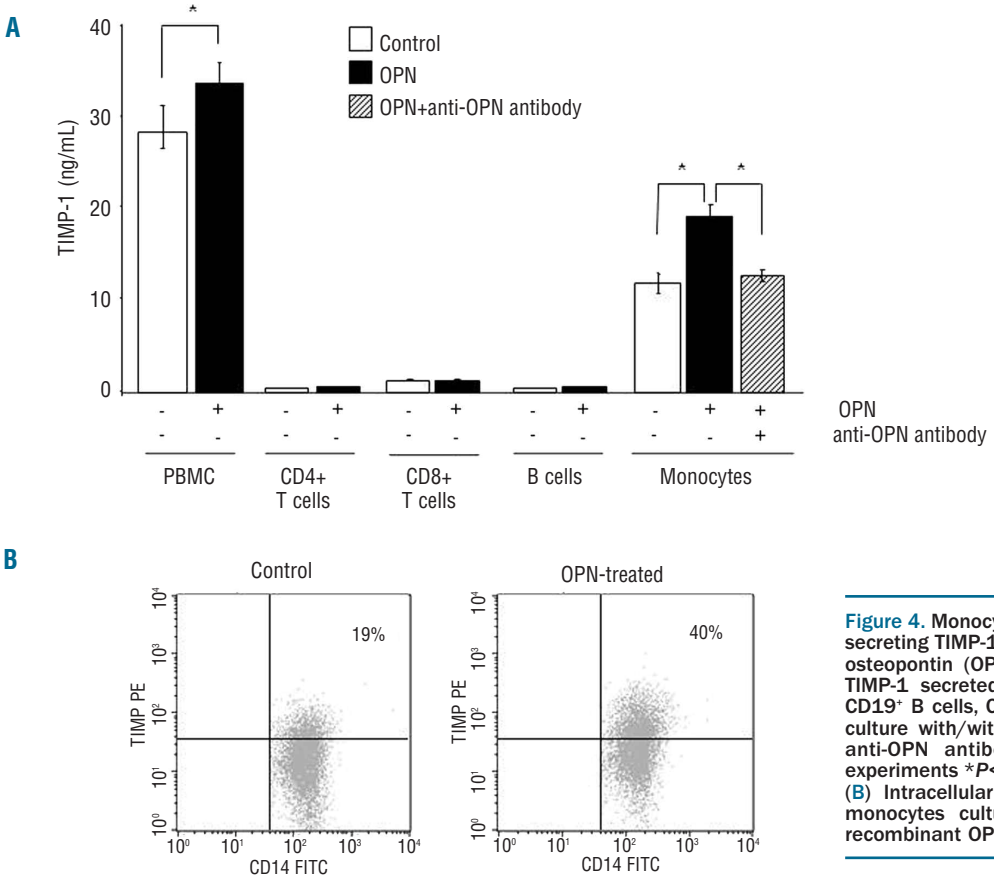

Figure 4. Monocytes are the main type of cell secreting TIMP-1 in response to recombinant osteopontin (OPN). (A) ELISA evaluation of TIMP-1 secreted by $CD4^+$ or $CD8^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes after 48 h culture with/without recombinant OPN and anti-OPN antibody. Means±SE from five experiments *P<0.05 (Mann-Whitney U test). (B) Intracellular TIMP-1 staining of $CD14^+$ monocytes cultured for 6 h with/without recombinant OPN.

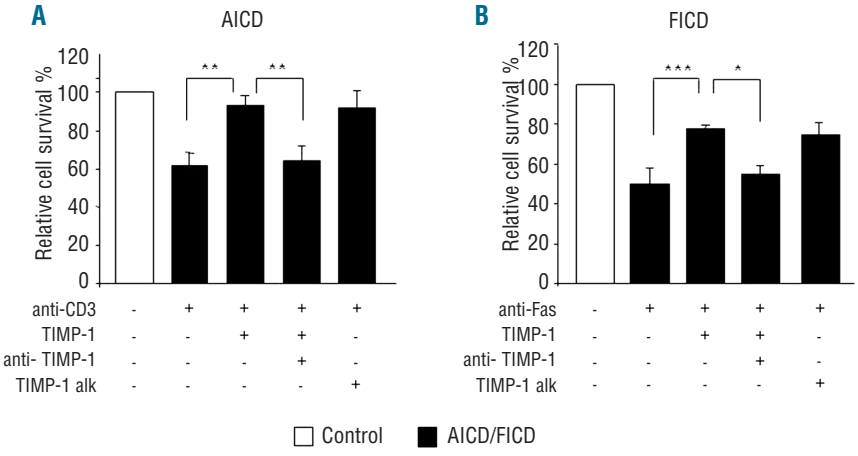

Figure 5. Recombinant TIMP-1 (rTIMP-1) inhibits AICD and FICD of T cells. AICD (A) and FICD (B) were induced in phytohemagglutinin-derived T-cell lines from healthy controls in the presence/absence of rTIMP-1 (0.25 μg/mL), alkylated rTIMP-1 (0.25 μg/mL), and anti-TIMP-1 antibody (3 μg/mL). Results are expressed as relative cell survival (%) and are the mean±SE of five experiments. **P*<0.05; ***P*<0.01; ****P*<0.001 (Mann-Whitney U-test).

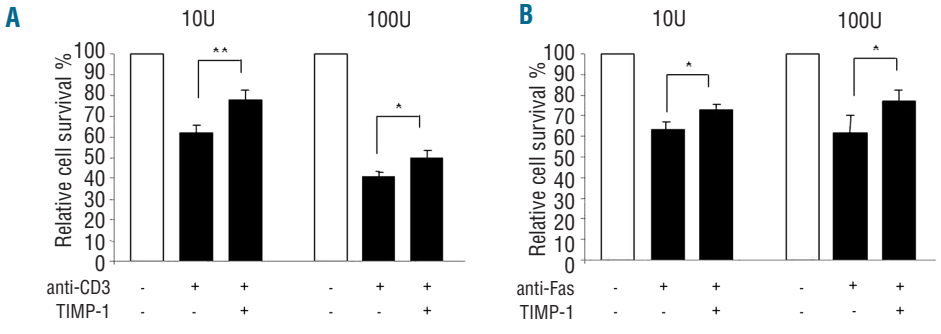

Figure 6. Recombinant TIMP-1 (rTIMP-1) inhibits AICD and FICD of T cells cultured in high dose interleukin-2. AICD (A) and FICD (B) were induced in phytohemagglutinin-derived T-cell lines from healthy controls, cultured in the presence of high levels (10 or 100 U/mL) of interleukin-2 in the presence/absence of rTIMP-1 (0.25 μg/mL). Results are expressed as relative cell survival (%) and are the mean±SE of six experiments. **P*<0.05; ***P*<0.01 (Mann-Whitney U-test).

CD63 may also be involved in the capacity of TIMP-1 to inhibit cell-mediated cytotoxicity, reported by other authors,[27] 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 the development of ALPS and DALD since cell-mediated cytotoxicity may partly compensate the apoptosis defect in these diseases.[14,27]

An alternative possibility is that the anti-apoptotic effect of TIMP-1 is mediated by triggering of CD44, which has been shown to form a ternary complex with pro-matrix metalloproteinase-9 and TIMP-1, inhibiting apoptosis of the UT-7 erythroid cell line.[28] This effect is independent of matrix metalloproteinase-inibitory activity. This molecular interplay is intriguing since osteopontin is known to up-modulate expression of CD44, which in turn is an osteopontin receptor.[29,30] In this scenario, osteopontin, TIMP-1, and CD44 may build up an anti-apoptotic network in which osteopontin up-modulates expression of both CD44 and TIMP-1, and CD44 triggers survival signals by interacting with both osteopontin and, through pro-matrix metalloproteinase-9, TIMP-1. It is noteworthy that the anti-apoptotic effect of osteopontin that we previously described in cultured T cells was probably independent of TIMP-1 since, unlike TIMP-1, osteopontin inhibited AIDC but not FICD; moreover, we found that osteopontin induced TIMP-1 secretion in peripheral blood mononuclear cells 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 have hypergammaglobulinemia and other authors have reported increased interleukin-10 levels.[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 interleukin-10.[16,32] Moreover, TIMP-1 serum levels have been correlated with hypergammaglobulinemia in patients with eosinophilic fasciitis.[33] However, we did not detect any significant correlation between TIMP-1 and interleukin-10 levels or hypergammaglobulinemia in our patients (*data not shown*).

In conclusion, these data suggest that the high serum levels of osteopontin in patients with ALPS 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

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