Research Article

Anti-glutathione S-transferase omega 1-1 (GSTO1-1) antibodies are increased during acute and chronic inflammation in humans

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Abstract

Glutathione S-transferase omega-1 (GSTO1-1) is a cytosolic enzyme involved in the modulation of critical inflammatory pathways as well as in cancer progression. Auto-antibodies against GSTO1-1 were detected in the serum of patients with esophageal squamous cell carcinoma and were proposed as potential biomarkers in the early detection of the disease. Our findings show that anti-GSTO1-1 antibodies can be found in a variety of inflammatory diseases, including autoimmune rheumatoid arthritis, infectious SARS-CoV-2, and trichinellosis. Our findings strongly suggest that anti-GSTO1-1 antibodies may be a marker of tissue damage/inflammation rather than a specific tumor-associated biomarker.

Graphical Abstract
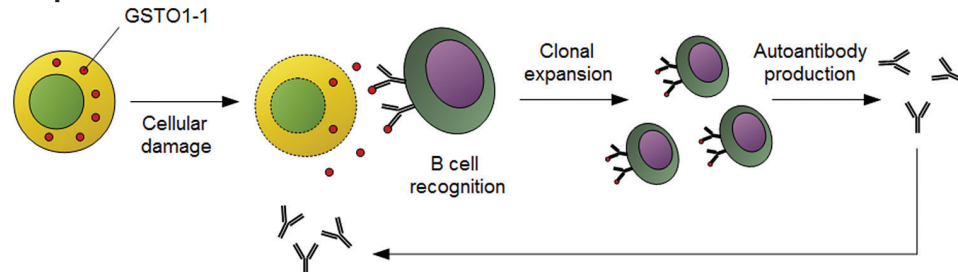
Keywords: glutathione S-transferase omega-1, auto-antibodies, inflammation, trichinellosis, rheumatoid arthritis, COVID-19
Abbreviations: COVID-19: coronavirus disease 2019; ELISA: enzyme-linked immunosorbent assay; GSTO1-1: glutathione S-transferase omega 1-1; RA: rheumatoid arthritis.

Introduction

Glutathione S-transferase omega-1 (GSTO1-1) is a cytosolic enzyme with uncommon reductase as well as thioltransferase activity [1, 2]. Some studies documented an important role of GSTO1-1 in modulating pathophysiological processes through the regulation of NF-κB [3] as well as NLRP3 pathways [4, 5], and the activation of survival pathways upon exposure to chemotherapeutic drugs [6] was also demonstrated. Increased levels of GSTO1-1 and/or its polymorphisms are also associated with inflammatory conditions [7–9] as well as with cancer progression [10, 11], and the involvement of hypoxia-inducible factors (HIFs) [12], Nrf2-ARE pathway

[13] and the RAS family of proto-oncogenes [14] has been proposed. Overall, these findings indicate that GSTO1-1 can play a role in modulating innate immune response as well as cancer cell survival.

In a study on esophageal squamous cell carcinoma (ESCC), Li and colleagues demonstrated the overexpression of GSTO1-1 in esophageal tissues along with detectable autoantibodies against GSTO1-1 in serum, the latter possibly representing a potential biomarker for the early detection of ESCC. Indeed, the frequency of detectable autoantibodies in ESCC subjects was 44.8% as compared with a 6.7% of positivity in normal serum [11]. On the other hand, in another

study on thyroid cancer, the overexpression of GSTO1-1 was not associated with relevant levels of autoantibodies in serum [14].

Based on the studies mentioned above, it can be envisaged that high levels of anti-GSTO1-1 autoantibodies could be detectable in the serum of subjects with inflammatory diseases. With the aim to evaluate this hypothesis, we set up a specific enzyme-linked immunosorbent assay (ELISA) for the detection of anti-GSTO1-1 antibodies in serum and analyzed samples from patients with different types of autoimmune and infectious diseases.

Materials and methods

Serum samples

The study has been conducted in accordance with the Helsinki Declaration as revised in 2013. Sera obtained from *n.* 20 patients (11 male, 9 female) recovered in the Intensive Care Units of the Pisa University Hospital, who were positive for SARS-COV-2 PCR nasopharyngeal swab and clinical symptoms of coronavirus disease 2019 (COVID-19), were collected within 15 days of disease onset. The median age was 65.7 (IQR: 60.5–72.8). The sera from *n.* 15 consecutive patients (6 male, 9 female) with the diagnosis of rheumatoid arthritis (RA) according to the ACR/EULAR revised criteria [15] followed in the Clinical Immunology Unit of the Pisa University Hospital, were also collected. The median age was 59.5 (IQR: 54–69.5). Sera from *n.* 15 *Trichinella spiralis*-infected subjects (median age 42 (IQR: 34.0–55.0); see [16] for details) and *n.* 32 *Trichinella britovi*-infected subjects (median age 47.0 (IQR: 37.0–64.0); see [17] for details) were also analyzed. Finally, sera from *n.* 19 healthy subjects were collected and used as controls.

The studies were approved by the respective institutional Ethical Committee for pathological samples and by the Committee on Bioethics of the University of Pisa for samples from healthy controls. The privacy of the patients was protected by ensuring anonymity and confidentiality in both data management and reporting. No identifying information is contained in the manuscript.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was optimized in our laboratory according to Lim *et al.* [18]. Briefly, 96-well polystyrene microtiter plates (Greiner Bio-One) were coated overnight at 4°C with purified recombinant GSTO1-1, previously produced in our laboratory [10], diluted (10 μg/ml) in phosphate-buffered saline (PBS). GSTO1-1-free wells were also prepared by using PBS alone.

Plates were blocked with the synthetic polymer Ficoll PM400 (4% w/v in PBS) for 1 h, at room temperature (RT). Antigen-coated and antigen-free wells were incubated for 2 h with 50 μl of sera diluted at 1:100 in PBS, 1% w/v Ficoll PM400, 0.2% v/v Tween 20. After three washes, wells were incubated for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody (Jackson) and washed three times again. Anti-GSTO1-1 antibodies were finally detected with 3,3′,5,5′-tetramethylbenzidine (TMB). The reaction was stopped by adding 2N sulfuric acid and absorbance was measured at 450 nm using a plate reader (BioTek). A calibration curve was routinely added to each plate by using serial dilutions of a rabbit anti-GSTO1-1

antiserum produced previously in our laboratories [10] and an HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). Absorbances obtained from patient samples were subtracted from the corresponding blanks (antigen-free wells) and used to interpolate their values from the calibration curve. The data were expressed as an arbitrary units of standard dilutions (AUSD).

Dot-blot analysis

Blots (3 μl) of purified recombinant GSTO1-1 were made on strips of nitrocellulose membrane. Membranes were blocked with 5% dry milk in TBS-T buffer (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4), incubated with 1:100 diluted sera (1h, RT), and finally treated with an HRP-conjugated goat anti-human IgG antibody (1h, RT; Jackson). Analysis of blots was obtained using the ECL detection system (Roche, Basel, Switzerland) and a Bio-Rad ChemiDoc apparatus equipped with the Quantity One software.

CRISPR-Cas9 method

HeLa cell line (Clontech) was grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine (Sigma-Aldrich) at 37°C with 5% CO_2 incubation. To knockout the GSTO1-1 gene, cells were transfected with GSTO1 Double Nickase Plasmid (h) (Santa Cruz Biotechnology, INC). Double Nickase Plasmid was designed to disrupt gene expression by causing highly specific Cas9-mediated double nicking of the target gene [19]. Cells were transfected with 1 ug of GSTO1 Double Nickase Plasmid using UltraCruz Transfection Reagent (Santa Cruz Biotechnology) following the manufacturer's instructions. Selection with puromycin was started after 72 h of the transfection. Cells were screened for GSTO1-1 expression by western blot analysis (data not shown). A single clone with no GSTO1-1 expression (HeLaGSTO1-) was thus obtained.

Immunofluorescence assay

Representative samples of sera from AR and trichinellosis patients were used to check their reactivity against cellular GSTO1-1. Sera were diluted (1:100) and preincubated with purified recombinant GSTO1-1 protein overnight at 4°C. Wild type HeLa, GSTO1-1 knock-out HeLa (HeLaGSTO1-), and HepG2 cells (ICLC—Genova) were seeded in 96 well plates. After 24 h samples were processed as described [10]. Briefly, cells were fixed with 4% v/v paraformaldehyde and blocked with phosphate-buffered saline (PBS) integrated with 8% w/v milk and 1% v/v Triton X-100 in PBS (30 min, RT). Cells were then incubated with untreated or GSTO1-1 preadsorbed sera (60 min, RT) and, after washing, all samples were incubated with Alexa Fluor 488 conjugated anti-human IgG (Life Technologies) as a secondary antibody. Samples incubated with a rabbit anti-GSTO1-1 antiserum [10] and Alexa Fluor 468 conjugated anti-rabbit IgG antibody were used as positive controls. DAPI was used for nuclear counterstaining. Images were acquired under identical conditions with a Leica microscope (DM750) equipped with an ICC50 camera and LAS EZ software.

Statistical analysis

Statistical analysis of data was performed by Kruskal–Wallis test followed by Dunn's post-test for multiple comparisons (GraphPad Prism Software).
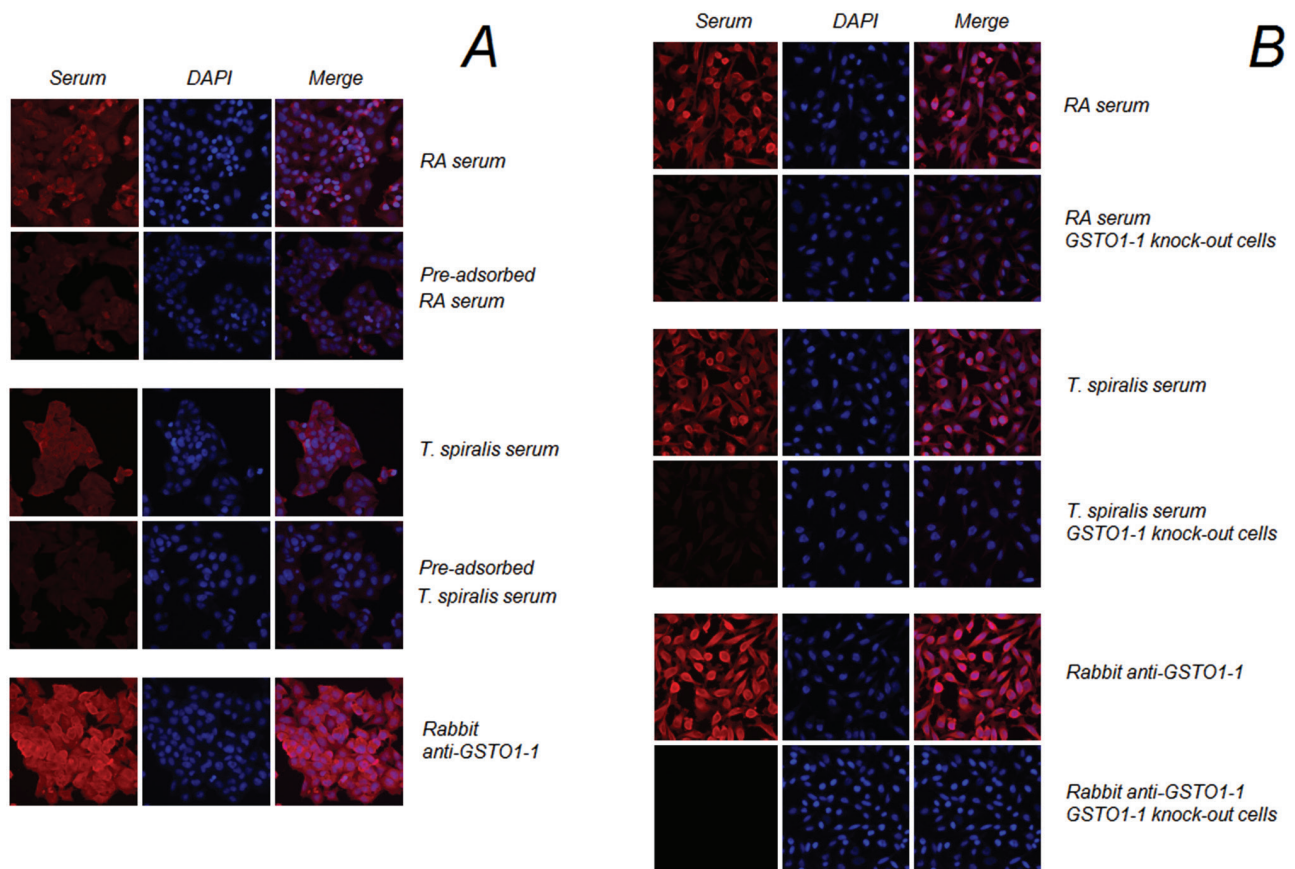
Figure 1: immunocytochemical analysis of sera reactivity against GSTO1-1. Sera from RA and *T. spiralis* infected subjects were incubated with HepG2 cells (A) and with wild type and GSTO1-1 knockout HeLa cells (B). The reddish staining was markedly reduced by both pre-incubating sera with a purified GSTO1-1 protein (A) and silencing GSTO1-1 expression (B), confirming the specificity of the detection. A rabbit anti-GSTO1-1 serum was used as a positive control (A,B).

Results and discussion

Anti-GSTO1-1 antibodies are detectable in sera of patients with inflammatory diseases

Sera from patients diagnosed with different types of inflammatory diseases were analyzed for the presence of anti-GSTO1-1 antibodies. Patients with confirmed diagnoses of coronavirus disease 2019 (COVID-19) and rheumatoid arthritis (RA) were selected as models of short-term, acute inflammation and long-term, an autoimmune chronic inflammatory disorder, respectively. In addition, subjects diagnosed with late trichinellosis (2–8 years of infection) by *T. spiralis* [16] and early infection by *T. britovi* [17], respectively, were selected as models of parasite-induced inflammation.

The presence of anti-GSTO1-1 antibodies was first evaluated by immunocytochemistry on human HepG2 and HeLa cell lines. As shown in Fig. 1A, sera from both RA and *T. britovi*-infected patients produced a red fluorescence staining in the cytoplasm of HepG2 cells, which was significantly reduced when sera were pre-adsorbed with purified GSTO1-1 protein. The red staining was also detectable in HeLa cells, but it was significantly reduced when GSTO1-1 knockout cells were incubated with the same sera (Fig. 1B).

Levels of anti-GSTO1-1 antibodies are higher in pathological samples

We optimized an ELISA procedure for the detection of anti-GSTO1-1 levels in serum. A highly reproducible calibration
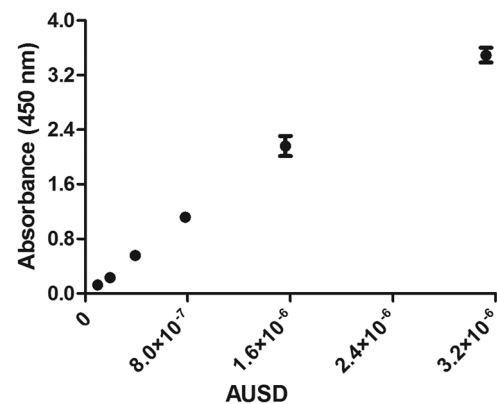

Figure 2: a representative calibration curve used for serum anti-GSTO1-1 quantification by ELISA. Serial dilutions of a rabbit anti-GSTO1-1 serum were used to create suitable calibration curves for anti-GSTO1-1 quantification in sera. Data are expressed as an arbitrary units of standard dilutions (AUSD) and are means ± SD of three values.

curve with serial dilutions of a rabbit anti-GSTO1-1 antiserum was used as a reference to quantify unknown anti-GSTO1-1 levels in serum samples (Fig. 2). A purified recombinant GSTO1-1 protein was immobilized on the microplate and its suitability as the target antigen was also confirmed by dot-blot with patients' sera (data not shown).

As shown in Fig. 3A, auto-antibodies against GSTO1-1 were detectable in all pathological samples, whereas about

40% of healthy subjects used as controls presented with very low/undetectable levels of anti-GSTO1-1. Moreover, 35%, 46%, and 60% of COVID-19, *T. spiralis* infected- and RA-samples, respectively, presented with levels of

Figure 3: anti-GSTO1-1 antibodies in patients sera. (A) Anti GSTO1-1 levels in sera from patients with the diagnosis of rheumatoid arthritis (RA; *n* = 15), COVID-19 (*n* = 20) and trichinellosis by *T. spiralis* (*n*.15). Data are expressed as an arbitrary units of standard dilutions (AUSD) of the reference standard. (*) *P* < 0.05. (B) Anti-GSTO1-1 levels in sera of patients after 4, 8, and 16 weeks (w) from the infection by *T. britovi* (*n*.32). Data are expressed as an arbitrary units of standard dilutions (AUSD) of the reference standard. (*) *P* < 0.05 as compared to healthy controls; (**) *P* < 0.05 as compared to healthy controls and " *T. britovi* (4 w)" samples.

anti-GSTO1-1 antibodies higher than the 98th percentile of the control group. Accordingly, the statistical analysis confirmed that levels of anti-GSTO1-1 antibodies were significantly higher in all pathological sera as compared to the control group (*P* < 0.05).

We then analyzed anti-GSTO1-1 levels in the second group of patients diagnosed with trichinellosis caused by *T. britovi* and for whom sequential samples over a period of 16 weeks from the serological diagnosis were available. Interestingly, we previously demonstrated that GSTO1 is overexpressed during the nurse cell formation in *T. spiralis* infection in mice and that *T. spiralis* excretory/secretory products from muscle larvae induce GSTO1-1 expression in the pro-monocytic human cell line U937 [20]. As shown in Fig. 3B, a progressive increase of anti-GSTO1-1 antibodies was detectable from 4 weeks to 16 weeks after the infection, possibly reflecting the progressive increase of GSTO1-1 expression in the inflamed tissue upon Trichinella infection and its release in the extracellular compartment. In support of this hypothesis, patients with increased levels of LDH after 10–30 days of infection (see [17] for details) presented with significantly higher levels of anti-GSTO1-1 antibodies at 16 weeks (*P* < 0.05; Fig. 4A), suggesting a more relevant myositis [21]. Similarly, a significant correlation was also observed between anti-GSTO1-1 antibodies and LDH levels in COVID-19 patients sera (*P* < 0.05; Fig. 4B). Interestingly, it was recently demonstrated that plasma from critically ill COVID-19 patients contains broadly auto-reactive immunoglobulins [22]. The release of GSTO1-1 could thus stimulate an immune response against the same GSTO1-1 protein and leads to an increase in anti-GSTO1-1 antibodies.
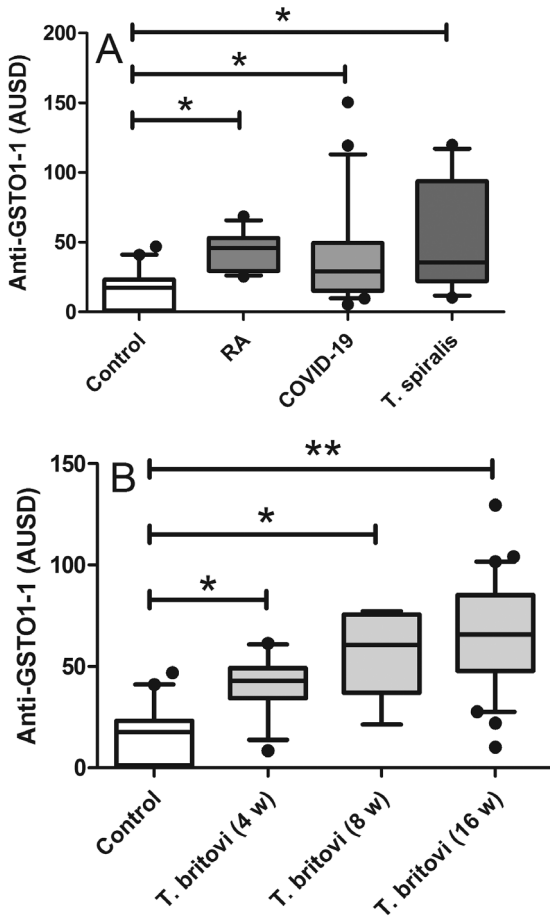
Concluding remarks

Our data demonstrate that anti-GSTO1-1 antibodies can be detectable in different types of inflammatory diseases, namely, the acute inflammation induced by SARS-CoV-2 infection, the chronic inflammation associated with RA, and the parasite-induced inflammation by different *Trichinella* species, both during the early infection and in late-stage patients.

GSTO1-1 is overexpressed in some types of malignancies [10, 11, 23] where it is involved in promoting cell survival and proliferation, and in immune cells where it takes part in the modulation of some major signalling pathways.
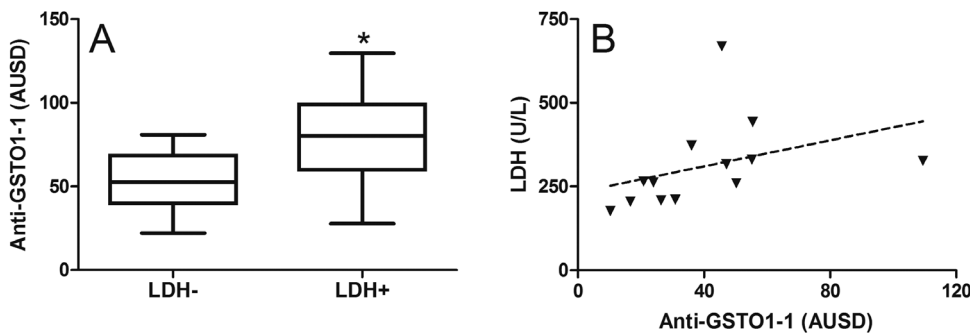

Figure 4: anti-GSTO1-1 antibodies and LDH levels. (A) Patients with increased levels of LDH (LDH+) after 10–30 days of infection by *T. britovi* (see [17] for details) present with significantly higher levels of anti-GSTO1-1 antibodies; (*) *P* < 0.05. (B) Anti-GSTO1-1 antibodies correlate with LDH levels in COVID-19 patients sera (*P* < 0.01; *r*. 0.692). Data are expressed as an arbitrary units of standard dilutions (AUSD) of the reference standard.

Nevertheless, the modulation of the Toll-like receptor 4-mediated pathway [3], the NLRP3 inflammasome activation [4], and the consequent production of proinflammatory cytokines [24] are critically involved in cancer progression as well. Our observation that anti-GSTO1-1 antibodies correlate with LDH levels suggests that their increase might result from an abnormal expression and release of GSTO1-1 upon cellular damage in the extracellular compartment, where it could be sensed by the immune system as a foreign antigen.

The presence of anti-GSTO1-1 antibodies in different types of inflammatory as well as oncologic diseases thus suggests that these antibodies could represent—at best—a marker of tissue damage/inflammation rather than a type of specific tumor-associated biomarker.

Conflict of interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

S.P., E.L., and A.C. performed biochemical determinations; F.P. and P.M. reviewed the clinical records and interpreted data; S.P., F.P., and A.C. drafted the manuscript; A.P. and F.B. reviewed the clinical records and the manuscript.

Ethics approval

The study has been conducted in accordance with the Helsinki Declaration as revised in 2013. The study was approved by the institutional ethics committee and by the Committee on Bioethics of the University of Pisa for samples of healthy controls. All subjects involved gave written informed consent.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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