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RESEARCH ARTICLE

Silica-induced fibrosis: an ancient response from the early metazoans

Marina Pozzolini[1,*], Sonia Scarfì[1], Lorenzo Gallus[1], Sara Ferrando[1], Carlo Cerrano[2] and Marco Giovine[1]

ABSTRACT

Exposure to crystalline silica particles causes silicosis, an occupational disease leading to an overproduction of collagen in the lung. The first step of this pathology is characterized by the release of inflammatory mediators. Tumour necrosis factor (TNF) is a pro-inflammatory cytokine directly involved in silica-induced pulmonary fibrosis. The marine demosponge *Chondrosia reniformis* is able to incorporate silica grains and partially dissolve the crystalline forms apparently without toxic effects. In the present work, *C. reniformis* tissue explants were treated with fine quartz dust and the expression level of fibrogenic genes was assayed by qPCR, demonstrating an overexpression of a fibrillar and a non-fibrillar collagen and of prolyl-4-hydroxylase enzyme. The deposition of new collagen could also be documented in quartz-treated sponge explants. Furthermore, TNF pro-inflammatory cytokine overexpression and involvement in silica-induced sponge collagen biosynthesis was demonstrated in quartz-treated explants as compared with controls by means of specific TNF inhibitors affecting the fibrogenic gene response. As no documentable detrimental effect was observed in treated explants, we conclude that the *C. reniformis* unique quartz engulfment and erosion is physiological and beneficial to the animal, leading to new collagen synthesis and strengthening of the body stiffness. Thus, we put forward the hypothesis that an ancient physiological behaviour from the lowest of the Metazoa, persisting through evolution via the same molecular mediators such as TNF, may have become the cause of disease in the specialized tissues of higher animals such as mammals.

KEY WORDS: Collagen, TNF, Porifera, Silicosis, Evolution

INTRODUCTION

Chondrosia reniformis is a marine sponge, common in the Mediterranean Sea, belonging to the Demospongiae class. As it does not possess an internal skeleton, to strengthen its structure, this sponge usually incorporates solid particles like little stones and sand into its body (Cerrano et al., 2007). In particular, it is able to engulf silica sand particles (quartz crystals) in its ectosome, and partially dissolve them through ascorbic acid-driven surface erosion, leading to an increase in the concentration of soluble silicates in the surrounding water (Bavestrello et al., 1995). Indeed, ascorbic acid is an abundant vitamin present in *C. reniformis* tissues. This unique reaction has been demonstrated to be specific for the crystalline form of silica (quartz), leaving the amorphous form quite untouched (Fenoglio et al., 2000). In *C. reniformis*, in fact, amorphous silica, in the form of spicules deriving from other demosponges, is also engulfed but is retained inside the sponge to strengthen the body structure. The retention of the spicules is due both to the fact that the ascorbic acid-driven erosion is ineffective on this form of silica and to the fact that *C. reniformis* does not possess silicases, the enzymes that specifically dissolve amorphous silica and are only present in demosponges able to produce siliceous spicules (for a review, see Ehrlich et al., 2010a). Histological analysis reveals that the engulfed crystalline silica grains are enveloped by tight collagen fibres associated with ectosomal cells, revealing that this particular process of incorporation/erosion of quartz is cell mediated (Pozzolini et al., 2016a). Despite the ecological relevance of soluble silica turnover to the marine environment, the physiological significance of this peculiar process in *C. reniformis* is still unclear. However, it is well known that inhalation of crystalline silica particles in mammals causes severe respiratory diseases such as pulmonary inflammation, fibrosis and lung cancer (Ding et al., 2002). Silica-induced fibrosis, termed silicosis, is characterized by an imbalance of extracellular matrix deposition, resulting in the accumulation of collagen fibres in the lung tissue. Although silicosis is a widespread occupational disease and has been recognised for many years, the molecular bases that drive this pathology are not completely elucidated. It is known that one of the first molecular events is the overproduction of inflammatory mediators (Hamilton et al., 2008). Among these humoral factors, tumour necrosis factor alpha (TNF) plays a fundamental role in the pathogenesis of silica-induced lung injury (Piguet et al., 1990). The pro-fibrotic role of TNF seems to be especially related to silica-induced fibrosis (Piguet et al., 1990), but it is not yet entirely clear what kind of molecular events make TNF a pro-fibrotic cytokine in silica-mediated diseases. An accurate knowledge of this pathway would provide new perspectives on possible therapies of silicosis.

Given the fibrotic effect of quartz dust in mammalian models, it has been speculated that crystalline silica sand incorporation and erosion by *C. reniformis* could be causal to sponge collagen biosynthesis (Giovine et al., 2013). The identification by immunostaining of the enzyme involved in collagen maturation, prolyl-4-hydroxylase (P4H), around the numerous sand grains usually incorporated by this sponge strongly supports this hypothesis (Pozzolini et al., 2015). A functional relationship between collagen and silica has already been described in glass sponges (Hexatinellida). In fact, in *Hyalonema seboldi*, a fibrillar collagen with an unusual double-hydroxylated motif, has been found in the axial filaments of the metre-long siliceous spicules of this sponge and it has been speculated by Ehrlich et al. (2010b) that this collagen, with its peculiar hydroxylated motifs, serves as a template for silica precipitation during the formation of the spicule itself. These findings suggest a close interaction between this protein and various forms of silica.

Quite recently, a full-length cDNA coding for a non-fibrillar collagen type was described and cloned from *C. reniformis* and

[1]Department of Earth, Environment and Life Sciences (DiSTAV), University of Genova, 16132 Genoa, Italy. [2]Department of Life and Environmental Sciences (DiSVA), Polytechnic University of Marche, 60131 Ancona, Italy.

*Author for correspondence (marina.pozzolini@unige.it)

M.P., 0000-0001-6867-3816

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expression of this gene is positively regulated by soluble silicates (Pozzolini et al., 2012). Furthermore, a TNF homologue was characterized from *C. reniformis* (chTNF) and is involved in collagen and P4H gene expression (Pozzolini et al., 2016b).

In the light of the recently acquired molecular information on *C. reniformis*, and thanks to its ability to incorporate quartz without any detectable biological damage, this animal may constitute an extraordinary model to understand the molecular evolution of mammalian silica-induced fibrosis.

In the present work, in order to evaluate the relationship between quartz incorporation and collagen synthesis in *C. reniformis*, we evaluated the expression levels of fibrogenic genes (collagen and P4H) in tissue explants of this animal treated with quartz dusts. Moreover, in order to verify a possible chTNF involvement in this process, the gene expression levels of this cytokine were measured in quartz-stimulated *C. reniformis* fragmorph models and the expression of fibrogenic genes was analysed in the presence of specific TNF inhibitors.

MATERIALS AND METHODS

Chemicals

All reagents were acquired from Sigma-Aldrich (Milan, Italy), unless otherwise stated.

Experimental animals

Specimens of *Chondrosia reniformis* Nardo 1847 were collected in the area of the Portofino Promontory (Liguria, Italy) at depths of 10–20 m. During sampling and transport, the temperature was maintained at 14–15°C. Short-term stabilization was performed as described in Pozzolini et al. (2014). Briefly, the sponges were stored at 14°C in 200 l aquaria containing natural seawater collected in the same area of the Portofino Promontory with a salinity of 37‰ and equipped with an aeration system.

Chondrosia reniformis fragmorph model preparation

Fragmorph models were prepared as previously described (Pozzolini et al., 2012). Briefly, specimens of *C. reniformis* were taken from the aquarium and immediately transferred to plastic containers filled with filtered natural seawater (FNSW). The sponges were cut in cylindrical fragments of 7 mm diameter (fragmorphs) using a sterilized brass borer, then quickly transferred to 12-well plates and stored in the aquarium for approximately 1 month, until complete surface healing and attachment to the plastic plates. For each set of experiments, fragmorphs obtained from a single large sponge were used.

Quartz dust suspension preparation

Quartz dust (Min-U-Sil 5, US Silica, Berkeley Spring Plant, WV, USA) was dry sterilized. A suspension of 10 mg ml^{-1} in FNSW was prepared and sonicated just before use.

Experimental conditions

Mature fragmorph samples (n=3, 1 month old) were removed from the aquarium, washed twice in FNSW, then incubated in 6 ml of FNSW and stimulated by injection of 100 µl per fragmorph of 10 mg ml^{-1} quartz dust suspension at 14°C for 18 h, 48 h and 7 days. Control fragmorph samples (n=3) were injected with 100 µl of FNSW per fragmorph.

In a second set of experiments, in order to establish the role of chTNF on quartz collagen and P4H gene regulation, fragmorphs were incubated in the same conditions and for the above-specified times, in the presence of two different TNF inhibitors: pentoxifylline (PTX, 5 mmol l^{-1} final concentration) or SPD304 (22 µmol l^{-1} final concentration) as described in Pozzolini et al. (2016b).

Gene expression analysis

Total RNA was extracted using Isol-RNA Lysil (5′-Prime, Eppendorf srl, Milan, Italy), according to the manufacturer's instructions, from *C. reniformis* fragmorphs stimulated as described above. Subsequently, the poly-A fraction was isolated using FastTrack® MAG mRNA isolation kit (Life Technologies, Milan, Italy). The sponge cDNA was obtained by reverse transcription with iScript cDNA Synthesis kit (Bio-Rad Laboratories, Milan, Italy), according to the manufacturer's protocol, using 200 ng of purified mRNA. GAPDH (accession number KM217385) was used as reference gene for sample normalization. Each PCR reaction was performed in 20 µl containing: 1× master mix iQ SYBR®Green (Bio-Rad), 0.2 µmol l^{-1} of each primer and 0.8 µl of synthesized cDNA. All samples were analysed in triplicate. The following conditions were used: initial denaturation for 3 min followed by 45 cycles with denaturation at 95°C for 15 s, annealing and elongation at 57.7°C for 60 s. Fluorescence was measured at the end of each elongation step. The next step was a slow heating (1°C s^{-1}) of the amplified product from 55 to 92°C in order to generate a melting temperature curve. All primers (Table 1) were designed using the software Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA, USA) and obtained from TibMolBiol (Genova, Italy). Data analysis was carried out using the DNA Engine Opticon® 3 Real-Time Detection System Software program (3.03 version). In order to detect the relative gene expression of chTNF, fibrillar and non-fibrillar collagen (F- and NF-collagen, respectively), P4H and the anti-apoptotic gene Bcl-2, each compared with an untreated (control) calibrator sample, the comparative threshold Ct method (Aarskog and Vedeler, 2000) was used with the software Gene Expression Analysis for iCycler iQ Real-Time Detection System (Bio-Rad) (Vandesompele et al., 2002).

Collagen assay

Chondrosia reniformis fragmorph specimens treated with quartz dust for 7 days, as described in 'Experimental conditions' (above), were cut into small pieces and a solution of 50 mmol l^{-1} Tris-HCl and 1 mol l^{-1} NaCl (pH 7.5) in a 1:10 ratio (tissue mass:solvent)

Table 1. Sequence of oligonucleotides used as primers for PCR reactions

Name	Sequence (5′–3′)	Position
FP4H (forward)	5′-AGAGGAGGAACGATTAGGAGAAC-3′	187–209[1]
RP4H (reverse)	5′-CCGCATAATGACAGCCAAGG-3′	294–313[1]
Fgapd (forward)	5′-AAGCCACCATCAAGAAGG-3′	882–899[2]
Rgapd (reverse)	5′-CCACCAGTTTCACAAAGC-3′	1023–1040[2]
Ftnf (forward)	5′-AGAAATCGCCAGAAGCAAGTTG-3′	210–231[3]
Rtnf (reverse)	5′-GATGAGCACATTGATAGCAGACC-3′	264–286[3]
FColch (forward)	5′-CCTTGTGTGGTCTGTAATG-3′	1932–1950[4]
RColch (reverse)	5′-ATTGTTGTCTTTCTTCCTTCG-3′	2060–2080[4]
FColf (forward)	5′-GTCATCCAGGTCGTCAAG-3′	1306–1323[5]
RColf (reverse)	5′-TAGGTCAATCTCTAATTCAGC-3′	1482–1502[5]
27F (forward) (Lane, 1991)	5′-AGAGTTTGATCCTGGCTCAG-3′	–
1492R (reverse) (Lane, 1991)	5′-GGYTACCTTGTTACGACTT-3′	–
FBcl-2	5′-ATATTCACCAAGCCAATGC-3′	366–384[6]
RBcl-2	5′-CCAGACACCACTCTATCG-3′	525–542[6]

[1]With respect to JQ699291.1 sequence. [2]With respect to KM217385.1 sequence. [3]With respect to KR072662 sequence. [4]With respect to DQ874470.3 sequence. [5]With respect to KR072663.1 sequence. [6]With respect to MF380423 sequence.

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was added. The samples were continuously and vigorously stirred overnight at 4°C. The solution, devoid of debris, was than centrifuged (15,000 ***g*** for 60 min at 4°C) to obtain a transparent solution containing the soluble collagen fraction. The solution was concentrated 10 times with Amicon filters with a molecular cut-off of 12,000 kDa (VWR International, Milan, Italy). Collagen content was quantified using the Sircol Assay (Biocolor Ltd, Carrickfergus, County Antrim, UK) according to the manufacturer's instructions. A solution of 1 mg ml^{-1} mouse type I collagen was used for construction of a calibration standard curve. Collagen content was then normalized to the wet mass of the fragmorph.

Histological analyses

Chondrosia reniformis fragmorphs were gently detached from the plastic containers and fixed overnight at 4°C in 4% paraformaldehyde (Carlo Erba Reagents, Milan, Italy) in 0.1 mol l^{-1} phosphate-buffered solution (pH 7.4) at 4°C, embedded in Paraplast (McCormick, IL, USA) at 56°C and cut into 6 µm-thick sections. Slides for light microscopy were stained with Haematoxylin and Eosin (HE), Picro Sirius Red (PSR) staining for collagen (Junqueira et al., 1979) and Masson's trichrome (MT) used to highlight the internal anatomy of wild *C. reniformis*. The PSR method was used for its ability to differentially stain the various collagen fibres; in particular, in bright-field microscopy, collagen appears red, but when examined through cross-polarized light microscopy, the larger collagen fibres are bright yellow or orange by birefringence, corresponding in vertebrates to Type I collagen, while the thinner ones, including the reticular fibres, are green by birefringence, corresponding to vertebrate Type III collagen (Junqueira et al., 1979). All the stained sections were visualized through a Leica DMRB light and epifluorescence microscope (Leica Microsystems, Milan, Italy) equipped with cross-polarizers and differential-interference contrast filters (Nomarski) and using a 40× Leica air objective with 0.65 numerical aperture. The images were acquired with a Leica CCD camera (DFC420C, Leica Microsystems), and analysed by the ImageJ open source software (Rasband, 1997-2016).

We compared the percentage of large- and small-calibre collagen fibres in five acquired images for each condition by the use of ImageJ software. In particular, the yellow/red and green areas within each image stained by PSR were quantified by the number of pixels of each coloured region of interest (ROI). For the yellow/red areas, the hue was set from 0 to 63 and the brightness from 150 to 255; for the green areas, the hue was set from 30 to 195 and the brightness from 150 to 255. The sum of pixels of the green areas in each image was then expressed as a percentage of the total number of pixels of the image (2592×1944). Data were graphed as the mean±s.d. of three image calculations for each condition (control, and quartz treatment for 18 h and 7 days).

Soluble silicate assay

Soluble silicates were assayed in the medium of fragmorph specimens stimulated as described in 'Experimental conditions' (above). All media (6 ml each) were recovered and centrifuged at 10,000 ***g*** at room temperature. Soluble silicate concentration was measured on 100 µl of the cleared medium by a Silicate Kit (Merck, Sharp and Dohme SpA, Rome, Italy), quantifying the silicic acid through the ammonium molybdate colorimetric method (McGavack et al., 1962), according to the manufacturer's instructions.

Symbiont bacteria quantification

Genomic DNA was extracted from *C. reniformis* fragmorphs, stimulated as described in 'Experimental conditions' (above), using the nucleoSpin Tissue kit (Machery-Nagel GmbH, Düren, Germany), according to the manufacturer's instructions. The relative amount of bacterial genes in different samples was analysed by qPCR using a Chromo 4 instrument (MJ Research, Bio-Rad). *Chondrosia reniformis* GAPDH was used as the reference gene. Amplification of the prokaryotic DNA was performed using the universal set of primers 338F/518R (Lane, 1991). Each PCR reaction was performed in 15 µl containing: 1× master mix iQ SYBR®Green (Bio-Rad), 0.2 µmol l^{-1} of each primer and 4.5 ng of purified genomic DNA or negative control. All samples were analysed in triplicate. The following conditions were used: initial denaturation for 3 min, followed by 45 cycles with denaturation at 95°C for 15 s, annealing and elongation at 60°C for 60 s. Fluorescence was measured at the end of each elongation step. The next step was a slow heating (1°C s^{-1}) of the amplified product from 55°C to 92°C in order to generate a melting temperature curve. All primers were designed using the software Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA, USA) and obtained from TibMolBiol (Genova, Italy). Data analysis was carried out using the DNA Engine Opticon® 3 Real-Time Detection System Software (v.3.03). In order to detect the relative gene copy number compared with the sample with the lowest DNA content, the comparative threshold Ct method was used with the Gene Expression Analysis Software for iCycler iQ Real-Time Detection System (Bio-Rad).

Statistical analyses

Statistical analysis was performed using one-way ANOVA plus Tukey's post-test (GraphPad Software, Inc., San Diego, CA, USA). $P<0.05$ was considered significant.

RESULTS

Effect of quartz dust on *C. reniformis* collagen biosynthesis

Gene expression analysis

In order to evaluate the fibrogenic effect of quartz dust in *C. reniformis*, fragmorph tissue explants were treated with Min-U-Sil quartz for 18 h, 48 h and 7 days. The gene expression levels of NF- and F-collagen, and of P4H enzyme were assayed by qPCR (Fig. 1A). At 18 h post-injection, no significant variation of NF- and F-collagen expression levels was observed, while at this time point, P4H mRNA expression was 2.1-fold higher than the control ($P<0.0001$). Conversely, at 48 h, expression of both collagen genes was upregulated in quartz-stimulated fragmorphs compared with control (1.74-fold increase for NF-collagen and 1.95-fold for F-collagen, $P<0.0001$ and $P<0.001$, respectively), while no significant variation of P4H mRNA was observed. Finally, for the longest quartz stimulation time (7 days), only F-collagen expression was upregulated, with a 3.36-fold increase compared with the control ($P<0.001$). Overall, these data indicate that quartz dust treatment in fragmorphs induces a significant upregulation of fibrogenic gene expression.

Collagen assay

Collagen production was assayed in *C. reniformis* fragmorphs after 7 days of quartz dust stimulation using the colorimetric Sircol assay on the fraction of newly synthesized tropocollagen. The results, displayed in Fig. 1B, show that in silica-treated specimens the levels of salt-soluble tropocollagen (collagen of recent synthesis) were significantly higher (2-fold increase, $P<0.001$) with respect to control, untreated explants, confirming the fibrogenic effect of quartz particles on *C. reniformis* tissues.

Soluble silica quantification

The release of soluble silica from *C. reniformis* fragmorphs injected with quartz dust was quantified in the specimen media after

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treatment at 18 h, 48 h and 7 days using a colorimetric test based on the ammonium molybdate method reacting with silicic acid.

As shown in Fig. 1C, at 18 h of treatment, no significant differences were observed in the media of the quartz-treated fragmorphs with respect to control, untreated specimens. Conversely, at both 48 h and 7 days of treatment, a slight, but significant increase of soluble silicates was measured in the media of the quartz-treated fragmorphs with respect to the controls (1.37±0.108- and 1.44±0.233-fold increase, respectively, $P<0.05$ for both), indicating the ability of *C. reniformis* to partially dissolve silica from the quartz particles. Finally, no significant differences between the media of the control fragmorphs and FNSW alone were ever detectable.

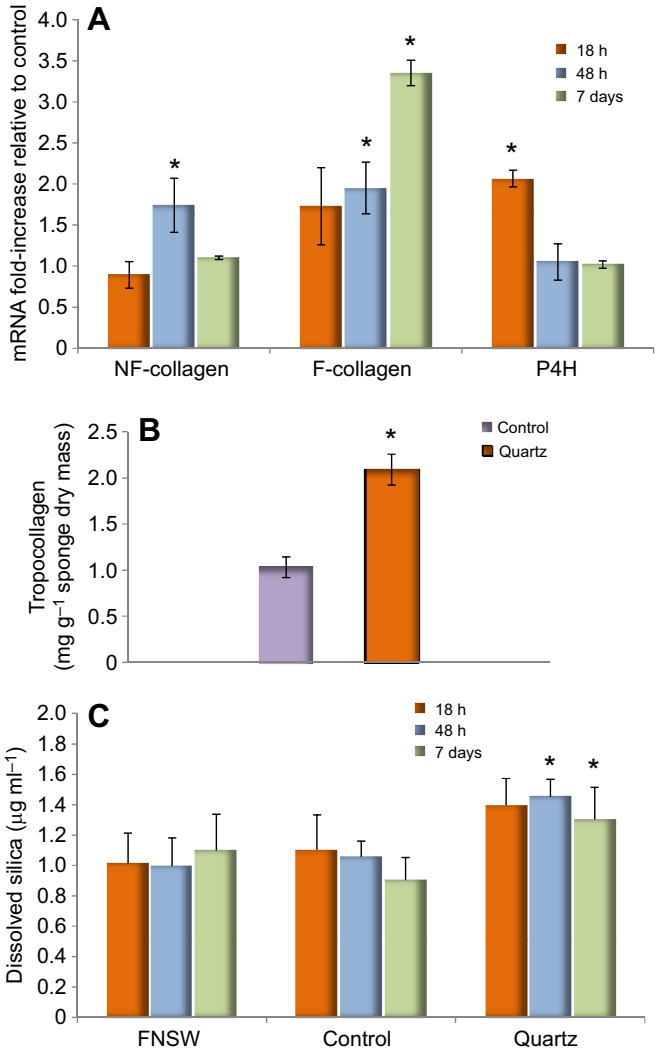

Fig. 1. Fibrogenic gene and protein expression and silicate quantification in *Chondrosia reniformis*. (A) Quantitative PCR gene expression analysis of healed and plate-attached *C. reniformis* fragmorphs treated with 100 µl of 10 mg ml^{-1} quartz dust suspension for 18 h, 48 h and 7 days. NF-collagen, non-fibrillar collagen; F-collagen, fibrillar collagen; P4H, prolyl-4-hydroxylase. Data are expressed as fold-increase relative to control at the same end-point and normalized to GAPDH housekeeping gene expression. Each bar represents the mean±s.d. of three independent experiments performed in triplicate. Asterisks indicate significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: NF-collagen 48 h versus control, $P<0.0001$; F-collagen 48 h and 7 days versus control, $P<0.001$ for both; P4H 18 h versus control, $P<0.0001$). (B) Salt-soluble tropocollagen quantification measured using the Sircol colorimetric method in plate-attached fragmorphs treated or not (control) with 100 µl of 10 mg ml^{-1} quartz dust suspension for 7 days. Each bar represents the mean ±s.d. of three experiments performed in duplicate. The asterisk indicates significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: quartz versus control, $P<0.001$). (C) Soluble silicate quantification measured by the ammonium molybdate colorimetric assay in the medium of plate-attached fragmorphs treated or not with 100 µl of 10 mg ml^{-1} quartz dust suspension for 18 h, 48 h and 7 days. FNSW, filtered natural seawater. Each bar represents the mean±s.d. of three experiments performed in duplicate. Asterisks indicate significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: quartz 48 h and 7 days versus control, $P<0.05$ for both).

Histological analyses

The collagen in the fragmorphs was highlighted by PSR staining, while HE staining was used to identify the cells embedded in the extracellular matrix. Sections of control and quartz-treated samples at 18 h and 7 days were stained, observed and photos were taken accordingly (Fig. 2).

PSR staining revealed an abundance of thick collagen fibres in yellow/red areas and thin fibres in dark green areas (Fig. 2A–C), which were also quantitatively graphed in Fig. 2G. This technique allowed us to observe an increase of the thin calibre fibres (green ROIs graphed as a percentage of total pixels for each condition in Fig. 2G) over time from 18 h to 7 days of quartz treatment with

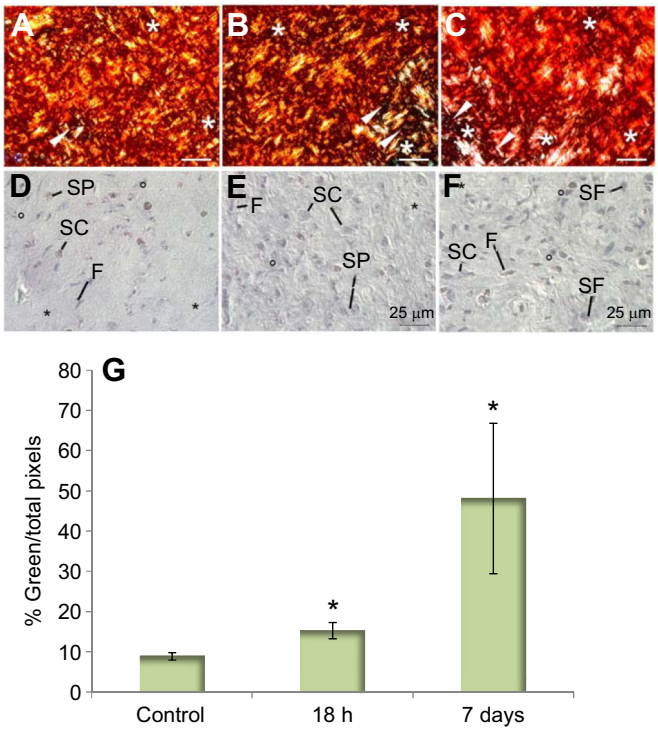

Fig. 2. Histological analysis and quantification of collagen deposition in *C. reniformis*. Histological sponge explant sections in control (A,D) and quartz-treated specimens (B,E: 18 h; C,F: 7 days) shown by Picro Sirius Red (PSR, A–C) and Haematoxylin–Eosin (HE, D–F) staining. Sections were prepared and analysed by a Leica DMRB microscope equipped with a 40× air objective as explained in Materials and methods. Arrow heads, thin fibres (green); Asterisks, collagen rarefaction. SC, spherulous cells; SP, cytoplasmic granules; F, fibroblasts or lophocytes; small circles indicate morphologically unidentifiable cells. Scale bars, 25 µm. (G) Quantification of the thin calibre collagen fibres shown in dark green in the PSR-stained panels (A–C) in the sponge explants treated or not (control) with quartz dust for 18 h or 7 days. Data are shown as the percentage of all the green pixel regions of interest identified in each acquired image with respect to the total image pixels and are the means±s.d. of three different image calculations for each condition. The asterisks indicate significance in Tukey's test (ANOVA: $P<0.05$; Tukey: 7 days and 18 h versus control, $P<0.05$).

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respect to the control (Fig. 2B,C versus A, and Fig. 2G, $P<0.05$ for both treatments versus control). As the thin calibre collagen fibres in the literature (Junqueira et al., 1979) are usually associated with newly deposited collagen, these histological data confirm the biochemical quantification of new collagen deposition at 7 days of quartz treatment in the sponge explants (Fig. 1B).

The HE sections (Fig. 2D–F) show that in the fragmorph model the typical original structure of the sponge appears greatly simplified when compared with the original (Fig. S1). There are no more choanocyte chambers filling the mesohyl. These chambers are normally crossed by the canals and supported by collagen arranged to better wrap the filtering structure. Regarding the cells, it was possible to identify spherulous cells (SC), the largest in size, and their residues after the expulsion of cytoplasmic granules (spherules, SP), the fibroblasts or lophocytes (F) and finally a group of morphologically unidentifiable cells indicated by a small circle. No differences in cell density and/or cell-type predominance were observed in the quartz-treated explants with respect to the control (Fig. 2E,F versus D). Finally, it was not possible to identify the presence of the fine quartz dust (Min-U-Sil) added during the experiments except in a few small areas of the observed sections.

Symbiont quantification

Porifera host a great variety of microorganisms that in many cases develop mutualistic relationships with sponges, sharing part of the metabolic pathway (Arillo et al., 1993) and having a profound impact on host biology. For example, microorganisms may contribute to host defence via the production of biologically active metabolites (Unson et al., 1994; Schmidt et al., 2000). In order to establish whether quartz treatment could affect quantitatively the bacterial component of the sponge body, a qPCR analysis was performed on fragmorph genomic DNA from treated and untreated samples at 18 h, 48 h and 7 days, using a couple of universal primers designed on a 16S conserved bacterial region (Lane, 1991). The amplified prokaryotic genomic DNA was then normalized to the amplified DNA of the GAPDH sponge gene and expressed as a fold-increase with respect to the relative control at each time point. As shown in Fig. 3, while no significant differences in the amount of the bacterial population were observed in quartz-stimulated fragmorphs after 18 and 48 h with respect to the controls at the same time points, after 7 days of treatment the relative bacterial gene copy number was 2-fold higher ($P<0.05$) than the respective control, indicating that quartz treatment stimulates sponge bacterial symbiont growth.

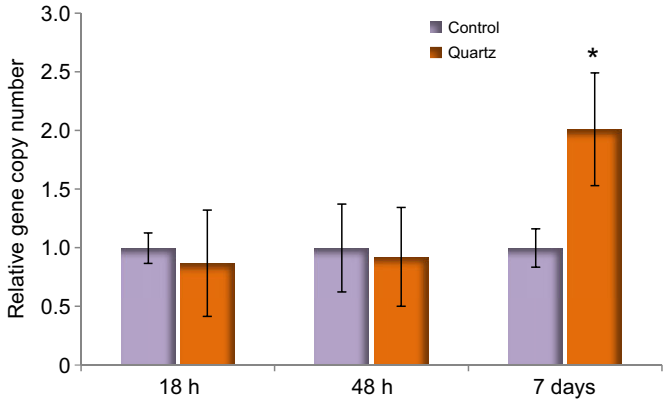

Fig. 3. DNA quantification of prokaryotic symbiont population in *C. reniformis*. Analysis of sponge prokaryotic symbiont population by qPCR DNA quantification in plate-attached fragmorphs treated or not (control) with 100 µl of 10 mg ml^{-1} quartz dust suspension for 18 h, 48 h and 7 days. Data are expressed as prokaryotic DNA gene copy number relative to control and normalized to the gene copy quantification of the sponge GAPDH housekeeping gene. Each bar represents the mean±s.d. of three independent experiments performed in triplicate. Asterisks indicate significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: quartz versus control, $P<0.0001$).

TNF involvement in quartz-induced sponge fibrogenesis

In order to investigate a possible TNF involvement in the quartz-mediated fibrogenesis in *C. reniformis*, the cytokine transcript levels of this sponge (chTNF) were initially quantified in the fragmorphs injected or not with quartz after 18 h, 48 h and 7 days (Fig. 4A). The data obtained indicate that a significant, transient overexpression of

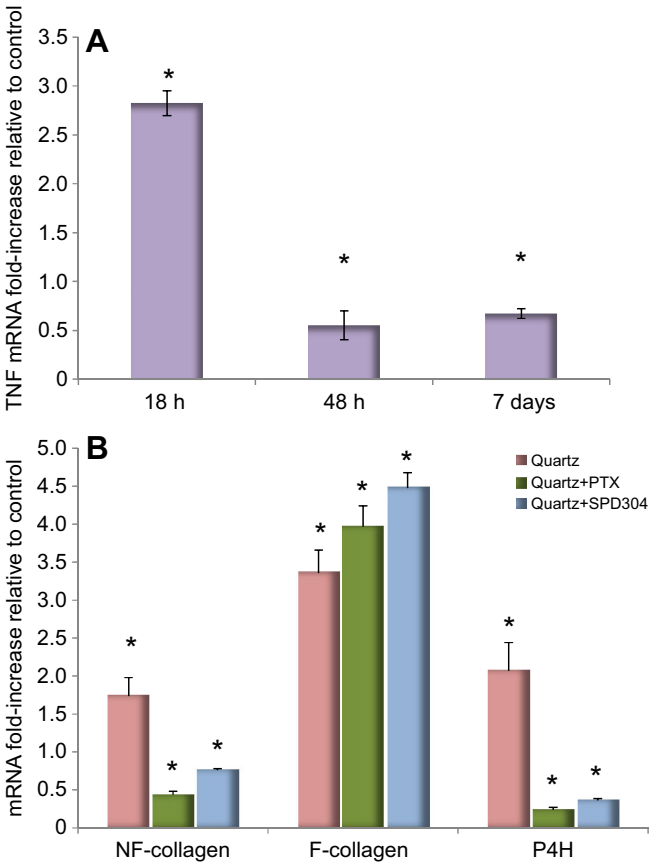

Fig. 4. Involvement of TNF gene in quartz stimulation in *C. reniformis*. (A) *Chondrosia reniformis* TNF gene expression analysis by qPCR on healed and plate-attached fragmorphs treated or not (control) with 100 µl of 10 mg ml^{-1} quartz dust suspension for 18 h, 48 h and 7 days. Data are expressed as fold-increase relative to control and normalized to expression of the GAPDH housekeeping gene. Each bar represents the mean±s.d. of three independent experiments performed in triplicate. Asterisks indicate significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: versus control, $P<0.0001$ for all three). (B) P4H, F-collagen and NF-collagen gene expression analysis by qPCR on healed and plate-attached fragmorphs treated or not (control) with 100 µl of 10 mg ml^{-1} quartz dust suspension alone or in the presence of 5 mmol l^{-1} pentoxyfilline (PTX) or 22 µmol l^{-1} SPD304. Data are expressed as fold-increase relative to control and normalized to expression of the GAPDH housekeeping gene. Each bar represents the mean±s.d. of three independent experiments performed in triplicate. Asterisks indicate significance in Tukey's test (ANOVA: NF-collagen, $P<0.000001$; Tukey: quartz versus control, $P<0.01$; quartz+PTX and quartz+SPD304 versus quartz, $P<0.0001$ for both; ANOVA: F-collagen, $P<0.0001$; Tukey: quartz versus control, $P<0.01$; quartz+PTX and quartz+SPD304 versus quartz, $P<0.05$ for both; ANOVA: P4H, $P<0.000001$; Tukey: quartz versus control, $P<0.0001$; quartz+PTX and quartz+SPD304 versus quartz, $P<0.0001$ for both).

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chTNF was detectable in quartz-treated fragmorphs at 18 h of treatment with respect to the control (2.83-fold increase, $P<0.0001$). Conversely, at longer times of quartz treatment, a slight but significant decrease of chTNF expression was measured compared with controls at the same time points (0.55- and 0.67-fold increase at 48 h and 7 days, respectively, $P<0.0001$ for both).

NF- and F-collagen, as well as P4H gene expression levels were then evaluated in quartz-treated fragmorphs in the presence or absence of two different TNF inhibitors, namely PTX and SPD304 (Fig. 4B). Pentoxifyllin is a phosphodiesterase inhibitor able to inhibit TNF production in several patho/physiological conditions (Poulakis et al., 1999), while SPD304 is a specific inhibitor determining the disassembly of the TNF trimeric form with the consequent impairment of TNF signalling (He et al., 2005).

The data obtained indicate that for P4H expression after 18 h of quartz stimulation, the presence of both TNF inhibitors strongly repressed the silica-induced mRNA upregulation (Fig. 4B, $P<0.0001$ for both). Similar results were observed for NF-collagen expression after 48 h of stimulation; in this case too, the mRNA upregulation elicited by the quartz dust was abolished by both TNF inhibitors (Fig. 4B, $P<0.0001$ for both). Finally, for F-collagen gene expression levels after 7 days of quartz stimulation, the presence of the two TNF inhibitors slightly increased the quartz-induced mRNA upregulation (1.18- and 1.33-fold increase in the presence of PTX and SPD304, respectively, compared with quartz alone, $P<0.05$ for both, Fig. 4B). These data indicate that chTNF is an early upregulated gene in *C. reniformis* upon quartz stimulation, and that its inhibition impairs P4H and NF-collagen upregulation, while concomitantly stimulating F-collagen deposition.

Cell proliferation and apoptosis

In mammalian models, quartz dust exposure induces cell death in macrophages (Scarfì et al., 2009) while it concomitantly stimulates cell survival and growth in fibroblasts (Pozzolini et al., 2016c). In order to establish what kind of cellular outcome arises from quartz treatment in the *C. reniformis* sponge model, the cell proliferative state during quartz treatment in fragmorph tissues was analysed by a BrdU incorporation assay while apoptosis was investigated by the quantification of expression levels of the anti-apoptotic gene Bcl-2 by qPCR.

The first set of experiments showed that no BrdU incorporation was observable either in the control or in the quartz-treated fragmorphs (not shown), after 48 h of treatment.

Subsequently, qPCR quantification of Bcl-2 mRNA expression in quartz-treated fragmorphs compared with controls was performed. The results (Fig. 5) showed that a peak of expression of this gene was reached after 48 h of treatment with a 1.86-fold increase with respect to the control ($P<0.0001$). Overall, these data indicate that quartz is not detrimental to the *C. reniformis* system and may promote cell survival rather than cytotoxicity and cell death as conversely observed in mammals.

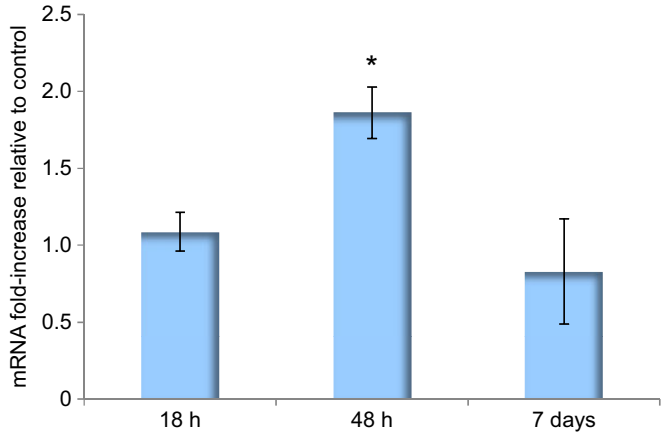

Fig. 5. Apoptosis quantification in quartz-stimulated *C. reniformis* explants. *Chondrosia reniformis* Bcl-2 gene expression analysis by qPCR on healed and plate-attached fragmorph samples treated or not (control) with 100 µl of 10 mg ml^{-1} quartz dust suspension for 18 h, 48 h and 7 days. Data are expressed as fold-increase relative to control and normalized to expression of the GAPDH housekeeping gene. Each bar represents the mean±s.d. of three independent experiments performed in triplicate. The asterisk indicates significance in Tukey's test (ANOVA: $P<0.000001$; Tukey: versus control, $P<0.0001$).

DISCUSSION

Occupational and environmental inhalation of quartz causes various pulmonary diseases (Ding et al., 2002) characterized by early lung inflammation mediated by pro-inflammatory mediators such as TNF, a cytokine that plays a key role in pulmonary fibrosis (Piguet et al., 1990). In contrast, in Porifera, the oldest phylum in the Animalia kingdom, the *C. reniformis* species is able to actively incorporate siliceous sediment, including quartz sand, in its ectosome without any detrimental effect. Compared with the elevated cytotoxicity observed in higher organisms (Hamilton et al., 2008), the unique quartz incorporation and erosion in *C. reniformis* is extremely interesting, and understanding the reasons and the physiological advantages behind this peculiar sponge behaviour could help us to better elucidate the molecular events triggering silica cytotoxicity and pulmonary fibrosis in higher animals. Quartz erosion in this sponge was suggested to be related to collagen biosynthesis (Giovine et al., 2013), but these speculations were never confirmed at the molecular level.

In the present work, for the first time, clear molecular evidence of the fibrogenic effect of crystalline silica in *C. reniformis* is showcased. Performance of this study required us to consider some important aspects regarding the choice of the appropriate experimental model and conditions. Studies focusing on the elucidation of molecular mechanisms *in vivo* need reproducible and controlled systems, and this is extremely difficult to achieve when dealing with marine sponges, which are very difficult to rear in aquaculture; concomitantly, individuals within the same species show extreme variability in shape, pigmentation, dimensions and developmental stages. Although optimized protocols to obtain *in vitro* sponge cell cultures have been described (Custodio et al., 1998; Mussino et al., 2013), they overall fail on *C. reniformis* species as a result of a low cell/extracellular matrix ratio (not shown). However, tissue explants (fragmorphs) can be easily obtained and can be maintained successfully *in vitro* for several months (Nickel and Brümmer, 2003).

In order to evaluate the fibrogenic effect of quartz dust, *C. reniformis* fragmorphs were exposed to quartz particles (microcrystals) and the expression levels of fibrogenic genes, as well as the rate of new collagen deposition and the involvement of chTNF in this process were analysed. As the NF-collagen gene is physiologically upregulated in the external region of the fragmorph reconstituting the ectosomal tissue (Pozzolini et al., 2012), only completely healed fragmorphs were used; moreover, because *C. reniformis* is characterized by a body polarity in sediment incorporation (Bavestrello et al., 1998), only fragmorphs attached to the plastic plate were chosen for all the experiments. Finally, because crystalline silica grains at various stages of erosion are usually

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already present in the sponge ectosome, in the present study fragmorphs were experimentally exposed to high concentrations of fine silica microcrystals (Min-U-Sil), as used in rodent experimental models of pulmonary fibrosis (Oghiso et al., 1992; Becher et al., 2007).

The gene expression analysis performed by qPCR revealed that all fibrogenic genes were upregulated by silica stimulation, albeit with different kinetic profiles (Fig. 1). The data suggest that, after silica stimulation, the P4H enzyme involved in collagen maturation is upregulated early, while both collagen mRNAs only increase subsequently. The gene expression evidence was also confirmed by the increase of new collagen protein deposition in the quartz-treated fragmorphs compared with controls, demonstrated both biochemically and histologically (Figs 1B and 2). This sponge response to silica microcrystals anticipates the behaviour described in higher animals concerning both P4H (Jin et al., 2008) and F-collagen upregulation (Mäkelä and Vuorio, 1986; Ghosh, 2002), while in the case of NF-collagen, no correspondence can be found in mammals, suggesting that this upregulation is specific of the sponge response to quartz. The *C. reniformis* NF-collagen analysed in this model is a type IV-related short-chain collagen, which, until now, has been identified only in some invertebrates, such as nematodes (Johnstone, 2000). This protein is highly abundant in the ectosome (Pozzolini et al., 2012), where it is one of the constituents of spongin, a demosponge-specific set of collagenous proteins. NF-collagen is considered an exo-collagen involved in substrate adhesion (Exposito et al., 1990), the structural organization of which is not completely understood, but it is possible that the upregulation of this gene, restricted to 48 h post-injection of quartz, may be involved in an early contact/encapsulation of silica grains. It should be noted that the upregulation of genes involved in the formation of support structures by quartz interaction has previously been reported in another demosponge. Indeed primmorph models of *Petrosia ficiformis* grown on quartz rocks showed an increased expression of silicatein (involved in spicule formation) compared with those grown on other rocky substrata, such as marble (Pozzolini et al., 2010). Taken together, these data suggest that crystalline silica can affect positively the growth of these organisms acting at the gene regulation level.

As reported by Bavestrello et al. (1995), when whole specimens of *C. reniformis* are incubated in the presence of silica sand, it is possible to detect a time-dependent soluble silicate release in the seawater, demonstrating the active sponge-mediated erosion process. In the present work, showing a new experimental model of silica microcrystal-stimulated sponges, the data obtained indicate that in this system, a slight but significant difference in silicate concentration in the silica-treated fragmorph medium with respect to the control was also observable (Fig. 1C), confirming the crystalline silica dissolution by *C. reniformis* for this form of quartz (Min-U-Sil) too, not just for the silica sands as already published.

In mammalian models, silica-induced collagen deposition causing silicosis is directly controlled by cytokines, one of the most important being TNF-α (Piguet et al., 1990). A TNF homologue was recently identified in *C. reniformis* (Pozzolini et al., 2016b) and, in the present work, its possible involvement in quartz-induced fibrogenesis was investigated (Fig. 4).

qPCR analysis of fragmorph samples exposed to silica microcrystals indeed revealed a time-dependent TNF gene upregulation. In order to verify a direct involvement of TNF in quartz-induced collagen gene upregulation, fragmorph samples were stimulated with quartz in the presence of two different TNF inhibitors and the mRNA level of the fibrogenic genes was evaluated. The results indicate that the presence of both TNF inhibitors strongly repressed P4H and NF-collagen gene upregulation while, in contrast, it produced a slight but significant increase of F-collagen gene expression (Fig. 4). Taken together, these data indicate that in marine sponges, the increase of collagen synthesis induced by microcrystalline silica exposure, as in the mammalian model, is mediated by cytokine release. This observation suggests that during evolution, with the increase in tissue specialization, a physiological process involving crystalline silica engulfment and erosion in sponges became harmful in higher organisms, which are still able to perform the engulfment process but have lost the ability to dissolve the crystalline silica, resulting in the development of a chronic inflammatory response. However, it is important to note some differences between sponges and mammals. Firstly, while in mammals the TNF overexpression persists for a long time after the first silica stimulation, the kinetic profile of TNF gene expression in sponges is characterized by an early upregulation at 18 h followed by a prolonged downregulation. Furthermore, there is a significant difference in the collagen gene targets that are stimulated by TNF. In mammals, in fact, collagen type I is typically upregulated by TNF, while in sponges, as demonstrated with the experiments in presence of TNF inhibitors, it is NF-collagen. Indeed, in mammals it is known that TNF can act in different tissue compartments alternatively reducing or enhancing type I collagen expression (Verrecchia and Mauviel, 2004). Notably, NF-collagen is a specific sponge collagen sharing some structural features with type IV collagen (Pozzolini et al., 2012), and in mammals it is apparently not involved in silicotic disease; however, the transcription of its gene is controlled by TNF in some specific tissues (Siu et al., 2003).

The qPCR analysis of the quantitative variation of the sponge bacterial component indicates that at 7 days after quartz dust treatment, the total bacterial population was increased with respect to the controls (Fig. 3). As the quartz-treated fragmorphs did not show any signs of deterioration or rotting during prolonged observation (more than 2 weeks; not shown), we conclude that the increase of prokaryotic DNA derives from an increment of the sponge bacterial symbiont population. Because an increase of microbial symbionts is usually considered metabolically beneficial to the sponge host (Webster and Taylor, 2012), this phenomenon also seems to indicate that quartz engulfment and erosion in *C. reniformis* is a positive event for the physiology of this sponge. To further confirm our hypotheses, we also evaluated the presence of proliferative or apoptotic cells in quartz-treated fragmorphs compared with controls. Firstly, it was not possible to highlight any proliferation either in control or in quartz-treated fragmorphs by BrdU incorporation (not shown). Indeed, no differences in cell density were observed following HE staining of treated versus control explants (Fig. 2), indicating that sponge cells in the fragmorphs are in a quiescent state and that quartz is not able to induce any proliferation in this model. Secondly, the evaluation of Bcl-2 anti-apoptotic gene expression showed an upregulation in quartz-treated specimens compared with controls. These two facts clearly exclude an apoptotic effect of quartz in the exposed fragmorphs, as conversely is observed in mammals (Scarfì et al., 2009), and on the contrary indicate an increased cell survival ability.

The lack of an apoptotic response in this model could be explained by the ability of the sponge cells, unlike their mammalian counterparts, to chemically attack the crystalline silica grain through ascorbic acid and partially dissolve it (Bavestrello et al., 1995). *In vitro* interaction between ascorbic acid and quartz dust causes both the release of soluble silicates and a silica surface modification increasing $OH^{\bullet}$ radicals (Fenoglio et al., 2000); in fact, in mammalian

models of cell toxicity, quartz microcrystals pre-treated with ascorbic acid are significantly more cytotoxic than untreated crystals (Giovine et al., 2002) and are able to induce higher levels of inflammatory factors and cell responses (Scarfì et al., 2007, 2009; Pozzolini et al., 2016c). However, sponge cells seem able to handle efficiently the huge release of radicals during the silica erosion process, preventing its detrimental effects. Indeed, these animals are able to adapt their antioxidant efficiency in response to environmental stress (Regoli et al., 2004) and, moreover, Porifera and their symbionts are known to produce various antioxidant compounds that could act as scavengers of the radicals formed on the surface of the silica grains.

The absence of quartz cytotoxicity in sponges could also be explained by the fact that the sponge TNF receptor lacks a death domain; thus, in this phylum, TNF is not able to trigger an apoptotic signal, as described for *C. reniformis* (Pozzolini et al., 2016b) and also recently revised by Quistad and Traylor-Knowles (2016). Furthermore, the TNF gene expression profile in silica-treated fragmorphs is transient with respect to the silicotic models in higher animals. In the latter, in fact, the persistence of TNF gene overexpression is related to the inability of macrophages to clear the quartz particles, finally causing apoptosis and a recycling of the released crystals in a new macrophage-driven engulfment process, perpetuating the inflammation.

In conclusion, in the present work, we demonstrate for the first time at the molecular level that quartz microcrystal incorporation in lower invertebrates leads to a beneficial increase of collagen deposition, which is probably advantageous in terms of strengthening of the sponge. Similar to the mammalian silicotic models, in sponges this process appears to be mediated by the pro-inflammatory cytokine TNF, suggesting that a molecular pathway controlling a physiological response in lower animals, and persisting during evolution, may have become the cause of disease in some specialized tissues in higher animals.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.P., M.G.; Investigation: M.P., L.G., S.F.; Data curation: M.P., S.S.; Writing - original draft: M.P.; Writing - review & editing: S.S., C.C., M.G.; Supervision: M.G.; Funding acquisition: S.S., M.G.

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Supplementary information

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References

Aarskog, N. and Vedeler, C. (2000). Real-time quantitative polymerase chain reaction. A new method that detects both the peripheral myelin protein 22 duplication in Charcot-Marie-Tooth type 1A disease and the peripheral myelin protein 22 deletion in hereditary neuropathy with liability to pressure palsies. *Hum. Genet.* **107**, 494-498.

Arillo, A., Bavestrello, G., Burlando, B. and Sarà, M. (1993). Metabolic integration between symbiotic cyanobacteria and sponges: a possible mechanism. *Mar. Biol.* **117**, 159-162.

Bavestrello, G., Arillo, A., Benatti, U., Cerrano, C., Cattaneo-Vietti, R., Cortesognoi, L., Gaggero, L., Giovine, M., Tonetti, M. and Sarà, M. (1995). Quartz dissolution by the sponge *Chondrosia reniformis* (Porifera, Demospongiae). *Nature* **378**, 374-376.

Bavestrello, G., Benatti, U., Calcinai, B., Cattaneo-Vietti, R., Cerrano, C., Favre, A., Giovine, M., Lanza, S., Pronzato, R. and Sarà, M. (1998). Body polarity and mineral selectivity in the Demosponge *Chondrosia reniformis*. *Biol. Bull.* **195**, 120-125.

Becher, R., Bucht, A., Øvrevik, J., Hongslo, J. K., Dahlman, H. J., Samuelsen, J. T. and Schwarze, P. E. (2007). Involvement of NADPH oxidase and iNOS in rodent pulmonary cytokine responses to urban air and mineral particles. *Inhal. Toxicol.* **19**, 645-655.

Cerrano, C., Calcinai, B., Di Camillo, C. G., Valisano, L. and Bavestrello, G. (2007). How and why do sponges incorporate foreign material? Strategies in Porifera. In *Porifera Research: Biodiversity, Innovation & Sustainability, Serie Livros 28* (ed. M. R. Custodio, E. Hajdu, G. Lobo-Hajdu and G. Muricy), pp. 239-246. Rio de Janeiro: Museu Nacional.

Custodio, M. R., Prokic, I., Steffen, R., Koziol, C., Borojevic, R., Brümmer, F., Nickel, M. and Müller, W. E. G. (1998). Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death. *Mech. Ageing Dev.* **105**, 45-59.

Ding, M., Chen, F., Shi, X., Yucesoy, B., Mossman, B. and Vallyathan, V. (2002). Diseases caused by silica: mechanisms of injury and disease development. *Int. Immunopharmacol.* **2**, 173-182.

Ehrlich, H., Demadis, K. D., Pokrovsky, O. S. and Koutsoukos, P. G. (2010a). Modern views on desilicification: biosilica and abiotic silica dissolution in natural and artificial environments. *Chem. Rev.* **110**, 4656-4689.

Ehrlich, H., Deutzmann, R., Brunner, E., Cappellini, E., Koon, H., Solazzo, C., Yang, Y., Ashford, D., Thomas-Oates, J., Lubeck, M. et al. (2010b). Mineralization of the metre-long biosilica structures of glass sponges is templated on hydroxylated collagen. *Nat. Chem.* **2**, 1084-1088.

Exposito, J.-Y., Ouazana, R. Garrone, R. (1990). Cloning and sequencing of a Porifera partial cDNA coding for a short-chain collagen. *Eur. J. Biochem.* **190**, 401-406.

Fenoglio, I., Martra, G., Coluccia, S. and Fubini, B. (2000). Possible role of ascorbic acid in the oxidative damage induced by inhaled crystalline silica particles. *Chem. Res. Toxicol.* **13**, 971-975.

Ghosh, A. K. (2002). Factors involved in the regulation of type I Collagen Gene Expression: Implication in Fibrosis. *Exp. Biol. Med.* **227**, 301-314.

Giovine, M., Pozzolini, M., Fenoglio, I., Scarfi, S., Ghiazza, M., Benatti, U. and Fubini, B. (2002). Crystalline silica incubated in ascorbic acid acquires a higher cytotoxic potential. *Toxicol. Ind. Health* **18**, 249-255.

Giovine, M., Scarfi, S., Pozzolini, M., Penna, A. and Cerrano, C. (2013). Cell reactivity to different silica. *Prog. Mol. Subcell. Biol.* **54**, 143-174.

Hamilton, R. F., Sheetal, A. and Thakur, S. A. (2008). Silica binding and toxicity in alveolar macrophages. *Free Rad. Biol. Med.* **44**, 1246-1258.

He, M. M., Smith, A. S., Oslob, J. D., Flanagan, W. M., Braisted, A. C., Whitty, A., Cancilla, M. T., Wang, J., Lugovskoy, A. A., Yoburn, J. C. et al. (2005). Small-molecule inhibition of TNF-alpha. *Science* **310**, 1022-1025.

Jin, Z., Liu, B., Feng, D., Chen, C., Li, X., Hu, Y., Peng, J., Liu, Y., Du, J., Fu, C. et al. (2008). Identification of differentially expressed genes in rat silicosis model by suppression subtractive hybridization analysis. *Acta Biochim. Biophys. Sin.* **40**, 740-746.

Johnstone, I. L. (2000). Cuticle collagene genes. Expression in *Caenorhabditis elegans*. *Trends Genet.* **16**, 21-27.

Junqueira, L. C. U., Bignolas, G. and Brentani, R. R. (1979). Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem. J.* **11**, 447-455.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics* (ed. E. Stackebrandt and M. Goodfellow), pp. 115-175. New York: Wiley.

Mäkelä, J. K. and Vuorio, E. (1986). Type I collagen messenger RNA levels in experimental granulation tissue and silicosis in rats. *Med. Biol.* **64**, 15-22.

McGavack, T. H., Leslie, J. G. and Tang Kao, K.-Y. (1962). Silicon in biological material. I. Determinations eliminating silicon as a contaminant. *Proc. Soc. Exp. Biol. Med.* **110**, 215-218.

Mussino, F., Pozzolini, M., Valisano, L., Cerrano, C., Benatti, U. and Giovine, M. (2013). Primmorphs cryopreservation: a new method for long-time storage of sponge cells. *Mar. Biotechnol. (NY)* **15**, 357-367.

Nickel, M. and Brümmer, F. (2003). In vitro sponge fragment culture of *Chondrosia reniformis* (Nardo, 1847). *J. Biotechnol.* **100**, 147-159.

Oghiso, Y., Yamada, Y. and Shibata, Y. (1992). Effects of instilled fibrogenic particles on the clonal growth of murine pulmonary alveolar macrophages. *Environ. Health Perspect.* **97**, 159-161.

Piguet, P. F., Collart, M. A., Grau, G. E., Sappino, A.-P. and Vassalli, P. (1990). Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* **344**, 245-247.

Poulakis, N., Androutsos, G., Kazi, D., Bastas, A., Provata, A., Bitsakou, C., Kontozoglou, T., Polyzogopoulou, C. and Tassiopoulou, A. (1999). The differential effect of pentoxifylline on cytokine production by alveolar macrophages and its clinical implications. *Resp. Med.* **93**, 52-57.

Pozzolini, M., Valisano, L., Cerrano, C., Menta, M., Schiaparelli, S., Bavestrello, G., Benatti, U. and Giovine, M. (2010). Influence of rocky substrata on three-dimensional sponge cells model development. *In Vitro Cell. Dev. Biol. Anim.* **46**, 140-147.

Pozzolini, M., Bruzzone, F., Berilli, V., Mussino, F., Cerrano, C., Benatti, U. and Giovine, M. (2012). Molecular characterization of a nonfibrillar collagen from the marine sponge *Chondrosia reniformis* Nardo 1847 and positive effects of soluble silicates on its expression. *Mar. Biotechnol. (NY)* **14**, 281-293.

Pozzolini, M., Mussino, F., Cerrano, C., Scarfi, S. and Giovine, M. (2014). Sponge cell cultivation: optimization of the model *Petrosia ficiformis* (Poiret 1789). *J. Exp. Mar. Biol. Ecol.* **454**, 70-77.

Pozzolini, M., Scarfi, S., Mussino, F., Ferrando, S., Gallus, L. and Giovine, M. (2015). Molecular cloning, characterization, and expression analysis of a prolyl 4-hydroxylase from the marine sponge *Chondrosia reniformis*. *Mar Biotechnol (NY)* **17**, 393-407.

Pozzolini, M., Ferrando, S., Gallus, L., Gambardella, C., Ghignone, S. and Giovine, M. (2016a). Aquaporin in *Chondrosia reniformis* Nardo, 1847 and its possible role in the interaction between cells and engulfed siliceous particles. *Biol. Bull.* **230**, 220-232.

Pozzolini, M., Scarfi, S., Ghignone, S., Mussino, F., Vezzulli, L., Cerrano, C. and Giovine, M. (2016b). Molecular characterization and expression analysis of the first Porifera tumor necrosis factor superfamily member and of its putative receptor in the marine sponge *Chondrosia reniformis*. *Dev. Comp. Immunol.* **57**, 88-98.

Pozzolini, M., Vergani, L., Ragazzoni, M., Delpiano, L., Grasselli, E., Voci, A., Giovine, M. and Scarfi, S. (2016c). Different reactivity of primary fibroblasts and endothelial cells towards crystalline silica: a surface radical matter. *Toxicology* **361-362**, 12-23.

Quistad, S. D. and Traylor-Knowles, N. (2016). Precambrian origins of the TNFR superfamily. *Cell Death Discov.* **2**, e16058.

Rasband, W. S. (1997-2016). *ImageJ*. Bethesda, MD, USA: U.S. National Institutes of Health. https://imagej.nih.gov/ij/.

Regoli, F., Nigro, M., Chierici, E., Cerrano, C., Schiapparelli, S., Totti, C. and Bavestrello, G. (2004). Variations of antioxidant efficiency and presence of endosymbiotic diatoms in the Antarctic porifera *Haliclona dancoi*. *Mar. Environ. Res* **58**, 637-640.

Scarfi, S., Benatti, U., Pozzolini, M., Clavarino, E., Ferraris, C., Magnone, M., Valisano, L. and Giovine, M. (2007). Ascorbic acid-pretreated quartz enhances cyclo-oxygenase-2 expression in RAW 264.7 murine macrophages. *FEBS J.* **274**, 60-73.

Scarfi, S., Magnone, M., Ferraris, C., Pozzolini, M., Benvenuto, F., Giovine, M. and Benatti, U. (2009). Ascorbic Acid pre-treated quartz stimulates TNF-α release in RAW 264.7 murine macrophages through ROS production and membrane lipid peroxidation. *Respiratory Res.* **10**, 25-40.

Schmidt, E. W., Obraztsova, A. Y., Davidson, S. K., Faulkner, D. J. and Haygood, M. G. (2000). Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel delta-proteobacterium, 'Candidatus *Entotheonella palauensis*'. *Mar. Biol.* **136**, 969-977.

Siu, M. K. Y., Lee, W. M. and Cheng, C. Y. (2003). The interplay of collagen IV, tumor necrosis factor-alpha, gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloproteases-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. *Endocrinology* **144**, 371-387.

Unson, M. D., Holland, N. D. and Faulkner, D. J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar. Biol.* **119**, 1-11.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034.

Verrecchia, F. and Mauviel, A. (2004). TGF-beta and TNF-alpha: antagonistic cytokines controlling type I collagen gene expression. *Cell. Signal.* **16**, 873-880.

Webster, N. S. and Taylor, M. W. (2012). Marine sponges and their microbial symbionts: love and other relationships. *Environ. Microbiol.* **14**, 335-346.

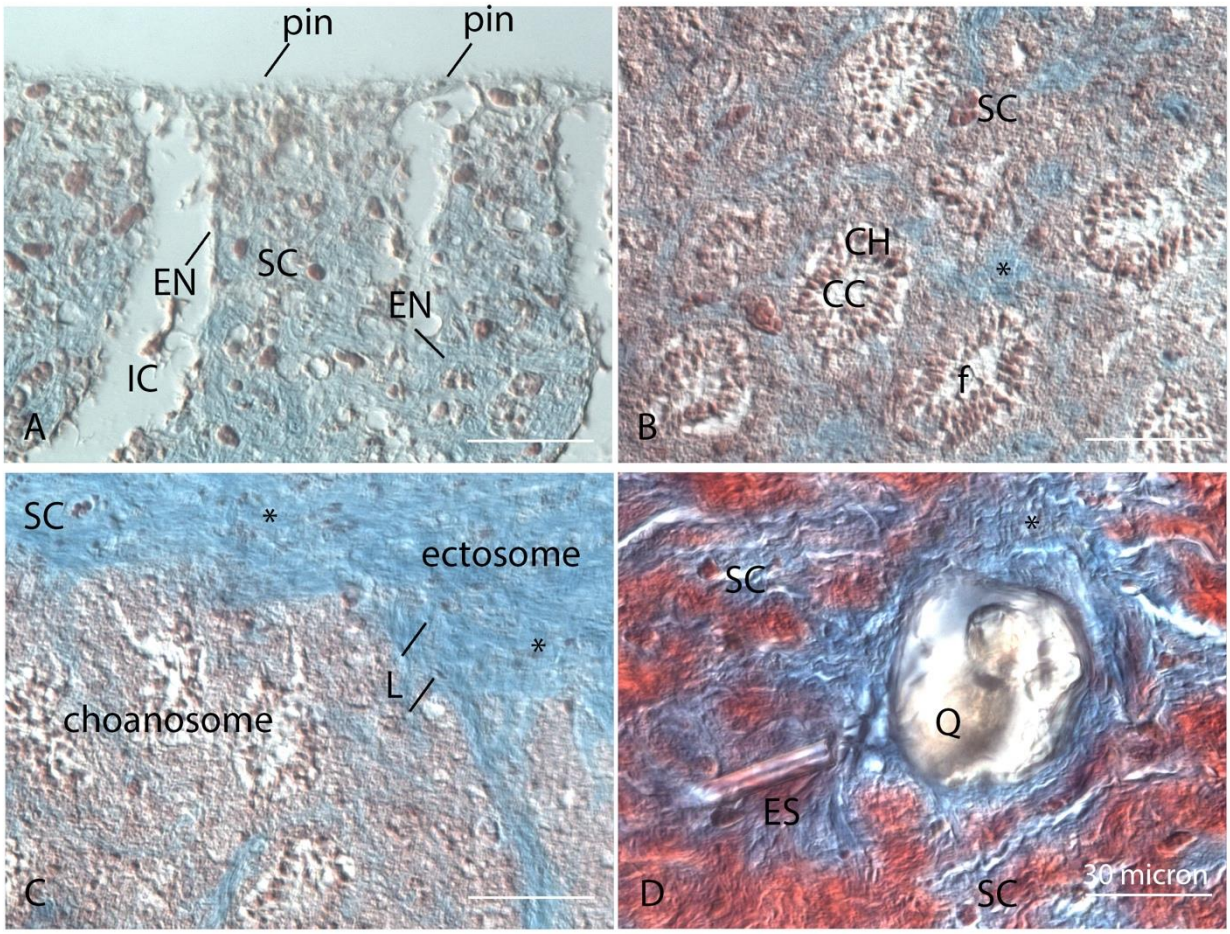

Figure S1

Internal structure of the *Chondrosia reniformis* marine sponge highlighted by the Masson's trichrome staining observed by differential-interference contrast filters (DIC). Images were acquired with a Leica DMRB microscope equipped with a 40 × air objective. Nuclei are in black, cytoplasm in red and dense collagen in blue. (*dense collagen); (CH: choanocyte) (CC: choanocyte chamber); (f: flagellum); (EN: endo pinacocytes); (ES: exogenous spicule); (IC: inhalant channel); (PC: pinacocyte); (pin: pinacoderm) (Q: quartz); (SC: spherulouse cell). Bars span 30 µm. Fig 1A: external portion of ectosome. B: particular of choanocite chamber clusters. C: border between ectosome (pinacoderm and mesohyl), and choanosome. D: particular of a crystalline grain and an exogenous spicule embedded by collagen.