Oxidative Stress Response in Regulatory and Conventional T Cells: A Comparison Between Patients with Chronic Coronary Syndrome and Healthy Subjects

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Research

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Abstract

Background

Inflammation and oxidative stress form a vicious circle in atherosclerosis. Oxidative stress can have detrimental effects on T cells. A unique subset of $CD4^+$ T cells, known as regulatory T (T_{reg}) cells, has been associated with atheroprotective effects. Reduced numbers of T_{reg} cells is a consistent finding in patients with chronic coronary syndrome (CCS). However, it is unclear to what extent these cells are sensitive to oxidative stress. In the present study, we tested the hypothesis that oxidative stress might be a potential contributor to the T_{reg} cell deficit in CCS patients.

Methods

Thirty patients with CCS and 24 healthy controls were included. T_{reg} ($CD4^+CD25^+CD127^-$) and conventional T ($CD4^+CD25^-$, T_{conv}) cells were isolated and treated with increasing doses of H_2O_2. Intracellular ROS levels and cell death were measured after 2 and 18 h, respectively. The expression of antioxidant genes was measured in freshly isolated T_{reg} and T_{conv} cells. Alxo, total antioxidant capacity (TAC) was measured in fresh peripheral blood mononuclear cells.

Results

At all doses of H_2O_2, T_{reg} cells accumulated more ROS and exhibited higher rates of death than their T_{conv} counterparts, $p < 0.0001$. T_{reg} cells also expressed higher levels of antioxidant genes, including thioredoxin and thioredoxin reductase-1 ($p < 0.0001$), though without any differences between CCS patients and controls. T_{conv} cells from CCS patients were, on the other hand, more sensitive to oxidative stress *ex vivo* and expressed more thioredoxin reductase-1 than T_{conv} cells from controls, $p < 0.05$. Also, TAC levels were lower in patients, 0.97 vs 1.53 UAE/100 μg, $p = 0.001$.

Conclusion

T_{reg} cells isolated from either CCS patients or healthy controls were all highly sensitive to oxidative stress *ex vivo*. There were however signs of oxidant-antioxidant imbalance in CCS patients and we thus assume that oxidative stress plays a role in the reduction of T_{reg} cells *in vivo*.

Background

Oxidative stress and chronic inflammation are closely linked phenomena that can perpetuate each other and easily form a vicious circle in chronic disease. The simultaneous existence of oxidative stress and low-grade inflammation in atherosclerosis is well established. Oxidative stress, defined as chronic overproduction of reactive oxygen species (ROS) or rather an imbalance between oxidants and antioxidants in favor of the oxidants [1], plays a major role in the development of atherosclerosis, as shown in experimental animal models [2, 3]. In humans, a variety of circulating markers of oxidative

stress, including oxidative modification of low density lipoprotein (LDL), have shown associations with cardiovascular disease [4, 5]. It has also been shown that oxidized LDL can elicit a robust immune response in atherosclerosis, involving activation of $CD4^+$ T cells [6].

Although moderate levels of ROS are necessary for the proper regulation of T cell activation, large quantities of ROS may have detrimental effects on T cells, such as decreased viability [7]. Moreover, oxidative stress contributes to immunosenescence, or aging of the immune system [8]. This may be an area of special interest in understanding the T cell perturbations associated with coronary artery disease. The systemic T cell activation that occurs in many patients with acute coronary syndrome (ACS) does not normalize over time but becomes persistent, despite clinical stability and medical treatment [9–11]. Features of immunosenescence, such as shorter telomere length, are consistently found in patients with chronic coronary syndrome (CCS), a term defining those with stable symptoms [12]. In these patients, senescent T cells capable of secreting large amounts of proinflammatory cytokines are reportedly expanded [13].

The perturbed T cell repertoire in CCS patients is also associated with a decrease of regulatory T (T_{reg}) cells, a specialized subpopulation of $CD4^+CD25^+$ T cells. T_{reg} cells constitute 5–10% of all peripheral $CD4^+$ T cells and have an important role in maintaining immune homeostasis and self-tolerance by suppressing effector T cell functions. Consequently, T_{reg} cell impairment has been reported in a number of human autoimmune disorders [14]. In experimental models of atherosclerosis, T_{reg} cells have been associated with atheroprotective effects [15] and, in line with this, clinical studies have shown a numerical and functional deficit of T_{reg} cells both in patients with ACS and in patients with CCS compared to healthy individuals [10, 16–18]. Moreover, baseline levels of T_{reg} cells in the population have been shown to be associated with increased risk of myocardial infarction [19]. The underlying mechanisms of the T_{reg} cell deficit are not clarified but in a previous study, Mor *et al* [17] demonstrated that the numbers of $CD4^+CD25^+$ T_{reg} cells were markedly reduced after incubation with oxidized LDL while the effect on conventional $CD4^+CD25^-$ T (T_{conv}) cells was negligible. Interestingly, they also found that T_{reg} cells from ACS patients were more sensitive to oxidized LDL compared to T_{reg} cells from CCS patients or controls with normal coronary angiograms. The finding indicating that T_{reg} cells were more sensitive to oxidative stress compared to T_{conv} cells was however contradicted by Mougiakakos *et al* [20]. These authors showed that T_{reg} cells from healthy volunteers were more resistant to hydrogen peroxide (H_2O_2)-induced cell death compared to T_{conv} cells, leading to the speculation about a protective mechanism that enables T_{reg} cells to survive within an inflammatory milieu.

The extent to which oxidative stress affects T_{reg} cell survival is thus far from clarified. In the present study, we tested the hypothesis that oxidative stress might be a potential contributor to the T_{reg} cell deficit in CCS patients. Our first aim was to perform a comparison between T_{reg} and T_{conv} cells with respect to sensitivity towards oxidative stress. A second aim was to investigate whether T_{reg} and T_{conv} cells from CCS patients and healthy controls differed in their sensitivity towards oxidative stress. Different aspects

of oxidative stress response, including H_2O_2-induced ROS production and cell death *ex vivo*, were measured in separate fractions of T_{reg} and T_{conv} cells from CCS patients and healthy controls. To further elucidate the oxidative stress response *in vivo*, we also measured the expression of antioxidant genes in freshly isolated T_{reg} and T_{conv} cells from patients and controls.

Methods

Study population

The study population consisted of patients (n = 30) recruited from the Department of Cardiology, University Hospital, Linköping, Sweden, as well as control subjects with approximately equal sex and age distribution (n = 24). All patients had angiographically verified coronary artery disease and a history of coronary event, i.e. ACS and/or revascularization with either percutaneous coronary intervention or coronary artery bypass grafting. For the control group, individual residents of Linköping were randomly selected from the Swedish Population Register and invited to participate in the study. Individuals who accepted the invitation were included as controls if they were anamnestically healthy and had normal routine laboratory tests. Use of lipid-lowering or antihypertensive drugs for primary prevention of cardiovascular disease was allowed in the control group.

Study subjects were excluded if they suffered from severe heart failure, immunological disorders, neoplastic disease, had evidence of acute or recent (< 2 months) infection or major trauma, had undergone surgery/revascularization procedure (< 2 months) or received regular treatment with immunosuppressive or anti-inflammatory agents (except low-dose aspirin).

Cell isolation

To prepare peripheral blood mononuclear cells (PBMCs), diluted sodium heparinized whole blood, approx. 30–40 mL was layered on Ficoll-Paque Density Gradient Medium (ThermoFisher Scientific) and centrifuged for 40 min at 400 x g with low acceleration and no brake. PBMCs were collected from the interphase and thereafter washed with PBS. The cells were then resuspended in PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA), and 0,5% fetal bovine serum (FBS). Cells were counted using a Countess II FL Automatic Cell Counter (ThermoFisher).

To isolate the T_{reg} and T_{conv} cell fractions, an EasySep Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation Kit (STEMCELL Technologies) was used according to manufacturer´s instructions on PBMCs resulting in two cell fractions; T_{reg} cells defined as $CD4^+CD127^{low}CD25^+$ and T_{conv} cells defined as $CD4^+CD127^+CD25^-$ T cells.

Following isolation, all cells were resuspended in complete RPMI (Fisher Scientific) cell medium with 10% FBS (Fisher Scientific) and 2% Penicillin Streptomycin solution (Fisher Scientific).

Purity checks

To ascertain the purity of cell fractions, cells were stained with a Human Regulatory T Cell Sorting Kit cocktail including FITC CD45RA-FITC, CD127-Alexa Fluor647, CD25-PE, and CD4-PerCP-Cy5.5 (BD Biosciences) and analysed on a FACS aria sorter (BD Biosciences). Cells were separately stained for FoxP3 expression using a Human FoxP3 Buffer Set (BD Biosciences). Briefly, cells were stained with CD4 FITC for 20 min, then washed and fixed with Buffer A. After fixation and wash, the cells were permeabilised with Buffer C. After permeabilisation and wash, the cells were stained with FoxP3-V450 antibodies and incubated for 30 min. Following a final wash, the cells were analysed in a 3-laser Gallios flow cytometer (Beckman Coulter). Illustrative results are shown in Supplementary Fig. 1.

H_2O_2-induced cell death of T_{reg} and T_{conv} cells

A total of 40 000 T_{reg} or T_{conv} cells were added to each well in a round-bottom 96-well plate. Cells were treated with 5, 10, 20, or 30 µM H_2O_2 or left untreated in a total volume of 200 µL. The cells were incubated for 18 h in a humified incubator at 37 °C and 5% CO_2. The incubation time was chosen based on previous similar studies [20, 21]. Following incubation, cells were washed and 100 µL Annexin-V Binding Buffer (BD Biosciences) was added followed by the addition of Annexin-V PE and CD4-BV510 antibodies (BD Biosciences). After 15 min incubation additional 400 µL Annexin-V Binding Buffer and SYTOX Red Dead Cell Stain (ThermoFisher) was added. After vortexing and incubation for another 10 min, cells were analyzed with a Gallios Flow Cytometer to monitor cell death and data was collected within 1 hour.

Staurosporin-induced cell apoptosis of T_{reg} and T_{conv} cells

In the same manner as described above, T_{reg} and T_{conv} cell fractions were also treated with 2.5 µM staurosporin (STS) (Streptomyces sp. Origin, Sigma-Aldrich) as an alternative way to induce apoptosis.

H_2O_2-induced intracellular ROS levels in T_{reg} and T_{conv} cells

The ROS generation within T_{reg} and T_{conv} cells was determined after 2 h incubation with H_2O_2. A total of 40 000 cells per well were seeded into a round-bottom 96-well plate and treated with the following concentrations H_2O_2, 30 µM, 60 µM and 120 µM at 37 °C. After 1 h, CellROX Green (Thermofisher) to a final concentration of 495 nM was added and cells were incubated for another hour. Thereafter, the T cell subsets were transferred to flow cytometry tubes, washed with 3 x volume (300 µL) PBS + 0.5% FBS and centrifuged at 500 x g for 5 min in room temperature. After removal of supernatants, cells were resuspended in 100 µL PBS + 0.5% FBS. SYTOX Red Dead Cell Stain, CD4BV510 and 10 µL Brilliant Stain Buffer (BD Biosciences) were added in each sample prior to 15 min incubation in the dark at room temperature. Finally, mean fluorescence intensity (MFI) of CellROX Green was recorded with a 3-laser Gallios Flow Cytometer. The proportions of living cells, defined as cells negative for Annexin-V and SYTOX, and normalized based on untreated cells (incubated with medium only).

Flow cytometry analyses

Flow cytometry data were analyzed with Kaluza Analysis Software 2.1 (Beckman Coulter). Cells were gated in a lymphocyte gate according to size and granularity and thereafter $CD4^+$ cells only were gated. Cells negative for Annexin-V and SYTOX were considered viable. For the oxidative stress assay, cells were gated in a lymphocyte gate and the CellROX Green signal was recorded in $CD4^+$ cells that were negative for SYTOX, i.e. living cells.

Oxidative stress gene expression in circulating T_{reg} and T_{conv} cells

In order to assess mRNA expression of oxidative stress-associated genes in circulating cells, T_{reg} and T_{conv} cells were isolated from PBMCs using the same kit from STEMCELL as described above. Cell lysates were collected directly after isolation. Total RNA was isolated from T_{reg} and T_{conv} cell fractions using Qiagen total RNA isolation kit (Thermofisher). The RNA (total 21,6 ng) was converted to cDNA using high capacity cDNA reverse transcription kit with an RNAse inhibitor (Life Technologies) according to manufacturer's instructions. cDNA was amplified by RT-PCR reactions with TaqMan™ Fast Universal PCR Mastermix (Life Technologies) in 96-well plates on an ABI 7500 Sequence Detector with 7500 v 2.3 software. The following TaqMan Gene Expression Assay kits (Life Technologies) were used: Hs00167309_m1 for superoxide dismutase 2 (SOD2), Hs00156308_m1 for catalase (CAT), Hs00757844_m1 for oxidation resistance 1 (OXR1), Hs04194449_s1 for glutathione peroxidase-7 (GPX7), Hs00828652_m1 for thioredoxin (Trx), Hs00917067_m1 thioredoxin reductase (TrxR1). Eukaryotic 18S rRNA (Part number: 4352930E) with an amplicon length of 187 bp served as endogenous control. The levels of gene expressions were calculated relative to the amount of rRNA in each sample. The expression of all genes was calculated with the comparative CT method where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{\Delta\Delta CT}$ according to the user bulletin no 2 (Applied Biosystems, Life Technologies). Results are presented as arbitrary units. Each sample was run in duplicates and a maximum deviation of 10% was allowed.

TrxR1 in cell supernatants

The concentration of TrxR1 in cell supernatants from T_{reg} and T_{conv} cells after treatment with or without 30 μM H_2O_2 for 18 h was measured with an ELISA kit (Abcam, UK) according to manufacturer´s instructions. The range of the standard curve was 0.196–25 ng/mL. Samples were assayed in duplicates and a maximal deviation of 15% was allowed. Undetected samples were given half the value of the lowest standard point.

Total antioxidant capacity in PBMCs

Total antioxidant capacity (TAC) in PBMCs was measured using Cell Biolabs, Inc. OxiSelect Total Antioxidant Capacity Assay Kit. This assay is based on the principle that Cu^{2+} ions are reduced into Cu^+ ions in the presence of antioxidants. The reduced Cu + ion chelates with a colorimetric probe, giving a broad absorbance peak at 570 nm, which is proportional to the TAC. Results are expressed as units of uric acid equivalents (UAE).

In brief, cell pellets of 3 millions PBMCs were snap frozen and stored in -80 °C for no longer than 2 weeks. Before analysis, the frozen PBMCs were lysed by thawing on ice followed by water-bath sonication for 10 min. Samples were kept on ice immediately after lysis. The TAC assays were then performed according to manufacturer's instructions. The TAC results were also normalized by protein amount measured in PBMC using Thermo Scientific's Coomassie Plus (Bradford) Assay Kit according to the manufacturer's instruction. The final TAC results were calculated according to the equation below:

$$\text{TAC} = (\text{UAE}/100\ \mu\text{g}) = \frac{UAE}{Protein\ Amount\ Assayed\ (\mu g)} \text{ x } 100$$

Statistics

Statistical analyses were calculated in IBM SPSS Statistics 25. Groups and independent variables were compared using independent Mann-Whitney U tests, Fishers Exact Tests, and a Chi Square test. Wilcoxon rank sum test or paired t-test were used to compare paired data. Correlations were calculated using Spearman rank correlation test. Numeric data are presented as median and inter-quartile range. A p value < 0.05 was considered significant.

Results

Characteristics of study population

The basal characteristics of the study population are presented in Table 1. There were no differences between CCS patients and controls with respect to age, gender, body mass index, current smoking, use of anti-hypertensive medication or laboratory variables, including high density lipoprotein (HDL) cholesterol, triglycerides, creatinine or fasting glucose levels. Seven (23%) patients had type 2 diabetes. The prevalence of former smoking was higher among patients. Statins were used by all patients (except for one) resulting in significantly lower LDL levels in this group. The TAC levels in PBMCs were significantly lower in patients compared to controls, $p = 0.001$. When the 7 patients with diabetes were excluded, the TAC levels were still significantly lower in patients, $p = 0.002$.

Table 1
Basal characteristics of CCS patients and controls.

		CCS patients (n = 30)	Controls (n = 24)	*p*
Age, years		67 (62–73)	73 (67–75)	0.053
Males		23 (77)	17 (71)	0.429
Body mass index, kg/m^2		27 (24–30)	25 (24–28)	0.192
Smoking	Current	1 (3.3)	0 (0)	0.556
	Former	15 (50)	3 (13)	**0.004**
Use of anti-hypertensive drugs		14 (47)	8 (33)	0.239
Use of statin		29 (97)	7 (29)	**< 0.0001**
LDL cholesterol, mmol/L		1,6 (1.4–1.9)	3 (2.2–3.5)	**< 0.0001**
HDL cholesterol, mmol/L		1,4 (1.0-1.7)	1,4 (1.0-1.7)	0.662
Triglycerides, mmol/L		1,0 (0.7–1.5)	1.3 (0.8–1.6)	0.398
Creatinine, µmol/L		88 (78–97)	79 (73–92)	0.134
Fasting glucose, mmol/L		5,8 (5.3–6.6)	5,7 (5.2–6.3)	0.403
TAC in PBMCs, UAE/100ug		0.97 (0.28–1.48)	1.53 (1.20–2.09)	**0.001**

Values are given as n (%) or median (interquartile range). CCS, chronic coronary syndrome, LDL, low density lipoprotein, HDL, high density lipoprotein, TAC, total antioxidant capacity, PBMC, peripheral blood mononuclear cell, UAE, uric acid equivalents. Bolded *P*-values represent statistical significance.

H_2O_2- and STS-induced cell death of T_{reg} and T_{conv} cells

T_{reg} and T_{conv} cells were isolated from 34 subjects (20 CCS patients and 14 controls) and treated with medium only or increasing concentrations of H_2O_2. Before treatment, the viability was > 90% in both cell types. After 18 h incubation with medium only, the proportions of living T_{reg} cells were significantly lower compared with T_{conv} cells, 71 (64–76) % *vs* 90 (86–93) %, $p < 0.001$. At all concentrations of H_2O_2, T_{reg} cells were more susceptible to H_2O_2-induced cell death than T_{conv} cells (Fig. 1A). There was a clear dose-response relationship with very low percentages of living T_{reg} cells at 20 and 30 µM H_2O_2.

T_{reg} cells were also more susceptible to cell death compared to T_{conv} cells when STS, a classical inducer of the intrinsic apoptotic pathway, was used. The proportions of living cells were 46 (37–54) % and 88 (79–95) %, $p < 0.001$, for T_{reg} and T_{conv} cells, respectively, after treatment with 2.5 µM STS

There were no significant differences in susceptibility to H_2O_2-induced cell death between T_{reg} cells from CCS patients and T_{reg} cells from controls at any concentration of H_2O_2 (Table 2). Neither were there any differences in STS-induced cell death in T_{reg} cells between patients and controls. On the other hand, the percentages of living T_{conv} cells were significantly lower in patients than in controls when T_{conv} cells were exposed to H_2O_2 at lower concentrations (5 or 10 μM) or to STS (Table 2).

Table 2
Percentages of living regulatory T (T_{reg}) cells and corresponding conventional T (T_{conv}) cells from CCS patients and controls, defined as negative for Annexin-V and SYTOX and normalized based on untreated cells (medium only), after 18 h treatment with different concentrations of H_2O_2 or 2,5 μM STS.

	CCS patients (n = 20)	**Controls (n = 14)**	
Treatment	**T_{reg} cells**, % living cells		p
5 μM H_2O_2	74 (69–88)	88 (77–94)	0.246
10 μM H_2O_2	50 (44–58)	58 (41–79)	0.338
20 μM H_2O_2	9.6 (2.8–11)	14 (8.8–29)	0.159
30 μM H_2O_2	2.7 (2.2–4.2)	3.9 (2.8–8.9)	0.106
2.5 μM STS	42 (30–52)	47 (40–56)	0.174
	T_{conv} cells, % living cells		
5 μM H_2O_2	94 (92–99)	98 (97–100)	**0.046**
10 μM H_2O_2	85 (92–99)	93 (87–98)	**0.030**
20 μM H_2O_2	39 (35–67)	69 (45–84)	0.110
30 μM H_2O_2	24 (11–41)	39 (21–57)	0.199
2.5 μM STS	80 (69–91)	91 (88–95)	**0.014**

Values are given as median (inter-quartile range). CCS, chronic coronary syndrome, H_2O_2, hydrogen peroxide, STS, staurosporin. Bolded *P*-values represent statistical significance.

H_2O_2-induced intracellular ROS accumulation in T_{reg} and T_{conv} cells

H_2O_2-induced intracellular ROS levels were measured in T_{reg} and T_{conv} cells after 2 h treatment in medium with or without increasing concentrations of H_2O_2. Treatment with medium only or with 10 μM H_2O_2 did

not reveal any differences between T_{reg} and T_{conv} cells regardless of subject types while the ROS accumulation was significantly larger in T_{reg} cells treated with 30 μM H_2O_2 or more (Fig. 1B). When we compared CCS patients and controls, the ROS production in T_{reg} and T_{conv} cells did not show any significant differences (Supplementary Figs. 2A and B.

The in vivo expression of oxidative stress-associated genes in T_{reg} and T_{conv} cells

In order to compare the oxidative stress response *in vivo*, we measured the expression of a number of oxidative stress-associated genes in freshly isolated T_{reg} and T_{conv} cells from 20 subjects, 10 CCS patients (8 males, 2 females, median age 67) and 10 controls (8 males, 2 females, median age 68). As shown in Fig. 2A, the mRNA levels of CAT, OXR1, Trx and TrxR1 were significantly higher in T_{reg} cells than in T_{conv} cells regardless of subject types while mRNA levels of GPX7 and SOD2 expression did not differ between the two T cell fractions (Fig. 2B).

When comparing CCS patients and controls, the T_{reg} cells did not show any significant differences in gene expression (Table 3). On the other hand, the T_{conv} cells from CCS patients showed significantly higher expression of TrxR1 than those from controls ($p = 0.033$). Also, the Trx and SOD2 mRNA levels tended to be higher in T_{conv} cells from patients ($p = 0.104$ and $p = 0.091$, respectively).

Table 3
The mRNA expression of thioredoxin reductase-1 (TrxR1), thioredoxin (Trx), glutathione peroxidase 7 (GPX7), oxidation resistance 1 (OXR1), catalase (CAT) and superoxide dismutase 2 (SOD2) in regulatory T (T_{reg}) cells and corresponding conventional T (T_{conv}) cells from CCS patients and controls.

	CCS patients (n = 10)	**Controls (n = 10)**	
Gene	**T_{reg} cells**		P
TrxR1	3.7 (2.6–4.4)	3.0 (2.6–3.9)	0.973
Trx	7.9 (4.6–10)	6.2 (4.0-6.7)	0.445
GPX7	2.0 (1.2–2.2)	1.6 (1.3–2.4)	0.621
OXR1	2.9 (1.8–3.3)	2.5 (1.7–2.7)	0.681
CAT	3.6 (2.4-4.0)	3.1 (2.8–3.9)	0.929
SOD2	1.9 (1.1-2.0)	1.7 (1.2–2.1)	0.949
	T_{conv} cells		
TrxR1	1.7 (1.4-2.0)	1.4 (1.0-1.6)	**0.033**
Trx	2.8 (2.2–3.2)	1.9 (1.5–2.4)	0.104
GPX7	1.4 (1.4–2.1)	1.5 (1.4–1.7)	0.267
OXR1	1.8 (1.4–1.9)	1.7 (1.4–1.9)	0.601
CAT	2.4 (1.9–2.7)	1.7 (1.4–2.8)	0.204
SOD2	1.6 (1.4–2.1)	1.3 (0.8–1.5)	0.091

Values are given as median (interquartile range). CCS, chronic coronary syndrome, Bolded P-value represents statistical significance.

Secretion of TrxR1 by T_{reg} and T_{conv} cells

In order to further confirm the changes in gene expression, we measured TrxR1 protein levels in supernatants from T_{reg} and T_{conv} cells collected from 32 subjects (18 CCS patients and 14 controls). The reason for measuring TrxT1 relied on a previous study by Xie *et al* [22]. The Trx1 levels were generally low and almost undetectable in untreated samples. However, both T_{reg} and T_{conv} cells secreted significantly more TrxR1 after H_2O_2 treatment, $p < 0.0001$ in both cell types. Also, TrxR1 levels were significantly higher in supernatants from T_{reg} cells compared to T_{conv} cells, $p = 0.003$ (Fig. 2C). There were no differences between CCS patients and controls (Supplementary Fig. 3).

Correlations

There were significant inverse relationships between H_2O_2-induced intracellular ROS levels and % of living cells after 18 h, in particular for T_{conv} cells (Table 4). There was no correlation between intracellular ROS levels and STS-induced cell death. We were not able to study correlations between susceptibility to H_2O_2 *ex vivo* and antioxidant gene expression since these analyses were performed in different study subjects.

Table 4
Relationships between H_2O_2-induced intracellular ROS levels after 2 h treatment with 60 μM H_2O_2 and % of living cells after 18 h with increasing doses of H_2O_2 in regulatory T (T_{reg}) cells and corresponding conventional T (T_{conv}) cells.

Treatment	T_{reg} cells	T_{conv} cells
5 μM H_2O_2	-0.091	-0.067
10 μM H_2O_2	-0.422	-0.571*
20 μM H_2O_2	-0.336	-0.609*
30 μM H_2O_2	-0.530*	-0.652**
Relationships are presented as Spearman correlation coefficients. * $p < 0.05$, ** $p < 0.01$.		

TAC levels in freshly isolated PBMCs were measured in all study subjects and correlated inversely with intracellular ROS levels after 2 h treatment with 60 μM H_2O_2 in T_{reg} cells, $r = -0.480$, $p = 0.037$; and T_{conv} cells, $r = -0.511$, $p = 0.030$, but showed no correlations with H_2O_2-induced cell death *ex vivo* or with antioxidant gene expression.

Discussion

The main findings from the present study are that human T_{reg} cells exhibit markedly increased sensitivity to H_2O_2-induced ROS production and cell death compared to T_{conv} cells in both CCS patients and healthy controls. T_{reg} cells were also more sensitive to spontaneous cell death when cultured in medium overnight and to cell death induced by STS, a potent inducer of apoptosis. Hitherto, the impact of oxidative stress on human T_{reg} cells has been far from clarified. The existing literature is both sparse and inconsistent. Mor *et al* [17] reported that the number of T_{reg} cells was reduced to a considerably larger extent than the number of T_{conv} cells after *in vitro* incubation with oxidized LDL. They further showed that this effect was attenuated in the presence of caspase inhibitor suggesting that apoptosis contributed to the loss of cells. Others demonstrated that T_{reg} cells were more prone to apoptosis than T_{conv} cells when they were cultured in medium only [23, 24]. On the other hand, Mougiakakos *et al* [20] used a protocol that was similar to ours, i.e. incubation with 5, 10 or 20 μM H_2O_2 for 18 h, but obtained completely opposite results. They found that T_{reg} cells were significantly more resistant to H_2O_2-induced cell death compared to T_{conv} cells. Moreover, they found that naïve T_{reg} cells were more resistant than memory T_{reg} cells. One

possible explanation for the contradictory results may be the choice of study subjects. Mougiakakos *et al* [20] used cells from a few healthy donors of unknown age while we used cells from sick and elderly subjects. Ageing and cardiovascular disease are both linked to elevated oxidative stress. We previously showed that naïve T_{reg} cells constituted only 15% of all T_{reg} cells in elderly CAD patients as opposed to 25% in age-matched controls, and also that the function of naïve T_{reg} cells was impaired in patients [10]. Differences in naïve T_{reg} cell pool size and function as well as individual variations in oxidative status may thus contribute to the disparity in results between our study and Mougiakakos *et al* [20].

In an attempt to assess intrinsic ability of these cells to counteract oxidative stress, we measured the mRNA expression of several main antioxidant genes, which all contribute to inactivate ROS, from freshly isolated T_{reg} and T_{conv} cells from both patients and controls. Collectively, the expression of catalase was significantly higher in T_{reg} cells than in T_{conv} cells and so was the expression of OXR1, a protein that has been described as a cellular oxidative stress sensor regulating the expression of several antioxidant enzymes [25]. Also, mRNA levels of the two main proteins, Trx and TrxR1, in the antioxidant Trx system were markedly upregulated in T_{reg} cells. Our findings partly agree with the previous study by Mougiakakos *et al* [21], who reported that Trx expression was higher in T_{reg} cells compared with T_{conv} cells while, on the other hand, catalase expression did not differ in their small study group of volunteers. An earlier genomic and proteomic screening study examined the H_2O_2-induced gene and protein expression in human skin fibroblasts and reported that TrxR1 was the only oxidation-related candidate with elevated levels at both the mRNA and protein level, the latter measured in cell lysates [22]. Another study by Söderberg *et al* [26] provided evidence that TrxR1 was secreted by human PBMCs upon inflammatory stimulation. Here, we were able to show that TrxR1 was secreted into the cell supernatant to a greater extent by T_{reg} cells than by T_{conv} cells upon H_2O_2 treatment. Altogether, our findings indicate that T_{reg} cells have a higher endogenous antioxidant capacity than T_{conv} cells in both CCS patients and controls.

Several studies have reported reduced levels of T_{reg} cells in peripheral blood of patients with ACS [10, 16–18]. It has also been proposed that oxidative stress is involved in T_{reg} cell depletion. Mor *et al* [17] showed that T_{reg} cells from ACS patients were more sensitive to oxidized LDL than T_{reg} cells from CCS patients or patients with normal coronary angiograms. A later study by Zhang *et al* [18] showed that the spontaneous apoptosis of T_{reg} cells was pronounced in ACS patients and further demonstrated that oxidized LDL induced apoptosis of human T_{reg} cells *in vitro*, yet without comparing patients and controls. Recently, we found that the numerical and functional T_{reg} cell deficit in ACS patients was not merely a transient phenomenon but that it remained after clinical stabilization [10]. In the present study, we therefore focused on the potential role of oxidative stress in the depletion of T_{reg} cells in CCS patients. However, there was no proof that T_{reg} cells from patients were more prone to H_2O_2-induced cell death than T_{reg} cells from controls, when assessed *ex vivo*. Moreover, the expression of antioxidant genes in freshly isolated T_{reg} cells from patients and controls indicated that T_{reg} cells in CCS patients and controls had similar levels of endogenous antioxidant enzymes *in vivo*. However, the significantly lower intrinsic

TAC levels in PBMC from CCS patients pointed toward an oxidant-antioxidant imbalance implicating the possible impact of exogenous environmental factors, such as sedentary lifestyle and unhealthy diet. The TAC assay is a copper-based assay which measures a large range of lipophilic or thiol-based antioxidants and antioxidant macromolecules but not antioxidant enzymes discussed above. [27, 28] Therefore, given their high susceptibility to oxidative stress, it is reasonable to assume that T_{reg} cell numbers are affected by the prooxidant state in CCS patients.

Interestingly, our results indicate that T_{conv} cells from CCS patients were affected to a greater extent by oxidative stress than T_{conv} cells from controls. T_{conv} cells from CCS patients were more prone to undergo cell death when treated with H_2O_2 or STS and they also expressed significantly higher levels of TrxR1. It is well documented that the proatherogenic T cell response in atherosclerotic lesions is reflected in peripheral blood [9]. In ACS patients, systemic T cell activation is associated with plaque instability and thrombus formation but there is also consistent evidence that the T cell activation persists after clinical stabilization [9–11]. T cell activation is accompanied by the release of ROS which in turn leads to an upregulation of endogenous antioxidants [29]. Moreover, the induction of Trx and TrxR1 in T cells upon activation was recently suggested to be a critical pathway controlling T cell activation and expansion [30]. We believe that the increased susceptibility to oxidative stress in T_{conv} cells from patients reflects a $CD4^+$ T cell activation that remains in CCS patients despite clinical stability and medical treatment.

A couple of limitations should be considered in our study. One limitation is the limited number of patients and controls per experimental set-up, permitting only cautious conclusions about differences between the two groups. The yield levels of T_{reg} cells in the subjects, particularly in the CCS patients, were not high enough to allow *ex vivo* experiments and gene expression analyses to be carried out in the same individuals, nor to perform functional assays. Gene expression analyses were therefore performed in a separate group of subjects, 10 patients and 10 controls, though with similar characteristics as the other subjects. Another potential limitation is that the *ex vivo* model, where isolated fractions of T cell subsets are exposed to increasing doses of H_2O_2 for a relatively short period of time, may not reflect chronic inflammation.

Conclusion

To summarize, we demonstrate that T_{reg} cells were highly susceptible to oxidative stress and cell death *ex vivo*. T_{reg} cells also expressed and secreted high levels of antioxidants indicating a high endogenous capacity to counteract oxidative stress in both CCS patients and controls. Still, there was clear evidence of an oxidant-antioxidant imbalance in CCS patients and we therefore postulate that oxidative stress is a potential contributor to the T_{reg} cell deficit *in vivo*. Whether treatments for oxidative stress reduction lead to normalized levels of circulating T_{reg} cells is speculative and warrants further investigation.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki, and the research protocol was approved by the Ethical Review Board of Linköping, Sweden (Dnr: …..). Written informed consent was obtained from all study participants.

Consent for publication

Not applicable.

Availability of data and materials

All datasets used and analyzed throughout the study are available from the corresponding author based on sensible request.

Competing interests

The authors declare that they have no competing interests.

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Author contribution

Anna K Lundberg: Methodology, Data analysis, Data curation, Project administration, Writing – review and editing. **Rosanna Chung**: Methodology, Data analysis, Writing – review and editing. **Louise Zeijlon**: Methodology, Data analysis. **Gustav Fernström**: Methodology, Data analysis. **Lena Jonasson**: Data analysis, Writing – original draft, Writing – review and editing, Project administration, Funding acquisition.

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Conflict of Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

References

1. Jones DP: **Redefining oxidative stress.** *Antioxid Redox Signal* 2006, **8:**1865-1879.

2. Kattoor AJ, Pothineni NVK, Palagiri D, Mehta JL: **Oxidative Stress in Atherosclerosis.** *Curr Atheroscler Rep* 2017, **19:**42.
3. Madamanchi NR, Vendrov A, Runge MS: **Oxidative stress and vascular disease.** *Arterioscler Thromb Vasc Biol* 2005, **25:**29-38.
4. Ho E, Karimi Galougahi K, Liu CC, Bhindi R, Figtree GA: **Biological markers of oxidative stress: Applications to cardiovascular research and practice.** *Redox Biol* 2013, **1:**483-491.
5. Patel RS, Ghasemzadeh N, Eapen DJ, Sher S, Arshad S, Ko YA, Veledar E, Samady H, Zafari AM, Sperling L, et al: **Novel Biomarker of Oxidative Stress Is Associated With Risk of Death in Patients With Coronary Artery Disease.** *Circulation* 2016, **133:**361-369.
6. Gisterå A, Hansson GK: **The immunology of atherosclerosis.** *Nat Rev Nephrol* 2017, **13:**368-380.
7. Yarosz EL, Chang CH: **The Role of Reactive Oxygen Species in Regulating T Cell-mediated Immunity and Disease.** *Immune Netw* 2018, **18:**e14.
8. Cannizzo ES, Clement CC, Sahu R, Follo C, Santambrogio L: **Oxidative stress, inflamm-aging and immunosenescence.** *J Proteomics* 2011, **74:**2313-2323.
9. Ammirati E, Moroni F, Magnoni M, Camici PG: **The role of T and B cells in human atherosclerosis and atherothrombosis.** *Clin Exp Immunol* 2015, **179:**173-187.
10. Hasib L, Lundberg AK, Zachrisson H, Ernerudh J, Jonasson L: **Functional and homeostatic defects of regulatory T cells in patients with coronary artery disease.** *J Intern Med* 2016, **279:**63-77.
11. Lundberg AK, Jonasson L, Hansson GK, Mailer RKW: **Activation-induced FOXP3 isoform profile in peripheral CD4+ T cells is associated with coronary artery disease.** *Atherosclerosis* 2017, **267:**27-33.
12. Haycock PC, Heydon EE, Kaptoge S, Butterworth AS, Thompson A, Willeit P: **Leucocyte telomere length and risk of cardiovascular disease: systematic review and meta-analysis.** *Bmj* 2014, **349:**g4227.
13. Bergström I, Backteman K, Lundberg A, Ernerudh J, Jonasson L: **Persistent accumulation of interferon-γ-producing CD8+CD56+ T cells in blood from patients with coronary artery disease.** *Atherosclerosis* 2012, **224:**515-520.
14. Grant CR, Liberal R, Mieli-Vergani G, Vergani D, Longhi MS: **Regulatory T-cells in autoimmune diseases: challenges, controversies and–yet–unanswered questions.** *Autoimmun Rev* 2015, **14:**105-116.
15. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, Merval R, Esposito B, Cohen JL, Fisson S, et al: **Natural regulatory T cells control the development of atherosclerosis in mice.** *Nat Med* 2006, **12:**178-180.
16. Emoto T, Sasaki N, Yamashita T, Kasahara K, Yodoi K, Sasaki Y, Matsumoto T, Mizoguchi T, Hirata K: **Regulatory/effector T-cell ratio is reduced in coronary artery disease.** *Circ J* 2014, **78:**2935-2941.
17. Mor A, Luboshits G, Planer D, Keren G, George J: **Altered status of CD4(+)CD25(+) regulatory T cells in patients with acute coronary syndromes.** *Eur Heart J* 2006, **27:**2530-2537.

18. Zhang WC, Wang J, Shu YW, Tang TT, Zhu ZF, Xia N, Nie SF, Liu J, Zhou SF, Li JJ, et al: **Impaired thymic export and increased apoptosis account for regulatory T cell defects in patients with non-ST segment elevation acute coronary syndrome.** *J Biol Chem* 2012, **287:**34157-34166.
19. Wigren M, Björkbacka H, Andersson L, Ljungcrantz I, Fredrikson GN, Persson M, Bryngelsson C, Hedblad B, Nilsson J: **Low levels of circulating CD4+FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke.** *Arterioscler Thromb Vasc Biol* 2012, **32:**2000-2004.
20. Mougiakakos D, Johansson CC, Kiessling R: **Naturally occurring regulatory T cells show reduced sensitivity toward oxidative stress-induced cell death.** *Blood* 2009, **113:**3542-3545.
21. Mougiakakos D, Johansson CC, Jitschin R, Böttcher M, Kiessling R: **Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress.** *Blood* 2011, **117:**857-861.
22. Xie L, Pandey R, Xu B, Tsaprailis G, Chen QM: **Genomic and proteomic profiling of oxidative stress response in human diploid fibroblasts.** *Biogerontology* 2009, **10:**125-151.
23. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN: **Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population.** *Eur J Immunol* 2001, **31:**1122-1131.
24. Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, Rustin MH, Taams LS, Beverley PC, Macallan DC, Akbar AN: **Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo.** *J Clin Invest* 2006, **116:**2423-2433.
25. Zhang X, Zhang S, Liu X, Wang Y, Chang J, Zhang X, Mackintosh SG, Tackett AJ, He Y, Lv D, et al: **Oxidation resistance 1 is a novel senolytic target.** *Aging Cell* 2018, **17:**e12780.
26. Söderberg A, Sahaf B, Rosén A: **Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma.** *Cancer Res* 2000, **60:**2281-2289.
27. Apak R, Güçlü K, Ozyürek M, Karademir SE, Altun M: **Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: the CUPRAC method.** *Free Radic Res* 2005, **39:**949-961.
28. Rubio CP, Hernández-Ruiz J, Martinez-Subiela S, Tvarijonaviciute A, Ceron JJ: **Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update.** *BMC Vet Res* 2016, **12:**166.
29. Belikov AV, Schraven B, Simeoni L: **T cells and reactive oxygen species.** *J Biomed Sci* 2015, **22:**85.
30. Muri J, Heer S, Matsushita M, Pohlmeier L, Tortola L, Fuhrer T, Conrad M, Zamboni N, Kisielow J, Kopf M: **The thioredoxin-1 system is essential for fueling DNA synthesis during T-cell metabolic reprogramming and proliferation.** *Nat Commun* 2018, **9:**1851.

Figures

Figure 1

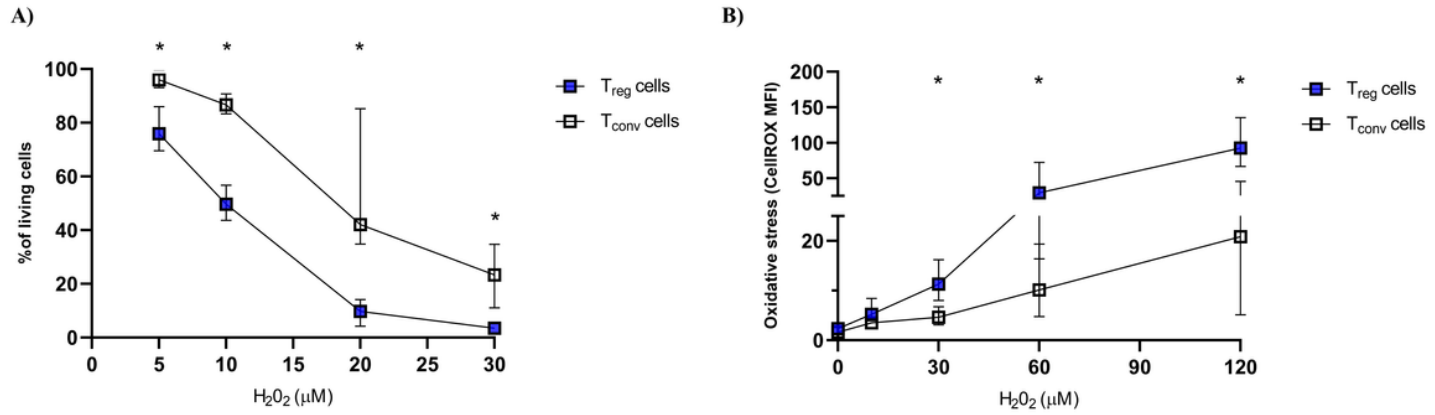

Figure 1

The response to oxidative stress ex vivo in Treg and Tconv cells. (A) Percentages of living Treg and Tconv cells, following treatment with 5, 10, 20, or 30 μM H2O2 for 18 h. % of living cells is defined as negative for Annexin-V and SYTOX and normalized based on untreated cells (medium only). Asterisks indicate significant differences between Treg and Tconv cells, all p < 0.0001. Number of study subjects per dose of H2O2 were n=19 for 5 μM, n=33 for 10 μM, n=22 for 20 μM, n=34 for 30 μM. (B) The levels of oxidative stress, measured as mean fluorescence intensity (MFI) of CellROX Green, is shown in Treg and Tconv cells following treatment with 0, 10, 30, 60 or 120 μM H2O2 for 2h (B). Asterisks indicate significant differences between Treg and Tconv cells, all p < 0.0001 (except for 60 μM H2O2, p < 0.001). Number of study subjects per dose of H2O2 were n=22 for 0 μM, n=3 for 10 μM, n=20 for 30 μM, n=20 for 60 μM, n=12 for 120 μM. Box plots display median and i-q range values.

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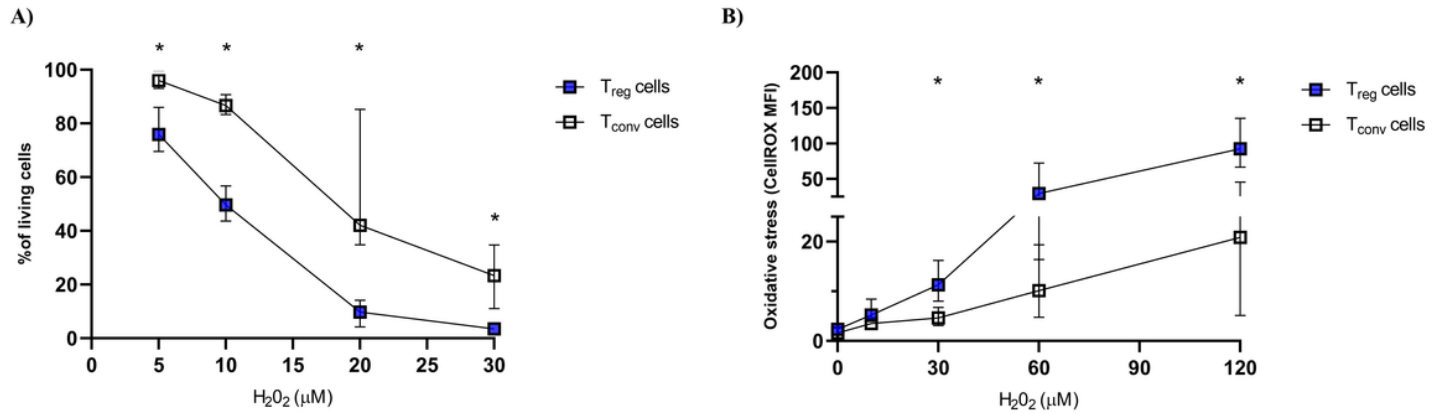

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Figure 2

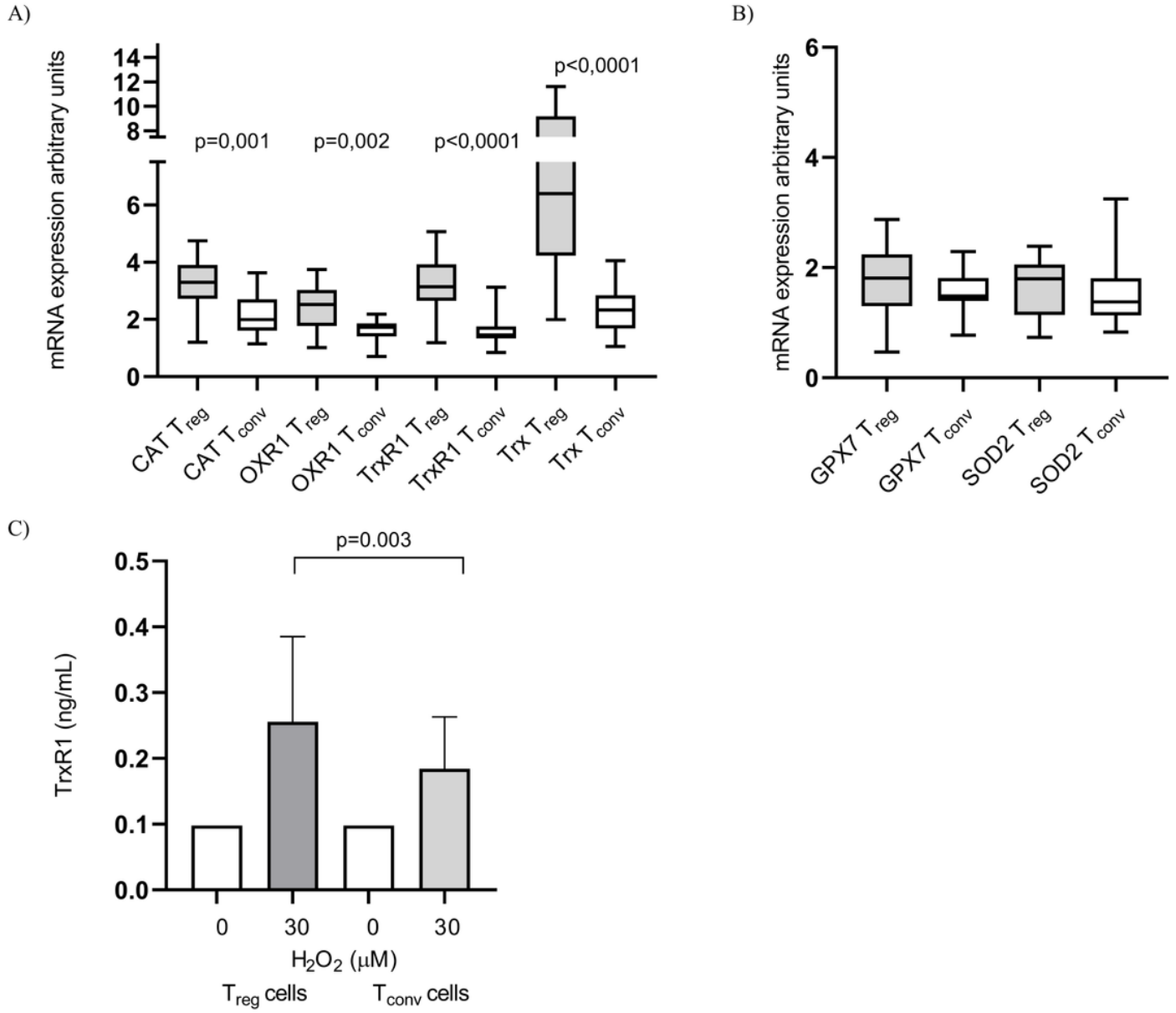

Figure 2

The expression of oxidative stress-associated genes in Treg and Tconv cells from 20 study subjects (10 CCS patients and 10 controls). (A) Genes with significant differences between patients and controls included catalase (CAT), oxidation resistance 1 (OXR1), thioredoxin reductase-1 (TrxR1) and thioredoxin (Trx). (B) Genes with non-significant differences included glutathione peroxidase 7 (GPX7) and superoxide dismutase 2 (SOD2). Graphs depict arbitrary values normalized against internal control and 18S rRNA in each individual sample. (C) Thioredoxin reductase 1 (TrxR1) (ng/mL) levels in cell supernatants of Treg and Tconv cells treated with either 0 or 30 μM H2O2. Number of study subjects per dose of H2O2 were n=32 for 0 μM and n=32 for 30 μM. In all figures, box plots and bars display median and i-q range values

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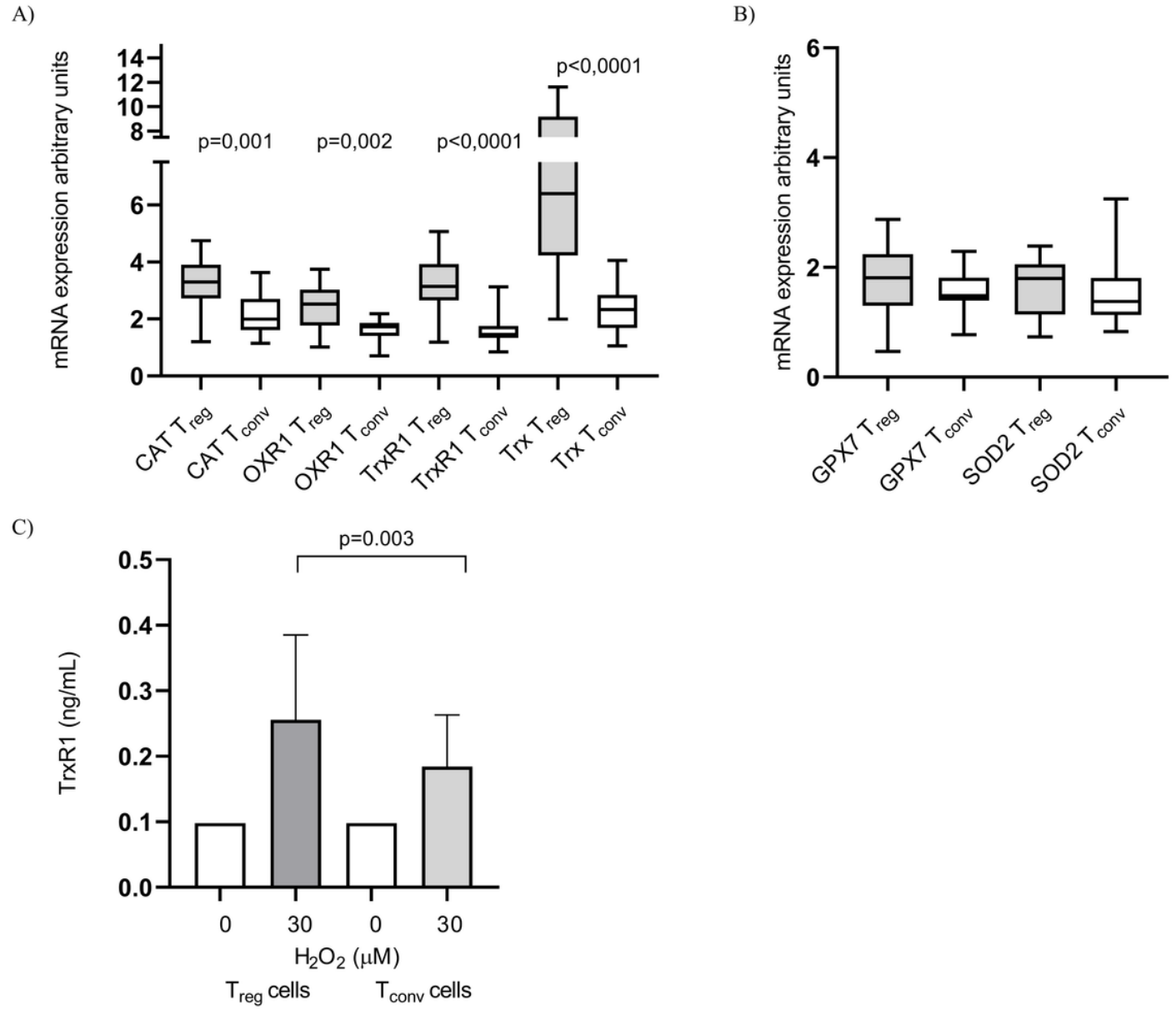

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