Biochimica et Biophysica Acta 1843 (2014) 265–274

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

T cell activation induces CuZn superoxide dismutase (SOD)-1 intracellular re-localization, production and secretion

Giuseppe Terrazzano [a,b,1], Valentina Rubino [b,1], Simona Damiano [c], Anna Sasso [c], Tiziana Petrozziello [c], Valentina Ucci [c], Anna Teresa Palatucci [b], Angela Giovazzino [b], Mariarosaria Santillo [c], Bruna De Felice [d], Corrado Garbi [e], Paolo Mondola [c,*], Giuseppina Ruggiero [b,**]

[a] Dipartimento di Scienze, Università della Basilicata, 85100 Potenza, Italy
[b] Dipartimento di Scienze Mediche Traslazionali, Università di Napoli "Federico II", 80131 Napoli, Italy
[c] Medicina Clinica e Chirurgia, Università di Napoli "Federico II", 80131 Napoli, Italy
[d] Dipartimento di Scienze e Tecnologie Ambientali Biologiche e Farmaceutiche, II Università di Napoli, 81100 Caserta, Italy
[e] Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli "Federico II", 80131 Napoli, Italy

ARTICLE INFO

Article history:
Received 6 June 2013
Received in revised form 21 October 2013
Accepted 24 October 2013
Available online 31 October 2013

Keywords:
Human T lymphocyte
Intracellular localization
Microvesicle secretion
SOD-1
T cell activation
TCR triggering

ABSTRACT

Reactive oxygen species (ROS) behave as second messengers in signal transduction for a series of receptor/ligand interactions. A major regulatory role is played by hydrogen peroxide (H_2O_2), more stable and able to freely diffuse through cell membranes. Copper–zinc superoxide dismutase (CuZn-SOD)-1 is a cytosolic enzyme involved in scavenging oxygen radicals to H_2O_2 and molecular oxygen, thus representing a major cytosolic source of peroxides. Previous studies suggested that superoxide anion and H_2O_2 generation are involved in T cell receptor (TCR)-dependent signaling. Here, we describe that antigen-dependent activation of human T lymphocytes significantly increased extracellular SOD-1 levels in lymphocyte cultures. This effect was accompanied by the synthesis of SOD-1-specific mRNA and by the induction of microvesicle SOD-1 secretion. It is of note that SOD-1 increased its concentration specifically in T cell population, while no significant changes were observed in the "non-T" cell counterpart. Moreover, confocal microscopy showed that antigen-dependent activation was able to modify SOD-1 intracellular localization in T cells. Indeed, was observed a clear SOD-1 recruitment by TCR clusters. The ROS scavenger N-acetylcysteine (NAC) inhibited this phenomenon. Further studies are needed to define whether SOD-1-dependent superoxide/peroxide balance is relevant for regulation of T cell activation, as well as in the functional cross talk between immune effectors.

1. Introduction

T cell activation is a complex phenomenon in which intracellular signals, mediated by the engagement of TCR, are integrated by a variety of ligand/receptor interactions whose outcome is to finely tune antigen-dependent T cell response [1]. T lymphocytes play a pivotal role in the orchestration of the immune response and TCR-mediated signaling is a critical event to properly channeling the immune response and to obtain pathogen control and self-tolerance [2].

Several studies have been suggesting that TCR-dependent T cell activation induces ROS production [3–5]. Different enzymatic sources, such as the mitochondrial respiratory chain [6], lipooxygenases, NADPH oxidases NOX2 and DUOX1 [7,8], have been described to contribute to ROS generation upon TCR triggering. In the light of these observations, the involvement of multiple anti-oxidant enzymes in fine tuning of antigen-dependent T cell response can be hypothesized.

TCR stimulation generates both H_2O_2 and superoxide anion [8,10] and antioxidant enzymes specific for H_2O_2 enhance and/or prolong TCR-dependent ERK activation, while those specific for superoxide anion have no effect [11].

ROS include oxygen superoxide, hydrogen peroxides, hydroxyl radicals and peroxides. They represent a normal product of cellular metabolism and play relevant roles in innate defense against pathogens [12]. Several receptor/ligand interactions, as represented by TGF-beta [13], insulin [14], angiotensin II [15] and EGF [16] have been correlated to the presence of ROS. In this context, ROS appear to act as key second messenger regulating several crucial cellular responses, as protein kinase activation, gene expression and cell proliferation/apoptosis [17].

* Correspondence to: P. Mondola, Dipartimento di Medicina Clinica e Chirurgia, Università di Napoli "Federico II", Via Pansini, 5, 80131 Napoli, Italy. Tel.: +39 081 7463225; fax: +39 081 7463494.
** Correspondence to: G. Ruggiero, Dipartimento di Scienze Mediche Traslazionali, Università di Napoli "Federico II", Via Pansini, 5, 80131 Napoli, Italy. Tel.: +39 081 7463311; fax: +39 081 7463252.
E-mail addresses: mondola@unina.it (P. Mondola), giruggie@unina.it (G. Ruggiero).
[1] GT and VR equally contributed to this work.

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.10.020

H_2O_2 is more stable than other short-lived ROS molecules (1 minute half-life). It is electrically neutral and it can diffuse inside the cell and freely through cell membranes. In addition, H_2O_2 can be rapidly generated and easily scavenged by numerous mechanisms, thus sharing several features with well-known second messengers [18–20].

SOD molecules mediate scavenging of ROS, to H_2O_2 and molecular oxygen. They belong to a large family of isoenzymes that mediate cellular response to oxidative stress and represent the main enzymatic source of peroxides [21]. All mammalian cells express both the intra-mitochondrial Mn-SOD and the cytosolic dimeric CuZn-SOD (SOD-1), while the tetrameric extracellular CuZn-SOD isoenzyme seems to be selectively expressed by specific cell populations [22,23]. Hyperoxia and copper availability accelerate both the synthesis and activity of Cu,Zn SOD [24]. Significant control of ROS signaling depends on its spatially restricted production at intracellular sites, where redox-regulated signal occurs [25]. In this context, SOD-1 recruitment has been described in redox-dependent TNF-alpha and IL-1 receptor-induced endosomes [26,27]. In addition, SOD-1 associates with Rac-1-regulated NADPH oxidase complexes in different mouse tissues and cell lines [28].

SOD-1 may be released *in vitro* by fibroblasts, hepatocytes [29], human neuroblastoma cells [30] and Sertoli cells [31]. The extracellular release of such enzyme is related to specific stress conditions [32]. ER/Golgi involvement in SOD-1 secretion has been described [33–36], while it is unclear how this cytosolic protein can be targeted into the ER/Golgi network.

SOD-1 is constitutively secreted by microvesicles in some cell lines through an ATP dependent mechanism [37]. The intracellular increase of the enzyme can be observed in neuroblastoma SK-N-BE cells when they are exposed to oxidative stress [37]. Recently, it has been shown that SOD-1 secretion is induced by high level of extracellular K^+ in GH3 rat pituitary cells [38] and that the enzyme interacts with membrane of neuroblastoma SK-N-BE cells activating a phospholipase/protein kinase C pathway, able to increase intracellular calcium [39,40].

Receptor–ligand interactions, involving members of hematopoietin receptor super family and EGF, have been described to mediate extracellular H_2O_2 generation [41,42]. Moreover, exogenously added H_2O_2 is able to induce signals in the absence of ligands, whereas catalase is able to inhibit such effect [43,44]. A role for SOD-1 in modulating ROS-dependent intra-cellular and inter-cellular signaling might be hypothesized.

Communication between immune cells involves the secretion of several proteins, like the cytokines, and the presence of their receptors on neighboring cells. This type of intercellular "dialog" may involve the release of membrane vesicles, like exosomes. These vesicles can affect cell physiology inducing intracellular signaling and conferring them new biological properties [45,46]. Peripheral blood human T cells, T cell clones and Jurkat T cells are able to release microvesicles in the culture medium. The microvesicle production is finely regulated and, notably, it increases upon TCR triggering [47].

In previous papers, we showed that cytosolic SOD-1 is secreted by several cell types [29,30,37] and it is also released in primary lymphoid organs, as represented by human thymus [48]. These observations suggest a paracrine role for SOD-1.

Multiple cytokines have been observed to regulate the expression of the tetrameric form of extra-cellular SOD-1 [49], while no data are available on the role of dimeric, cytosolic SOD-1 in functional adaptive immune effectors. Therefore, the role for SOD-1 in ROS-dependent signaling as well as in the communication between immune effectors needs to be addressed.

This study is aimed to investigate whether cytosolic SOD-1 might be part of the molecular network involved in TCR triggering. With this purpose SOD-1 intracellular level and localization, as well as SOD-1 microvesicle secretion have been investigated in TCR-triggered human T lymphocytes.

2. Material and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 healthy donors, after informed consent, by centrifugation of peripheral blood on Ficoll-Paque cushion (GE Healthcare, Uppsala, Sweden) gradient. T cells have been isolated from PBMC by using a negative isolation kit (Invitrogen Corporation, Carlsbad, CA, USA) and following the manufacturer's instructions. PBMC or T cells (1×10^6/ml) were cultured in 96 well flat-bottomed plates (Falcon) in RPMI 1640 medium with 2% FCS (Invitrogen, Carlsbad, CA, USA). TCR triggering was performed by anti-CD3 mAb (Becton Dickinson, Mountain View, CA, USA) at 5 ng/ml or by using anti-CD3/anti-CD28 beads (Invitrogen), at 0.3 bead/cell. This activation strategy has been largely demonstrated to mimic antigen-dependent T cell triggering. To analyze TCR-dependent SOD-1T cell export, distinct experiments were performed in the presence of Brefeldin-A, (BFA) at 5 μg/ml or of 1 mM methylamine, all purchased from Sigma-Aldrich (Milan, Italy), as described [37]. Cell viability was evaluated by using Propidium Iodide (PI) (Sigma-Aldrich) labeling and flow cytometry detection [37] as well as by analyzing lactate dehydrogenase (LDH) activity in culture supernatants by using the Roche Molecular Biochemical LDH kit (Mannheim, Germany). Written informed consent (model n. 5526 of Azienda Ospedaliera Universitaria "FEDERICO II") was obtained from each donor at the time of venous peripheral blood donation. All the experiments done by using blood donations were performed and analyzed anonymously, without any biographical reference to donors.

2.2. ELISA

The quantitative detection of human SOD-1 in medium of cultured PBMC was carried out using the Bender Med System kit (Bender Med System Diagnostic, Vienna, Austria), as described [37]. Results were always normalized for total protein content of the tested sample. SOD-1 ELISA detection has been always performed on culture supernatants immediately frozen at −80 °C. Protein concentrations were determined according to the method of Lowry et al. [50] using BSA, as standard.

2.3. RNA preparation, semi-quantitative RT-PCR and DNA sequencing

Analysis of SOD-1 specific RNA has been performed, as described [51]. Briefly, total RNA was extracted with High Pure RNA isolation kit (Roche Italia, Milano, Italia), according to the manufacturer's instructions. Traces of contaminated DNA were removed with DNAse I treatment. Quantification was achieved in a single reaction by using the housekeeping β-actin gene as internal standard. To rule out genomic DNA contamination we performed a negative control that contained RNA instead of cDNA. The signal intensities of PCR products were separated on a 1.2% agarose gel and were visualized by ethidium bromide staining. The products' signal intensities were determined by computerized densitometric analysis using Fotoplot software. The expression of SOD-1 was normalized to β-actin mRNA levels. To check the specificity of the amplified products, DNA bands were eluted from the gel and purified; sequence analysis was determined by the Big Dye Terminator Cycle Sequencing method (ABI-PRISM Sequencer 310 Perkin-Elmer).

2.4. Microvesicle isolation and western blotting for SOD-1 detection

To purify the membrane microvesicle-containing fraction, supernatants were collected immediately after culture and treated, as described [52]. Briefly, they were sequentially centrifuged at 500 *g* for 15 min to remove cellular debris and again at 10,000 *g* for 20 min. The obtained supernatant was collected and further centrifuged at 100,000 *g* for

2 h. The resulting pellet was then collected and considered to represent the enriched membrane vesicle fraction. Western blotting analysis of the purified material was performed as previously described [37]. Comparative analysis of SOD-1 was performed by using 40 μg of total proteins.

2.5. Immunofluorescence and flow cytometry analysis

Intracellular SOD-1 content was evaluated anti-SOD-1 mAb and FITC labeled anti-mouse IgG secondary antiserum (Sigma-Aldrich) staining of permeabilized cells and immunofluorescence technique. A commercial fixing/permeabilization kit, purchased from Becton Dickinson was always employed, following the manufacturer's instructions. For the analysis of SOD-1 content in distinct cell subsets and to evaluate T cell activation after TCR triggering, co-staining with FITC, PerCP or APC labeled anti-CD3, anti-CD45 and anti-CD69 mAb was performed. Labeled antibodies and isotype-matched controls were purchased from Becton Dickinson. T cell staining and activation were performed by anti-CD3 mAb recognizing different CD3 epitopes. Cell death was always less than 5% as evaluated by using PI (Sigma-Aldrich) staining. Immunofluorescence, flow cytometry and data analysis were performed by using a two laser equipped Becton–Dickinson FACSCalibur flow cytometer and the Cell Quest analysis software.

2.6. Fluorescence microscopy

PBMC or purified T cells (0.5×10^6) were adhered to polylysine-coated glass slides for 16–18 h at 37 °C. When indicated, the above populations were stimulated with anti-CD3 mAb CLB-CD3/4E at 1:100 ascites dilution or anti-CD3/anti-CD28 beads (at 0.3 bead/cell) and 1 mM NAC [53]. Cells were incubated at 37 °C and immediately fixed with 3% paraformaldehyde solution. Fixed cells were incubated with FITC labeled anti-CD3 and anti-human Cu,Zn SOD-1 rabbit antibody (Santa Cruz Biotechnology, CA, USA) for 45 min in a humidified chamber, washed three times with PBS and incubated with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Life Technologies) for additional 45 min at 37 °C in the same conditions. After 3 washes with PBS the glass slides were mounted using a 50% solution of glycerol in PBS and examined with a Zeiss LSM 510 confocal microscope with a 63× oil immersion objective (N.A. 1,4) at room temperature. Pictures were taken from selected fields of control and treated samples.

2.7. Cell to cell aggregate evaluation

To evaluate cell aggregation, PBMCs were cultured in 96 wells flat bottomed microtiter plates (Falcon) in the presence of Medium, anti-CD3 and 1 mM NAC (Sigma-Aldrich), as indicated. This NAC concentration was demonstrated in preliminary experiments to completely block ROS formation, as described [54]. Contrast phase microscopy analysis was performed with a Leitz DIAVERT microscope with a 10× objective at room temperature. Pictures were taken from selected fields by using a digital Nikon Coolpix Camera. NAC treatment was unable to significantly affect T cell viability and proliferation, as evaluated by PI staining after 1 to 5 h of culture and ^{3}H thymidine incorporation after 72 h of culture. Quantification has been performed by counting the number of cell aggregates (identified by the presence of at least 8 clustered cells) in the cell culture of 1×10^5 PBMC plated on the flat-bottomed microtiter wells.

2.8. Statistical analysis

Statistical evaluation of data, by InStat 3.0 software (GraphPad Software Inc., San Diego, California, USA), has been performed by means of the Mann–Whitney test or Paired *t* test, as indicated. Two-sided p values of less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. TCR triggering induces both intracellular increase and BFA-dependent secretion of SOD-1 in human T lymphocytes

To investigate whether antigen-dependent T cell activation induces SOD-1 production and extracellular secretion, we measured the release of this enzyme in supernatants of PBMC cultured from 15 min to 18 h in presence of Medium alone or with anti-CD3 mAb, that induces the TCR triggering mimicking antigen-dependent activation of T lymphocytes. As shown in Fig. 1A, human PBMC cultures secreted small amount of SOD-1 that remained substantially stable from 15 min till to 18 h of culture. Anti-CD3 treatment slightly, but significantly ($p < 0.05$), increased such basal secretion. The increment was detectable after 4 h of culture and reached the highest level after 18 h of activation. This effect was independent on cell damage, always evaluated by PI labeling (Fig. 1B) and significantly correlated with TCR-dependent activation, as revealed by the up-regulation of the activation molecule CD69 (Fig. 1C). Therefore, TCR-triggering was associated with the induction of SOD-1 secretion in human lymphocytes.

To ascertain whether such secretion was sustained by up-regulation of SOD-1 gene transcription, we analyzed SOD-1 specific mRNA. As shown in Fig. 2, an increase of more than 70% of SOD-1 specific mRNA has been observed in the cultures treated with anti-CD3 mAb as compared with resting cells. Therefore, antigen-dependent T cell activation induced SOD-1 secretion that was sustained by the increase of SOD-1 transcription in the whole PBMC population.

To evaluate whether SOD-1 export might be part of micro-vesicle production upon TCR triggering, we purified the micro-vesicle fraction from the supernatants of 18-hour cultures of PBMC and purified T cells. TCR triggering of PBMC was performed with anti-CD3 mAb. In order to mimic costimulatory signals, usually mediated by accessory cells, a combination of anti-CD3/anti CD28 beads was used to fully activate purified T cells, as described [1]. Fig. 3 shows western blotting analysis of SOD-1 in the enriched membrane vesicle fractions. Fig. 3A–B reports one representative experiment, while Fig. 3C refers the analysis of data obtained in all the six experiments performed in PBMC cultures. The comparison revealed the occurrence of a mean 25% increase of SOD-1 content in the enriched membrane vesicle fraction obtained from anti-CD3 treated lymphocytes ($p < 0.01$). As shown, in the enriched membrane vesicle fraction isolated from anti-CD3/anti-CD28 treated T cells, a more consistent increase of SOD-1 content was observed (Fig. 3D–F). Indeed, a mean increase >120% of SOD-1 content was evidenced in three experiments performed with purified T lymphocytes ($p < 0.05$).

To investigate whether other cell populations, present in PBMC, contribute to SOD-1 production and secretion in response to TCR-triggering, we analyzed intra-cellular SOD-1 levels in the T cell subset and in the "non-T" counterpart in a mixed context. This evaluation was performed by the combination of immune fluorescence and flow cytometry detection, to preserve the biological complexity of antigen-dependent T cell response and allow specific detection of SOD-1 in the T cell subset and in "non-T" population (Fig. 4A). As shown (Fig. 4B), very low amount of intracellular SOD-1 was observed in all the "resting" lymphocytes (T and non-T cells).

After TCR-triggering, the up-regulation of SOD-1 intracellular level was observed only in T lymphocytes if compared to non-T cells (Region 1 *versus* Region 2 in Fig. 4C).

To investigate the pathway involved in SOD-1 secretion, we analyzed intracellular SOD-1 retention in T cells in the presence of BFA (Fig. 4D) or methylamine (Fig. 4E) described to block ER/Golgi intracellular network and cell endocytosis, respectively [51,52]. In this regard, BFA but not methylamine treatment induced significant increase of

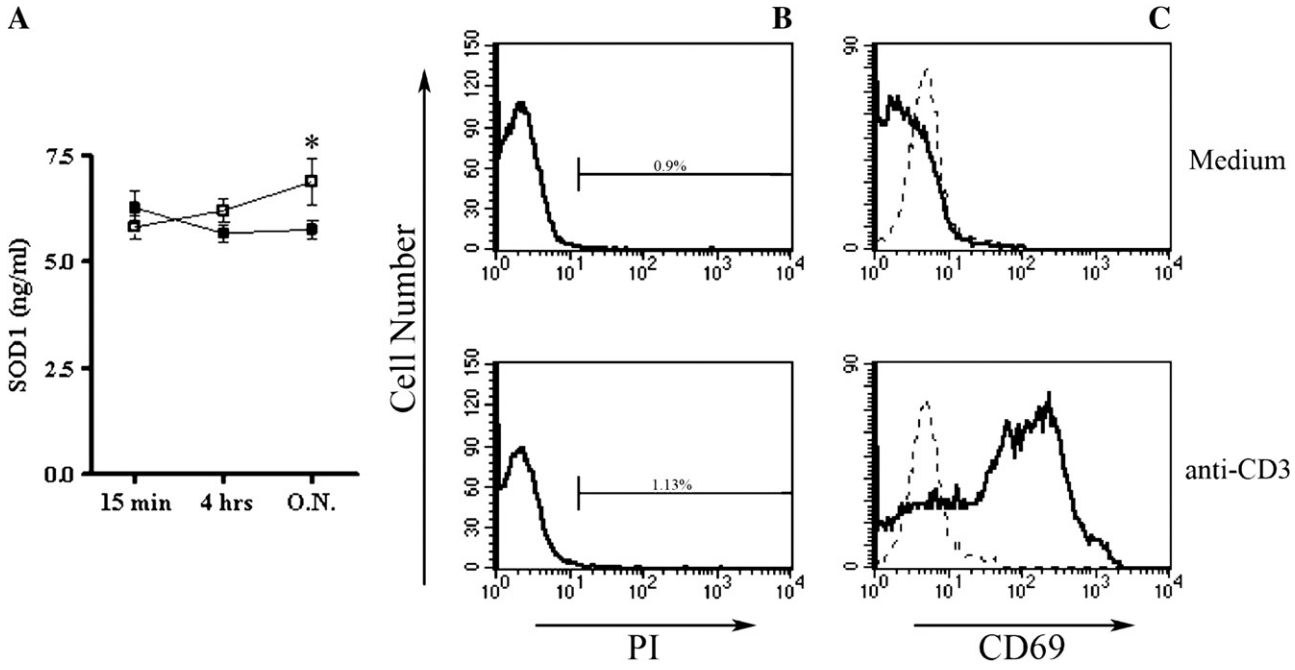

Fig. 1. SOD-1 concentration in anti-CD3 triggered cultures of human lymphocytes. (*A*) SOD-1 amount in supernatants of PBMC cultured in the presence of Medium (black squares) or anti-CD3 (white squares). SOD-1 concentrations were analyzed in undiluted samples by using ELISA assay; results were normalized for total protein content of the tested sample. Each point refers mean value obtained in five independent experiments; error bars indicate SEM. * indicates the occurrence of statistically significant ($p < 0.05$) higher SOD-1 concentration in anti-CD3 treated cultures. (B and C) PI and CD69 labeling of PBMC cultured O.N. with Medium or anti-CD3, as indicated. Results refer to one of five independent experiments. As shown (B), no significant differences have been observed in PI staining profiles of PBMC cultured with Medium and anti-CD3; (C) CD69 staining profile (bold line) of PBMC cultured with Medium or anti-CD3, as indicated; broken line indicates isotype control; as shown, activated PBMCs were characterized by significant increase of the activation molecule CD69.

the enzyme content in T cells. No significant changes in intracellular levels of SOD-1 were observed in "non-T" cell population, in the same experimental conditions (Fig. 4F). Notably, none extracellular SOD-1 increase was detected by ELISA in anti-CD3 cultures incubated with BFA (data not shown).

Fig. 4G–I reports the statistical comparisons of SOD-1 intracellular amount in PBMC (Fig. 4G), in T cells (Fig. 4H) and "non-T" cells (Fig. 4I), as evaluated by considering the mean fluorescence intensity (MFI) values obtained in all the 4 experiments performed. As shown, no significant changes in SOD-1 levels were observed in the absence of TCR triggering. Thus, SOD-1 amount was strictly dependent on antigen-mediated T cell activation. Indeed, anti-CD3 treatment significantly increased SOD-1 intracellular level ($p < 0.005$) in the whole lymphocyte population (Fig. 4G). This effect specifically characterized the T cell subset ($p < 0.005$; Fig. 4H), while no differences were observed in the "non-T" population (Fig. 4I). Moreover, the block of ER/Golgi network, mediated by BFA treatment, was observed to mediate significant ($p < 0.05$) intra-cellular SOD-1 retention only in T lymphocytes (Fig. 4H). Similar results have been obtained by anti-CD3/anti-CD28 triggering of purified T cells (data not shown). As control, intracellular accumulation of Interferon-gamma was specifically detected in TCR triggered cultures treated with BFA (not shown).

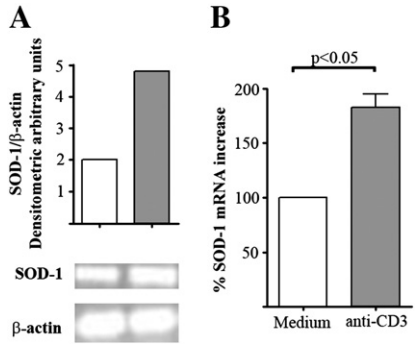

Fig. 2. Anti-CD3 treatment induces significant increase of SOD-1 mRNA in human lymphocytes. (A) Densitometric arbitrary units ratio between SOD-1 and beta actin in Medium and anti-CD3 treated cultures. Results refer one of five independent experiments. mRNA was measured by RT-PCR, as detailed in Material and methods section. (B) Comparative analysis of SOD-1 mRNA percent increase in all the five experiments performed. For each experiment mRNA amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, a mean increase of more than 70% of SOD-1 specific mRNA has been observed in anti-CD3 treated PBMC. Error bars indicate SEM. Statistical analysis has been performed by using Paired *t* test.

3.2. TCR and SOD-1 co-localize and cluster after TCR-triggering in human T cells

We analyzed SOD-1 and TCR cellular localization by confocal microscopy after 2 min of culture in the presence of Medium alone or with anti-CD3. Fig. 5 shows TCR and SOD-1 co-staining after 2 min of culture with Medium alone (Fig. 5A) or anti-CD3 (Fig. 5B and C). As expected, the homogeneous surface TCR distribution observed in resting T cells (Fig. 5A) was completely changed by anti-CD3 triggering (Fig. 5B and C). Indeed, significant TCR clustering (Fig. 5B and C) characterized activated T cells. SOD-1 staining revealed a quite homogeneous intracellular distribution of the enzyme in resting T cells; staining profiles also confirmed the presence of SOD-1 at very low levels in human T lymphocytes (see Fig. 4).

Notably, confocal microscopy revealed that TCR triggering was able to induce a clustered distribution of SOD-1 enzyme (Fig. 5B and C). Merged images clearly showed that TCR clusters have been recruiting intracellular SOD-1, whose localization strictly reflected TCR distribution (Fig. 5B and C). TCR/SOD-1 co-localization disappeared 20 min after anti-CD3 treatment (not shown). To preserve the physiological complexity, we chose to perform the analysis in PBMC population as a whole and we identified T cells by labeling with specific antibodies. In this model, TCR triggering is allowed by the physiological cross talk between T cells and autologous antigen

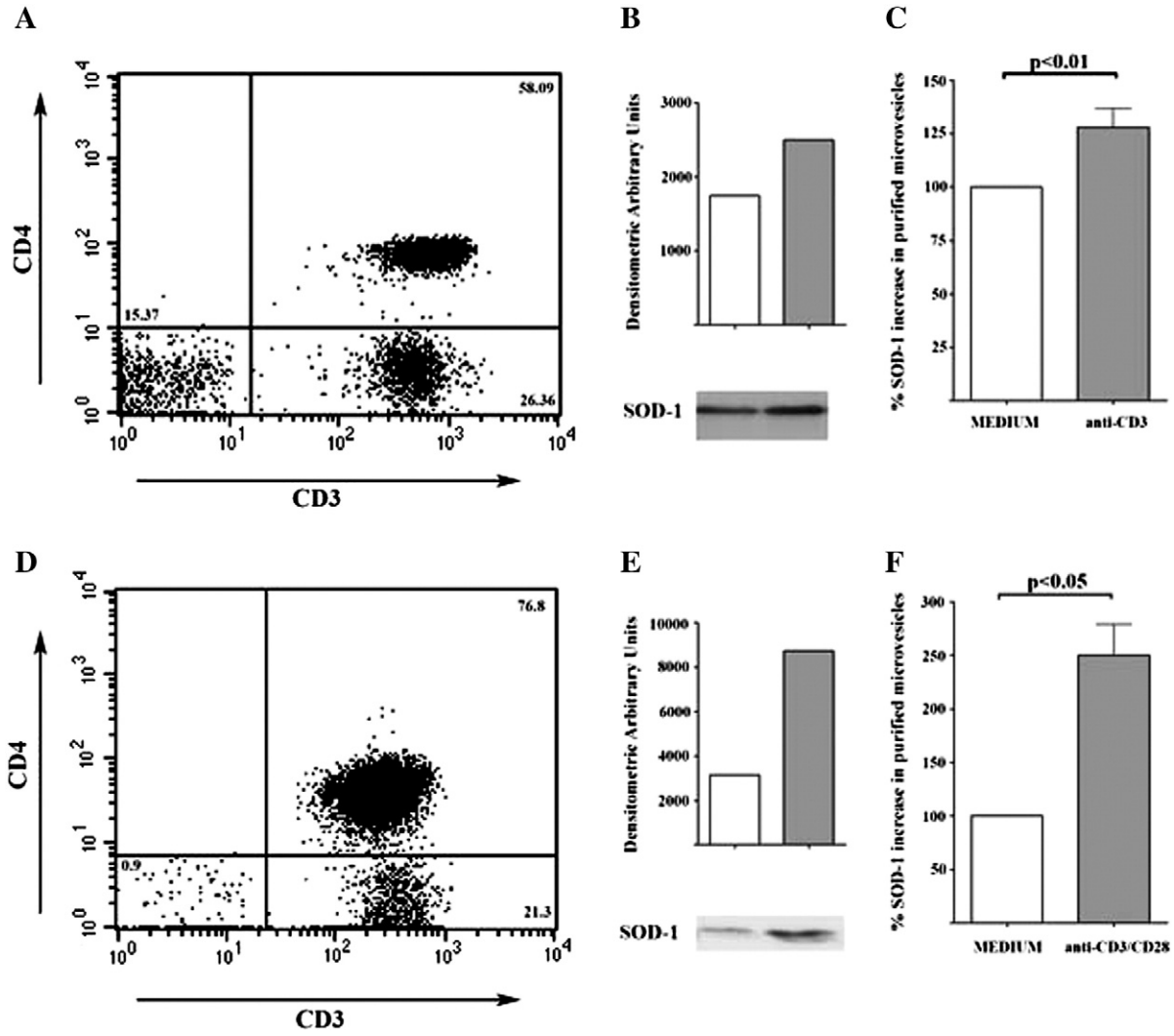

Fig. 3. TCR-dependent T cell activation increases SOD-1 containing microvesicle secretion by human T lymphocytes. (A) Flow cytometry analysis of PBMC population. As shown, T lymphocytes ($CD3^+$ cells) represent less than 85% of the total population; (B) western blot of enriched membrane vesicle fractions, isolated from culture supernatants of Medium (white column) and anti-CD3 treated PBMC (gray column), as detailed in Material and methods section. Densitometric analysis shows increased presence of SOD-1 in TCR triggered PBMC. Results refer one representative experiment of the six performed; (C) Analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3 treated PBMC in six experiments. For each experiment, SOD-1 amount in Medium cultured PBMC was considered the reference value (100) for calculation of percent increase in the anti-CD3 treated culture. As shown, a mean increase of 25% was observed in the microvesicle-enriched fraction obtained from the supernatants of anti-CD3 treated PBMC. (D) Flow cytometry analysis of a typical purified T cell population isolated by using negative selection strategy, as indicated in the Material and methods section. As shown, T lymphocytes ($CD3^+$ cells) represent more than 98% of the total population; (E) western blot of enriched membrane vesicle fractions, isolated from culture supernatants of Medium (white column) and anti-CD3/antiCD28 treated T cell cultures (gray column), as detailed in Material and methods section. Densitometric analysis shows increased presence of SOD-1 in the sample obtained from TCR triggered T cells. Results refer one representative experiment of the three performed; (F) analysis of percent increase of SOD-1 containing microvesicle in supernatants derived from anti-CD3/anti-CD28 treated T lymphocytes in three experiments. For each experiment, SOD-1 amount in Medium cultured T cells was considered the reference value (100) for calculation of percent increase in the anti-CD3/anti-CD28 treated cultures. As shown, an increase of more than 120% was observed in the microvesicle enriched fraction obtained from the supernatants of anti-CD3/anti-CD28 treated purified T cells. Error bars indicate SEM. Western blot comparative analysis of SOD-1 has been always performed on aliquots of enriched microvesicle fractions containing 40 μg of total proteins. Statistical analysis has been performed by using Paired *t* test.

presenting cells (APC). Notably, we never observed a TCR clustering decoupled from SOD-1 co-localization. No significant changes in SOD-1 intracellular localization were observed in "non-T" population after anti-CD3 triggering (not shown). Notably, SOD-1/TCR intracellular co-clustering was observed also in anti-CD3/anti-CD28 triggered purified T cells (not shown).

To investigate whether SOD-1/TCR co-localization in anti-CD3 activated T cells is dependent on ROS bioavailability, we performed experiments in the presence of the ROS scavenger NAC at 1 mM concentration. In this condition, TCR clustering was significantly reduced (from 70 to 95% in NAC/anti-CD3 co-cultures). As shown, NAC significantly inhibited both TCR and SOD-1 clustered localization (Fig. 5D). Indeed, TCR was homogeneously distributed on cell membrane, similarly to what was observed in resting condition (Fig. 5A). SOD-1 co-staining in anti-CD3/NAC treated lymphocytes also resembled basal images with the presence of small areas of faint cytosolic accumulation (Fig. 5D). Merged images revealed a clear-cut distinct distribution of TCR and SOD-1 in anti-CD3/NAC treated T cells. Thus, ROS availability significantly affected activation-dependent TCR/SOD-1 re-localization in human T cells.

To ascertain whether ROS availability might also affect the cell-to-cell aggregation dependent on TCR triggering, we analyzed the effect of NAC incubation on early cell clustering (usually detectable after 45 min of incubation with anti-CD3). As shown in Fig. 6, anti-CD3-sitimulation was able to induce cell aggregation after 1 h of treatment (Fig. 6C). Such effect became more evident after 3 h of incubation (Fig. 6G). Anti-CD3/NAC co-treatment severely impaired anti-CD3 induced cell clustering after 1 h of incubation (Fig. 6D).

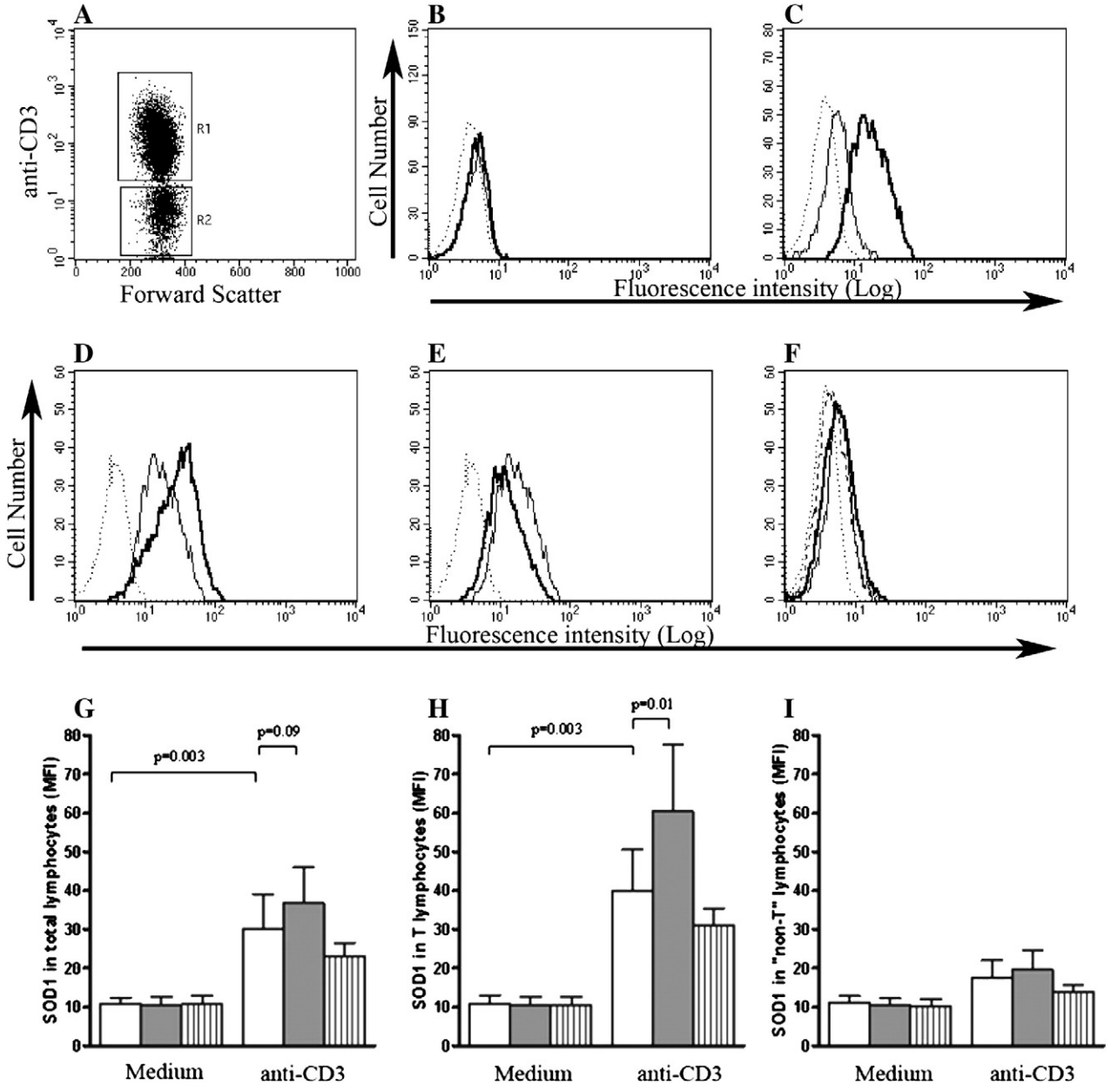

Fig. 4. Significant increase of intracellular SOD-1 and BFA-dependent SOD-1 export can be specifically demonstrated in anti-CD3 triggered human T cells. (A) Gating criteria for the identification of the "T cell subset" (R1) and of the "non-T population" (R2) in a PBMC culture; (B) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in O.N. Medium cultured PBMC. Dotted lines show the isotype control. As shown, similar, very low amount of intracellular SOD-1 characterizes both population. (C) SOD-1 staining profile of T cells (bold line) and "non-T population" (plain line) in anti-CD3 cultured PBMC. Dotted lines show the isotype control. As shown, specific increase of SOD-1 intracellular content can be observed in the T cell subset (bold line) as compared with the "non-T" counterpart (plain line); (D) SOD-1 staining in T cell population (R1) cultured with anti-CD3 alone (plain line) or in the presence of BFA (bold line). Dotted lines show the isotype control. As shown, anti-CD3/BFA co-culture increases intracellular SOD-1 content in T cell population. (E) SOD-1 staining in T cell population (R1) cultured with anti-CD3 alone (plain line) or in the presence of methylamine (bold line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular levels can be observed in anti-CD3/methylamine co-cultures. (F) SOD-1 staining profiles of "non-T population" (R2) in anti-CD3 cultures (plain line), in BFA/anti-CD3 co-cultures (bold line) or in methylamine/anti-CD3 cultures (broken line). Dotted lines show the isotype control. As shown, no significant changes in SOD-1 intracellular content have been observed. Results refer one of 4 independent experiments. (G–I) Column graphic reports the means of SOD-1 fluorescence intensities observed in 4 independent experiments in the whole PBMC population (G), in the T cells (H) and in the "non-T" population (I). White columns indicate SOD-1 levels in cells, cultured as indicated; gray columns indicate SOD-1 in BFA co-treated cultures; striped columns refer SOD-1 in methylamine co-treated cultures.

Quantification has been performed by direct counting of cell aggregates, identified by the presence of at least 8 clustered cells, in the microtiter wells. Comparative analysis showed a cell aggregate inhibition of 83.64 ± 1.62 in anti-CD3/NAC co-treated cultures in 6 independent experiments; ($p < 0.05$). This inhibition was transient and progressively decreased, likely mirroring the ROS scavenging activity of NAC. Indeed, after 3 h of NAC/anti-CD3 incubation a percentage of clustering inhibition of 55.35 ± 2.23 was observed in 6 independent experiments (Supplementary Table S1). The inhibiting effect of NAC co-incubation completely disappeared after 6 h of anti-CD3/NAC co-treatment. NAC co-incubation was unable to mediate significant effects on cell viability and proliferation (not shown).

4. Discussion

This study revealed that SOD-1 is part of the network of molecules involved in antigen-dependent T cell response. SOD-1 was recruited by antigen triggered TCR and its intracellular content was specifically upregulated in human T cells after 16–18 h of anti-CD3 incubation. Moreover, SOD-1 was secreted by a BFA-dependent

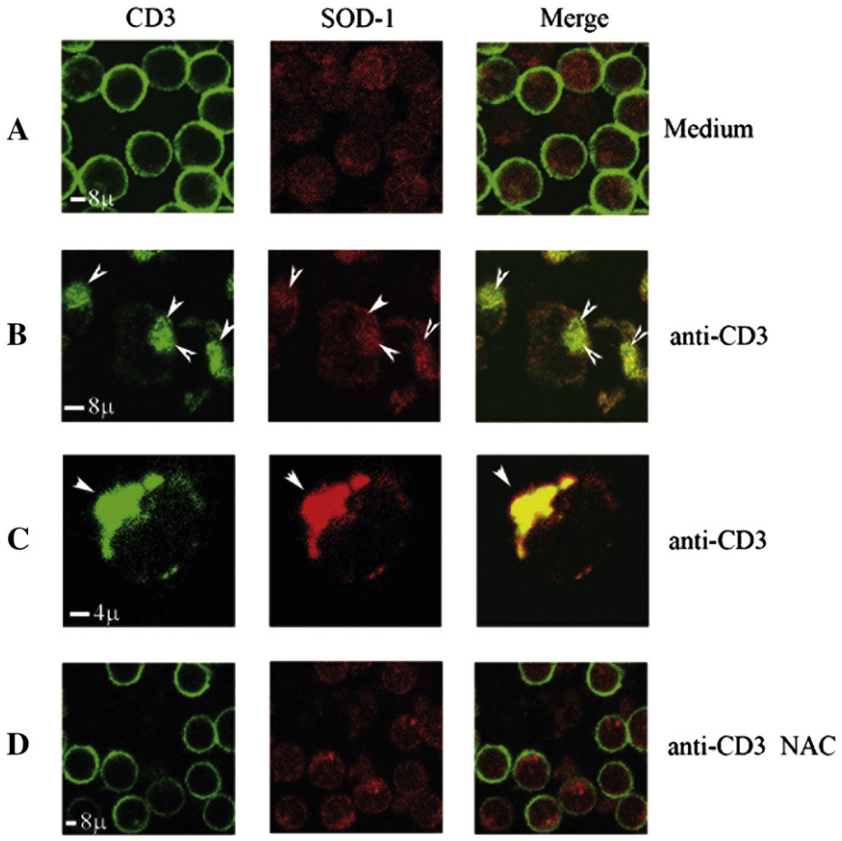

Fig. 5. Anti-CD3 triggering induces ROS-dependent TCR and SOD-1 co-clustering in activated lymphocytes. (A) Confocal microscopy image of CD3 (green) and SOD-1 (red) in resting T cells. A homogeneous, distinct, membrane and intracellular distribution of the TCR and SOD-1 can be appreciated. (B and C) After 2 min of anti-CD3 treatment a clustered distribution of TCR can be observed (white arrows). SOD-1 distribution becomes strongly clustered and resembles that of TCR (white arrows). This cell re-localization is better showed in panel C where a single cell has been focused. Merged images show that TCR clusters recruit intracellular SOD-1, whose localization strictly reflects TCR distribution (white arrows). (D) anti-CD3/NAC co-incubation induces a homogeneous TCR surface distribution. SOD-1 localization also resembles basal images, with the presence of small areas of faint cytosolic accumulation. Merged images reveal a clear-cut distinct distribution of TCR and SOD-1.

microvesicle pathway by TCR triggered T cells. These effects have been observed maintaining the biological complexity of antigen-dependent T cell response and confirmed in purified T cells activated by anti-CD3/anti-CD28 beads.

We showed that extra-cellular SOD-1 is increased in PBMC cultures after anti-CD3 treatment. This effect was accompanied by both the induction of SOD-1 mRNA and increase of SOD-1 containing micro-vesicles in culture supernatants. Moreover, we identified the T cell population as the specific target for SOD-1 induction and extra-cellular export. Therefore, TCR-dependent activation behaves as a triggering element for SOD-1 production and secretion by human T cells.

SOD-1 production is induced in neuroblastoma SK-N-BE cells after oxidative stress [30,37]. Moreover, other and our data showed that cytosolic SOD-1 is secreted by many cell lines carrying out a paracrine modulatory role [30,31,37] and SOD-1 extracellular export was by us described in primary lymphoid organs [48].

Induction of extra-cellular export of SOD-1 after TCR-triggering proposes a more complex physiological involvement of such enzyme in T cell activation. In this study, we described that a BFA-dependent secretion mechanism [54] characterized SOD-1 micro-vesicle inter-cellular trafficking upon antigen-dependent immune response. No effect has been observed in the presence of methylamine that impairs cell endocytosis [55]. Thus, a major involvement of endocytic recycling pathways might be excluded. Such mechanism, previously described in neuronal model [30–34,37], represents an intriguing issue for further investigations.

SOD-1 is a cytosolic protein lacking signal peptide and consequently considered to be excluded from ER translocation. The small amount of wild type SOD-1 detected in ER–Golgi apparatus [36], does not support the direct involvement of this organelles in SOD-1 secretion. Moreover, the possible interference of BFA, a classical inhibitor of ER–Golgi dependent protein secretion, in vesicular pathways not directly involving ER–Golgi apparatus cannot be excluded. In this context, our data propose that SOD-1 could be part of the micro-vesicle-dependent pathways functioning as secondary messenger between immune cells [45–47].

A number of data indicate that exogenously added H_2O_2 induced signals in the absence of ligands, whereas catalase is able to inhibit such effect [42,43,56]. Moreover, the observations that production of catalase characterizes many pathogens [57] and that viral infection modulates H_2O_2 production [58] confirm the multiple roles played by extracellular H_2O_2 in the activation processes of lymphocytes [59].

We consistently found that antigen-dependent T cell triggering mediated changes in the intra-cellular localization of SOD-1 that was observed to co-localize with clustered TCR. This event was dependent on ROS availability since it was impaired by NAC co-treatment. ROS production is an essential component in signaling cascades that mediate actin cytoskeleton rearrangements. Small G protein Rac, a key element in the network assembly of actin in lamellipodia [60–62] participates in activation-dependent ROS production by different cell types [63,64]. Moreover, Rac-mediated ROS production results in the downmodulation of Rho activity thus regulating cellular morphology and migratory behavior [65]. SOD-1 associates with Rac-1 regulated NADPH oxidase complexes in different mouse tissues and cell lines [28]. In this context, massive ROS scavenging is expected to disrupt ROS-dependent regulation of cell contractility and motility. This event could account for the TCR/SOD-1 intracellular redistribution (Fig. 5). The impairment of early cell aggregation in

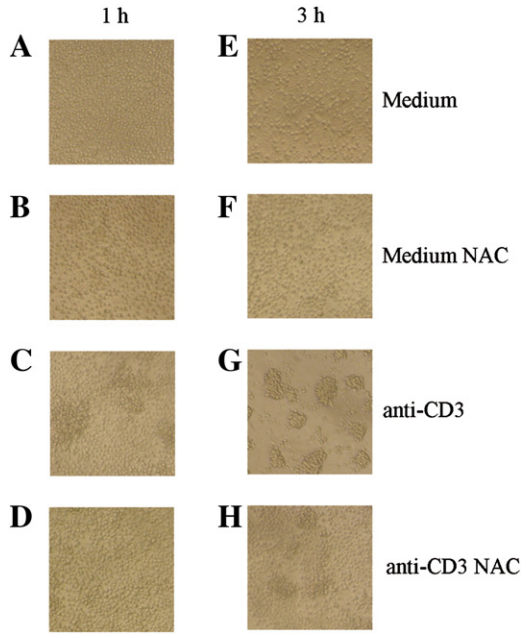

Fig. 6. NAC treatment inhibits early activation-induced aggregation of TCR triggered lymphocytes. Contrast phase microscopy images showing 1 and 3 hour cultures of PBMC incubated with Medium (A and E), NAC (B and F), anti-CD3 (C and G) or anti-CD3 and NAC (D and H). As shown, significant cell aggregation is observed in anti-CD3 triggered cells. Lymphocyte clustering is significantly inhibited in the anti-CD3/NAC co-treated cultures; (D) after 1 h of incubation. Quantification is performed by counting cell aggregates, identified by the presence of at least 8 clustered cells. Comparative analysis (see Supplementary Table S1) shows a 83.64 ± 1.62 inhibition of cell aggregates in anti-CD3/NAC co-treated cultures in 6 independent experiments; ($p < 0.05$). (H) After a 3 hour period the inhibiting effect of NAC treatment is observed to be lowered (55.35 ± 2.23 inhibition of cell aggregates in 6 independent experiments). Quite normal clustering of anti-CD3 treated PBMC is observed at longer culture time in anti-CD3/NAC co-treated cells (not shown). Results show one representative experiment of the six performed.

presence of anti-CD3/NAC co-treatment (Fig. 6) strongly supports such hypothesis.

Compelling evidences indicate that ROS, together with their essential role in innate antimicrobial defense [12], are critically involved in the regulation of antigen-dependent response of adaptive immune effectors [10,11,66–69]. Exposure of T cells to oxidant agents, such as pervanadate or H_2O_2, induces and/or enhances TCR signaling during T cell activation [70–72]. TCR-dependent signaling generates both superoxide anion and H_2O_2 that selectively regulate antigen-dependent proliferation and Fas ligand expression by T effectors [10]. An oxidative signal implies its tight regulation and transient character. Thus, in the presence of multiple intracellular ROS sources [6–9], the involvement of multiple anti-oxidant mechanisms in fine tuning of antigen-dependent T cell response can be hypothesized. Indeed, Mn-SOD/SOD-2 a major mitochondrial antioxidative enzyme has been consistently associated with T cell activation [73] and a role of catalase, glutathione and thioredoxin has been also proposed [74].

In the models of ROS generation upon stimulation of receptors it has been shown that H_2O_2 is the relevant oxidant species that regulate signaling [18–20]. Notably, H_2O_2 has a short half-life in the reducing environment of the cytosol, and it acts close to its site of production. Thus, an important aspect of ligand-dependent TCR activation might be the rapid translocation of receptors to a source of H_2O_2 or, *vice versa*, the clustering of such a source to the receptor. To this regard, our data strongly support the hypothesis that SOD-1 intracellular localization in antigen-triggered T cells could provide H_2O_2 generation in the cell compartment specifically involved in tuning antigen-dependent signals.

A number of data suggested the role for H_2O_2 as key modulator of protein phosphorylation on either serine–threonine and tyrosine residues [75]. Indeed, all protein tyrosine phosphatases (PTPs) contain an essential cysteine residue in the signature active enzyme site motif that has been demonstrated to be target of specific H_2O_2 oxidation. The H_2O_2-mediated inhibition of PTP activity is expected to result in a shift of protein tyrosine kinases toward protein phosphorylation. The involvement of SOD-1 in such regulatory pathways has been suggested [76].

Our data on SOD-1 intracellular re-localization upon TCR-triggering suggests that SOD-1 could directly modulate kinase/phosphatase activity related to proximal TCR signaling. The evidence [10] that anti-CD3 induced ERK phosphorylation requires H_2O_2 but is independent on superoxide anion, strongly supports such hypothesis. Thus, subcellular compartmentalization of H_2O_2 generating enzymes (like SOD-1) could represent a relevant element in achieving the superoxide/peroxide balance required to optimize antigen-dependent T cell response.

Taken in all, our data suggest that SOD-1 is part of the molecular network involved in antigen-dependent T cell response. At the best of our knowledge, this is the first observation revealing a relationship between SOD-1 secretion/intracellular re-localization and the antigen dependent T cell activation. Further studies are needed to investigate on the involvement of SOD-1 in the regulation of TCR signaling cascades as well as in the functional cross talk between immune effectors.

5. Conclusion

This study reports for the first time that SOD-1, a major physiological regulator of cytosolic superoxide/peroxide balance, is part of the molecular network involved in antigen-dependent T cell activation. Indeed, we observed: i. mRNA induction and increased levels of extra-cellular SOD-1 containing micro-vesicles in anti-CD3 triggered cultures; ii. increase of intra-cellular SOD-1 and BFA-dependent SOD-1 microvesicle secretion in TCR-triggered T cells; iii. TCR/SOD-1 co-localization was observed in anti-CD3 treated T cells.

Further studies are needed to establish whether SOD-1 is involved in modulating ROS-dependent intra-cellular and inter-cellular signaling in antigen triggered human T cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.10.020.

Acknowledgements

This work was supported by Italian MIUR, PRIN grant.
Dedicated to the memory of Prof. Serafino Zappacosta.

References

[1] J.E. Smith-Garvin, G.A. Koretzky, M.S. Jordan, T cell activation, Annu. Rev. Immunol. 27 (2009) 591–619.
[2] I.D. Fraser, R.N. Germain, Navigating the network: signaling cross-talk in hematopoietic cells, Nat. Immunol. 10 (2009) 327–331.
[3] C. Sekkat, J. Dornand, M. Gerber, Oxidative phenomena are implicated in human T-cell stimulation, Immunology 63 (1988) 431–437.
[4] M.S. Williams, P.A. Henkart, The role of reactive oxygen intermediates in TCR-induced death of T cell blasts and hybridomas, J. Immunol. 157 (1996) 2395–2402.
[5] S. Tatla, V. Woodhead, J.C. Foreman, B.M. Chai, The role of reactive oxygen species in triggering proliferation and IL-2 secretion in T cells, Free Radic. Biol. Med. 26 (1999) 14–24.
[6] L.A. Sena, S. Li, A. Jairaman, M. Prakriya, T. Ezponda, D.A. Hildeman, C.-R. Wang, P.T. Schumacker, J.D. Licht, H. Perlman, P.J. Bryce, N.S. Chandel, Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling, Immunity 38 (2013) 225–236.
[7] M. Los, H. Schenk, K. Hexel, P.A. Baeuerle, W. Droge, K. Schulze-Osthoff, IL-2 gene expression and NF-kappa B activation through CD28 requires reactive oxygen production by 5-lipoxygenase, EMBO J. 14 (1995) 3731–3740.
[8] S.H. Jackson, S. Devadas, J. Kwon, L.A. Pinto, M.S. Williams, T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation, Nat. Immunol. 5 (2004) 818–827.
[9] J. Kwon, K.E. Shatynski, H. Chen, S. Morand, X. de Deken, F. Miot, T.L. Leto, M.S. Williams, The nonphagocytic NADPH oxidase Duox1 mediates a positive feedback loop during T cell receptor signaling, Sci. Signal. 3 (2010) ra59.

[10] S. Devadas, L. Zaritskaya, S.G. Rhee, L. Oberley, M.S. Williams, Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression, J. Exp. Med. 195 (2002) 59–70.
[11] J. Kwon, S. Devadas, M.S. Williams, T cell receptor-stimulated generation of hydrogen peroxide inhibits MEK–ERK activation and lck serine phosphorylation, Free Radic. Biol. Med. 35 (2003) 406–417.
[12] F.C. Fang, Antimicrobial reactive oxygen and nitrogen species: concepts and controversies, Nat. Rev. Microbiol. 2 (2004) 820–832.
[13] V.J. Thannickal, R.M. Day, S.G. Klinz, M.C. Bastien, J.M. Larios, B.L. Fanburg, Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGFh1, FASEB J. 14 (2000) 1741–1748.
[14] K. Mahadev, A. Zilbering, L. Zhu, B.J. Goldstein, Insulin stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade, J. Biol. Chem. 276 (2001) 21938–21942.
[15] M. Ushio-Fukai, R.W. Alexander, M. Akers, Griendling KK, P38 mitogen-activated protein kinase is a critical component of the redox-sensitive signaling pathways activated by angiotensin II. Role in vascular smooth muscle cell hypertrophy, J. Biol. Chem. 273 (1998) 15022–15029.
[16] Y.S. Bae, S.W. Kang, M.S. Seo, I.C. Baines, E. Tekle, P.B. Chock, S.G. Rhe, Epidermal growth factor (EGF)-induced generation of hydrogen peroxide: role in EGF receptor-mediated tyrosine phosphorylation, J. Biol. Chem. 272 (1997) 217–222.
[17] R.H. Burdon, Control of cell proliferation by reactive oxygen species, Biochem. Soc. Trans. 24 (1996) 1028–1032.
[18] S.G. Rhee, Y.S. Bae, S.-R. Lee, J. Kwon, Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation, Sci. STKE 10 (2000) 1–6.
[19] M. Reth, Hydrogen peroxide as second messenger in lymphocyte activation, Nat. Immunol. 3 (2002) 1129–1134.
[20] S.G. Rhee, H_2O_2, a necessary evil for cell signaling, Science 312 (2006) 1882–1883.
[21] A.F. Miller, Superoxide dismutases: ancient enzymes and new insights, FEBS Lett. 586 (2012) 585–595.
[22] S.L. Marklund, Human copper containing superoxide dismutase of high molecular weight, Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 7634–7638.
[23] L. Tibell, K. Hjalmarsson, T. Edlund, G. Skogman, A. Engstrom, S.L. Marklund, Expression of human extracellular superoxide dismutase in Chinese hamster ovary cells and characterisation of the product, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 634–638.
[24] E.D. Harris, Regulation of antioxidant enzymes, FASEB J. 6 (1992) 2675–2683.
[25] M. Ushio-Fukai, Localizing NADPH oxidase-derived ROS, Sci. STKE 349 (2006) re8.
[26] L. Qiang, N.Y. Spencer, F.D. Oakley, G.R. Buettner, J.F. Engelhargt, Endosomal Nox2 facilitates redox-dependent induction of NF-kB by TNF-alpha, Antioxid. Redox Signal. 11 (2009) 1249–1263.
[27] F.D. Oakley, R.L. Smith, J.F. Engelhargt, Lipid rafts and caveolin-1 coordinate Interleukin-1 beta (IL-1 beta)-dependent activation of NFkB by controlling endocytosis of Nox2 and IL-1 beta receptor 1 from the plasma membrane, J. Biol. Chem. 284 (2009) 33255–33264.
[28] M.M. Harraz, J.J. Marden, W. Zhou, Y. Zhang, A. Williams, V.S. Sharov, K. Nelson, M. Luo, H. Paulson, C. Schöneich, J.F. Engelhargt, SOD1 mutations disrupt redox-sensitive Rac regulation of NADPH oxidase in a familial ALS model, J. Clin. Invest. 118 (2008) 659–670.
[29] P. Mondola, T. Annella, M. Santillo, F. Santangelo, Evidence for secretion of cytosolic CuZn superoxide dismutase by HEPG2 cells and human fibroblast, Int. J. Biochem. Cell Biol. 28 (1996) 677–681.
[30] P. Mondola, T. Annella, R. Seru, F. Santangelo, S. Iossa, A. Gioielli, M. Santillo, Secretion and increase of intracellular CuZn superoxide dismutase content in human neuroblastoma SK-N-BE cells, Brain Res. Bull. 45 (1998) 517–520.
[31] D. Mruk, C.H. Cheng, Y.H. Cheng, M.Y. Mo, J. Grima, B. Silvestrini, W.M. Lee, C.Y. Cheng, Rat testicular extracellular superoxide dismutase: its purification, cellular distribution, and regulation, Biol. Reprod. 59 (1998) 298–308.
[32] T. Ookawara, N. Imazeki, O. Matsubara, T. Kizaki, S. Oh-Ishi, C. Nakao, Y. Sato, H. Ohno, Tissue distribution of immunoreactive mouse extracellular superoxide dismutase, Am. J. Physiol. 275 (1998) C840–C847.
[33] B.J. Turner, J.D. Atkin, M.A. Farg, D.W. Zang, A. Rembach, E.C. Lopes, J.D. Patch, A.F. Hill, S.S. Cheema, Impaired extracellular secretion of mutant superoxide dismutase 1 associates with neurotoxicity in familial amyotrophic lateral sclerosis, J. Neurosci. 25 (2005) 108–117.
[34] H. Kikuchi, G. Almer, S. Yamashita, C. Guégan, M. Nagai, Z. Xu, A.A. Sosunov, G. McKhann II, S. Przedborski, Spinal cord endoplasmic reticulum stress associated with a microsomal accumulation of mutant superoxide dismutase-1 in an ALS model, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 6025–6030.
[35] M. Urushitani, A. Sik, T. Sakurai, N. Nukina, R. Takahashi, J.-P. Julien, Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis, Nat. Neurosci. 9 (2006) 108–118.
[36] M. Urushitani, S.A. Ezzi, A. Matsuo, I. Tooyama, J.-P. Julien, The endoplasmic reticulum–Golgi pathway is a target for translocation and aggregation of mutant superoxide dismutase linked to ALS, FASEB J. 22 (2008) 2476–2487.
[37] P. Mondola, G. Ruggiero, R. Seru', et al., The Cu,Zn superoxide dismutase in neuroblastoma SK-N-BE cells is exported by a microvesicles dependent pathway, Brain Res. Mol. Brain Res. 110 (2003) 45–51.
[38] M. Santillo, A. Secondo, R. Serù, S. Damiano, C. Garbi, E. Taverna, P. Rosa, S. Giovedì, F. Benfenati, P. Mondola, Evidence of calcium- and SNARE-dependent release of CuZn superoxide dismutase from rat pituitary GH3 cells and synaptosomes in response to depolarization, J. Neurochem. 102 (2007) 679–685.
[39] P. Mondola, M. Santillo, R. Serù, S. Damiano, C. Alvino, G. Ruggiero, P. Formisano, G. Terrazzano, A. Secondo, L. Annunziato, Cu, Zn superoxide dismutase increases intracellular calcium levels via a phospholipase C-protein kinase C pathway in SK-N-BE neuroblastoma cells, Biochem. Biophys. Res. Commun. 324 (2004) 887–892.
[40] A. Secondo, M. De Mizio, L. Zirpoli, M. Santillo, P. Mondola, The Cu–Zn superoxide dismutase (SOD1) inhibits ERK phosphorylation by muscarinic receptor modulation in rat pituitary GH3 cells, Biochem. Biophys. Res. Commun. 376 (2008) 143–147.
[41] G.U. Bae, D.-W. Seo, H.-K. Kwon, H.Y. Lee, S. Hong, Z.W. Lee, K.S. Ha, H.W. Lee, J.W. Han, Hydrogen peroxide activates p70(S6k) signaling pathway, J. Biol. Chem. 274 (1999) 32596–32602.
[42] Y.S. Bae, J.-Y. Sung, O.-S. Kim, Y.J. Kim, K.C. Hur, A. Kazlauskas, S.G. Rhee, Platelet-derived growth factor-induced H_2O_2 production requires the activation of phosphatidylinositol 3-kinase, J. Biol. Chem. 275 (2000) 10527–10531.
[43] H. Konishi, M. Tanaka, Y. Takemura, H. Matsuzaki, Y. Ono, U. Kikkawa, Y. Nishizuka, Activation of protein kinase C by tyrosine phosphorylation in response to H2O2, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 11233–11237.
[44] G.J. DeYulia, J.M. Carcamo, O. Borquez-Ojeda, C.C. Shelton, D.W. Golde, Hydrogen peroxide generated extracellularly by receptor–ligand interaction facilitates cell signalling, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 5044–5049.
[45] S. Mathivanan, H. Ji, R.J. Simpson, Exosomes: extracellular organelles important in intercellular communication, J. Proteomics 73 (2010) 1907–1920.
[46] C. Thery, M. Ostrowski, E. Segura, Membrane vesicles as conveyors of immune responses, Nat. Rev. Immunol. 9 (2009) 581–593.
[47] N. Blanchard, D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, C. Hivroz, TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ζ complex, J. Immunol. 168 (2002) 3235–3241.
[48] V. Cimini, G. Ruggiero, T. Buonomo, R. Serù, S. Sciorio, C. Zanzi, F. Santangelo, P. Mondola, CuZn-superoxide dismutase in human thymus: immunocytochemical localisation and secretion in thymus-derived epithelial and fibroblast cell lines, Histochem. Cell Biol. 118 (2002) 163–169.
[49] P. Strålin, S.L. Marklund, Multiple cytokines regulate the expression of extracellular superoxide dismutase in human vascular smooth muscle cells, Atherosclerosis 151 (2000) 433–441.
[50] O.H. Lowry, N.J. Rosenbrough, A.L. Farr, R.J. Randal, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[51] M. Russo, S. Cocco, A. Secondo, A. Adornetto, A. Bassi, A. Nunziata, G. Polichetti, B. De Felice, S. Damiano, R. Serù, P. Mondola, G. Di Renzo, Cigarette smoke condensate causes a decrease of the gene expression of Cu–Zn superoxide dismutase, Mn superoxide dismutase, glutathione peroxidase, catalase, and free radical-induced cell injury in SH-SY5Y human neuroblastoma cells, Neurotox. Res. 19 (2011) 49–54.
[52] C. Thery, S. Amigorena, G. Raposo, A. Clayton, Isolation and characterization of exosomes from cell culture supernatants and biological fluids, Curr. Protoc. Cell Biol. 3 (2006) 22–30.
[53] H. Karlsson, S. Nava, M. Remberger, Z. Hassan, M. Hassan, O. Ringde, N-acetyl-L-cysteine increases acute graft-versus-host disease and promotes T-cell-mediated immunity in vitro, Eur. J. Immunol. 41 (2011) 1143–1153.
[54] L. Orci, M. Tagaya, A. Amherdt, A. Perrelet, J.G. Donaldson, J. Lippincott-Schwartz, R.D. Klausner, J.E. Rothman, Brefeldin A, a drug that blocks secretion, prevents the assembly of nonclathrin-coated buds on Golgi cisternae, Cell 64 (1991) 1183–1195.
[55] F.R. Maxfield, M.C. Willingham, P.J. Davies, I. Pastan, Amines inhibit the clustering of a2-macroglobulin and EGF on the fibroblast cell surface, Nature 277 (1979) 661–663.
[56] M. Sundaresan, Z.-X. Yu, V.J. Ferrans, K. Irani, T. Finkel, Requirement for generation of H2O2 for platelet-derived growth factor signal transduction, Science 270 (1995) 296–299.
[57] G. Wang, P. Alamuri, R.J. Maier, The diverse antioxidant systems of *Helicobacter pylori*, Mol. Microbiol. 61 (2006) 847–860.
[58] E. Peterhans, Reactive oxygen species and nitric oxide in viral diseases, Biol. Trace Elem. Res. 56 (1997) 107–116.
[59] M.D. Kraaij, N.D.L. Savage, S.W. van der Kooij, K. Koekkoek, J. Wang, J.M. van den Berg, T.H. Ottenhoff, T.W. Kuijpers, R. Holmdahl, C. van Kooten, K.A. Gelderman, Induction of regulatory T cells by macrophages is dependent on production of reactive oxygen species, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 17686–17691.
[60] A.J. Ridley, A. Hall, The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors, Cell 70 (1992) 389–399.
[61] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.
[62] J. Heo, Redox control of GTPases: from molecular mechanisms to functional significance in health and disease, Antioxid. Redox Signal. 15 (2011) 689–724.
[63] J.D. Lambeth, NOX enzymes and the biology of reactive oxygen, Nat. Rev. Immunol. 4 (2004) 181–189.
[64] K. Bedard, K.H. Krause, The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology, Physiol. Rev. 87 (2007) 245–313.
[65] A.S. Nimnual, L.J. Taylor, D. Bar-Sagi, Redox-dependent downregulation of Rho by Rac, Nat. Cell Biol. 5 (2003) 236–241.
[66] R.L. Lee, J. Westendorf, M.R. Gold, Differential role of reactive oxygen species in the activation of mitogen-activated kinases and Akt by key receptors on B-lymphocytes: CD40, the B cell antigen receptor and CXCR4, J. Cell Commun. Signal 1 (2007) 33–43.
[67] D. Purushothaman, A. Sarin, Cytokine-dependent regulation of NADPH oxidase activity and the consequences for activated T cell homeostasis, J. Exp. Med. 206 (2009) 1515–1523.

[68] S.M. Richards, E.A. Clark, BCR-induced superoxide negatively regulates B-cell proliferation and T-cell-independent type 2 Ab responses, Eur. J. Immunol. 39 (2009) 3395–3403.
[69] P.J. Nadeau, A. Roy, C. Gervais-St-Amour, M.-E. Marcotte, N. Dussault, S. Neron, Modulation of CD40-activated B lymphocytes by N-acetylcysteine involves decreased phosphorylation of STYAT-3, Mol. Immunol. 49 (2012) 582–592.
[70] J.P. Secrist, L.A. Burns, L. Karnitz, G.A. Koretzky, R.T. Abraham, Stimulatory effects of the protein tyrosine phosphatase inhibitor, pervanadate, on T-cell activation events, J. Biol. Chem. 268 (1993) 5886–5893.
[71] C. Cenciarelli, K.G. Wilhelm Jr., A. Guo, A.M. Weissman, T cell antigen receptor ubiquitination is a consequence of receptor-mediated tyrosine kinase activation, J. Biol. Chem. 271 (1996) 8709–8713.
[72] S.P. Hehner, R. Breitkreutz, G. Shubinsky, H. Unsoeld, K. Schulze-Osthoff, M.L. Schmitz, W. Dröge, Enhancement of T cell receptor signaling by a mild oxidative shift in the intracellular thiol pool, J. Immunol. 165 (2000) 4319–4328.
[73] M.M. Kamiński, D. Röth, S. Sass, S.W. Sauer, P.H. Krammer, K. Gülow, Manganese superoxide dismutase: a regulator of T cell activation-induced oxidative signaling and cell death, Biochim. Biophys. Acta 1823 (2012) 1041–1052.
[74] P. Kesarwani, A.K. Murali, A.A. Al-Khami, S. Mehrotra, Redox regulation of T-cell function: from molecular mechanisms to significance in human health and disease, Antioxid. Redox Signal. 18 (2013) 1497–1534.
[75] J.S. Fetrow, N. Siew, J. Skolnick, Structure-based functional motif identifies a potential disulfide oxidoreductase active site in the serine/threonine protein phosphatase-1 subfamily, FASEB J. 13 (1999) 1866–1874.
[76] I.C. Juarez, M. Manuia, M.E. Burnett, O. Betancourt, B. Boivin, D.E. Shaw, N.K. Tonks, A.P. Mazar, F. Doñate, Superoxide dismutase 1 (SOD1) is essential for H_2O_2-mediated oxidation and inactivation of phosphatases in growth factor signalling, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 7147–7152.