Regulation of NFAT by poly(ADP-ribose) polymerase activity in T cells

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Abstract
The nuclear factor of activated T cells (NFAT) family of transcription factors is pivotal for T lymphocyte functionality. All relevant NFAT activation events upon T cells stimulation such as nuclear translocation, DNA binding, and transcriptional activity have been shown to be dictated by its phosphorylation state. Here, we provide evidence for a novel post-translational modification that regulates NFAT. Indeed, NFATc1 and NFATc2 are poly(ADP-ribosyl)ated by poly-ADP-ribose polymerase-1 (PARP-1). Moreover, we have also found a physical interaction between PARP-1 and both NFATc1 and NFATc2. Interestingly, PARP is activated during T cell stimulation in the absence of DNA damage, leading to ADP-ribose polymers formation and transfer to nuclear acceptor proteins. Our data suggest that poly(ADP-ribosyl)ation modulates the activation of NFAT in T cells, as PARP inhibition causes an increase in NFAT-dependent transactivation and a delay in NFAT nuclear export. Poly(ADP-ribosyl)ation will expedite NFAT export from the nucleus directly or by priming/facilitating NFAT phosphorylation. Altogether, these data point to PARP-1 and poly(ADP-ribosyl)ation as a novel regulatory mechanism of NFAT at nuclear level, suggesting a potential use of PARP as a new therapeutic target in the modulation of NFAT.

Keywords: NFAT; Nuclear retention; Poly-ADP ribose polymerases; Poly(ADP-ribosyl)ation

1. Introduction
The NFAT family comprises four calcium-regulated members [NFAT1 (also known as NFATc2), NFAT2 (NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3)] and a non-calcium-regulated NFAT5 protein. Three of the four calcium-regulated members (NFAT1, 2 and 4) are expressed in T cells (Macian, 2005). All relevant NFAT activation events (nuclear translocation, DNA binding, and transcriptional activity) are dictated by its phosphorylation state, which is so far the best studied post-translational modification for NFAT. In resting T cells, NFAT proteins are heavily phosphorylated and reside in the cytosol, with the shuttling to the nucleus being precisely controlled. The calcium calmodulin-dependent phosphatase calcineurin is known to dephosphorylate NFAT, allowing its nuclear translocation. In the nucleus, NFAT binds to DNA either alone or in conjunction with other transcription factors, integrating at the nuclear level, calcium in puts with either stimulatory or inhibitory signals. Several kinases, including glycogen synthase kinase 3 (GSK3), casein kinase 1 (CK1), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and dual-specificity tyrosine-phosphorylation regulated kinases (DYRK), have been reported to be involved in the rephosphorylation of NFAT in the nucleus, thereby rendering them inactive and triggering their nuclear export (Beals et al., 1997; Chow et al., 2000; Gomez del Arco et al., 2000; Gwack et al., 2006; Okamura et al., 2004).

In addition to phosphorylation, there is increasing evidence for other post-translational modifications relevant for
the regulation of transcription factors at nuclear level such as poly(ADP-ribose)ylation. Poly(ADP-ribosyl)ation is a transient post-translational modification of proteins with a homopolymeric chain composed of linear and branched sequences of repeating ADP-ribose units linked together in a complex polymer. The synthesis of poly(ADP-ribose) (PAR) is catalyzed by the Poly-ADP-ribose polymerase (PARP) enzymes which constitute a family of 17 members with PARP-1 being the best known. PAR is then subjected to degradation by poly-ADP-ribose-glycohydrolase (Schreiber et al., 2006). Regulation by poly(ADP-ribosylation) has been described for different transcription factors such as YY-1 (Oei and Shi, 2001), RNA polymerase II-associated factors (Oei et al., 1998), p53 (Mendoza-Alvarez and Alvarez-Gonzalez, 2001), and NF-κB (Kameoka et al., 2000). Indeed, PARP-1 and poly(ADP-ribosyl)ation play a critical role regulating the expression of multiple NF-κB dependent genes involved in the inflammatory response (Carrillo et al., 2004). Likewise, PARP inhibitors have been found to be important for the control of systemic inflammatory processes (Cuzzocrea, 2005).

The aim of this study is to investigate the role of poly(ADP-ribosyl)ation in NFAT regulation upon T cells activation. Our results show that pharmacological inhibition of PARP activity delays NFAT nuclear export and, accordingly increases NFAT-dependent transactivation. Moreover, we show that NFAT is effectively co-immunoprecipitated with PARP-1, being also a substrate for poly(ADP-ribose)ylation. Altogether, these data point out to poly(ADP-ribosyl)ation as a novel mechanism that regulates NFAT at the nuclear level.

2. Materials and methods

2.1. Cells

Human peripheral blood mononuclear cells from healthy adult volunteer donors were isolated by centrifugation of venous blood on Ficoll-Hypaque density gradient (Amersham Pharmacia Biotech, Piscataway, NJ). To enrich T lymphocytes, the adherent cells and B cells were depleted by passage of the cell suspension through a nylon wool column. Jurkat and HeLa cell lines (American Type Culture Collection, Manassas, VA) were maintained in exponential growth in RPMI 1640 and DMEM medium, respectively (Biowhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, UK), 1 mM L-glutamine, 100 μM penicillin, and 10 μg/ml streptomycin (all from Sigma–Aldrich, St. Louis, MO).

2.2. Antibodies and chemicals

The following antibodies were used: mouse anti-NFATc1 (7A6), and mouse anti-NFATc2 (4G6-G5) were from BD Pharmingen (San Diego, CA); mouse anti-PAR monoclonal antibody (10H) and rabbit anti-PARP-1 polyclonal antibodies (288) were from Alexis (Lausen, Switzerland); rabbit anti-PAR polyclonal antibody was from Biomol (Exeter, UK); mouse anti-γH2AX (Ser 139) (JBW301) was from Upstate (Lake Placid, NY); anti-GFP (7.1 and 13.1) was from Roche Applied Science (Indianapolis, IN); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse was from DAKO (Glostrup, Denmark); horseradish peroxidase-conjugated goat anti-mouse IgG was from Promega (Madison, WI); mouse anti-rabbit immunoglobulin, and rabbit anti-glutathione-S-transferase were from Sigma–Aldrich.

The PARP inhibitors PJ34 (N-[(6-oxo-5,6-dihydropyridin-2-yl)-N,N-dimethylametamide HCl) and ANI (4-amino-1,8-naphthalimide) were purchased from Alexis; 3H-NAD+ and 32P-NAD+ were from American Radiolabeled Chemical Inc. (St. Louis, MO); glutathione sepharose 4B and sepharose A were from GE Healthcare (Uppsala, Sweden); CsA was from Novartis (Basel, Switzerland); 6-biotin-17-nicotinamide-adenine-dinucleotide was from R&D System (Minneapolis, MN). Other chemicals were of the highest purity available and came from Sigma–Aldrich.

2.3. Plasmids

The firefly luciferase gene driven by the distal NFAT site of the IL-2 promoter (NFAT3x-Luc) has been described previously (Hedin et al., 1997). The Renilla luciferase expression vector pRL-TK was from Promega. HA-tagged NFATc1 (pEFTAGm-NFATc1), and NFATc2 (pEFTAGmNFATc2) expression vectors encoding full length murine NFATc1 and NFATc2, respectively, with three tandem copies of the influenza virus haemagglutinin (3 × HA) epitope at its N-terminus, has been described previously (Luo et al., 1996). HA and GFP-tagged NFATc2 (amino acids 1–460) (pEGFPN1mNFATc2(1–460-GFP) was generated by subcloning the fragment coding for the first 460 N-terminal residues of murine NFATc2 in frame with a 3 × HA in its N-terminus and GFP in its C-terminus has been described previously (Aramburu et al., 1998). pBC vectors containing truncated forms of hPARP-1 have been described previously (Masson et al., 1998), pEGFP-XRCC1 and pEGFP-XRCC1-BRCT1 plasmids were generated by subcloning the EcoRI/EcoRI fragment from pCD2-XRCC1 plasmid.

2.4. PARP activity assay

PARP activity in T cells was determined as previously described (Muiras et al., 1998).

2.5. Transient transfection and luciferase activity

Jurkat cells were transfected by the liposome-mediated gene transfer method as previously described (Carrillo et al., 2004) with a firefly luciferase reporter plasmid under the control of a 3 × NFAT promoter element and the Renilla luciferase expression vector pRL-TK (ratio 20:1) as an internal control. Cells were cultured and Firefly and Renilla luciferase activity was measured by using the dual luciferase assay kit (Promega), as specified by the manufacturer. HeLa cells were transfected with JetPEI TM, as specified by the manufacturer (Polyplus transfection SA, Illkirch, France).
2.6. Immunocytochemistry

Jurkat cells were untreated or treated with PJ34 for 1 h before starting stimulation with a combination of the calcium ionophore ionomycin and phorbol 12-myristate 13-acetate (PMA) or vehicle for the indicated time. Cells were washed with PBS, centrifuged in a cytospin, fixed in ice-cold methanol/acetone and permeabilized with 1% Triton X100. NFATc1 was detected by using mouse anti-NFATc1, while PAR was detected by using mouse anti-PAR antibody, followed by rabbit anti-mouse FITC-conjugated antibody. Nuclear counterstaining was performed with DAPI. Where indicated, nuclear translocation of NFAT was inhibited by pre-incubation with 1 µM CsA. Immunofluorescence microscopy images were obtained with a Leica microscope.

2.7. Flow cytometry analysis for γH2AX

Jurkat cells were treated for 60 min with either PMA (50 nM) plus ionomycin (3 µM) or with etoposide (10 µg/ml) as positive control. Cells were then fixed in 1.5% paraformaldehyde for 15 min on ice and then kept in 70% ethanol at −20°C for 16 h. Fixed cells were permeabilized for 10 min and incubated for 2 h at room temperature with a mouse monoclonal anti-γH2AX antibody. Samples were raised and incubated for 2 h at room temperature with a FITC-conjugated rabbit anti-mouse antibody. Finally, cells were rinsed, incubated with Hoechst (5 µg/ml) for 30 min at room temperature and analysed with a LSR cytometer (BD, San José, CA).

2.8. Cell extracts, immunoprecipitation, GST pull-down and Western blotting

Cell extracts were prepared as previously described (Aceves et al., 2004). Immunoprecipitation, GST pull-down and Western-blot were performed as described previously (Schreiber et al., 2002).

2.9. Poly(ADP-ribose)ylation of NFAT

For poly(ADP-ribosyl)ation of endogenous NFAT analysis, 2 × 10^8 Jurkat cells were lysed in high salt buffer containing 55 mM Heps, 235 mM NaCl, 10% Glycerol, 2 mM DTT, 2.5 mM EDTA. Pellets containing DNA were resuspended in PARP adaptation buffer, sheared by sonication and used for PARP stimulation together with double stranded nicked DNA at 8.3 µg/ml. PARP stimulation buffer (HEPES pH 7.0, 117.5 mM NaCl, 28 mM KCl, 10 mM MgCl2, 1 mM DTT, 1.25 mM EDTA, 5% glycerol) was added to cell extracts together with 6-biotin-17-nicotinamide-adenine-dinucleotide (biotin-NAD+) and incubated for 45 min at room temperature. Streptavidin magnetic beads (BD) were added to separate biotinilated proteins, incubated for 15 min on ice and subsequently washed three times in a buffer containing 10 mM Tris–HCl (pH 7.5), 0.1% Nonidet P-40 and once in PBS. Bound proteins were resuspended in SDS laemml buffer and separated in a 10% SDS-PAGE gel. Control reactions were carried out in the presence of the PARP inhibitors PJ34 (30 µM). NFAT was identified with an anti-NFATc1 antibody.

In a second approach, HA-tagged NFATc1, NFATc2 and GFP-tagged truncated NFATc2 (amino acids 1–460) transient transfected HeLa cell total extracts were immunoprecipitated with an anti-HA or an anti-GFP monoclonal antibody and poly(ADP-ribosyl)ation by PARP-1 was determined as previously described (Schreiber et al., 2002).

3. Results

3.1. PARP activity is induced upon T cell activation

To investigate whether PARP activity is induced upon T cell activation, either human primary T cells or the Jurkat T cell line were stimulated with PMA + ionomycin, which together mimic many of the signals activated in antigen receptor stimulation in the presence of costimulation. In Fig. 1, we show that stimulation of T cells with PMA + ionomycin induces rapid PARP activation measured by the incorporation of 3H-NAD+ into proteins in permeabilized cells (Fig. 1a). PARP inhibitors, both PJ34 and ANI, dose-dependently inhibited PARP activity in Jurkat cells (Fig. 1b). PARP activation in stimulated Jurkat T cells was confirmed by immunofluorescent staining and Western blotting using a specific anti-PAR antibody (Fig. 1c and d). The absence of H2AX phosphorylation, an early sensitive indicator of DNA double strand-break (DSB), after PMA + ionomycin treatment suggests that PARP is activated upon T cell stimulation in the absence of DNA damage (Fig. 2).

3.2. Poly-ADP-ribose modulates NFAT-dependent transcriptional activity in T cells

To address the effect of poly(ADP-ribosyl)ation on T cell activation, we evaluated NFAT-dependent transcriptional activation in Jurkat cells, transiently transfected with a reporter construct containing the firefly luciferase gene driven by the distal NFAT site of the IL-2 promoter (NFAT3x-Luc) using a Renilla luciferase reporter (pRL-TK) for normalization. Twenty-four hours after transfection, cells were pre-treated for 1 h with increasing concentrations of either PJ34 or ANI PARP inhibitors before 6 h stimulation with PMA + ionomycin. Results from luciferase assays showed that PARP inhibitors significantly increase the NFAT-dependent transcriptional activity in a dose-dependent manner (Fig. 3).

3.3. NFAT is a substrate for poly(ADP-ribosyl)ation

To gain insight into the regulation of NFAT by PAR, we tested whether NFAT is poly(ADP-ribosyl)ated. We have taken advantage of the PARP substrate, biotin-NAD+ which is incorporated into proteins as biotinyl-ADP ribose moieties in a PARP dependent manner. Jurkat cell lysates were subjected to PARP mediated biotinylation and subsequently separated by means of a magnetically labeled streptavidin. NFATc1 was detected from the streptavidin bound fraction by Western-blot. To demonstrate the specificity of NFAT poly(ADP-ribosyl)ation, a control reac-
Fig. 1. PARP activity induction upon T cells activation. (a) Human primary T cells or the Jurkat T cell line were treated for the indicated times with 50 nM PMA and 3 μM ionomycin. In (b), different doses of either PJ34 or ANI PARP inhibitors were used in Jurkat cells. Permeabilized cells were incubated with 3H-NAD+ for 10 min. 3H-thymidine incorporation was measured by liquid scintillation counting and represented as cpm. Results represent the mean ± S.D. of three independent experiments carried out in triplicate. (c) Immunofluorescent staining of Jurkat cells treated for the indicated times with 50 nM PMA (P) and 3 μM ionomycin (I), for PAR formation. Nuclei are counterstained with DAPI. Treatment of cells with H2O2 for 10 min was used as control. Magnification ×50. (d) Jurkat cells were treated for the indicated times with 50 nM PMA (P) and 3 μM ionomycin (I), harvested and analysed by immunoblotting for PAR formation. The PARP inhibitor PJ34 was used as control of poly(ADP-ribosyl)ation. PARP-1 was used as loading control.

No NFAT was present in the streptavidin bound fraction when biotin-NAD⁺ was not added to the lysate (data not shown).

The ability of PARP-1 to poly(ADP-ribosyl)ate NFATc1 and NFATc2 was also evaluated using an alternative experimental approach. Total extracts from HA-tagged NFATc1 or NFATc2 transfected HeLa cells, were immunoprecipitated with an anti-HA antibody and then incubated in a reconstituted

Fig. 2. Effect of PMA and ionomycin on H2AX phosphorylation in Jurkat T cells as measured by flow cytometry. The cells were untreated (control) or treated with PMA (50 nM) plus ionomycin (I) (3 μM) for 60 min, then fixed, permeabilized and stained with a mouse monoclonal anti-γH2AX antibody. DNA counterstained was performed with Hoechst (5 μg/ml). As positive control, cells were treated with etoposide (10 μg/ml). The gating threshold marks the upper level of immunofluorescent from the untreated samples. Representative dot plots are shown. Percentage of cells from two independent experiments is indicated.
Fig. 3. Effects of PARP inhibitors in NFAT-dependent transcriptional activity. Jurkat cells were transiently co-transfected with a firefly luciferase reporter plasmid under the control of a 3× NFAT promoter element and the Renilla luciferase expression vector pRL-TK. Twenty-four hours after transfection, cells were pretreated for 1 h with the corresponding drugs or vehicle and stimulated for 6 h with 50 nM PMA and 3 μM ionomycin. Cell lysates were assayed for luciferase activity. Data are shown as the percent of activation of the transcriptional activity observed after preincubation for 1 h with vehicle alone. Results represent the mean ± S.D. of at least five independent experiments carried out in quadruplicate. *P < 0.05 compared with vehicle-treated cells.

poly(ADP-ribosyl)ating enzyme system containing recombinant pure PARP-1 enzyme, DNase I activated calf thymus DNA and 32P-NAD+ to label the modified proteins. Reaction products were analysed by SDS/PAGE. As shown in Fig. 4b, both NFATc1 and NFATc2 were found to be poly(ADP-ribosyl)ated by PARP-1. Moreover, NFATc2 was poly(ADP-ribosyl)ated on its regulatory region spanning amino acids 1–460 (Fig. 4c).

3.4. Physical interaction between PARP-1 and NFAT

As NFAT is poly(ADP-ribosyl)ated in a PARP-1 dependent manner, we studied whether there is a physical interaction between PARP-1 and either NFATc1 or NFATc2. Total extracts were obtained from HeLa cells transfected with HA-tagged NFATc1, NFATc2 or the truncated NFATc2 (amino acids 1–460) containing the NFAT regulatory region. Immunoprecipitation experiments were performed either with an anti-PARP-1 antibody or with an irrelevant antibody and the presence of NFATc1 or NFATc2 in the precipitate was revealed by Western-blot, using an anti-HA antibody. As shown in Fig. 5a, all NFAT isoforms tested were able to interact with PARP-1. We further examined the region within PARP-1 that interacts with NFAT, using GST fusion proteins in frame with serially truncated ver-

Fig. 4. Poly(ADP-ribosyl)ation of NFAT. (a) Total Jurkat cell lysates were incubated with bio-NAD+ and sheared DNA in the presence or absence of PARP inhibitor PJ34. Biotinylated proteins were separated by means of magnetically labeled streptavidin, and NFATc1 was detected by Western-blot from the streptavidin bound fraction. A control reaction was run in parallel in the presence of PARP inhibitor PJ34 (30 μM). Input, 5% of cell lysates were used as control. (b) Total extracts from HA-tagged NFATc1 or NFATc2 transfected HeLa cells were immunoprecipitated with an anti-HA antibody and incubated 4 min at 25 °C in activity buffer with or without hPARP-1 as indicated, in the presence of [%2P] NAD+ and DNase I activated DNA. Samples were analysed by autoradiography (upper panel) and Western-blot with an anti-HA antibody (lower panel). (c) Total extracts from NFATc2-GFP (amino acids 1–460) transfected HeLa cells were immunoprecipitated with an anti-GFP antibody and incubated 4 min at 25 °C in activity buffer with or without hPARP-1 as indicated above. Samples were analysed by autoradiography (upper panel) and Western-blot with an anti-GFP antibody (lower panel). Total extracts from pEGFP-XRCC1-BRCT1 transfected HeLa cells were used as control.
Fig. 5. Physical interaction between NFAT and PARP-1. Total extracts from HA-tagged NFATc1, NFATc2 or truncated NFATc2 (amino acids 1–460) transfected HeLa cells were immunoprecipitated with control (lanes 2, 4 and 6) or anti-PARP-1 (lanes 1, 3, 5 and 7) antibodies. (a) The immunoprecipitates were examined for the presence of NFAT or PARP-1 by immunoblotting. Input, 5% of cell lysates were used as control. (b) Schematic representation of hPARP-1. (c) Mapping of the interface PARP-1 domains. GST (lane 1) and GST-tagged deletion mutants of hPARP-1 (lanes 3–8) were expressed in HeLa cells; lane 2 and 9: crude extract of 10⁶ HeLa cells. Interacting endogenous proteins were extracted by GST-pull-down and analysed by Western-blot with successively anti-GST and anti-NFATc2 antibodies.

Fig. 6. PARP inhibition does not affect the nuclear import of NFAT. Jurkat cells were treated with 50 nM PMA and 3 μM ionomycin (I), in the absence or in the presence of PJ34 (30 μM). (a) Immunofluorescent staining (magnification ×20). (b) Nuclear extracts were analysed by Western-blot as described in Section 2. The data shown are representative of at least three independent experiments. The immunoblot was also stained with Ponceau S to confirm equal protein loading and transfer among the lanes.
Fig. 7. PARP inhibition delays nuclear export of NFAT. (a) Jurkat cells were preincubated for 1 h with PJ34 (30 μM) and then stimulated with 50 nM PMA and 3 μM ionomycin to induce dephosphorylation and nuclear translocation of NFATc1. Calcineurin activity was inhibited 15 min latter by addition of CsA (1 μM). Subcellular localization of endogenous NFATc1 was visualized by indirect immunofluorescence at the indicated time points. Magnification ×20 and in (b) magnification ×64. (c) Jurkat cells were treated as above and nuclear extracts were analysed by Western-blot with an anti-NFATc1 antibody. The immunoblot was also stained with Ponceau S to test protein loading and transfer among the lanes.
been implicated in the binding to several partners (Schreiber et al., 2002).

### 3.5. Inhibition of Poly-ADP-ribose polymerase activity delays NFAT nuclear export

NFAT activation is mainly regulated by its subcellular localization (Okamura and Rao, 2001). The amount of NFAT present in the nucleus is a net result of the rate of nuclear import/export, controlled in turn by the interplay of phosphatase calcineurin versus export kinases. To investigate whether poly(ADP-ribosylation) modulates the NFAT subcellular localization, nuclear translocation of NFATc1 was tested by immunocytochemistry in Jurkat cells in the absence or presence of the PARP inhibitor PJ34. NFATc1 was present in the nucleus of most PMA + ionomycin treated cells. No effect of PJ34 on NFATc1 nuclear translocation was observed (Fig. 6a). Similarly, Western-blot analysis of nuclear extracts from PMA + ionomycin stimulated Jurkat cells, showed similar nuclear import kinetic of NFATc1 in the absence or the presence of PJ34 (Fig. 6b). However, from those experiments it was apparent that NFATc1 was still present in the nuclear fraction of cells stimulated in the presence of PJ34 at later time points.

To further confirm the effect of PARP inhibition in NFAT nuclear retention, the calcineurin inhibitor cyclosporin A (CsA) was added to the cells 15 min after PMA + ionomycin stimulation to block nuclear import and synchronously initiate NFATc1 nuclear rephosphorylation and nuclear export of endogenous NFATc1. Immunofluorescence revealed a striking difference in the kinetics of NFATc1 nuclear export between PJ34-untreated T cells and those cells pre-treated with PJ34 for 1 h (Fig. 7a and b). After 45 min of CsA treatment, nuclear export of NFATc1 was essentially complete in PJ34-untreated cells, and only just starting in PJ34-treated cells. One hour after addition of CsA, NFATc1 nuclear export was complete in PJ34-untreated cells but still incomplete in PJ34-treated cells (Fig. 7a and b). Western-blot analysis of nuclear extracts from Jurkat cells treated as indicated above, confirmed the nuclear export delay of NFATc1 in cells treated with PJ34 (Fig. 7c).

### 4. Discussion

Activation of T lymphocytes initiates signal transduction pathways that lead to the induction of a complex array of kinases, phosphatases, calcium influx and downstream activation of transcription factors (Crabtree and Clipstone, 1994) that must be tightly regulated by a variety of mechanisms. Here, we provide the first evidence that activation of T cells leads to PAR formation which modulates the activity of the transcription factors NFAT. Likewise, the absence of H2AX phosphorylation, an early sensitive indicator of DSB (Rogakou et al., 1998), after PMA + ionomycin treatment suggest that PARP activation upon T cell stimulation takes place in the absence of DNA damage. Indeed, previous studies have demonstrated that PARP-1 could be activated in the absence of DNA damage by certain DNA structures (Kun et al., 2002), polynucleosomes (Kim et al., 2004), as well as phosphorylated Erk2 in neurons and cardiomyocytes (Cohen-Armon et al., 2007). Accordingly, Erk2 phosphorylation that takes place during T cell activation (Cantrell, 1996) could be responsible for the activation of PARP we have observed in the present work.

NFAT activation is mainly regulated by its subcellular localization (Okamura and Rao, 2001). NFAT nuclear localization is a net result of the rate of nuclear import/export, which so far has been shown to be ruled by phosphorylation, by means of interplay of calcineurin and export kinases (Okamura et al., 2000; Okamura et al., 2004). In this work, we have demonstrated that both NFATc1 and NFATc2 are poly(ADP-ribosylated) by PARP-1. Moreover, we have also found a physical association between PARP-1 and NFATc1 and NFATc2. To our knowledge, this is the first description that poly(ADP-ribosylation) is a post-translational modification of NFAT which could regulate their function. Interestingly, our data indicated that PARP inhibitors cause a delay in NFAT nuclear export indicating that poly(ADP-ribosylation) would facilitate NFAT nuclear export during the physiological T cell stimulation as soon as the calcium mediated import ends, preventing NFAT from lingering in the nucleus in the absence of a persistent calcium influx. That will affect NFAT ability to cooperate with partner transcription factors that have different kinetics of activation (Feske et al., 2000). The quantity of NFAT in the nucleus might influence not only the total number of promoter/enhancer sites occupied by NFAT, but also the extent of tandem occupancy of multiple sites in a single promoter/enhancer region and even could dictate which promoters would be occupied. Clearly, both parameters are important for achieving the long-range interactions among transcription factors to create a cooperative “enhanceosome” complex (Tjian and Maniatis, 1994). All of these are in keeping with the increased NFAT-dependent transactivation we observed in the presence of PARP inhibitor.

Whether poly(ADP-ribosylation) expedites NFAT export from the nucleus directly or by priming/facilitating NFAT phosphorylation remains to be elucidated. There are indications that favour either mechanism. In one hand, it has also been shown that poly(ADP-ribosylation) of HMGB1 regulates its translocation from the nucleus to the cytosol (Ditsworth et al., 2007). While poly(ADP-ribosylation) of PARP-1 has also been implicated in stabilizing the interaction of pErk2 with Elk1, thus increasing phosphorylation of Elk1 (Cohen-Armon et al., 2007). In an analogous manner, NFAT directly after poly(ADP-ribosylation) or through other nuclear protein modified by poly(ADP-ribosylation) following PARP activation, possibly by pErk2 in T cells, could stabilizes the interaction of NFAT with relevant kinases into a multi-component complex.

### 5. Conclusions

Taken together, these data support the importance of PARP-1 and poly(ADP-ribosylation) as a new regulatory mechanism of NFAT at nuclear level. Indeed, NFAT is largely regulated indirectly by drugs that impact upon signal transduction cascades. The pharmacological modulation of its transcriptional activity therefore represents an attractive therapeutic approach to many diseases associated with aberrant gene expression (Emery et al.,
2001; Lee and Park, 2006). Thus, our results suggest a potential use of PARP as a new therapeutic target in the modulation of NFAT.

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References

Cuzzocrea, S., 2005. Shock, inflammation and PARP. PharmacoI. Res. 52, 72–82.