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ORIGINAL ARTICLE

Expression analysis of genes involved in apoptosis, proliferation and endoplasmic reticulum stress in ionomycin/PMA treated Jurkat cells

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Summary

Purpose: *Activation of T cells by direct stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Io) results in numerous downstream signals that activate pathways enabling T cells to proliferate and produce cytokines. Inducible T cell activation is regulated predominantly at the transcriptional level. Therefore, we were interested to analyze the transcriptional activity of the 19 genes involved in the regulation of several important cellular processes.*

Methods: *Quantitative real-time (RT) PCR analysis was performed using mRNA-specific primers and SybrGreen for relative mRNA expression levels of all the examined genes.*

Results: *Our results showed c-kit expression in Jurkat cells, further confirmed by sequencing of c-kit mRNA-specific PCR product. The expected increased expression of interleukin (IL)-2 mRNA, together with moderate Ki-67 upregulation, indicate the proliferation of PMA/Io treated Jurkat cells. Significant upregulation of nuclear factor (NF)-κB, JNK and the prosurvival Bcl-2 was followed by activation of only one protein kinase RNA-like endoplasmic reticulum kinase (PERK) out of 3 main endoplasmic reticulum (ER) stress subpathways (ATF6 and spliced XBP were downregulated). NF-κB and JNK activation, as well as ERK downregulation were reactive oxygen species (ROS)-independent, shown by the lack of activation of antioxidative enzymes (SOD, NOS, GSTP1, gGCS and GR). C-kit was downregulated in the absence of exogenous SCF (c-kit ligand).*

Conclusion: *Based on these data it is concluded that the PMA/Io treatment of Jurkat cells induced increased expression of IL-2, followed by upregulation of prosurvival genes belonging to the Bcl-2 family. Neither c-kit nor the antioxidative system were activated, excluding their role in Jurkat T-cell activation in the absence of exogenous c-kit ligand SCF.*

Key words: apoptosis, endoplasmic reticulum stress, oxidative stress, T cell activation

Introduction

The cytokine milieu during T cell activation is one of the most important factors in determining T cell fate. IL-2 has a well known and dominant role in the promotion of T-cell growth, survival, and effector cell differentiation *in vitro*, together with its contribution to promoting primary and memory immune responses *in vivo* [1]. Therefore, IL-2 is the major cytokine contributing to the homeostasis of T cells, inducing an essential signal for T cell proliferation and differentiation [2]. Jurkat cells are an IL-2 producing lymphocyte cell line, commonly used to study T cell signaling. Established from the peripheral blood of a patient with T cell leukemia, Jurkat cells are also useful for studying acute T cell leukemia [3].

In the body, T cells are exposed to a panoply of external signals that, in turn, transmit signaling cascades that are integrated into the appropriate cellular response [4]. T lymphocytes can be activated *in vitro* by stimulation, with a combination of the T cell mitogen, PMA along with the calcium (Ca^{2+}) ionophore Io. Activation of T cells by direct stimulation with PMA and Io results in numerous downstream signals that activate pathways enabling T cells to proliferate and produce cytokines, such as IL-2. Inducible T cell activation is regulated predominantly at the transcriptional level [5]. This in-

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crease in protein production and protein folding activity enlarges the physiological demands on the cellular protein folding machinery [4]. Co-treatment of PMA and Io is a prototypic model for T cell activation [6].

ER calcium homeostasis plays an important role in the control of cell activation. Calcium signaling is essential for most of the biological T cell activities [6]. Calcium release from the ER and increase of intracellular calcium concentration may be induced by Io treatment leading to activation of key calcium-dependent enzymes involved in cell activation such as various protein kinase C isoforms (PKC), calcineurin or calmodulin dependent kinases. Because these enzymes critically modulate the activity of transcription factors such as NF-κB, NF-AT or AP1, calcium signaling, initiated by calcium release from the ER, plays important roles in cell survival, maturation and activation. In addition, normal ER calcium homeostasis is also required for the posttranslational modifications/chaperoning of nascent proteins in activated cells [7].

One of the most characteristic and highly conserved ER stress responses is the unfolded-protein response (UPR). Major cross talks exist between UPR and Ca^{2+} imbalance: Ca^{2+}-depletion or alteration in Ca^{2+} transport systems (SERCAs) can directly cause UPR. In turn, if ER stress is prolonged in time and ER resident chaperones are unable to counteract the accumulation of misfolded proteins, an ER-mediated apoptotic program is triggered through the activation of caspase-12 [8].

It is believed that GRP78 is the key molecule in the regulatory system that initiates a UPR because it complexes with the 3 resident ER membrane proteins that sense misfolded proteins in the ER lumen and activate the UPR [9,10]. These 3 sensors are: (1) PERK; (2) the proto-transcription factor, ATF6; and (3) the combined kinase/RNA ribonuclease, IRE-1. While the precise mechanism by which the 3 sensors are activated is not completely understood, it has been postulated that a higher affinity of GRP78 for unfolded proteins in the ER lumen causes dissociation of GRP78 from the luminal surface of the sensors, thereby allowing them to become activated [11]. GRP78 is constitutively expressed in B and T lymphocytes, but its expression is enhanced under a variety of stressful conditions including glucose deprivation, ER Ca^{2+} depletion, the blockade of glycosylation, oxidative stress, and hypoxia. Due to its antiapoptotic activity, induction of GRP78 is critical for maintaining the viability of cells subjected to such stress [12]. The results of Takano et al. [12], suggest that GRP78 is an essential molecular chaperone to maintain ER function and viability in activated T cells.

The ER lumen also has an oxidative environment, which is crucial for the formation of disulphide bonds mediated by protein disulphide isomerase (PDI) and for the proper folding of many proteins destined for secretion or for display on the cell surface [13]. Even a small fluctuation in the ER environment could dramatically affect cellular homeostasis, importantly regulated by the balance of ROS production and scavenging [14].

Recent studies indicate that NF-κB controls and prevents the proapoptotic accumulation of ROS in response to death stimuli. ROS play a critical role in programmed cell death (PCD) triggered by chemo- and radiotherapy. Downregulation of NF-κB activity contributes to enhanced efficacy of cytotoxic drugs, followed by decreased expression of transcriptional targets Bcl-XL and Bcl-2 [15]. ROS suppression represents the main mechanism by which NF-κB downregulates the JNK proapoptotic cascade [16]. UPR response and PERK activation mediate the increase of the nuclear expression of ATF4, GRP78 and Nrf2 by enhancing translation of their transcripts. In normal lung epithelial cells and in normal B cells, ATF4 and Nrf2 induce upregulation of genes encoding for the antioxidant enzymes heme oxygenase-1, glutathione S-transferase, glutathione peroxidase, superoxide dismutase (SOD) and nitric oxide synthase (NOS) [17,18]. Overexpression of SOD *in vitro* increases cell differentiation, and decreases cell growth and proliferation [19], therefore the T cell activation may preferentially require SOD mRNA downregulation in order to enable the signaling activation. Compelling evidence showed that ROS are crucial intracellular signaling molecules. Similarly, enzymatic or nonenzymatic antioxidant systems, upregulated to counteract ROS, may have a role in signaling activation [18].

Our hypothesis of c-kit involvement in Jurkat cell activation is based on results showing that numerous signal transducing pathways, such as PI3K/AKT and JNK pathway, are implicated in c-kit mediated biological effects [20]. C-kit belongs to type III tyrosine kinase receptors family. Cell proliferation, migration, survival and differentiation are mediated upon c-kit activation through ligand-induced oligomerization [21]. Binding of stem-cell factor (SCF) to c-kit induces receptor dimerization and its intrinsic tyrosine kinase activity, leading to phosphorylation of key residues, mediating downstream effectors activation [20]. C-kit expression is important for the growth and differentiation of haematopoietic stem cells, including myeloid, erythroid, megakaryocytic, natural killer and dendritic progenitor cells, as well as pro-B and pro-T cells and mature mast cells [20].

A selective accumulation of certain protein species, like the pro-apoptotic Bcl-2 relatives Bax and Bim, or the NF-κB counteractor IkBα, may facilitate death, additional stress being imposed by oxidative stress, UPR, ER calcium release, etc. These processes have been implicated in inducing cell death in a variety of systems [22-24].

Collectively, these stressful conditions may be linked in T cell activation by PMA/Io with the differential regulation of the particular cellular pathways, such as apoptosis, proliferation, redox regulation and endoplasmic reticulum stress. Therefore, we were interested to analyse the transcriptional activity of the 19 genes involved in the regulation of these important cellular processes.

Methods

Gene expression experiments were performed in Jurkat cells RNA (Stratagene Catalog No 540108). Jurkat cells were incubated for 10 h with 1.5 mM calcium chloride, 4 μM Io, and 0.1 mg/L PMA (Stratagene Catalog No 540111). The quality of RNA was analysed by the Bioanalyser 2100 (RIN > 7).

The synthesis of cDNA was performed using 1 μg of Jurkat and Jurkat PMA/Io RNA as template, using the cDNA synthesis kit (Superscript III, Invitrogen, USA). For the reverse transcription, we used the combination of the oligo-deoxythymidine (oligo-dT) and random hexamers, in order to achieve the optimal cDNA synthesis. Primers were designed using Primer Express 3.0 Software (Applied Biosystems, USA) and produced by Sigma (the sequence of primers for all 19 genes is shown in Table 1). cDNA was synthesised using the Invitrogen reverse transcription kit, according to the manufacturer's instructions. By means of the quantitative RT-PCR (qRT-PCR), we measured the gene expression levels. The forward and reverse primers for all tested genes were designed using Primer 3 software, applying the strategy of exon spanning across an exon-exon boundary, in order to avoid false-positive signals, as described before [25]. For qRT-PCR, the primers were used in concentration of 1.2 μM each in a total qRT-PCR volume of 20 μl. qRT-PCR analysis was performed using the instrument StepOne Plus (Applied Biosystems, USA) for quantification with Sybr Green and the standard software PCR product fluorescence detection and analysis (Applied Biosystems StepOneTM Real-Time PCR software v. 2.0). Relative mRNA expression levels of all the examined genes were determined from the threshold cycle values and were normalized using the human ACTB gene as a reference gene (Applied Biosystems, USA) to yield the relative abundance.

Table 1. List of primers for qRT-PCR expression analysis

	Name of primer	*Sequence of primer*	*Name of the gene*
1	IL-2_F	GAATCCCAAACTCACCAGGA	Interleukin-2
	IL-2_R	CGTTGATATTGCTGATTAAGTCC	
2	Ki-67_F	CAAAAGGATTCCCTCAGCAA	Antigen Ki-67
	Ki-67_R	CAAAGGACACACGCCTTCTT	
3	JNK_F	GAGCAATGGTGAAACGGAAT	c-Jun N-terminal kinase
	JNK_R	CTGTTGTTCCGCTAGATGGAG	
4	ERK_F	CACACAGGGTTCCTGACAGA	Extracellular signal-regulated kinase
	ERK_R	CTGGAAAGATGGGCCTGTTA	
5	Bcl-2_F	GGTGACCAGTTCAACGGAGA	Bcl-2, B-cell CLL/lymphoma 2
	Bcl-2_R	CCAGGAATCAATCACCCAAT	
6	Bax_F	ACCAAGAAGCTGAGCGAGTG	Bax, Bcl2-associated X protein
	Bax_R	CCTCCCAGAAAAATGCCATA	
7	Bak_F	GTAGCCCAGGACACAGAGGA	BAK1 Bcl2-antagonist/killer 1
	Bak_R	GTCAGGCCATGCTGGTAGAC	
8	c-kit_F	TTTAGATCCTTAGATGAGT	proto-oncogene, tyrosine-protein kinase c-Kit
	c-kit_R	AGGGTGCCAGCTGTAGTCAC	
9	GADD34_F	TCTTATGCAAGACGCTGCAC	Growth arrest and DNA damage-inducible protein
	GADD34_R	GTAGCCTGATGGGGTGCTT	
10	PERK_F	GTCGCCAATGGGATAGTGAC	protein kinase RNA-like endoplasmic reticulum kinase
	PERK_R	GCAGCTTCCTGTTCTTCCAC	
11	ATF4_F	TCAAACCTCATGGGTTCTCC	Activating transcription factor 4
	ATF4_R	GTGTCATCCAACGTGGTCAG	
12	XBP1-s_F	TCTGCTGAGTCCGCAGCAGG	X-box binding protein 1 - spliced form
	XBP-1_R	CTCTAAGACTAGAGGCTTGG	
13	ATF6_F	CAGCGATAGCCCAGTGAAT	Activating transcription factor 6
	ATF6_R	TTCTGAGAGGGCAGCCTTTA	
14	SOD_F	CGTCACCGAGGAGAAGTACC	SOD, superoxide dismutase
	SOD_R	CTGATTTGGACAAGCAGCAA	
15	NOS_F	CCCTTCAATGGCTGGTACAT	NOS, nitric oxide synthase
	NOS_R	CAGCATCTCCTGGTGAAACA	
16	NFkB_F	CTGGAAGCACGAATGACAGA	Nuclear factor κB
	NFkB_R	TGAGGTCCATCTCCTTGGTC	
17	GSTP1_F	CTCTATGGGAAGGACCAGCA	Glutathione S-transferase π1
	GSTP1_R	GACAGCAGGGTCTCAAAAGG	
18	γGCS_F	ACCGCTCGTCTCTTTGACAT	Gamma-glutamylcysteine synthetase
	γGCS_R	AAGCTGGCAGAGATGGTGTT	
19	GR_F	CAATGATCAGCACCAACTGC	Glutathione reductase
	GR_R	CAGGCAGTCAACATCTGGAA	

Statistical considerations

All data were expressed as means of triplicate qRT-PCR measurements. Results were expressed as mean ± standard deviation of the mean value (SD). Differences among means were tested for statistical significance by Student's two-tailed t-test. Statistical significance was set at $p<0.05$.

Results

The expression analysis of PMA/Io treated Jurkat cells, showed the expected upregulation of IL-2 mRNA, followed by a moderate upregulation of Ki-67, indicating the proliferation of PMA/Io treated Jurkat cells (Figure 1). In the same cellular set, significant upregulation of NF-κB and JNK, together with prosurvival Bcl-2 was followed by the activation of only one (PERK) out of 3 main ER stress subpathways (ATF6 and spliced XBP were downregulated) (Figures 1-3). These results showed the predominant activation of prosurvival pathways.

The absence of the spliced XBP and ATF6 activation in PMA/Io treated Jurkat cells indicated that the only induced ER stress subpathway was PERK/eIF2, together with its specific target, the PP1-interacting protein GADD34. PERK upregulation induced a transient phenomenon of repressed translation, which was reversed in PMA/Io treated Jurkat cells by the activated GADD34. NF-κB and JNK activation as well as ERK downregulation were ROS-independent, shown by the lack of activation of the antioxidative enzymes SOD, NOS, GSTP1, gGCS and GR (Figures 1,3,4).

Our results of qRT-PCR using mRNA-specific primers showed c-kit expression in Jurkat cells, further confirmed by sequencing of c-kit mRNA-specific PCR product (Figure 5).

Discussion

The result of c-kit mRNA expression and sequencing enabled us to use PMA/Io-activated Jurkat cells as a model for deciphering the possible involvement of c-kit in Jurkat cell proliferation, which is in contrast to previous investigations carried out by other authors that used Jurkat cells (at the level of protein analysis) as a negative control for the c-kit expression [26]. In contrast to the SCF-mediated c-kit induced IL-2Rβγ activation in the absence of IL-2 and subsequent cancer cell proliferation, our results indicate that in the PMA/Io Jurkat cells, IL-2 upregulation resulted in c-kit downregulation in the absence of exogenous SCF [20].

Different Bcl-2 family members have distinct patterns of responsiveness depending on the specific stimuli and they can be activated either by transcriptional acti-

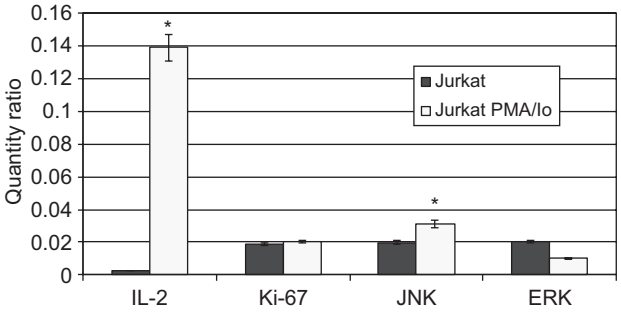

Figure 1. Expression of genes involved in proliferation of Jurkat cells. *p<0.05.

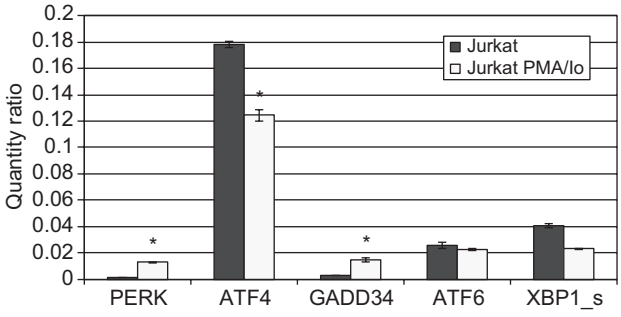

Figure 3. Expression of genes involved in endoplasmic reticulum stress. *p<0.05.

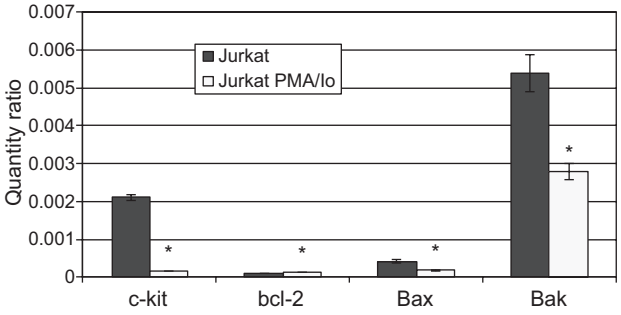

Figure 2. Expression of genes involved in proliferation and apoptosis of Jurkat cells. *p<0.05.

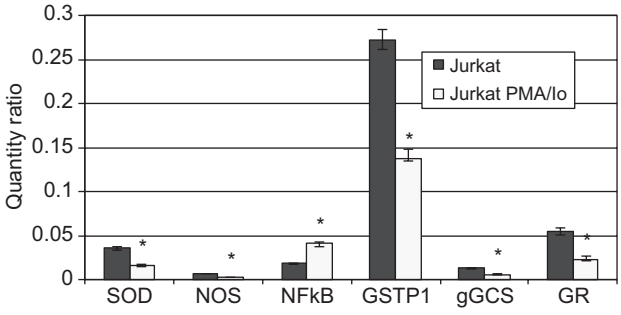

Figure 4. Expression of genes involved in antioxidative defence. *p<0.05.

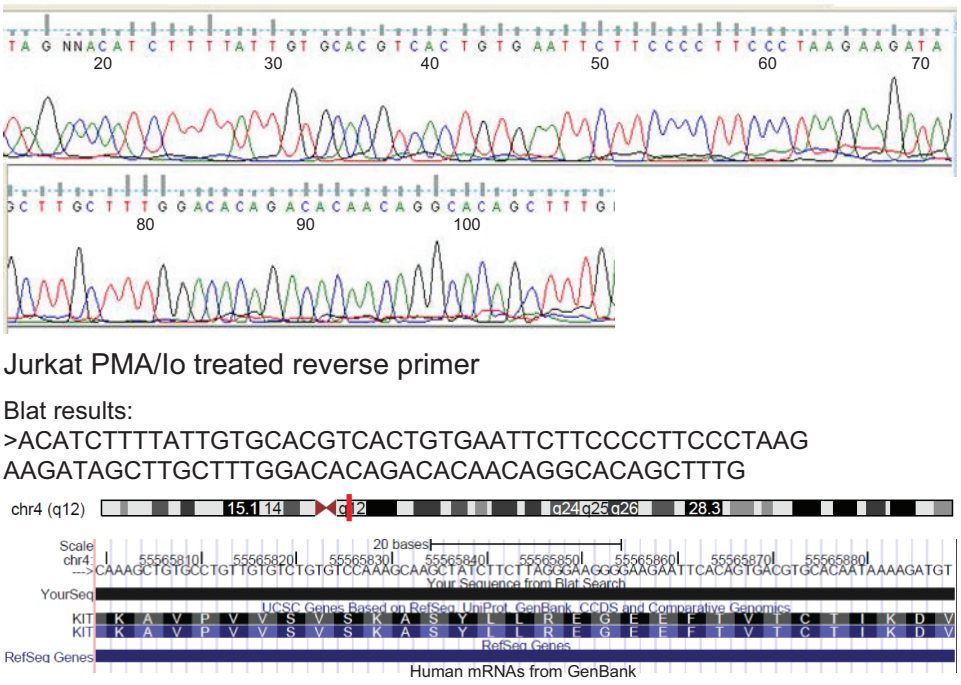

Figure 5. Sequencing of c-kit mRNA-specific PCR product.

vation or through post-translational modifications [27]. In this context, our expression data of predominant pro-survival pathways activation reflect the transcriptional events involved in PMA/Io Jurkat cell stimulation.

In breast cancer cells, PERK-mediated transient phosphorylation of eIF2α results in preferential translation of the ER stress regulated transcription factor ATF4, while the ATF4 mRNA levels remain constant [28]. Therefore, our results of ATF4 downregulation upon PMA/Io treatment of Jurkat leukemia cells are different both from the upregulated ATF4 in hypoxic cells [29] and from the results of Sequeira et al [28], who showed that the PERK activation influences the ATF4 levels at the level of the translational enhancement of ATF4 message, but not by overexpression.

In hypoxic cells, activation of eIF2α causes induction of CCAAT/enhancer binding protein homologous protein (CHOP) via upregulation of ATF4 [30]. The induction of ATF4 may be regulated at both the transcriptional and translational levels. However, in rat renal cells, treatment with 3′-deoxyadenosine did not trigger substantial expression of CHOP and ATF4, and rather suppressed the induction of these genes by ER stress, which is in accordance with our data of ATF4 mRNA downregulation [29].

Another more plausible mechanism by which PMA/Io induced the ATF4 mRNA downregulation is that ATF4 is a basic leucine zipper transcription factor that regulates the transcription of genes encoding proteins such as the growth arrest and DNA damage 34 (GADD34). ATF4-mediated upregulation of such transcripts is thought to play a key role in the recovery of the cell from the stress that induced eIF2a phosphorylation. Since we have used the mRNA of PMA/Io treated cells after 10 h of treatment, in our set of data GADD34 mRNA was already overexpressed. GADD34 targets the protein phosphatase 1 to eIF2, resulting in dephosphorylation of the protein. By promoting eIF2a dephosphorylation, GADD34 derepresses the eIF2B activity, resulting in increased ternary complex formation and resumption of translation of most mRNAs, thus providing a negative UPR feedback. Simultaneously, the translation of mRNAs with multiple upstream open reading frames, such as that encoding ATF4, is repressed [31].

The molecular chaperone GRP78 regulates the IRE1, ATF6, and PERK transduced signaling pathways of UPR; however, these signaling pathways may have distinctive sensitivities to fluctuations of the free GRP78 pool [32]. PMA/Io concentrations used to stimulate T cells in the study of Takano et al. [12], showing significant GRP78 upregulation during IL-2 transcriptional activation, are almost the same dosage conventionally used for T cell proliferation assay and IL-2 response (10 ng/ml PMA and 500 ng/ml ionomycin for 72 h). However, the doses required for the Jurkat malignant T cell activation and IL-2 production are higher, and the order of magnitude of the concentrations similar to our conditions [33,34] during the 10 h of treatment, taking into account that the IL-2 production in Jurkat cells peaks at 7 h during the PMA/Io stimulation [35].

ATF4 in conjunction with XBP1 upregulates the expression of specific UPR-regulated genes [36]. Splicing of XBP1 is supposed to mediate antiapoptotic signals, whereas the activation of JNK is assumed to be PERK/ATF4 mediated [30]. However, in our set of data, both ATF4 and XBP1 mRNA were downregulated, indicating that PERK activation did not upregulate the ATF4 mRNA levels (Figure 3). Upregulation of Bax and Bak is important for the efficient XBP1 splicing and in Bax/Bak deficient cells JNK phosphorylation and XBP1 splicing is downregulated [30,37]. In line with these results are our data in PMA/Io treated Jurkat cells, where both Bax and Bak and spliced XBP1 were downregulated (Figure 2).

NF-κB induction and translocation to the nucleus mediates the upregulation of the transcription of antiapoptotic genes, such as the inhibitors of apoptosis proteins (IAPs) and Bcl-2 [32], which is in accordance with our results in PMA/Io treated Jurkat cells. ER stress induces downregulation of Bcl-2, whereas in our set of data the mRNA expression of the antiapoptotic protein Bcl-2 was induced. Bcl-2 and Bcl-xL are known to be overexpressed in leukemia cells [38], and this effect was even more emphasized in PMA/Io treated malignant Jurkat cells.

The activation of all three MAPK cascades (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK) occurs in cells undergoing ER stress, inducing the programmed cell death [32]. In PMA/Io activated Jurkat cells we detected a concerted upregulation of both NF-κB and JNK. Despite its well documented ability to trigger PCD, JNK activation in most cell types occurs without significant induction of death, since it must signal chronically, as during inhibition of NF-κB [15]. Therefore, based on our dataset we may suggest that the upregulation of both JNK and NF-κB was the antiapoptotic, prosurvival and proliferative signal, resulting in IL-2 overexpression and downregulation of genes coding for the enzymes involved in the antioxidative defense.

NF-κB regulates the transcription of many genes involved in stress remediation, cell growth, and apoptosis [35]. T lymphocyte specific IL-2 gene transcription and protein accumulation is induced by the nuclear expression of NF-κB in T cells [39]. UPR signaling can directly intersect with inflammatory pathways including NF-κB, JNK and TLR-mediated signaling and production of ROS [30]. ER UPR and inflammatory pathways are interrelated by the generation of ROS and NO (nitric oxide). Increased protein folding due to ER UPR activation and formation of disulfide bonds may result in the accumulation of ROS and oxidative stress [30,40]. ROS inhibition reestablishes the Bcl-2 and Bcl-xL antiapoptotic effects and downregulates the Bax and Bad expression [40].

Our data add to previous observations, indicating a mutual interdependence between the UPR and ROS generation or in other words that ROS play a role both downstream and upstream of UPR targets [41]. A recent study by Santos et al. [41] in tunicamycin stimulated vascular smooth muscle cells showed (a) normal eIF2a phosphorylation, which peaks at 2 h and sustained until 8 h; (b) marked decrease in phosphorylation after full-length GADD34 transfection; and (c) significant prevention of ROS generation after UPR interruption with full-length GADD34.

In our set of data in mRNA of Jurkat cells isolated 10 h after PMA/Io treatment, we observed downregulation of the mRNA coding for the antioxidative enzymes SOD, NOS, glutathione S transferase π1/GSTP1, gamma glutamyl cysteinyl synthetase/gGCS, and glutathione reductase/GR), and this result is in line with the ATF4 downregulation in the same cellular set. ATF4 regulates a number of genes involved in redox reactions like heme oxygenase-1, glutathione peroxidase, periredoxin and inducible nitric oxide synthase (iNOS) [14]. Therefore, since ATF4 is responsible for much of the cellular response to combat ROS production upon the oxidative folding, downregulated transcription of ATF4 may influence the inhibition of ROS scavenging enzymes expression. Taken together, these data argue that although both PERK and GADD34 were upregulated and that GADD34 removes the PERK-mediated translational block [38], PMA/Io treatment of Jurkat cells contributed to the transcriptional block of numerous genes involved in apoptosis induction and antioxidative ROS scavenging.

In view of the results from Pizzola et al. [42], who showed that the ROS secretion by antigen presenting cells oxidizes T cell membrane proteins and thereby downregulates the excessive T cell activation involved in the etiopathogenesis of autoimmune diseases, the proposed hypothesis is that in situations with lower ROS production (auto)immunity may develop as a result of increased T cell activation. Therefore the downregulation of the ROS scavenging enzymes in PMA/Io treated Jurkat T cells may be the immuno-modulatory mechanism by which activated cells prevent the ROS scavenging and increased T cell activation.

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