REGULAR ARTICLE

Activation of human T lymphocytes under conditions similar to those that occur during exposure to microgravity: A proteomics study

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A number of experiments, conducted under microgravity conditions, *i.e.* in space shuttle biolaboratories or in ground based systems simulating the conditions occurring in microgravity, show that in hypogravity, *in vitro* human lymphocyte activation is severely impaired. However, very early stimulation steps of T lymphocytes are not compromised, since CD69 receptor, the earliest membrane activation marker, is expressed by T cells at a level comparable to that observed on 1 *g* activated lymphocytes. Since CD69 engagement, together with submitogenic doses of phorbol esters, transduces an activation signal to T lymphocytes, we undertook a comparative study on the stimulation mediated through this receptor on human CD3+ cells cultured under conditions similar to those which occur during exposure to microgravity, *i.e.* in clinorotation, or at 1 *g*. During the early hours of activation, increased levels of intracellular calcium and increased mitochondrial membrane potential were detectable in clinorotating as well as in 1 *g* cells. However, after 48 hours clinorotation, interleukin 2 production by T lymphocytes was significantly reduced and cell proliferation was greatly decreased. By means of a differential proteomics approach on T cells activated in clinorotation or at 1 *g* for 48 hours, we were able to detect statistically significant quantitative protein alterations. Seven proteins with modified expression values were identified; they are involved in nucleic acids processing, proteasome regulation and cytoskeleton structure.

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Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine; **Fluo3-AM**, 1-[2 amino 5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-[2-amino-5-methyl-phenoxy]ethane-N,N,N,N′-tetra-Am; **IL**, interleukin; **JC-1**, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide; **PMA**, phorbol 12-myristate 13-acetate

1 Introduction

In the past years, the effects of microgravity on lymphocyte physiology have been analyzed by *in vitro* experiments performed either in laboratory space facilities or on the ground, under conditions similar to some of those which occur in microgravity, by means of devices such as the Rotating Clinostat or Random Positioning Machinery [1]. Under these conditions, resting lymphocytes are induced to undergo apopto-

sis by 5-lipooxygenase-mediated mitochondrial uncoupling and cytochrome *c* release [2]. Furthermore, a dramatic decrease in mitogen mediated cell proliferation has been observed compared to the proliferation of lymphocytes activated at 1 *g* [3–6]. Since T cell activation plays a crucial role in many cell mediated immunological responses and in cytokine synthesis, the effect of microgravity on this process has been studied in detail. On the ground, in clinotostat, lymphocytes activated either by mitogens or by anti-CD3 mAbs show the early signaling events required for internalization of T cell receptor, intercellular interactions and interleukin(IL)-1 release by monocytes [6]. However, expression of IL-2 and its receptor were reduced [3–6]. Since IL-2 receptor expression and IL-2 synthesis are required for cell cycle entry and DNA synthesis [7], their low expression may be related to decreased lymphocyte proliferation.

In T lymphocytes cultured in the absence of accessory cells, signaling mediated by T cell receptor can be reinforced by the aggregation and engagement of T cell surface accessory molecules such as CD28, thereby promoting a proliferative response [6, 8]. Alternatively, activation can be triggered by phorbol esters and calcium ionophore, since the former agents directly activate protein kinase C [9], while calcium ionophore promotes a sustained increase in intracellular calcium levels, which is necessary for prolonged activation signaling [10]. By studying these pathways, Hashemi *et al.* [6] have found that the activation responses are differently regulated in clinorotating and in spaceflight microgravity cell cultures, since the latter fail to express the IL-2 receptor γ chain, CD25, which, in contrast, is up-regulated in clinorotating cultures at a level comparable to control cells activated at 1 *g*.

However, in spaceflight cultures, some of the very early activation steps seem to occur normally, since T cells stimulated by phorbol esters and calcium ionophore or insolubilized antiCD3/antiCD28 antibodies express CD69 [6], an early activation marker which appears on the cell membrane in 2–3 hours following addition of the signaling agents [11].

CD69 is a type II membrane protein with a calcium dependent lectin domain in the extracytoplasmic region, and is detectable as a glycosylated homodimer on activated T, B, natural killer, and granulocyte cell surfaces. Furthermore, CD69 is expressed constitutively by monocytes, platelets, epidermal Langerhans cells, CD3+ thymocytes, and bone marrow myeloid precursor cells indicating a general functional role for this molecule in the haematopoietic lineages [11]. Although the natural ligand of CD69 is still poorly defined, the signaling function of this molecule has been investigated by means of different anti-CD69 mAbs such as MLR3, AIM, Leu 23, BL-Ac/p26, FN50, which are able to mimic the ligand [12 and http://www.ncbi.nlm.nih.gov/prow/guide].

In vitro, interaction of CD69 with anti-CD69 antibodies, in combination with protein kinase C activation, may stimulate resting T cells and B cells to proliferate [13–14]. Furthermore, anti-CD69 antibodies can trigger cytotoxic activity of monocytes, natural killer cells and γδ+ T lymphocytes [15]. Recent data on CD69 deficient mice indicate that *in vivo* CD69 sig-

naling is involved in the regulation of lymphocyte apoptosis, anti-tumor immune responses and inflammatory responses through transforming growth factor β synthesis induction and modulation of inflammatory cytokines and chemokines such as IL-1 and monocyte chemoattractant protein-1 [16].

In view of these observations, we studied the effect of clinorotation on the activation of human purified T cells triggered by an anti-CD69 antibody in combination with the protein kinase C activator phorbol myristate acetate (PMA). Our data show that under these conditions T cells synthesize IL-2 at a lower level than cells activated in normal gravity conditions. Accordingly, a very low percentage of them proceed through the cell cycle.

In an attempt to understand the unresponsiveness of clinorotating T cells to the activation signals, we used a differential proteomics approach, which allowed a comparative study of proteins expressed by stimulated cells in clinorotation and in normal gravity. We found that the level of expression of several proteins is significantly altered under the different gravitational conditions and we identified some of these proteins by MS analysis.

2 Materials and methods

2.1 Chemicals and antibodies

All chemicals were of analytical grade and, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA). Anti-CD69 (Leu23) mAb was purchased from Becton Dickinson (San Josè, CA, USA). FITC conjugated anti-mouse immunoglobulin F(ab)'2 polyclonal antibody was from Euroclone (Devon, UK). Anti-CD69 mAb MLR3 (IgG2a) was from our laboratory [17] and was purified from ascitic fluid by affinity chromatography on a protein-A sepharose column (Pharmacia, Uppsala, Sweden). FITC conjugated anti-bromodesoxyuridine mAb was purchased from Chemicon (Temecula, CA, USA). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) and 1-[2 amino 5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)phenoxy]-2-[2-amino-5-methyl-phenoxy]ethane-*N,N,N',N'*-tetra-Am(Fluo-3 AM) were from Molecular Probes (Leiden, The Netherlands).

2.2 Lymphocytes purification and culture conditions

Human peripheral blood from healthy male donors (age range 28–40 years) was collected in standard 250 mL collection blood bags (Baxter Italia, Roma, Italy) containing citrate phosphate adenine as anticoagulant, according to the regulations of the Italian blood transfusion service. Each donor had signed an informed consent form. Blood was diluted three times with PBS and mononuclear lymphocytes were isolated by Ficoll/Hypaque (Pharmacia) density gradient centrifugation. After washing with PBS, the cells were resuspended in RPMI 1640 culture medium (Gibco, Paisley, UK)

supplemented with 10% fetal calf serum (Gibco), 50 IU/mL penicillin, 50 µg/mL streptomycin and 2 mM L-glutamine (Gibco). The lymphocytes were separated from adherent cells by incubation of the mononuclear suspension (2×10^6 cells/ mL) for 45 min at 37°C in petri dishes, then CD3+ cells were obtained with positive immunomagnetic selection by using anti-CD3 coupled magnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany) and following the procedure suggested by the manufacturer. After purification, T cell suspensions (10^6/mL) were stored for at least 12 h at 6°C to allow recovery from stress due to centrifugation and immunomagnetic separation. More than 95% of the separated cells were CD3+ cells, as assessed by FITC conjugated anti-CD3 staining and flow cytometry analysis performed on the CD3+ cell population after separation. The activation was triggered by one of the following stimuli: Phytohemagglutinin-A (10 µg/mL), A23187 calcium ionophore (1 µM) and anti-CD69 mAb (MLR3; purified Ig, 10 µg/mL). A23187 and the anti-CD69 antibody were used in combination with PMA (10 nM/L). T cell cultures were set up in 1.2 mL cryovials (Corning, NY, USA), which were filled to capacity and incubated at 37°C under conditions similar to those which occur during exposure to microgravity, *i.e.* in a fast rotating clinostat (CCN, Neunen, Netherlands), for 48 h, with the exception of the kinetics experiments, when the incubation lasted as indicated in Section 3. In clinostat the vials were positioned horizontally and rotated at 90 rpm along the longitudinal axis. The rotation speed was assessed so that gravity force on cells was minimized according to the method described by Cogoli *et al.* [4, 18] and produced a force of gravity of 4×10^{-3} *g* at the center of the rotation. HEPES (40 mM) and sodium bicarbonate (5 mM) were added to the incubation medium to compensate for the lack of CO_2 supply. Control cell cultures were set up in the same medium and placed beside the clinostat at 37°C.

2.3 Determination of CD69 expression, intracellular calcium concentration, mitochondrial membrane potential changes and cell viability by flow cytometry analysis

To investigate CD69 expression, T cells were harvested at the times indicated after activation, washed twice and incubated for 30 min at 4°C with an anti-CD69 mAb (MLR3) or with isotype matched irrelevant mouse immunoglobulin, then washed and stained with FITC conjugated anti-mouse immunoglobulins. In most assays, CD69 expression was evaluated also with the anti-CD69 Leu 23 mAb and no relevant discrepancy was observed with respect to the assay with MLR3 antibody (data not shown). Cell fluorescence was assessed by flow cytometry with a FACScan cytometer (Becton Dickinson) equipped with a 15 mW 488 nm argon ion laser and analyzed with Cell Quest software. Cytoplasmic free calcium was measured by means of the fluorescent calcium indicator Fluo-3 AM. Purified CD3+ cells were loaded with the probe as already described [19]. Then they were stimulated in clinorotation or at 1 *g* for different times. The Fluo-3 Am

fluorescence of 10 000 cells/sample was recorded at 530 nm by means of the cytofluorimeter and the fluorescence mean value of each cell population was determined with Cell Quest software. The concentration of intracellular calcium was calculated from the fluorescence mean value with a standard equation [20] and with a Fluo-3 AM dissociation constant of 390 nM [21]. Maximum and minimum values were the fluorescence of non-activated cells treated with 5 µM A23871 for 1 min and with 10 mM EGTA and A23871 for 5 min. Mitochondrial membrane potential was determined by loading T cells with the potentiometric probe JC-1 at a concentration of 5 µM, as described [22]. Then the cells were treated with PMA and anti-CD69 at 1 *g* or in clinorotation for different time intervals up to 3 h. In order to examine cells cultured for longer time intervals, the lymphocytes were loaded with the probe at the end of the culture period. Cells (10 000 *per* sample) were examined by flow cytometry. Green fluorescence was collected through a 530 nm band pass filter, while red fluorescence was recorded through a 585 nm long pass filter. The cells were visualized on a FL-1(green fluorescence) *versus* FL-2 (red fluorescence) dot plot, and the data were analyzed with Cell Quest software. Treatment with 50 µM/L carbonylcyanide-*4*-(trifluoromethoxy)phenylhydrazone for 45 min at 37°C induced a sharp decrease of the red fluorescence, *i.e.* a mitochondrial depolarization in more than 90% of the cells and served as a control to set the marker defining the cell population with mitochondrial membrane depolarized. Cell viability was evaluated by determining the percentage of propidium iodide permeable cells by flow cytometry.

2.4 Determination of DNA synthesis

After 47 h activation, cell cultures were pulsed with 100 µM 5-bromo-2'-deoxyuridine (BrdUrd) for 1 h, harvested, washed in 3 mL of cold PBS, fixed with cold 70% ethanol for at least 60 min, then split into two aliquots for DNA content and BrdUrd uptake determination. For the former assay, the cells were washed with PBS and treated for 60 min with PBS solution containing 50 µg/mL of propidium iodide, 0.2 mg/mL RNase and 2 mM EDTA, before flow cytometry analysis. The incorporation of BrdUrd was assessed by a modification of the protocol described by Holm *et al.* [23]. After fixation, the cells were washed in PBS-EDTA (4 mM) and treated with 2 N HCl for 15 min, to obtain partial DNA and protein denaturation. Afterwards, the nuclei were washed in 0.1 M Na tetraborate, pH 8.5, washed in PBS-EDTA (4 mM) and resuspended in PBS containing 1% BSA, Tween-20 (0.5%) and FITC conjugated anti-BrdUrd at the concentration recommended by the manufacturer. After 60 min incubation in the dark at room temperature, DNA was stained for 30 min with propidium iodide solution (10 µg/mL in PBS). Cells (20 000/sample) were analyzed by flow cytometry as described above. To exclude overlapping of red and green fluorescence in the double stained cell populations, compensation was set so that in the parallel cell populations treated with propidium iodide only, the percentage of green fluorescent cells never exceeded 0.8%. Cell

debris and aggregates were excluded from the analysis by appropriate gating. Recording the fluorescent cells on biparametric dot plot histograms allowed the BrdUrd positive cells in the G1-S phase cell population to be visualized.

2.5 2-DE and gels analysis

T cells purified from peripheral blood from six donors and activated for 48 h at 1 *g* or in clinorotation in separate experiments, were harvested at the end of stimulation, centrifuged at 200 × *g* and the supernatants were stored at −20°C to assay IL-2 release. The cell pellets (1–2 × 10^6 cells *per* sample) were washed once with PBS, then stored at −20°C. For proteomic analysis, they were pooled and solubilized in 200 μL of lysis buffer containing 7.0 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 1% pH 3–10 L or pH 4–7 IPG Buffers (Amersham Biosciences, Piscataway, NJ, USA), 1% v/v β-mercaptoethanol and 40 mM Tris-HCl. Aliquots of the cell lysates were analyzed by SDS-PAGE, followed by silver-staining of the gels and densitometric scanning, in order to normalize the protein amount to be loaded onto IEF strips. An aliquot of each sample (10–50 μL of total cell extracts) containing an average amount of 30 μg of protein was loaded onto 13 cm, pH 3–10 L or pH 4–7 IPG strips. IEF was conducted using a IPGPhor II system (Amersham Biosciences) according to the manufacturer's instructions. Focused strips were equilibrated with 6.0 M urea, 26 mM DTT, 4% SDS, 30% glycerol in 0.1 M Tris-HCl (pH 6.8) for 15 min, followed by 6.0 M urea, 0.38 M iodoacetamide, 4% SDS, 30% glycerol, and 0.01% bromophenol blue in 0.1 M Tris-HCl (pH 6.8) for 10 min, then applied directly to 10% SDS-polyacrylamide gels and separated at 130 V. After silver-staining, gels were scanned with an Image Master 2-D apparatus (Amersham Biosciences) that allowed the relative differences in spot intensities for each represented protein to be estimated.

2.6 Evaluation of differentially-represented spots

For each sample (cell lysate from pooled 1 *g* or clinorotating lymphocyte populations) two 2-DE were performed, then a comparative analysis was conducted, which included matching of spots present in the two sets of gels with a different staining intensity (quantitative differences) and identification of spots present in only one of the two samples (qualitative difference). Due to the different representative levels of the proteins, gel analysis was performed by cropping the region under 120 kDa. Protein spots in the cropped images were identified and individual spot volume values were obtained according to the program instructions. The staining intensities of matching spots between gels of the same sample were measured using Image Master 2-D software. For each cropped image, the total volume of matched spots were set equal to 1.0 using the program's volume normalization function and a normalized volume value was calculated for each matched spot. An analysis was performed on about 250 different matched spots from each sample. The normalized volume

values (relative intensity values) for the various samples were visualized as histograms representing the ratios of the intensities of matched spots from clinorotating lymphocytes *versus* control cells. Mean and median of logarithmic ratios of the intensity values for each pair of matched spots was determined. These latter values were clustered around 0, as expected if the mean error of our analysis was normally distributed. The normal distribution was further demonstrated by the Kolmogorov-Smirnov test (performed using the Instant 3.0 computer software; GraphPad, San Diego, CA, USA). Since the Kolmogorov-Smirnov parameter was close to 0, SDs of logarithmic ratios distributions were a valid parameter to assess the variability of our analysis and the significance of the quantitative differences measured. A logarithmic ratio > 0.27, which was three times above the maximal value of logarithmic ratios standard deviations (0.09), was used as a cutoff criterion to identify differentially expressed proteins with a *p* value < 0.01. In the region under 120 kDa no qualitative differences were found.

2.7 MS analysis

Spots from 2-DE gels were excised, triturated and washed with water. Proteins were in-gel reduced, *S*-alkylated and digested with trypsin as previously reported [24]. Specific samples were alternatively digested with endoprotease AspN. Digested aliquots were removed and subjected to a desalting/concentration step on μZipTipC18 (Millipore, Bedford, MA, USA) using ACN as eluent before MALDI-MS analysis. Peptide mixtures were loaded on the MALDI target, using the dried droplet technique and CHCA as matrix, and analyzed using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Internal mass calibration was performed with peptides derived from enzyme autoproteolysis. Protein Prospector and PROWL software (http:/prowl.rockefeller.edu) packages were used to identify spots unambiguously from the NCBI and Swiss-Prot nonredundant sequence databases. Identification did not include modified peptide search options. Candidates from peptide matching analysis were further evaluated by comparison with their calculated mass and p*I* using the experimental values obtained from 2-DE. In the case of extensive peptide mapping analysis, assignment of the recorded mass values to individual peptides was performed on the basis of their molecular mass, protein sequence and protease specificity, using GPMAW 4.23 software (Lighthouse Data, Odense, Denmark).

2.8 Determination of IL-2 production

The release of IL-2 into the supernatants was assessed by an instant-ELISA assay on each supernatant in duplicate following the procedure suggested by the manufacturer (Bender Medsystems, Vienna, Austria). OD values were measured by a microplate reader at a wavelength of 450 nm with 620 nm as the reference wavelength.

2.9 Statistics

Unless otherwise indicated, the data reported represent the mean of at least three independent experiments. Results were analyzed using the Student's *t*-test. Differences between data from clinorotating and 1 *g* cells were considered significant at $p < 0.01$.

3 Results

3.1 Expression of the activation marker CD69 by clinorotating T cells

To analyze the expression of CD69 on T cells activated in clinorotation, we studied the kinetics of CD69 appearance on CD3+ lymphocytes activated with PMA (10 nM/L) or phytohemagglutinin (10 μg/mL) by immunofluorescence and cytofluorimetric analysis.

In the presence of either the former or the latter signaling agent, a low percentage of CD69+ cells was detectable as early as 3 h after activation. Activation and the number of CD69+ cells increased in the following hours (Fig. 1A and B). In CD3+ cell populations activated with PMA, no relevant dis-
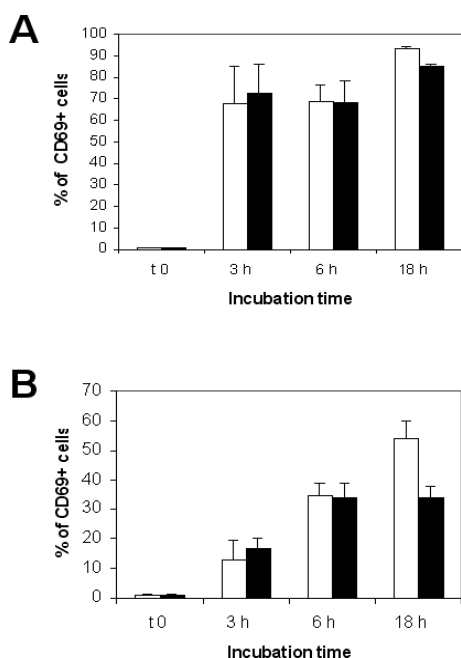

Figure 1. CD69 expression by activated lymphocytes in clinorotation. CD3+ lymphocytes from human peripheral blood from three different donors were incubated at 1 *g* (open bars) or in clinostat (closed bars) with (A) PMA (10 nM/L), or (B) phytohemagglutinin (10 μg/mL), for different time intervals as described in Section 2.3. At the indicated times, CD69 expression was determined by indirect immunofluorescence with the anti-CD69 mAb MLR3, followed by fluorimetric analysis. Mean values ± SD of the percentage of positive cells are reported. In unstimulated cell populations the percentage of CD69+ cells was lower than 3%.

crepancy was observed in clinorotating as compared to control populations either with respect to the number of CD69+ cells (Fig. 1A) and the level of CD69 expression as measured by fluorescence intensity (data not shown). In clinorotating lymphocytes stimulated with phytohemagglutinin, the pattern and kinetics of CD69 expression was comparable to those observed in control cells, with the exception of the lower expression at 24 h (Fig. 1B). The unchanged capability of lymphocytes to express CD69 when activated in clinorotation, prompted us to explore the activation pathway mediated by this molecule. We cultured T cells in clinorotation in the presence of a combination of PMA (10 nM/L) and anti-CD69 mAb (MLR3, 10 μg/mL) and monitored the activation in the short and long term effects over a time period of 48 h. We analysed some of the electrochemical changes occurring in the early steps of activation, namely the increase in intracellular calcium concentration, which is a very early step of stimulation mediated by either the T cell receptor or CD69 [10, 25–26] and the hyperpolarization of mitochondrial membrane potential, which occurs in CD3-CD28 activated lymphocytes [27]. Furthermore, we investigated the lymphocytes capability to produce IL-2 and to proceed through the cell cycle.

3.2 Cytosolic calcium increase and hyperpolarization of mitochondrial membrane potential in clinorotating T cells activated through CD69

In order to monitor the changes in intracellular calcium concentration we loaded T cells with Fluo-3-AM immediately before activation at 1 *g* or in clinorotation, then we assessed the mean fluorescence of samples containing 10 000 cells after different times of incubation. While over the first hour only a slight increase of intracellular calcium levels was observed in clinorotating as well as in 1 *g* activated cells, after 3 h, a significant rise in $[Ca^{2+}]_i$ was detectable in clinorotating cultures, which was even higher than in control cells (281 *versus* 216 nM) (Fig. 2). The intracellular calcium increase is not surprising, since 3 h after the onset of stimulation 60–80% of the cells were CD69 positive, and, as it has been already reported [26], interaction of the receptor with the antibody trigger calcium influx, thereby amplifying and sustaining the low plateau generated by PMA. The mitochondrial membrane potential showed parallel variations.

Over the first 2 h of activation, a moderate hyperpolarization of mitochondrial membrane potential with respect to resting cell populations was detectable in T cells activated under both gravity conditions, as indicated by the increase of mean red fluorescence (data not shown). A significant increase in red fluorescence in clinorotation and at 1 *g*, after 3 or 6 h of activation, when CD69 was expressed by the majority of the cells was evident (Fig. 3A, B, E and F), possibly reflecting a consequence of massive metabolic activation of the cells. In 1 *g* lymphocytes, this effect was transient and after 20 h, in the majority of the cells the mitochondrial membrane potential was comparable to the one observed in
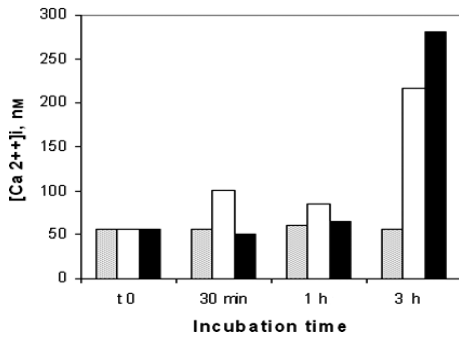
Figure 2. Effect of CD69 and PMA mediated activation on intracellular calcium level in clinorotating CD3+ lymphocytes. CD3+ lymphocytes from human peripheral blood were loaded with Fluo-3-Am and then incubated in the absence of activatory signals (gray bars) or activated at 1 *g* (open bars) or in clinostat (closed bars) with 10 µg/mL anti CD69 and 10 nM PMA. At the indicated times, the fluorescence of the cells was measured by cytofluorimetric analysis. Aliquots of non-activated lymphocytes were treated with 5 µM A23187 and 10 mM EGTA and the intracellular calcium concentration was calculated as reported in Section 2.3. A representative experiment from the three performed is shown.

the starting cell population as suggested by the similar mean fluorescence of the two cell populations (715 and 756 arbitrary units, respectively; Fig. 3C). In contrast, in clinorotating cultures, mitochondrial hyperpolarization was more prolonged since after 20 h activation, the peak channel and the mean fluorescence values (respectively 827 and 1553, arbitrary units) were higher than those in activated static lymphocytes (715 and 1165; Fig. 3G). Moreover, hyperpolarization was followed by depolarization in a fraction of the cell population at 36 h (Fig. 3H). This effect was not detectable in the cells activated at 1 *g* (Fig. 3D).

3.3 Determination of IL-2 production and cell cycle analysis in clinorotating T cells activated through CD69

To further explore the capability of clinorotating T cells to proceed through the CD69 mediated activation process, we investigated IL-2 release and DNA synthesis in T lymphocytes from six distinct donors activated with anti-CD69 and PMA for 48 h.

As indicated in Fig. 4A, clinorotation significantly inhibited ($p < 0.01$) IL-2 production, since the amount of the cytokine released in the supernatants was lower than in those from parallel, 1 *g* cell cultures. Furthermore, the ability to proliferate was impaired in the majority of cells activated for 48 h in clinorotation ($p < 0.01$), as assessed by cytofluorimetric analysis of BrdUrd uptake during the S phase of the cell cycle (Fig. 4B). The cell growth arrest was not due to an ongoing apoptotic process since the cytofluorimetric analysis of DNA content failed to reveal any hypodiploid peak which would indicate the presence of apoptotic cells. Fur-
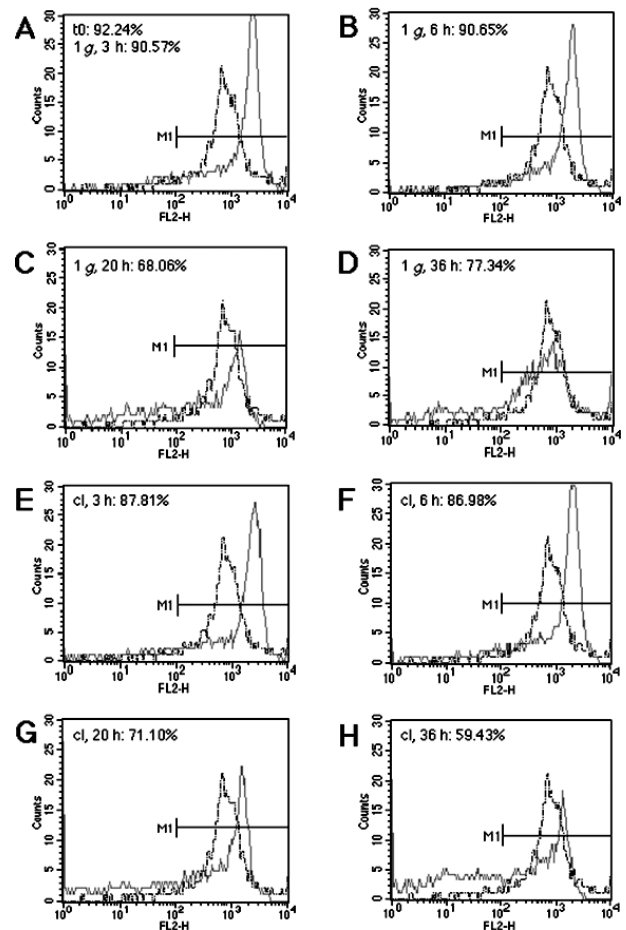

Figure 3. Effect of CD69 and PMA mediated activation on mitochondrial membrane potential in clinorotating CD3+ lymphocytes. The potentiometric probe JC-1 was used to assess changes of mitochondrial membrane potential during activation as described in Section 2.3. After different times of culture in clinostat or at 1 *g* with anti-CD69 and PMA, red and green fluorescence was measured by cytofluorimetric analysis. Overlaid histograms showing the distribution in the red fluorescence channels of t_0 control cells and cells activated for the indicated times are reported. (A-D) Cells at 1 *g* and (E-H) clinorotating cells for 3, 6, 20 and 36 h, respectively. The percentage given in each panel indicates the fraction of cells included in the region defined by marker M1, which excluded 90% of the cells treated with carbonylcyanide-*4*-(trifluoromethoxy)phenylhydrazone. A representative example from four separate experiments is shown.

thermore, as shown by the viability assay, the clinorotating lymphocytes populations included only a low percentage of propidium iodide permeable cells after 48 h activation (6% ± 6), which was similar to the number of cells stained by the dye in control cell cultures (6% ± 5). In T cells cultured under both gravity conditions, proliferation and IL-2 release was triggered by the combination of the calcium ionophore A23187 and PMA, which, when used alone, induced low IL-2 production and DNA synthesis in 1 *g* cells and failed to activate those in clinorotating (data not shown and [6]).
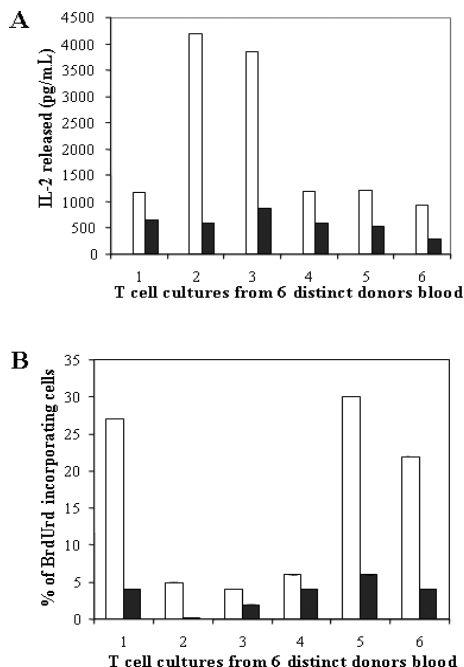
A

B

Figure 4. IL-2 production and BrdUrd uptake by activated CD3+ lymphocytes in clinorotation. Lymphocytes were incubated with anti-CD69 and PMA as in Fig. 3 for 48 h at 1 *g* (open bars) or in clinorotation (closed bars). In the last hour of the culture period, 100 μM/L BrdUrd was added to the cells as described in Section 2.4. Then, IL-2 released in the culture supernatants was assessed by immunoELISA (A) and BrdUrd uptake was evaluated by cytofluorimetric analysis (B). In both (A) and (B) bars 1–6 refer to data of CD3+ lymphocytes cultures from six distinct healthy donors.

3.4 Analysis of proteins expressed by T cells activated at 1 *g* and in clinorotation

In an attempt to obtain some mechanistic information on clinorotating lymphocytes, we undertook a comparative study of the protein expression profile in T cells activated at 1 *g* and in clinorotation. We used aliquots of the above mentioned six cell populations analyzed for IL-2 release and BrdUrd uptake. The cells were lysed and analyzed by 2-DE as described in Section 2.5. Figure 5A shows representative silver-stained 2-DE performed on two different pH gradients. Computer-assisted comparative analysis of the silver-stained spots performed on about 250 matched spots allowed the presence of differently expressed proteins to be estimated. As several experimental conditions may occasionally affect the protein staining, which are not related to a different level of expression, we used standardized methodology in performing the analysis and, to rule out any possible artefacts, the analysis of the quantitative differences was validated by the Kolmogorov-Smirnov test. By this method, we were able to detect 18 proteins whose spot intensities showed statistically significant quantitative differences. Six of these proteins were more represented in clinorotating cells while twelve were more abundant in 1 *g* cells (Fig. 5B). The differently expressed spots were then excised from the gel, digested with trypsin and analyzed by MALDI-TOF MS. Peptide mass fingerprint analysis and nonredundant sequence database matching allowed the unambiguous identification of seven of the analysed species. Figure 5C and Table 1 report the nature of each identified spot, the measured 2-DE coordinates

Table 1. List of spots/protein species sensitive to clinorotation in activated lymphocytes, detected by 2-DE and identified by peptide mass fingerprint analysis

Spot no.	Protein	Exp. Mass (apparent mass, kDa)	Exp. p*I*	Intensity (%)	Coverage (%)	Ref.
61	ATP-dependent DNA helicase II, 80 kDa subunit / *Lupus erythematosus* Ku autoantigen (P13010)	85	5.5	52	15	[28, 29]
83	Moesin / anaplastic lymphoma kinase fusion protein (P26038)	70	6.05	228	22	[34, 35]
118	Ras-GTPase-activating protein binding protein 1 (Q13283)	52	5.28	49	19	[31]
189	Nuclear RNA helicase, DECD variant of DEAD box family (Q9BVP6)	49	5.5	35	20	[30]
258	26S proteasome non-ATPase regulatory subunit 6 (Q15008)	50	5.40	30	24	[32]
356	Annexin A4 (P09525)	35	5.55	205	24	[36, 37]
372	Proteasome activator complex subunit 3 / *Lupus erythematosus* Ki autoantigen (Q12920)	25	5.52	47	31	[33]

The spot number, protein description, accession number (Swiss-Prot entry), experimental molecular mass, experimental p*I*, intensity variation (expressed as percentage of the 1 *g* control) and sequence coverage are listed.
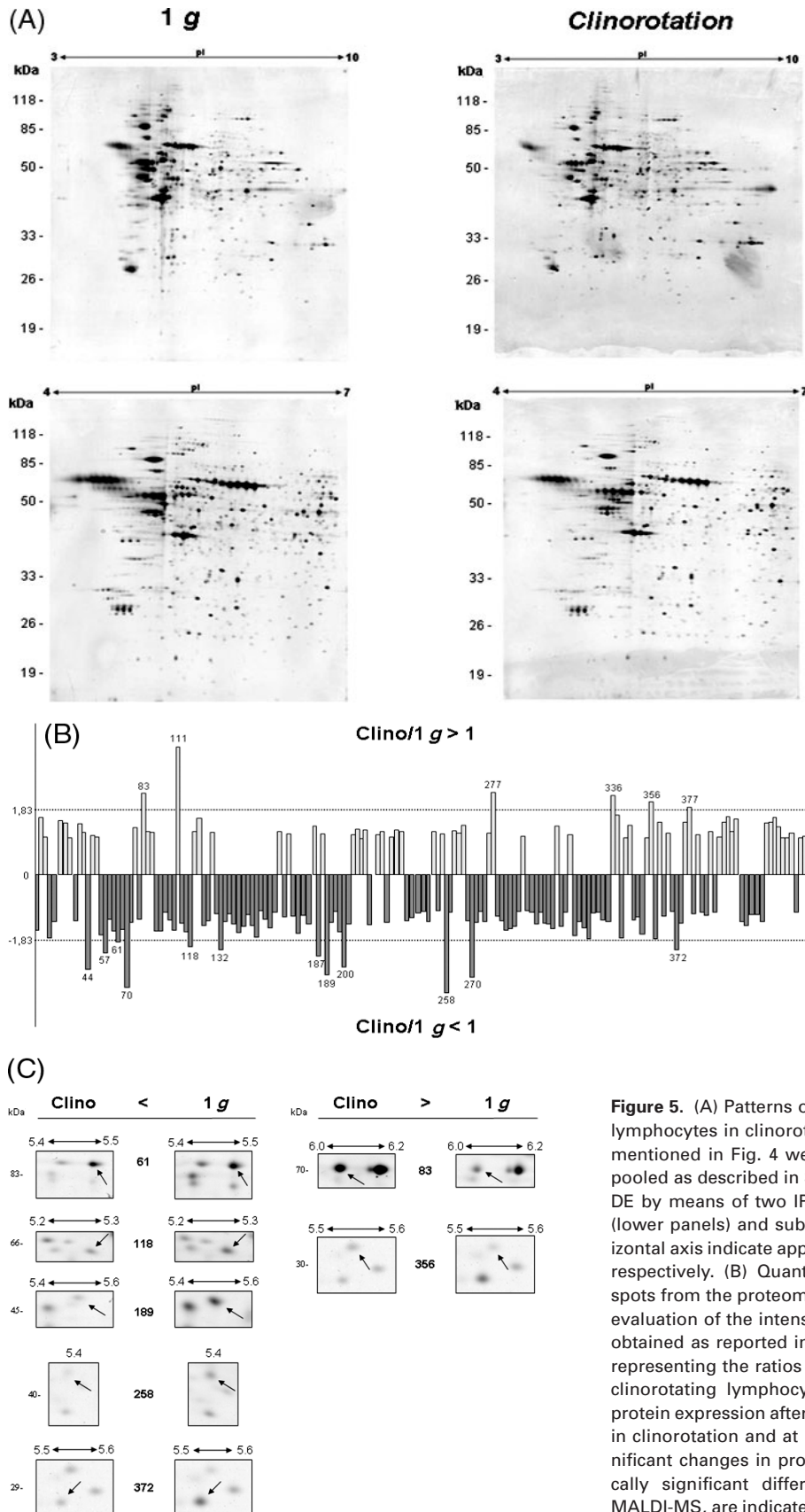
Figure 5. (A) Patterns of protein expression in stimulated CD3+ lymphocytes in clinorotation and at 1 *g*. Cells from cultures 1–6 mentioned in Fig. 4 were pelleted by centrifugation, lysed and pooled as described in Section 2.5. Proteins were analysed by 2-DE by means of two IPGs: pH 3–10 (upper panels) and pH 4–7 (lower panels) and subsequent silver-staining. Vertical and horizontal axis indicate apparent molecular mass (kDa) and p*I* values respectively. (B) Quantitative analysis performed on matched spots from the proteomes of the cell cultures in (A). Quantitative evaluation of the intensity values of each of the matched spots, obtained as reported in Section 2.6, is reported as a histogram representing the ratios of the intensities of matched spots from clinorotating lymphocytes *versus* control cells. (C) Pattern of protein expression after 2-DE analysis of stimulated lymphocytes in clinorotation and at 1 *g*. Regions comprising statistically significant changes in proteome repertoire were cropped. Statistically significant different spots, subsequently identified by MALDI-MS, are indicated by arrowheads and numbers.

and relative sequence coverage. The nature of the mass signals occurring in the spectra excluded the simultaneous presence of different protein species in each analysed spot.

Two of the proteins differentially expressed (ATP-dependent DNA helicase II and nuclear RNA helicase) are involved in DNA repair [28–29] and RNA processing [30]. The former enzyme plays a primary role in immunoglobulin class switching and in overall genome maintenance [28–29]. The latter is 90% identical to the DExH/D box protein UAP56, and is involved in splicing and nuclear export of mRNA [30]. Ras GTPase-activating protein protein binding 1 is an endoribonuclease activity which, as a component of the Ras transduction pathway, regulates mRNA decay through external signals. In particular it promotes the assembly of stress granules and controls mRNA metabolism during cellular stress [31]. The decreased expression of these three enzymes is likely related to the reduced entry of clinorotating T cells into the cell cycle. Finally, two proteins involved in the immunoproteasome machinery (26S proteasome non-ATPase regulatory subunit 6 and proteasome activator complex subunit 3, [32–33]) were down-regulated.

Among the protein species overexpressed in clinorotating condition, we identified moesin and annexin A 4. Moesin is a cytoskeletal adaptor element which is involved in T cell receptor mediated changes in cytoskeletal organization, together with ezrin and radixin [34–35]. Annexin A 4 is a calcium/phospholipid-binding protein which promotes membrane fusion and causes the membrane leaflet to become more rigid, reducing water and proton permeability, and lateral lipid diffusion in the bilayer [36–37]. The remaining spots could not be identified because of the scanty amount of protein sample.

4   Discussion

The microgravity environment affects the immune system, as shown by the observed immunodepression of astronauts during and after space flight. This partial immune deficiency has been related to the stress hormones released during space flight or landing, and to cosmic radiations, to which leukocytes are particularly sensitive, rather than to a direct effect of microgravity on lymphocyte physiology [1]. However, the observed immunodepression *in vivo* seems to well parallel the failure of *in vitro* cultured lymphocytes to respond to activatory stimuli in microgravity, which is detectable even in some of the early phenotypical and biochemical events preceding cell cycle entry such as CD25 expression and IL-2 production [4].

The present data on cells cultured in clinorotation with PMA or phytohemagglutinin confirm previous reports on the ability to these cells to express CD69 [6]. While the appearance of CD69 in clinorotating cells as early as 3 h after the addition of the mitogen is likely due to mobilization of intracellularly stored CD69 molecules [6, 38], its expression in activated cells after 24 h clinorotation, suggests that CD69

up-regulation is not transient and the ability of T cells to enter into a preliminary to commitment step is likely not compromised. The aim of this study was to investigate the signaling capabilities of CD69 in human T lymphocytes under conditions similar to those which occur in cells exposed to microgravity and, by means of a proteomics study, to gain some mechanistic information on T cell physiology. To evaluate the activation potential of CD69 signaling, we used the mAb anti-CD69 MLR3 in combination with PMA, which activates protein kinase C [9]. In *in vitro* cultured T cells, CD69, through interaction with anti-CD69 antibody may trigger a biochemical pathway, which has been investigated in detail, and ultimately leads to the expression of CD25, synthesis of cytokines such as IL-2, tumor necrosis factor, interferon γ and proliferation [11].

Our data show that some of the early events which occur in T cell activation, such as calcium influx [25–26] and mitochondrial potential increase [27], take place also in clinorotation. However, the following steps leading to lymphocyte proliferation are impaired, since over the last 24 h of the 48 h culture, the activation response seems to be down-regulated, as shown by reduced IL-2 synthesis and BrdUrd uptake, and by the decline in mitochondrial membrane potential in a fraction of the cells. In this regard, we cannot rule out that the mitochondrial depolarization in a fraction of the cells at 36 h (see Fig. 3H) is related to progressive apoptosis affecting T cells in clinorotation. Cell death has already been shown to occur when no activation stimulus is added to lymphocyte cultures in conditions similar to those occurring in microgravity [2]. Studies conducted on the leukemic T cell line Jurkat in space have shown that microgravity causes cell growth arrest and apoptosis [39]. However, as mentioned in Section 3, after 48 h activation in clinostat, T cell populations did not show a high percentage of dead cells. Although we have not formally investigated the possibility of apoptosis in clinorotating cells over the 48 h activation period, we are confident that in most cells, this process was not the primary cause of unresponsiveness to the activation signal.

Since our goal is to perform and extend these investigations by means of experiments conducted in the real microgravity experienced in space biolaboratories, the methods and experimental approach applied in the present study were designed on the basis of the constraints imposed either by the use of clinostat or by the requirements of experiments performed during space missions. Therefore, in the present proteomics study we analyzed whole cell extracts from a limited number of lymphocytes and the resolution power of our comparative analysis was restricted to protein species characterized by major expression differences. However, the spectrum of protein alterations underscores the amplitude of the impact that conditions simulating microgravity, have on T lymphocyte physiology and these changes may be related to the observed functional impairment. The overexpression of two cytoskeleton proteins is likely due to the stress caused by gravitational unloading. By means of DNA microarray technology, Lewis *et al.* [40] have already shown that in the

human Jurkat T cell line cultured in microgravity, *i.e.* in spaceflight, for 48 h, ten cytoskeletal genes were over-expressed as compared to ground controls. Changes in cytoskeletal protein expression may in turn affect signal transduction pathways [41], thereby causing lymphocyte incompetency to proliferate.

The low expression of DNA helicase II and of nuclear RNA helicase in clinorotating *versus* 1 *g* cells, is likely related to the cells failure to enter into the cell cycle and to a reduced level of nuclei acid processing and turn over. Also the downregulation of Ras GTPase-activating protein protein binding 1 is likely related to the impairment in cell cycle progression. Finally, our analysis revealed changes in the expression of two proteins associated with the proteasome machinery, which are likely sensitive to the clinorotation condition, although we have not formally studied the significance of their low expression.

5   Concluding remarks

In summary, our study adds CD69 to the signaling receptors whose function is impaired in human T cells exposed to conditions similar to those which occur in microgravity. Although more studies are needed to understand the molecular mechanisms of the activation suppression, the general role played by CD69 in the hematopoietic system makes CD69 mediated activation a helpful model to investigate this issue. The variety of the functions and cellular localizations of the differentially expressed proteins identified in the present study, suggests the need for a comprehensive proteome analysis on all subcellular compartments to better understand lymphocytes anergy in microgravity

6   References

[1] Cogoli, A., *J. Gravit. Physiol.* 1996, *3*, 1–9.

[2] Maccarrone, M., Battista, N., Meloni, M., Bari, M. *et al.*, *J. Leukoc. Biol.* 2003, *73*, 472–481.

[3] Schwarzenberg, M., Cossu, G., Cogoli-Greuter, M., Meloni, M. A. *et al.*, *J. Gravit. Physiol.* 2000, *7*, 9–11.

[4] Walther, I., Pippia, P., Meloni, M. A., Turrini, F. *et al.*, *FEBS Lett.* 1998, *436*, 115–118.

[5] Cooper, D., Pellis, N. R., *J. Leukoc. Biol.* 1998, *63*, 550–562.

[6] Hashemi, B. B., Penkala, J. E., Vens, C., Huls, H. *et al.*, *FASEB J.* 1999, *13*, 2071–2082.

[7] Nelson, B., Willerford, D. M., *Adv. Immunol.* 1998, *70*, 1–81.

[8] Carreno, B. M., Collins, M., *Annu. Rev. Immunol.* 2002, *20*, 29–53.

[9] Farago, A., Nishizuka, Y., *FEBS Lett.* 1990, *268*, 350–354.

[10] Rachmilewitz, J., Lanzavecchia, A., *Trends Immunol.* 2002, *23*, 592–595.

[11] Testi, R., D'Ambrosio, D., De Maria, R., Santoni, A., *Immunol.Today* 1994, *15*, 479–483.

[12] Schwarting, R., Niedobitek, G., Stein, H. in: Knapp, W., Doerken, B., Gilks, W. R., Rieber, E. P. *et al.* (Eds.) *Leukocyte Typing IV; Cluster Report: CD69*, Oxford University Press, Oxford, England 1989, pp. 428–445.

[13] Cebrian, M., Yague, E., Rincon, M., Lopez-Botet, M. *et al.*, *J. Exp. Med.* 1988, *168*, 1621–1630.

[14] Risso, A., Cosulich, M. E., Rubartelli, A., Mazza, M. R., Bargellesi, A., *Eur. J. Immunol.* 1989, *19*, 323–328.

[15] Moretta, A., Poggi, A., Pende, D., Tripodi, G. *et al.*, *J. Exp. Med.* 1991, *174*, 1393–1398.

[16] Esplugues, E., Sancho, D., Vega-Ramos, J., Martinez, C. *et al.*, *J. Exp. Med.* 2003, *197*, 1093–1106.

[17] Cosulich, M. E., Rubartelli, A., Risso, A., Cozzolino, F., Bargellesi, A., *Proc. Natl. Acad. Sci. USA* 1987, *84*, 4205–4209.

[18] Cogoli, A., Valluchi-Morf, M., Mueller, M., Briegleb, W., *Aviat. Space Environ. Med.* 1980, *5*, 29–34.

[19] Greimers, R., Trebak, M., Moutschen, M., Jacobs, N., Boniver, J., *Cytometry* 1996, *23*, 205–217.

[20] Tsien, R. Y., Pozzan, T., Rink, T. J., *J. Cell. Biol.* 1982, *94*, 325–334.

[21] Minta, A., Kao, J. P., Tsien, R. Y., *J. Biol. Chem.* 1989, *264*, 8171–8178.

[22] Risso, A., Braidot, E., Sordano, M. C., Vianello, A. *et al.*, *Mol. Cell. Biol.* 2002, *22*, 1926–1935.

[23] Holm, M., Thomsen, M., Hoyer, M., Hokland, P., *Cytometry* 1998, *32*, 28–36.

[24] Talamo, F., D'Ambrosio, C., Arena, S., Del Vecchio, P. *et al.*, *Proteomics* 2003, *3*, 440–460.

[25] Cantrell, D., *Annu. Rev. Immunol.* 1996, *4*, 259–274.

[26] Testi, R., Phillips, J. H., Lanier, L. L., *J. Immunol.* 1989, *143*, 1123–1128.

[27] Gergely, P. Jr., Niland, B., Gonchoroff, N., Pullmann, R. Jr. *et al.*, *J. Immunol.* 2002, *169*, 1092–1101.

[28] Schaffer, A., Kim, E. C., Wu, X., Zan, H. *et al.*, *J. Biol. Chem.* 2003, *278*, 23141–23150.

[29] Zhu, C., Bogue, M. A., Lim, D. S., Hasty, P., Roth, D. B., *Cell* 1996, *86*, 379–389.

[30] Pryor, A., Tung, L., Yang, Z., Kapadia, F. *et al.*, *Nucleic Acids Res.* 2004, *32* , 1857–1865.

[31] Tourriere, H., Chebli, K., Zekri, L., Courselaud, B. *et al.*, *J. Cell Biol.* 2003, *160*, 823–831.

[32] Ren, S., Smith, M. J., Louro, I. D., McKie-Bell, P. *et al.*, *Oncogene* 2000, *19*, 1419–1427.

[33] Nikaido, T., Shimada, K., Shibata, M., Hata, M., *Clin. Exp. Immunol.* 1990, *79*, 209–214.

[34] Delon, J., Stoll, S., Germain, R. N., *Immunol. Rev.* 2002, *189*, 51–63.

[35] Chu, P., Pardo, J., Zhao, H., Li, C. C. *et al.*, *J. Biol.* 2003, *2*, 21.

[36] Kaetzel, M. A., Mo, Y. D., Mealy, T. R., Campos, B. *et al.*, *Bio-chemistry* 2001, *40*, 4192–4199.

[37] Hill, W. G., Kaetzel, M. A., Kishore, B. K., Dedman, J. R., Zei-del, M. L., *J. Gen. Physiol.* 2003, *121*, 413–425.

[38] Risso, A., Smilovich, D., Capra, M. C., Baldissarro, I. *et al.*, *J. Immunol.* 1991, *146*, 4105–4114.

[39] Lewis, M. L., Reynolds, J. L., Cubano, L. A., Hatton, J. P. *et al.*, *FASEB J.* 1998, *12*, 1007–1018.

[40] Lewis, M. L., Cubano, L. A., Zhao, B., Dinh, H. K. *et al.*, *FASEB J.* 2001, *15*, 1783–1785.

[41] Vicente-Manzanares, M., Sanchez-Madrid, F., *Nat. Rev. Immunol.* 2004, *4*, 110–122.