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EFFECTS OF MICROGRAVITY AND OXIDATIVE STRESS ON HUMAN T LYMPHOCYTES: DIFFERENT APPROACHES TO STUDY THEIR PROTEIN EXPRESSION AND POST-TRANSLATIONAL MODIFICATIONS

Tesi di dottorato

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ABSTRACT

The aim of this research was to study the protein expression and post-translational modifications of human T cells undergone to microgravity and oxidative stress.

During spaceflights the constant influence of weightlessness leads to several modifications of many physiological cellular processes such as proliferation, differentiation, growth, signal transduction, cytoskeletal architecture, motility and gene expression. Several experiments in real and simulated microgravity conditions have shown that immune system function, particularly T lymphocyte function, is altered in more than 50% of space crew members. Although significant data has been accumulated, microgravity's effects on signal transduction need to be elucidated.

We analysed components of the IL2/IL-2R signalling system and cytoskeletal components, known to be negatively affected. Little information is available regarding changes of phosphorylation and their functional consequences in T cells subjected to microgravity. It has the potential to be a valuable indicator of the impact of microgravity on cellular metabolism. As a result of the whole study, clinical problems related to disturbed immune cells functions of astronauts during long-term space flights could be anticipated and prevent.

T lymphocytes are associated with several immune diseases such as lymphoid leukemia and lymphoma. Many pathologies appear to be directly associated or otherwise related to oxidative phenomena, which influence protein phosphorylation changes. This kind of responses is considered as molecular switch in the pathways that regulate cell proliferation and carcinogenesis.

In this thesis T cells response in extreme conditions was evaluated to better clarify the chemical complexity of lymphocytic proteome. Particularly, I focused the attention on the Spleen Tyrosine Kinase family components, Syk and Zap-70, already known in the literature for their behaviour in pathological states and as possible treatment of some forms of leukemia and lymphoma. Western blot of total proteins of T cells treated in different ways showed a strong phosphorylative response and a consistent expression of Syk kinase.

1. INTRODUCTION

1.1 Space biology

Gravity is the force of attraction by which terrestrial bodies tend to fall toward the Earth and it is one of the four fundamental forces of physics along with electromagnetic force, strong nuclear force and weak nuclear force. The strength of terrestrial gravity depends on the mass of the body being affected and it is inversely proportional to the square of the distance between the object and the Earth. In space, living organisms are confronted with two new important factors: microgravity and cosmic radiation. Microgravity is the result of free fall: essentially, a compensation of the force of gravity by means of an accelerating force. In fact, space systems orbiting the Earth at about 400 km are free fall systems in which the force of gravity is balanced by the accelerating force. Considering all terrestrial life evolved for millions of years in an environment affected by constant gravity of 9.81 m/s^2, it can be hypothesized that changing the gravitational environment can result in important alterations in the biological behaviour of organisms, cells included.

The ultimate goal of research in space biomedicine is the knowledge of the limits of organisms in microgravity conditions, in order to maximize human stay in space in the future. Using microgravity as a model of study will lead to a better life on Earth. In fact, conducting experiments in real and simulated microgravity conditions allows scientists to study the phenomena that play a key role in different fields such as biology, physics and chemistry by removing the effects of gravity. The first experiments in space were carried out thanks to the American National Aeronautics and Space Administration (NASA) and the Russian Federal Space Agency (RKA). Their works established the foundation that allowed the scientific community to develop applications for space technology, explore space and the solar system, and investigate the effects of microgravity and cosmic radiation on living organisms.

In Europe, the gateway to space is the European Space Agency (ESA), an international organisation with 19 Member States, including Italy. ESA's job is to draw up the European space programme and carry it through. The programmes are designed to find out more about Earth, its immediate space environment, the Solar System and the Universe, as well as to develop satellite-based technologies and services. ESA's Italian partner is the Agenzia Spaziale Italiana (ASI), the third contributor after Germany and France.

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The European Low Gravity Association (ELGRA) is a non-profit international society of multidisciplinary nature. ELGRA is dedicated to the promotion of research under various gravity conditions in Europe. The organization provides a platform for all scientists interested in life and physical sciences and technology in space or on ground.

In Russia, the Institute of Biomedical problems (IMBP), in addition to supporting basic and applied research, provides biomedical support for space flights. IBMP is responsible for performance of fundamental and applied scientific research, experimental design and technological activities in various areas.

In Italy, the promotion of space biomedicine is carried out by the Italian Society for Space Biomedicine and Biotechnology (ISSBB), an apolitical, scientific association that occupies a prominent position in the scientific community. ISSBB's aim is to study and deepen our knowledge of biomedicine and biotechnology as related to the particular environmental conditions encountered in space.

Over the past three decades, space life science has made great progress: many facilities have been developed, giving the scientific community several opportunities for space flights. The obtained data has great influence on the current way of perceiving the role of gravity. As a consequence of the interest in exploration, time spent in space has increased.

The development of space biology can be divided in four phases. The first, from the early seventies to the mid eighties, mainly involved studying living organisms randomly in an effort to understand how they were affected by the space environment. The second phase, up until the mid 90s, was characterised by the discovery of important effects microgravity has on cellular mechanisms. In the third phase, experiments in space were useful tools for basic research. The major topics were gene expression, cell-cell interactions, cytoskeletal changes and signal transduction. In the last ten years, space biology studies have been aimed at developing biotechnological processes and for possibly finding new strategies for therapeutic intervention on various diseases. Obviously, cellular space biology develops in parallel with the progress of scientific achievements and analytical techniques like microarrays, flow cytometry and proteomics.

The experiments can be done in real microgravity through several vectors and for different spans of time. The choice of a vector depends on the length of the experiment and the kind of information the scientist is interest in. The Shuttle, with its orbiting lab Spacelab, has allowed us to study microgravity effects up to 16 days. These vectors were useful for

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studying the most complex biochemical and physiological mechanisms. Nowadays, in the labs on board of the International Space Station (ISS), it is possible to study the effects of microgravity and cosmic radiation for longer periods (several months). ISS is one of the most extensive civil engineering projects ever carried out. It is the principal platform for scientists to gain access to the environmental conditions in space for undertaking projects across all scientific domains. The ISS is a cooperative programme between US, Russia, Canada, Japan and 11 ESA member States (Belgium, Denmark, France, Germany, Italy, Netherlands, Norway, Spain, Sweden, Switzerland and the United Kingdom). It orbits the Earth approximately every 90 minutes at an altitude of 350-400 km.

An essential facility for the cellular experiments on board of ISS is the KUBIK incubator, which can function as an incubator and a cooler, with a temperature range between +6°C and +38°C. It accommodates biological samples and, thanks to a centrifuge insert, it is possible to have 1xg in flight run in parallel with samples in weightlessness.

Another important facility is the Italian Mice Drawer System (MDS), used for in vivo experiments using mouse models for long periods (from 100 to 150 days) to investigate the genetic mechanisms leading to bone mass loss and other microgravity effects on different tissues such as muscles, glands, and the brain. Research conducted with the MDS is an analogue to the human research program, which has the objective to safely extend human presence beyond Earth's low orbit.

Moreover, the European Columbus Laboratory is ESA's biggest contribution to the ISS and it is equipped with a suite of facilities offering extensive research capabilities. Among them, 'BIOLAB' houses experiments on micro-organisms, cell and tissue cultures, small plants and small animals, whereas 'The European Physiology Modules Facility (EPM)' is used to investigate the effects of long-duration space-flight on the human body, contributing to a better understanding of conditions like osteoporosis and balance disorders on Earth.

Life and material sciences can also be studied on board of sounding rockets (Texus, Maser and Maxus), that provide 6 to 12 minutes of microgravity. A majority of sounding rockets launches takes place at the European Space Center (ESC) Esrange, located outside the town of Kiruna, in northern Sweden. It is a base for scientific research with high altitude balloons, investigation of the aurora Borealis, sounding rocket launches, and satellite tracking. Located 200 km north of the Arctic Circle and surrounded by a vast wilderness, its geographic location is ideal for many of these purposes. The sounding rocket program is developed and

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managed by the Swedish Space Corporation (SSC), with ESA as a regular participant. It is a complete, cost-effective concept for short duration microgravity research with international users participation. The first TEXUS rocket was launched in 1977, the first MASER in March 1987 and the first MAXUS in 1991. Sounding rockets are a sort of ballistic missile able to launch to altitude up to 700 km, with nearly vertical ascent and descent trajectories (Ceglia E. et al., 2005). Excellent weightless conditions are met during the free-fall phase of the rocket's payload once the rocket motors have exhausted their thrust and separated from the payload. The rocket's payload continues to rise due to the momentum built up during the launch phase before falling back towards Earth. The period of weightlessness ends just prior to its re-entry phase. The free-fall ends with the re-entry into the Earth's atmosphere and, finally, the deployment of the parachute that lowers the payload to the ground at appropriate impact speed (Beysen D. et al., 2011). The choice of the sounding rocket is suitable for studying the early physiological and molecular events involved in the cell physiology. The availability of real-time data allows experimenters to follow and, if necessary, direct the course of their experiments. Launch preparation activities allow for late access to the experiment modules (up to 4 hours before takeoff).

Parabolic flights conducted on specially configured aircraft provide repetitive periods of microgravity up to 20 seconds long. They are used to conduct short-term scientific and technological investigations in microgravity. In Europe, this service is supplied by NoveSpace in Bordeaux (France) using an Airbus A300 0xg aircraft. During a campaign of three individual flights, 30 parabolas are flown on each flight. On each parabola, there are periods of hyper gravity (1.8-2xg) that last about 20 seconds, immediately prior to and following a 20 second period of weightlessness.

Finally, aerostatic balloons are useful facilities for studying the effects of cosmic radiation on biological samples. They make trans-Mediterranean flights, form the ASI's base in Milo (TP, Italy) to Spain, reaching 30-40 km of altitude. On board, dosimeters measure the cosmic radiation components, such as x- and γ–rays, thermal and fast neutrons and allow a reliable evaluation of dose and composition of the cosmic radiation. There also trans-Oceanic balloons and other types of balloons launched from the Arctic and Antarctic poles, where the magnetic terrestrial field is minimal, allowing scientists to carry out specific physical experiments.

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The high cost of experiments on board of space facilities and the limited number of doable experiments do not allow scientists to give continuity to the studies in real microgravity. For this reason, it was considered necessary to develop simulation systems on Earth. Currently, it is possible to carry out studies in simulated microgravity conditions thanks to different and advanced facilities such as the "Bed Rest", the "Rotating Wall Vessel" (RWV) or the bidimentional (Fast Rotating Clinostat) and tridimentional clinostat or "Random Positioning Machine" (RPM) (Dutch Space). The latter is able to simulate microgravity almost perfectly, but on a different scale (FRC 10^{-2}xg and RPM 10^{-3}xg). In fact, experiments carried out in space are from 10^{-6} to 10^{-4}xg, whereas the simulations on Earth are in the order of 10^{-3} to10^{-2}xg. Bed rest studies are precious for simulating the effects of space-flight on the human body with regard to the cardiovascular and musculoskeletal systems and feeding. Lying in a bed tilted at 6° for an extended period with the head lower than the feet produces bone and muscle mass loss and fluid shift very similar to those seen in real microgravity.

The RWV uses rotation to annul gravitational pull; it is useful for studying the effects of microgravity on cell cultures and animal embryos. The RPM (Fig. 3) is a very well established paradigm for microgravity simulation. A prototype was described for the first time by Scano in the early sixties (Scano A., 1963). The modern RPM represents an important qualitative and quantitative improvement of the conditions simulating low-g on ground. This apparatus, located in a room at 37°C, has two rotating frames, driven by two separate motors. It rotates in such a way as to simulate weightlessness by removing the effects of gravity in any specific direction. As the rotation is autonomous and with random speeds and directions, it is termed "Random Positioning Machine" (van Loon J.J.W.A, 2007). A panel for lodging the samples is located in the inner frame and the samples have to be put as close as possible to the centre of the panel, where the microgravity is 10^{-3} xg.

1.1.1 Effects of microgravity on living organisms

Since the first human space-flights, many discoveries on living organisms' capability to adapt to microgravity have been made; nevertheless, our knowledge of gravitational changes effects is still partial.

Understanding the mechanisms of spaceflight-induced pathological states will help to prepare humans for long-duration missions on the ISS and may serve to better understand and

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treat diseases occurring on Earth, such as muscle atrophy, cardiovascular diseases, osteoporosis and immune disorders and to provide new strategies for therapeutic interventions.

To date, hundreds of experiments in space and on Earth have been carried out. The feeling is that cells from all the evolutionary steps can live and proliferate in space, but important changes occur anyway. The observations described so far indicate the notable tolerance of the organisms, perhaps due to homoeostatic mechanisms of balance and adaptation. Nevertheless, microgravity leads to several alterations associated with physiopathological aspects. The effects on bone were seen for the first time during the short flights Vostok III and IV in 1962, where increased urinary calcium in the bones of crew members was observed. Subsequent experiments showed that exposure to microgravity resulted in an accelerated bone loss (1 to 1.5 % a month). Skeletal changes in astronauts after exposure to space flight were similar to those observed after immobilization and/or in ageing on Earth. (Spector E.R. et al., 2009). Microgravity affects muscles as well, leading to loss of muscle mass, strength and endurance, especially in the lower extremities (Zobel B.B. et al, 2012). The neurovestibular system is also affected by gravitational changes, with orthostatic intolerance, motion sickness in space as well as disorientation, nausea and decreased coordination upon return from prolonged missions (Homick J.L., 1979; Clement G. et al., 2005). It has also been demonstrated that space-flights affect the cardiovascular system, causing the redistribution of body fluids away from the extremities, which results in facial fullness and puffy appearance (Broskey J. et al, 2007). The underlying mechanism for these phenomena might lie within cells at the molecular level. Thus, the aim of space cell biology is to understand the cellular and molecular mechanisms operating in the perception of microgravity. Numerous studies in space and on Earth have shown that *in vitro* cells behave differentially under modified gravity conditions as compared to 1xg controls. In particular, essential cellular processes such as proliferation, cytoskeletal architecture, gene expression, growth rate and signal transduction are modified under microgravity conditions (Maccarrone 2003; Hammond T.G., 1999; Ingber D., 1999; Guignandon A. et al., 2001; Cubano L.A. et al., 2001). For example, considering that Mesenchymal Stem Cells (MSCs) contribute to healthy bone maintenance, understanding their response to microgravity will help to identify the mechanisms behind physiological changes occurring during space-flights. It has been demonstrated that in simulated microgravity obtained through RPM human MSCs decrease

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their proliferation with low osteoblast differentiation under osteogenic stimulation. Moreover, a study on human bone marrow cell lines (characterized as osteoclastic precursors) incubated in RPM for three days, showed a clear resorption of the bone, whereas no changes were detected in the control samples (Monici M. et al., 2006).

Epidermial growth factor (EGF) plays a key role in the proliferation of various mammalian cells. Pioneering experiments detected epidermal growth factor (EGF)-induced *c-jun* and *c-fos* oncogenes expression decreased in epidermic cells subjected to real and simulated microgravity (deGroot R.P. et al., 1990 and 1991; Boonstra J., 1999). In addition, cells showed an increased cell rounding, determined by an increased polymerization of actin (Boonstra J., 1999).

Modifications of gene expression, with increased expression of MMP-1 and IL-6, were observed in human fibroblasts. The current hypothesis is that microgravity affects cell architecture, the Rho GTPase function and their cellular signalling (Servotte S. et al., 2005).

In addition, dysfunctions of vascular endothelial cells (ECs) have been described (Versari S. et al., 2007; Mariotti M. et al., 2008). ECs play an important role in homeostasis, blood tissue exchange and vasotonic regulation and they are very sensitive to the physical environment. In particular, simulated microgravity was shown to influence Human Umbelical Vein EC proliferation and differentiation and the results have been validated in space experiments. Despite the considerable amount of data accumulated thus far, the effects of microgravity on living organisms still need be elucidated. The findings obtained so far and future experiments on the alterations of cell behaviour in weightlessness may be exploited for biotechnological and biomedical applications. As a result of further research in this field, clinical problems related to disturbed cell function in astronauts during long-term space flights could be anticipated and prevented.

1.1.2 Microgravity and the immune system

Over the last three decades, evidence has increasingly indicated that weightless conditions can affect single immune cell functions, as well as global immune dysfunctions, caused by alteration of stress-sensitive auto-, para-, and neuro-endocrine mechanisms.

Experiments conducted by American, Russian, and European investigators in dedicated space missions as well as in simulations on Earth have shown that mammalian cells are sensitive to gravitational changes. Among others, the cells of the immune system are

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severely affected by microgravity. A decrease in the functional activity of phagocytes, natural killer cells, T lymphocytes and their receptors was observed after the first space-flights and experiments in simulated microgravity conditions. In addition, this down-regulation of the human immune system can facilitate the growth and virulence of several pathogens, leading to a possible higher risk of infection for the crew members.

Due to their key role in cellular immunity and the complexity of their activation, T lymphocytes have been the object of intense investigation. The events that bring to their complete activation are very complex and take approximately 70 hours. The whole process is characterised by important morphological changes and involves various components such as membrane proteins and their receptors, cytoskeletal proteins, protein kinases, transcriptional factors and oncogenes. Some aspects are still disputed and studied. The molecular mechanisms that lead to the end point of *in vitro* T lymphocytes proliferation is based on three different signals:

1.The first signal, triggered by the TCR/CD3 complex and corresponding to the antigen recognition, can be released by the antigen presenting cell (APc), by anti-CD3 or by the mitogen Concanavalin A (ConA). The latter is a lectin that carries four binding sites for α-glucosides of membrane glycoproteins, miming the *in vivo* first signal and leading to a clustering described as 'patching' and 'capping' of the glycoconjugates. Alternatively, activation can be triggered by phorbol esters and calcium ionophore, since the former agents directly activate protein kinase C, while calcium ionophore promotes a sustained increase in intracellular calcium levels, which is necessary for prolonged activation signalling. After the recognition of the first signal the activation process starts: G proteins are induced to activate the phospholipase C (PCL). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). DAG activates the protein kinase C (PKC) that increases its affinity for Ca^{2+}. IP3 stimulates the release of Ca^{2+} from endoplasmic reticulum to cytosol. This ion induces a conformational change in various enzymes, such as MAP kinases Ras and Rac, that activate nuclear factors such as AP-1 and NF-kB;

2. The secretion of interleukin-2 (IL-2) and the interaction between T lymphocytes and accessory cells (ACs, in general monocytes) through CD28/B7 triggers the production and secretion of interleukin-1 (IL-1), required as the second activation signal (Landis R.C. et al.,

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1991). IL-1 activates Rap which itself activates PKC, thus triggering the second activation cascade.

DAG contributes, probably synergistically with IL-1, to activate PKC. The second signal is processed in the cytoplasm by phosphorylation of cytoplasmatic proteins. The end point of this cascade is the increase of IL-2 production and secretion and the subsequent production of its receptor IL-2R, inserted in the cell membrane (Gaulton G.N. and Williamson P., 1994).

3. The third signal is triggered by the interaction between IL-2 and its specific receptor IL-2R; it is an autocrine signal in which the IL-2 bond to its membrane receptor is considered the key step in the whole activation, leading the cells from G0 phase to G1 and hence to mitosis. Therefore, cells start to divide into two populations, the effectors and the memory cells with a maximum 72h after the addition of the mitogen. The IL-2/IL-2R interaction triggers the full activation of the lymphocyte through the JAK/STAT pathway (Janus Tyrosine Kinase/Signal Transducers and Activators of Transcription). The kinase function of JAK is activated and it can autophosphorylate itself. The STAT protein then binds the phosphorylated receptor, where STAT is phosphorylated itself. The phosphorylated STAT protein binds to another phosphorylated STAT protein, forming a dimer, and translocates into the cell nucleus. Here, it binds DNA and promotes transcription of target genes responsive to STAT, with DNA replication and mitosis of the lymphocyte.

A near total inhibition of T cells activation by ConA, determined by the mitotic index measuring the tritiated thymidine incorporated into DNA, was discovered in an experiment conducted in Spacelab 1 (Cogoli A. et al., 1984). The results from Spacelab 1 were then confirmed in 1985 in the Spacelab D1 mission. From this start point, three lines of experiments have been conducted on T cells to try to clarify how microgravity affects cell activation: *in vitro*, *ex vivo* and *in vivo* studies. *In vitro* experiments are based on immune cells isolated from the peripheral blood of healthy donors (not necessarily astronauts), cultured in a standard medium in the presence of mitogen, sent in space and compared to ground controls.

Ex vivo experiments are based on blood samples of space crew members drawn before, during and after the flight, suspended in a suitable medium and incubated in the presence of mitogen. *In vivo* studies consist of the application of antigens onto the skin of the crew members in order to determine hypersensitivity. The objective of *ex vivo* and *in vivo* experiments is to evaluate the efficiency of the immune system in humans exposed to the

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stress of a space flight. The objective of the *in vitro* experiments is to investigate the molecular and cellular mechanisms of T cells activation under the influence of gravitational changes.

Several techniques have been used in space experiments, from the simple determination of the proteins present in the cell cultures to the measurement of gene expression through the reverse transcriptase-polymerase chain reaction (RT-PCR) and the more modern and sophisticated cDNA microarray analysis and the proteomic approach.

Since 1984, the strategy based on the study of the transduction of the three signals has led to interesting results. In particular, experiments conducted on board of sounding rockets have shown that:

- the binding of ConA to the membrane glycoproteins is not affected at 0xg;

- the subsequent steps of patching and capping are slightly delayed;

- interaction between cells and aggregation occurs at 0xg, but less than at 1xg;

- cytoskeletal structure is sensitive to gravitational variations and its alterations influence the bond of ConA to its ligands, compromising signal transduction. In particular, vimentin and tubulin filaments are subjected to significant modifications as early as 30 second exposure at 0xg.

Over the last 30 years, several experiments have been performed in Spacelab and sounding rockets. Such experiments were accompanied by extensive investigations conducted in the ground laboratories in simulated microgravity conditions. T lymphocytes taken from healthy donors on Earth and activated *in vitro* during the space flights with ConA show a lower reactivity and a drastic reduction of their proliferative capability when compared to the ground controls. This inability in proliferation was observed in early experiments done with T lymphocytes of astronauts before, during and after the flight, as demonstrated by pioneering results from Russian labs (Konstantinova I.V. et al., 1973; Talas M. et al., 1984). Analogous observations were then reported by US scientists with an experiment on board of Skylab in 1975; lymphocytes taken from crew members prior to and after the flight were activated with mitogen and a significant decrease of RNA synthesis was reported (Kimzey S.L., 1977).

Experiments using the RT-PCR technology in simulated microgravity conditions showed that the expression of IL-2 is depressed in mitogen activated T cells (Walther I. et al., 1998). In detail, the genetic expression of the α-subunit of the IL-2 receptor (IL-2Rα) was depressed, whereas the β-subunit remained unaffected.

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More recent experiments suggest that microgravity induces apoptosis, as shown in an experiment in RPM: simulated microgravity induced a 6-fold increase of apoptotic bodies and DNA fragmentation, together with an early 4-fold increase in 5-lipoxigenase (5-LOX) activity (involved in the initiation of apoptosis) and a 5-fold decrease in mitochondrial membrane potential and increase in cytochrome c release. Apoptosis was significantly increased as early as 24h in RPM and reached maximum levels between 24 and 48 hours of incubation (Maccarrone M. et al., 2003). Later, the ROALD experiment flown on the International Space Station as part of the BIO-4 mission of the ESA showed that exposure of human lymphocytes to microgravity for 48 hours remarkably increased apoptotic hallmarks, such as DNA fragmentation (∼3-fold compared to ground-based controls) and cleaved-poly (ADP-ribose) polymerase (PARP) protein expression (∼3-fold), as well as mRNA levels of apoptosis-related markers such as p53 (∼3-fold) and calpain (∼4-fold); these changes were paralleled by an early increase of 5-LOX activity (∼2-fold) (Battista N. et al., 2012).

Recent studies in simulated microgravity conditions (RPM) with cDNA microarrays and quantitative real time RT-PCR technologies have revealed an impaired induction of early genes regulated primarily by transcription factors such as NF-kB, CREB, ERK, AP-1 and STAT compared with normal gravity controls. After activation of human T lymphocytes with ConA and anti-CD28 and loading for 4 hours onto the RPM, a significant differential gene expression was identified. The most highly differentially expressed genes encoded cytokines, chemokines and, as cited above, members of the tumor necrosis factor superfamily as well. (Boonyaratanakornkit J.B. et al., 2005).

A recent experiment conducted on board of ISS investigated the global gene expression pattern of human T lymphocytes after 1.5 hours of activation by ConA and anti-CD28 in order to identify the immediate early genes whose transcription may be inhibited in microgravity. The analysis reported that 47 genes significantly down-regulated in microgravity samples. In particular, transactivation of Rel/NFkB, CREB and SRF gene targets were down-regulated. An analysis of gene connectivity indicated that the tumor necrosis factor (TNF) pathway is likely a major early downstream effector pathway inhibited in microgravity and may lead to an ineffective pro-inflammatory host defence during space-flights (Chang T.T. et al., 2012).

Understanding gene expression and especially protein expression could be the key to uncovering the mechanisms through which microgravity affects the immune and other

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systems and eventually to finding countermeasures. The studies cited above used different and valued tools such as fluorescent microscopy, RT-PCR, flow cytometry and microarrays to evaluate the effects of microgravity on gene and protein expression, but very few works have been done to study changes in the protein profile. A systematic investigation of protein expression is still missing and proteomics undoubtedly represents an innovative approach.

The depression of the immune response does not place the astronaut in a pathological state, nor does this phenomenon put his/her life at risk. Nevertheless, it is a fundamental problem that must be solved before longer duration missions (i.e. to Mars and back) can be attempted. In this light, in vitro cell studies in simulated microgravity could be helpful. Future research should focus on delineating the specific mechanisms of how microgravity causes dysregulation of the signal transduction pathways, in order to further clarify the molecular basis of space-flight immunosuppression and find possible countermeasures.

1.2 Cellular stress response

Stress is a term commonly used today but increasingly difficult to define. It shares common meanings in both biological and psychological sciences. The concept of stress was introduced for the first time by Robert Hooke (1635–1703) as a primary mechanistic term for certain physical principles. In biology "stress" came to signify the response of the body to different noxious loads or stressors. It was argued that no external event is stressful in itself, although many events will induce psychological distress in most people (Lazarus R.S. et al., 1985). Later, the term "homeostasis" was coined, referring to a set of acceptable ranges of values of internal variables (Cannon W.B., 1932). In an organism, during homeostasis there is an equilibrium between the net growth rate and the net death rate of cells. The biochemical processes are aimed at maintaining a state of equilibrium. Environmental factors, internal or external stimuli, continually disrupt homeostasis. Every condition that deviates from homeostasis and hence from an optimal condition for living, can be interpreted as stress.

Thanks to numerous pioneering studies (Burchfield SR., 1979; Borysenko M. and Borysenko J., 1982; Chrousos G.P. and Gold P.W: 1992), today it is well known that an organism can be subjected to environmental stress such as heat, UV light, high metals, allergens, or to physiopathological states, such as tissue injury and bacterial or viral infections. Mitochondria are the principal mediators of cellular stress systems. Apart from providing most of the body's energy requirements, they have numerous other functions

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including crucial roles in lipid metabolism, calcium regulation, intracellular signalling, and initiation of cell death pathways (Manoli I. et al., 2007).

Depending on the severity of the stress, cells can respond in a variety of ways ranging from the activation of pathways that promote survival, to initiating programmed cell death if the stress is beyond cell's tolerance (Fulda S. et al. 2010). In the first case cells can count on different defence mechanisms. On the one hand, the inactivation or down-regulation of many genes will occur and hence the down regulation of most of the proteins. In particular, in case of glucose starvation, inhibition of glycosilation, disturbance of Ca^{2+} homeostasis and oxygen deprivation, a set of pathways known as Unfold Protein Response (UPR) is activated.

On the other hand, stress genes are selectively activated to rapidly synthesize 'stress proteins' necessary for cellular repair. This class of proteins includes the glucose-regulated proteins (GRPs), the heat shock proteins (HSPs) and the ubiquitin (Moseley P., 2000). Many of the stress proteins are present continuously (constitutive expression), whereas the expression of others is increased by stress (stress inducible). GRPs are the most studied UPR targets and were originally identified as proteins produced in glucose starvation conditions (Shiu R.P.C. et al., 1977). More recent studies highlight how GRPs promote cells survival in hypoxia, ischemia and neurodegeneration (Tamatani M. et al., 2001; Tanaka S. et al., 2000; Uehara T. et al., 2006).

The HSPs are so-called because they were originally described as the biochemical response of cells to mild heat stress in Drosophila tissues (Lindquist S., 1986). It was later found that HSPs belong to the molecular chaperone family of proteins and that they refold other cellular polypeptides with aberrant conformations (Ellis R.J., 2007). .Their presence can be found across the biological spectrum from bacteria to humans.

Nowadays it is well-known that many stimuli, i.e. oxidative stress and heavy metals, can increase HSP synthesis drastically. In general, they are pro-survival and anti-apoptotic molecules. The inducible regulation of HSPs genes transcription is mediated by a family of heat shock factors (HSF) that responds to a number of pathological states (Morimoto R.I. et al., 1996). In particular, in eukaryotic cells the ability to respond to stress by increasing the HSP levels is due to a unique Heat shock factor (HSF1) that can bind the nucleotide sequence in the 5' sequence which works as a promoter for mRNA synthesis (Elliott T. 1998). Inactive HSF1 is maintained in a monomeric form in the cytoplasm through the interaction with HSP90 and when the cell is exposed to the stress stimulus, the factor is released and can

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translocate to nucleus and bind to DNA. Abnormal levels of HSPs have been found in several disorders, including atherosclerosis, autoimmune diseases, tumours and heart failure. Not surprisingly, they represent dominant antigens in many infectious and autoimmune diseases that induce strong humoral and cellular immune responses. Indeed, HSPs have been shown to be taken up by dendritic cells (DCs), the most efficient APCs (Antigen Presenting Cells) (Steinman R.M. et al., 1983; Heath W.R. and Carbone F.R., 2009).

1.2.1 T cells response under stress conditions

Stressful events have deep effects on a variety of immunological mechanisms. Pathogens have attempted to penetrate organisms for millennia. Consequently, the mechanisms activated for defence have evolutionarily developed to rely upon structures that bar their entry, or track and destroy them. The immune system, together with the nervous system, is the main protagonist in this adaptive process (Dragoş D. et al., 2010). Several studies on stress-associated immune dysregulation focused on the interactions between the Central Nervous System (CNS), the endocrine system and the immune system. Lymphocytes, monocytes and granulocytes exhibit receptors for many neuroendocrine products that can cause alterations in proliferation, cytokines secretion, antibody production and cytolytic activity (Madden K.S. et al., 1995). Both animal and human studies have provided convincing evidence that these immune alterations have substantial implications for human health. Numerous studies have shown that a brief exposure to stress increases the number of circulating CD8 suppressor/cytotoxic T cells, whereas chronic stress decreases lymphocytes' proliferative ability in response to mitogens. Stress may also reduce the percentages of total T lymphocytes and of T helper lymphocytes in the body (Naliboff B.D. et al., 1991; Kiecolt-Glaser J.K. et al., 1991; Andersen B.L. et al., 1998). Therefore, in individuals who produced delayed, weaker and shorter-lived immune responses to vaccines, it is reasonable to assume that a slower immune response to other pathogens would also be developed (Padgett D.A. and Glaser R., 2003). Based upon this hypothesis, further investigation showed that adults with poor response to vaccines also experienced high rates of illness as well as long-lasting infectious episodes (Burns E.A. and Goodwin J.S. 1990; Patriarca P.A. 1994).

Although it is now clear that stress triggers complex response mechanisms and that the immune system plays a key role in an organism's response to a stressful condition, the mechanisms linking stress with immune mediators, including T lymphocytes, are not fully

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understood. A literature review reveals that few works have been carried out to clarify changes in T cell protein expression and in their subsequent modifications under stress conditions. It is well-known that T cell activation is controlled by a coordinated web of tyrosine and serine kinases. It is also estimated that one third of all human proteins are phosphorylated (Cohen P., 2001). The combinatorial action of specific kinases and phosphatases determines the cells' response to different stimuli. To date, an interesting comparative study has been conducted to identify and quantitatively assess tyrosine phosphorylation events in response to IL-2 stimulation in a human T cell line (Osinalde N. et al., 2011). The most over-represented protein categories were cellular growth and proliferation, cellular development and cell death, showing that apart from its immunostimulatory effect, IL-2 is involved in other essential processes. Moreover, phosphotyrosine networks triggered by CD3 and CD28 co-stimulation were analyzed. Results showed an increased tyrosine phosphorylation of CD28, MAPKs, PLCγ, and a few adaptor proteins in comparison with cells activated with either CD3 or CD28 (Kim J.E. and White F.M., 2006).

1.3 Oxidative stress: general aspects

Oxidation is an important condition presents in many physiological and pathological states. Reactive oxygen species can induce and modulate a variety of biological responses, including gene expression, cytokine production, differentiation, proliferation and apoptosis. The most likely mechanism of redox regulation is the post-translational modification of protein thiols. A number of proteins involved in cell signalling pathways contain critical thiol groups whose oxidation alters their activity properties. These include some kinases, phosphatases and transcription factors. While individual proteins have been identified as redox-sensitive, it is not clear how many proteins become oxidized in a cell during signalling, or which changes are critical to the subsequent response (Baty J.W. et al., 2002). Research is devoting more and more attention to oxidation phenomena because they seem associated not only with some physiological states but also with the onset and course of certain diseases.

In healthy conditions, there is a balance between oxidant production and antioxidant defence. The body generates about 5 g of reactive oxygen species (ROS) per day by leakage from the electron transport chain during oxidative phosphorylation (Stein T.P. 2002). The ROS are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), single oxygen, hydroxyl radical

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(OH$^{\cdot}$), peroxy radical and nitric oxide (NO$^{\cdot}$) that reacting with O$_2^{\cdot-}$ forms peroxynitrite (ONOO^{-}). Normally, they are metabolized by enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidise and glutathione (GSH). ROS production plays a vital role in cell signalling, mitogen response, cell differentiation and apoptosis.

From the physiological viewpoint, the important role of redox signalling between mitochondria and NADPH oxidase has been demonstrated (Daiber et al , 2010), as well as the association between changes in oxidative homeostasis and skeletal muscle (Musarò et al., 2010). The importance of oxidative phenomena in relation to cardiovascular homeostasis system have also been described (Olson et al , 2010).

Recent research has focused on elucidating the exact function of cysteine oxidation in physiological conditions (Leonard S.E. and Carroll K.S., 2011). Indeed it has been demonstrated that oxidized cysteine residues play a key role in metabolism and stress signalling in prokaryotic and eukaryotic organisms and can be considered as a new reversible type of regulatory post-translational modification (Klomsiry C., Karlplus P.A., Poole L.B., 2011).

In spite of their important role in physiological states, ROS are among the most potent threats for cells. Some of the ROS can be converted by redox reactions with transition metals or other redox cycling compounds into more aggressive radical species that can cause extensive cellular damage (Valko M. et al., 2005). Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and the ability of cells to detoxify the reactive intermediates with specific antioxidants or to repair the resulting damage. The antioxidants can interact with and stabilize free radicals to protect cells and may prevent some of the damages free radicals might cause. They have been considered promising therapy for prevention and treatment of several diseases, especially given the links observed between diets high in fruits and vegetables and decreased risks for cancer. The purpose of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high level within a cell causing a damage. The tripeptide glutathione (GSH) is the major sulfhydryl antioxidant (Anderson M.E. and Meister A., 1983). Cellular antioxidants may be enzymatic; noteworthy examples include catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD). They can also be nonenzymatic (the above mentioned glutathione, coenzyme Q 10, lipoic acid, some vitamins and metals, or phytochemicals such as polyphenols, selenium and flavanoids) (Seifried H.E. et al., 2007). Antioxidants are also classified into two broad

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divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation (Sies H., 1997). These compounds may be synthesized in the body or obtained from many foods (Vertuani S. et al., 2004). Their use to prevent disease is controversial (Meyers D.G. et al., 1996). In fact, while the generation of ROS was once viewed as unfavourable to the health of the organism, advances in research have shown that ROS play crucial roles, as above cited, in normal physiological processes. In spite of these beneficial functions, aberrant production of ROS activity has been demonstrated to contribute to the development of some prevalent diseases.

More recent papers describe the presence of a close relationship between pathologies and oxidative stress, which influence protein phosphorylation changes (Olson K.R. et al., 2010). It has been shown how several haemolytic disorders are related to the increases in oxidative damage to membranes, such as β-thalassemia, G6PD deficiency, sickle-cell anemia and malaria-infected RBCs, and also Alzheimer's disease (James S.A. et al., 2012), Parkinson's disease (Wersinger C. and Sidhu A. 2006), Crohn's disease (Ibbora M. et al., 2011) and cardiovascular diseases (Sawyer D.B., 2011). In addition, some experimental evidence shows that oxidative phenomena occurring at the mitochondrial level are related in the maintenance and proliferation of cancer cells and resistance to certain drugs (Solaini et al., 2011).

1.3.1 Oxidative stress response in T cells

Oxidative stress seems to also be associated with different types of leukemias and lymphomas and further study in this direction should be promising for the understanding of these disease states and their possible treatment (Hole P.S. et al., 2011; Zhou F.L. et al., 2010; Lyu B.N. et al., 2008). Among the most notable examples of immune alterations related to oxidative conditions we can also cite autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (Chauhan A.K. and Moore T.L., 2012) and chronicle inflammation diseases as Rheumatoid Arthritis (Kundu S. et al., 2012) as well as allergies (Tanou K. et al., 2009).

From an immunological point of view, many studies have been carried out on B and T Lymphocytes. In 1994 Staal et al. showed how the intracellular GSH concentration in T cells

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provides a central mechanism for modulating responses elicited by antigen and cytokine signals. They suggested that the defective T-cell function in human immunodeficiency virus-infected individuals could be due, at least in part, to GSH-regulated changes in the signals transduced following cytokine and antigenic stimulations. Subsequent studies using an in vitro model of HIV infection have shown that HIV-infected cells have decreased ability to dethiolate glutathionylated proteins (Ghezzi P. et al., 2002). Recently some studies have investigated the consequences of accumulation of ROS in various immune cells, including T cells and dendritic cells (DC). The relevant effects in T lymphocytes were studied within co-culture experiments using activated macrophages/granulocytes as sources of NO or H_2O_2 or upon treatment with exogenous H_2O_2, respectively (Mougiakakos D. et al., 2009). In particular, numerous investigators have shown that the regulation of the cellular processes in T cells is markedly influenced by the redox state of these cells. In addition, other works have underscored that oxidative stress in this type of cell is not only associated with cancer but also represents an important immune escape mechanism in chronic inflammation (Hofseth L.J.. et al., 2003). Moreover, the work by Baty J.W. and his team on Jurkat cells treated with H_2O_2 showed that sensitive proteins had different functions, such as energy metabolism, protein degradation, structure maintenance and signalling (Baty J.W. et al., 2005). Above all, they found peroxiredoxin 2 and GAPDH as the most oxidant-sensitive proteins. Recently, Lichtenfels R. and collaborators used a 2-DE based approach to better define the key pathways and proteins involved in the response to oxidative stress in the presence and absence of low dose of H_2O_2 in naïve CD45RA^{+} and memory CD45RO^{+} T cells (Lichtenfels R. et al., 2012). The latest studies seem to be promising for the understanding of immune disease and for finding possible therapies (Hole PS et al., 2011).

In order to better understand the redox signalling pathways in T cells that seem to be associated with the onset of different immune diseases and in order to identify new targets for the development of possible therapies, it is advantageous to study the phosphorylated phenomena implicated in oxidative stress conditions. Kinases probably act as molecular switches that carry many signal cascades, particularly in relation to physiological and pathological stress, resulting in a massive phosphorylation of several cellular proteins.

Specific tyrosine kinases are involved in different phenomena that support the development of malignancy in oncological diseases such as proliferation, apoptosis resistance, invasion, angiogenesis and metastasis (Kolch W. et al., 2010). Among the kinases involved in

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the phosphorylation of proteins, the Spleen Tyrosine Kinase (Syk) family is present in abundance in a extensive range of hematopoietic cells such as B cells, T cells (in form of its homologous Zap-70), mast cells, macrophages and neutrophils (Zhou F. et al., 2006). Moreover, Syk is also found in various non immune cells such as fibroblasts, epithelial cells, hepatocytes, neuronal cells and endothelial cells (Yanagi S. et al, 2001). This family of non-receptor protein kinase is characterized by a basic structure that includes two in tandem highly conserved SH2 domains (Src Homology 2) that are essential for regulating the activity of these kinases. There are an interdomain A, a region of about 50 aminoacids between the two SH2 domains and an interdomain B, a region of connection (about 80-100 aminoacids). It is located between the second SH2 domain and the catalytic SH1 domain. The latter consists of about 300 aminoacids. It includes the binding sites for ATP and 2 autophosphorylation sites (Bradshaw J.M. 2010).

Many papers describe Syk kinase as a therapeutic target in inflammatory response (Mourão-Sá D. et al., 2011) and in several diseases such as rheumatoid arthritis (Coffey G. et al., 2011; Yazici Y. et al., 2011), allergies (Lu Y. et al., 2011) and various kinds of cancer (Coopman P.J., 2006). Furthermore, Syk has been widely studied in the red blood cell model and in its association with the band 3 membrane protein and several hematological pathologies (Rosse W.F. et al., 2004; Bordin L. et al., 2005; De Franceschi L. et al., 2008; De Franceschi L. et al., 2011).

Different behaviours of Syk based on different natural stimuli have been recognized. Very preliminary results have shown that Syk acts in different pathways with a pro-apoptotic or anti-apoptotic activities, depending on osmotic and oxidative stress. In the latter condition, Syk may act in ERK or JUNK (leading to inhibition of apoptosis) or on the BCR pathway (Qin S. et al., 1998). The behaviour of Syk in oxidizing conditions and the pathways in which this kinase could be involved remains to be clarified and the functional meaning of post-translational phosphorylation it causes is still controversial.

Accordingly, phosphoproteomic analysis can be a powerful tool for evaluating functional cell status in connection to oxidative conditions. Quantitation in proteomics experiments facilitates the comparison of proteins across various cellular states such as in a receptor stimulation time course experiment. Therefore, it can be a versatile approach to discover the critical nodes leading to cell decisions. Over the last few years, redox phosphoproteomics seems to have become a really promising methodology. It has been used

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to identify the proteins of T lymphocytes that undergo glutathionylation in oxidative stress conditions (Fratelli M. et al., 2002). This approach has shown that many proteins can undergo this modification if treated with either diamide or H_2O_2. Moreover, in 2009 Nguyen V. and collaborators combined the Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) and label-free quantitation through the automated IMAC/nano-LC/ESI-MS system. The aim was to dissect the tyrosine phosphoproteome of the human Jurkat T cell. Among the 178 total unique phosphorylation sites identified in this analysis, 105 were found to be novel. Furthermore, of the 105 newly discovered sites, 69 were changed significantly with Zap-70 removal. This data provides a wealth of information about the hypothetical placement of these sites relative to Zap-70 and the TCR and it highlights the importance of studying the kinetics of the phosphoproteomes of isogenic mutants (Nguyen V. et al., 2009). It has long been known that T-cell antigen receptor (TCR)-stimulated signal transduction results in the activation of transcription factors that enable the expression of genes involved in the activation of T cells. The induction of some of these transcription factors such as NF-KB (Staal F. J. T. et al, 1990; Schreck, R. et al, 1990) and AP-1 (Devary, Y. et al, 1991; Devary, Y. et al, 1992) is redox regulated. It has been demonstrated that phosphorylative redox responses are also decisive in the activation of several pathways, such as p53, BCR, JNK/ERK, p38-MAPK, FcγRI, phosphoinositide 3-kinase (PI3K)/AKT, and JAK/STAT (Rivera A. et al., 2005; Gao Y. et al., 2009; Shen H.M. et al., 2006; Kim W.H. et al., 2008).

In light of current knowledge, it is clear that much remains to be learned about the signalling pathways that involve protein oxidation and the pathophysiological role of redox-based posttranslational modifications. It will therefore be promising to study the tyrosine phosphorylation involved in the oxidative signalling in T lymphocytes and to investigate their functional consequences. Given Syk and Zap-70's different behaviour in conditions of oxidative stress and their importance in regulating signal transduction, part of the focus of this Ph.D. thesis is on their expression in T cells under extreme conditions.

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2. AIM OF THE THESIS

The aim of this Ph.D. thesis is to evaluate the response of T lymphocytes to real and simulated microgravity conditions and to oxidative stress in order to better clarify the complexity of the lymphocytic proteome.

In particular, regarding the effects of real microgravity, the study focused on the expression of cytoskeletal proteins, cytokines and their receptors. Thus, during the second and third years of the Ph.D. programme, I participated in the pre-flight preparation and the setting up of an experiment on board of the sounding rocket Maser 12.

Regarding the simulated microgravity conditions obtained through the RPM and the oxidative stress caused using the two reagents diamide and H_2O_2, attention was focused upon the possible changes in protein expression and on possible different post-translational modifications, such as tyrosine and serine phosphorylations, as well as on specific tyrosine kinases involved in this phenomenon.

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3. MATERIALS AND METHODS

3.1 Cell cultures

The experiments carried out in this thesis were performed using both Jurkat (Jk) cells and T lymphocytes isolated from whole peripheral blood or buffy coat.

3.1.1 Jurkat Cells

Jurkat cell E6.1 clones are an immortalized line of T lymphocyte cells established in the late 1970s from the peripheral blood of a 14 year old boy with T cell leukemia. Cells were maintained in RPMI 1640 (Glutamax-GIBCO, Paisley, UK), traditionally used for growth of human lymphoid cells, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO), 20mM HEPES, 10 ml/l penicillin/streptomycin (GIBCO) at 37°C in a humidified atmosphere 5% CO_2. The cells were subcultured every 3 days at the concentration of $1.5x10^7$ cells/75 cm^2. For each experiment they were suspended at a density of $9x10^5$/ml in fresh medium.

3.1.2 Human T lymphocytes

Experiments in real microgravity conditions were performed after isolating T lymphocytes from male donors' whole peripheral blood (45 ml per donor, age range 25-45) from Kiruna Hospital.

Experiments in simulated microgravity and stress oxidative conditions were performed after isolating T lymphocytes from buffy coat (45 ml per donor) from Sassari Hospital's male donors. The buffy coat is the fraction of an anti-coagulated blood sample that contains most of the white blood cells and platelets following density gradient centrifugation of the blood.

Peripheral Blood Leucocytes (PBLs) were purified by density gradient centrifugation, using the separation medium HISTOPAQUE 1077 (Sigma-Aldrich) according to the Böyum method (Böyum A., 1968 and 1976). Subsequently, T lymphocytes were purified using high affinity CD3+ T-cell enrichment columns (Fig. 1) (R&D Systems, Minneapolis; MN). These columns are designed to purify human T cell populations via high affinity negative selection. PBLs ($3x 10^8$ maximum per column) were resuspended in 2 ml of column wash buffer and

loaded onto T Cell Enrichment Columns. Via F(ab)-surface Immunoglobulin (Ig) interactions, B cells bind to glass beads coated with anti-Ig while monocytes bind to the glass beads coated with Ig via Fc interactions. The resulting column eluate contained highly enriched T cell populations. Total CD3+ cell recovery ranges between 37% and 54% and the purity of recovered cells ranges between 87% and 95%.
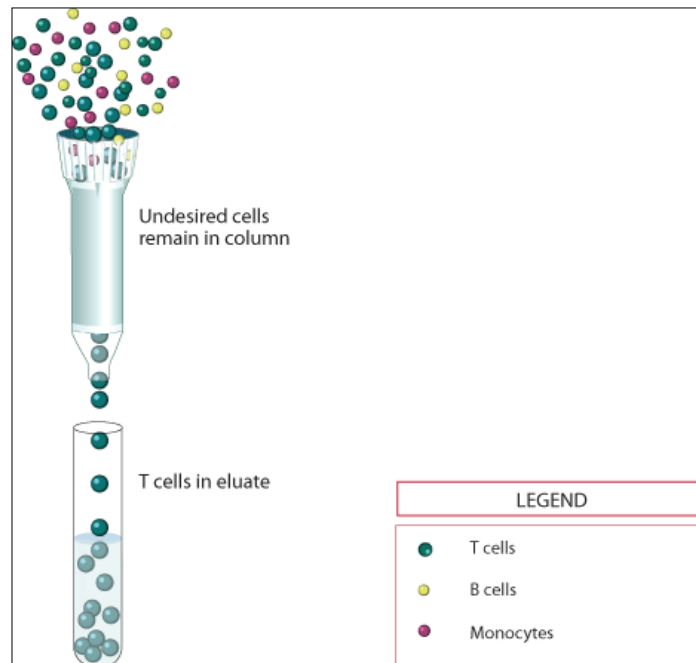

Fig. 1: T Cell Enrichment Columns Assay Principle.

After isolation, T lymphocyte viability was evaluated through a Trypan Blue exclusion test and cells were maintained in RPMI 1640 and supplemented with 10% (v/v) heat-inactivated FBS, 20mM HEPES, 10 ml/l penicillin/streptomycin at 37°C. T cell suspensions were stored for at least 12 h at 37°C to allow recovery from stress due to centrifugation and separation. For the experiments, cells were suspended at the concentration of 9 x 10^5/ml in fresh medium.

3.1.3 T lymphocyte activation

T lymphocytes from blood donors are in G0 (resting phase) but their dividing phase can be activated in vitro by using the mitogen ConA or anti-CD3 and anti-CD28 antibodies. In the experiments presented in this Ph.D. thesis, T cells were activated in different ways:

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- injecting Con A only, in 10 μg/ml concentration. ConA is used not only for its mitogenic activity in T cells but also because it increases synthesis of cellular products,
- injecting 10 μg/ml Con A and 4 μg/ml anti-CD28,
- injecting 4 μg/ml anti-CD3 and 4 μg/ml anti-CD28.

The anti-CD28 is used in a homogeneous culture of lymphocytes to mimic the 'B7' ligand of monocytes. The anti-CD3 is the co-stimulator signal fundamental for the T cells' proliferation.

3.2 Experiments on oxidative stress

This preliminary work has the aim to clarify the role of oxidative stress induced by treating Jurkat cells and T lymphocytes with diamide, reported to be of use as a thiol oxidizing agent, and H_2O_2, physiologically produced by cells and reduced to H_2O and oxygen, at different concentrations. Changes in the polypeptides' functions that accompany protein phosphorylation are also investigated. Diamide is a sulfhydryl reagent which oxidizes sulfhydryl groups to the disulfide form. It has been used to titrate protein glutathiolation to discriminate from other oxidative protein modifications. Treatment increased protein glutathiolation in a concentration-dependent manner and had comparably little effect on protein-protein disulfide formation. Diamide and H_2O_2 were first tested in different dose response experiments in order to decide the concentration to use thereafter. Diamide 0.4 mM (Sigma-Aldrich) or H_2O_2 0.1 mM were added in RPMI 1640 medium to the T cell suspension obtained above. The mixture was incubated at 37°C for different time intervals in order to assess a phosphorylative dose response and then washed four times with Phosphate Buffer Saline (PBS) 1X (150xg, 10 min). Viability of the cells was examined through a Trypan blue dye exclusion test.

3.3 Experiments in microgravity conditions

The experiments relating to the effects of microgravity on T cells were conducted in real microgravity conditions during the space mission on board Maser 12 sounding rocket and in simulated microgravity conditions through the use of the RPM.

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3.3.1 Simulated microgravity: the Random Positioning Machine (RPM)

In the experiments for this thesis the RPM was used to test the microgravity effects on post-translational modification of proteins extracted from Jk cells and T lymphocytes isolated from buffy coat. The RPM at the Dept. of Biomedical Sciences of the University of Sassari was developed by Fokker Space (Leiden, The Netherlands). It is located in a 37°C room and it is under the control of a computer with a dedicated program. Fig. 2 shows the effects of microgravity on the activation of human T lymphocytes and particularly how the simulation with the RPM is comparable with the effects of real microgravity, especially if compared with the results obtained with the old bidimensional clinostats (FRC).
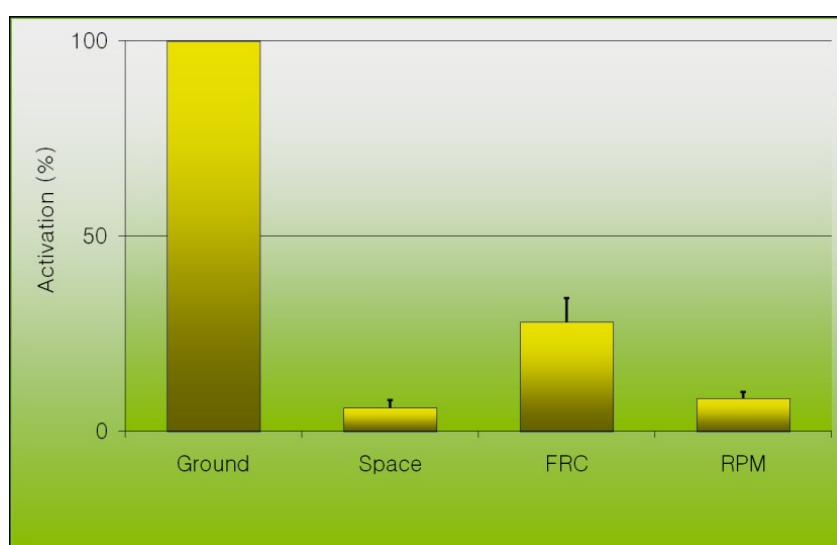
Fig. 2: Comparison between the effects of real microgravity and simulated microgravity obtained through old bidimentional clinostat (FRC) and Random Positioning Machine (RPM).

Before each experiment, T cell viability was evaluated through a Trypan Blue exclusion test. The cells were then resuspended in fresh RPMI 1640 to obtain 1.5x107 cells for each experimental point. First, T cells were activated with ConA only and subjected to 0xg in simulated microgravity for different time intervals (15', 30', 60', 120' and 180'). The rotation velocity of the frames was 60°x s-1. Control T lymphocyte cultures were installed in the basement of the RPM (1xg static controls) (Fig. 3). All the experiments included non-activated T cells as negative controls at 0'and 180'.
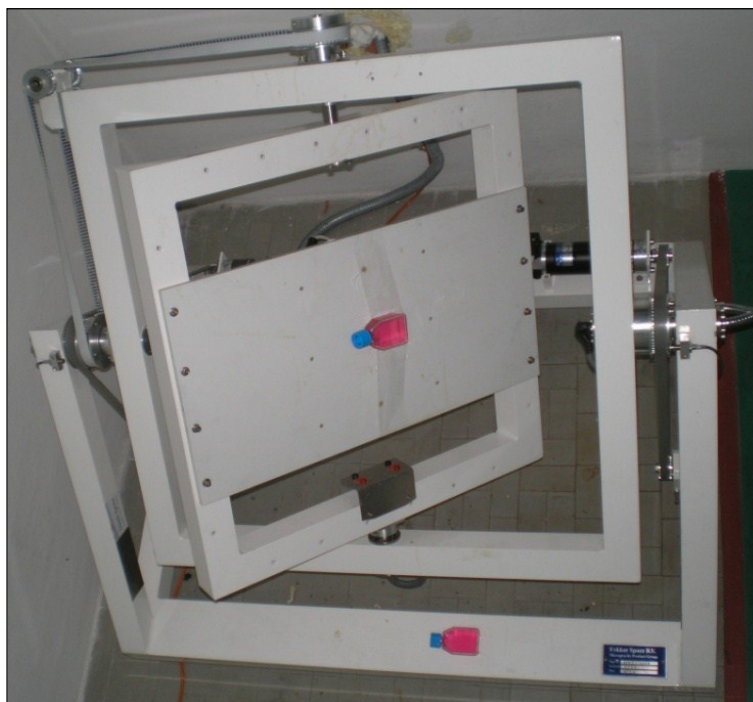
Fig. 3: T lymphocyte cultures subjected to simulated microgravity
in the inner frame of RPM and static controls on its basement.

3.3.2 Real microgravity: 'Maser 12' space mission

Part of the work of this thesis was focused on the study of the effects of real microgravity on T lymphocytes. Thus, during the second and third years of the Ph.D. I participated in the pre-flight preparation and the setting up of an experiment on board the sounding rocket Maser 12. The experiment STIM (Signal Transduction In Microgravity) had the aim to evaluate the expression and function of cytoskeletal proteins, chemokines, cytochines and their receptors. The launch took place the 13th of February 2012 at the Esrange Space Center and the microgravity lasted 6 minutes and 30 sec.

For the experiments, T lymphocytes were isolated from fresh blood donors and then cultured in RPMI 1640 supplemented with 10% FBS.

The experimental plan scheduled the use of 24 hardware devices (Fig. 4) (or 'units'), developed by the Dutch company CCM - Centre for Concepts in Mechatronics.

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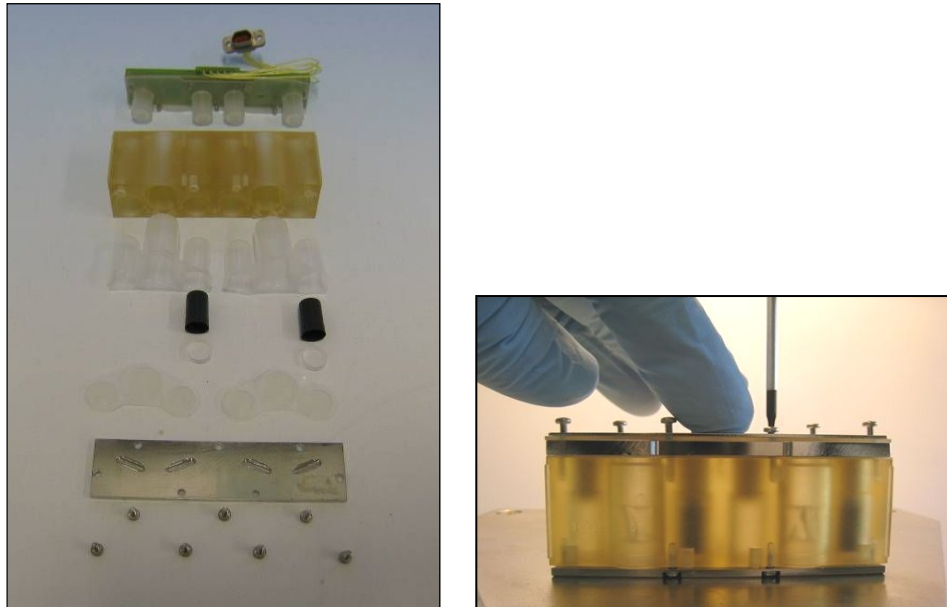
Fig. 4: Left: Components of a mix-unit with electronic activation mechanism (on top) developed by CCM. Right: One of the units ready to be loaded onto the sounding rocket.

Each unit accommodated two experiment mix chambers (Fig. 5, **a** and **b**). Each mix chamber contained 1 ml of T lymphocytes ($5x10^6$/ml) in the culture compartment (the central one). The other two storage compartments were for the mix of activators (0.8 ml, fig. 5b) on the left of the culture compartment) and for the fixative (0.8 ml, fig. 5b) on the right of the culture compartment). Each mix chamber also contained a rubber membrane (silicone or Viton®) (Fig. 5c) in which the fixative formalin was stored, because of its toxicity for cells.

a   b   c
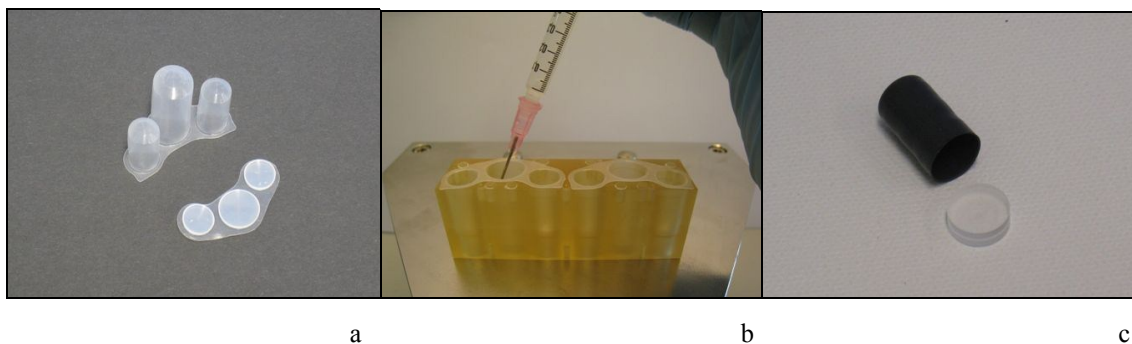
Fig. 5: a) Silicon membrane with storage/culture compartments and silicon membrane bottom. b) Filling the membranes with T cell cultures. c) Viton and silicon bottom filled with formalin.

During the flight, one automated plunger activation mechanism initiated the confluence between the activators and the cells, while a second plunger initiated that between fixative and activated cells in a subsequent phase (Fig. 6).

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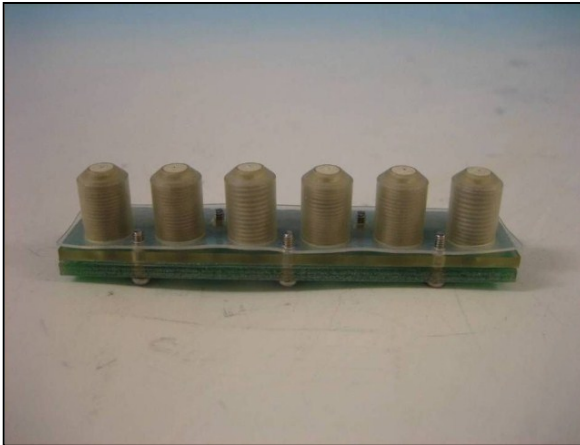
Fig. 6: Electronic version of the plunger activation mechanism.

There were 24 units in total, eight of them in static conditions in the rocket at 0xg, and an additional eight in a centrifuge on board (in flight 1xg control). Eight control experiments were on ground (1xg) in the laboratory (Figures 7 and 8).
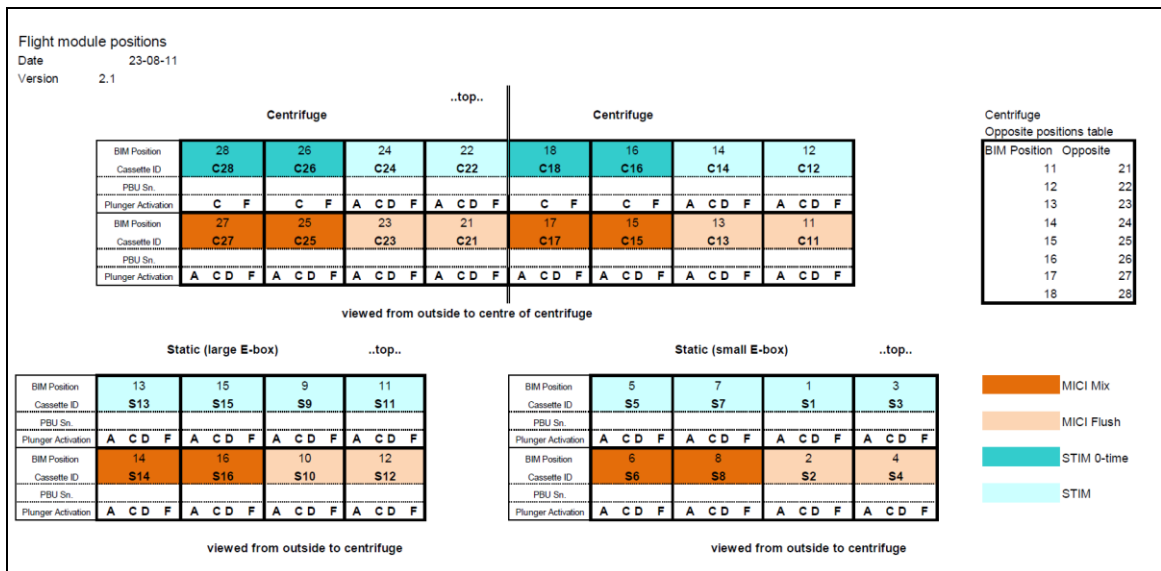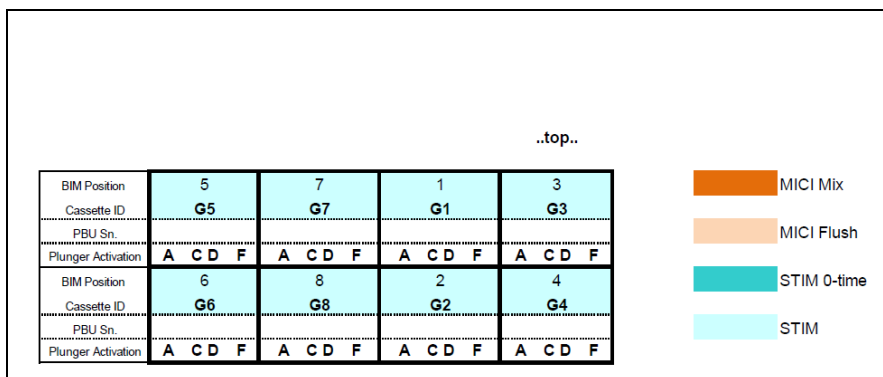

Fig. 7: Units position on board of Maser 12.

Fig. 8: Ground controls.

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Four hours before the launch, the 'in flight' units were housed inside a LAI (Late-Access Insert), a thermally isolated compartment inserted into the cylindrical module of the rocket (Fig. 9a).

a   b   c
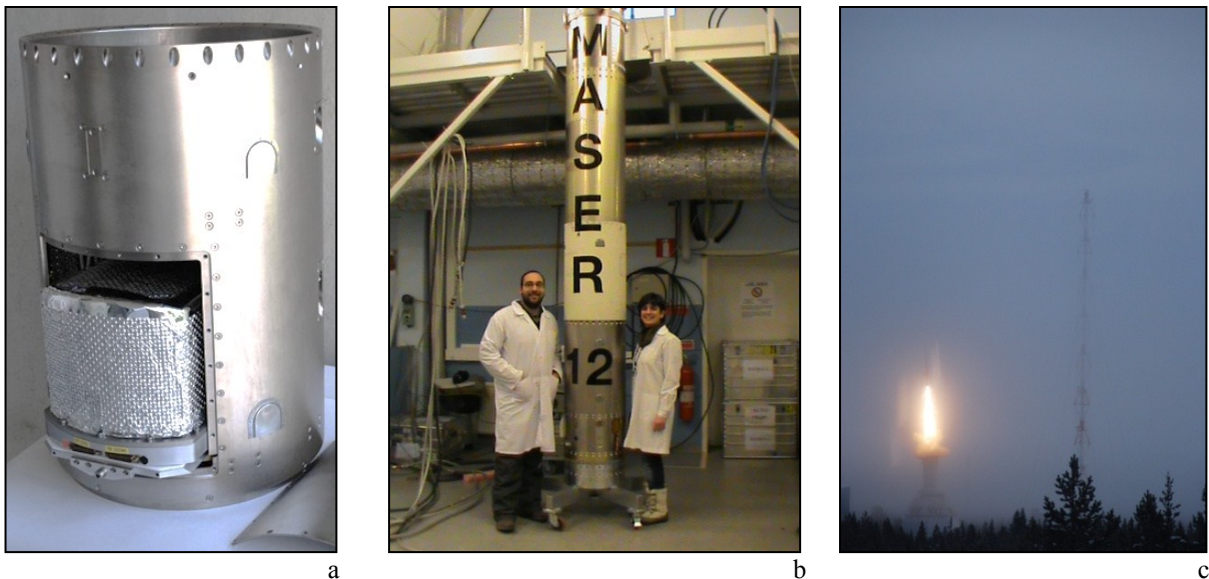
Fig. 9: a) The BIM (Biology in microgravity) experiments were housed inside a LAI (Late-Access Insert), a thermally isolated compartment inserted into the cylindrical module. b) The sounding rocket Maser 12 ready to be moved to the launch tower. c) Lift off of the rocket.

At the beginning of the microgravity period, the T lymphocytes in the eight units at 0xg were activated with the mix of ConA and anti-CD28. T lymphocytes of four of the eight units at 1xg were activated as well, whereas the cells in the other four devices were fixed at the beginning of microgravity period, without being activated (Fig.7, 0-time baseline controls). At the end of the period of microgravity, the activated cells were fixated with formalin. The ground controls were activated and fixed in synchrony with the samples on the rocket. The rocket landed 756 seconds after the launch and was returned to the launching area 2 h 26 after lift-off (Fig. 10). The hardware devices were transported to the laboratory for sample collection (Fig. 11).

Fig. 10: The payload of the sounding rocket after landing.

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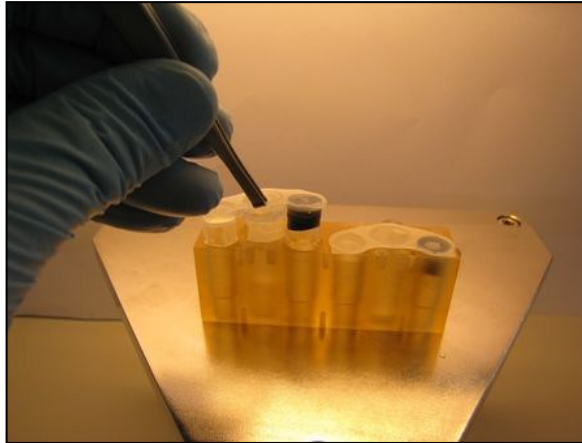
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Fig. 11: Collection of the samples.

After retrieval of the hardware devices from the rocket, all samples were centrifuged (300xg, 5 min), supernatants were removed and cells were resuspended in Stain-buffer (BD Biosciences). Each pellet was split into 20 subsamples. The post-flight FACS analysis was carried out by our mission partners (University of Zurich).

3.4 Study of protein expression by flow cytometry

The analyses of the samples flown on board Maser 12 were performed by flow cytometry. Unstained cells for FACS analysis were discarded.

For surface staining of CD3 and IL-2R, samples were not permeabilized, while for all other stainings cells were permeabilized (intracellular staining) with Perm Buffer III (BD) according to the manufacturer's instructions. Cells were centrifuged, supernatants were removed and cells were resuspended in 80 µl Stain Buffer containing Alexa Fluor® 488-conjugated antibodies.

Antibodies were used in the following dilutions: CD3 (PromoCell, PK-AB913-144)1:80; IL-2R (PromoCell,PK-AB913-104), 1:40; β-Tubulin (Cell Signaling, 3623), 1:640; Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) (Cell Signaling, 4344), 1:160; Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, 4374), 1:160; α-Tubulin (Cell Signaling, 8058), 1:640; Zap-70 (D1C10E) (Cell Signaling, 9473), 1:80; Acetyl-Histone H3 (Lys9) (Cell Signaling, 9683), 1:400; Phospho-Histone H3 (Cell Signaling 9708), 1:40; Vimentin (Cell Signaling, 9854), 1:320; Mouse anti-LAT (pY171) (BD Bioscience, 558519), 1:8; Mouse (MOPC-21) mAb IgG1 Isotype Control (Cell Signaling, 4878), 1:80; Rabbit (DA1E) mAb IgG Isotype Control (Cell Signaling, 2975), 1:40. After 30

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min of incubation 250 µl Stain Buffer were added, and cytometry was performed using a FACSCanto II (BD) or the CyFlow (Partec). FlowJo software (TreeStar) was used for the data analysis. Only cells that appeared single and alive according to forward- and sideward-scatter were analysed. Results are expressed as the relative fluorescent intensity (RFI) which is calculated by dividing the median fluorescent intensity (MFI) of the test antibody by the MFI of the isotype-and species-matched unspecific control antibody.

3.5 Study of the post-translational modifications by proteomic approach

A proteomic approach was utilized in the simulated microgravity and oxidative stress experiments to studying the post-translational modifications..

3.5.1 SDS-PAGE

Total protein content was quantified using DC Protein Assay (Biorad), a colorimetric assay for protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay (Lowry O. H. et al., 1951). The assay is based on the reaction of protein to an alkaline copper tartrate solution and Folin reagent. Proteins reduce the Folin reagent by causing a loss of 1, 2, or 3 oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm. Protein concentration was determined as instructed by the manufacturer, turning out to be about 3 µg/µl in Jk cells and in T lymphocytes as well.

In order to perform one dimensional electrophoresis (1-DE), proteins were solubilised in a Laemmli Buffer (Laemmly U.K., 1970) in a volume ratio of 1:1. 2% DTT was added to the solution. The samples were heated for 5 min at 95°C and then separated on an 8% polyacrylamide mini gel (7 cm), with acrylamide-bisacrylamide in a ratio of 29:1. 10 µg total proteins were loaded for each lane. SDS-PAGEs were run in duplicate on the Bio-Rad mini-protean Tetra cell set up.

3.5.2 Western Blotting

For the western blotting analysis, 30 µg of proteins were loaded onto each lane of minigels. They were then transferred to nitrocellulose membranes (Towbin H. et al., 1979) as instructed by the manufacturer. Membranes were blocked in PBS/T (Na_2HPO_4 (8.5 mM),

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KH$_2$PO$_4$ (1.47 mM), KCl (2.68 mM), NaCl (137 mM), 0.1% Tween20). Proteins separated were probed with either anti-phosphotyrosine anti- phosphoserine antibodies (Santa Cruz, CA) or anti-Zap-70 (Cell signaling), all diluted 1:2000. Secondary antibodies conjugated to infrared fluorescent dyes excitable at 800 nm (IRDye 800 CW, Li-COR-USA) were then used in a 1:2500 dilution to visualize the desired antigens using an 800 nm double laser scanner (Odyssey, Licor, USA).

3.5.3 Mass spectrometry

In parallel, the second gels were coloured with Coomassie Blue Brilliant mass-compatible (Barbero G. et al., 2006) and bands of interest were cut to allow the identification in mass spectrometry by peptide mass finger-printing. In detail, Coomassie stained bands were excised from gels and proteins were digested with trypsin. Each piece of gel was de-stained by way of several washes in 5 mM NH$_4$HCO$_3$/acetonitrile (50/50 v/v) and successively dried with pure acetonitrile. The gel slices were rehydrated for 45 min at 4°C in 20 µl of a 5mM NH$_4$HCO$_3$ digestion buffer containing 10 ng/µl of trypsin. Excess protease solution was removed and the volume adjusted with 5 mM NH$_4$HCO$_3$ to cover the gel slices. Digestion was allowed to proceed overnight at 37°C (Barbero G. et al., 2006). Samples were loaded onto a MALDI target using 1 µL of the tryptic digests mixed 1:1 with a solution of alpha-Cyano-4-hydroxycinnamic acid [10 mg/mL in acetonitrile/trifluoroacetic acid 0 1%, 40/60]. MS analysis of peptides were performed with a MALDI-TOF Micro MX (Micromass, Manchester, UK) operating the instrument in reflectron modality according to the tuning procedures suggested by the manufacturer. Peak lists were generated with Proteinlynx Data Preparation using the following parameters: external calibration with lock mass using mass 2465,1989 Da of ACTH fragment 18-39 (Sigma A8346) background subtract with adaptive mode, performing deisotoping with threshold 3%.

For peptide mass fingerprinting (PMF) analysis of MS spectra were converted into pkl files using Mass Lynx 4 0. Peak lists containing the 20 most intense peaks of the spectrum were sent to MASCOT PMF search (http://www.matrixscience.com) using the Swiss-Prot database (release 50 0 dating to 30-May-2006). Search settings allowed one missed cleavage with the trypsin enzyme selected, oxidation of methionine as potential variable modification and a peptide tolerance of 50 ppm. Only protein identifications with significant Mascot scores (p< 0.05) were taken in consideration.

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4. RESULTS AND DISCUSSION

Oxidative stress

All the gels presented in this thesis were performed three times at 8% polyacrylamide and run under reduction conditions. At the beginning, the phosphorylation responses in activated and non-activated T cells were tested. The experiments were carried out on Jurkat cells, routinely used as a T cell leukemia model, and on T lymphocytes isolated from buffy coat. Where specified, T cells were activated with 10 μg/ml of ConA (as cited above, a lectin used for its mitogenic activity). Coomassie gels showed that following ConA activation there was no detectable change in the Jurkat cells' proteic pattern with respect to the control samples (Fig. 12). After injection of ConA, and treatment with 0.4 mM diamide, a marked time dependent response was observable with an increase which reached its maximum at 60 minutes. A subsequent decrease in the phosphorylation signal was observed with a complete reversal to basal conditions after 180 minutes (Fig. 13, panel A). This trend is parallel to the findings related to the viability of cells (Fig. 13, panel B), which showed a decrease at 60 minutes from the treatment followed by a gradual recovery.
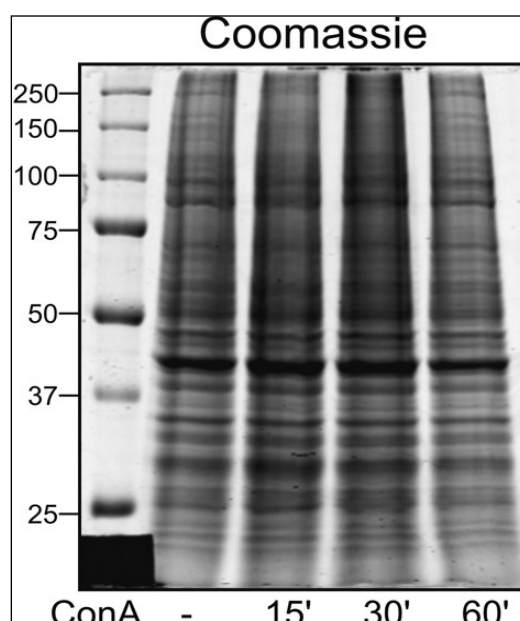
Fig. 12: 8% Gel stained in Coomassie of ConA activated Jurkat Cell total proteins.

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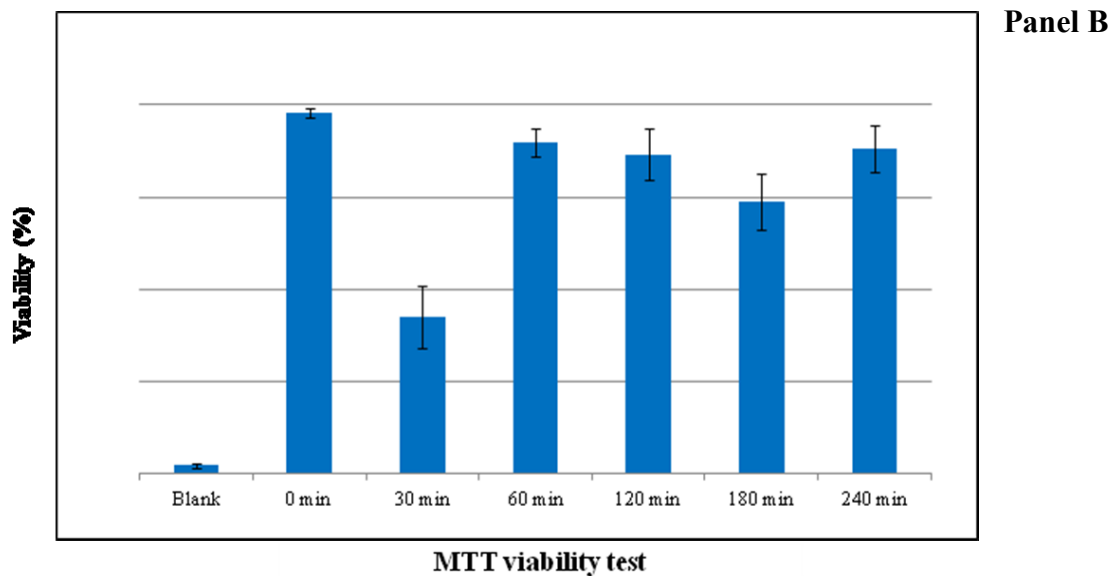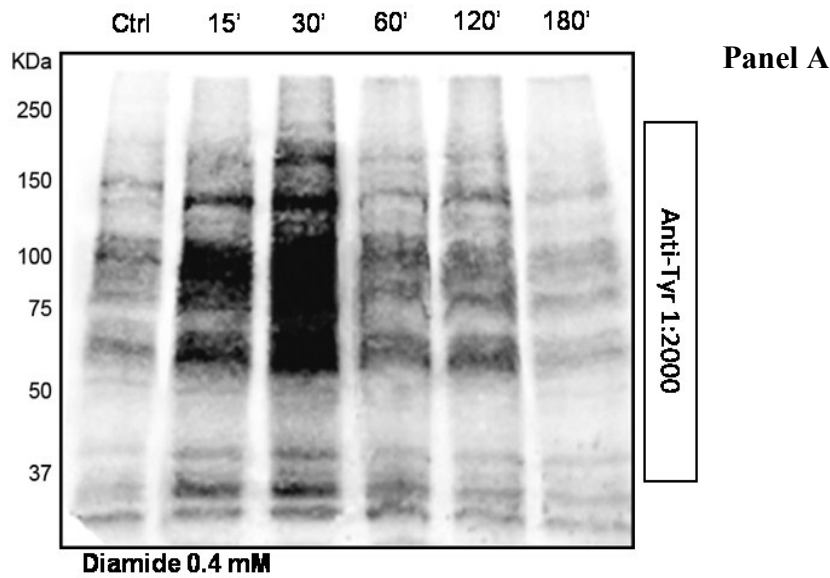
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Fig. 13: Panel A: Western blot of ConA activated Jurkat Cell total proteins treated with diamide 0.4 mM and stained with anti-phosphotyrosine antibody (1:2000). Panel B: MTT viability test.

In order to evaluate the response of Jurkat cells under stress conditions, they were treated with diamide (as mentioned above, reportedly used as a thiol oxidizing agent) at different incubation times and concentrations. Fig. 14 shows that diamide induces a strong tyrosine protein phosphorylation in a dose dependent way and that the phosphorylation signal seems to be dependent upon the time of incubation. In fact, we noticed an intense response (especially at 0.5 and 0.4 mM) detectable within 15 minutes showing a decrease at 30

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minutes, perhaps due to the natural consumption of ATP. Moreover, the same signal becomes weak at lower concentrations.
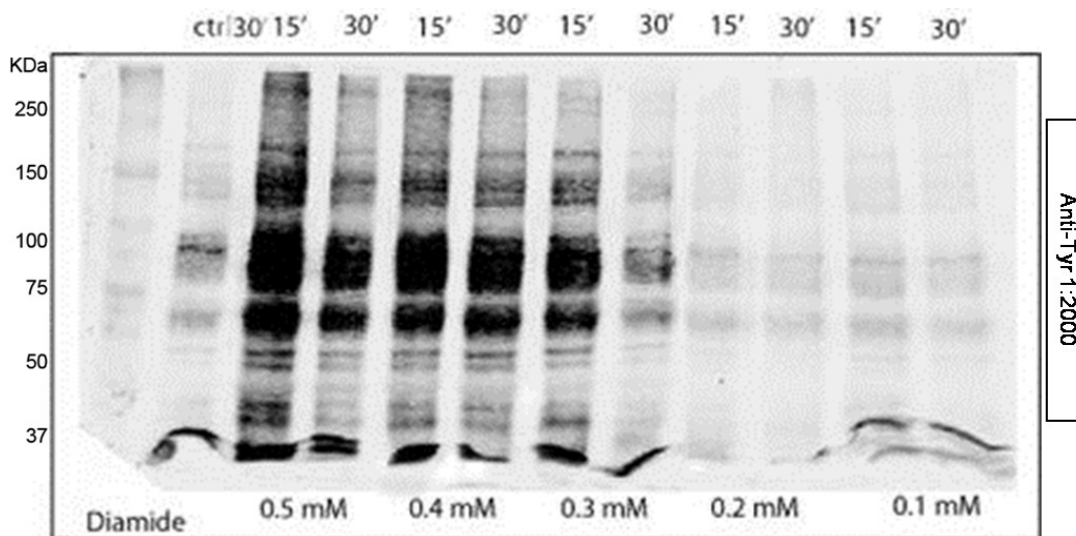
Fig. 14: Western blot of Jurkat Cell total proteins treated with different concentrations of diamide (0.5-0.1 mM) stained with anti-phosphotyrosine antibody (1:2000).

After these tests were performed, we decided to set up experiments treating Jurkat cells and T lymphocytes with diamide 0.4 mM for longer incubation times (from 15 to 90 min) (Fig. 15). In particular, in Jurkat cells the tyrosine phosphorylation signal reached its peak between 15 and 30 minutes and then decreased, possibly due to the metabolic reduction of diamide. On the other hand, in T lymphocytes the phosphorylative signal started at 15 minutes and was maintained for long time (90 min). This may be possibly linked to the fact that Jurkat cells' metabolism is higher than that of T cells in a resting state.

Western blot with anti-phosphoserine did not evidence marked serine phosporylation changes under the same conditions (data not shown).

We then performed a western blot on Jurkat cells and T lymphocytes after diamide treatment in order to investigate the role of Syk kinase (a not-receptorial protein that plays a critical role in T-cell signalling, leading to phosphorylation of its target proteins) during oxidative stress conditions. In Jurkat cells, Syk appears to be constitutively expressed and the signal is maintained over time (Fig. 16a), perhaps because Jurkat cells are a cell line. Conversely, in non-activated T lymphocytes, Syk does not seem to be expressed, perhaps reflecting their physiological condition (Fig. 16b).

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In order to explore redox sensitive protein phosphorylative changes in T lymphocytes (Fig. 17), a Maldi- Tof analysis was performed. Table 1 shows the identified proteins. It is notable that Heat shock proteins, which are typically abundant in stress conditions, and cytoskeletal proteins were found to be most phosphorylated.
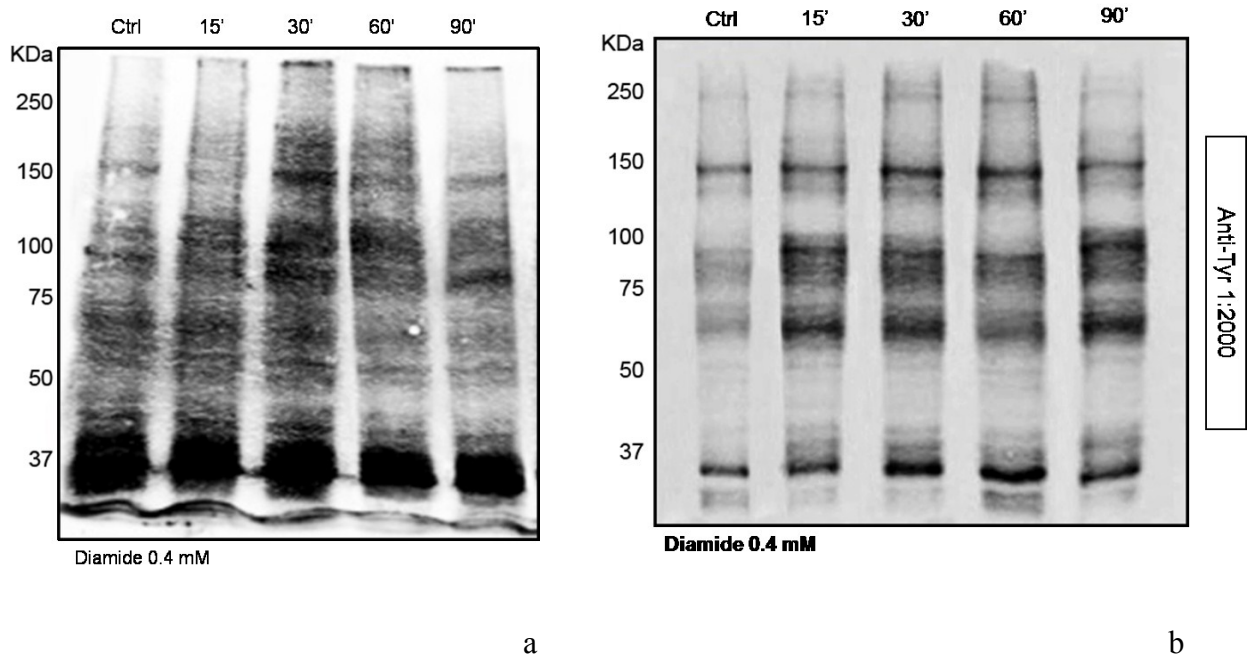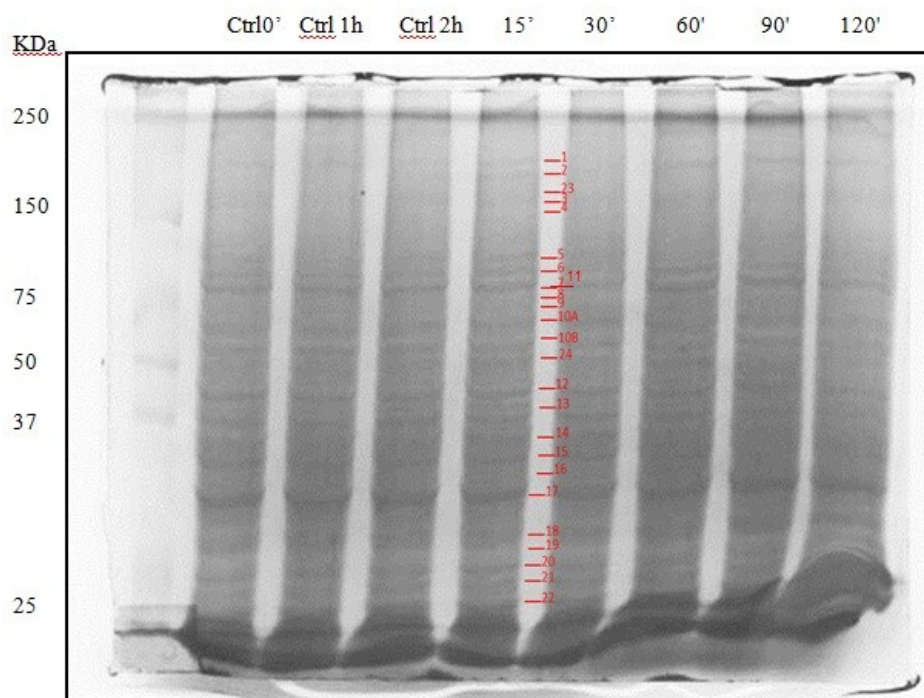

a   b

Fig. 15: Western blots of Jurkat Cell (a) and T lymphocyte (b) total proteins treated with diamide 0.4 mM stained with anti-phosphotyrosine antibody (1:2000).

a   b
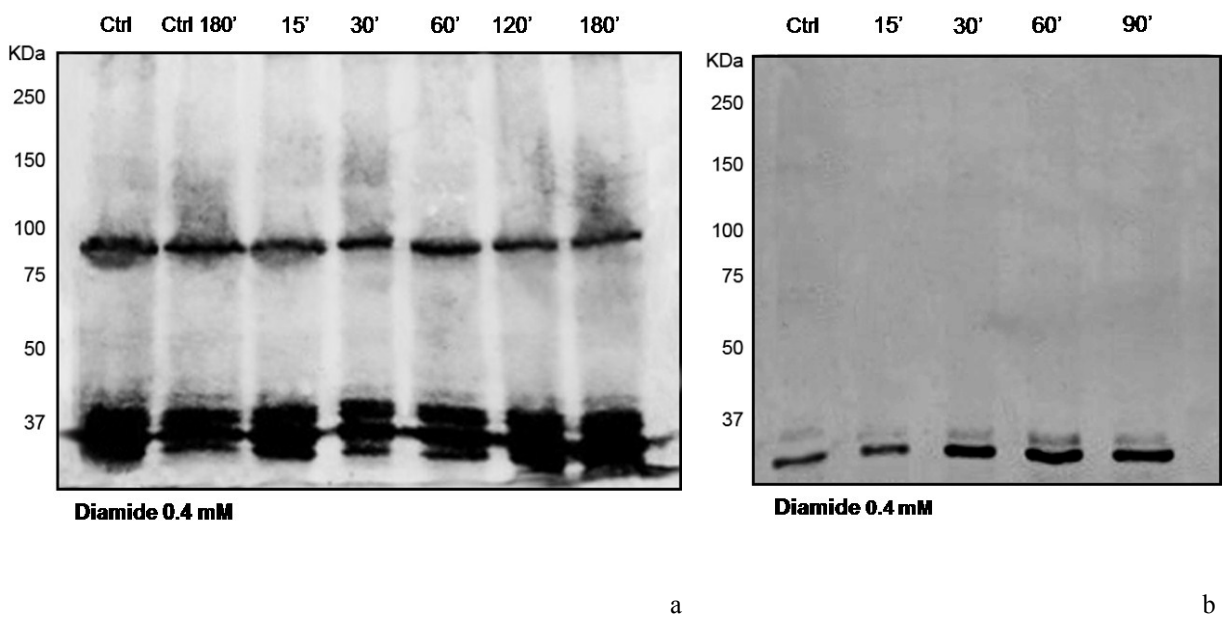
Fig. 16: Western blot of Jurkat Cell (a) and non-activated T lymphocyte (b) total proteins treated with diamide 0.4 mM stained with anti-Syk antibody (1:2000).

Fig. 17: Coomassie gel of T lymphocytes total proteins treated with diamide 0.4 mM.

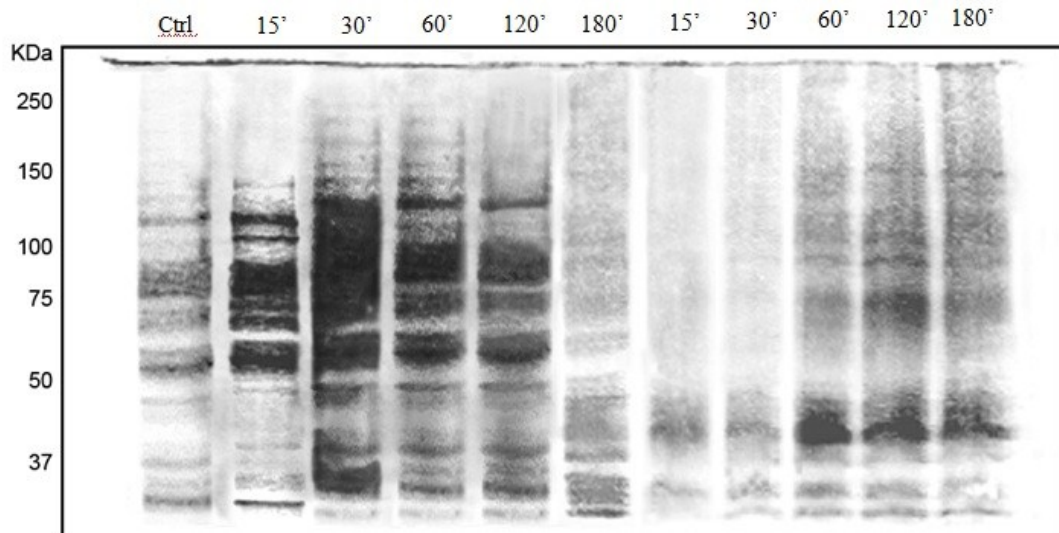| Number of band	Score	Matched Pept.	Name	Mass (Da)	Accession Number
5	57	11	Endoplasmin	92411	HSP90B1_HUMAN
7	96	16	Heat shock protein HSP 90-alpha	84607	HSP90AA1_HUMAN
7	77	14	Heat shock protein HSP 90-beta	83212	HS90A_HUMAN
7	59	4	Putative metallothionein MT1DP	4979	MT1DP_HUMAN
10 b	63	11	78 kDa glucose-regulated protein	72288	GRP78_HUMAN
11	52	11	Heat shock cognate 71 kDa	70854	HSP7C_HUMAN
11	73	11	Stress-70 protein, mitochondrial	73635	GRP75_HUMAN
12	95	12	60 kDa heat shock protein, mitochondrial	61016	CH60_HUMAN
15	68	8	Protein disulfide-isomerase A3	56747	PDIA3_HUMAN
16	58	9	ATP synthase subunit beta, mitochondrial	56525	ATPB_HUMAN
20	87	9	Glyceraldehyde-3-phosphate dehydrogenase	36030	G3P_HUMAN
21	74	10	Heterogeneous nuclear ribonucleoproteins A2/B1	37407	ROA2_HUMAN
23	112	24	Clathrin heavy chain 1	191493	CLH1_HUMAN
18	75	7	Actin, cytoplasmic 1	41710	ACTB_HUMAN
18	75	7	Actin, cytoplasmic 2	41766	ACTG_HUMAN

Table 1: T lymphocyte proteins identified by Maldi – ToF.

In order to investigate whether Syk kinase is actually involved in this kind of stress response, Jurkat cells were treated with diamide 0.4 mM in presence of 10 μM Syk inhibitors
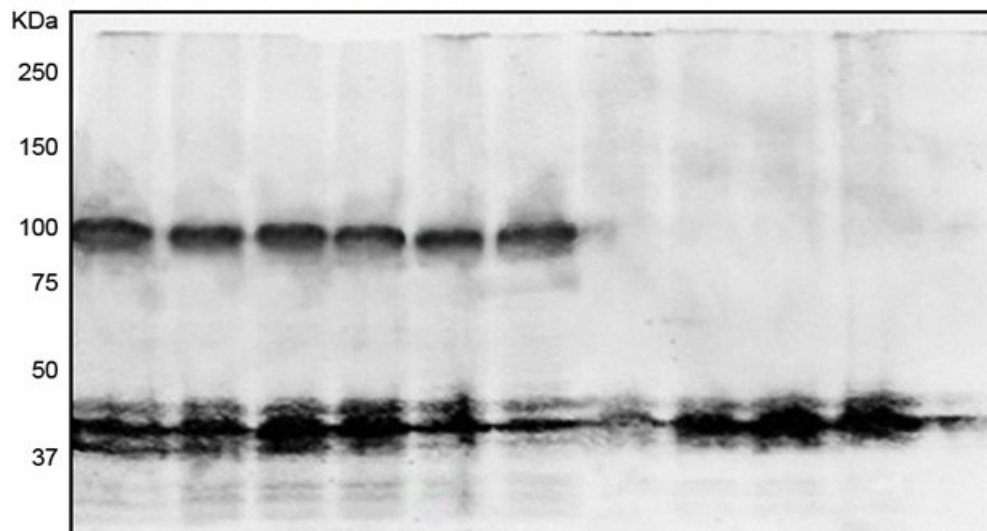
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and they were compared with cells treated in the same way but without the addition of inhibitors. Total proteins were run on 1D gel under reduction conditions and anti-phosphotyrosine western blots were performed. Fig. 18 shows that the tyrosine phosphorylation signal in Jurkat cells treated with diamide 0.4 mM decreases in the presence of Syk Inhibitors, especially in the first 30 minutes.

Panel A

Panel B

Fig. 18: Panel A: Western blot of Jurkat Cell total proteins treated with diamide 0.4 mM at different incubation times in presence of Syk inhibitors. Anti-phosphotyrosine antibody 1:2000. Panel B: Western Blot stained with anti-Syk antibody 1:2000.

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At this point, it was interesting to examine whether stimulation of resting T lymphocytes could lead to activation of pathways in which Syk is involved. For this reason, non-activated T lymphocytes were treated with 0.4 mM diamide in presence or absence of Syk inhibitors (Fig. 19). This result confirms the constant signal previously observed (Fig. 15b).
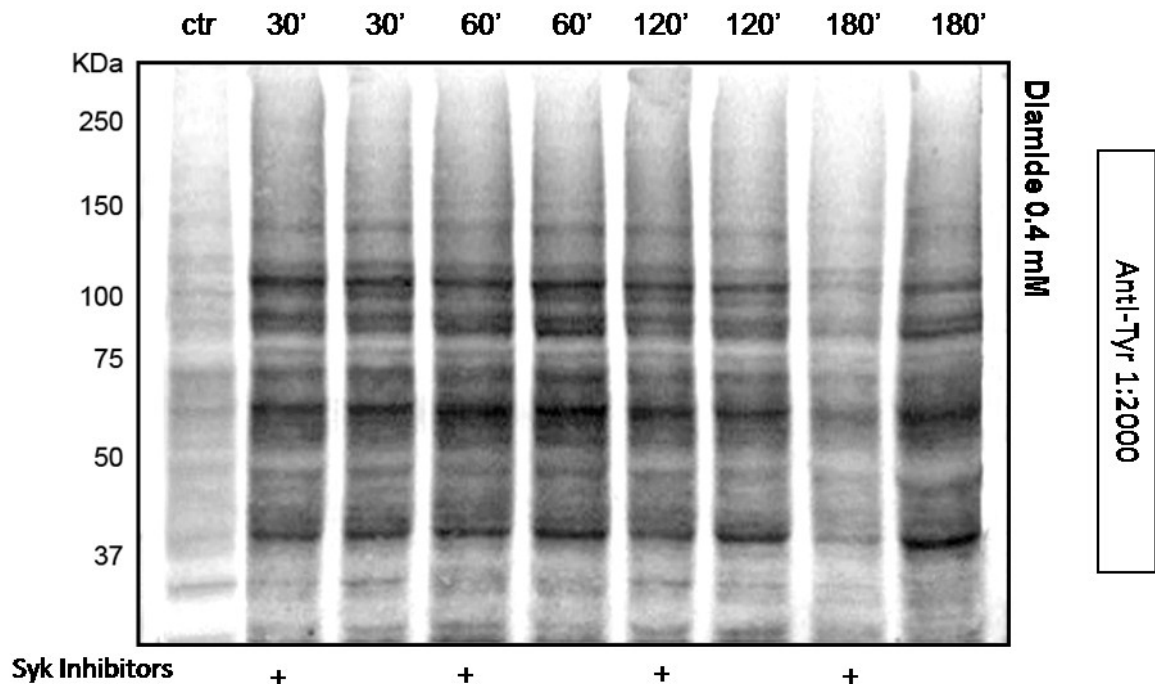

Fig. 19: Western blotting stained with anti-phosphotyrosine antibody (1:2000) of T lymphocyte total proteins treated with diamide 0.4 mM, in presence or in absence of Syk inhibitors.

Then, T lymphocytes were activated with anti-CD3 and anti-CD28 and anti-Syk and anti-phosphotyrosine western blots were performed. As shown in fig. 20 it could be argued that activation leads to a high phosphorylation signal and, more specifically, to the expression of Syk in T lymphocytes, which is not present in resting cells (Fig. 16b). Fig. 21 shows activated T cells behave differently compared to non-activated T lymphocytes in oxidative conditions (Diamide 0.4 mM): the tyrosine phosphorylation signal starts at 30 minutes and it is maintained over time (3 hours). Moreover, activated T lymphocytes were incubated with Syk inhibitors and an anti-phosphotyrosine western blot analysis was performed. It seems that Syk kinase could really be involved in tyrosine phosphorylation of downstream proteins, as the addition of Syk inhibitors leads to a decrease of the phosphorylative signal (Fig. 22).

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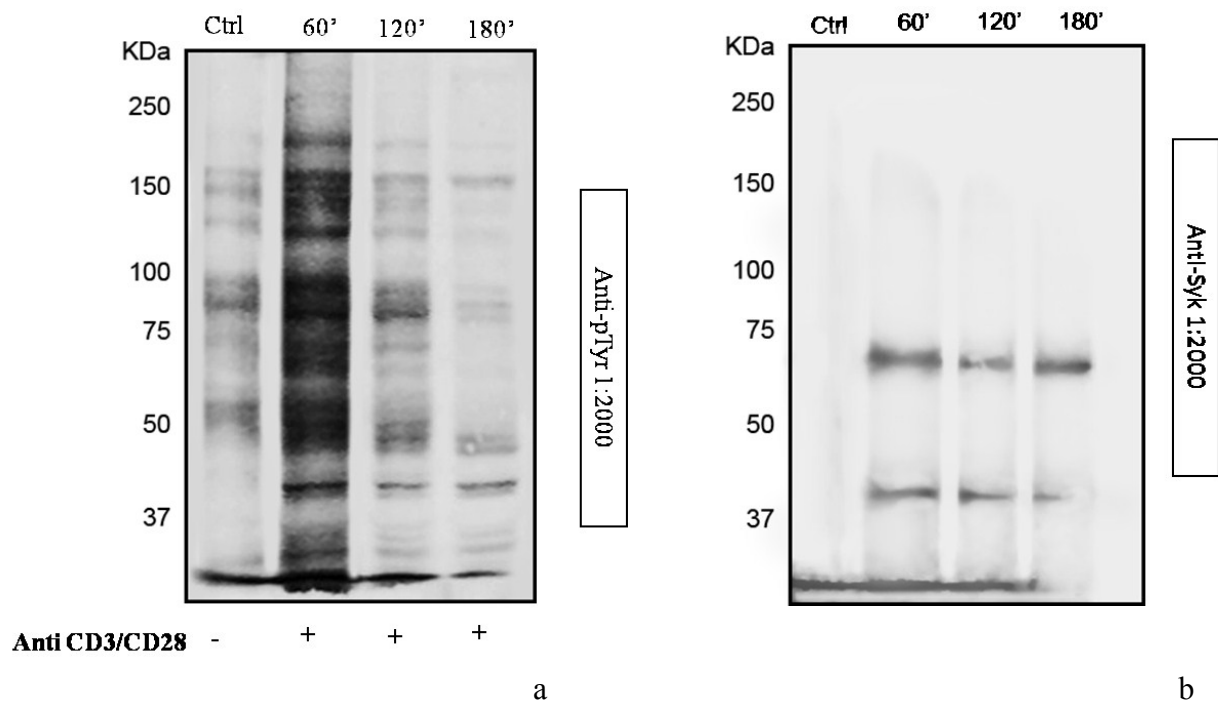
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Fig. 20: a) Western blot stained with anti-phosphotyrosine antibody (1:2000) of T lymphocyte total proteins activated with anti-CD3 and anti-CD28. b) Western blot stained with anti-Syk antibody (1:2000).
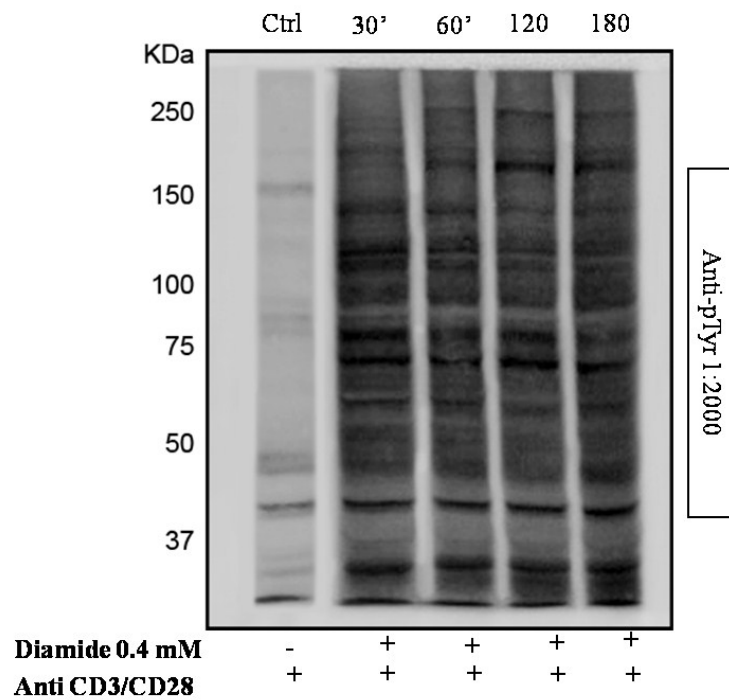

Fig. 21: Western blot of activated T lymphocytes total proteins treated with diamide 0.4 mM and incubated with anti-phosphotyrosine antibody 1:2000.

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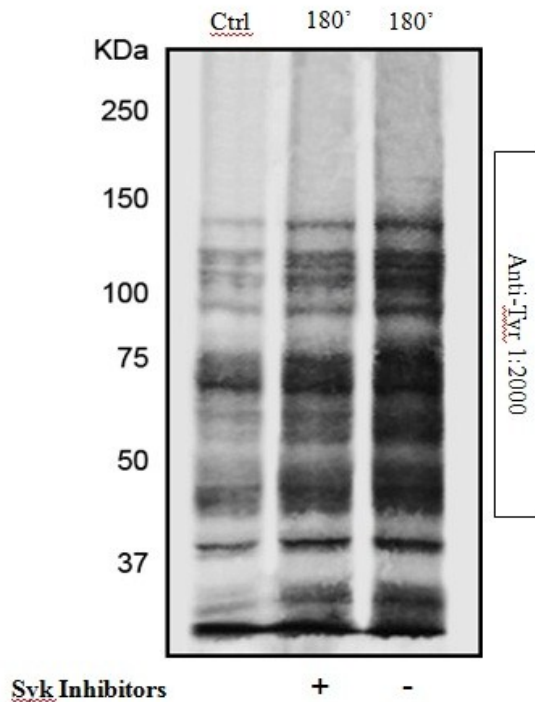
42

Fig. 22: Western blot of T lymphocyte total proteins activated with anti-CD3 and anti-CD28 and treated with Syk inhibitors. Anti-phosphotyrosine antibody 1:2000.

As cited above, an evaluation of oxidative stress on Jurkat cells was also performed by using H_2O_2 at different concentrations. An anti-phosphotyrosine Western blot analysis showed an intense dose dependent response detectable after 30 minutes, especially at 0.1 mM concentration (Fig. 23).
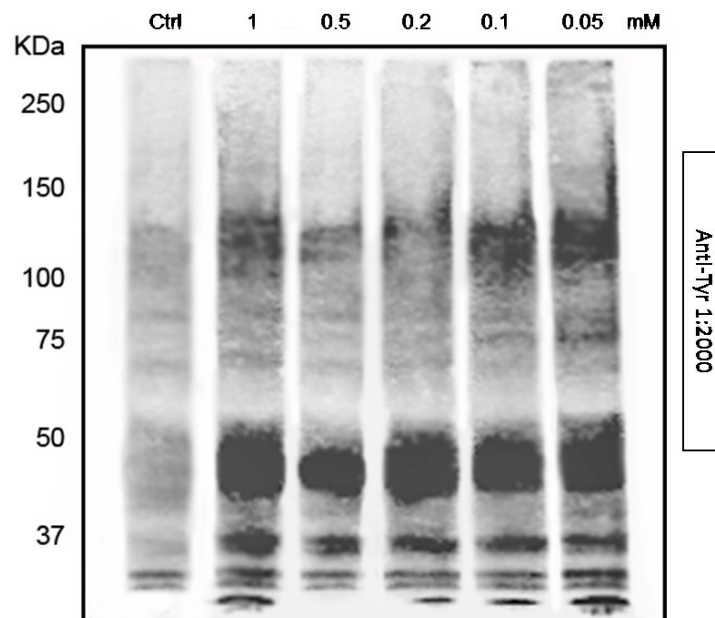

Fig. 23: Western blot of Jurkat cell total proteins treated with different concentrations of H_2O_2 (1- 0,05 mM) stained with anti-phosphotyrosine antibody 1:2000.

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On the basis of our preliminary data, it can be argued that Jurkat cells show a response to ConA , as well as to oxidative stress caused by diamide and H_2O_2 treatment in the form of intense tyrosine phosphorylation. The response lasts approximately 1 hour and appears to be primarily mediated by Syk kinase. Syk seems to be constitutively expressed in Jurkat cells, whereas it does not seem to be expressed in non-activated T lymphocytes.

Some of the proteins found to be phosphorylated belong to the class of cytoskeletal proteins that are particularly abundant in cells. As expected, other identified proteins include the heat shock proteins HSP60, HSP70 and HSP90, known in the literature to be implicated in cellular response to stress conditions. Finally, in Jurkat cells phosphorylation signal decreases in presence of Syk inhibitors.Serine phosphorylation response was not measurable.

T lymphocytes activated with Anti-CD3/anti-CD28 showed a different phosphorylative pattern than resting T cells; oxidative conditions (Diamide) appear to increase the phosphorylation signal.

To date, studies on T cells in the literature have evaluated changes in protein expression associated with cell activation. However, there is a lack of studies on protein expression of activated T cells under stress conditions.

In light of these obtained results, it will be necessary to better clarity the functional roles of the tyrosine kinases involved in the signal transduction process. It is also essential to better identify the protein targets and their phosphorylation sites. It is also important to carry out further research to elucidate whether the Syk Inhibitors could down-regulate phosporylative response in activated T cells. Although the observed modifications could play a central role in a large number of processes, additional knowledge is clearly required.

The technique we used can help identify proteins specifically modified under pathological conditions. This preliminary study will provide helpful information on protein patterns and behaviours involved in the molecular processes underlying the inhibitory effect of oxidative stress on human T cells.

Simulated microgravity

Previous experiments in real and simulated microgravity have shown that human T-lymphocyte proliferation and activation are reduced by more than 90%, under these conditions, with a relevant effects on the IL-2 and its receptor (IL-2Rα) expression.

As information on protein profile alterations is unavailable, our attention was focused on post-translational modifications in T cells (Jurkat cells and T lymphocytes). All the simulated microgravity experiments presented in this thesis were repeated three times. Total proteins were separated on 1D gels and analysed via western blotting using specific antibodies and by Coomassie staining following mass spectrometry.

In the first experiments, Jurkat cells were activated with ConA, subjected to microgravity at 37°C for different time periods (from 15 to 180 min), and compared with the static controls. Proteins obtained from 1-DE were transferred to a nitrocellulose membrane and probed with an anti-phosphotyrosine antibody 1:2000. Fig. 24 shows that microgravity seems to increase the phosphorylation of the proteins when compared to static controls (1xg). Furthermore, it can be observed that activated cells have an intense phosphorylation signal compared to non-activated cells. These results are comparable with those obtained from activated T lymphocytes subjected to simulated microgravity for the same time intervals (Fig. 25). In fact, the phosphorylative protein response seems to increase in this cellular model as well.
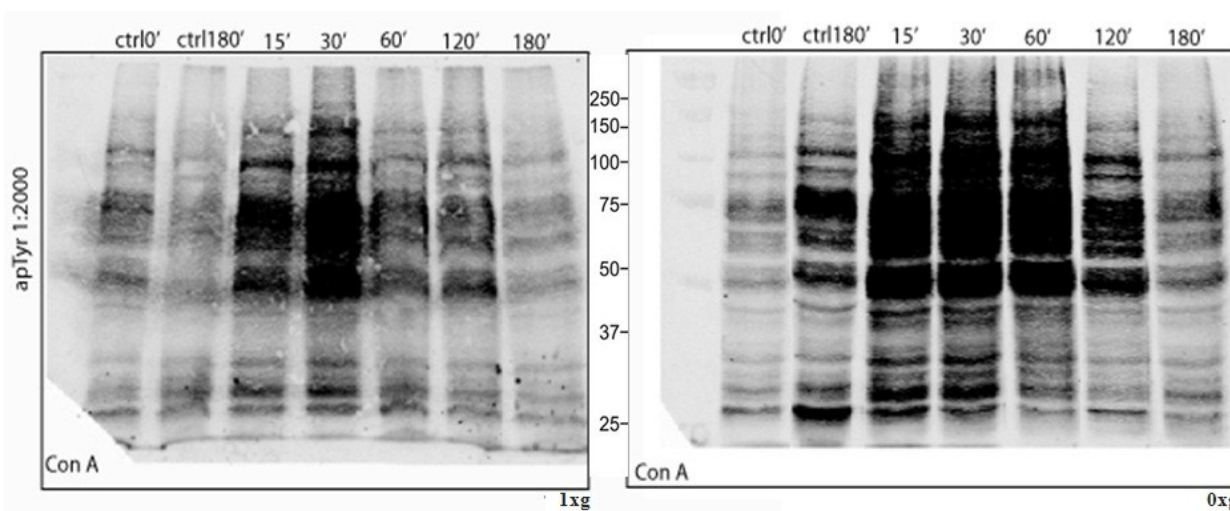

Fig. 24: Jurkat cells activated with ConA and subjected to simulated microgravity (0xg), compared to static controls (1xg). Western blotting anti-phosphotyrosine antibody 1:2000.

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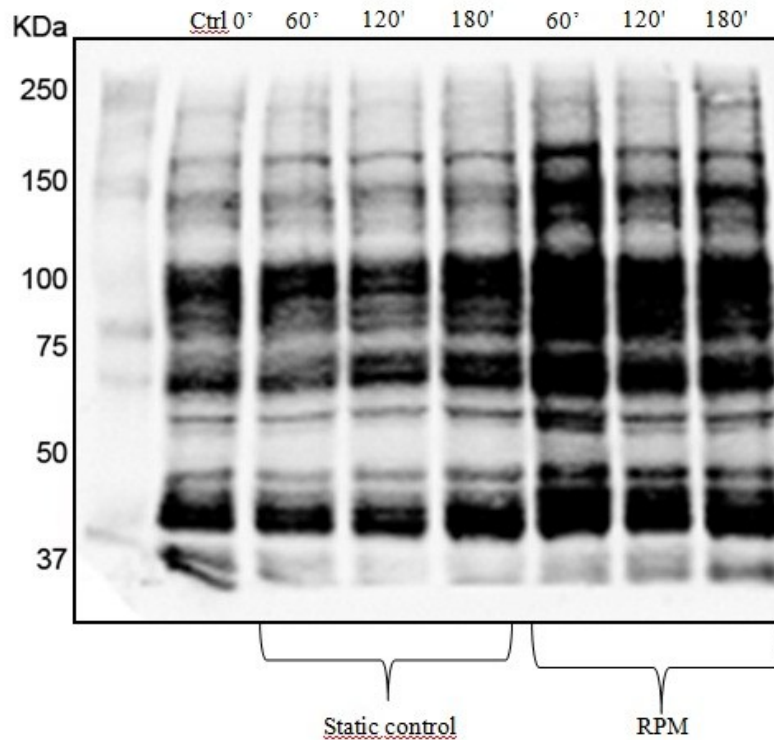
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Fig. 25: T lymphocytes activated with ConA and subjected to simulated microgravity, compared to static control (1xg). Western blot anti-phosphotyrosine antibody 1:2000.

Coomassie gels of T lymphocyte total proteins were run in parallel with the previous ones. Activation was induced by ConA or ConA and anti-CD28. It seems that the total protein pattern does not change in microgravity conditions compared to non-activated controls (Fig. 26). Bands were cut and digested with trypsin. Mass spectrometry (Table 2) revealed that the phosphorylated proteins mainly belong to the class of the heat shock proteins typically abundant in stress conditions, and to cytoskeletal proteins. These results are comparable with those obtained from identification of Jurkat cell proteins treated in the same way.
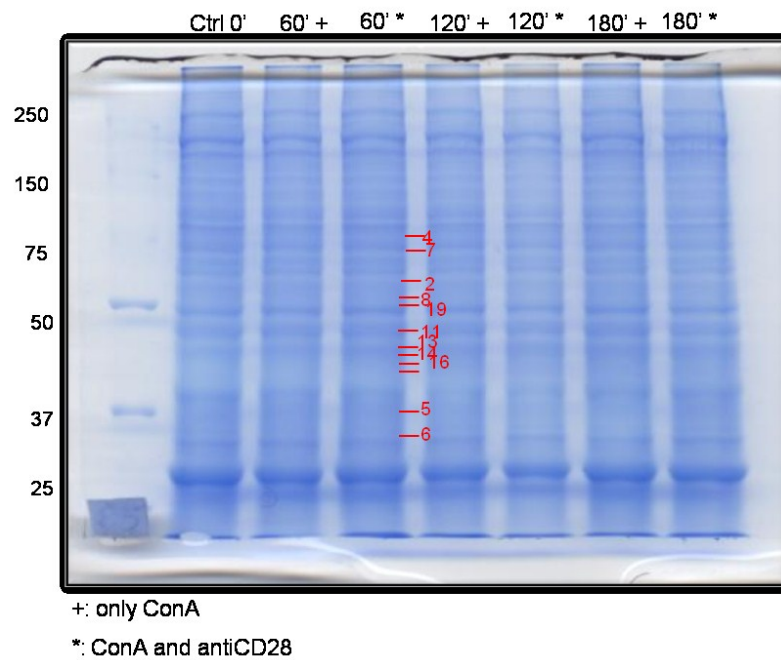
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+: only ConA

*: ConA and antiCD28

Fig. 26: T lymphocytes activated with ConA only

or ConA and anti-CD28 subjected to simulated microgravity.

Number of band	Score	Matched Pept.	Name	Mass (Da)	Accession Number
2	56	7	GRB2-associated-binding protein	74411	GAB2_HUMAN
4	64	14	Zinc fingers and homeoboxes protein 2	92250	ZHX2_HUMAN
5	56	5	Mitochondrial uncoupling protein 3	34193	UCP3_HUMAN
6	60	6	RNA-binding protein PNO1	27907	PNO1_HUMAN
7	146	15	Heat shock protein HSP 90-beta	83212	HS90B_HUMAN
8	79	9	78 kDa glucose-regulated protein	72288	GRP78_HUMAN
11	139	14	Tubulin beta chain	49639	TBB5_HUMAN
12	80	8	Alpha-enolase	47139	ENOA_HUMAN
13	92	8	Actin, cytoplasmic 1	41710	ACTB_HUMAN
14	80	7	Fructose-bisphosphate aldolase A	39395	ALDOA_HUMAN
15	59	5	Heterogeneous nuclear ribonucleoproteins A2/B1	37407	ROA2_HUMAN
16	73	12	Annexin A2	38580	ANXA2_HUMAN
19	66	8	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	72645	DHSA_HUMAN

Table 2: T lymphocytes proteins identify by Maldi – ToF.

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Moreover, anti-phosphotyrosine and anti-Syk western blot analyses were performed on T lymphocytes activated in the same way. Obtained results show that after 60 minutes in microgravity conditions the phosphorylation signal is quite similar for both activation with ConA only and ConA combined with antiCD28. However, the addition of a co-stimulator (anti-CD28) leads to a higher phosphorylation in longer incubation periods, particularly after 120 minutes after activation (Fig. 27a). Furthermore, Syk seems to be constitutively expressed (Fig. 27b).

In the subsequent experiments, T lymphocytes were activated using a mix of anti-CD3 and anti-CD28 in order to mimic the physiological interaction between monocytes and lymphocytes. Activated cells were subjected to simulated microgravity and compared with activated static (1xg) controls. As shown in fig. 38, while after 60 min a marked phosphorylation signal is visible both at 0xg and 1xg, phosphorylation is higher at 0xg as time passes (120 and 180 min). It is also notable how this pathway leads to Syk activation (Fig. 28b).
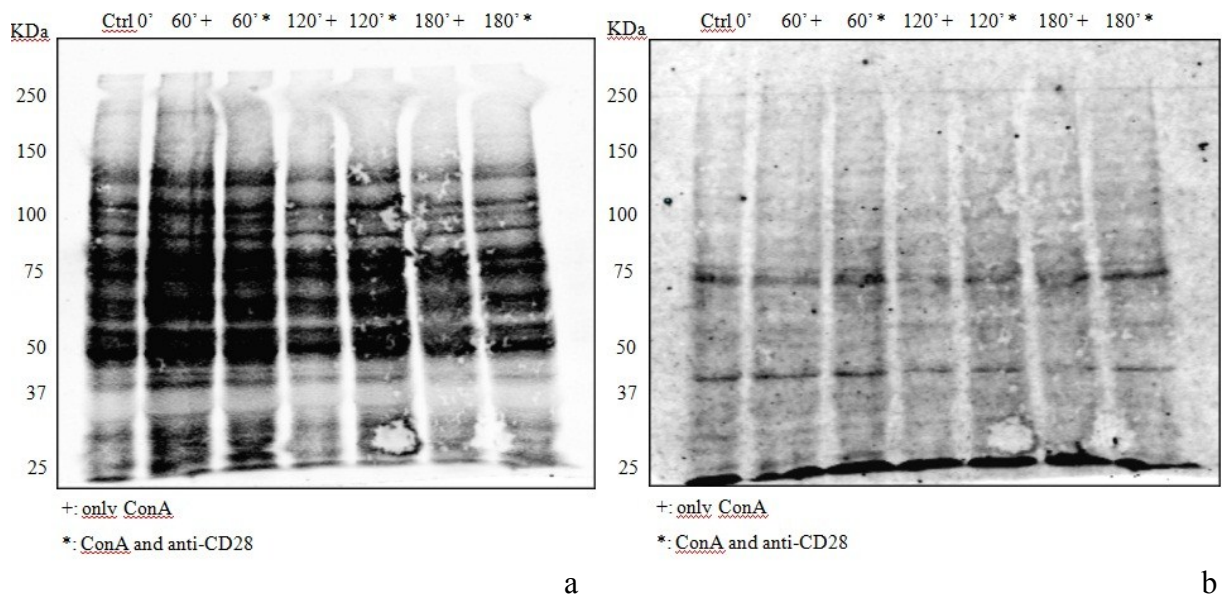

Fig. 27: a): T lymphocytes activated with ConA only or ConA and anti-CD28 and subjected to simulated microgravity. Western blot stained with anti-phosphotyrosine antibody 1:2000. b) Western blot stained with anti-Syk antibody 1:2000.

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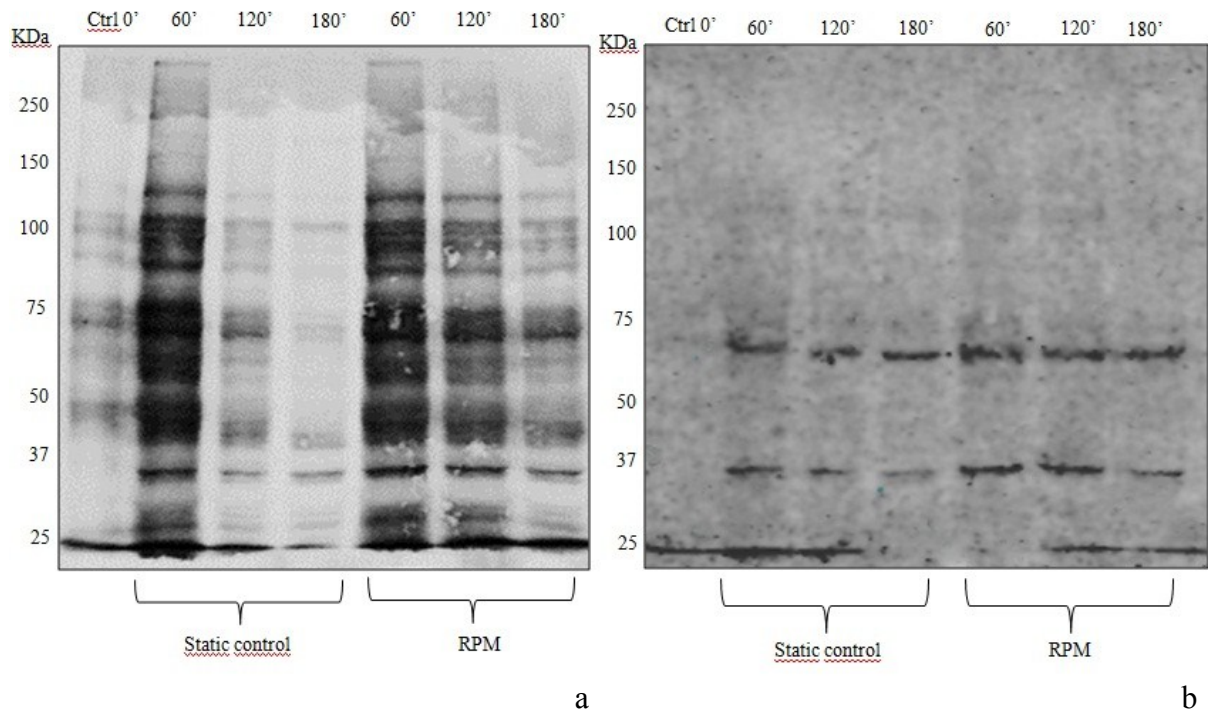
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Fig. 28: a) T lymphocytes activated with a mix of anti-CD3 and anti-CD28. Western blot stained with anti-phosphotyrosine antibody 1:2000. b): Western blot stained with anti-Syk antibody 1:2000.

As highlighted by the figures, after a strong starting signal in RPM and static controls as well, the signal decreases as time passes but remains higher in simulated microgravity conditions. In addition, Syk seems to be expressed by this pathway, whereas no signal is present in non-activated controls.

To summarize, this work constitutes a comprehensive study of tyrosine phosphorylative changes in T cell proteins performed via differential analysis by 1-DE.

From the obtained data, microgravity appears to influence the phosphorylative response in T cells. Western blot testing with anti-phosphotyrosine and anti Syk antibodies turned out to be very sensitive and accurate. T cell proteins respond to ConA and even more to anti-CD3/anti-CD28 activation with an intense tyrosine phosphorylation response. The response lasts approximately 2 hours and Syk kinase does not appear to be primarily involved in non-activated T lymphocytes.

Some of the proteins found to be oxidazed in simulated microgravity conditions belong to a class of cytoskeletal proteins that are particularly abundant in cells. Additional identified proteins are the heat shock proteins.

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Further studies are definitely required to clarify which pathways are affected by microgravity and, in particular, which molecular targets are involved.

Experiments in real microgravity

Initially the aim of the STIM (Signal Transduction In Microgravity) experiment on board Maser 12 was to study the effect of real microgravity on the expression and the function of chemokines, cytochines and their receptors and epigenetic alterations in human T lymphocytes. Technical problems related to the materials used to set up the hardware devices led the team to shift the focus of the experiment. The membranes were made with a silicone that did not allow the use of the Trizol reagent, fundamental for studying the gene expression and the epigenetic of the above mentioned molecules in microgravity conditions. Therefore, the chosen fixative for this type of hardware was formalin. Hence, the experiment was oriented towards the study of real microgravity effects on the expression of proteins hypothetically involved in the first minutes after T lymphocytes activation. Our attention focused on membrane and cytoskeletal proteins because it had already shown been that longer period of microgravity on board ISS affected their expression and functionality.

Pre-flight analysis: part of my Ph.D. work has been dedicated to the analysis of all materials used in the hardware devices. They were tested individually for their biocompatibility and for ensuring the absence of leakage from each compartment. It was also shown that Trizol was unsuitable because it is corrosive for the membranes. Finally, the viability of the cells inside the units was evaluated up to 36 h (Fig. 29). In fact, in case of unexpected changes in the launch schedule due to unfavourable weather conditions, there would not have been enough time to re-open the hardware devices and fill them again with fresh cultures of cells.
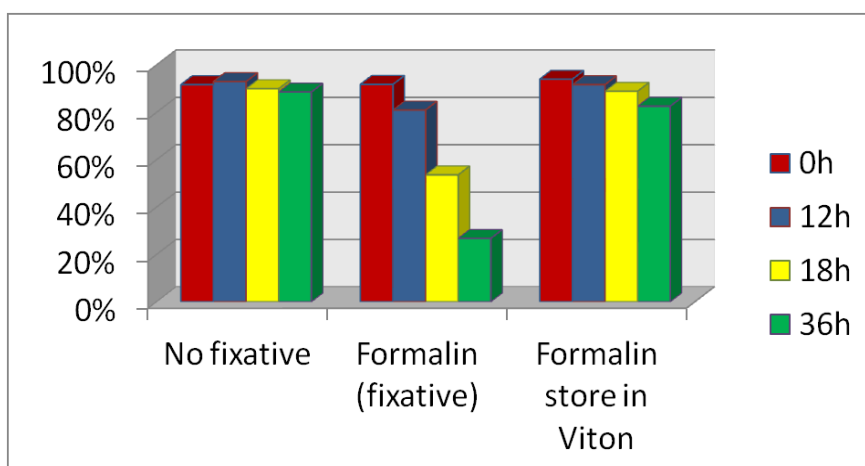

Fig. 29: Viability tests on T lymphocytes inside the hardware devices up to 36 h.

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Post-flight analysis: The samples flown on board Maser 12 were analysed for IL2/IL-2R signalling pattern and cytoskeletal components by FACS-analysis. Microgravity samples (0xg), on board-centrifuge 1xg samples (1xg), on board baseline-controls (BL) and ground controls (GC) were compared. This analysis should shed light on the question of which cellular components are affected within minutes by microgravity and can be causally involved in the often observed depressed activation and proliferation of T lymphocytes in microgravity. Our results seem to confirm that T cells are sensitive to gravitational changes. Undoubtedly, there is a hyper-g effect on several parameters measured. In fact, a comparison between the protein expression of ground controls, baseline samples, 1xg and 0xg samples of molecular targets showed a depression in the baseline samples after exposure to the launch hyper-g.

As shown in Fig. 30, in-flight non-activated T lymphocytes (BL) showed a significant reduction of IL-2Rα in comparison with ground controls (GC) (*$p < 0.005$). ConA/CD28 activated T lymphocytes subjected to 1xg conditions on board show a significantly higher expression of IL-2Rα compared with the cells of the baseline (BL) samples (**$p < 0.001$). The expression of IL-2Rα in the 1xg samples could constitute a reactivation of IL-2Rα after the rocket launch, which is negatively influenced by microgravity. 0xg samples did not show a significant difference with BL samples (n.s.). A direct comparison of IL-2Rα staining between 0xg samples and 1xg samples did not reveal a significant difference (n.s.).

With regard to β-tubulin samples, in flight non-activated T lymphocytes (BL) also showed a decrease of the protein expression in comparison to the cells of the ground controls (GC), even if it was not significant (n.s.). A significantly higher expression of β-tubulin was found when comparing non-activated T lymphocytes (BL) with both activated on-board centrifuge (1xg) (*$p = 0.005$) and microgravity T lymphocyte samples (0xg) (**$p < 0.005$) (Fig. 31).
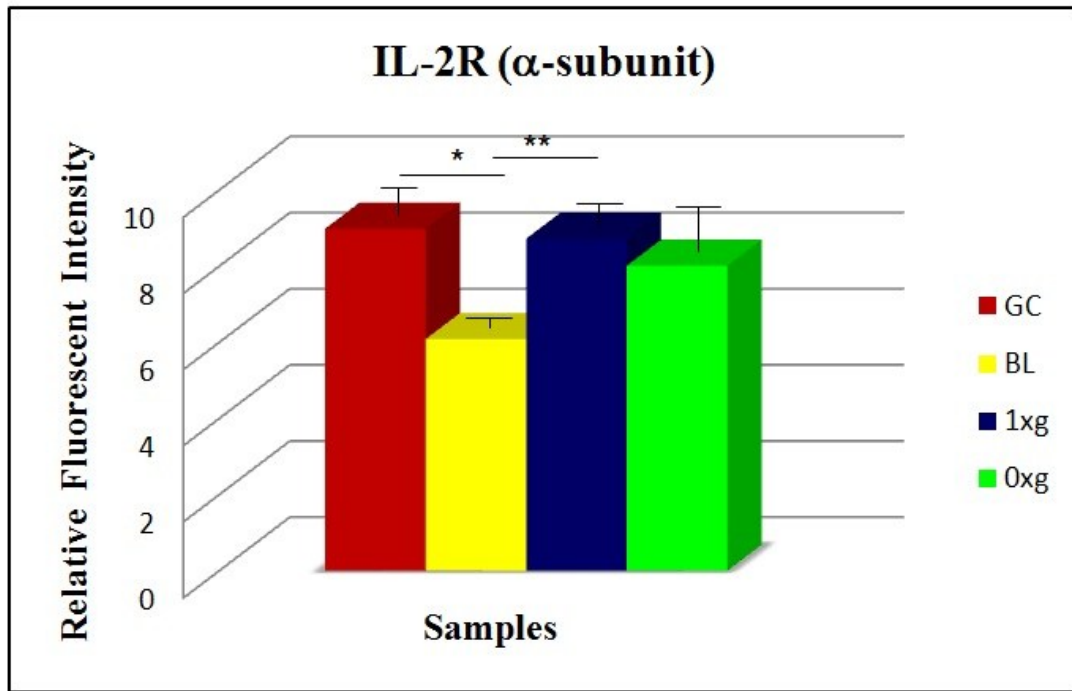
Fig. 30: IL-2Rα expression in T lymphocytes on board Maser 12 and in ground controls
(*p<0.005 and **p<0.001 after two-tailed Mann-Whitney-U-Test; n=3).
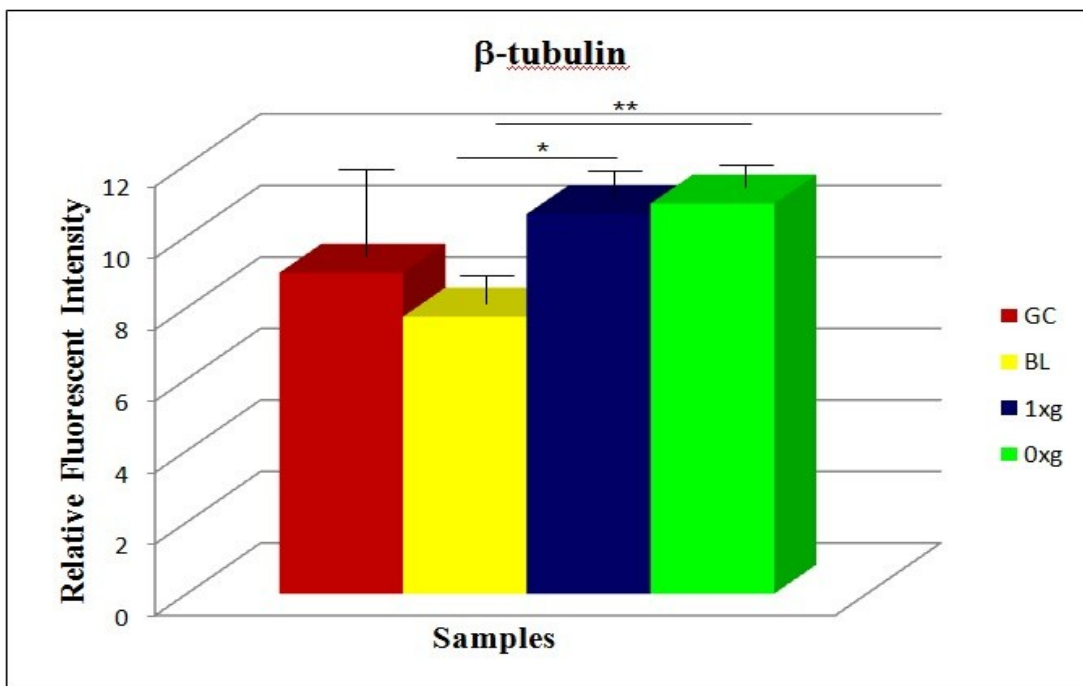

Fig. 31: β-tubulin expression in T lymphocytes on board Maser 12 and in ground controls
(*p= 0.005 and **p<0.005 after two-tailed Mann-Whitney-U-Test; n=3).

Vimentin expression was particularly low in the in- flight non-activated T lymphotes (BL) compared to ground controls (*p<0.0001). Compared to the BL samples, both activated T lymphocytes samples, 1xg and 0xg, showed a higher expression of this protein (**p=0.05 and ***p<0.01 respectively), even if the signal was lower than in ground controls, confirming the negative effect of microgravity on cytoskeletal organisation. The direct comparison between 0xg samples and 1xg samples showed no significant difference (n.s.).

The most interesting result concerns the effect of real microgravity on protein phosphorylation in tyrosine residues. In fact, in- flight non-activated T lymphocytes (BL) displayed much lower phosphorylation signal levels than the ground controls (GC)(*p=0.05). Conversely, the comparison of non-activated cells (BL) with the activated T lymphocytes showed significantly higher levels of the signal in both 0xg samples (**p<0.05) and 1xg samples (**p<0.05). Direct comparison of 1xg samples and 0xg samples showed a tendency towards a lower signal in 1g samples, although not at a significant level (n.s.). It can be hypothesized that this is due to the cells' ability of to recover and adapt to microgravity conditions (Fig. 33). These results are comparable to the data obtained in simulated microgravity conditions.

Regarding Zap-70 expression, baseline samples had a significantly lower staining than ground controls (*p<0.05). No differences were observed when staining intensities in baseline samples were compared with either 0xg samples (n.s.) or 1xg samples (n.s.). In addition, a direct comparison of 0xg with 1g samples revealed no significant difference (n.s.) (Fig. 34).

The exposure of T cells to microgravity in MASER 12 was too short to detect clear effects on the expression of proteins. In fact, data collected on activated samples did not always show significant differences in the protein expression of the targets of interest. For this reason, further studies will be fundamental to clarify which molecules are directly affected.

Clinical problems related to disturbed immune cells functions of astronauts during long-term space flights could be anticipated and prevented thanks to progresses in this field of research. Moreover, identifying gravisensitive signal transduction pathways will help to provide new strategies for therapeutic intervention in disturbed immune cell functions on Earth.
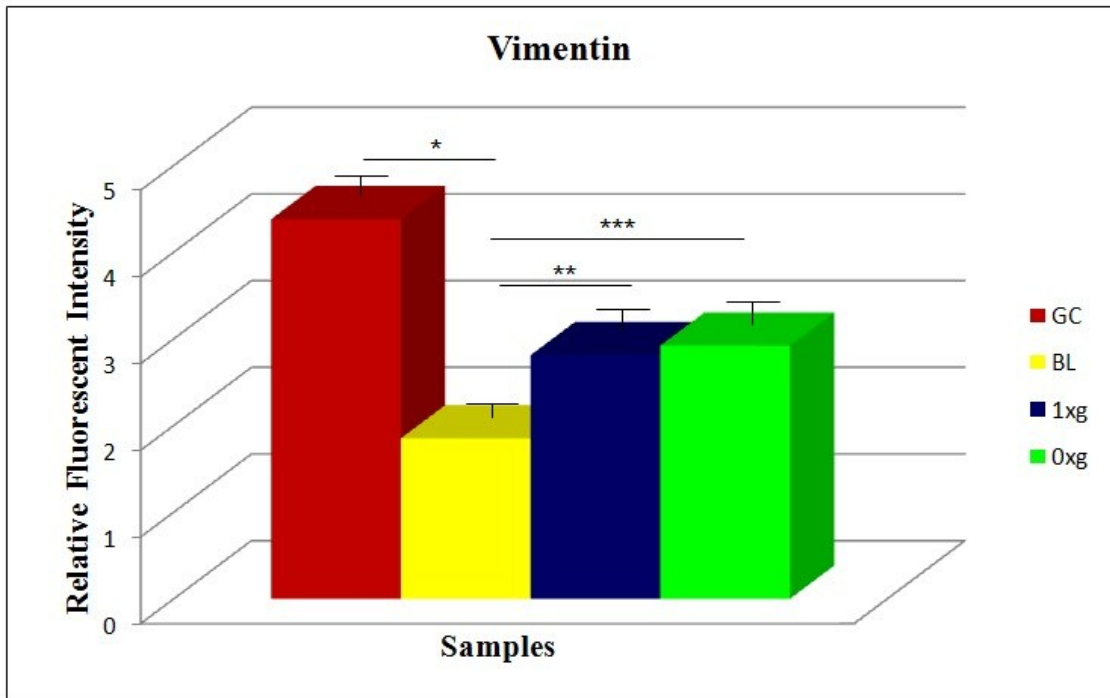
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Fig. 32: Vimentin expression in T lymphocytes on board Maser 12 and in ground controls
(*p<0.0001, **p=0.05 and ***p<0.01 after two-tailed Mann-Whitney-U-Test; n=3).

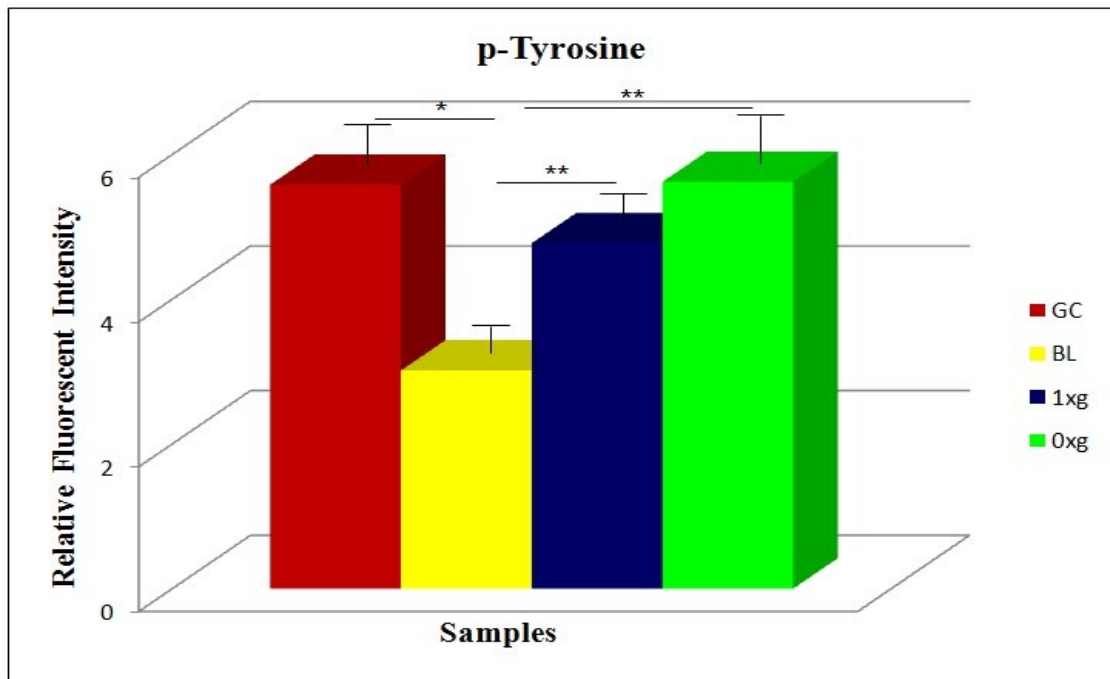Fig. 33: Tyrosine phosphorylation in T lymphocytes on board Maser 12 and in ground controls
(*p=0.05 and **p<0.05 after two-tailed Mann-Whitney-U-Test; n=3).

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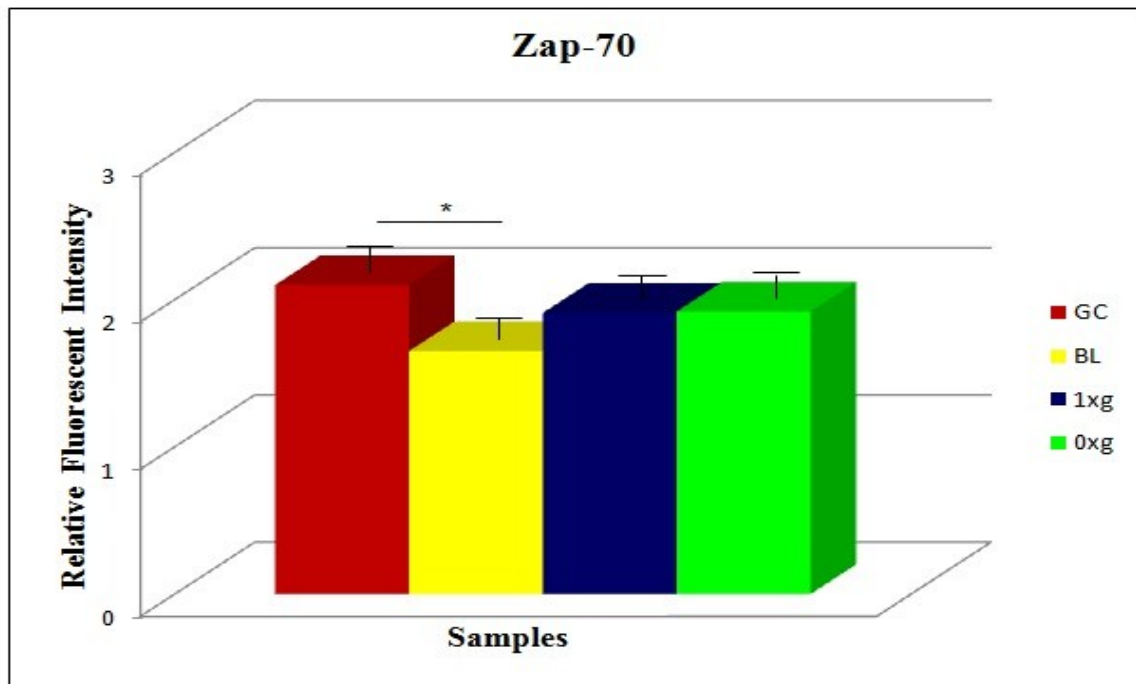
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Fig. 34: Zap-70 expression in activated T lymphocytes on board Maser 12 and in ground controls (*p<0.05 after two-tailed Mann-Whitney-U-Test; n=3).

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5. CONCLUSIONS

On the basis of the data obtained from experiments on oxidative stress, it can be argued that this condition leads to a strong phosphorylative response in activated T cells. The next step will be undertaking the study of the functional meaning of the phosphorylative response, the confirmation of identification of the tyrosine kinases involved in the process, and an enhanced identification of the protein targets and of their phosphorylation sites. Further studies should help to determine the different susceptibility of the various proteins to different degrees of oxidative stress. This preliminary study will provide helpful information on protein pattern involved in the molecular processes underlying inhibitory effect of oxidative stress on human T cells.

Simulated microgravity seems to affect the T cells with a mechanisms similar to that seen with oxidative stress. Future experiments are also required to clarify the molecular basis negatively influenced by microgravity and how to prevent its effects. Although the observed modifications could play a central role in a large number of processes, additional knowledge is clearly required.

As cited above, the analysis of samples on board Maser 12 did not reveal strong effects of microgravity on analysed molecules or differences in phosphorylations. In fact, there was no statistically significant difference between the 0xg samples and 1xg centrifuge samples. The rocket's departure phase undoubtedly had a strong impact on the investigated molecules; almost all of them were down-regulated by the exposure to hyper-gravity in the first part of the flight. These effects might mask microgravity effects which could not be detected in this experiment. Therefore, in flight baseline controls (0-time samples) are very important and must be taken into account when interpreting data gathered in rocket experiments. This factor might also explain why results from rocket experiments might differ from those observed in simulated microgravity conditions without the additional stress of being subjected to a hyper-g phase.

Our immune system protects us from infection and disease, yet T cells lose their adaptability in spaceflight. The physical mechanisms by which the T cells respond to gravity remain an intriguing enigma. In the future, the ISS could offer abundant opportunities for both in vitro and in vivo studies in order to further clarify the molecular basis of spaceflight immunosuppression.

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