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MICROGRAVITY AND IMMUNE SYSTEM: PHOSPHORYLATIVE MODIFICATIONS RELATED TO THE RECOGNITION OF APOPTOTIC CELLS BY PHAGOCYTES IN SIMULATED MICROGRAVITY

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INDEX

1. ABSTRACT

This thesis deals with experiments on phosphorylative changes in peripheral blood mononuclear cell cultures grown under simulated microgravity conditions and in normal conditions. Proteomic techniques have been used to evaluate phosphorylative post-translational modifications and to compare the protein pattern of cells grown in a microgravity environment simulated by the Random Positioning Machine. Monodimensional electrophoresis was used to visualize the proteins of interest obtained from peripheral blood mononuclear cells; Blue Coomassie staining was performed to visualize proteins. MALDI-ToF mass spectrometry was also employed to characterize the same proteins. Western Blotting with specific antibodies: antiphosphotyrosine and anti-Syk was prepared to study phosphorylative differences between cells grown in normal conditions (1xg), those grown under simulated microgravity (0xg) and activated by a gravisensitive mitogen such as Concanavalina A.

Microgravity seems to influence monocytes with an increase of tyrosine phosphorylations of several proteins; also T lymphocytes and monocytes proteins are extensively modified after Con A, particularly after Random Position Machine exposition. Treatment in the presence of Syk inhibitors decreases the phosphorylation levels in all conditions. Mass spectrometry analysis revealed that some phosphorylated proteins belonged to the class of cytoskeletol proteins. Moreover other identified proteins are HSPs, typically abundant in stress conditions.

2. INTRODUCTION

Gravitation is a natural phenomenon by which all physical bodies attract each other. It is most commonly experienced as the agent that gives weight to objects with mass and causes them to fall to the ground when dropped. So, weight is a manifestation of gravitation itself. Gravitation is one of the four fundamental interactions of nature (together with electromagnetism, weak and strong nuclear forces) which, for macroscopic phenomena and most physical situations, can be expressed by Newton's law of universal gravitation: the gravity force is proportional to the masses of interacting bodies and inversely proportional to the square of the distance between them. The term gravity is commonly used synonymously with gravitation, but whereas the latter is the attractive force acting to draw any body together, gravity indicates that force in operation between Earth and other bodies, i.e., the force acting to draw bodies towards Earth. The force tending to hold objects to Earth's surface depends not only on Earth's gravitational field but also on other factors, such as Earth's rotation, latitude, altitude, tides, atmospheric and/or hydrostatic pressure, air resistance, planets movements. Thus, the acceleration due to gravity, symbolized as *g,* provides a convenient measure of the strength of Earth's gravitational field at different locations. The value of *g* varies from about 9.832 m/s^2 at the poles to about 9.780 m/s^2 at the equator, but for ordinary calculations an average value of 9.8 m/s^2 is commonly used, and for our purposes we can use the gravity acceleration as a unit of measurement, so that on Earth it is equal to 1x *g*. In space an object will experience a situation of microgravity, or weightlessness, when gravity is about 0x*g*. It is true that gravity decreases with distance, so it is possible to be far away from a planet or star and feel less gravity. But that does not account for the weightless feeling that astronauts experience in space. The reason that astronauts feel weightless actually has to do with their position compared to their spaceship: any ship in orbit around Earth is falling slowly

towards Earth. Since the ship and the astronauts are falling at the same speed under the only gravitational force, they experience what is called a “free fall”. Technically, an object is in free fall when its acceleration compensates with the gravity force, even when moving upwards or instantaneously at rest at the top of its motion. Examples of objects in free fall include: a) A spacecraft (in space) with propulsion off (e.g. in a continuous orbit at 400 km, or on a suborbital trajectory (ballistics) going up for some minutes, and then down, like in parabolic flights), b) An object dropped from the top of a drop tower, c) An object thrown upward, like a sounding rocket. Microgravity is a condition when zero-g is approached, but still in a state where weightlessness is not perfectly attained, due to small residual forces, as air drag or solar pressure. Microgravity is expressed as a fraction of *g* (from 10^{-2} *g* to 10^{-6} *g*). Every single terrestrial organism has evolved and adapted its body to cope with gravity (i.e. the composition and shape of the skeleton, the position and functioning of the organs, etc.), thus microgravity is expected to affect any living being under many aspects, although a fast adaptation to low gravity environment was shown by many organisms. Ever since the first manned long term space missions, bone loss, muscle atrophy, immune system deficiencies in otherwise healthy astronauts have been evidenced, and following experiments in microgravity conditions confirmed these observations.

2.1 Microgravity conditions

Knowing the limits of life in microgravity conditions will be indispensable for designing long term manned missions in space, going from the ISS stationing around the Earth to the futuristic travel to Mars and terraforming activities. But space biosciences can give useful information also to improve life even on Earth. In fact, conducting experiments in real and simulated microgravity conditions allows the scientists to study phenomena and mechanisms in different fields such as biology, physiology and chemistry by removing the effects of gravity.

Obviously the earliest experiments of this kind were performed by the organizations involved in the so-called "space conquest", characterized by a strong competition between the American National Aeronautics and Space Administration (NASA) and the Russian Federal Space Agency (RKA). But afterwards also academic researchers joined in the study of microgravity and cosmic radiation effects, and their work was supported by a number of Space Agencies, offering both facilities and technologies.
Besides NASA and RKA, in Europe, the gateway to space is the European Space Agency (ESA), an international organization involving 19 Member States, including Italy, whose Italian partner is the Agenzia Spaziale Italiana (ASI), the third contributor after Germany and France. [1].

Also the European Low Gravity Association (ELGRA), a non-profit international society of multidisciplinary nature, supports research under various gravity conditions. 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) provides biomedical support for space flights. In Italy, this kind of research is implemented by the Italian Society for Space Biomedicine and Biotechnology (ISSBB).

Space biomedicine studies can be performed both in real and simulated microgravity conditions.

Real conditions involve the use of space vectors and can be designed to last for a short or long time. Orbiting Stations are surely the most valuable tools for such a research. The Shuttle, with its Spacelab, allowed microgravity studies up to 16 days, but since its missions ended in July 2011 all the experiments had to be moved on the International Space Station (ISS,NASA,RKA, ESA, JAXA, CSA), with the improvement that now they can be conducted for longer periods (up to several months). The ISS is equipped with the KUBIK incubator (ESA's Space Research) [2] 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 1x*g* in flight run in parallel with samples in weightlessness.

Another important facility on board is the Italian Mice Drawer System (MDS) [3,4] which can host 6 mice up to 100-180 days, used for in vivo experiments, to investigate the genetic mechanisms leading to bone mass loss and other microgravity effects on different tissues such as muscles, glands and the brain. Finally, ESA's major contribution to the ISS is the European Columbus Laboratory where the BioLab [5] performs experiments on micro-organisms, cells and tissue cultures, small plants and small animals, and the European Physiology Modules (EPM) [6] is used to study the effects of long-duration space-flight on the human body, contributing to a better understanding of terrestrial problems like ageing, osteoporosis and balance disorders as well as muscle wastage.

Sounding rockets (Texus, Maser and Maxus) German-Swedish rocket programme at Esrange for ESA microgravity research programmes, on the other hand, are able to provide 6 to 13 minutes of microgravity, so compared to orbiting space stations their applications are quite limited, but of course less expensive and still valuable for many

purposes, such as studying the early biological and molecular events involved in cell physiology. Sounding rockets are ballistic, instrument-carrying missiles designed to take measurements and perform scientific experiments during their sub-orbital flight. The rockets are used to carry instruments from 50 to 1.500 kilometres above the surface of Earth, with nearly vertical ascent and descent trajectories [7,8]. The weightless conditions are met during the free fall phase of the rocket payload once the rocket motors have exhausted their thrust and have dropped. The rockets 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 freefall ends with the deployment of the parachute that lowers the payload to the ground with appropriate impact speed [9]. Sounding rockets launches take place at the European Space Center (ESC) Esrange, located outside the town of Kiruna, in northern Sweden, within a program developed and managed by the Swedish Space Corporation (SSC), with ESA as a regular participant.

Another way to reach temporary 0x*g* conditions is to perform parabolic flights on specially configured aircrafts. They provide repetitive periods of microgravity lasting up to 20 seconds, allowing only short-term experiments. Such flights are operated by the NASA Reduced Gravity Research Program, in Europe by NoveSpace (Bordeaux, France) using an Airbus A300 aircraft known as Zero-G, and in Italy by the Italian Space Agency contracts SpaceLand, an Italian parabolic flight and sub-orbital flight engineering and operational agency to support research, development and educational flight campaigns. The aircraft (Fig.1) gives its occupants the sensation of weightlessness by following an (approximately parabolic) elliptic flight path relative to the centre of the Earth, so that the aircraft and its payload are in free fall at certain points of its flight path. Weightlessness begins while ascending until the craft reaches a downward pitch angle of 30 degrees, lasting about 20

seconds. At this point, the craft is pointed downward at high speed, and must begin to pull back into the nose-up attitude to repeat the manoeuvre (20 seconds). The forces are then roughly twice that of gravity on the way down (1.8-2.0 x*g*), at the bottom. Each parabola lasts about 65 sec. and can be repeated up to 30-60 times during one flight.

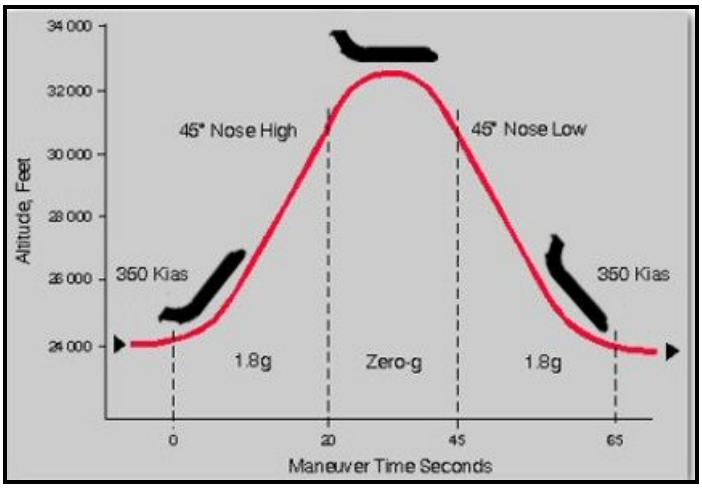

Fig.1: Scheme of parabolic flight.

These kinds of vectors are obviously highly expensive and their cost is seldom affordable by an academic research laboratory. Thus, simulated microgravity conditions have to be achieved on the ground by using devices as the Rotating Wall Vessel (RWV) (Fig.2), the 2D Clinostat, the Random Positioning Machine (RPM) built by Dutch Space (Leiden, NL). The Rotating Wall Vessel **(**RWV) has been used by the National Aeronautics and Space Administration (NASA) since the early 1990s. The design and principle of the RWV is as follows: a chamber, completely filled with culture medium and a defined number of cells, rotates around an axle and doing this movement subjects the cells to a continuous free fall. The rotation speed regulates the fall velocity. Due to the continuous free fall, cells are not able to attach to the chamber surface but they attach to each other and form three-dimensional constructs.

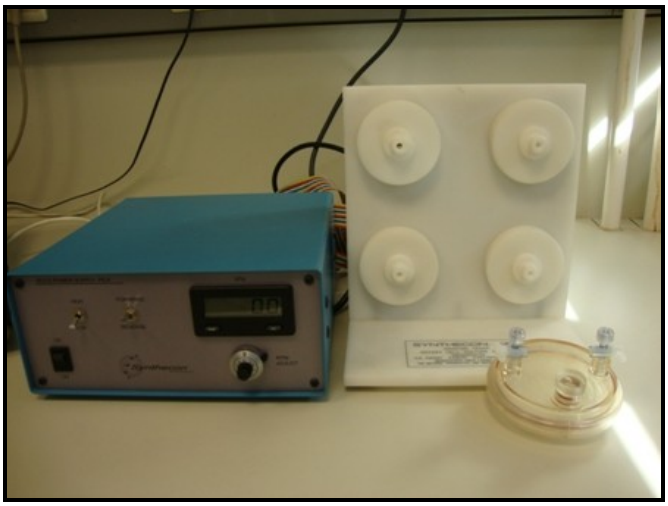

Fig. 2: Rotating Wall Vessel (RWV)

A similar design principle is exhibited by the 2D Clinostat (Fig. 3-4). The rotation takes place around an axle perpendicular to the gravity force vector. In contrast to the RWV, weightlessness is obtained through vector averaging of the gravity force vector and results below 1g when the clinostat rotates fast enough (40-100 rpm). Rotation samples like cultured cells are centered in the middle of the Clinostat. However, a big drawback of the 2D Clinostat is the rotation velocity. If the velocity too high the Clinostat turns into a centrifuge. Furthermore, just cells or plants small enough to fit centered into the rotation axis can be rotated with the Clinostat, because any part of a sample larger than the rotation axis would experience centrifugal forces.

Fig. 3: 2D clinostat: Fast Rotating Clinostat with Camera (DLR, Cologne)

Fig.4: 2D Clinostat: Fast Rotating Clinostat (DLR, Cologne)

Random Positioning Machine (RPM) (Fig.5) simulates low gravity conditions (microgravity) almost perfectly (10^{-3}x*g*, while experiments carried out in space are from 10^{-6} to 10^{-4}x*g*). It was developed by T. Hoson in Japan and manufactured by Dutch Space (former Fokker Space). The basic principle consists of an inner and an outer frame rotating independently from each other in random direction. The samples in the centre of the machine experience low gravity as the gravity vector is averaged to zero over time.

The "3-D clinostat" Random Positioning Machine (RPM), hosted and operated at the Life and Physical Sciences Instrumentation and Life Support (LIS) Laboratory of ESTEC, ESA's technical centre located in the Netherlands, in collaboration with the Dutch Experiment Support Centre (DESC), is a tool able to simulate microgravity conditions on the ground and is fully available to scientists. The RPM is an affordable means to perform gravitropism-related experiments and it has demonstrated its value for stand-alone research studies as well as for preparing flight experiments. The RPM is relevant for fundamental biological research on animals or plants and single cells. It is an instrument designed to provide an experiment with continuous random orientation changes in 3-dimensional space relatively to Earth's gravity vector. The RPM is able to recreate on the ground effects close to those of a real microgravity environment. The principle of the RPM relies on "gravity-vector averaging" (*i.e.* there is no dominant orientation during the performance of an experiment) and on the fact that random changes in direction of the platform are faster than the response time to the gravitational stimulus of the studied system.

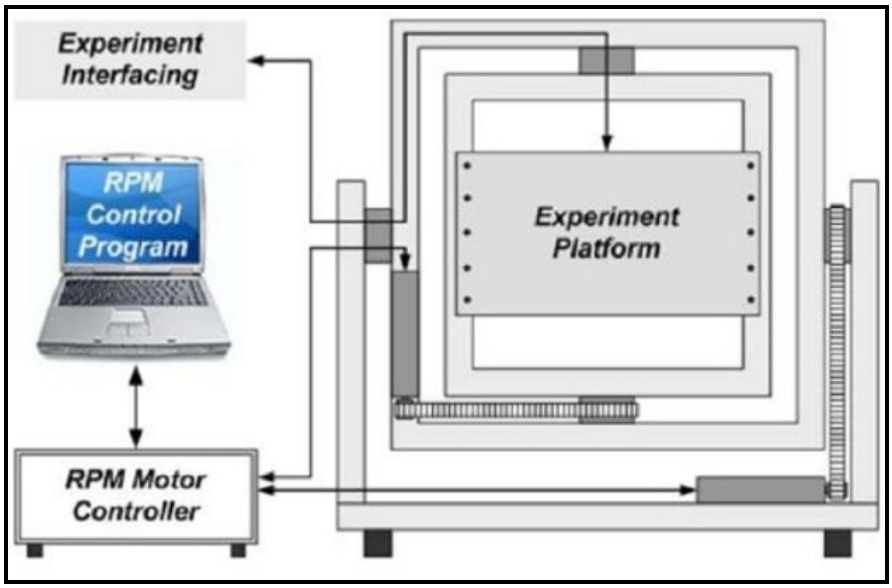

Fig. 5: Random Positioning Machine

The design of the RPM consists of two cardanic frames and a platform accommodating the experiment. The movements of the frames are generated via belts and two electro-motors. Both motors are controlled on the basis of feedback signals generated by encoders, mounted on the motor-axes, and by 'null position' sensors on the frames. The RPM is controlled by a computer hosting dedicated software.

2.2 Microgravity effects on human

The observed effects of the space environment on the human body are numerous: the first that becomes evident is the one on blood circulation. On Earth, the heart distributes blood throughout the body and gravity helps draw blood to the lower limbs. In space the force to pull body fluids down is missing, causing a fluid shift (Fig.6) that induces in the astronauts a characteristic facial edema, whereas legs became thinner because blood can only be pumped there by the heart with no help from gravity [10].

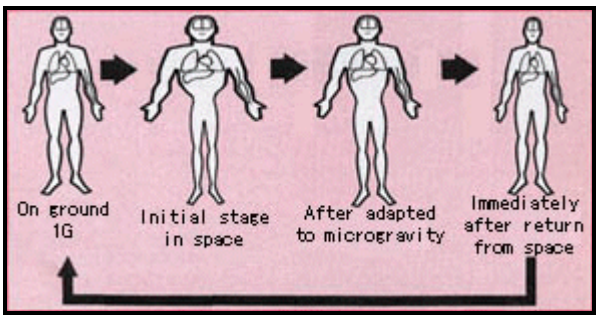

Fig. 6: Fluid shift

These changes in fluid shift during a space mission are considered as short term effects since the body quickly tends to adapt to the new conditions, and generally disappear within few hours on return on Earth.

Some studies showed a decrease in heart rate in rats [11] as well as in humans [12]. Other reported effects on blood circulation including a decreased plasma volume [13,14] followed by a post-flight hypovolemia [15] and a post-flight postural hypotension [12].

Space stationing can alter the delicate balance of homeostasis of weight-bearing bones in normal Earth gravity environment [16,17] and be the cause of a spaceflight osteopenia [18]. It has been reported that after a 180-days spaceflight the principal bone formation parameters were decreased while bone resorption markers were increased [19] especially in weight-bearing bones [16,17,18]. Further studies showed that microgravity caused an increase in osteoclast-mediated resorption of bone, resulting in an increased osteoblast apoptosis [18]. Skeletal muscle is another tissue strongly affected by microgravity. In 2008 Tesch and coworkers found that, even after a short time in orbit, a reduction of muscle function was already detectable and that the longer the mission time was extended, the more the muscle function worsened. Furthermore, the near-zero gravity environment has been reported to affect gene transcription and protein translation in the muscle cells [20,21]. An important consequence of the microgravity exposure detectable during the post-flight period is an increased muscle fatigue. Persistent fiber necrosis, interstitial edema, and activation of macrophages have been observed even weeks after landing [21].

During a space flight mission, astronauts do not receive the same stimuli from the surrounding environment as on Earth. The central nervous system processes involved in the development of the astronaut's sense of direction are misleading and for astronauts it is difficult to adapt to the microgravity environment. The near zero gravity mainly affects the vestibular apparatus inside the inner ear: this condition usually leads to a deconditioning of motion sensors as well as of the somatosensory system, to an altered orientation perception and to a loss of balance. This disorientation is the main symptom of a temporary disease

known as Space Adaptation Syndrome (SAS), the main cause of Space Motion Sickness (SMS). As a matter of fact, SAS is caused by a sensory conflict between inputs from visual and tactile senses and inputs from the vestibular organs [22]. Thus, in order to reorganize the orientation apparatus, some adaptive changes occur in the neural strategies to perceive spatial information. Visual cues and references become fundamental to astronauts since in space ‘‘down’’ is where the astronaut feet are. Other tissues and organs for which alterations due to a microgravity exposure have been reported include: effects on inner ear [23] as well as on the formation of renal stones after spaceflight [24].

2.3 Microgravity effects on immune system

The observed deterioration of the immune system results in a secondary immunodeficiency that can lead to increase infections or autoimmunity [25] is an important issue in spaceflight consequences. Studies on the immune system performed by Gridley and colleagues showed a reduction of mice T-lymphocytes after 13 days in space, thus demonstrating that microgravity has a significant effect on T cell distribution, function, and gene expression after a short-term spaceflight. Experiments performed on thymus revealed an effect on the expression of cancer related genes, indicating a possible increase of carcinogenesis [26]. Moreover, the potential for astronauts to develop cancer could be enhanced by alterations in natural killer cell function, since after space flight the number and cytotoxic activity of natural killer cells was reduced [25]. Furthermore, other studies have shown a decrease in the production or function of specific cytokines and chemokines, such as interferon [27] and tumour necrosis factor alpha (TNF-a) [28]. In addition, the impairment of the immune system during spaceflight can also dramatically alter the body ability to repair itself following a cutaneous or intrinsic wounding [29]. The

negative shifts in the congenital immunity system in some volunteers at the end of immersion and during recovery can be considered as a warning about depletion of the system reserve and increase of the risk of infectious diseases such as those caused by normal microflora which typically does not provoke pathological reactions of the host [30]. Immune system dysregulation has been demonstrated to occur during and immediately after spaceflight. As the initial bias and magnitude for an immune response is heavily influenced by monocyte/macrophage secreted cytokines, this study investigated monocyte phenotype and cytokine production patterns following short-duration spaceflight.

Peripheral monocyte percentage values were unaltered postflight. Constitutive monocyte expression of both CD62L and HLA-DR was reduced following spaceflight in a mission-specific fashion [31]. Loss of these molecules indicates a functional disability of monocytes, either by inhibition of adhesion and tissue migration (CD62L) or by impaired antigen presentation (HLA-DR). These data indicate that changes in monocyte constitutive phenotype and inflammatory cytokine production occur after short-duration spaceflights, which may impact overall crewmember immunocompetence. Also, monocyte/macrophage function may be highly sensitive to mission specific parameters [31].

Certain functions of immune cells in returning astronauts are known to be altered. A dramatic depression of the mitogenic in vitro activation of human lymphocytes was observed in low gravity [32]. T-cell activation requires the interaction of different type of immune cells as T-lymphocytes and monocytes. Cell motility based on a continuous rearrangement of the cytoskeletal network within the cell is essential for cell-cell contacts. In this investigation on the International Space Station has been studied the influence of low gravity on different cytoskeletal structures in adherent monocytes and their ability to migrate. Cell motility relies on an intact structure of different cytoskeletal elements. The highly reduced motility of monocytes in low gravity must be attributed to the observed

severe disruption of the cytoskeletal structures and may be one of the reasons for the dramatic depression of the in vitro activation of human lymphocytes [32].

Previous studies demonstrated that cell activity in immune system is severely dysregulated during microgravity conditions [33]; however, the underlying molecular aspects have not been elucidated yet. The identification of gravity-sensitive molecular mechanisms in cells of the immune system is an important and indispensable prerequisite for the development of counteractive measures to prevent or treat disturbed immune cell function of astronauts during long-term space missions. Moreover, their sensitivity to altered gravity renders immune cells an ideal model system to understand if and how gravity on Earth is required for normal mammalian cell function and signal transduction.

Ullrich et al. investigated the effect of simulated weightlessness (2D clinostat) and of real microgravity (parabolic flights) on key signal pathways in a human monocytic and a T lymphocyte cell line [33,34]. In their paper the authors reported that cellular responses to microgravity strongly depend on the cell-type and the conditions in which the cells are subjected to microgravity. In Jurkat T cells, enhanced phosphorylation of the MAP kinases ERK-1/2, MEK and p38 and inhibition of nuclear translocation of NF-kB were the predominant responses to simulated weightlessness, in either stimulated or non-stimulated cells.

Cogoli et al. demonstrated that non-stimulated monocytic cells U937t responded to simulated weightlessness with enhanced overall tyrosine-phosphorylation and activation of c-jun, whereas PMA-stimulated U937 cells responded the opposite way with reduced tyrosine-phosphorylation and reduced activation of c-jun, compared with PMA-stimulated 1g controls. P53 protein was rapidly phosphorylated in microgravity [33].

2.4 Microgravity effects on lymphocytes and monocytes

Investigations performed in space have shown that gravity changes affect important cellular mechanisms like proliferation, differentiation, genetic expression, cytoskeletal architecture, and motility in lymphocytes, monocytes, and other mammalian cells. In particular a dramatic depression of the mitogenic in vitro activation of human peripheral blood lymphocytes at low gravity has been demonstrated [35]. In this work the authors also showed a reduced interaction between T lymphocytes and monocytes, essential for the second signalling pathway. This hypothesis might be one of the reasons for the observed depression of the in vitro activation of human lymphocytes. Cell motility and a continuous rearrangement of the cytoskeletal network within the cell are essential for cell-to-cell contacts [35]. Whereas nonactivated lymphocytes in suspension are highly motile at low gravity, no data are available so far on the motility of adherent monocytes. It thus can be argued that impaired monocyte locomotion and cytoskeletal changes could be responsible for a reduced interaction of monocytes with T lymphocytes. Pippia et al. demonstrated that the locomotion ability of J-111 cells, an adherent monocyte cell line, in the Random Positioning Machine as earth based model of spaceflight [36] was severely reduced compared with that of controls and the structures of actin, tubulin, and vinculin were affected [35,36].

Crucian et al. demonstrated in lymphocyte subsets exhibiting space flight-induced the presence of alterations in cytokine production [37]. In the same paper they indicated significant physiologic stresses in astronauts following space flight. Altered peripheral leukocyte subsets, altered serum and urine stress hormone levels, and altered T cell cytokine secretion profiles were all observed postflight. In addition, there appeared to be differential susceptibility to space flight regarding cytokine secretion by T cell subsets. These alterations may be the result of either microgravity exposure or the physiologic

stresses of landing and re-adaptation to unit gravity. Future studies, including in-flight analysis or sampling, will be necessary to determine the cause of these alterations [37].

2.5 Apoptosis and phagocytosis

Apoptosis, or programmed cell death, is a critical process in natural tissue homeostasis and results in immediate removal of the dying cell. As cells undergo apoptosis, they are rapidly phagocytosed by professional phagocytes, such as macrophages and dendritic cells [38, 39, 40] or by semi-professional phagocytes in the surrounding tissue such as mesangial cells [41, 42]. In this way, despite the high turnover of cells on a daily basis, tissues are protected from harmful exposure to the inflammatory or immunogenic contents of dying cells by phagocytic clearance. Should cells die by necrosis and disintegrate in situ, release of their contents may exacerbate the local inflammatory response and trigger further leukocyte influx. Therefore, phagocytic removal of apoptotic leukocytes is a prerequisite to restore normal tissue function and plays a critical role in the resolution of inflammation [38, 39, 40, 43, 44]. In addition to removing cells before they undergo lysis, it is proposed that ingestion of apoptotic cells results in potent anti-inflammatory and immunosuppressive effects through the production of anti-inflammatory cytokines such as TGF-β1 and PGE2 and the suppression of release of pro-inflammatory mediators, including IL-8, TNF-α and TXA2, from activated macrophages. This proposal is based on in vitro studies [45, 46]. More recently, in vivo models of inflammation have been utilized to demonstrate that clearance of apoptotic cells in inflammatory lesions such as thioglycollate-stimulated peritonea and endotoxin-stimulated lungs leads to accelerated resolution of inflammation, mediated by the increased production of TGF-β1 [47]. In

addition, apoptotic cell instillation in endotoxin-stimulated lungs reduced pro-inflammatory chemokine levels [47].

Phagocytosis is a phylogenetically ancient process that is an essential feature of the immune response. It was first observed by Elie Metchnikoff [48], a Russian biologist who in the late 19th century noted that "microphages" were englobed by macrophages. Since the recognition by Metchnikoff of the biological importance of phagocytosis, investigators have strived to unravel the molecular basis of this process. Today phagocytosis is defined as the cellular engulfment of large particles (> 0.5 μm).

The initial event in phagocytosis is the recognition of the target. Successful engulfment requires that apoptotic cells expose an "eat me signal" on their cell surface [39]. The phagocyte recognizes this signal and transduces it to the cell machinery required for engulfment. Recognition of apoptotic cells by phagocytes depends on rearrangement of the lipid portion of the plasma membrane on the target cells. Apoptosis leads to disruption of the normal asymmetric distribution of phospholipid across the plasma membrane that generates ligands on the cell surface [49], facilitating recognition by specific receptors on the phagocytes. Phagocytes show significant redundancy in recognition strategies and are able to use many receptors at the same time [38, 40]. Subsequent to recognition mediated through tethering, specific cellular responses culminating in the transduction of a phagocytic signal are generated [40]. These signals target the submembranous cytoskeleton facilitating changes that lead to engulfment, followed by the process of internalization [50]. Finally, the ingested particle enters the lysosomal system in the phagocyte where it is degraded [51].

Apoptotic cells are rapidly phagocytosed by macrophages, a process that represents a critical step in tissue remodeling, immune responses, and the resolution of inflammation [52]. In 1998, Peter Henson, Donna Bratton, and colleagues at National Jewish Health

demonstrated that phagocytosis of apoptotic cells actively suppresses inflammation by inhibiting the production of inflammatory cytokines and inducing production of anti-inflammatory factors, including TGF-β and prostaglandin E2.

Removal of apoptotic cells by phagocytes plays an important role in many biological processes, including embryological development and tissue remodeling. In addition, it has become apparent that one of the key mechanisms for the successful resolution of inflammation is the orchestrated clearance of apoptotic inflammatory cells by phagocytes (e.g., macrophages and dendritic cells) and other cells known to have phagocytic capacity (e.g., hepatocytes, endothelial cells, epithelial cells, etc.).

Furthermore, phagocytosis of apoptotic cells is an active and highly regulated process that not only serves to remove potentially histotoxic cells from the inflammatory milieu, but also directs the phenotype of the phagocytic cell to be anti-inflammatory. Convincing evidence has been presented that reduced or dysregulated phagocytosis of apoptotic cells contributes to the development and propagation of inflammatory disorders. Conversely, enhanced phagocytosis of apoptotic cells may be exploited for therapeutic gain. Indeed, powerful anti-inflammatory drugs such as the glucocorticoids have been shown to augment clearance of apoptotic cells which may contribute to their therapeutic effectiveness [53].

2.6 Apoptosis and phagocytosis in microgravity

The deleterious effects of microgravity on lymphocytes have been demonstrated in previous studies [54,55]. However, research on the effects of microgravity on human natural killer (NK) cells remains exceedingly limited. In this study, we demonstrated that NK cell cytotoxicity was significantly decreased under simulated microgravity (SMG)

conditions ($p < 0.05$). Several processes, including apoptosis, receptor expression, and cytokine secretion, were investigated in human NK cells under SMG. In this study a decreased cytotoxicity was observed, concurrent with increased apoptosis and necrosis, in NK cells after exposure to SMG ($p < 0.05$). A combination of IL-12 and IL-15 may be useful as a therapeutic strategy for overcoming the effects of microgravity on human NK cells during long space missions [54].

Cogoli et al. investigated short-term and long-term effects of clinorotation on human endothelial cells using a three-dimensional random positioning machine [55]. Moreover, the impact of vascular endothelial growth factor (VEGF) was addressed. Immediately, within one hour and after four and twenty-four hours an increase of apoptotic cells was detected. VEGF significantly inhibited the amount of apoptotic endothelial cells. In addition, a clear increase of extracellular matrix proteins such as osteopontin and fibronectin was measured [55].

The functional adaptation of the immune system to the surrounding environment is also a fundamental issue in space [56]. It has been suggested that a decreased number of lymphocytes might be a cause of immunosuppression, possibly due to the induction of apoptosis. Early activation of 5-lipoxygenase (5-LOX) might play a central role in the initiation of the apoptotic program. The goal of the role of apoptosis in lymphocyte depression (ROALD) experiment, flown on the International Space Station as part of the BIO-4 mission of the European Space Agency, was to ascertain the induction of apoptosis in human lymphocytes under authentic microgravity, and to elucidate the possible involvement of 5-LOX. Pippia et al. demonstrate that exposure of human lymphocytes to microgravity for 48 h onboard the ISS 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). The findings provide a molecular background for the immune dysfunction observed in astronauts during space missions, and reveal potential new markers to monitor health status of ISS crew members. This data were also confirmed in studies carried out by the same group in simulated microgravity (RPM) [56].

As part of the systematic evaluation of the innate immune system for long duration missions, Kaur et al. focused the experiments of the antimicrobial functions of monocytes in astronauts participating in spaceflight. The phagocytic index was significantly reduced following spaceflight when compared to control values. This reduction in phagocytosis was accompanied by changes in the expression of two surface markers involved in phagocytosis, CD32 and CD64 [57].

Previously performed in vitro study suggested that gravitational stress may alter functions of immune cells [58]. In this study the authors investigated the in vivo effects of parabolic flight manoeuvres as a short-term model of micro- and hyper-gravity on the cytotoxic and microbicidal polymorphonuclear leucocyte (PMN) functions as the key element of innate immunity.

Parabolic flight induced an increase in leucocyte number with a significant elevation of the PMN fraction. The spontaneous H_2O_2 production by PMNs did not change; however, the capability of PMNs to produce H_2O_2 in response to soluble stimuli [N-formyl-methionyl-leucyl-phenylalanine (fMLP), fMLP and TNF-alpha, calcium ionophore (A23187), phorbol myristate acetate (PMA)] was increased. Adhesive and phagocytic properties of PMNs were not altered. Regarding priming cytokines, IL-8 and G-CSF were significantly elevated [58].

Exploration class human spaceflight missions will require astronauts with robust immune systems. Innate immunity will be an essential element for the healthcare maintenance of astronauts during these lengthy expeditions.

Kaur et al. investigated neutrophil phagocytosis, oxidative burst, and degranulation of 25 astronauts after four space shuttle missions and in nine healthy control subjects. The mean values for phagocytosis of Escherichia coli and oxidative burst capacity in neutrophils from astronauts on the 5-day mission were not significantly different from those observed in neutrophils from the control subjects. Before and after 9 to 11 day missions, however, phagocytosis and oxidative burst capacities were significantly lower than control mean values. No consistent changes in degranulation or expression of surface markers were observed before or after any of the space missions. This study indicates that neutrophil phagocytic and oxidative functions are affected by factors associated with space flight and this relationship may depend on mission duration [59].

2.7 Phosphorylation changes, Syk and microgravity

Investigations in space have shown that gravity changes affect important cellular mechanisms like proliferation, differentiation, genetic expression, cytoskeletal architecture, and motility in lymphocytes, monocytes and other mammalian cells. Phosphorylation is an important posttranslational modification that modulates cell life. In particular Tyrosine 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.

In the last decade researches focused their efforts to better understand the pathways associated with the onset of different immune diseases and to identify new targets for the development of possible therapies.

Protein tyrosine phosphorylation is important in cell signaling and is involved in many physiological activities, such as tissue differentiation and growth, sugar and fat metabolism, and the immune response [64,65]. Protein tyrosine phosphatase 1B (PTP1B), also known as PTPN1, is the first purified mammalian PTP and is considered as reference prototype for following PTP studies [63]. Protein tyrosine phosphorylation is precisely regulated by the balance of PTKs and PTPs [60]. Spleen tyrosine kinase (SYK) was first discovered and cloned in porcine splenocytes in 1991 [61,64].

SYK is a 72-kDa nonreceptor tyrosine kinase that contains two SRC homology 2 domains and a kinase domain. It was initially shown to be critical for the signaling of immunoreceptors, such as FcR and BCR, in association with ITAMs in hematopoietic cells [61]. More recent studies demonstrate the expression of SYK in nonhematopoietic cells, such as fibroblasts, mammary epithelial cells, hepatocytes, synoviocytes, and vascular endothelial cells. It is also involved in cell signaling downstream of diverse cellular stimuli, including IL-1, TNF-α, LPS, and β_1-integrin in cells, that do not contain conventional ITAMs [62, 66, 65].

Consequently, the development of Syk kinase inhibitors could conceivably treat these disorders and so they have become a major focus in the pharmaceutical and biotech industry. Syk kinase inhibition is suggested as a powerful tool for the therapy of different pathologies [69, 70].

Data in the literature show that the receptor complex CD36, β1 and/or β2 integrins, and the tetraspanins CD9 and/or CD81, serves to link CD36 to the adaptor FcRγ, which bears an immunoreceptor tyrosine activation motif [62]. By coupling to FcRγ, CD36 is

able to engage Src-family kinases and Syk, which in turn drives the internalization of CD36 and its bound ligands. Thus, Syk could be involved in the cascade of events related to their programmed cell death, identifying the molecular components involved in the signal transduction pathway of activated CD36 [62].

We investigated tyrosine phosphorylated proteins in response to simulated microgravity. Gravity has been a constant force throughout evolutionary history on Earth. Thus, one of the fundamental biological questions is how the architecture and function of human cells are related to the gravitational force and thereby adapted to life on Earth. MASER-12 experiment could identify signal molecules, which are sensitive to altered gravity, and indicates that gravity is obviously not only a requirement for transcriptional processes as described before, but also for specific phosphorylation/dephosphorylation of signal molecules and surface receptor dynamics (MASER-12) [71].

3. AIM OF THE THESIS

Phosphorylation is an important posttranslational modification that modulates cell life. In particular Tyrosine 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. Microgravity could play a role in an increase of physiological stress.

The aim of this thesis is to investigate the phosphorylative modifications related to the recognition of apoptotic cells by phagocytes under simulated microgravity and also to analyze the role of p72 Syk in phosphorylative changes.

4. MATHERIALS AND METHODS

4.1 Cell cultures

The experiments performed in this work were carried out using primary cultures as monocytes, human T lymphocytes isolated from buffy coat of donors kindly supplied by the "Centro Immunotrasfusionale, Azienda Sanitaria, Santissima Annunziata" Sassari, Italy.

4.1.1 Peripheral Blood Mononuclear Cells (PBMCs)

Primary cultures of Peripheral Blood Mononuclear Cells (PBMCs) have been prepared starting from buffy coats of healthy donors. The buffy coat has been slowly reconstructed with Hanks' Balance Salts (cell culture tested and modified, without $CaCl_2$, $MgSO_4$, phenol red and $NaHCO_3$, used at 9.5 g/l, pH 7.6) in a 1:10 (v/v) ratio. The mixture has been stratified over an isovolume of a solution containing polysucrose and sodium diatrizoate, adjusted to a density of 1.077 g/ml (Histopaque 1077, Sigma-Aldrich St. Louis, USA). This medium facilitates the recovery of large numbers of viable mononuclear cells. The Peripheral Blood Mononuclear Cells (PBMCs) have been separated according to the Böyum method [72, 73]. Thus, 20 ml of reconstructed buffy coat have been stratified over 20 ml of Histopaque 1.077 for each 50 ml conical polypropylene tube. The resulting mixture was centrifuged at 400 x g for 30 minutes at room temperature. The Histopaque 1.077 layer is able to separate the different phases, helping recover the limpho-monocytes ring which was afterward diluted to a volume of 50 ml with Hanks' Salts. The samples have been centrifuged at 250 x g for 10 minutes at room temperature, eliminating the supernatant and resuspending the pellet in another 40 ml of Hanks' Salts. The excess erythrocytes have been eliminated by resuspending the obtained pellet with 10 ml of ACT

(Tris-buffered Ammonium Chloride solution) lysing solution, keeping it incubating for 10 minutes at room temperature, then suspending it in 40 ml of Hanks' Salts to block the ACT activity. The samples have been centrifuged again at 250 xg for 10 minutes at room temperature, and this procedure has been repeated three times in order to collect all cell pellets, so that it was all transferred to a single tube. The overall pellet has been resuspended in 30 ml of RPMI 1640 (Glutamax-GIBCO, Paisley, UK) culture medium containing 20 mM HEPES and 50 μg/ml of gentamicin. The cell count was conducted according to the trypan blue method 400-600 x 10^6 total PBMCs have been obtained, and then added to 10% Fetal Bovine Serum (FBS, Gibco Paisley, UK) heat-inactivated at 56° C for 30 minutes. The PBMCs pellet was kept in incubator at the constant temperature of 37° C in a 5% CO_2 humidified atmosphere, and underwent periodic changes of growth medium until the use. The pellet was employed to obtain purified T lymphocytes and adhesion monocytes.

4.1.2 T lymphocytes

The purified T lymphocytes were obtained using high affinity Human T-cell enrichment columns (R&D Systems, Minneapolis, USA). These columns are designed to purify human T cell populations via high affinity negative selection. 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 contains 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%.
Processed cells that are to be loaded onto the column (300 million maximum) are suspended in 2 ml of column wash buffer and loaded onto T Cell Enrichment Columns. The column is placed in a column rack. The column content is then washed with a total of

8 ml of column wash buffer (1X), after the column buffer has drained down to the level of the white filter, then the 2 ml cell suspension is applied to the top of the column. The cells suspended in the column are incubated at room temperature for 10 minutes and subsequently eluted from the column with 4 aliquots of 2 ml column wash buffer. The collected cells are centrifuged at 250 xg for 5 min.

The overall pellet from the cell count according to the trypan blue method are resuspended in $1x10^6$/ml/75cm^2 flask of RPMI 1640 (Glutamax-GIBCO, Paisley, UK) culture medium containing 20mM HEPES and 50μg/ml Gentamicin (solution in deionized water, Sigma-Aldrich St. Louis, USA) without Fetal Bovine Serum (FBS Gibco Paisley, UK) to induce apoptosis in cells. After verifying apoptosis in human T cells, it was possible to combine the two cell cultures to form a co-culture of human T cells and macrophages.

4.1.3 Monocytes/macrophages

To get the best adhesion monocytes culture, cells should be seeded at $1x10^6$ cell/cm^2/ml in plates or wells with RPMI 1640 (Glutamax-GIBCO, Paisley, UK) added with 10% Fetal Bovine Serum (FBS Gibco Paisley, UK) containing 20mM HEPES and Gentamicin 50 mg/ml (solution deionized water, Sigma), and kept in incubator at the constant temperature of 37° C in a 5% CO_2 humidified atmosphere.

Cells must remain inside the thermostat without shaking for 48 hours [74] then the culture medium is replaced by slow and careful removal of the supernatant, and the plate is washed with PBS (Phosphate buffered saline, Sigma-Aldrich, St.Louis, USA) at room temperature. The cells adhering to the plate are reconditioned with maximum 15 ml of growth medium and kept in incubator till the next medium change, at least after 48 additional hours. Around the sixth day an initial differentiation of the monocytes into

macrophages will start (so the culture medium should be changed once more) which will be stabilized with fully differentiated macrophages on the eighth day.

4.1.4 Co-culture of T lymphocytes and monocytes/macrophages

The co-culture of human T apoptotic cells and macrophages was formed combining the differentiated macrophages apoptotic cells that are phagocytosed in 20 hours. Firstly the supernatant must be eliminated from the flask of macrophages using BD Falcon serological pipettes (Becton Dickinson and Company, Franklin Lakes, USA) and then washed several times with PBS; subsequently the human T apoptotic cells are placed in a ratio of 1:20 and put in the incubator for 15-20 hours to induce phagocytosis and the formation of the co-culture. All solutions and equipment coming into contact with cells must be sterile, and aseptic techniques should be used accordingly. All culture incubations should be performed in a humidified 37° C, 5% CO_2 incubator unless otherwise specified.

4.1.5 Random Positioning Machine (RPM)

Conditions similar to those occurring during exposure of cells to microgravity [75] were created by the three-dimensional clinostat developed by Fokker Space (Leiden, The Netherlands), also called RPM. The RPM consists of a frame that rotates within a second rotating frame, and each frame is driven by a separate motor. Rotation of each frame is random, autonomous, and regulated by computer software. Each frame rotates at a speed of 60 s^{-1}, which corresponds to a force of gravity of approximately 2 x 10^{-3} *g* at the centre of the frame. The RPM was located in a room at 37° C, and a box containing the cell cultures, tissue culture flasks, was placed at the centre of the inner frame. Control (static) cultures were placed at the basement of the RPM. To avoid the presence of air bubbles, which could lead to shear force damage of the cells on the RPM, the tissue culture flasks were

completely filled [76]. Before each experiment, tissue culture flasks were evaluated through a Trypan Blue exclusion test. The cells were then suspended in fresh RPMI 1640 to obtain 8 millions cells for each experimental point. RPM was used to test the microgravity effects on post-translational modification of proteins extracted and how the simulation with the RPM is comparable with the effects of real microgravity. Then 1 μl phosphatase inhibitors 2-3 and protease (Sigma-Aldrich, St.Louis, USA) was added to all samples and stored at - 20° C in freezer.

4.1.6 Treatment in RPM

T cells and monocytes were activated with 10μg/ml of ConA (Concanavalina A is a mitogenic agent extracted from the plant named *Canavalia ensiformis* and is specific for this kind of cells) and subjected to 0 xg in simulated microgravity for different interval times (1, 2, 3h) [77]. The rotation speed of the frames was 60 s^{-1}. Control T cell and monocytes cultures were installed in the basement of the RPM (1xg static controls). Secondly T cells monocytes, macrophages and co-culture were subjected to 0 x g in simulated microgravity for different interval times (0 h and 2 h). The rotation speed of the frames was 60 s^{-1} and the same cultures were installed in the basement of the RPM (1 xg static controls). After each step all adherent cells were removed by gently scraping with a plastic cell scraper and all types of cells transferred to a 15 ml conical tube and centrifuged 10 minutes at 250 xg room temperature, to remove the PBS solution.

Parameters adopted until this moment in *in vitro* experiments on human T lymphocytes demonstrate that RPM is a reliable tool to simulate microgravity, which is attested approximately the 10^{-3}-10^{-2} xg (Fig.7).

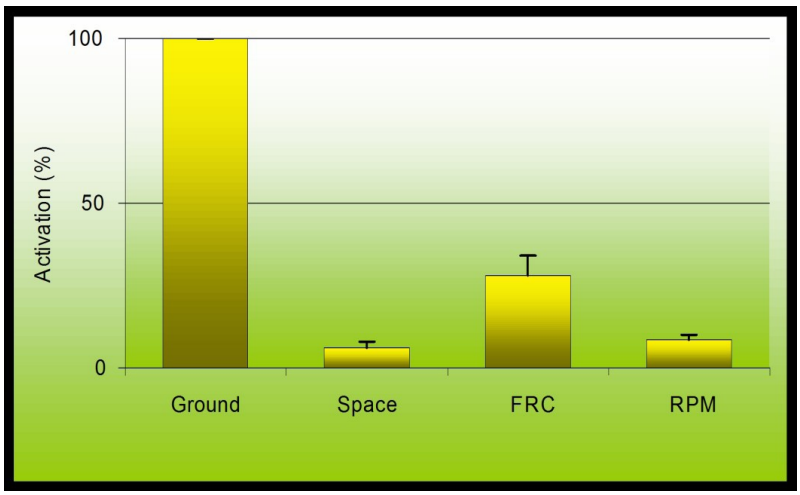

Fig. 7: **Effects of microgravity on activated human T lymphocytes**

Microgravity culture and ground controls were observed using an Olympus IX 71 inverted microscope equipped with a 20X and 40X Plan Apo objectives. A Nomarsky Differential Interference Contrast illumination was used to better investigate morphology differences between samples. Digital images were acquired with a Magnafire monochrome cooled CCD camera (Optronics, Goleta, Ca, USA) and processed with ImageJ software (Fig.8).

Fig. 8: Inverted microscope

4.1.7 SDS-PAGE

The total protein content was measured according to the DC Protein Assay (Bio-rad in the U.S.), a colorimetric assay for protein concentration after detergent solubilisation. Protein concentration was determined as instructed by the manufacturer, turning out to be about 3 g/l in monocytes, macrophages and co-culture as well as in human T cells.

Proteins were checked through mono-dimensional electrophoresis (1-DE), so a previous solubilisation was required in a Laemmli Buffer [77], with 2% DTT in a volume ratio of 1:1. The samples have been heated for 5 minutes at 95° C and then separated on 8% polyacrylamide mini-gel (6.5 cm), with acrylamide-bisacrylamide in a ratio of 29:1.10 μg of total proteins were loaded for each lane. SDS-PAGEs were run in duplicate on the Bio-Rad mini-protean Tetra cell set up.

4.1.8 Western Blotting

30 μg of proteins were loaded onto each lane of mini-gels then transferred to nitrocellulose membranes [78] following the instructions by the manufacturer. Membranes were blocked in PBS/Tween [Na_2HPO_4 (8.5 mM), KH_2PO_4 (1.47 mM), KCl (2.68 mM), NaCl (137 mM), 0.1% Tween 20]. The separated proteins were probed by both anti-phosphotyrosine (SC 7020 Santa Cruz tecnology, CA) and anti–Syk antibodies (Cell Signaling Technology, Inc Danvers, MA) all diluted 1:2000. To visualize the desired antigens, secondary antibodies conjugated to infrared fluorescent dyes excitable at 800 nm (IRDye 800 CW, Li-COR-USA) were employed in a 1:25000 dilution, and measured in an 800/700 nm double laser scanner (Odyssey, Licor, USA).

4.1.9 Mass spectrometry

In a parallel test, the second series of gels was coloured with Coomassie Blue Brilliant mass-compatible [78] and the bands of interest were cut to be identified through mass spectrometry by peptide mass finger-printing. For this purpose, the Coomassie stained bands were cut from gels, and proteins were digested with trypsin. Each slice of gel was de-stained through several washes in 5 mM NH_4HCO_3/acetonitrile (50/50 v/v) and successively dried in pure acetonitrile. The gel slices were then rehydrated for 45' at 4° C in 20 µl of a 5mM NH_4HCO_3 digestion buffer containing 10 ng/µl of trypsin. Excess protease solution was removed and the volume adjusted with 5 mM NH_4HCO_3 to fully cover the gel slices and digestion was allowed to continue overnight at 37° C [78]. Samples were characterized by mass spectroscopy. For this purpose they 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 was performed with a MALDI-TOF Micro MX (Micromass, Manchester, UK) operating in reflection modality and 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 the mass of 2465,1989 Da corresponding to the ACTH fragment 18-39 (Sigma A8346) background subtract with adaptive mode, performing deisotoping with threshold 3%.

For peptide mass fingerprinting (PMF) analysis, the MS spectra were converted into pkl files using Mass Lynx 4 0. Peak lists containing the 20 most intense peaks of the spectrum were conveyed 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 missing 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 into account.

5. RESULTS AND DISCUSSION

The purpose of this thesis was to characterize phosphorilative changes related to the recognition of apoptotic cells by phagocytes under simulated microgravity, and to analyze the role of p72 Syk in phosphorylative changes using as a model PBMCs for which data in the literature are still not clear.

Proteomic techniques have been used to evaluate phosphorylative post-translational modifications and to compare the protein pattern of cells in simulated microgravity and in normal conditions. All the gels presented were performed 3 times at 8% of polyacrylamide and run under reduction conditions. As a preliminary experiment we tested the phosphorylation responses both in non-activated T cells isolated from donors. For each experiment, proteins were extracted using a specific solubilisation buffer. 1 DE, Coomassie stain blue and Anti-phosphotyrosine or Anti- Syk Western Blot were performed. Coomassie gels (Fig.9) showed a uniform protein pattern of T lymphocytes, monocytes, macrophages and co-cultures in all conditions employed.

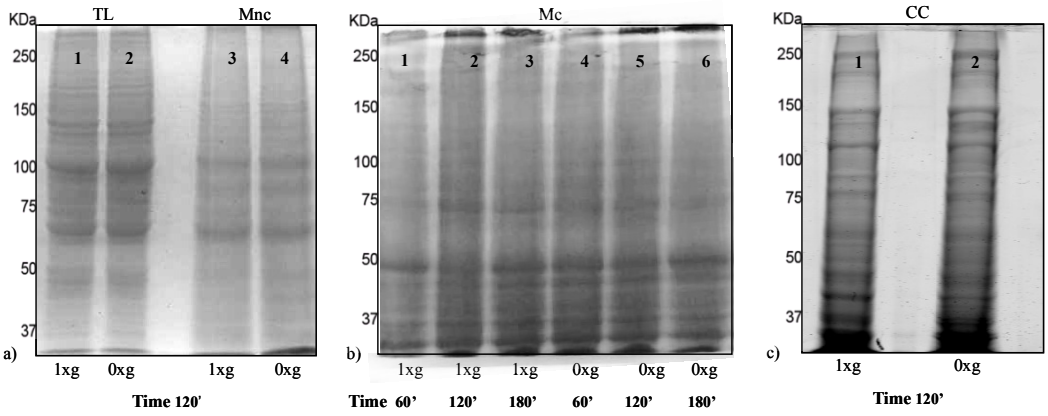

Fig. 9: Gel coomassie stain blue a) **1** T lymphocytes (TL) static control (1xg); **2** T lymphocytes (TL) RPM (0xg); **3** Monocytes (Mnc) static control (1xg), **4** Monocytes (Mnc) RPM (0xg). b) **1** Macrophages (MC) static control (1xg); **2** Macrophages (MC) static control (1xg); **3** Macrophages (MC) static control (1xg) ; **4** Macrophages (MC) RPM (0xg); **5** Macrophages (MC) RPM (0xg); **6** Macrophages (MC) RPM (0xg). c) **1** Co-Culture (CC) static control (1xg); **2** Co-Culture (CC) static control (1xg).

In order to investigate the microgravity effects and tyrosine phosphorilative response in T cells and in monocytes, anti-phosphotyrosine immunoblots were performed. Fig.10 shows that in resting T lymphocytes the phosphorylative signal is not present, instead we found an increase of tyr-phosphorylation in monocytes particularly after 120 minutes of RPM.

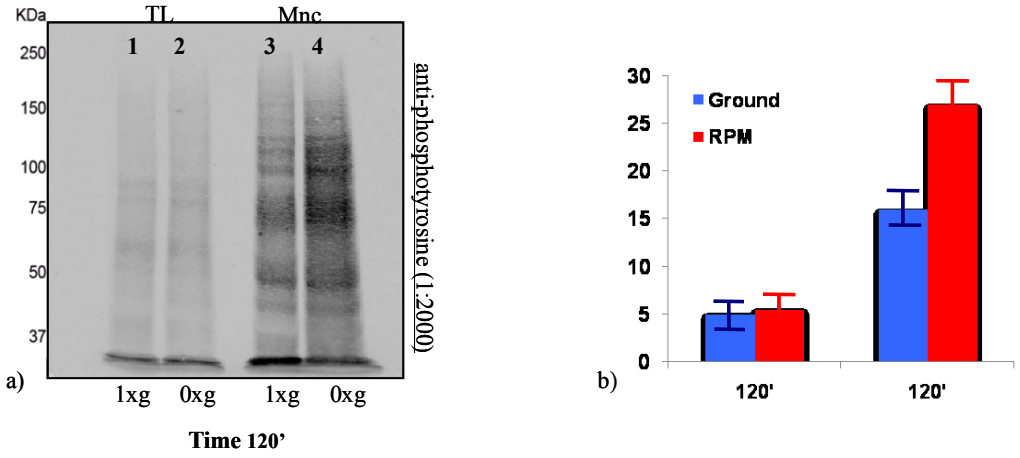

Fig. 10: a) **Western Blotting anti-phosphotyrosine** (1:2000). **1** T lymphocytes (TL) static control (1xg); **2** T lymphocytes (TL) RPM (0xg); **3** Monocytes (Mnc) static control (1xg) , 4 Monocytes (Mnc) RPM (0xg) recorded at times shown respectively on each line. **b) Densitometry analysis.**

Then we focused our attention on the protein tyrosine kinase Syk (p72Syk), already known in the literature for its different role in stress conditions and as possible treatment for several forms of leukemia and lymphoma. Western Blot was performed incubating membranes with anti-Syk antibody. Fig 11 shows that this kinase in isolated resting T cells after 120 minutes in RPM does not seem to be present, on the contrary in monocytes Syk appears to be constitutively expressed.

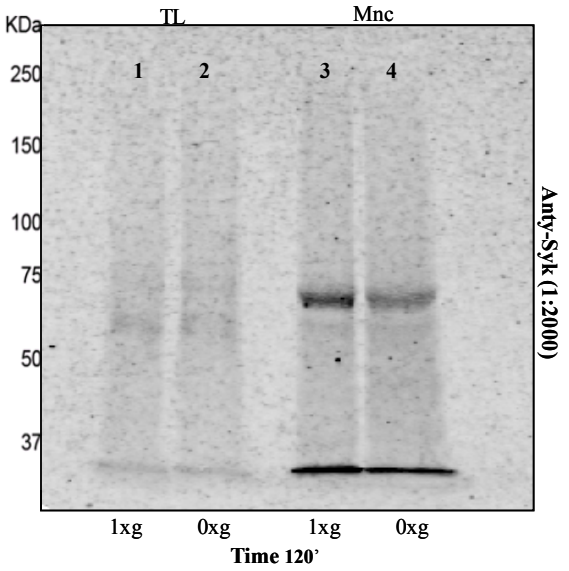

Fig. 11: **Western Blotting Anti-Syk** (1:2000). **1** T lymphocytes (TL) static control (1xg); **2** T lymphocytes (TL) RPM (0xg); **3** Monocytes (Mnc) static control (1xg); **4** Monocytes (Mnc) RPM (0xg) recorded at times shown respectively on each line.

We chose to deepen the study of the T cells under microgravity conditions. Therefore, to stimulate the activation of the T cell we used 10 μg/ml of Concanavalin A (ConA) activator. Activated T cells in normal conditions (Fig. 12) after Concavanalin A treatment showed a marked time dependent response observable with an increase which reached the maximum at 120 minutes and a subsequent decrease of phosphorylation with a complete reversal after 180 minutes; the tyrosine phosphorylation signal was strongly higher in activated T cells undergone to RPM.

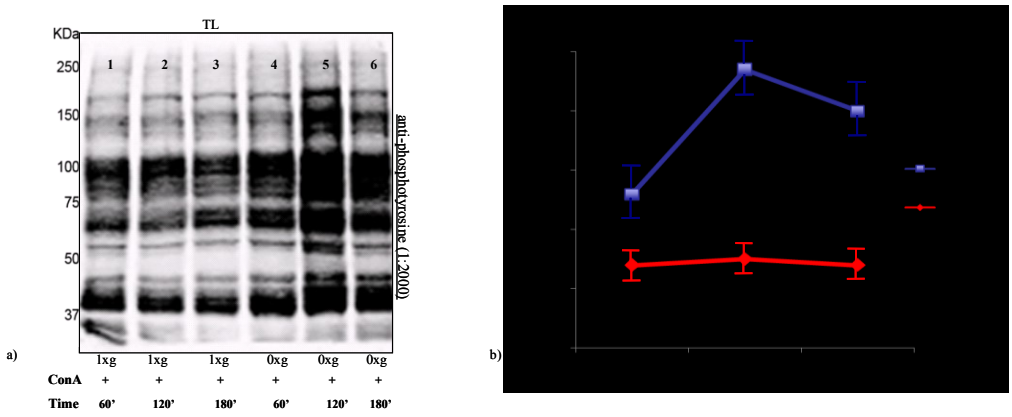

Fig. 12: Western Blotting anti-phosphotyrosine (1:2000) activated with Concanavalin A (ConA) a) **1** T lymphocytes (TL) static control (1xg); **2** T lymphocytes (TL) static control (1xg); **3** T lymphocytes (TL) static control (1xg); **4** T lymphocytes (TL) RPM (0xg) ; **5** T lymphocytes (TL) RPM (0xg); **6** T lymphocytes (TL) RPM (0xg); **7** T lymphocytes (TL) static control (1xg) recorded at times shown respectively on each line; **b) Densitometry analysis.**

We also tested the phosphorylation response in monocytes undergone in microgravity conditions at different incubation time. Fig. 13 shows a marked phosphorylative response with a signal increase which reached the maximum in RPM after 120 minutes. Control monocytes were less phosphorylated in comparison with samples submitted in microgravity conditions. Data were confirmed by densitometry analysis.

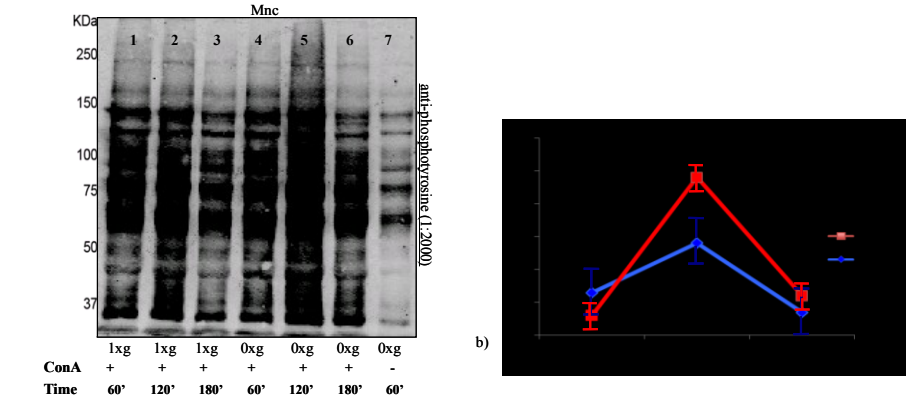

Fig.13: Western Blotting anti-phosphotyrosine (1:2000) activated with Concanavalin A (ConA) a) **1** Monocytes (Mnc) static control 1xg; **2** Monocytes (Mnc) static control 1xg; **3** Monocytes (Mnc) static control 1xg; **4** Monocytes (Mnc) RPM 0xg; **5** Monocytes (Mnc) RPM 0xg; **6** Monocytes (Mnc) RPM 0xg; **7** Monocytes (Mnc) control 0xg recorded at times shown respectively for each line; **b) Densitometry analysis**.

Moreover, we decided to incubate isolated T lymphocytes with ConcanavalinA and under microgravity conditions for 120 minutes. Anti-Syk immunoblot showed that Syk is not expressed in T lymphocytes at 0xg, but it is present in activated T lymphocytes (Fig.14).

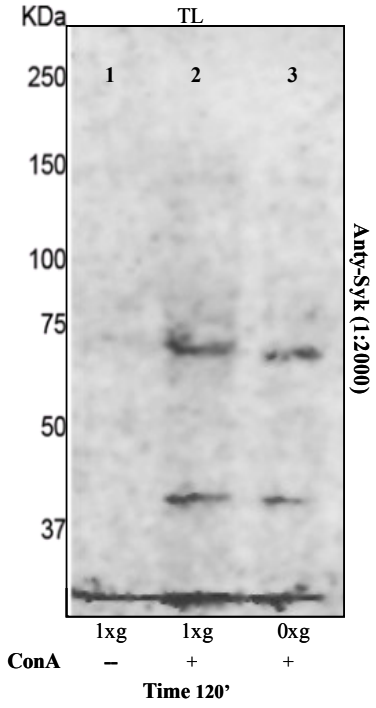

Fig.14: Western Blotting anti-Syk (1:2000) activated with Concanavalin A (ConA). **1** T lymphocytes (TL) static control (1xg) without ConA; **2** T lymphocytes (TL) static control (1xg); **3** T lymphocytes (TL) RPM (0xg) recorded at times shown respectively on each line.

In order to observe if Syk Kinase is involved in Tyrosine phosphorylation signal, we decided to incubate isolated monocytes with Syk Inhibitors under microgravity conditions for 120 minutes. Monocytes total protein immunoblot incubated with Anti phosphor-Tyr (1:2000) showed a decreased phosphorylation signal in the presence of Syk Inhibitors (fig.15). In the absence of Syk Inhibitors under the same conditions the Tyrosine phosphorylation signal is persistent.

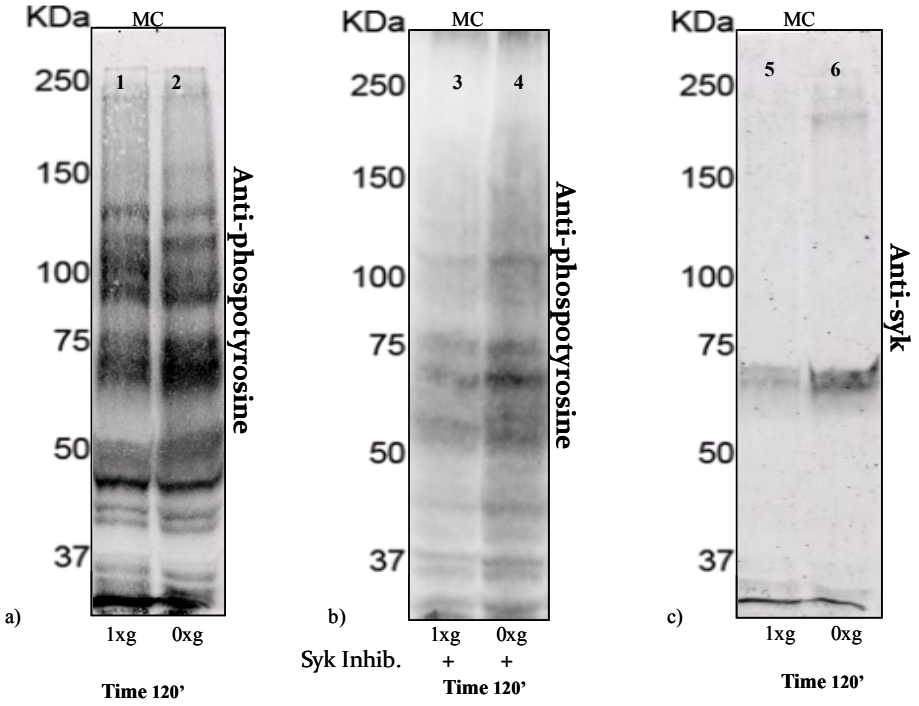

Fig. 15: Macrophage Western Blotting. a) Anti-phospotyrosine: **1** static control **(**1xg); **2** RPM (0xg); b) Anti-phospotyrosine Syk Inhibitor: **3** static control (1xg); **4** RPM (0xg); c) Anti-syk: **5** static control (1xg); **6** RPM (0xg) recorded at times shown respectively for each line.

Furthermore, immunoblots of the co-cultures (Fig.16) total proteins were performed and stained with anti-phosphotyrosine and anti-Syk. It was evident a decrease of the phosphorylation signal in microgravity conditions. Moreover Syk did not appear expressed at 0xg. These two phenomena could be associated to a degradation of Syk. In fact the decline of tyrosine phosphorylation concomitant with the absence of Syk expression showed that this kinase involved in the first steps of these events.

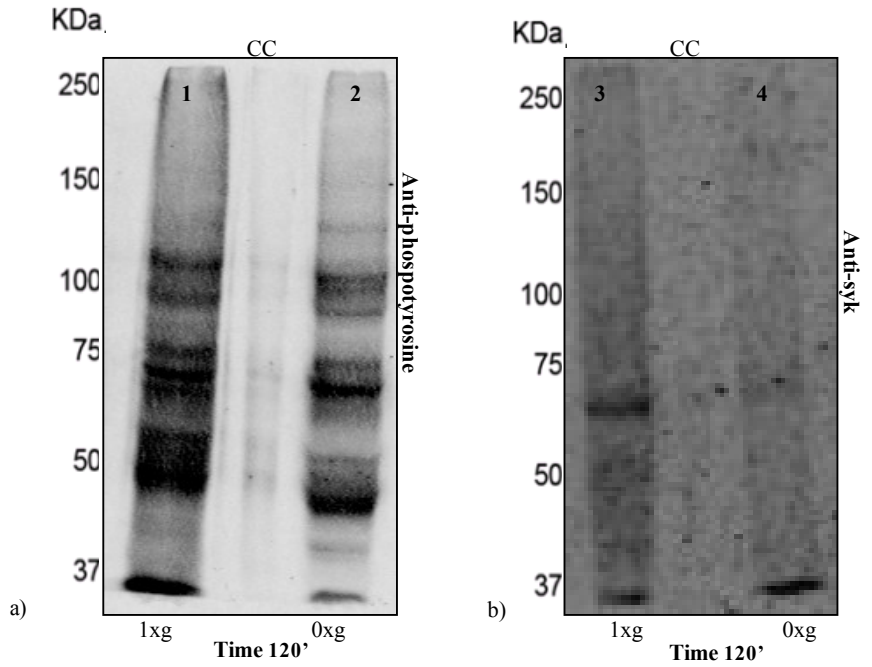

Fig. 16: Co-culture (CC) Western Blotting. a) anti-phosphotyrosine (1:2000): **1** static control (1xg); **2** RPM; b) Anti-syk: **3** static control (1xg); **4** RPM (0xg) recorded at times shown respectively on each line.

In addition, experiments were carried out using MALDI-TOF mass spectrometry to identify the proteins involved with these phenomena.

Mass spectrometry identification in Table 1,2 revealed that several proteins found to be phosphorylated belonged to the class of cytoskeletal proteins. Other identified proteins are HSPs, typically abundant in stress conditions, such as mechanical or pathological stress. They appear to be present also in microgravity conditions.

TABLE 1: Identification of human T lymphocyte proteins

Score	Matched Pept.	Name	Mass (Da)	Accession Number
57	11	Endoplasmin	92411	HSP90B1_HUMAN
96	16	**Heat shock protein HSP 90-alpha**	**84607**	**HSP90AA1_HUMAN**
77	14	**Heat shock protein HSP 90-beta**	**83212**	**HS90A_HUMAN**
63	11	78 kDa glucose-regulated protein	72288	GRP78_HUMAN
52	11	Heat shock cognate 71 kDa	70854	HSP7C_HUMAN
73	11	**Stress-70 protein, mitochondrial**	**73635**	**GRP75_HUMAN**
95	12	**60 kDa heat shock protein, mitochondrial**	**61016**	**CH60_HUMAN**
68	8	Protein disulfide-isomerase A3	56747	PDIA3_HUMAN
58	9	ATP synthase subunit beta, mitochondrial	56525	ATPB_HUMAN
87	9	Glyceraldehyde-3-phosphate dehydrogenase	36030	G3P_HUMAN
75	7	Actin, cytoplasmic 1	41710	ACTB_HUMAN
75	7	Actin, cytoplasmic 2	41766	ACTG_HUMAN

TABLE 2: Identification of monocytes proteins

Score	Matched Pept.	Name	Mass (Da)	Accession Number
55	7	GR2- associated - binding -protein	74411	GAB2-HUMAN
56	5	**Heat shock protein HSP 90-alpha**	**84607**	**HSP90AA1_HUMAN**
77	14	**Heat shock protein HSP 90-beta**	**83212**	**HS90A_HUMAN**
146	15	**Heat shock protein HSP 90-beta**	**83212**	**HS90A_HUMAN**
79	9	78 kDa glucose-regulated protein	72288	GRP78_HUMAN
139	14	Tubulin beta chain	49639	TBB5_HUMAN
80	8	Alpha-enolase	47139	ENOA_ HUMAN
92	9	Actin, cytoplasmic1	41710	ACTB_HUMAN
58	10	ATP synthase subunit beta, mitochondrial	56525	ATPB_HUMAN
87	9	Glyceraldehyde-3-phosphate dehydrogenase	36030	G3P_HUMAN
74	10	Annexin A 2	38580	ANXA2_HUMAN
74	7	Actin, cytoplasmic 2	41766	ACTG_HUMAN

At each cell stage, both in natural conditions and in simulated microgravity (RPM), pictures were taken with inverted microscope and specific images from three different experiments were selected. Fig 17-a and 17-b show T lymphocytes both in normal (1xg) and simulated microgravity conditions (0xg). No differences in cell number and morphology were detected following simulated microgravity for 120 min. Instead relevant differences can be observable in monocytes, especially under microgravity conditions, compared to controls with a decrease in adherent cell number.

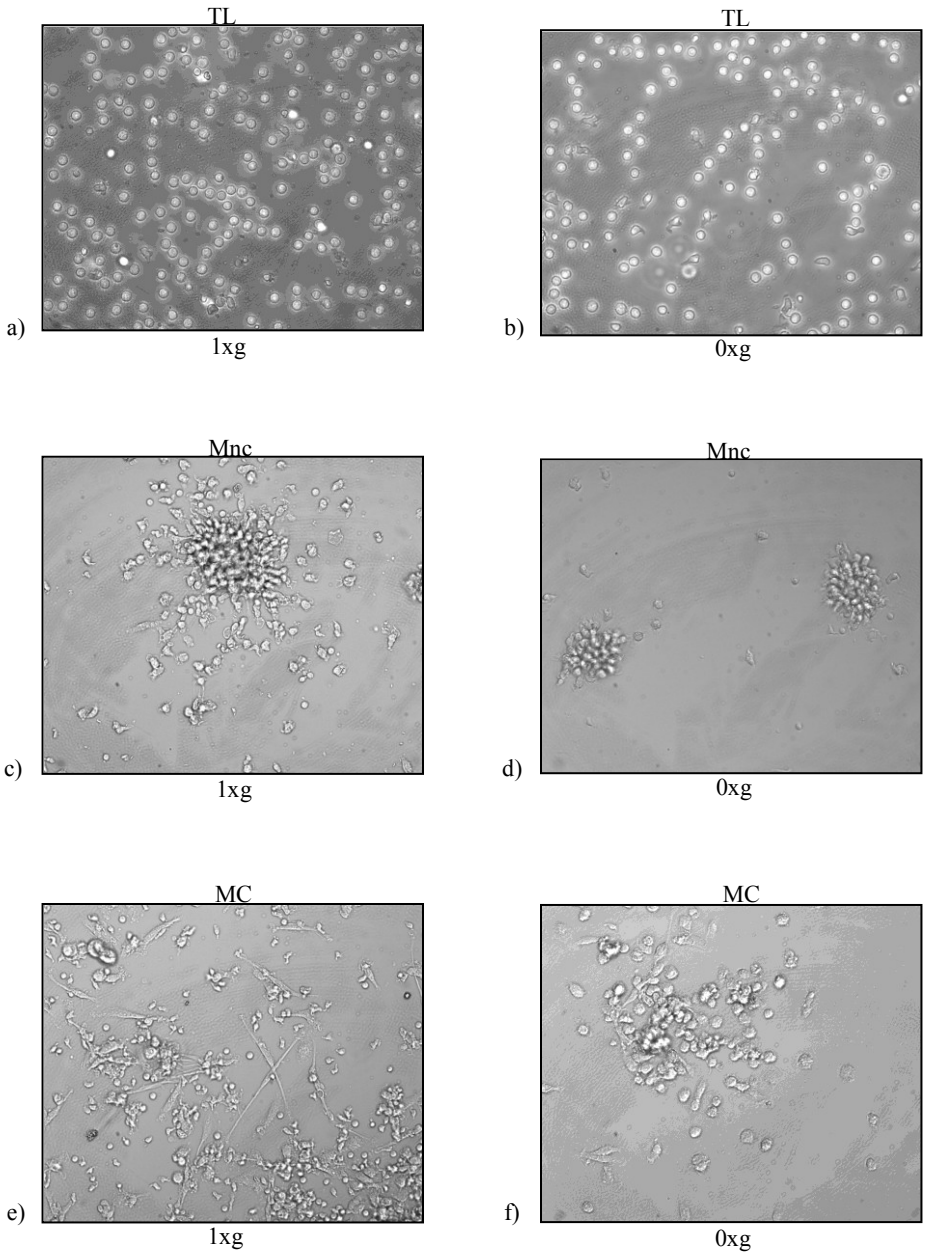

Fig. 17: T lymphocytes (TL) a) static control **(**1xg); b) RPM (0xg)**;** monocytes c) static control (1xg); d) RPM (0xg); macrophages e) static control (1xg); f) RPM (0xg).

In macrophages undergoing RPM (Fig. 17) filopodia start to detach, leading to an adherence loss. Cells get a round shape and are not differentiated compared to the control macrophages that instead result as oblong and dynamic cells.

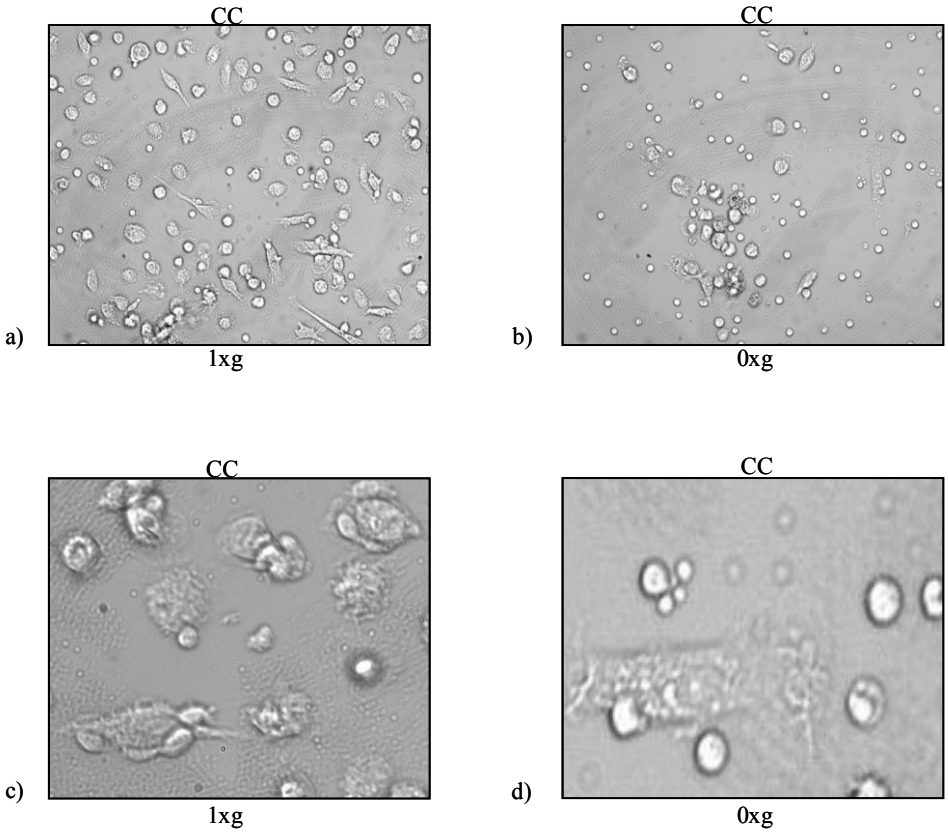

Fig. 18: Co-Culture (CC) a) static control (1xg); b) RPM (0xg); c) detail static control (1xg); d) detail RPM (0xg).

In static control co-cultures of apoptopic T lymphocytes and macrophages we noted that cells are still adherent and had a good phagocytic response (Fig.18-c). A different cellular aspect could be observed in RPM co-cultured cells. Filopodia were detached and cells did phagocytate. Fig.18-d shows co-cultured cells having spheric shape and lack of phagocytosis. Unfortunately interference microscope did not provide intracellular vision.

6. CONCLUSION

We have performed a series of experiments designed to evaluate the effects of simulated microgravity on monocytes, macrophages, human T lymphocytes and on co-cultures of apoptotic T cells and macrophages. We showed that all these cells are actually influenced by reduced gravity and the effects they experienced are reflected in their phosphorylation process.

Microgravity seemed to influence monocytes, macrophages, and co-cultures of apoptotic T cells and macrophages, showing a time dependent response with an increase which reached the maximum at 120 minutes and a subsequent decrease of phosphorylation with a complete reversal after 180 minutes.

T lymphocytes proteins are extensively modified after ConA stimulation at different incubation times. Syk Kinase could be involved in a better internalization of CD36.

Mass spectrometry identification revealed that some proteins found to be phosphorylated belonged to the class of cytoskeletal proteins. Other identified proteins are HSPs, typically abundant in stress conditions especially in RPM in monocytes, macrophages and co-cultures. T cells, macrophages and all primary cultures with the increase of tyrosine phosphorylation of several proteins.

The results here obtained are interesting under the point of view of the response of the immunity system to reduced gravity conditions, casting the bases for more deepened studies on this remarkable topic which can be of interest also in view of long term manned space missions.

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