Consequences of space stressors on immunity

Evaluation of immunological changes using space-simulated *in vitro* models

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Summary

As human beings we are defined by our thirst for knowledge and our drive to venture beyond the horizon. At the risk of quoting a famous starship captain, among all the adventures we have undertaken throughout history, space can without a doubt be considered our final frontier. From the moment our presence in space became a reality - over 50 years ago - we have continuously challenged ourselves to keep going further. We have conquered the Moon and have established a permanent human presence in low-Earth orbit (LEO) by means of the International Space Station (ISS) which not only represents one of the greatest technological and scientific achievements of mankind, it is a symbol of international cooperation and peace. More recently, the involvement of the private industry in the space sector has increased and several companies are paving the road to space tourism. Meanwhile, the major space agencies have begun focusing their efforts on the next, logical step… extending our presence beyond LEO.

Unfortunately, our experience in space so far has already confronted us with one major obstacle: human beings are not made to dwell in space. Life as we know it has evolved for millions of years in the presence of gravity and in the safe and stable environment our planet provides. In space, we have to survive without Earth's gravity, without high-level radiation-protection and without a natural ecosystem tending to our needs. Hence, it is not surprising that astronauts are confronted with a broad array of stressors (microgravity, radiation, psychological stress) affecting their physiological and psychological health.

The physiological effects of space travel on a human being are diverse. Microgravity affects our cardiovascular, musculo-skeletal and neuro-vestibular system, while radiation originating from the Sun and outer space as well as secondary particles can cause extensive damage at the cellular level (DNA) which in turn can lead to cell death, an increased risk for cancer and cataract. Although it seems less obvious, spending time in space also has a detrimental effect on our immune system, particularly the mechanisms responsible for initiating a protective response. Since expanding our presence in space will translate into longer flights and several bacteria, viruses and fungi are known to thrive and become more pathogenic in space, it is of the upmost importance to understand how our (cellular) immune system evolves in space and to find ways of counteracting the negative effects of space travel.

In order to allow close monitoring of the astronaut's cellular immunity in space, this master thesis evaluated the use of an *in vitro* delayed-type hypersensitivity (DTH) reaction using whole blood samples. This work was part of the preparatory phase of the ESA accepted flight experiment "Monitoring the Cellular Immunity by *in vitro* DTH assay on the ISS" (MoCISS) with a flight foreseen in 2014-2015.

Several preliminary experiments confirmed functioning of the *in vitro* DTH test in both vented and sealed containers. In addition, a test was performed to evaluate the effect of a major physical space stressor, i.e. microgravity. Although the latter experiment confirmed that a lack of gravity had a negative effect on our cellular immune response towards antigens (using sealed tubes), some differences in response could be observed between the sealed and vented containers. This suggests that gas-exchange is an important requirement for this particular assay and as a result additional effort was put in finding a container that would allow this exchange as well as avoid the formation of air bubbles during microgravity experiments.

Future work will focus on further testing different containers and optimising the *in vitro* DTH protocol. The applicability of the test will also be evaluated by performing experiments on a larger population and including a broader panel of stimuli. Finally, the scope of the assay will be extended to additional space simulated stressors such as ionizing radiation or psychological stress, as well as combinations of these to detect potential synergistic or antagonistic effects.

List of Abbreviations

APC	Antigen-Presenting Cell
CD	Cluster of Differentiation
CME	Coronal Mass Ejection
CMI	Cell-Mediated Immunity
CMV	CytoMegaloVirus
CNSA	China National Space Agency
DLR	Deutsche zentrum für Luft- und Raumfahrt
DMEM	Dulbecco's Modified Eagle's Medium
DSB	Double-Stranded Break
EBV	Epstein Barr Virus
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESA	European Space Agency
eV	ElectronVolt
FISH	Fluorescence In Situ Hybridization
GCR	Galactic Cosmic Ray
Gy	Gray
HIV	Human Immunodeficiency Virus
HZE	High-energy particles of high atomic number (HZE)
IFN	InterFeroN
Ig	ImmunoGlobulin
IL	InterLeukin
ISS	International Space Station
LEO	Low Earth Orbit
LTE	High-Linear Energy
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MoCISS	Monitoring the Cellular Immunity by *in vitro* delayed type hypersensitivity assay on the ISS
NASA	National Aeronautics and Space Administration
PBS	Phosphate-Buffered Saline
rpm	Revolutions Per Minute
RPM	Random Positioning Machine
RPMI	Roswell Park Memorial Institute
RWV	Rotating Wall Vessel
SAA	South Atlantic Anomaly
SMS	Space Motion Sickness
SPE	Solar Particle Event
Sv	Sievert
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor

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Chapter 1

Introduction

1 Introduction

Although space confronts us with a vast array of extremely hazardous conditions, such as vacuum, intense heat and radiation due to direct sun exposure, low temperatures due to exposure to cold deep space and potential impact of micro-meteoroids, mankind is defined by its will to explore it. Over fifty years ago, the human presence in space became a reality and since that time our goals in space exploration have become more ambitious. While during the last decade efforts focused on ensuring our continuous presence in low-Earth orbit (LEO) by means of the International Space Station (ISS), today's leading space agencies are looking beyond for our next challenge, in particular returning to the Moon and possibly establishing a permanent base as well as setting the first foot on Mars. Obviously, these endeavors will require a fair amount of ingenuity, innovation and global cooperation.

The future exploration of our Solar System and beyond is mainly driven by the search for habitability and life beyond Earth. Although it is clear that robots will always precede humans to characterize extra-terrestrial environments, assess the risks connected to human missions and identify potential resources for life support and technology purposes, exploration without humans lacks an essential societal and scientific aspect. With the exception of the Apollo missions, all exploratory missions performed beyond LEO so far were unmanned. The main reason for this is that humans have a dramatic impact on the mission profile, in particular in terms of safety requirements, accommodation and mission duration as well as the need for a mandatory return to Earth. As a result, most of the space exploration-enabling research has been geared towards robotic technologies rather than human factors. However, given our ambition to extend human presence in space, there will be a need for a clear roadmap in this area.

During the last decade, several studies have been performed to assess the research effort required for sustainable human space exploration, e.g. the European Space Agency's (ESA) HUMEX study (2001), the National Aeronautics and Space Administration's (NASA) Bioastronautics Roadmap (later "Human Research Roadmap", 2005-2012), the Global Exploration Roadmap by the International Space Exploration Coordination Group (ISECG, 2011), the US Space Studies Board's Decadal Survey on Biological and Physical Sciences in Space (2011) and the European Union's THESEUS Roadmap (*Towards Human Exploration of Space: a EUropean Strategy*, 2012).

These initiatives discuss possible mission scenarios and suggest an approach to deal with mission aspects related to science, technology, economy and culture. Here, the overall goal is to provide a phased, priority-based working strategy for exploration in the field of human physiology, human psychology, habitat management and health care (including countermeasures against e.g. space radiation). In addition, the roadmaps promote a cross-disciplinary and multi-factorial approach and point out the added value of a synergy between space and terrestrial activities.

1.1   Factors affecting human health during spaceflight

Previous missions have shown that spaceflight has a dramatic impact on almost all our physiological systems, causing muscle atrophy, bone demineralisation, cardiovascular and metabolic changes, impaired cognitive processes, reduced immunological competence and reduced nutrition intake and metabolism. An important prerequisite in our future quests in space will be to understand the long-term effects of deep-space travel on humans and to provide the necessary countermeasures. Unfortunately, our experience in space is still rather limited and outer space remains mainly an unexplored territory. Today the ISS forms an interesting evaluation platform, although we have to realize that in this case we limit ourselves to a "safe place" close to our home planet, in contrast to more hostile environments such as the Moon, Mars or deep-space. Nevertheless, our experience in space so far has already confronted us with several aspects of space travel - the so-called "space stressors" - which can have an effect on both our physiological and psychological well-being.

1.1.1   Microgravity

Life on Earth has evolved in the constant presence of gravity, from the first organic molecules to humans. Hence, the most obvious stressor for the human body in space is microgravity which is caused by the leveling out of the gravitational pull towards Earth by the centrifugal force as the result of the orbital motion of the spacecraft. In other words, the weightlessness experienced by astronauts on e.g. the ISS is not related with them being in space per se, but rather caused by their falling motion around our planet. An overview of the different physiological systems affected by microgravity during a long-term stay in space is given in Figure 1.
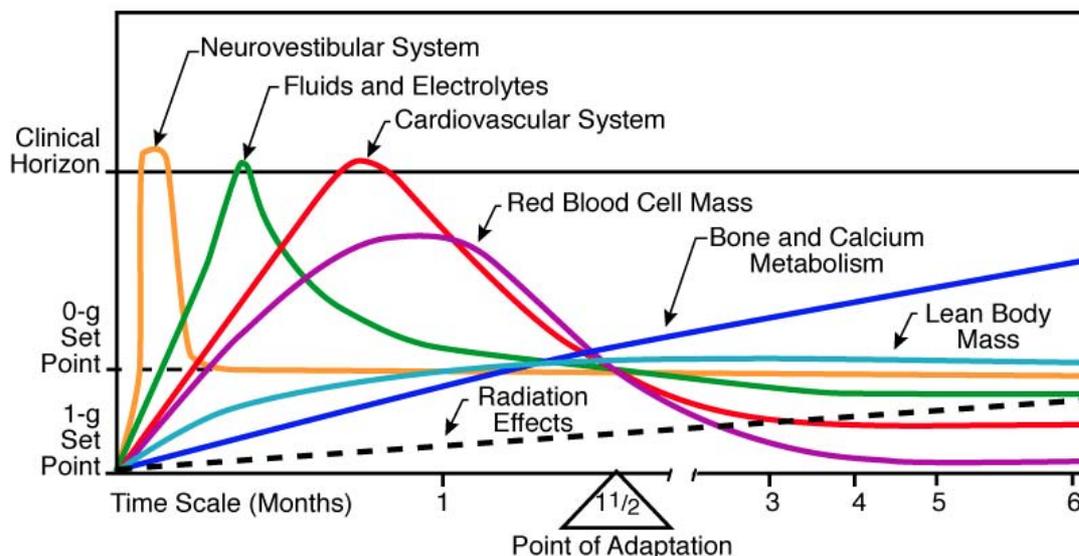

Figure 1. Changes of various body systems during adaptation to microgravity (taken from Blanc *et al*., 2013).

Microgravity has a profound effect on human physiology (Pietsch *et al*., 2011; http://www.nsbri.org). Since the body no longer experiences the downward pull of gravity responsible for distributing the blood and other body fluids to the lower part of the body (particularly the legs), fluids are redistributed to the upper part of the body. This **fluid shift** to the head causes the so-called "puffy face" and bird-legs syndrome, and can result in a feeling of nose congestion.

While the overall function of our **cardiovascular** system is not impaired in space, the microgravity environment does lead to deconditioning of the heart causing cardiac atrophy. Other changes include a decrease in heart rate, an increasing occurrence in arrhythmias and weakening of the veins and arteries in the legs. Although these changes are not considered an issue during flight, they can become problematic upon return to Earth. As the ability of arteries and veins to constrict normally has been compromised, the astronaut body has problems in maintaining an adequate blood pressure when suddenly confronted with a 1 g environment. As this hampers the normal blood flow to the upper parts of the body (brains) it can lead to dizziness and even fainting. This condition is known as orthostatic intolerance (or hypotension) and is observed in up to half of the astronauts on short-term missions (two weeks or less) and nearly all astronauts on long-term missions (4-7 months) (Aubert *et al*., 2005; Behnke *et al*., 2012).

The lack of gravity also has a profound effect on the **musculo-skeletal** system of the astronaut. Since the bones in the lower body no longer bear the weight of the upper body, they become weaker due to the progressive loss of bone mass. In addition, the calcium released during this process will be reabsorbed by the body and poses an increased risk of kidney stone formation. The overall bone loss observed in astronauts onboard the ISS is quite severe: while postmenopausal women can lose 1.0-1.5% of bone mass over one year, astronauts lose the same amount in only one month. An additional effect of weightlessness is that the disks between the vertebrae of the spinal column are less compressed than in Earth. This causes the spine of the astronaut to increase in length (astronauts are taller in space!) and can be accompanied by continuous back pain.

Another obvious consequence of microgravity is the reduced load on the muscles, in particular leg and back muscles. Extended periods in space can cause muscle weakening or atrophy and can compromise the safety of the astronauts during exploration missions. Hence, astronauts are required to exercise regularly in space to maintain their muscle mass. These exercises depend on equipment specifically designed to mimic the gravitational stress as perceived on Earth in the form of a modified treadmill, bicycle, "weight" lifting devices, etc. So far these countermeasures have been proved to be quite successful, although one has to take into account the fact that the training schedule is fairly intense (2-2.5h mandatory exercise per day) which has a negative effect on the astronaut's psychological health, particularly for long-term missions (to be discussed later). Current research is focusing on how nutritional interventions or pharmaceutical drugs could improve the efficacy of exercise.

On Earth, a complex set of neural circuits is responsible for maintaining our balance, monitoring our orientation and stabilizing our vision, i.e. the **neuro-vestibular** system (also referred to as the inner-ear and balance system). Information received from our eyes, inner ear, muscles and joints is interpreted by our brain to give us an idea of orientation. However, as soon as astronauts are in space and experience microgravity and their senses no longer

function as originally designed: the pattern of the information received is dramatically altered. This can cause disorientation and impaired motor control early in the mission. Depending on the extent of the effect, these changes can result in nausea and vomiting (also known as space motion sickness, SMS). While the overall discomfort is typically limited to the first days in space, one has to take into account the potential impact on operational activities such as approach, docking, remote manipulation, extravehicular activity and landing. Medication is available to counter SMS - e.g. promethazine (Phenergan®) - although natural adaptation is generally preferred over the potential side effects of medication.

1.1.2   Radiation

In space astronauts are subjected to a dose of ionizing radiation which differs from and is significantly higher than that experienced on Earth (Hellweg and Baumstark-Khan, 2007). In contrast to microgravity, the effect of space radiation on human health is less obvious and - as a result - poorly understood. This can mainly be attributed to the fact that most spaceflights so far have been rather limited in time and - with exception of a single, high dose radiation events - low-dose radiation-induced effects will typically render symptoms after long-term exposure. An interesting example here is the occurrence of **cataract** in astronauts. Until recently, it was assumed that radiation cataract is a deterministic event requiring threshold doses generally greater than 2 Gy. However, newer findings strongly suggest dose-related lens opacification at significantly lower doses (reviewed by Kleiman, 2013).

For the LEO environment, the primary ionizing radiation consists of (1) **solar particle events** (SPEs), (2) **galactic cosmic rays** (GCRs) and (3) particles trapped in Earth's radiation belts (so-called **Van Allen Belts**) (Figure 2).
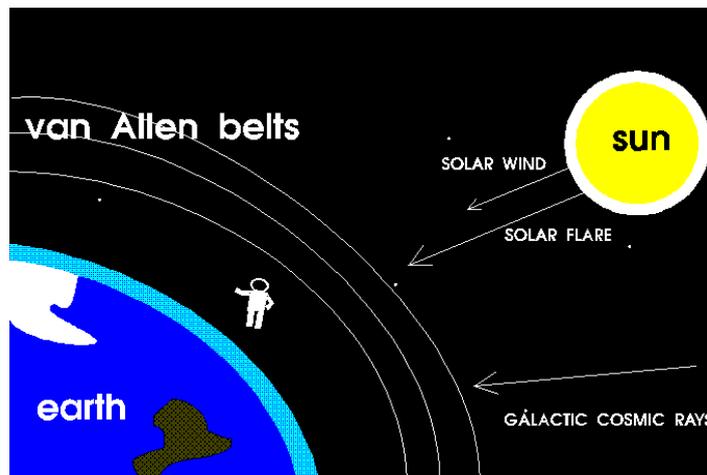
Figure 2. Schematic representation of the main radiation sources for humans in LEO (taken from http://home.bway.net/rjnoonan/humans_in_space).

SPEs are energetic particles emitted by the Sun during solar flares and - to an extreme extent - coronal mass ejections (CMEs) and mainly consist of high-energy protons, electrons and heavy ions. Although SPEs only occur occasionally (related to solar activity) and are short-lived (hours to days), their potential impact on the astronaut health should not be

underestimated, particularly the relatively abundant and energetic protons (1 MeV for solar storms and up to 1 GeV for solar flares).

GCRs represent a population of charged particles originating from outside our Solar System. They mainly consist of protons (85%) and helium ions (14%, i.e. alpha particles) as well as trace amounts of heavy ions (Reitz, 2008). Their average energy is around 1 GeV, although individual energies can vary between 10 MeV and 10^{12} MeV. These latter particles are often referred to as high-energy particles of high atomic number (HZE) or high-linear energy (LET) particles. They are important to monitor when dealing with space radiation given their high energy and penetration potential and lack of shielding solutions, particularly iron ions which are the most abundant high Z ions in space. Finally, it is important to note that GCRs are attenuated by their interaction with the solar wind and the Earth's magnetic field (Badwhar, 1997), e.g. the GCR flux is significantly lower when the solar activity is high and vice versa.

A third important source of radiation in LEO are Earth's **Van Allen Belts**. The Van Allen Belts are two torus-shaped layers of energetic charged particles (protons and electrons) around the planet, held in place by its magnetic field and distorted by the solar wind (Figure 3). The inner belt is formed by a combination of protons (150-250 MeV) and electrons (<5 MeV) centered at an altitude of 2,000-5,000 km, while the outer belt is formed by electrons (<7 MeV) and centered at 15,000-20,000 km. In addition, the belts contain lesser amounts of other nuclei, such as alpha particles. It is thought that most of the particles that form the belts come from solar wind (outer belt) or cosmic rays (inner belt). Under stable solar conditions the Van Allen Belts are constant in size and density (adiabatic equilibrium). In case the speed of the entering particle is high, it will pass through the belts and will be absorbed by the atmosphere. In case the particle speed is low, it will be captured by the belts and will show (1) gyration around magnetic field line, (2) bouncing between poles and (3) a longitudinal drift, meaning as the particles gyrate and bounce from pole to pole in the magnetic field, they will also drift to the east around the Earth if they are positively-charged (protons and ions) and west if they are negatively-charged (electrons).
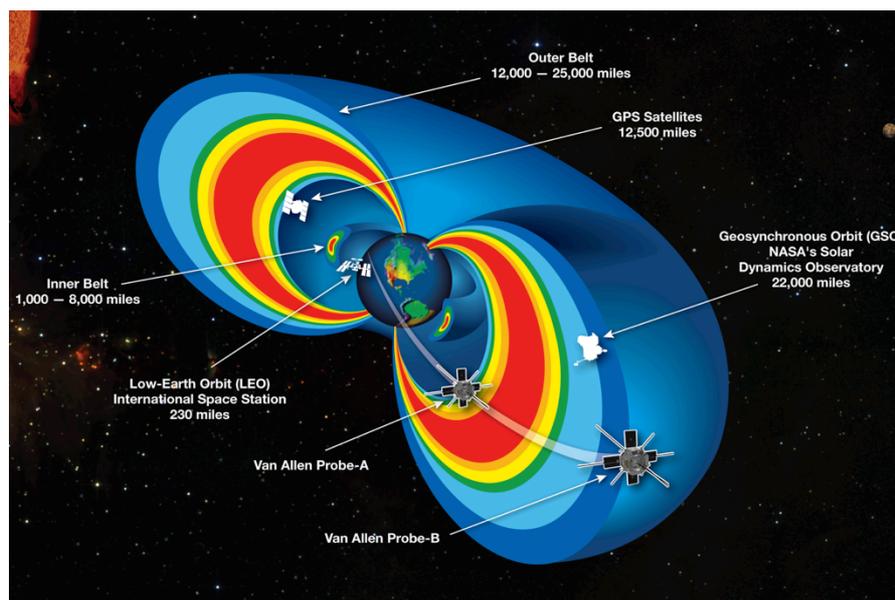

Figure 3. Earth's inner and outer Van Allen Belts (taken from www.nasa.gov).

The South Atlantic Anomaly (SAA) is an area of the coast of Brazil where the Earth's inner Van Allen Belt comes closest to its surface, dipping down to an altitude of 200 km. The effect is caused by the non-concentricity of the Earth and its magnetic dipole and the SAA is the near-Earth region where the Earth's magnetic field is weakest. This leads to an increased flux of energetic particles in this region and exposes orbiting spacecraft and their crew to higher than usual levels of radiation. The ISS (orbiting with an inclination of 51.6°) requires extra shielding to deal with this particular problem and astronauts are also affected by this region which is said to be the cause of peculiar 'shooting stars' (phosphenes) seen in their visual field.

In LEO the daily radiation dose is about 180 micro-Gray (µGy) (equivalent dose = 407 micro-Sievert (µSv)) (Petrov *et al.*, 2002; Spurny *et al.*, 2002; Goossens *et al.*, 2006; Cucinotta *et al.*, 2008). The low-LET dose rate (γ-rays) is around 157 µGy/day (equivalent dose = 157 µSv/day), while the high-LET dose (protons, neutrons, HZE) is around 23 µGy/day (equivalent dose = 250 µSv/day). Astronauts are subjected to a dose that is about 160 times higher than the dose received on Earth (2.5 µSv/day), so it is not surprising that they are officially classified as radiation workers. It is also important to note that over 80% of the organ dose equivalents on the ISS are from GCRs (neutrons and HZE) while only a small contribution is from γ-rays and trapped protons.

The main effects of ionizing radiation have been studied in depth at the cellular level and typically results in DNA damage (Todd, 2004). Here, double-stranded breaks (DSBs) are considered most critical, although most are repaired automatically by the cell. While an excess of DSBs typically triggers apoptosis, DSBs occurring at multiple sites can result in mutations and chromosomal aberrations. These aberrations can be monitored using a multicolor fluorescence in situ hybridization (FISH) technique (Figure 4) and are known to increase in the blood lymphocytes of astronauts after spaceflight (Cucinotta *et al.*, 2008).
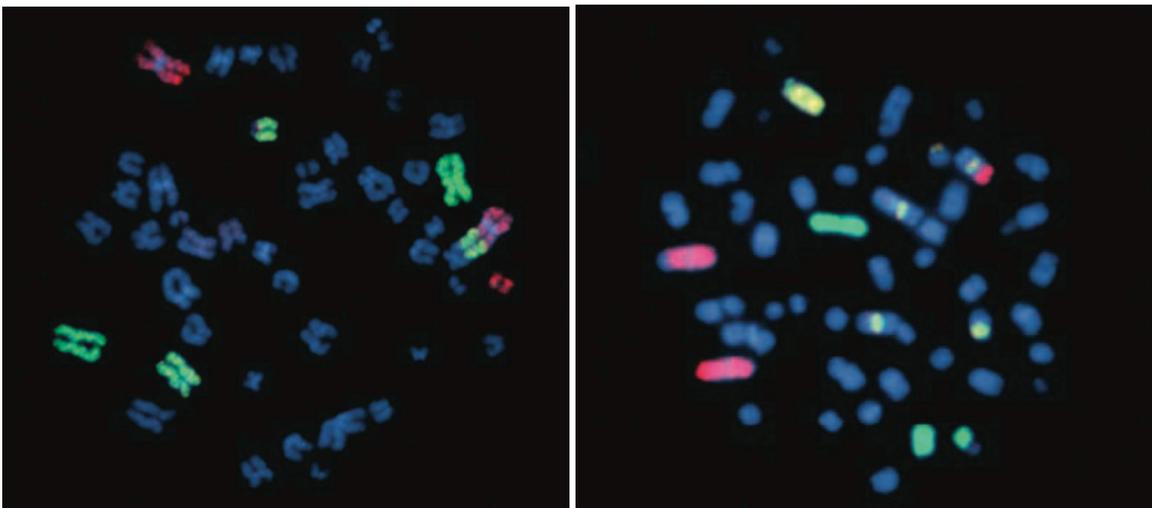

Figure 4. Examples of two complex aberrations involving three or more chromosomes observed in ISS astronauts post-flight (short-term flight). Chromosomes were hybridized with painting probes for chromosome 1 (red), chromosome 2 (green) and chromosome 4 (yellow). All other chromosomes were counterstained with DAPI (blue) (taken from Cucinotta *et al*, 2008).

DNA damage can lead to cell death or can result in mutagenesis, which in turn increases the risk for cancer and cataract (Cucinotta *et al.*, 2008). Some studies have also reported a positive correlation between radiation dose and Epstein-Barr virus (EBV) reactivation in lymphocytes, although this effect could only be significantly demonstrated after exposure to 2-4 Gy (Ferrieu *et al.*, 2003). Furthermore, there is a growing concern that space radiation beyond LEO may impact our bone metabolism, our immune system and our cardiovascular system (e.g. by affecting endothelial cells which might initiate or accelerate coronary heart disease). The main problem when assessing the potential biological effect of radiation in space is the lack of accurate data on radiation quality and dose range. To add to this complexity, studies performed so far show significant individual differences in terms of radiation susceptibility rendering a high degree of variation in the data gathered and making it difficult to draw strong conclusions (Figure 5). Furthermore, the damage accumulated due to radiation in space can take years before resulting in clinical symptoms (e.g. cataract).

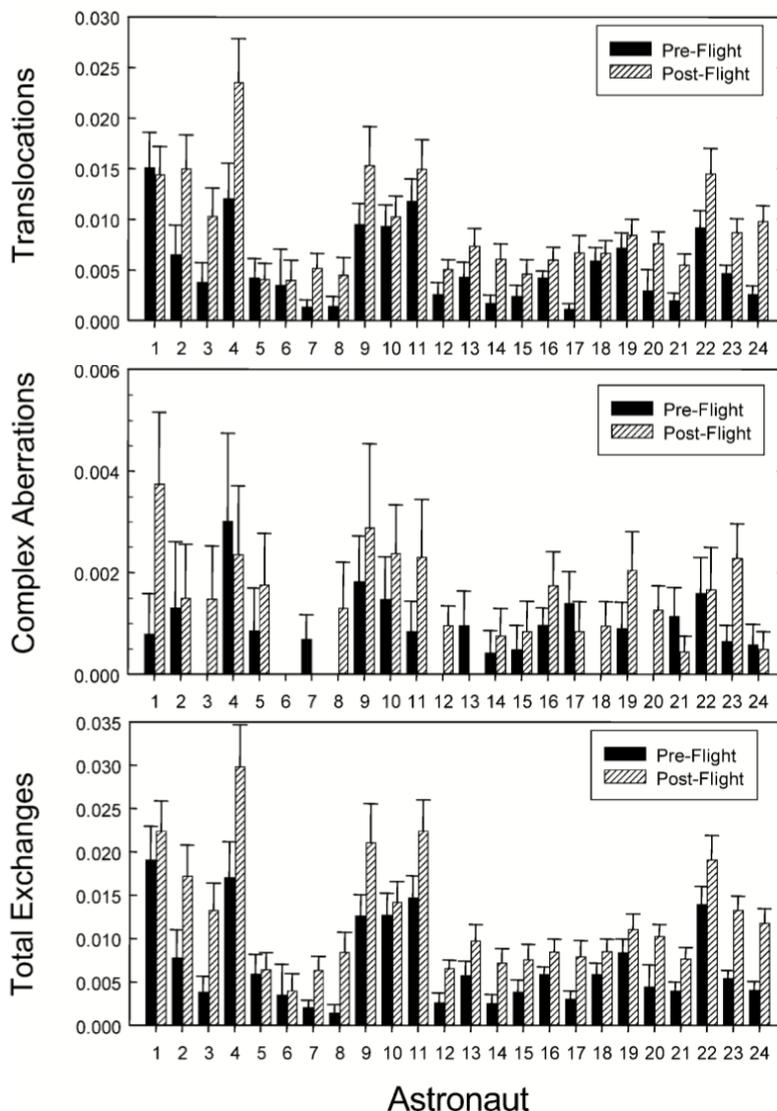**Figure 5. Frequency of translocations, complex aberrations or total chromosome exchanges measured in each astronaut's blood lymphocytes before and after his/her respective space mission onboard the ISS, Mir or STS. Increases in total exchanges were observed for all astronauts. Translocations (22 of 24) and complex aberrations (17 of 24) were increased in the majority of astronauts (Cucinotta *et al*, 2008).**

Another side effect of space radiation is the interaction of primary particles with the spacecraft and the subsequent production of secondary particles (e.g. protons, alpha-particles, heavy ions, neutrons and γ-rays) that can also cause biological damage. Hence, in space it is difficult to link a certain effect to a specific dose threshold. Furthermore, the total dose to which astronauts are subjected should be evaluated in a cumulative manner rather than a time-limited event, especially when considering space travel beyond LEO. For instance, a trip to Mars and back would result in a total equivalent dose of 1 Sv (Figure 6).
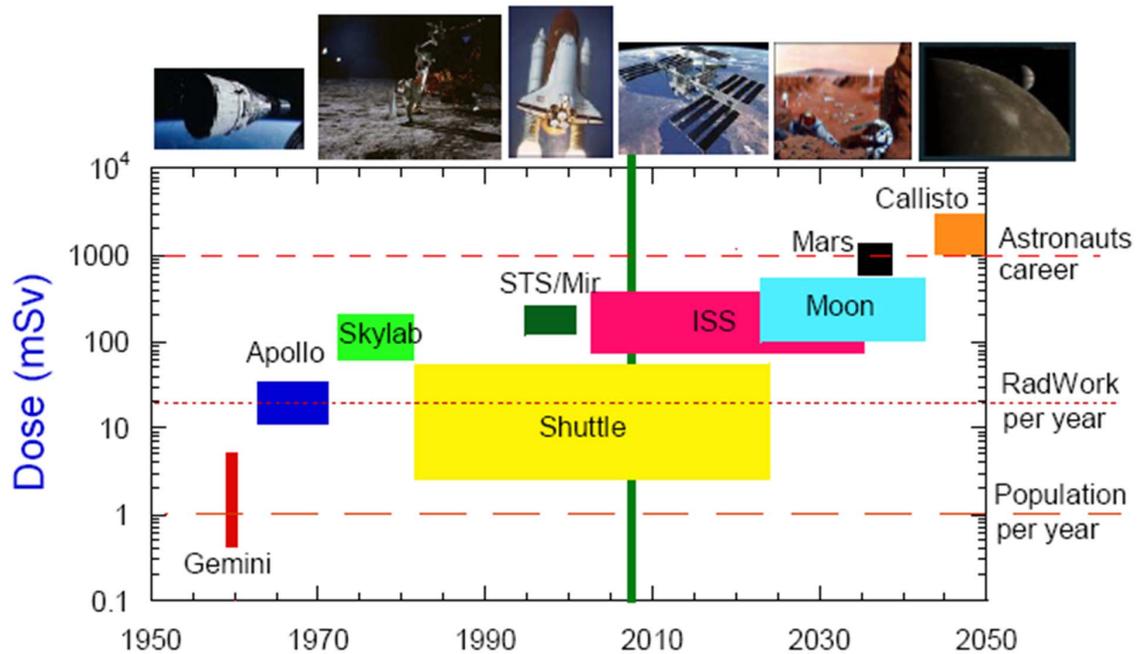
Figure 6. Estimated equivalent radiation doses for astronauts for past, present and future space missions (taken from "Life Science & Biology in Space" course, Chapter 12 "The Radiation Issue in Space", KU Leuven - UGent, Master of Science in Space Studies, Professor S. Baatout).

1.1.3 Psychological stressors

In addition to microgravity and radiation, astronaut health is also affected by psychological stress. Although this kind of stress is difficult to define, it can have a major impact on the functioning of the crew. An astronaut has to reside for a long period in an isolated, confined and noisy environment where he/she is confronted with a heavy workload, a small crew and the continuous threat of working in a hazardous environment. Furthermore, due to the orbital parameters of LEO-bound spacecraft, one day-night cycle onboard the ISS lasts about 90 min (16 sunsets every 24h!) which has a profound effect on the quality of astronaut sleep (circadian disruption).

Taking into account the physiological effects of microgravity discussed earlier, it is not surprising that humans in space are not feeling 100%. In addition, the psychological stressors are not limited to space: astronauts are subjected to an extensive training regimen prior to their mission and have to endure the stress of pre-launch quarantine, launch, docking and eventually the re-entry. In conclusion, the psychological status of an astronaut is maintained by the central nervous system which can trigger a cascade of stress hormones that indirectly can affect other physiological systems.

1.2   Space research on Earth

The stressors affecting human health in space are highly diverse and can have a significant effect on astronaut performance. While many investigations have been performed during past 30 years, it remains difficult to draw sound conclusions concerning the clinical risks of space travel. The main bottleneck is the limited size of the study population so far (about 600 astronauts in total), the large variety in mission length (from several days up to 1 year), the lack of conformity and standardization between the tests performed by different research groups and the individual differences reported between astronauts in terms of biological response. Furthermore, multiple stressors act simultaneously throughout the mission making it difficult to investigate cause-effect relationships (Blanc *et al*., 2013).

Since it is not acceptable - both ethically and financially - to merely increase our astronaut population as well as their time in space in the near future, it is important to develop well-controlled, large population-sized, cost-effective experiments using relatively cheap ground-based space-analogs in an attempt to broaden our understanding of space health effects. *In vitro* studies focusing on cell and tissue cultures can make use of devices capable of approximating microgravity conditions, such as a fast rotating clinostat, a rotating wall vessel (RWV) bioreactor or a random positioning machine (RPM) (Schwarzenberg *et al*., 1998, 1999; Walther *et al*., 1998, 1999; Uva *et al*., 2002; Nickerson *et al.*, 2003; Strollo *et al*., 2004; Sonnenfeld, 2005; Infanger *et al*., 2006; Simmons *et* al., 2006; Patel *et al*., 2007; Fitzgerald *et al*., 2009; Grimm *et al*., 2009; Ulbrich *et al*., 2010a, 2010b; Grosse *et al*., 2012; Beck *et al*., 2012). *In vivo* alternatives for human spaceflight include head out-of-water immersion, dry immersion and parabolic flight campaigns - to monitor short-term effects of microgravity on human physiology, e.g. cardiovascular changes - as well as long-term head-down bed rest studies - which in addition trigger bone loss, muscle atrophy and subtle immune alterations (Crucian *et al.*, 2009a; Feuerecker *et al.*, 2013a; Kelsen et al., 2012). The psychological stressors of space travel - e.g. isolation, confinement, high workloads - can be studied in environments that are either artificially created (e.g. the Mars500 study performed by ESA, Roscosmos and China National Space Agency (CNSA), and NASA's underwater NEEMO project) or naturally occurring (e.g. ESA's underground CAVES training program and the French-Italian research station Concordia located in Antarctica) (Figure 7). In addition, the German Aerospace Centre (Deutsche Zentrum für Luft- und Raumfahrt, DLR) is currently finalizing the construction of their new :envihab facility which will allow the allocation of up to 12 test persons exposed to equal and controlled environmental conditions (Figure 8).

Figure 7. Different Earth-bound analogs for human spaceflight: (A) head out-of-water immersion (University of Occupational and Environmental Health, Department of Environmental Physiology, Japan), (B) dry immersion (Roscosmos, Russia), (C) parabolic flights (ESA), (D) head-down bedrest (CNSA), (E) Mars500 (http://news.cnet.com/2300-11386), (F) NEEMO (NASA), (G) CAVES (http://www.infuture.ru/article/7036) and (H) Concordia (http://www.vikdhillon.staff.shef.ac.uk). Pictures (B), (C) and (D) taken from course "Advanced Topics in Life Sciences in Space" course, Lecture 10 "Simulation", KU Leuven - UGent, Master of Science in Space Studies, Professor A. Aubert).
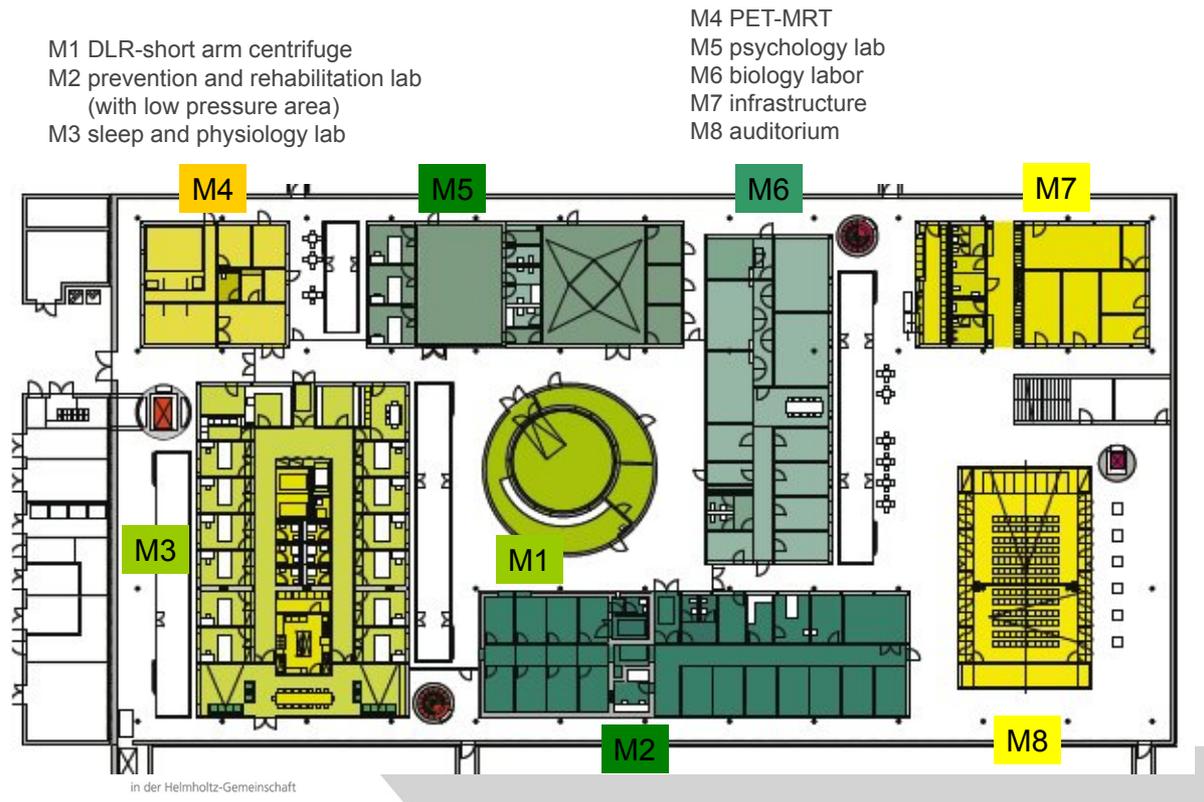
Figure 8. Scheme of DLR's :envihab facility (taken from http://www.dlr.de/envihab/en).

1.3 Our immune system in space

1.3.1 The importance of the immune system

Our immune system is a complex network of organs, specialized cells and chemicals responsible for protecting us from foreign as well as local damaging material based on layered mechanisms (Choukèr *et al.*, 2008). The first line of defense are the **surface barriers** responsible for preventing pathogens from entering our body, e.g. skin, mucus, acids in the stomach and enzymes in saliva and tears. The second line - the so-called **innate immune system** - consists of phagocytes (white blood cells) that are able to engulf and destroy foreign cells (e.g. bacteria and viruses) and is located in tissues most vulnerable to invasion (e.g. lungs and intestine). The innate immunity triggers a generalized, non-specific response typically resulting in redness and swelling of local tissues. This system can be found in both lower and higher life forms (e.g. fungi, plants, starfish, vertebrates). The third level of defense is specific to higher vertebrates and is called **acquired or adaptive immunity**. Here, exposure to a microorganism will trigger specific response which targets that particular organism and will allow a more rapid and more efficient action and elimination in case of future exposures.

The most important characteristic of our immune system is that it is capable of distinguishing between self and non-self. Anything that can trigger an immune response is called an antigen, which can be bacteria, viruses, fungi, parasitic worms, but also other foreign molecules such as toxins, chemicals, drugs and foreign bodies, and even cancer cells, and distinguish them from the organism's own healthy tissues. Tissues or cells from another person (except an identical twin) also carry non-self markers and act as antigens. This explains why tissue transplants may be rejected.

Our entire immune system contains over 4 trillion cells and weighs more than our brain and liver together. It is made up of primary, central organs (bone marrow and thymus) which are responsible for the production and maturation of the workhorses of the immune system (i.e. immune cells or leukocytes) and secondary, peripheral organs (lymph nodes and spleen) which store mature naive lymphocytes and filter blood and body fluids (Figure 9).
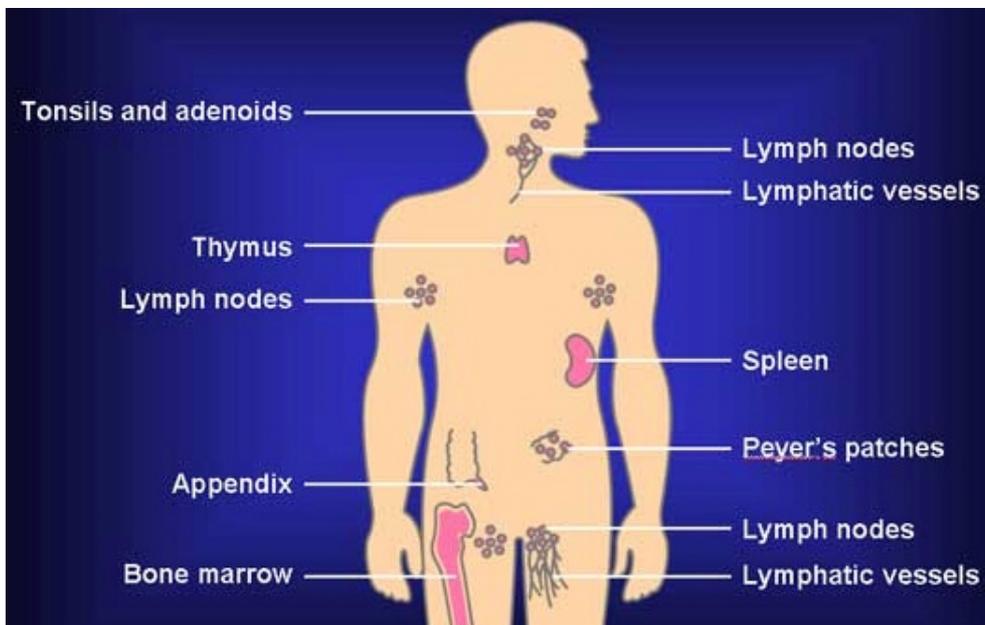
Figure 9. The organs of our immune system (taken from http://www.web-books.com/eLibrary/ Medicine/Physiology/Immune/05MB8.html).

At the cellular level, the immune system consists of a wide range of cell types, each with their own particular appearance and function. For sake of completeness, an overview of the different cells is given in Figure 10. In addition to plasma, red blood cells and platelets, the cells of the immune system also form an important component of our blood (i.e. white blood cells). Two types of white blood cells can be distinguished, including agranular cells (T lymphocytes, B lymphocytes, natural killer cells and monocytes) and granular cells (basophils, eosinophils and neutrophils). As a result, blood samples can give a valuable insight in the status of our immune system. For instance, infection or leukemia typically coincide with an excessive number of white blood cells, while a human immunodeficiency virus (HIV) infection results in a drastic reduction.
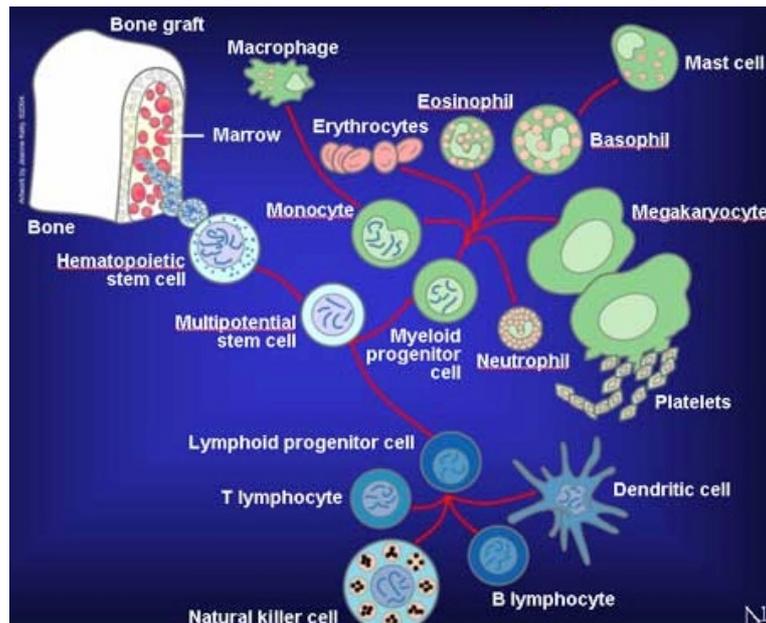
Figure 10. The cells of our immune system (taken from http://www.web-books.com/eLibrary/ Medicine/ Physiology/Immune/08MB8.html).

Alternatively, the adaptive immune system can be divided into two functional parts, i.e. cellular immunity and humoral immunity.

Cellular immunity is managed by T lymphocytes equipped with receptors capable of recognizing antigens (i.e. T cell receptors, TCRs) that are presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). Since the affinity of this binding is relatively low, the signal from the T cell complex is enhanced by simultaneous binding of the MHC molecules to a specific co-receptor, the so called cluster of differentiation (CD). While basically all T cells express CD3 on their cell surface, additional co-receptors will determine the eventual outcome of the immune response. As a result, T cells can either coordinate the immune response (T helper cells, CD4+)), target infected cells or tumor cells passing the lymphoid organs (cytotoxic killer cells, CD8+) or deal with tumor cells outside the lymphoid organs (natural killer cells).

Humoral immunity is mainly controlled by B lymphocytes which can mature into plasma cells and produce antibodies (so-called immunoglobulins, Ig's) capable of binding extracellular pathogens or toxins present in tissues and the bloodstream, hereby initiating the complement destroying cascade.

Within the orchestration of the immune response, an important role is fulfilled by the **cytokines** which are peptides, proteins or glycoproteins secreted by the immune cells. Cytokines are the chemical messengers responsible for most biological effects in our immune system, e.g. cell-mediated immunity and allergic type responses. They can promote cell proliferation, differentiation, apoptosis, antibody secretion as well as chemotaxis. Although the cytokine family is diverse, it can be functionally divided into two groups, namely those that are pro-inflammatory and those that are anti-inflammatory but promote allergic responses.

The major source of cytokines are the T lymphocytes. For CD4+ T cells (helper T cells) a clear distinction is made between **Th1 and Th2 cells**, which in turn produce Th1 and Th2 type cytokines (Berger, 2000).

Th1 type cytokines promote the inflammatory responses responsible for killing intracellular parasites and for maintaining autoimmune responses. Examples include interferon-γ (IFN-γ and transforming growth factor β (TGF-β). However, since an excessive proinflammatory response can result in uncontrolled tissue damage, a mechanism to counter this response is required. In excess, Th2 responses counteract the Th1 mediated microbicidal action and are therefore considered anti-inflammatory. **Th2 type cytokines** favor an antibody base response. Examples include interleukin 4 (IL-4), IL-5, IL-10 and IL-13. Hence, under optimal conditions the human body should maintain a balanced Th1/Th2 response. For instance, an allergy is regarded as a Th2 weighted imbalance. Hence, scientists are investigating ways to redirect the immune system to a Th1 type response (e.g. by high dose exposure to an allergen or using of mycobacterial vaccines in early life).

1.3.2   Effects of space travel on our immune system

Space travel confronts the human body with a multitude of stressors - both physical and psychological - which can have profound effects on our immune system and can lead to significant **immune suppression**. Both genuine and simulated spaceflight experiments have shown effects at the cellular, organ and system level. The consequences for this reduced immune-competence are even more severe in space since several pathogenic microorganisms are known to be more proliferative and more pathogenic or virulent under space conditions. Furthermore, studies have reported that drug efficacy (e.g. antibiotics) is typically lower in space. Therefore, it is of the upmost importance to understand how space alters our immune system and what factors could lead to clinical risks during long-term space travel. The current section provides a brief overview of the most important observations made to date.

Studies performed on cells (Earth-based models)
Numerous studies have shown that T cell activation is dramatically impaired in the presence of microgravity stress (Cogoli *et al.,* 1984, Cogoli, 1996; Cogoli *et al.*, 1996; Pippia *et al.*, 1996; Hashemi *et al.*, 1999; Cooper *et al.*, 2001; Sastry *et al.*, 2001; Simons *et al.*, 2006; Fitzgerald *et al.*, 2009). Data gathered so far suggest that microgravity is interfering with cell-cell communication which can affect the signaling pathways of the immune system (reviewed by Ullrich *et al.*, 2008). Several parts of our white blood cells are sensitive to gravity (or lack thereof), such as the nucleus (directly involved in gene regulation, affecting cell differentiation and proliferation), the cytoplasm (e.g. protein kinase C intracellular signaling pathway) and the cell surface (e.g. IL-2 and IL-2 receptor involved in T cell differentiation and proliferation). Experiments performed on peripheral leukocytes showed that microgravity and radiation (γ-ray exposure of 0.2-2 Gy) can cause a dramatic change in the expression of genes related to hemopoiesis, cytokine production, immune system development and lymphocyte differentiation, and typically results in a shift in the early T cell response from Th1 to Th2 (Girardi *et al.*, 2012). A recent study has also shown that the limited proliferation and activation of T cells as a result of microgravity can be attributed to the inhibition of the Rel/NF-κB signaling pathway which in turn affects cell cycle regulation, apoptosis,

cytoskeleton functioning and the expression of several surface molecules and cytokines (Chang *et al.*, 2012). It is believed that the lack of gravity has an effect on the overall organization of a cell hereby effecting intracellular transport and communication. However, the molecular mechanisms of gravisensitivity in mammalian cells are widely unknown and their impact on cellular pathways still remains to be elucidated.

Studies performed on animals models (space conditions vs. Earth-based models)
Animal models can be a useful tool when studying human biology. Elucidation of biological pathways and effect of different stressors is possible by selecting proper models with an adequately sized, homogenous test population which can be placed in a well-controlled environment. The first observations concerning the effect of space travel on the mammalian immune system date back more than 50 years (described by the Hungarian endocrinologist Hans Selye). For example, mice subjected to a 13-day mission showed a significant lower number of leukocytes as well as a reduction in spleen and thymus mass compared to control mice kept on Earth (Gridley *et al*, 2009). Furthermore, isolated white blood cells were found to be less responsive to bacterial antigen challenge. In addition, microgravity simulation by hindlimb unloading of mice reported an increased susceptibility to bacterial infections (Aviles *et al.*, 2003). Meanwhile, the overall effects of microgravity have been demonstrated in terms of T cell distribution and function as well as gene expression patterns - in particular genes involved in stress, glucocorticoid receptor metabolism and T cell signaling - which typically result in immunosuppression (Baqai *et al.*, 2009).

In addition to cellular immunity, space flight experiments using amphibian and insect models have shown an effect on antibody production after antigen stimulation, e.g. IgA homolog expression was increased after flight, in correspondence with previous reports on Russian astronauts (Konstantinova *et al.*, 1993; Marcu *et al.*, 2011). The effects were also detected on gene level such as the expression of specific Ig heavy chain variable region (VH) gene subgroups (Boxio *et al.*, 2005), individual VH genes (Bascove *et al.*, 2009) and somatic hyper-mutations (Bascove *et al.*, 2011). Hence, the data gathered clearly indicate impairment of the immune system at several levels as a result of space stressors (Lebsack *et al.*, 2010).

Studies performed on humans (space conditions vs. Earth-based models)
Lessons learnt from cells and animal models can be further explored in humans in an attempt to maintain human health in space. Early reports on dysregulation of the human immune system in space already date back to the Soyuz and Skylab missions in the seventies (Konstantinova *et al.*, 1973; Kimzey, 1977). Additional studies performed so far range from short-term (from several hours to several weeks) to long-term space missions (4-7 months onboard the ISS). Tests focus on saliva, urine and blood samples to obtain a comprehensive view of the astronaut overall health status. While short-term missions are characterized by an adrenocortical stress response, long-term missions typically result in glucocorticoid-mediated changes. Furthermore, both acute gravitational stress and space travel activate the **endocannabinoid system** involved in a variety of physiological processes including appetite, pain sensation, mood and memory. In correspondence with results obtained for cell cultures and animals, human studies have demonstrated a dramatic modulation of the immune response during space missions (Crucian *et al.*, 2009b; Blanc *et al.*, 2013). Due to practical issues, the majority of the studies have focused on comparing pre- and post-flight data. These clearly show an effect on cytokine expression (Th2 shift), leukocyte distribution and activation, monocyte, natural killer cell and granulocyte functioning,

as well as neuroendocrine responses (Crucian *et al.*, 2008, 2013). However, these effects are the result of flight-associated variables as well as the psychological stress associated with the launch and afterwards the high-gravity re-entry and subsequent re-adaptation. So if we want to understand the in-flight effects, more data should be gathered onboard the ISS. An earlier study reported changes in cellular immunity in certain individuals during spaceflight (Gmünder *et al.*, 1994). This phenomenon was further investigated in a more recent study dealing with blood samples taken during flight. Findings included changes in leukocyte distribution, T cell functioning and cytokine expression profiles (Crucian *et al.*, 2013). Furthermore, white blood cells showed an abnormal reproduction rate, reduced numbers and effectiveness when cultured in microgravity and lost almost all ability to respond to foreign challenge with proliferation potentials <3% compared to ground lymphocytes (Girardi *et al.*, 2012). This weakening of the cell-mediated immunity combined with the impaired hygiene conditions and increased virulence in space can become a health risk for the crew. Although no unusual microbial hazards have been identified so far, several astronaut studies report asymptomatic reactivation of latent EBV, cytomegalovirus (CMV) and varicella zoster virus in astronauts (Payne *et al.*, 1999; Mehta *et al.*, 2000, 2004; Pierson *et al.*, 2005; Wilson *et al.*, 2007; Cohrs *et al.*, 2008 Crucian *et al.*, 2009a; Brinley *et al.*, 2012). In contrast, activated lymphocytes and produced Igs have been shown to function normally in microgravity (Taylor and Dardano, 1983; Walther *et al.*, 1998; Stowe *et al.*, 1999; Choukèr *et al.*, 2010, 2011; Chang *et al.*, 2012).

Multiple Earth analog studies suggest stress related to the experimental conditions (e.g. confinement, simulated microgravity) - typically resulting in an increased production of epinephrine or cortisol - can negatively influence the immune system (Stowe *et al.*, 2003; Crucian *et al.*, 2008; Kaufmann *et al.*, 2009; Clément *et al.*, 2011; Morukov *et al.*, 2010; Choukèr *et al.*, 2010; Chereshnev *et al.*, 2012). Furthermore, extended periods of stress are known to result in increased blood levels of cortisol and catecholamine which increase blood pressure and blood sugar levels while downregulating our immune response (Choukèr *et al.*, 2008).

1.3.3   Monitoring the health status of our immune system

In view of future long-term space travel one of the questions that remains to be answered is whether the reduced immune response observed in spaceflight so far will continue to deteriorate over time or whether our immune system will find a new balance. Some studies have already indicated that our immune system can adapt to space stressors to some extent, e.g. radio-adaptation (Horneck, 1999) and bedrest adaptation (Kelsen *et al.*, 2012) although these effects are still a subject of debate.

An interesting approach for evaluating the health status of our cell-mediated immune system is assessing its response when challenged with recall antigens of bacterial, fungal or viral origin, i.e. the so-called **delayed-type of hypersensitivity (DTH)**. This response is a T cell-mediated defense mechanism against microorganisms that survive within phagocytes or infect non-phagocytic cells. The reaction was discovered in 1882 by Robert Koch and in the 1940s Landsteiner and Chase showed that it was cell-mediated in nature. The first skin DTH test described used the tuberculin antigen to test if prior exposure to *Mycobacterium tuberculosis* had occurred (referred to as the Mendel-Mantoux test, Pirquet test or PPD test).

However, the scope of the assay was broadened to include reactions to other bacterial and viral antigens, pure proteins with adjuvant or haptens as well as host responses to allografts.

Although the overall mechanisms of the reaction are understood, the exact pathways of the reaction are still a subject of debate. Upon exposure, the introduced antigens are engulfed by phagocytes. Phagocytosed antigens either enter the **exogenous pathway** and are processed for presentation on the MHC class II molecules to CD4+ T cells, or remain in the cytoplasm and are processed by the **endogenous pathway** for presentation on MHC class I molecules to CD8+ T cells (Poulter *et al*., 1982; Gibbs *et al*., 1984; Yang *et al*., 2010). This presentation will trigger a cascade of different cytokines, such as IL-2, IFN-γ and TNF-α (to be discussed later in detail) promoting the local recruitment of macrophages and the infiltration of inflammatory immune cells. This will eventually lead to inflammation, edema and erythema of local tissues. The DTH reaction typically peaks 1-3 days after challenge and is dependent on the presence of memory T cells. In case of T cell **anergy** - i.e. inability to mount a complete response against a target - the reaction is basically non-existing, hereby confirming the role of Th1 cells. Nevertheless, several studies have shown that Th2 cells may also be involved in certain types of proinflammatory cell-mediated immunity (Dannenberg, 1991; Black, 1999; Vukmanovic-Stejic *et al*., 2006).

The DTH test was previously performed *in vivo* by means of the **cell-mediated immunity (CMI) skin test** (Merieux multitest) which involves challenging the forearm intracutaneously with seven different antigens (tetanus and diphteria toxoid, Streptococcus, Tricophyton, Proteus, Candida and tuberculin antigen) and one diluent (negative control) and assess the response in terms of skin indurations by measuring the diameter of the induced inflammation sites (Figure 11). This way it was possible to evaluate the extent of immunodeficiency e.g. as a result of distress (Smith *et al*., 2004), in intensive care patients, heart-transplant patients, people infected with the human immunodeficiency virus, but also people subjected to extreme living and working conditions such as Antarctic workers (Williams *et al*., 1986) and astronauts before, during and after flight (Taylor and Janney, 1992). However, since 2002 the Merieux test has been taken out of the market alternative assays needed to be developed.

Figure 11. Principle of CMI (Merieux) multitest: introduction of antigens on the skin and measuring the diameter of the induced inflammation sites.

More recent efforts in monitoring the efficacy of our cell-mediated immune response consider *in vitro* whole blood samples as a feasible alternative to the skin test, e.g. reactions to metals described by Rustemeyer *et al*. (2004) and Minang *et al*. (2006). In short, blood is taken from the subject at regular intervals and challenged *ex vivo* with a broad array of stimuli, e.g. diphtheria, tetanus and pertussis toxoid, tuberculin and lysate from Candida albicans, CMV, EBV and Trichophyton lysate (Kelsen *et al.*, 2012; Feuerecker *et al.*, 2013a). APCs will present the foreign antigen on their surface MHCs to surrounding T cells. Upon recognition by their TCR, these T cells will be primed and will stimulate CD4+ and CD8+ T cells. This cascade of antigen induced immune response will trigger secretion of IL-2 and the expression of IL-2 receptors (IL-2Rs) whose interaction will further stimulate differentiation. Next, the IL-2 triggered action will promote the growth and differentiation of antigen-selected cytotoxic T cells. Moreover, when activation is complete, the CD4+ T helper cells will start to proliferate. The T helper cells receiving both signals of activation will become Th0 cells that secrete IL-2, IL-4 and IFN-γ. Finally, the Th0 cells will then differentiate into Th1 or Th2 cells. This differentiation is dependent on the cell cytokine environment: e.g. IFN-γ enhances a Th1 cell production while the IL-10 inhibits it.

The incubation of blood with these antigens can be performed over a period of up to 48h to be able to monitor late cell-mediated immune reactions. The performance of the assay can be evaluated by challenging blood samples with polyclonal mitogens (e.g. concanavalin A or pokeweed mitogen (PWM)). PWM acts as a strong polyclonal activator which induces mitosis in lymphocytes in a non-receptor specific manner. One of the major benefits of this approach is that the assay is relatively simple, can be standardized and include antigens which could be too harmful to introduce to human subjects (e.g. viruses). Also, the final read-out can be tailored and can focus on cytokines which are most representative for T cell-mediated immunity, i.e. IL-2, IFN-γ, tumor necrosis factor (TNF) α and IL-10 (Feuerecker *et al*., 2013a).

IL-2
Human IL-2 (also referred to as T cell growth factor) is a Th1 cytokine that is produced by T cells (mainly CD3+) after stimulation with antigens or mitogens (Feuerecker *et al.*, 2013a). IL-2 mainly promotes T cell proliferation and maturation, but has also been shown to promote growth and differentiation of B cells (Ig production), natural killer cells, lymphokine-activated killer cells, monocytes and oligodendrocytes, while inhibiting granulocyte-macrophage colony formation (Hoyer *et al.*, 2008). IL-2 signals through various pathways including the JAK/STAT5, RAS/MAPK, and PI3 kinase pathways, and it known to be a potent inductor of IFN-γ (Seder *et al.*, 1994). Through the STAT5 pathway IL-2 also functions as a regulator of the Th2 response. Furthermore, IL-2 has been identified as an important biomarker in several pathologies such as cancer, multiple sclerosis, systemic lupus erythematosus rheumatoid arthritis and type I diabetes. Post-flight studies and microgravity simulations focusing on T cell functioning have reported a significant drop in IL-2 and IL-2R production which can ultimately lead to T cell anergy (Walther et al., 1998; Lewis *et al.*, 2001; Crucian *et al.*, 2008; Gridley *et al.*, 2009). Also, the production of IFN-γ and TNF-α (to be discussed later) is closely linked to IL-2 expression and is downregulated during bedrest studies (Kessel *et al.*, 2012). It is important to note that in addition to microgravity, increased body cortisol levels reflecting stress - e.g. as a result of the confinement or heavy work load related to space missions - can also have a negative effect on IL-2 production (Feuerecker *et al.*, 2013a).

IFN-γ

IFN-γ (also referred to as type II interferon) is a Th1 promoting / Th2 inhibiting cytokine that is produced by activated T cells (CD8+), B cells and natural killer cells as a result of an infection (Feuerecker *et al.*, 2013a). It has anti-viral and anti-parasitic properties and can act in synergy with other cytokines such as TNF-α. IFN-γ is a potent activator of vascular endothelial cells, neutrophils, mononuclear phagocytes, can promote endocytosis and phagocytosis by monocytes, and trigger macrophages to kill tumor cells. Its expression is typically upregulated as a result of infections, autoimmune diseases, allergies and diabetes. IFN-γ is known to be downregulated in bed rest studies (Kelsen *et al.*, 2012) and has been also identified an important cytokine involved in mediating skin DTH reactions (McKarns and Schwartz, 2008). Furthermore, as IFN-γ expression is regulated by IL-2 the overall kinetics in a response to an antigen challenge are comparable (Crucian *et al.*, 2008; Feuerecker *et al.*, 2013a).

TNF-α

TNF-α is a Th1 cytokine that is mainly secreted by monocytes, macrophages and T cells but can also be produced by neutrophils, natural killer cells, mast cells, endothelial cells, activated lymphocytes as well as certain cancer cells. It plays a key role in both innate and adaptive immunity and is able to initiate and enhance T cell signaling and activation (McKarns & Schartz, 2008). Expression at damaged sites leads to recruitment and activation of pro-inflammatory cells and induction of various cytokines. In addition to its immunomodulatory activities, TNF-α can induce cell proliferation and differentiation, tumorigenesis, apoptosis and affect lipid metabolism, coagulation and endothelial function. It has been associated with multiple diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis and multiple sclerosis. Both microgravity simulations and bedrest studies have reported a drop in TNF-α titers (Kelsen et al., 2012) as well as a downregulation in gene expression pathways where TNF-α is the central player (Chang *et al.*, 2012).

IL-10

In contrast to the previous cytokines describes, IL-10 is a Th2 cytokine with anti-inflammatory characteristics. It is expressed by a broad spectrum of immune cells such as Th2 cells, B cells, dendritic cells, neutrophils, eosinophils, macrophages, natural killer cells and mast cells. The main function of IL-10 is to inhibit an immune response against pathogens in order to avoid extensive tissue damage to the host, e.g. when dealing with parasitic or viral infections. As a result, it is an important biomarker for several diseases and can be used for therapeutic purposes and to suppress graft or organ rejections after transplantation. IL-10 levels were found to be less affected in bedrest studies (Kelsen *et al.*, 2012). However, the reduction in IFN-γ titers results in a drop in IFN-γ / IL-10 ratio and shifts the immune system's balance towards Th2 (Crucian *et al.,* 2008).

Hence, the results obtained from earlier experiments in space confirm that spaceflight can have a huge effect on our immune system. Although the contribution of the psychological stress to which astronauts are subjected cannot be neglected and the long-term effects of space radiation are still poorly understood, microgravity appears to play an important role. Unfortunately, the test population of astronauts is still relatively small, mission conditions can vary extremely (e.g. in total mission time, EVA vs. non-EVA) and the extent of the effect is related to the susceptibility of the individual. As a result, all the information we have gathered

so far does not allow us to exclude the potential effect of radiation or a combination of all the stressors mentioned earlier, which could work in a synergistic or antagonistic manner (Manti, 2006; Baatout *et al.,* 2012; Moreels *et al*, 2012a; Moreels *et al*., 2012b). Although some studies did not immediately reveal additive effects (Horneck *et al*., 1996, 1997; Kiefer and Pross, 1999; Pross *et al.,* 2000; Manti *et al*., 2005, 2006) other have reported synergistic interactions between microgravity and radiation (Bucker *et al*., 1983; Horneck, 1992; Horneck *et al*., 1999; Mosesso *et al*., 2001; Canova *et al*., 2005; Mognato *et al*., 2009). Examples include the slower repair of γ-radiation induced DNA DSBs in human peripheral blood lymphocytes subjected to microgravity (Mognato *et al*., 2009). This delay in DNA repair increases the risk of gene mutations and chromosomal aberrations in space which can have consequences for long-term flights.

In conclusion, if we want to safeguard the health of our astronauts during their time in space it will be important to continuously monitor their immune system, understanding how and when changes become a health risk, what kind of countermeasures could be implemented or whether intervention - drug or vaccine based - is preferred. In order to achieve this goal, an innovative, easy-to-handle, minimal-invasive assay based on a limited sample size will have to be developed. An *ex vivo* assay based on blood samples and challenge antigens as described by Feuerecker *et al.* (2013a) could prove to be a valuable tool. Therefore, this approach will be further investigated in ESA's "Monitoring the Cellular Immunity by *in vitro* delayed type hypersensitivity assay on the ISS" (MoCISS) flight study in which the Radiobiology Unit of SCK•CEN is actively contributing.

Objective

Objective

Studies focused on cell and animal models as well as data gathered in humans have clearly demonstrated the negative impact of space flight on the functioning of our immune system (in particular the initiation of cellular immunity). As space confronts the astronaut with a broad array of stressors acting simultaneously (microgravity, radiation, psychological stress) the impact of each of these on the immune system is still poorly understood. In order to ensure the safety of our astronauts in future flights beyond LEO it is important to measure the effect of each stressor (or a combination thereof) in an objective manner in an attempt to develop the necessary countermeasures for immune system dysregulation.

This master thesis is part of the preparatory phase of the ESA accepted flight experiment "Monitoring the Cellular Immunity by *in vitro* delayed type hypersensitivity assay on the ISS" (MoCISS). This flight is foreseen in the time frame of 2014-2015. MoCISS will provide the astronauts with a tool to monitor the overall health status of their cellular immunity *ex vivo*. These analyses will improve our understanding of the impact of physiological and psychological space stressors on the immune system - as well as the mechanisms involved - in preparation of future long-term space missions beyond LEO.

The goal of this master thesis is to study changes in cellular immunity induced by simulated space conditions - in particular microgravity - on blood cell cultures taken from healthy male volunteers. The work will consist of evaluating the feasibility of the approach and optimizing the overall working protocol (e.g. sample handling, sample containers, antigen challenge and cytokine analysis). Cellular immune (dys)function will be monitored by means of the *in vitro* delayed-type hypersensitivity (DTH) test which includes stimulation of white blood cells with mitogens/antigens of bacterial, fungal and viral origin. The outcome of these tests will be assessed by determining the cytokine expression profile of the blood culture after up to 48h incubation with different stimuli. The following cytokines will be investigated: IL-2 (Th1, pro-inflammatory), IL-10 (Th2, anti-inflammatory), IFN-γ (Th1, pro-inflammatory) and TNF-α (Th1, pro-inflammatory) - mainly secreted by activated T lymphocytes - using two different detection techniques (Luminex, ELISA).

Chapter 2

Materials & Methods

2   Materials & Methods

The current chapter provides an overview of the protocols applied to generate the experimental data for this master thesis. For each procedure, the required materials and methods are discussed in detail.

2.1   Collection of whole blood from healthy volunteers

Blood samples used in the different experiments were obtained from healthy male volunteers (25-35 years of age, non-smokers) by venipuncture and collected in either ethylene diamine tetraacetic acid (EDTA) tubes (Terumo, Venosafe® Plastic Tubes, VG-054SDK, purple cap) or lithium heparin tubes (Terumo, Venosafe® Plastic Tubes, VF-109SHL, green cap) to prevent blood clotting (Figure 12). All subjects were asked for their vaccination history and no recent diseases or infections were reported (normal blood formula). Blood collections were performed in the morning and subjects were not subjected to overnight fasting prior to collection.
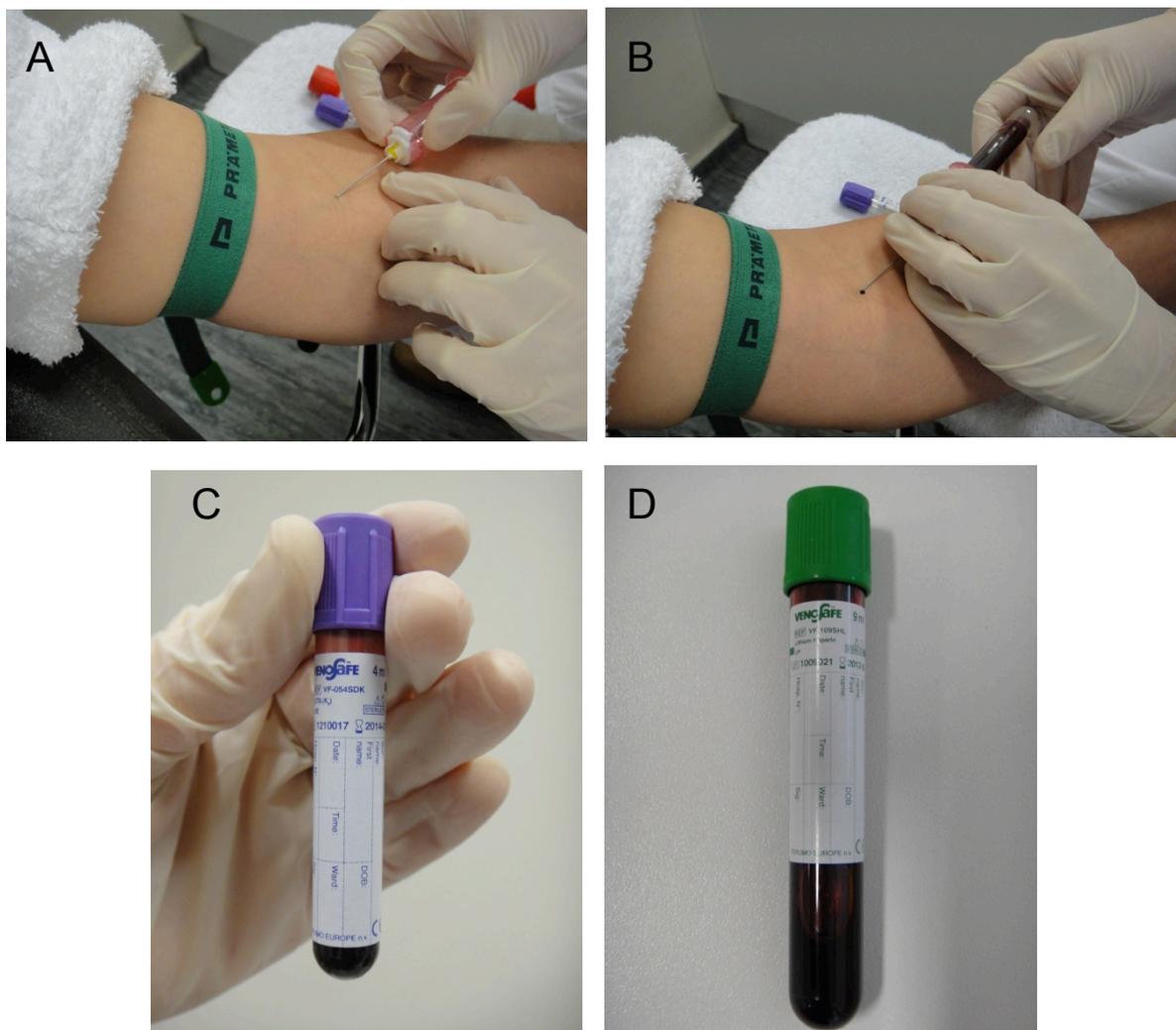

Figure 12. Blood collections performed at SCK•CEN on healthy male volunteers (A+B) in EDTA (C) or lithium heparin treated (D) tubes.

Each tube contained a maximum 9 mL of blood. Not more than 4 tubes per person were taken at a given time depending on the amount of blood required for the experiment in question. After collection, tubes containing blood were placed on a rocking platform at room temperature to prevent cell sedimentation prior to further sample treatment which can cause hypoxia.

2.2 *Ex vivo* treatment of blood samples

All handlings performed on blood samples prior to incubation were done under sterile conditions in a laminar flow cabinet. At the start of each experiment, blood samples were pooled for each individual donor before further handling and preparation to ensure sample homogeneity. Next, samples were diluted 1:1 with Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, R0883-1L) or Dulbecco's Modified Eagle's Medium (DMEM) nutrient mixture (F-12 HAM; Sigma-Aldrich) with or without additional stimuli (mitogens or antigens) (see below) and mixed gently by inversion. Finally, the prepared cultures were incubated for up to 48h at +37°C (5% CO_2) under different (space simulated) conditions.

2.2.1 Sample containers

The incubation of blood cultures and medium was performed using several container types with total volumes varying from 1.5 to 10 mL. Both sealed containers and containers allowing gas-exchange were included to assess their feasibility for microgravity experiments and potential effects on cell survival, cell stimulation and cytokine secretion. An overview of the containers tested is given in Table 1 and Figure 13.

Table 1. Overview of different containers tested in incubation experiments.

container	total volume (mL)	gas exchange ?	catalog number / reference	figure
Polypropylene/polyethylene CryoTubes™ with external thread	1.5		Nunc - 37529	Figure 13A
Polystyrene tube (round-based) with low density polypropylene two-position fit ventilation cap	5.0	X	Sarstedt - 55.476.013	Figure 13B
Opticell 1100 culture system with gas-permeable, polystyrene membranes	10.0	X	Nunc - 155330	Figure 13C
Polystyrene 12.5 cm² Falcon™ Cell Culture Flask with vented cap	33.0	X	BD - 53107	Figure 13D
Self-made miniaturised Falcon™ Cell Culture Flask with vented cap	2.5	X	(in-house design)	Figure 13E
Rotating wall vessel (RWV) with ventilation membrane	10.0	X	Cellon - D-410	Figure 13F
SCK•CEN module with biofilm membrane	1.0	X	(in-house design)	Figure 13G
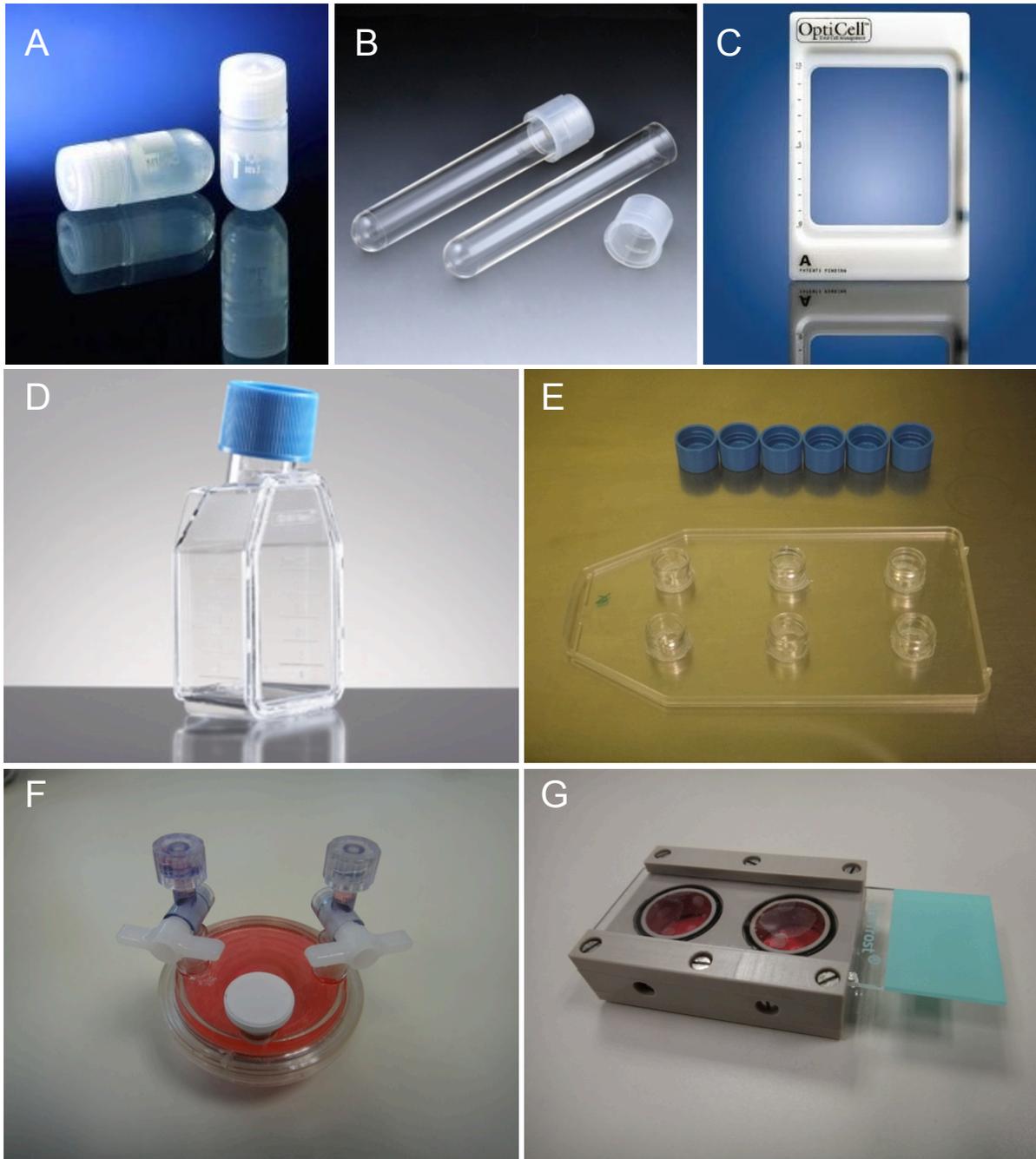
Figure 13. Overview of different containers tested in incubation experiments: (A) cryotubes, (B) tubes with two-position fit ventilation cap, (C) Opticell 1100 culture system, (D) cell culture flask with vented cap, (E) self-made miniaturised cell culture flasks with vented cap, (F) rotating wall vessel and (G) SCK•CEN module with biofilm membrane. Pictures (A), (B), (C) and (D) obtained from corresponding manufacturers (refer to Table 1).

2.2.2   Mitogen/antigen challenge

During incubation blood cultures were challenged with different stimuli to initiate an immune cascade and subsequent cytokine expression. These stimuli were introduced by mixing blood with medium (1:1) containing either PWM (positive control) or an antigen of bacterial, viral or fungal origin. An overview of the different mitogens/antigens tested is given in Table 2. For each experiment a negative control was included which consisted of a 1:1 mixture of blood with blank culture medium.

Table 2. Overview of stimuli used for white blood cell stimulation.

Stimulus type	Stimulus	Concentration	Manufacturer
Bacterial	Diphteria, tetanus & pertussis toxoid (Boostrix®)	1%	GlaxoSmithKline, Munich, Germany
Viral	Cytomegalovirus (CMV) lysate	10 µg/mL	ABI, Columbia, SC, USA
	Epstein-Barr virus (EBV) lysate	10 µg/mL	ABI, Columbia, SC, USA
	Alpha-Rix®	1%	GlaxoSmithKline, Munich, Germany
	Influvac®	1%	Solvay, Hannover, Germany
Fungal	Candida and Trichophyton lysate	10 µg/mL	Allergopharma, Reinbeck, Germany
Plant	Pokeweed mitogen (PWM)	5 µg/mL	Sigma-Aldrich, St.-Stallen, Switserland

2.2.3   Microgravity simulation

Blood cultures challenged with or without different stimuli were subjected to simulated microgravity using a desktop random positioning machine (RPM) from Dutch Space B.V. (EADS-Astrium, Leiden, The Netherlands) (Figure 14).

Figure 14. Picture of the RPM used for simulation of microgravity on Earth (taken from http://www.dutchspace.nl).

The RPM is a laboratory instrument capable of continuously rotating biological samples along two independent axes in a randomized manner (Grimm *et al*., 2011; Grosse *et al*., 2012). The system is based on the principle of "gravity-vector-averaging" in which the samples are constantly put in different positions, hereby reducing the effect of gravity. A desktop RPM is particularly interesting for testing the effect of simulated microgravity on cell cultures (Schwarzenberg *et al*., 1998, 1999; Walther *et al*., 1998, 1999; Uva *et al*., 2002; Strollo *et al.*, 2004; Infanger *et al*., 2006; Patel *et al*., 2007; Grimm *et al*., 2009; Ulbrich *et al*., 2010a, 2010b; Grimm *et al*., 2011; Grosse *et al*., 2012; Beck *et al*., 2012). For our experiments, the instrument was placed in an incubator allowing accurate control of temperature and CO_2/O_2 levels. A typical setup of an RPM experiment is shown in Figure 15.
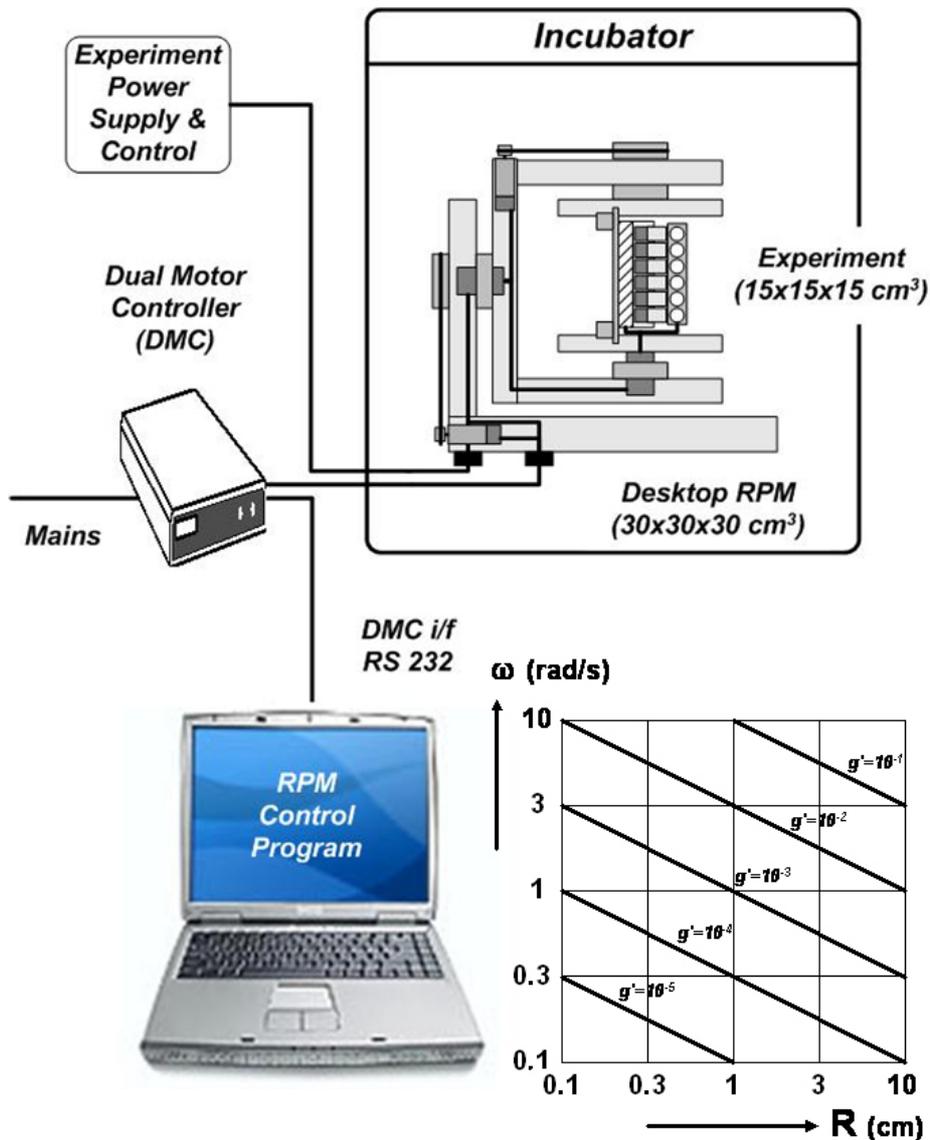

Figure 15. Typical setup of an RPM experiment including a desktop RPM unit with power source, controlling unit, computer and incubator (taken from http://www.dutchspace.nl).

The following steps were performed to initiate RPM experiments with blood cultures:

1. connect all cables provided with RPM system (RPM, controller, gravity sensor, PC)
2. connect/switch on power sources
3. open program "RPM controller" (this will launch the software needed for control)
4. click "Go online" (connection with RPM will be established)
5. click "Motion mode" → "Real random" → 72°/min + 55°/min
6. click "G sensor" → select port "COM4"
7. click "Hold frame" (required to enable RPM operation)
8. attach samples to centre plate using rubber straps
    REMARK: it is important to mount the samples as close as possible to the centre of the device to ensure an optimal microgravity simulation is obtained
9. reset average g value
10. click "Run"
11. check RPM movement (no obstructions, samples secured)

After incubation, the RPM system was shut down by clicking "Finish" and "Release frame".

2.2.4   Collection of culture supernatant (plasma)

After incubation blood cultures were taken out of the incubator and centrifuged (15 min at 1,500 g) to allow separation of plasma from cells. The supernatant (plasma) was transferred to eppendorf tubes (typically around 800 µL per sample) and stored at -80°C until further analysis.

2.3   Determination of pH and dissolved oxygen level

The pH of test samples was monitored before and after incubation using a calibrated micro pH meter (InoLab WTW). The amount of dissolved oxygen present was measured with a Knick Portamess® dissolved oxygen meter equipped with Knick SE302 oxygen sensor (0-60 mg/mL detection).

2.4   Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood for cell counting purposes or to assess the effect of different stimuli on cytokine secretion profiles.

First, 7 mL of collected blood was gently transferred onto 7 mL Histopaque-1077 (Sigma-Aldrich, 10771) without disturbing the blood-medium interface. To achieve this, the pipette tip was placed against the side of the tube and the blood was allowed to run along the side of the tube. Next, the tube was centrifuged at 400 g for 30 min at room temperature (disabling both acceleration and braking) rendering gentle separation of the blood into different layers, with a well-defined central white PBMC layer.

The layer of PMBCs was transferred to an empty 15 mL falcon tube. The aim was to collect as many PMBCs as possible within a limited volume (several mL). The cells were then washed with 10 mL of 1X phosphate-buffered saline (PBS) solution and centrifuged for 10 min at 250 g (including acceleration and braking). Next, the supernatant was discarded and the remaining cell pellet resuspended in the remaining buffer by flicking the tube. The latter wash and resuspension step was repeated two more times prior to final resuspension. The resuspended cells were counted using a Moxi Z^{TM} cell counter with Maxi Z cassette (MXC002, ORFLO Technologies, Figure 16). Cell viability was monitored by trypan blue staining, i.e. by incubating 50 µL cell suspension with 50 µL trypan blue stock solution (final concentration = 0.5%) followed by immediate read-out under a microscope. Two hundred cells were checked for color (white = alive; blue = dead) and the relative cell viability was reported for a given cell culture.

Figure 16. Moxi Z^{TM} cell counter from ORFLO Technologies.

2.5 Cytokine quantification using multiplex assay (Luminex®)

The concentration of Th1 cytokines (IL-2, IFN-γ, TNF-α) and Th2 cytokines (IL-10) was performed using the MILLIPLEX® xMAP technology from Millipore (HCYTOMAG-60K.MPX) based on a 96-well plate design and human cytokine magnetic bead panel with a sensitivity threshold of 0.7-1.0 pg/mL.

The following antibody immobilized beads (4-plex) were used during analysis:
- anti-human IL-2   (HIL2-MAG, bead region 48)
- anti-human IFN-γ (HCYIFNG-MAG, bead region 25)
- anti-human TNF-α (HCYTNFA-MAG, bead region 75)
- anti-human IL-10   (HCYIL10-MAG, bead region 27)

Prior to use all reagents were allowed to equilibrate to room temperature and samples were vortexed extensively. As the beads are light sensitive all incubation steps were performed in the absence of light by covering the assay plate with aluminum foil.

2.5.1   Preparation of immobilized beads

Each individual vial with beads was sonicated for 30s and vortexed for 1 min, after which 60 µL of each antibody bead vial was added to the mixing bottle provided. The mixture was brought to a final volume of 3.0 mL using bead diluent and vortexed again.

2.5.2   Preparation of quality controls

Before use, Quality Control 1 and Quality Control 2 was reconstituted with 250 µL deionized water. The vial was inverted several times and vortexed. Next, the vial was allowed to settle for 10 min and the content was transferred to appropriately labeled polypropylene microcentrifuge tubes.

2.5.3   Preparation of standards

Prior to use the human cytokine standard was reconstituted with 250 µL deionized water to give a 10,000 pg/mL concentration of standard for all analytes. The vial was inverted and vortexed, allowed to sit for 10 min and transferred to an appropriately labeled polypropylene microcentrifuge tube. This was used as the 10,000 pg/mL standard.

Five polypropylene microcentrifuge tubes were labeled 2,000, 400, 80, 16, and 3.2 pg/mL. Two-hundred µL of assay buffer was transferred to each of the five tubes. Serial dilutions were prepared by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2,000 pg/mL tube and repeatedly transferring 50 µL to the next tube (Figure 17). The 0 pg/mL standard (background) consisted of blank assay buffer. All prepared standards were used within 1h of preparation.
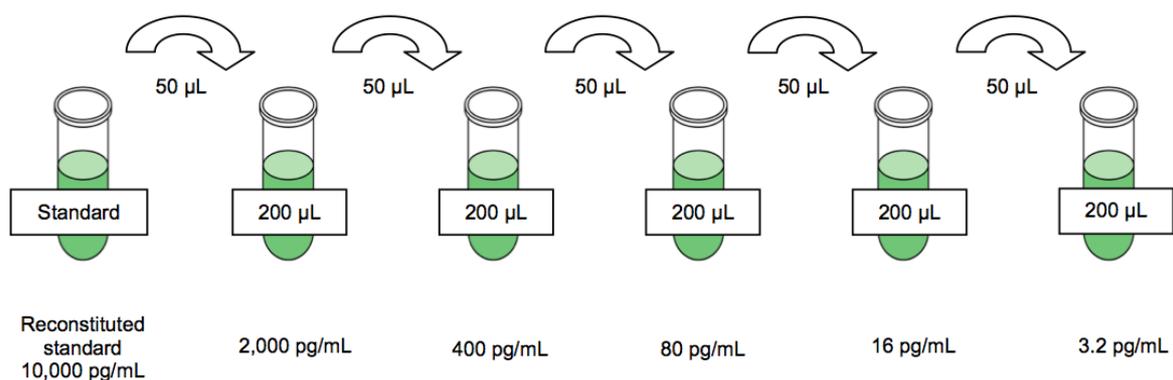

Figure 17. Preparation of standard cytokine serial dilution for assay calibration purposes.

2.5.4   Immunoassay procedure

Test samples were kept on ice prior to loading onto the plate. First, 200 µL wash buffer was added to each well, the plate sealed and mixed on a plate shaker for 10 min at room temperature (700 revolutions per minute, rpm). Afterwards, the wash buffer was decanted and the residual amount was removed from the wells by inverting the plate and tapping it gently onto absorbent towels several times. Twenty-five µL of each standard or control was transferred into the appropriate wells. Blank assay buffer was used for the 0 pg/mL condition (i.e. background). Next, 25 µL assay buffer was added to all sample wells as well as 25 µL of serum matrix solution (provided in the kit) to the background, standards, and control wells. Wells reserved for test samples were loaded with 25 µL sample (Figure 18). Finally, 25 µL premixed beads was added to each well, the plate was sealed, wrapped in foil and incubated on a plate shaker overnight at +4°C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	400 pg/mL Standard	QC-2 Control									
B	0 pg/mL Standard (Background)	400 pg/mL Standard	QC-2 Control									
C	3.2 pg/mL Standard	2,000 pg/mL Standard	Sample 1									
D	3.2 pg/mL Standard	2,000 pg/mL Standard	Sample 1									
E	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
F	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
G	80 pg/mL Standard	QC-1 Control	Etc.									
H	80 pg/mL Standard	QC-1 Control										

Figure 18. Example of typical plate layout when performing Luminex® analysis.

After incubation, the plate was washed 2 times. The wash step was performed using a hand-held magnet on which the plate could be mounted. The plate was allowed to rest on the magnet for 60s to ensure complete settling of magnetic beads. The well content was removed gently by decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Afterwards, the plate was washed with 200 µL of wash buffer by removing plate from magnet, adding wash buffer, shaking for 30s, reattaching to magnet, letting beads settle for 60s and removing well contents as previously described.

Next, 25 µL of detection antibodies were added into each well. The plate was sealed, covered with foil and incubated on a plate shaker for 1h at room temperature. After incubation, 25 µL streptavidin-phycoerythrin was added to each well followed by sealing and incubation for 30 min at room temperature. Next, the well content was gently removed and the plate was washed 2 times. Finally, 150 µL of sheath fluid was added to all wells and the beads were resuspended on a plate shaker for 5 min prior to read-out.

2.5.5   Analysis and calculation of cytokine concentrations

The plate was read on a Luminex® 100TM IS system equipped with xPONENT® 3.1 software (Figure 19). For each sample, at least 50 events were recorded for each bead type using 100 µL sample with gate settings 8,000 - 15,000 (for additional details on system settings refer to the manufacturer's instruction manual). The Median Fluorescent Intensity (MFI) data was analysed with a 5-parameter logistic curve-fitting method - using GraphPad software (GraphPad Software Inc., version 5) - for calculating cytokine concentrations in the different samples. For each sample, 2 biological replicates were analysed in triplicate (6 measurements in total). The average value was determined and confidence intervals were expressed based on the standard deviation.

Figure 19. Luminex® 100TM IS system with xPONENT® 3.1 software.

2.6   Cytokine quantification using ELISA

As an alternative to the Luminex assay, an enzyme-linked immunosorbent assay (ELISA) was performed for determining the IL-2 concentration in plasma samples, more specifically the validated human IL-2 ELISA kit from Millipore with ready-to-use reagents (EZHIL2). All reagents included in the kit were brought to room temperature prior to use. A 96-well plate pre-coated with IL-2 capture antibody (Part No. CS210048) is included and can be adjusted to fit the number of samples to be tested.

2.6.1   Preparation of standards

For each assay, a standard dilution series of an IL-2 stock was prepared. First, the lyophilized IL-2 standard provided (Part No. CS210050) was reconstituted in Assay Buffer B (Part No. CS210052) to obtain a stock solution at 20 ng/mL. The reconstituted standard was allowed to sit at room temperature for 15-20 min after which its was mixed by vortexing. Next, 500 μL of a 1000 pg/mL standard was made by diluting 25 μL of the stock solution in 475 μL of Assay Buffer B. Finally, six two-fold serial dilutions were made in separate tubes (again using Assay Buffer B) to obtain the following series: 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/ mL, 31.3 pg/mL, and 15.6 pg/mL (Figure 20). Assay Buffer B without IL-2 standard served as a blank (0 pg/mL).
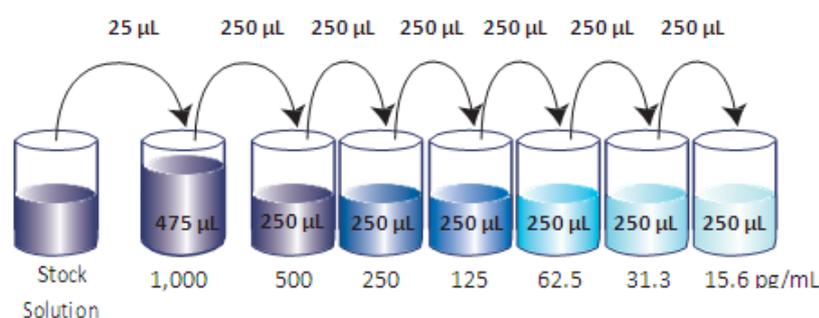

Figure 20. Preparation of standard IL-2 serial dilution for assay calibration purposes.

2.6.2   Preparation of plate and sample loading

Prior to sample loading, the 96-well plate was washed 4 times by adding 300 μL 1X Wash Buffer (Part No. CS210053) per well. The plate was decanted, the remaining buffer was removed by firmly tapping the plate upside down on absorbent paper and 50 μL of Assay Buffer B was transferred to each well to be used for analysis. Next, 50 μL standard solution or 50 μL test sample was added to the corresponding wells, the plate was closed with a Plate Sealer (Part No. CS210056) and incubated at room temperature for 2h while shaking at 200 rpm. All standards and samples were analysed in duplicate (2 wells/sample).

2.6.3   Detection of IL-2

After initial sample incubation, the content of the plate was discarded and the plate was washed 4 times with Wash Buffer. Next, 100 μL of Human IL-2 Detection Antibody solution (Part No. CS210049) was added to each well, the plate was sealed and incubated at room temperature for 1h while shaking (200 rpm). The content of the plate was again discarded followed by 4 wash steps. Next, 100 μL of Avidin-HRP A solution (Part No. CS210051) was added to each well, the plate was sealed and incubate at room temperature for 30 min while shaking (200 rpm). After discarding the plate content, the wells were washed 5 times, allowing a soaking period of 1 min for each washing step in order to minimize the

background signal. Next, 100 µL of Substrate Solution F (Part No. CS210054) was added to each well and the plate was incubated for 15 min in the dark. As a result, wells containing human IL-2 turn blue in color with intensity proportional to its concentration. The reaction was stopped by adding 100 µL of Stop Solution (Part No. CS210055) to each well hereby changing the color of the solution from blue to yellow.

Finally, the absorbance of the plate was measured using a plate spectrophotometer (Thermo Labsystems, Multiskan Ascent) at 450 nm. The absorbance at 595 nm was measured to allow subtraction from the 450 nm values. A schematic overview of the different steps of the IL-2 ELISA protocol is given in Figure 21.
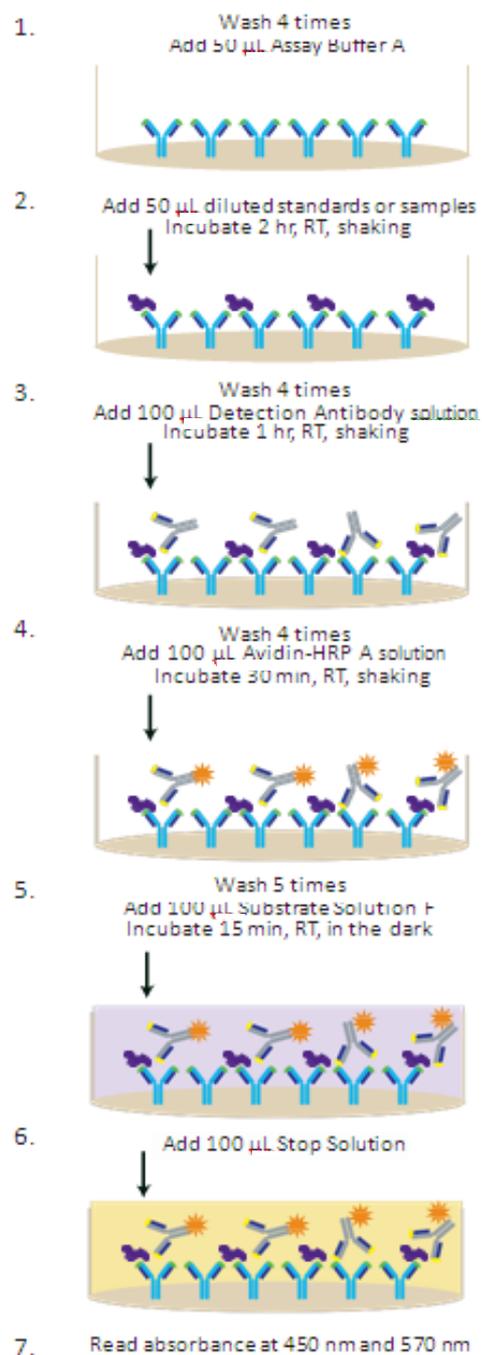

Figure 21. Schematic overview of IL-2 ELISA protocol (according to manufacturer).

2.6.4   Calculation of IL-2 concentrations

The data was processed in GraphPad (GraphPad Software Inc., version 5) using a 5-parameter logistics curve-fitting algorithm, i.e. a non-linear regression model used for prediction of the probability of occurrence of an event by fitting data to a logistic curve. It differs from the 4-parameter logistic model in that it is an asymmetric function providing a better fit for immunoassay data. As the name suggests, there are 5 parameters in the model equation:

$F(x) = A + (D/(1+(X/C)^B)^E)$
    A = mean fluorescence intensity (MFI) value for minimum asymptote
    B = hill slope
    C = concentration at inflection point
    D = mean fluorescence intensity value (MFI) for maximum asymptote
    E = asymmetry factor (if 1 than 5-PL equation = 4-PL equation)

The graphic representation of the model is shown in Figure 22. Parameters A (minimum asymptote) and D (maximum asymptote) are the limits of where you can interpolate or extrapolate your data. Any MFI values > D and MFI values < A cannot be calculated since they are out of the function range.
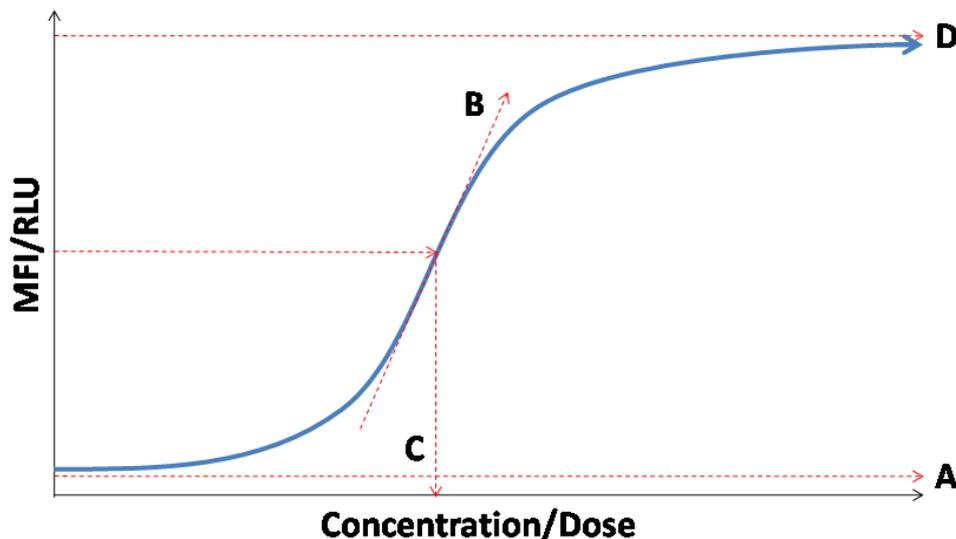

Figure 22. Graphic representation of 5-parameter logistic model with annotation of model parameters (taken from http://www.miraibio.com/blog/2009/02/5-pl-logistic-regression).

The obtained model described by the formula below was used to determine the IL-2 concentration of the different test samples. Ideally, the estimated concentrations are in the range of the standard series. In addition, concentrations can be estimated based on extrapolation - i.e. for points within the calculable limits (A < x < D) but outside of the range of the standard curve. However, since minor changes in MFI values result in significant changes in estimated concentration extrapolation should be treated with caution (Figure 23).
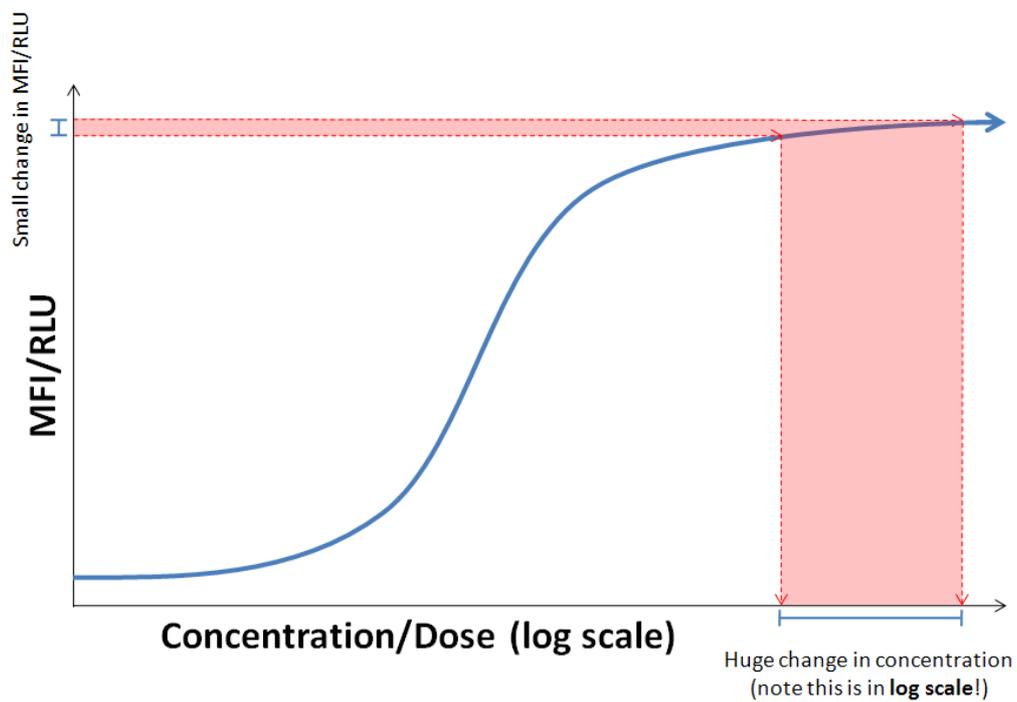
Figure 23. Risk of extrapolation using the 5-parameter logistic model (taken from http://www.miraibio.com/blog/2009/02/5-pl-logistic-regression).

For each sample, 2 biological replicates were analysed in duplicate (4 measurements in total). The average value was determined and confidence intervals were expressed based on the standard deviation.

Chapter 3

Results

3   Results

The following section provides an overview of the experimental data obtained. The overall aim of the experiments performed was to assess the feasibility of the *in vitro* DTH approach for measuring *ex vivo* cellular immune (dys)function under space-simulated conditions.

A first set of experiments dealt with the implementation and optimization of the *in vitro* DTH method, including antigen/mitogen challenge, the effect of sample container and effect of antigen handling (storage conditions). The outcome of these tests was assessed by analysis of the cytokine expression profile of the blood cultures after incubation with different stimuli, using Luminex® or ELISA technology. A second part further investigated alternative container types to allow gas-exchange while still remaining compatible with space-simulation experiments.

3.1   *Ex vivo* induction of cytokine response in vented container

The goal of the first experiment was to perform the *in vitro* DTH test in the same manner as described by Feuerecker *et al*. (2013a), i.e. by challenging a blood:medium mixture (1:1) stored at +37°C in a tube allowing gas-exchange (Figure 24A). This test would confirm proper functioning of the assay and its outcome would function as a reference for future experiments. The experiment included a challenge with 2 different stimuli, i.e. 1% PWM (a polyclonal mitogen often included in the *in vitro* DTH assay as a positive control) and 1% Alpha-Rix® (a viral antigen mix known to initiate the adaptive immune response (Moltedo *et al*., 2009) and comparable to the influenza challenge described by Feuerecker *et al*. (2013a)). A negative control was included in the experiment by mixing whole blood with blank RPMI medium (i.e. basal condition). After 48h incubation at +37°C (5% CO_2) all tubes were centrifuged to allow separation of plasma from blood cells (Figure 24B). The plasma was transferred to eppendorf tubes and cytokine concentrations were determined using the Luminex® assay. The results of the analysis are summarized in Figure 25.
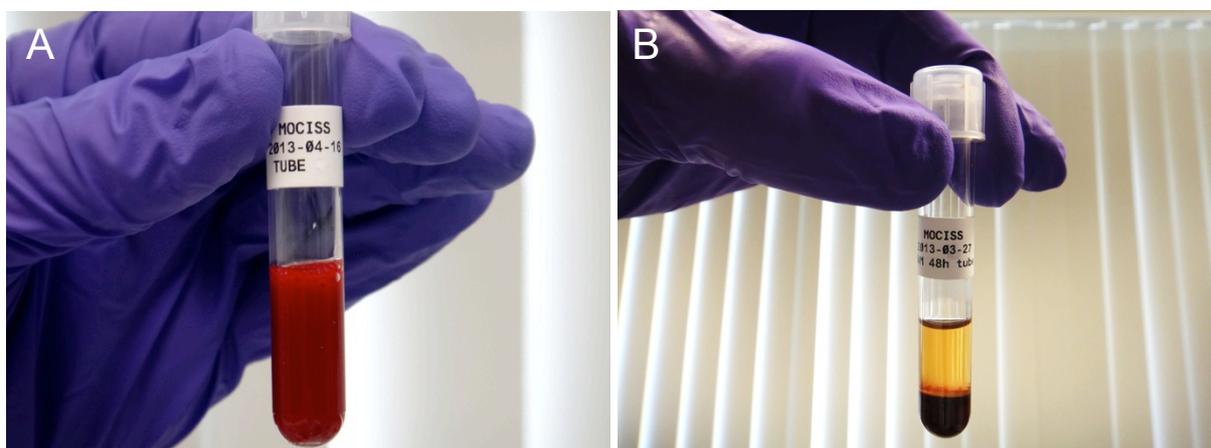

Figure 24. Picture of (A) vented tube containg a blood culture prior to centrifugation and (B) after centrifugation with dark red cell pellet (bottom) and yellow plasma phase (top).
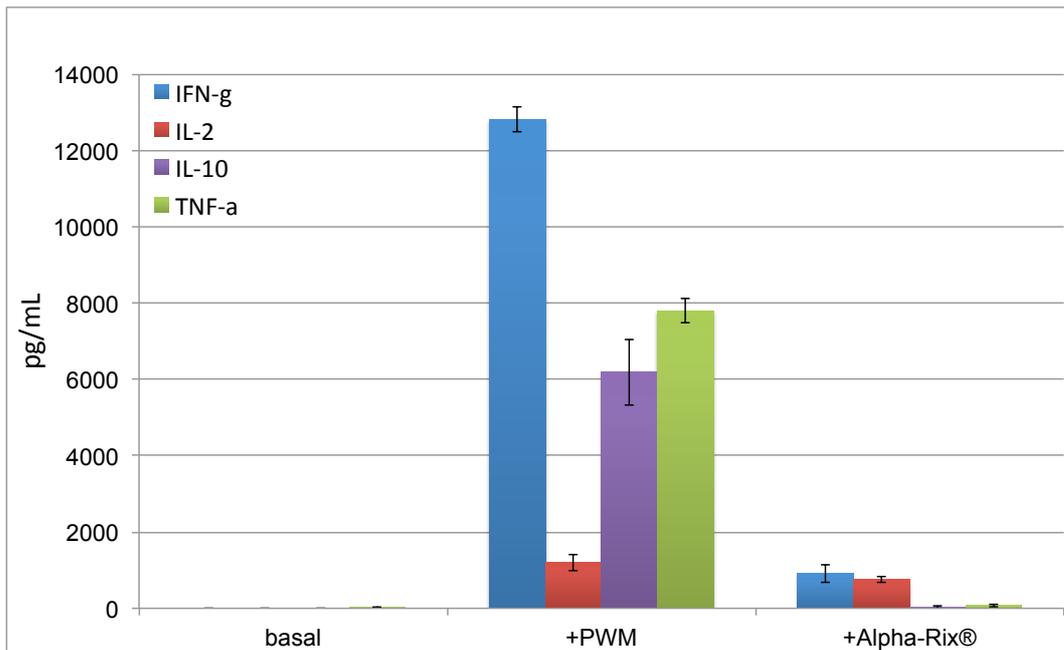
Figure 25. Cytokine levels measured in blood cultures after 48h challenge with PWM or Alpha-Rix® in a <u>vented tube</u>. Standard deviations are indicated. Basal = negative control.

The basal levels for all cytokines measured were found to be below the limit of quantification (<10 pg/mL) confirming that secretion of the cytokines monitored is non-existing under healthy conditions and is not triggered by *in vitro* incubation.

After incubation with PWM, a dramatic increase in IFN-γ concentration (12,800 pg/mL), IL-10 concentration (6,200 pg/mL) and TNF-α concentration (7,800 pg/mL) as well as an increase in IL-2 concentration (1,200 pg/mL) could be observed.

Challenging the blood culture with Alpha-Rix® rendered a less extreme cytokine profile compared to challenge with PWM, suggesting the former triggered a more specific immune response. While incubation with Alpha-Rix® did not have an effect on the IL-10 levels, an increase in the concentration of IFN-γ (910 pg/mL) and IL-2 (750 pg/mL) was detected.

These results confirm earlier findings from Feuerecker *et al*. (2013a), i.e. the possibility of *ex vivo* testing of blood cultures in vented tubes. Unfortunately, given its large head space and incomplete sealing mechanism, this container type does not allow to perform microgravity experiments on the RPM. Besides obvious leaking, air bubbles present in the tube would create a dynamic interface with the blood culture which would interfere with the free-fall motion of blood cells. Therefore, several types of alternative (sealed) containers - i.e. different eppendorf tubes and cryotubes - were briefly evaluated to see whether it was feasible to completely fill and seal a container while avoiding air bubbles.

Cryotubes were found to be best suitable for this type of experiments. Filling both the tube and internal contact surface of its screw cap with blood culture prior to sealing (≈1.8 mL/container) allowed us to close the tube without introducing air bubbles. Tightening of the screw cap forced any remaining air bubbles out of the container (example see Figure 26). The only two disadvantages of this approach are (1) that some blood culture was spilled during closure and (2) the container does not allow gas-exchange.

Figure 26. Picture of sealed, air bubble-free cryotube containing a blood culture (whole blood + RPMI).

3.2 *Ex vivo* induction of cytokine response in sealed container

As the *in vitro* DTH test described by Feuerecker *et al*. (2013a) was performed in vented tubes with significant head space, the next experiment investigated whether using a cryotube could have an effect on the outcome of the assay. Therefore, the experiment described in the previous section was repeated using cryotubes. Blood cultures from the same donor were incubated for 48h at +37°C in a sealed cryotube while being challenged with 1% PWM (positive control) or 1% Alpha-Rix® (Figure 27A). After incubation all samples were centrifuged (Figure 27B) and cytokine plasma levels were determined using the Luminex® assay. A graphical representation of the results obtained is shown Figure 28.

Figure 27. Picture of (A) sealed cryotube containing a blood culture prior to centrifugation and (B) cryotube after centrifugation with cell pellet (bottom) and plasma phase (top).
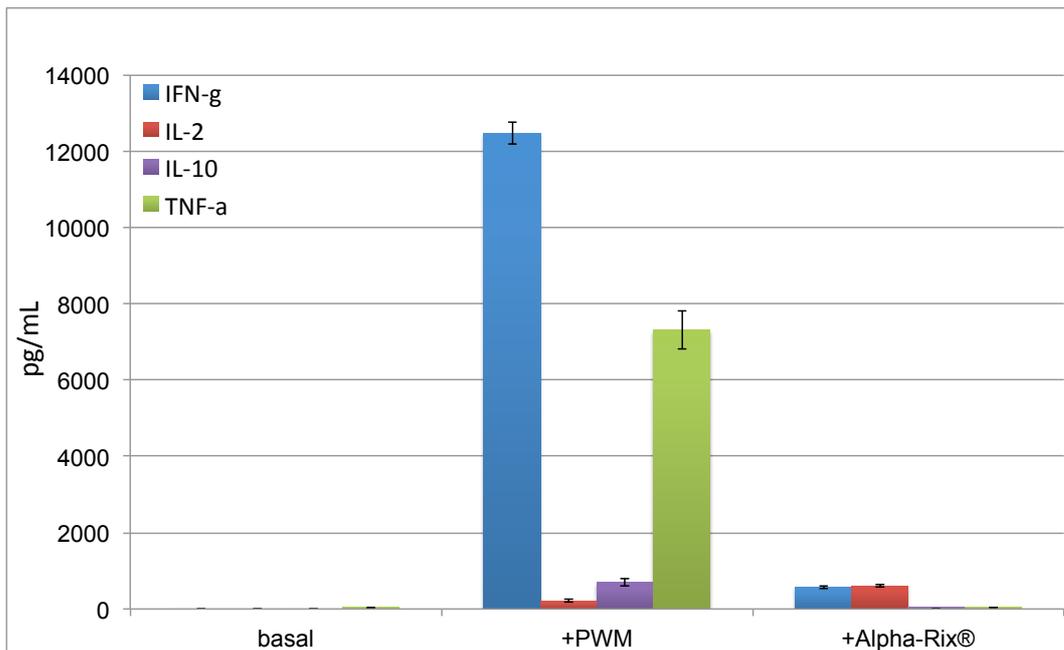
Figure 28. Cytokine levels measured in blood cultures after 48h challenge with PWM or Alpha-Rix® in a <u>sealed cryotube</u>. Standard deviations are indicated. Basal = negative control.

Again, basal levels for all cytokines measured were below the detection limit of the assay (<10 pg/mL). Similar as observed for the vented tubes, incubation with PWM caused a dramatic increase in IFN-γ concentration (12,500 pg/mL) and TNF-α concentration (7,300 pg/mL) as well as an increase in IL-2 concentration (220 pg/mL) and IL-10 concentration (690 pg/mL). In contrast, incubation with Alpha-Rix® did not have an effect on IL-10 and TNF-α levels, although an increase in the concentration of IFN-γ (570 pg/mL) and IL-2 (610 pg/mL) was detected.

When comparing the results obtained in the vented tube with those in the sealed cryotube, the overall cytokine levels were found to be higher in the vented tubes (Figure 29). When challenging with PWM (Figure 29A) this effect was most dramatic for IL-2 with a 6-fold increase (from 220 pg/mL to 1,200 pg/mL) and IL-10 with a 10-fold increase (from 700 pg/mL to 6,200 pg/mL). Correspondingly, incubation with Alpha-Rix® rendered higher cytokine levels in the gas-exchange capable container compared to the sealed container, although in this case the differences were minor.
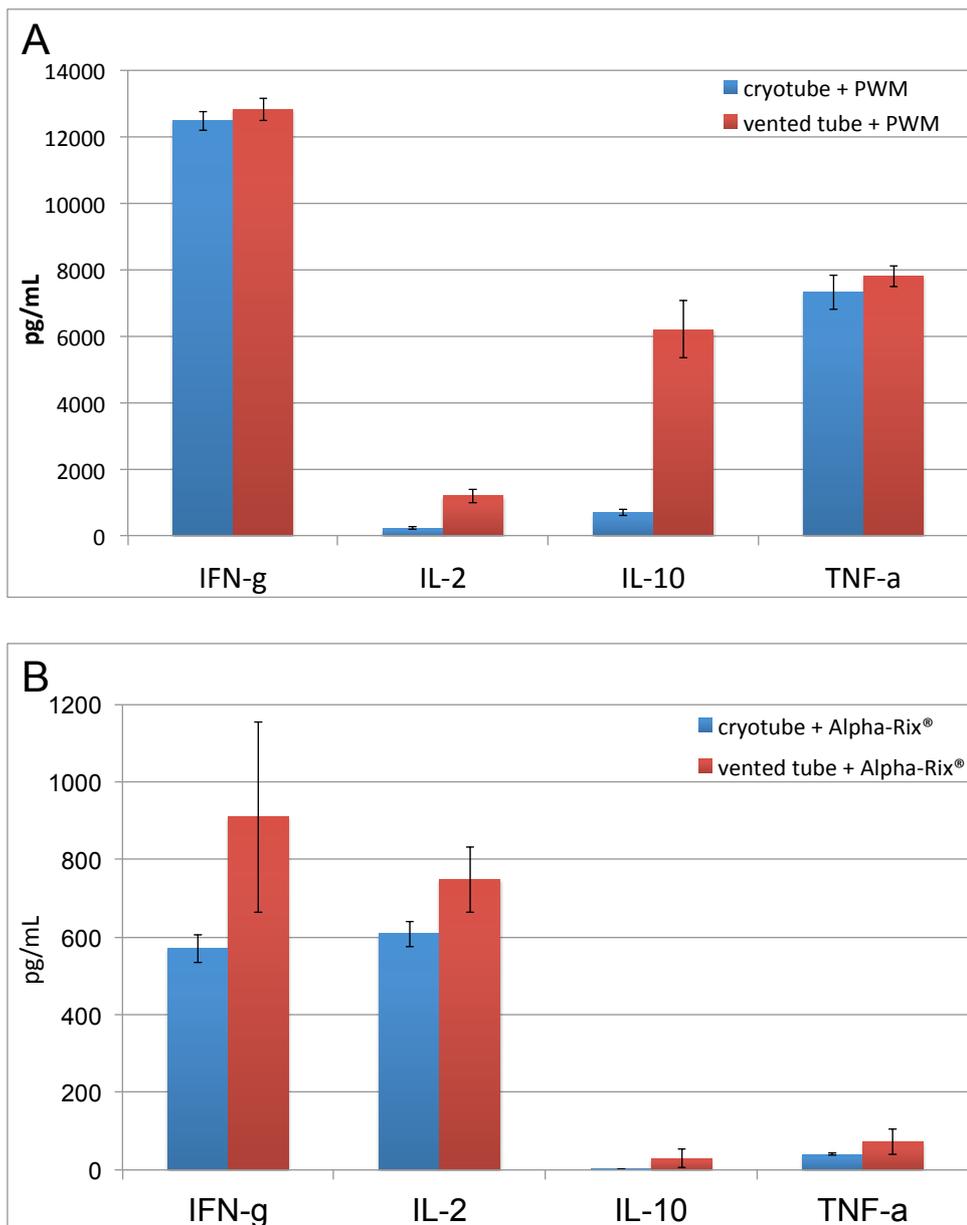
Figure 29. Cytokine levels measured in blood cultures after 48h challenge with PWM (A) or (B) Alpha-Rix® in sealed cryotubes vs. vented tubes. Standard deviations are indicated. Basal = negative control.

Hence, the data obtained - particularly for PWM - suggest an effect of container type on assay outcome. It is possible that incubation in sealed cryotubes without gas-exchange could have an effect on the pH and oxygen levels of the blood culture which in turn could affect cytokine expression. In order to confirm the data, the same samples were re-analysed using an IL-2 ELISA (data not shown). Unfortunately, the results of this assay were difficult to interpret: on one hand, a high background signal was observed, on the other hand the range of the standard was limited to 1,000 pg/mL. As a result, the signal obtained for most samples was either too low (high noise) or too high (signal saturation).

In addition, a test was performed to assess whether the lack of gas-exchange had an effect on the pH or the oxygen level of the blood culture. An unchallenged blood culture was incubated at +37°C in a cryotube or in a vented tube (Figure 30).
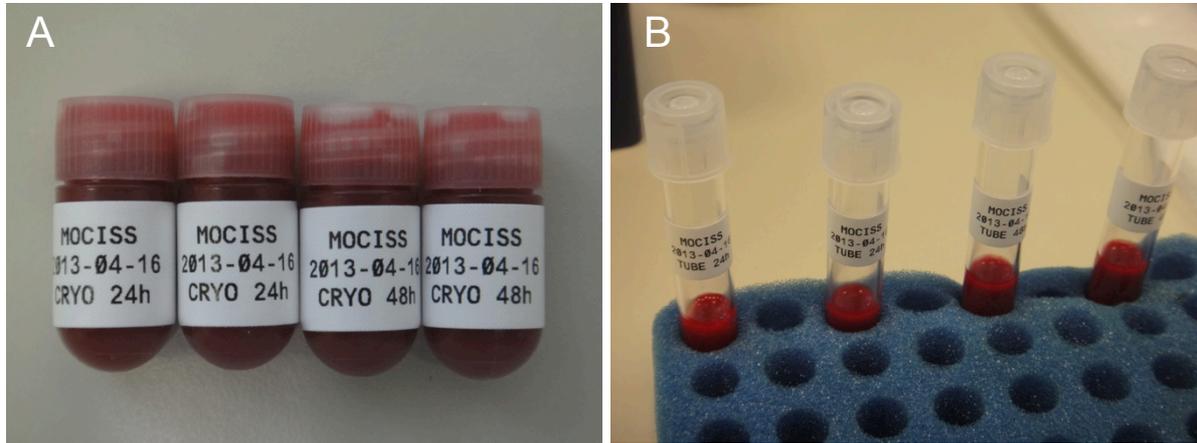

Figure 30. Picture of (A) sealed cryotubes and (B) vented tubes containing blood culture (whole blood + RPMI) for assessment of pH and oxygen levels after up to 48h incubation.

Samples were collected after 24h and 48h incubation followed by pH and oxygen level measurement. The results are summarized in Table 3.

Table 3. pH and oxygen levels measured for blood cultures incubated at +37°C in sealed vs. vented tubes. Values shown are average of 2 samples except those marked with asterix (single measurement).

container	sample	time	volunteer 1		volunteer 2 (test 1)		volunteer 2 (test 2)	
			pH	oxygen (mg/mL)	pH	oxygen (mg/mL)	pH	oxygen (mg/mL)
start material	RPMI	0h	8.05*	8.60*	N.D.	N.D.	N.D.	N.D.
	whole blood		7.50*	N.D.	7.64*	1.29*	N.D.	N.D.
	whole blood+RPMI		7.60*	N.D.	7.62*	1.13*	7.61*	N.D.
cryotube	whole blood+RPMI	24h	7.21	1.91	7.17	1.00	7.05	N.D.
	whole blood+RPMI	48h	7.13	1.02	7.17	0.62	6.92	N.D.
vented tube	whole blood+RPMI	24h	7.34	1.02	7.27	0.90	7.22	N.D.
	whole blood+RPMI	48h	7.25	1.09	7.27	0.63	7.18	N.D.

N.D. = not determined; red = volunteer 1; blue = volunteer 2 (test1) ; green = volunteer 2 (test 2)

During incubation a drop in pH could be observed which was slightly more pronounced for the cryotube. Overall, the oxygen levels were low and remained constant with the exception of a higher value measured for the cryotube after 24h (outlier?). Furthermore, no difference in cell viability (trypan blue staining, refer to section 2.4) could be observed between the cryotube and the vented tube (data not shown).

3.3   Challenge of whole blood from different individuals with influenza antigens

The aim of the next experiment was to assess the effect of influenza antigens on the cytokine profile of blood cultures obtained from two individuals. Subject 1 was vaccinated against the flue less than 3 months ago (using the same antigens as included in the test, i.e. Alpha-Rix®) while subject 2 was not.

After incubating the blood cultures in cryotubes for 48h at +37°C with either 1% Alpha-Rix® or blank RPMI medium (negative control) the samples were centrifuged, and plasma was transferred to eppendorf tubes and subjected to cytokine analysis using the Luminex® technology.

A graphical representation of the 4 cytokine concentrations measured for the different samples is given in Figure 31. For all samples IL-10 levels were below the detection limit and therefore these values were omitted from the graph. The control sample incubated with blank medium did not show significant cytokine expression. However, for both individuals a challenge with Alpha-Rix® resulted in an increase in IL-2 and IFN-γ concentration and to a minor extent TNF-α. Interestingly, the IL-2 and IFN-γ concentrations for subject 1 were found to be two-fold higher compared to subject 2.
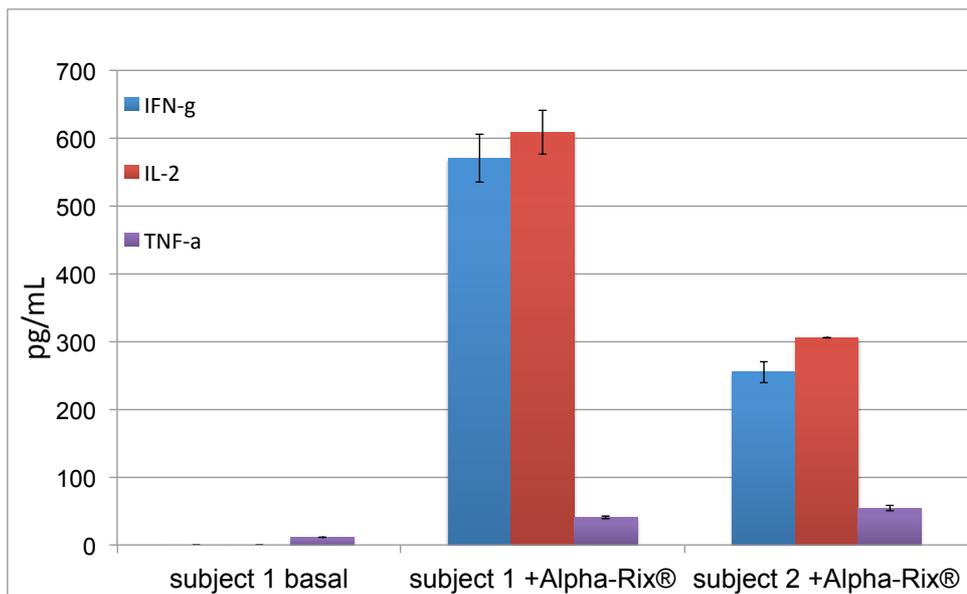

Figure 31. Cytokine levels measured in blood cultures from subject 1 and 2 after 48h challenge with Alpha-Rix®. Standard deviations are indicated. Basal = negative control.

3.4 Assessing effect of Alpha-Rix® storage conditions on cytokine response

Antigen mixtures used for challenging blood cultures are typically stored at +4°C or -20°C. However, for practical purposes aliquots of these products might be subjected to e.g. an additional freeze-thaw step and/or pre-dilution in culture medium.

In order to assess whether these additional handling steps could have an effect on the product's capability to trigger cytokine expression *ex vivo*, blood cultures in cryotubes were challenged with 1% Alpha-Rix® mixtures prepared from:
- a stock solution kept at +4°C (control, as prescribed by manufacturer)
- a stock solution kept at -20°C (i.e. additional freeze-thaw step)
- a predilution step in RPMI (1:50) followed by storage at +4°C
- a predilution step in RPMI (1:50) followed by storage at -20°C

After 48h incubation in sealed cryotubes the blood cultures were centrifuged and IL-2 plasma concentrations were determined using the ELISA-based assay. As the ELISA rendered a significant background signal (absorbance around 1) resulting in a limited dynamic range, it was not possible to calculate absolute IL-2 levels. Hence, the assay could only be interpreted in a qualitative manner by comparing relative absorbance values (Figure 32). All conditions rendered a similar signal response indicating that the handlings performed did not have a significant effect on Alpha-Rix® quality.
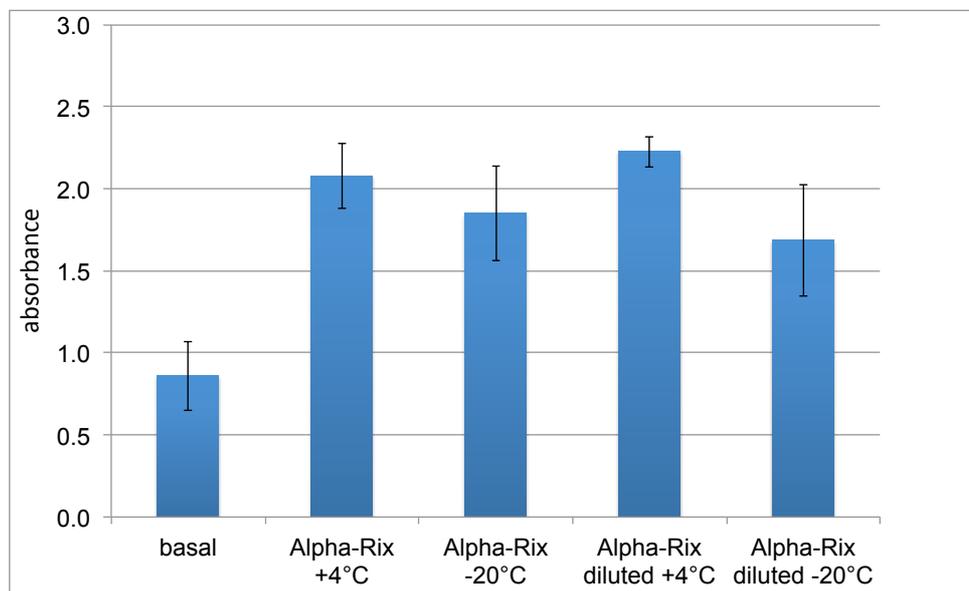

Figure 32. IL-2 absorbance levels measured in blood cultures after 48h challenge with Alpha-Rix®. Standard deviations are indicated. Basal = negative control.

3.5 Assessing cellular immunity under space-simulated conditions: effect of simulated microgravity on cytokine response in sealed containers after challenge with antigens/mitogen

The next set of experiments was aimed at evaluating the effect of simulated microgravity on the cytokine expression profile of challenged blood cultures.

The experiment included two different stimuli, i.e. PWM and Alpha-Rix® (1%). Blood cultures were prepared, transferred to cryotubes and mounted onto the centre plate of the RPM (placed in the incubator). Control samples were placed next to the RPM for the duration of the treatment (48h).

After incubation, plasma was obtained from all samples by centrifugation and cytokine levels were determined using the Luminex® assay (Figure 33 and Figure 34).

Samples challenged with Alpha-Rix® (Figure 34A) that were exposed to simulated microgravity (using the RPM) showed an 8-fold decrease in IFN-γ concentration (from 570 pg/mL to 70 pg/mL), a 6-fold decrease in IL-2 concentration (from 610 pg/mL to 110 pg/mL) and a 2-fold decrease in TNF-α levels (from 40 pg/mL to 20 pg/mL). These results clearly indicate that the cellular immune response was compromised.

In contrast, rather contradicting results were obtained for the blood cultures incubated with PWM (Figure 34B). Simulated microgravity resulted in a drop in IL-2 concentration, although this decrease was less pronounced compared to Alpha-Rix® challenge (from 220 pg/mL to 100 pg/mL). Furthermore, the IL-10 concentration decreased from 690 pg/mL to zero. These data indeed demonstrate that microgravity prevented a proper cellular immune response. However, no change in IFN-γ or TNF-α concentration was observed when blood was stimulated with PWM under microgravity conditions. A possible explanation for this deviating result could be the fact that the original concentrations for these particular cytokines were beyond the quantitative range of the Luminex® assay and consequently were underestimated. This could explain why no drop was observed after RPM treatment.

Figure 33. Cytokines level measured in blood cultures after 48h challenge with Alpha-Rix® or PWM in (A) control and (B) simulated microgravity conditions. Standard deviations are indicated. Basal = negative control.
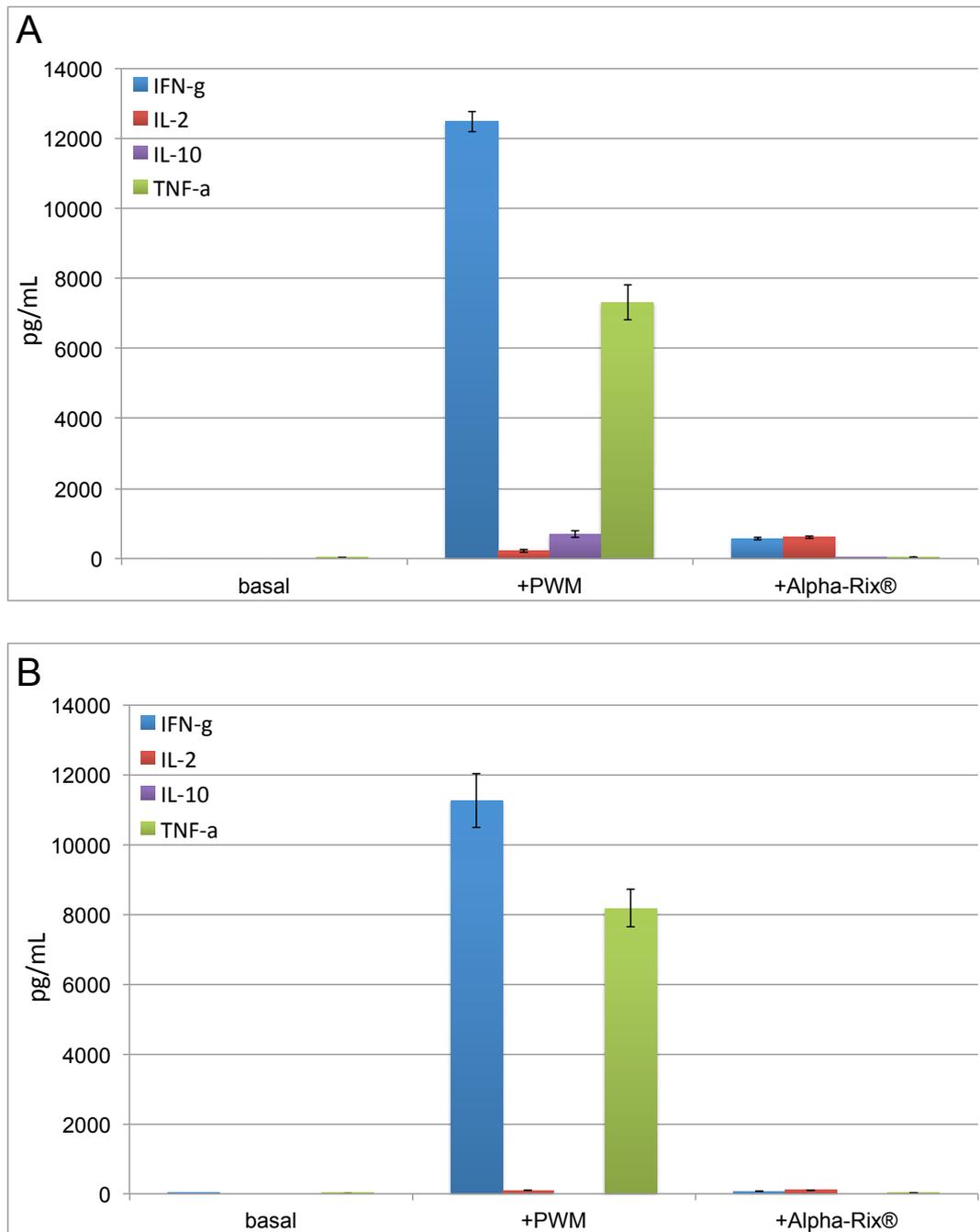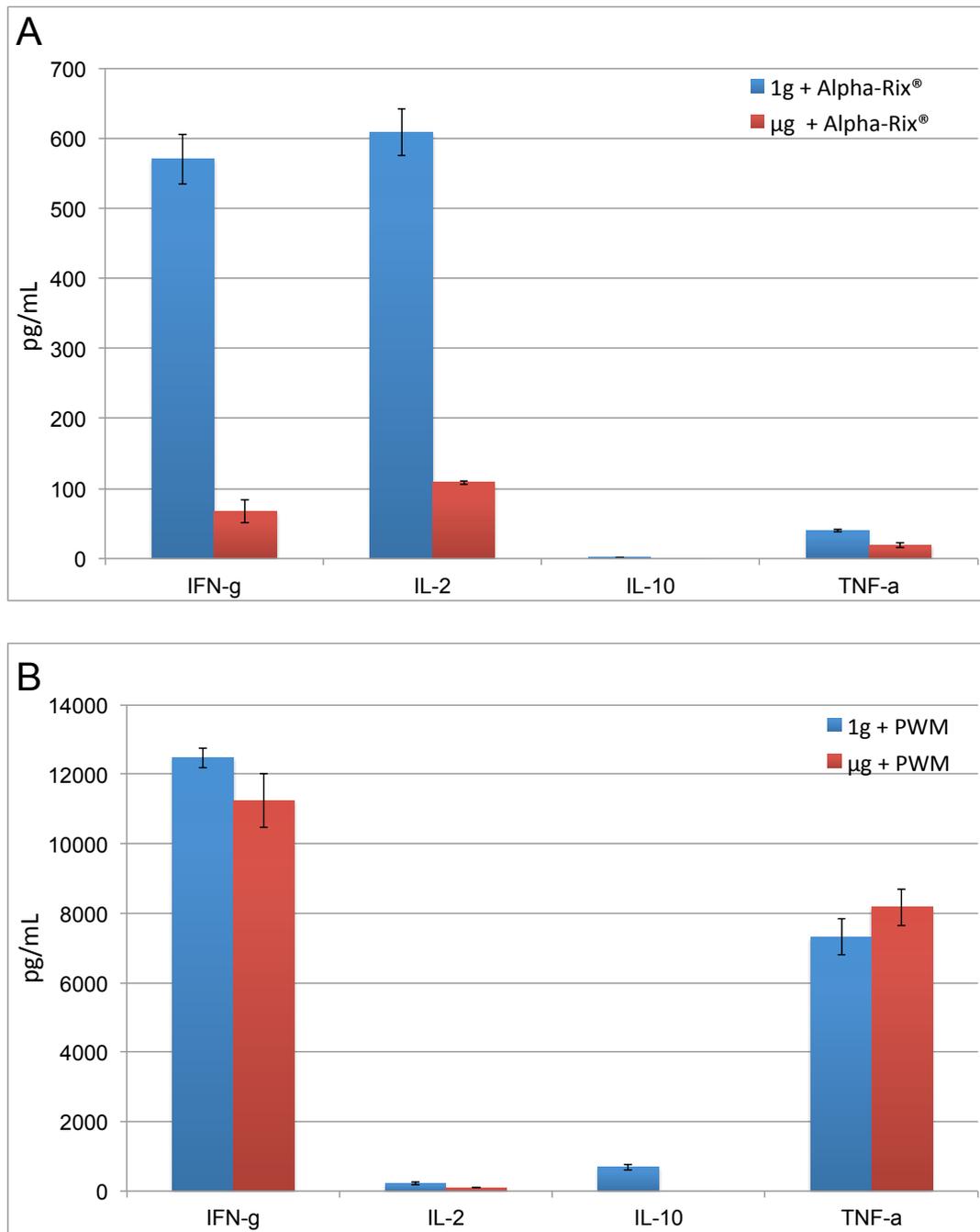
Figure 34. Cytokine levels measured in blood cultures after 48h challenge with (A) Alpha-Rix® or (B) PWM in control and simulated microgravity conditions. Standard deviations are indicated. Basal = negative control.

3.6 Technical challenge: evaluating different containers allowing gas-exchange

As described in previous experiments, different cytokine expression profiles were observed between blood cultures kept in sealed or vented containers. As gas-exchange is known to be beneficial for cell survival - e.g. by maintaining pH and oxygen levels during long incubation times - it was decided to further investigate possible alternatives to the sealed cryotubes for our simulated microgravity experiments. These containers should be low in volume (<10 mL) to limit the total amount of blood needed for testing, while equipped with a cap that allows gas-exchange without causing the formation of major air bubbles in a 48h time frame. In order to avoid an extensive requirement of blood, several containers were evaluated using blank RPMI medium. All containers were filled completely with medium without introducing air bubbles (Figure 35, A-D) and incubated at +37°C for 48h.
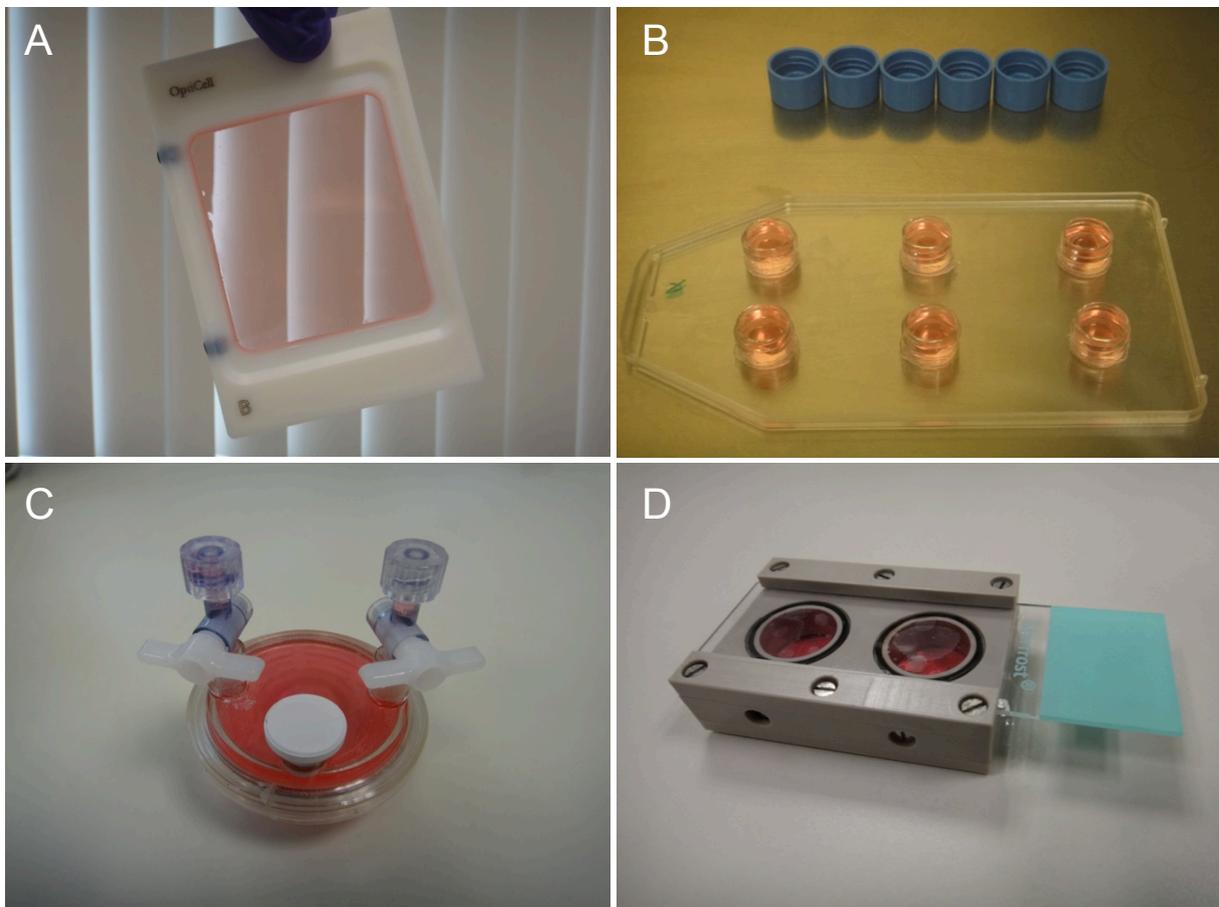

Figure 35. Overview of different containers evaluated for 48h incubation at +37°C; (A) Opticell 1100 culture system (10 mL), (B) self-made miniaturised cell culture flask with vented cap (5 mL), (C) rotating wall vessel (10 mL) and (D) SCK•CEN module with biofilm membrane (2 mL).

Although no issues were observed in terms of sample recovery for any of the containers tested (full recovery possible), all of them showed air bubbles after incubation (Figure 36).
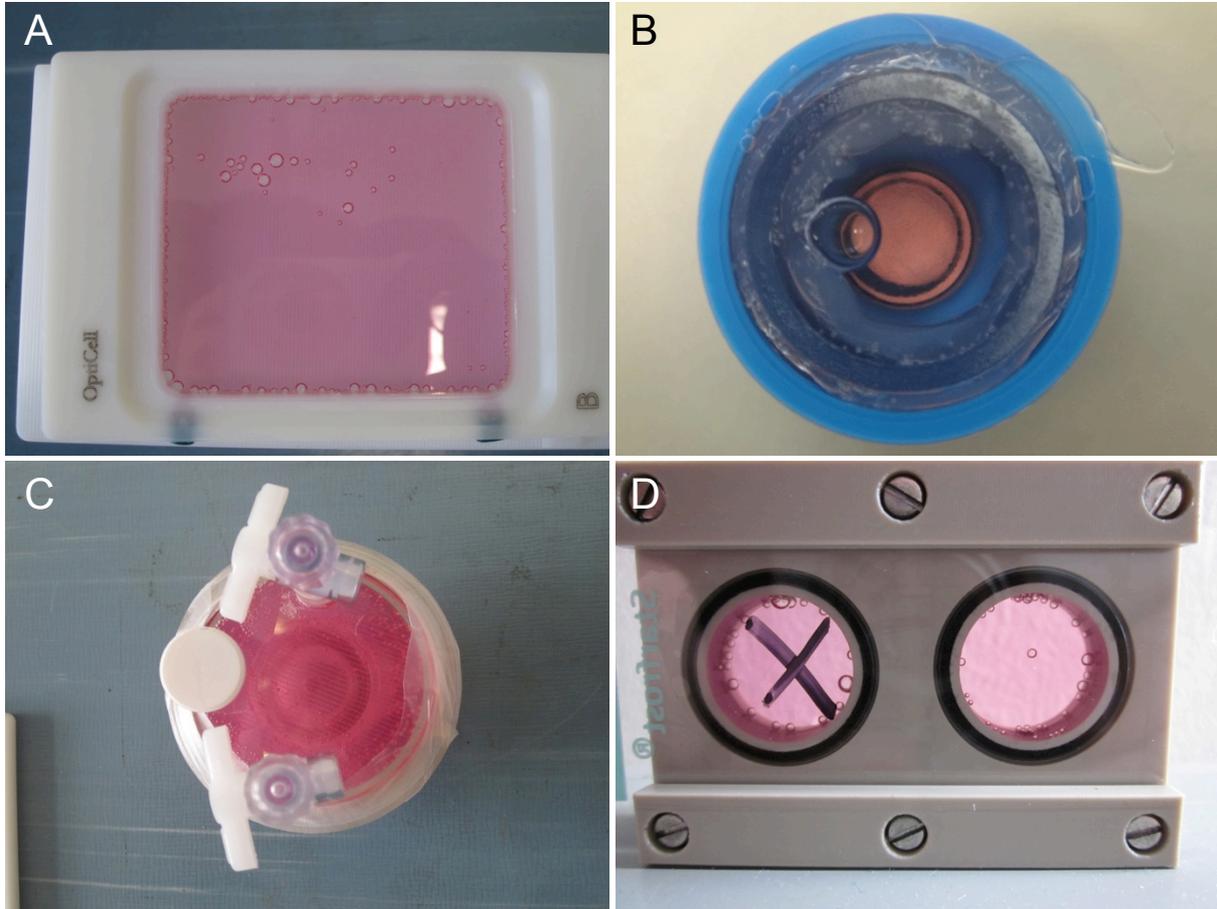
Figure 36. Overview of different containers after 48h incubation at +37°C; (A) Opticell 1100 culture system (10 mL), (B) self-made miniaturised cell culture flask with vented cap (5 mL, bottom view), (C) rotating wall vessel (10 mL) and (D) SCK•CEN module with biofilm membrane (2 mL).

The accumulation of air bubbles in the Opticell system (Figure 36A) and the RWV (Figure 36B) can most likely be attributed to the relatively large surface. The bubbles observed in the self-made miniaturized cell culture (Figure 36C) were probably the result of improper container sealing due to an incompatibility between the original cap and the dimensions of the modified flask neck. Since the original culture flasks were successfully used in previous long-term incubation experiments involving cell cultures, an attempt was made to limit the flask volume by filling it with inert, bio-compatible silicone (Soudal Aquatium Sillicone, 1474). The smallest flask available (total volume 33 mL) was filled with about 28 mL of silicone, rendering a container with an available volume of about 5 mL (Figure 37).
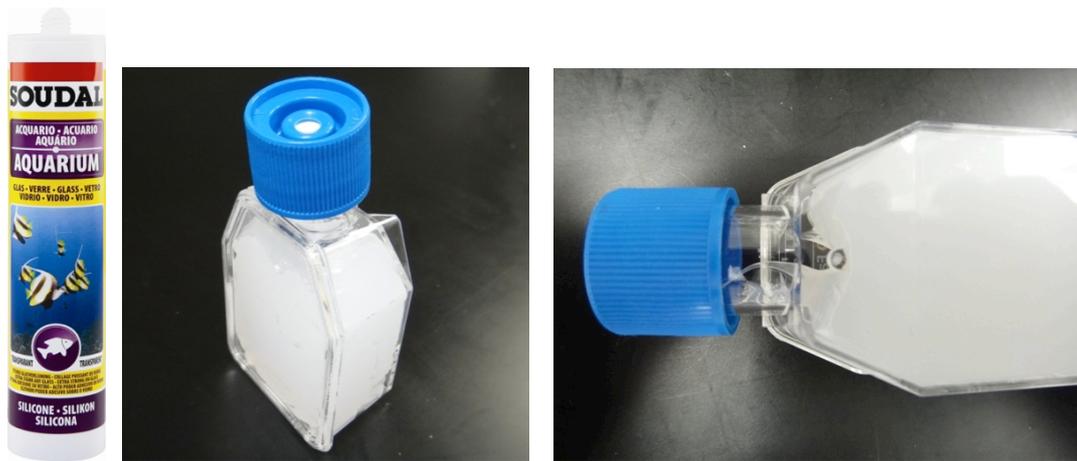
Figure 37. PS 12.5 cm² Falcon™ cell culture flask with vented cap filled with biocompatible silicone to obtain a 5 mL container with 5 mL

However, the downside of this approach is that the silicone adds significant weight to the container, hereby limiting the total number of units that can be loaded onto an RPM and the fact that the silicone was found to be incompatible with autoclaving. Also, it is not clear whether the silicone and acids released have a (toxic) effect on cell cultures. Finally, the in-house design module (Figure 36D) was found most promising in terms of total volume. However, also here there was an issue with air bubbles. Whether this is related to the type of biofilm used requires further investigation.

Chapter 4

Discussion & Perspectives

4 Discussion & Perspectives

The following section will further discuss the results obtained during this master thesis. The most important findings as well as issues observed will be highlighted and suggestions for future experiments will be given.

4.1 Implementation of *in vitro* DTH assay

A first set of experiments focused on confirming the results of the *in vitro* DTH test described by Feuerecker *et al*. (2013a). This assay makes it possible to assess the cellular immune response of an individual *ex vivo* against different pathogens by incubation with a panel of specific stimuli/recall antigens followed by quantification of the cytokines of interest (in this case IFN-γ, IL-2, IL-10 and TNF-α). A first test was set up using a similar type container as the one used by Feuerecker (equipped with ventilation cap) and included two stimuli, i.e. PWM as a positive control and the influenza vaccine Alpha-Rix®. The latter is a viral antigen mixture that is known to initiate an adaptive immune response (Moltedo *et al.*, 2009) and is similar to the Influvac® antigen (Solvay, Hannover, Germany) that is one of the three antigens present in the viral antigen mixture used by Feuerecker. A comparison of the cytokine levels detected by Feuerecker and at SCK•CEN is given in Table 4.

Table 4. Concentration of pro- and anti-inflammatory cytokines 48h after challenge with PWM or influenza antigens as determined by Feuerecker *et al*. (2013a) (average values).

cytokine level	basal (no challenge)		PWM (positive control)		viral antigens	
	Feuerecker (pg/mL)	SCK•CEN (pg/mL)	Feuerecker (pg/mL)	SCK•CEN (pg/mL)	Feuerecker (pg/mL)	SCK•CEN (pg/mL)
IFN-γ	5.98 (1.23)	0.00 (0.00)	6093.11 (1910.24)	12813.07 (329.81)	165.83 (108.43)	909.67 (246.19)
IL-2	0.18 (0.17)	0.00 (0.00)	3718.38 (2005.80)	1197.75 (210.63)	47.58 (12.63)	748.38 (84.95)
TNF-α	5.51 (0.95)	12.58 (0.23)	4123.07 (1472.80)	7803.33 (315.90)	55.96 (14.63)	72.38 (32.86)
IL-10	N.D.	0.00 (0.00)	N.D.	6195.93 (867.04)	N.D.	29.47 (23.05)

standard error is indicated between brackets
N.D. = not determined

At both sites basal cytokine levels were low, indicating that the blood donors were healthy - lacking an active adaptive immune response at the time of testing - and the assay has a low background signal, hereby allowing the detection of subtle increments in cytokine levels. Challenging the blood with PWM rendered a dramatic increase in IFN-γ, IL-2 and TNF-α levels which is in line with previous studies performed with PBMCs (Miller *et al*., 1991; Katial *et al*., 1998). PWM is known to promote the proliferation of and to stimulate B and T lymphocytes which would explain the increased levels of IL-2. The reason why PWM challenge causes the cells to produce different levels of cytokines is not clear. The initial increase in IL-2 levels could stimulate different PBMC subpopulations in a non-specific manner triggering a cytokine cascade. Some differences in cytokine ratios could be observed between the two test sites. These can probably be explained by the fact that blood was taken from different individuals (different genetic background). Also, the health status of the test person could affect the extent of the immune response and resulting cytokine levels.

The major difference between both sites was observed after challenge with influenza antigens. At SCK•CEN the increase in IFN-γ and IL-2 concentrations was higher compared to Feuerecker. It is important to note that here the vaccination history of the volunteer probably had a major effect on the outcome. The subject at SCK•CEN was vaccinated against influenza 3 months earlier (with Alpha-Rix®) which would explain the relatively strong cellular immune response towards these recall antigens. Furthermore, the antigen mixture used by Feuerecker also included EBV and CMV lysates which could have an attenuating effect on cytokine expression, e.g. by promoting a Th2 response.

The next experiment focused on performing the *in vitro* DTH test in an alternative (sealed) container that could be used for microgravity experiments, i.e. the cryotube. Using blood from the same donor a test was performed with PWM as a positive control and Alpha-Rix®. Hence, the data obtained could be compared head-to-head with that of the first experiment.

Interestingly, a similar response was observed for Alpha-Rix® in the cryotube although the cytokine concentrations were somewhat less compared to those for the vented tube. When challenging the blood with PWM however, lower IL-2 and IL-10 concentrations were detected. One possible explanation might be that in the sealed containers, changes in pH and oxygen can occur (e.g. after 48h the environment in the cryotubes can become hypoxic). To further investigate this, pH and oxygen levels in sealed and vented tubes were monitored. Although the outcome of this test was inconclusive in terms of oxygen levels, previous studies have shown that hypoxia can lead to an impaired activation of T lymphocytes (Otha *et al.*, 2011) and reduced expression of certain cytokines (e.g. IL-2, described by Zuckerberg *et al.*, 1994). Hence, the outcome of this test (oxygen/pH dependent secretion of cytokines) highlighted the importance of selecting the proper container for future *in vitro* DTH assays.

Given the differences observed between the blood cultures incubated in cryotubes and vented tubes, and the fact that some effect on pH could be demonstrated, a follow-up experiment was performed. For sake of comparison, the experiment focused on challenging blood cultures in cryotubes with the same panel of stimuli included in Feuerecker's test. To achieve this, pre-diluted aliquots of DMEM containing the different stimuli were provided by the Department of Anaesthesiology of Klinikum Großhadern (University of Munich, Munich), i.e.:

- Diphteria, tetanus & pertussis toxoid (Boostrix®)
- Cytomegalovirus (CMV) lysate
- Epstein-Barr virus (EBV) lysate
- Influvac®
- Candida and Trichophyton lysate
- Pokeweed mitogen (PWM)

The respective aliquots were mixed with whole blood (1:1), transferred to cryotubes (Figure 38) and incubated for 48h at +37°C. The analysis of these samples will be performed in the near future.

Figure 38. Picture of sealed cryotubes containing a blood culture (whole blood + DMEM) with different antigens/mitogens. Blank DMEM = negative control.

4.2 Monitoring effect of simulated microgravity on DTH cytokine profile

Although some differences could be observed between the cytokine profiles obtained in the vented tubes and the cryotubes, the DTH assay was able to successfully detect a cellular immune response in the sealed container. Furthermore, a test involving blood taken from 2 individuals could point out the most recently vaccinated person based on final cytokine levels, demonstrating that the discriminatory power of the assay was not compromised in cryotubes. Finally, cryotubes were found relatively easy to handle for RPM experiments (small size, reliable closing mechanism) particularly when trying to avoid the presence of air bubbles throughout the experiment.

Therefore, the use of cryotubes was extended to simulated microgravity experiments using the RPM. A first experiment clearly showed that the concentration of the 4 cytokines dramatically decreased in response to a viral challenge (Alpha-Rix®), i.e. in accordance with previously reported studies where a Th2 shift was observed (Baqai *et al.*, 2009; Crucian *et al.*, 2008; Gridley *et al*, 2009; Girardi *et al.*, 2012; Crucian *et al.*, 2013). In the case of PWM, this reduced immune response was only observed for IL-2 and IL-10, while IFN-γ and TNF-α levels were not affected. The cause for this selective reduction in cytokines remains unknown. In correspondence with the follow-up experiment performed for cryotubes earlier, the same blood cultures were challenged with Feuerecker's stimuli (cfr. section 4.1) while being subjected to simulated microgravity (Figure 39). Again, the results are expected as soon as new Luminex® reagents are available.
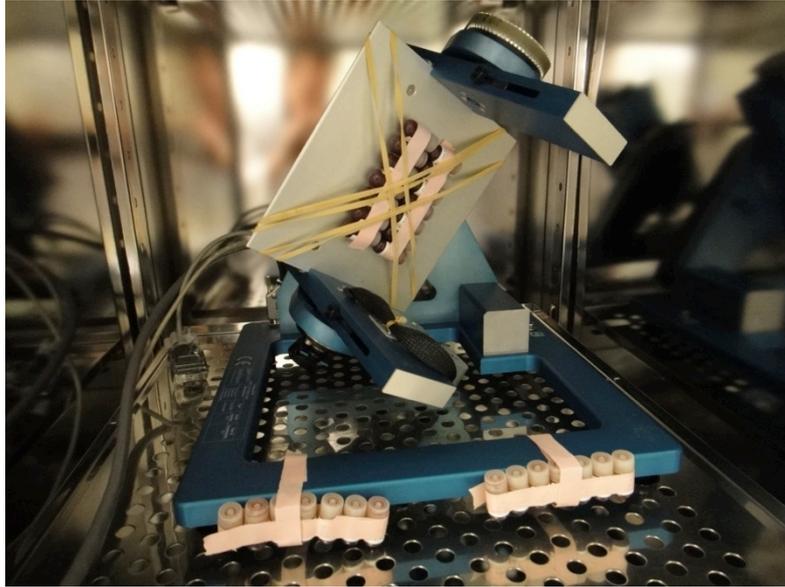
Figure 39. Picture of sealed cryotubes containing a blood culture (whole blood + DMEM) with different antigens/mitogens on the RPM. Blank DMEM = negative control. RPM control samples were mounted on the side of the device.

4.3 Comparison of Luminex® and ELISA based assay

One of the original goals of the current thesis was to systematically compare data generated with Luminex® technology with data obtained using an ELISA based assay. However, given the limited time available for experimental testing, the ELISA assay could only be performed once.

Unfortunately, the assay showed several unexpected results The major problem was the relatively high background signal observed in the blank wells which interfered with the setup of the calibration curve. As a result, the assay was not able to quantify cytokine levels accurately in the lower range of the standard curve and the overall dynamic range of the assay was limited (up to 1000 pg/mL). Hence, it was difficult to interpret the results and perform a direct comparison with the Luminex® data. The root cause for this background remains to be identified. It is possible that incomplete blocking of the plate wells prior to sample incubation was responsible for this result. However, as this problem was also observed in the blank wells and the standard included in the kit, the background can most likely be attributed to a problem with a specific reagent. In addition, replicate analysis occasionally showed high variations. Here, it could not be excluded that RPMI present in the samples had an effect on assay efficiency. This could be further investigated by including blank samples and standards prepared in RPMI in the future.

None of the issues mentioned for the ELISA were observed during Luminex® analyses. However, it should be noted that SCK•CEN has much more experience with this technology than with the ELISA. The major advantage of the Luminex® assay is that is able to measure multiple cytokines in a single sample, hereby nulling out possible matrix effects and rendering highly accuracy measurements. The ELISA measurements are more variable by nature (one measurement per well, plate effects, pipetting errors, etc.). Hence, in order to improve the performance of the ELISA the number of replicates would have to be increased dramatically. This in turn will limit the total amount of samples that can be analysed per plate and will come with an increased cost. Also, it is important to bear in mind that the ELISA is only capable of measuring one cytokine at a time. Assessing multiple cytokines would again increase the workload and cost of the analysis.

In conclusion, the results we have obtained so far favor the use of the Luminex® technology in future experiments as high-throughput, highly accurate and multi-cytokine screening assay.

4.4   Perspectives

The experiments performed in the current thesis - albeit limited - clearly indicate the potential of the *in vitro* DTH test for monitoring cellular immunity under simulated space conditions and eventually onboard the ISS (cfr. MoCISS project). Additional tests involving a diverse array of stimuli (bacterial, fungal and viral) will have to be performed with blood taken from several individuals to fully grasp the assay's potential. For these experiments it will be important to have a clear view on the vaccination history and health status of the test subjects in order to correctly interpret the outcome of the assay. Furthermore, one has to keep in mind that the assay will only be able to detect a response in case the test subject has been previously exposed to the challenge antigen earlier (recall antigen). Although this will be the case for most bacterial and viral antigens as a result of worldwide vaccination campaigns (e.g. tetanus) or natural exposure (e.g. EBV, CMV), the magnitude of the response to certain antigens can be quite different depending on the timing of vaccination (e.g. influenza) or first exposure. Hence, future work will include optimizing the antigens in question to customize them to the subject's need. Depending on its application the assay could also be extended to include other types of stimuli, such as cancer cells that can trigger both an innate or adaptive immune response (Rizvi *et al.*, 2011; Vesely *et al.*, 2011).

Further work on the assay would also include focusing on the kinetics of the cytokines of interest to determine optimal incubation times depending on the scope of the assay. A different onset of cytokine secretion has already been demonstrated by Feuerecker *et al.* (2013a): e.g. during incubation the IL-2 levels in the culture supernatant increased significantly after 24h and even more after 48h (i.e. strong, time-dependent Th1 response) while peak concentrations for TNF-α were already reported at <12h. An additional experiment to investigate the kinetics of the cytokine response in both sealed and vented containers was performed (i.e. by 24h to 48h incubation with PWM). Unfortunately, due to time restrictions these samples were not yet subjected to Luminex® analysis. The samples are currently being stored at -80°C and their analysis is planned.

Finally, it will be important to standardize the entire assay in order to allow comparison of data throughout different missions and making it simple to use and readily accessible to the astronauts. The reagents of the assay will require further investigation to ensure assay robustness (e.g. as was demonstrated by evaluating the effect of sample handlings on Alpha-Rix®).

So far, the show-stopper in the experimental work is finding the appropriate container that on one hand allows adequate gas-exchange and on the other hand limits air bubble formation for simulated microgravity experiments. Experiments still ongoing at SCK•CEN focus on improving the design of the in-house module, for instance by optimizing the seals and including a compatible biofilm surface. The final design of the module planned for the MoCISS experiments onboard the ISS has not yet been defined (hardware is currently being developed by ESA). However, it will have to be small (< 1 mL), equipped with a membrane to allow gas exchange and make it possible for the astronauts to draw blood and transfer it into the proper compartments for incubation without introducing air bubbles. This could for instance be achieved by using a pressurized tubing system equipped with channel ports. In addition, the experiment will have to include a centrifuge to exclude the effect of microgravity (KUBIK centrifuge onboard the ISS).

The space conditions simulated in the experiments described in this thesis were limited to microgravity. As soon as the appropriate container has been selected and the assay is functioning accordingly, tests will include other simulated space stressors, such as radiation (combination of high- and low-LET radiation) and psychological stress.

Simulating space radiation in an Earth-based model is not straightforward. Obtaining an environment that mimics cosmic radiation will require adequate radiation sources capable of exposing blood cultures to a combination of low and high LETs. A low chronic dose could be achieved by combined exposure to ^{137}Cs (mainly γ-rays, low-LET) and ^{252}Cf (mainly neutrons, high-LET). By optimizing the distance between the sources and the incubator a total dose comparable to a stay of several months onboard the ISS can applied within 48h (e.g. 56 mSv dose = long duration spaceflight of 3.5 months, Ghardi *et al.*, 2012). However, the major challenge will be to accurately determine the total dose for each sample during incubation. Since all samples will have to be stored in an incubator (+37°C), neutron radiation will lead to secondary particles and the ultimate dose will be difficult to determine. This problem could be solved to some extent by performing dry runs with dosimeters implemented in the respective containers. Future experiments could also include alternative types of cosmic radiation, such as heavy ions - which can be generated at the GSI (Gesellschaft für Schwerionenforschung Helmholtz Centre for Heavy Ion Research, Darmstadt, Germany) or GANIL (Grand Accélérateur National d'Ions Lourds, Caen, France).

Psychological stress - which can impair the overall functioning of the immune system - can be mimicked by adding increasing amounts of hydrocortisone (analog of cortisol) to an *in vitro* blood culture during incubation (Feuerecker *et al.*, 2013b). Cortisol is a steroid hormone produced by the adrenal glands in response to stress. It releases glucose into our bloodstream and increases blood pressure for increased physical activity and increased awareness (i.e. fight or flight response triggered by our sympathetic nervous system). Furthermore, the activity of our immune system is lowered to save energy for this physical

activity. Therefore, in future experiments involving cortisol, cortisol blood or saliva concentrations of the donors should be measured at the start of each experiment to determine "baseline" conditions.

The ultimate goal will be to study the effect of multiple space stressors combined and to investigate whether these work synergistically or not. The outcome of these experiments will be important for long-duration missions where in addition to microgravity, radiation exposure and psychological stress become more important (Manti, 2006; Baatout *et al.,* 2012; Moreels *et al.*, 2012a; Moreels *et al.*, 2012b).

Taking these suggestions into account, it should however be noted that Earth-based models only partially mimic actual space conditions. For instance, radiation experiments on Earth will never include the high-energy GCRs to which an astronaut will be subjected beyond LEO. Furthermore, the use of an RPM as a microgravity simulator for biological samples comes with several limitations. First of all, microgravity simulated by an RPM differs from the weightlessness experienced by astronauts onboard the ISS. While microgravity conditions at the ISS are of the order of 10^{-6} g, the RPM can only deliver up to 10^{-2} g. Second, microgravity conditions can only be achieved near the centre of the device. As a result, extensive RPM experiments require miniaturized sample containers (e.g. cryotubes, well-plates). Also, given the limited lifetime of cell cultures, RPM experiments are limited to several days or microgravity conditions have to be interrupted to refresh culture media.
Another example is the use of hormone analogs to mimic psychological stress *in vitro*. Including hydrocortisone in the assay allows for a fairly simple simulation of stress. However, we have to realise that cortisol represents only one parameter in the complex pathway induced by stress. Cortisol production in turn is regulated by a cascade of hormones (corticotropin-releasing hormone released by the brain and adrenocorticotropic hormone released by the pituitary gland). The events occurring during *in vivo* stress are highly complex, involving several stress hormones acting rather quickly on different body systems such as adrenaline and noradrenaline as well as those that can have on how a stress reaction evolves, e.g. estrogen, testosterone, dopamine and serotonin.

Conclusion

Conclusion

The current thesis was part of the preparatory phase of ESA's MoCISS study to allow *ex vivo* monitoring of the cellular immunity of astronauts onboard the ISS. Several exploratory experiments were performed to confirm functioning of an *in vitro* DTH test in both vented and sealed containers. For the latter system, some differences in cytokine response were observed compared to the vented tube and which requires further investigation. In addition, the negative effect of simulated microgravity on the cellular immune response to recall antigens was observed which shows the detrimental effect of the lack of gravity on the proper functioning of our immune system. Due to lack of time, the cytokine levels of several sample sets could not yet be determined. These analyses will be performed at SCK•CEN in the upcoming months. Future work will also focus on further evaluating different container types - to allow adequate gas-exchange in microgravity experiments - and optimising the *in vitro* DTH protocol. This cellular response will have to be assessed for a larger subject population and for a broad panel of stimuli. Findings obtained using whole blood can also be confirmed on purified PBMCs. A final set of experiments will include additional space stressors such as radiation or psychological stress as well as combinations of these to detect potential synergistic effects. All these experiments (and more) will be part of a PhD thesis which is expected to start Q3 of 2013.

Once the *in vitro* DTH test has been fully optimised and its potential onboard the ISS and future space missions has been demonstrated, the assay could also function as an important tool during astronaut selections (e.g. to evaluate the susceptibility of the candidate's immune system to space stressors). Obviously, it would be useful to detect a potential sensitivity to e.g. space radiation prior to subjecting the candidate to actual space travel. The assay could also prove to be useful for other applications on Earth, such as a screening tool to assess and follow up immune deficiencies in patients with a compromised resistance to infections, inherited and acquired immuno-deficiencies, trauma and burns (Kaufmann *et al.*, 2013). Finally, the assay can be applied to study changes in gene expression, elucidate underlying molecular pathways involved in the cellular immune response, identify key targets (biomarkers) of the immune system (e.g. to relieve people suffering from autoimmune diseases such as arthritis) and allow evaluation of the efficacy of certain countermeasures and therapies.

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