B-cell homeostasis is maintained during long duration spaceflight

Authors

Guillaume Spielmann, PhD.[1], Nadia Agha[2], Hawley Kunz[2,3], Richard J. Simpson[2,4], Brian Crucian[5], Satish Mehta[5], Mitzi Laughlin[2,6] and John Campbell[1,7].

Affiliation:

[1]School of Kinesiology, Louisiana State University, Baton Rouge, Louisiana, USA.
[2]Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Texas, USA.
[3]Division of Endocrinology and Metabolism, Mayo Clinic, College of Medicine, Rochester, Minnesota, USA.
[4]Department of Nutritional Sciences, Department of Pediatrics, Department of Immunobiology, The University of Arizona, Tucson, Arizona, USA.
[5]NASA-Johnson Space Center, Houston, Texas, USA.
[6] Fondren Orthopedic Research Institute, Fondren Orthopedic Group, Houston, Texas, USA
[7]Department for Health, University of Bath, Bath, UK.

Corresponding Author: Dr Guillaume Spielmann BSc, Ms, PhD, School of Kinesiology, Louisiana State University, Baton Rouge, Louisiana, USA, gspielmann@lsu.edu

Funding Sources:

This study was funded by a NASA Omnibus Grant NNX17AB16G to GS, a NASA Grant SMO 015 to BC and a NASA Grant NNX12AB48G to RS

Conflict of interest disclosure:

JC reports shares in Abingdon Health. Other authors report no conflicts of interest.

Abstract

Long duration spaceflights reportedly induce immune dysregulation, which is considered a risk to astronaut safety and mission success. Recent studies have examined the impact of spaceflight on markers of adaptive and innate immunity, but no study to date has comprehensively evaluated humoral immunity and serological markers of B-cell function. The aim of this study was to characterize changes in B-cell numbers and phenotypes, along with plasma immunoglobulins and polyclonal free light chains (FLC) – near 'real-time' biomarkers of immunoglobulin synthesis – in response to a ~6-month mission to the International Space Station (ISS). Whole blood samples were collected before flight, during ("Early flight", "Mid-flight" and "Late flight"), immediately upon return and during a recovery period (R+18, R+30/R+33 and R+60/R+66) from 23 ISS crewmembers. B-cell counts and phenotypes were measured throughout the duration of the mission, along with total plasma immunoglobulin (Ig) and FLC levels. There was no effect of spaceflight on the number and proportion of the different B-cell subsets. There was no difference in kappa FLC between pre-flight samples and either in-flight or recovery samples ($p>0.05$), and only a marginal reduction was observed in lambda FLC levels upon return to Earth ($p<0.05$). Furthermore, IgG and IgM remained unchanged during and after spaceflight, when compared to pre-flight values ($p>0.05$). Of note, plasma IgA concentrations were elevated *in-flight* when compared to baseline and recovery values ($p<0.05$). These results indicate that B-cell homeostasis is maintained during long duration spaceflight, advocating for potential *in-flight* vaccination as viable countermeasures against viral reactivation during exploration-class missions.

Keywords

Long-duration spaceflight; B-cell homeostasis; Free Light Chains; Immunoglobulins.

1. Introduction

Long duration spaceflights are associated with alterations to both innate and adaptive immunity (11, 24), including impaired cytokine response to antigenic stimuli (8, 49), NK cell cytotoxicity (46), neutrophil and monocyte oxidative burst capacity (30, 31), lymphocyte distribution and proliferative capacity (40). As exposure to pathogens (45) and rate of latent viral reactivations (35, 36) are known to increase in space, profound immune dysregulation would likely have great clinical and operational significance during perennial missions. Logistical constraints unique to spaceflight have compelled the majority of space immunology research to be collected using short-duration missions or by comparing pre to post-flight measures of immune function. While these studies provided valuable insights on the immune status of astronauts upon return on earth, the magnitude of immune alterations *during* spaceflight are less clear. Furthermore, few studies have attempted to comprehensively characterize B-cell and plasma cell homeostasis during orbital missions.

Effective humoral immunity is of fundamental importance to ensure adequate destruction of extracellular pathogens and the control of intracellular viral infections. As such, humoral immunity relies on the induction of different effector functions to antibody production, and any alteration in B-cell production, ability to differentiate, and in immunoglobulin output from plasma cells could lead to profound immune dysregulation and potentially endanger crew safety. The use of ground-based analogs to spaceflight, including hind-limb unloading, has highlighted significant reductions in B-cell progenitor cells in the bone marrow of mice which led to reduced B lymphopoiesis (32). Furthermore, short duration spaceflight missions have been shown to negatively impact cell phenotypes in the bone marrow (41) and splenic B-cell counts in mice (21), while simulated microgravity reduces hematopoietic stem cell proliferation rate (42). Although these changes appear to promote reductions in immunoglobulin output in animals following spaceflight (33), without affecting the breadth of the immunoglobulin repertoire (58), they may not translate to humans, as pre-flight immunoglobulin levels remain unchanged in cosmonauts following 6 months in the International Space Station (ISS) (46). Considering that it has been suggested for exploration crewmembers to receive *in-flight* vaccination against certain latent herpesviruses, in order to

maintain optimal protection throughout the duration of a ~3-year mission (13), optimal B-cell homeostasis is likely to be of paramount importance to preserve vaccine response and crewmember health. Unfortunately however, those studies failed to measure in-flight changes in B-cell phenotypes and immunoglobulin concentrations.

A limitation of measuring intact circulating immunoglobulins, such as total plasma IgG, IgA and IgM concentrations, is their slow clearance via cellular catabolism which confers them a relatively long biological half-life (1-3 weeks) (16). As this limits assessment of shorter term changes to Ig production, plasma Free Light Chains (FLC), with a short half-life of 2-6 hours (16), are conventionally used for the diagnosis, prognostication and monitoring of plasma cell dyscrasias (43, 44). In this context, plasma immunoglobulin FLC are considered to be a sensitive barometer of plasma cell activation and immune competency (28, 39). Immunoglobulin FLCs are produced by activated plasma cells during antibody synthesis, at a rate influenced by the magnitude of immune activation (28, 57). Elevated FLC levels are indicative of inflammation and have been associated with metabolic disorders and Type 2 Diabetes (4, 27), chronic low grade inflammation (6), myeloma (53), and mortality in the general population (17), while low levels identify immune suppression (28). Consequently, plasma immunoglobulin FLC are likely the ideal candidates to detect early immune-suppression in astronauts, and characterizing the effects of long duration spaceflight on plasma immunoglobulin FLC is of paramount importance.

The successful implementation of exploration-class missions to Mars or other near-Earth objects requires a better understanding of the impact of long-duration spaceflight on the immune system in order to evaluate the risks of crew adverse health events associated with immune dysregulations. The aim of this study was to assess the impact of long-duration spaceflight on B-cell and plasma cell homeostasis by comprehensively analyzing changes in B-cell number, phenotype and soluble markers of humoral function during a 6-month mission in the International Space Station.

2. Materials and Methods

2.1 Subjects and Study Design

Data for this study was collected from 2 independent NASA-funded studies: The "Integrated Immune" study conducted at NASA-JSC (PI: Crucian) and the "Salivary Markers" study conducted at the University of Houston (PI: Simpson). A total of 23 ISS crewmembers (15 from *Integrated Immune* and 8 from *Salivary Markers)* ranging in age from 37.0 to 57.0 years old (3 women, age 47.1 ± 5.6 years) were enrolled in this study. The ISS crew were affiliated with the National Aeronautics and Space Administration (NASA), European Space Agency (ESA), Japanese Aerospace Exploration Agency (JAXA) or Canadian Space Agency (CSA) and participated in a 6-month mission to the ISS. Data were collected over 18 separate ISS missions. Additionally, 6 ground-based controls (1 woman, age 33.0 ± 7.1 years) were enrolled in this study as part of *Salivary Markers* to ensure assay validity**.** The Committees for the Protection of Human Subjects (CPHS) at Louisiana State University, at the University of Houston and at NASA JSC approved the study, and informed consent was obtained from all subjects.

2.2 Sample Collection

For both studies, plasma samples were collected in lithium heparin tubes and whole blood samples were collected in Acid-Citrate Dextrose (ACD) tubes for cellular phenotyping during the *Salivary Markers* study (BD Vacutainers, Franklin Lakes, NJ, USA) before, during and after the 6-month mission in the ISS.

Specifically, plasma samples from *Integrated Immune* were collected at L-180 (180 days before launch), L-45, FD-10 (10 days after launch - "Early"), FD-90 ("Mid"), FD-180/R-1 (last day in space, "Late"), R+0 (Upon return on earth) and R+30 from 15 ISS crewmembers. Plasma samples collected during the *Salivary Markers* study were drawn at L-180, L-60, FD-10, FD-90, FD-180/R-1, R+0, R+18, R+33 and R+66 from 8 astronauts and 6 ground-based controls. Blood samples used for B-cell phenotyping were collected during *Salivary Markers* on 8 ISS crewmembers, in vacutainers supplemented with an acidified glucose nutrient solution (Acid-citrate dextrose) to maintain optimal cellular viability during sample return on Earth. Technical constraints did not allow for timely return of whole blood on earth at FD-10, and consequently no B-cell phenotype data were measured at that timepoint.

A temperature-controlled environment (temperature range: 6-24°C) was maintained during the 24-36 hours of sample storage/transport to the laboratories at NASA-JSC and the University of Houston. Upon arrival to the respective laboratories, the plasma samples were centrifugally separated from whole blood and stored at -80°C and B-cell phenotype was characterized using whole blood samples from the ACD tubes. Cryopreserved plasma samples were transported to the ImmunoEnergetics laboratory at Louisiana State University, and freshly isolated B-cells phenotypes were characterized on a BD Accuri C6 flow cytometer (BD Accuri, Ann Arbor, MI, USA) at the University of Houston. Lymphocyte counts were determined using a Mindray BC-3200 auto hematology analyzer (Mahwah, NJ, USA).

Urine and saliva samples were collected during the *Integrated Immune* study to determine CMV, EBV and VZV viral load in astronauts throughout the mission; results reported elsewhere (35, 36). In brief, aliquots of urine were sampled from a 24-hours urine pool at each of the aforementioned timepoint, and fasting saliva samples were collected using sterile Salivette cotton rolls (Sardstedt, Newton, NC) immediately upon awakening. The urine samples were frozen until return to Earth, while the salivettes were stored in stability buffer (0.5% SDS, 10mM Tris-Cl and 1mM EDTA) at room temperature for up to 2 weeks before return to Earth for subsequent analysis (37). Viral DNA was extracted and quantified by Dr. Mehta at NASA JSC by polymerase chain reaction as described previously (38).

2.3 B-cell phenotyping

B-cells were labeled with directly-conjugated monoclonal antibodies and analyzed using 4-color flow cytometry as previously described (54). Briefly, aliquots of 50µL of whole blood were incubated with 5µL of each mAb for 30 minutes at room temperature in the dark. The following mAb were used to stain the cells: anti-CD20-FITC clone #LT20, anti-CD43-PE clone #84-3C1 (eBioscience, San Diego CA USA), anti-IgD-PE clone #IA6-2, anti-IgM-PE clone #G20-127 (BD Pharmingen, San Diego CA USA), anti-IgG-PE clone #H2 (Southern Biotech, Birmingham AL USA), anti-CD27-PerCP clone #0323, anti-CD25-APC clone #CD25-4E3, anti-CD5-APC clone #L17F12 and anti-CD38-APC clone #HIT2 (eBioscience, San Diego CA USA). Using these mAbs, CD20+ B-cells were classified into immature

(CD20+/CD43-/CD27-/IgD-), naive/transitional (CD20+/CD43-/CD27-/IgD+), memory B-cells (CD20+/CD43+/CD27+/CD38-), plasmablasts/plasma cells (CD20+/CD43+/CD27+/CD38^{hi}), B1 cells (CD20+/CD43+/CD27+/CD5-), regulatory B-cells (CD20+/CD43+/CD27-/CD5+), IgG+ memory B-cells (CD20+/CD27+/IgG+) and IgM+ B-cells (CD20+/CD27+/IgM+) (**Table 1)**. Following incubation, erythrocytes were lysed by increased osmotic pressure induced by the addition of 500µL of RBC lysis buffer (eBioscience, San Diego, CA, USA) for 20 minutes at room temperature. Samples were then washed twice with PBS and resuspended in 250µL of PBS to be analyzed on a Accuri C6 flow cytometer. Flow cytometry analysis was conducted on the Accuri proprietary flow cytometry analysis software. Total cell numbers of each B-cell subset were determined by multiplying the percentages of cells expressing each marker of interest by the total lymphocyte count.

2.4 Immunoglobulin analyses

Plasma samples from the 23 crewmembers (N "integrated immune"=15; N "salivary markers"=8) were thawed and a total volume of 100µL were analyzed for Kappa and Lambda free light chains (FLC) using commercially-available enzyme linked immunosorbent assays (ELISA) (Seralite®, Abingdon Health, Oxford, UK) using previously published methods (7). Briefly, diluted plasma samples were incubated in 96 well plates pre-coated with either anti-Kappa or anti-Lambda FLC monoclonal antibodies at room temperature for 60 minutes. Following initial incubation, the wells were washed four consecutive times, and incubated with HRP-labeled anti-Kappa or anti-Lambda detection antibody for 30 minutes at room temperature. After another washing step, the presence of Kappa or Lambda FLC in the plasma samples were detected using a colorimetric reaction and read on a SpectraMax i3x plate reader (San Jose, CA, USA). The color intensity was directly correlated with the Kappa and Lambda FLC concentration in the samples. Plasma cystatin-C was measured with commercially-available ELISA (R&D Systems, Minneapolis, MN, USA) and used to calculate estimated glomerular filtration rate (eGFR) throughout the missions based on an established algorithm (47). This estimate of renal function was used to account for changes in renal clearance of FLC in response to spaceflight.

Total IgA, IgM and IgG were measured in a total of 150µL of thawed plasma sample from all astronauts and corresponding controls using commercially available ELISA kits (eBioscience, San Diego, CA, USA).

2.5 Statistical Analysis

A longitudinal, repeated measures design was utilized to determine the effects of long-term exposure to microgravity on proportions and numbers of the different B-cell subsets, Kappa and Lambda FLC and immunoglobulin levels. Linear mixed models were used to evaluate potential differences in the main and interaction effects of time (L-180, L-45 to 60, early flight, mid-flight, late flight, R+0, R+1, R+18, R+30 to 33, and R+66) after controlling for potential change in eGFR. When a significant time effect was observed, post-hoc tests were performed with Bonferroni correction. Data is presented as means ± standard error. All statistical analyses were performed using SPSS version 24 (IBM Corp., Armonk, NY) and significance was set at $p < 0.05$.

3. Results

3.1 Effect of long-duration spaceflight on circulating B-cell subsets

Cell counts for the different B-cell subsets isolated from crewmembers throughout the *Salivary Markers* study are presented in **Table 2.** There was no change in the percentages of total B-cells within the lymphocyte population or in the total number of B-cells during the 6 months mission ($F_{B\text{-cell Frequency}}=1.603$; $p_{B\text{-cell Frequency}}=0.142$ and $F_{B\text{-cell count}}=0.248$; $p_{B\text{-cell count}}=0.972$). Furthermore, long duration spaceflight was not associated with any statistically significant change in Plasma cells, Immature, Naïve/Transitional, Memory, Regulatory and B1-cells ($p>0.05$) in crewmembers.

3.2 Long-duration spaceflight and blood immunoglobulins

3.2.1 Plasma intact immunoglobulins concentration during a 6-month mission in the ISS

There was no change in plasma IgG and IgM concentrations in astronauts throughout the mission ($p>0.05$). The impact of long-duration spaceflight on total plasma immunoglobulin concentrations is presented in **Table 3.** Astronauts exhibited an increase in plasma IgA during flight, when compared to baseline values (L-60/45) ($F=7.077$; $p<0.001$). Upon return on Earth, plasma IgA concentrations decreased from in-flight levels, and were back to pre-flight values (L-60/L-45) during recovery (R+30) ($p=0.047$). All changes withstood adjustment for latent viral reactivation status and DNA load, along with eGFR.

3.2.2 Plasma FLC concentration during a 6-month mission in the ISS

The effects of long-duration spaceflight on Kappa (κ) and Lambda (λ) FLCs, along with the ratio of κ/λ and total FLC are presented in **Figure 1**. There was no effect of spaceflight on plasma κ FLC ($p>0.05$), and only a minor decrease in the concentration of plasma λ FLC was observed immediately upon return on Earth (R+0) in crewmembers when compared to in-flight plasma λ FLC concentrations (Early: $p=0.03$; Mid: $p=0.005$ and Late/R-1: $p=0.012$). The preferential reduction in plasma λ FLC at landing without any change in plasma κ FLC concentration led to a minor decrease in κ/λ ratio at the Mid-flight timepoint when compared to baseline L-60/L-45 and return R+0 and R+30 κ/λ ratio ($p_{L-45}= 0.029$; $p_{R+0}= 0.037$; $p_{R+18}=0.053$; $p_{R+33}=0.037$). As plasma FLC levels can be impacted by altered production from plasma cells and/or impaired clearance from renal metabolism, Cystatin C was measured to calculate estimated glomerular filtration rate (eGFR) (1) and account for variation in renal function during spaceflight. There was no change in kidney function during flight ($p>0.05$), however post-flight eGFR values (R+30) were significantly lower than pre-flight values (L-60/L-45) ($p=0.015$). Subtle changes in plasma FLC withstood adjustment for eGFR.

In related analyses, we explored whether latent viral reactivation was associated with FLC levels in blood. Cytomegalovirus (CMV), Epstein-Barr Virus (EBV) and Varicella Zoster Virus (VZV) reactivation status and viral DNA load from a subset of samples (*Integrated Immune* study) were included in the model as covariates. The rate and magnitude of latent viral reactivation observed in the *Integrated Immune* cohort is described elsewhere (51). In brief, 47% (7/15) of astronauts exhibited CMV reactivation at any point during the mission, 73%

(11/15) of astronauts exhibited EBV reactivation at any point during the mission and 60% (9/15) of astronauts exhibited VZV reactivation at any point during the mission. There was no difference in plasma κ and λ FLC concentrations in astronauts who exhibited CMV and EBV reactivation at any point during the mission ($p>0.05$), and there was no association between CMV and EBV DNA load and plasma FLC at any timepoint ($p>0.05$). When VZV DNA load was included in the model, the greater plasma λ FLC concentration observed in-flight when compared to post-flight values was associated with the magnitude of VZV reactivation ($F_{VZV\ DNA\ load} = 4.937$; $p_{VZV\ DNA\ load} = 0.029$).

4. Discussion

The rapid progress made in aerospace technologies over the past 50 years has dramatically increased the scope and duration of manned space exploration missions. In addition to providing novel technological challenges, the safe implementation of exploration-class missions requires a profound understanding of the impact that prolonged exposure to space environments has on astronaut's biology and health. In particular, numerous studies have raised concerns about the potential clinical risks associated with reported immune impairments observed during spaceflight (10, 11, 14, 50), such as reduced T-cell (25, 26, 34) and NK-cell functions (46), sustained production of pro-inflammatory cytokines (15, 35), altered neutrophil and monocyte microbicidal activity (30, 31), diminished anti-microbial protein concentration and increased rate and magnitude of latent viral reactivation (36, 51). However no study to date has attempted to comprehensively characterize the impact of long-duration spaceflight on humoral immunity in humans. The goal of this study was to identify changes in a broad range of phenotypically distinct B-cell subsets, along with secreted immunoglobulins and free light chains in astronauts who completed 6-months on the ISS. We found no change in the number of total B-cells in response to spaceflight, however there was a trend for increased Memory B-cells during spaceflight, when compared to baseline values. Contrary to supposition, only marginal changes were observed in soluble biomarkers of B-cell homeostasis during spaceflight. Indeed, we found no effect of spaceflight on plasma κ FLC,

IgG and IgM levels, and only modest changes to plasma λ FLC concentrations. Interestingly, long-duration spaceflight increased plasma IgA levels, which considering the correlation between mucosal and plasma IgA (55), may represent alterations in mucosal immunity.

Spaceflight has been shown to alter human and animal leukocyte distribution (12, 48), but the majority of studies were focused on T-cells and NK-cells (22, 46), and were limited to either short-duration missions (8-15 days) (23, 52) or to comparisons between pre- and post-flight. Our data are in accordance with previous findings showing that total B-cell counts and proportion within the lymphocyte compartment are not affected by long-duration spaceflight (46). However, the more advanced and detailed phenotypic analysis performed in this study highlighted for the first time that spaceflight does not have a detrimental impact on the phenotypic composition of the B-cell compartment. Indeed, memory B-cell counts changed modestly in-flight, while the number of naive/transitional and regulatory B-cells remained unchanged during the mission. Animal studies have shown that short-duration spaceflight (41) and ground-based analogs to spaceflight such as hind limb unloading (32) had deleterious impacts on immune cell phenotypes in the bone marrow and led to a reduction in *de novo* generation of B-cells (32) and total splenic B-cells number (21). As both bone marrow progenitor cells (18) and B-cells are known to respond to acute stressors, such as intense exercise (54), it can be hypothesized that the changes in B-cell lymphopoiesis observed in animals immediately after short-duration spaceflight may be due to a variety of factors other than microgravity, including landing-associated stressors, rather than exposure to the space exposome.

Effective humoral responses rely on B-cell activation, differentiation and antibody output. Although there was no effect of spaceflight on the number of circulating plasma cells in astronauts, it is interesting to note that they had a greater amount of B1 cells than ground-based controls (data not shown). As B1 cells have recently been characterized as pre-plasmablasts (9), a greater circulating number could be associated with increased capability to produce antibodies following activation. Conflicting data exist on the effect of spaceflight on immunoglobulin outputs, with some studies showing a reduction in *in vitro* IgG production in response to altered gravity (19), while others showed no change from baseline in IgG

311 production after a 10-15 days flight (52, 56), and no alteration in the B-cell repertoire of
312 unimmunized animals (58). Our data are in partial agreement with the current literature, as
313 we found no difference in total plasma IgG and IgM at any point in the mission, even after
314 controlling for latent viral reactivation and DNA load. Interestingly however, astronauts
315 exhibited an increase in total plasma IgA during flight. Elevated plasma IgA have been
316 observed in the urodele amphibian *Pleurodeles waltl* exposed to microgravity for 6 months
317 (5), but also in rodents and human subjects under chronic psychological and physiological
318 stress (20, 60), similar to those experienced by the astronauts on board the ISS. Furthermore,
319 although the *in-flight* increases in plasma IgA concentrations were statistically significant
320 when compared to baseline values, astronauts' plasma IgA levels did not exceed the normal
321 clinical range observed on Earth (59). Consequently, the fluctuations in plasma IgA observed
322 in this study are likely attributable to long-duration exposure to spaceflight-associated
323 psychological and operative stressors rather than to alterations in B-cell function.

324 The immune impairments associated with prolonged exposure to the myriads of stressors
325 specific to the spaceflight environment are known to result in increased latent CMV, EBV
326 and VZV reactivation (35, 36, 51). However, no study to date has attempted to determine
327 whether potential alterations in B-cell activation and function could play a role in latent viral
328 reactivations in space. While our data did not show any biologically-relevant change in total
329 plasma immunoglobulin concentrations in response to spaceflight, it could be argued that the
330 relatively long half-life of intact plasma Ig (around 21 days) curtails their sensitivity at
331 detecting changes in B-cell function (16). As such, we sought to determine changes in κ and λ
332 immunoglobulin Free Light Chains, a more sensitive barometer of immune competency with
333 short half-life (κ: 2-4 hrs; and λ: 3-6 hrs) due to rapid renal clearance (16). Plasma κ FLC
334 levels remained unchanged during the missions, however there was a slight decrease in the
335 concentration of plasma λ FLC immediately upon return on Earth (R+0) in crewmembers
336 when compared to in-flight plasma λ FLC concentrations. While this reduction in λ FLC at
337 R+0 when compared to in-flight values reached statistical significance, no crewmember
338 reached clinically significant low-levels of λ FLC (29). As there was no change in kidney
339 function *in-flight*, this preferential reduction in plasma λ FLC is not due to increased renal

clearance. However, while there was no effect of CMV and EBV reactivation on λ FLC concentrations, the magnitude of VZV reactivation during flight was associated with the greater levels of λ FLC observed at the same timepoints. This discrepancy in response between plasma κ and λ FLC, could be explained by the difference in size between both light chains. Indeed, although κ FLC are produced in greater quantities than λ FLC during antibody synthesis (57), the larger size of the dimeric λ FLC impedes their clearance by the kidney (16). Our results suggest that B-cell ability to produce FLC and consequently immunoglobulins in response to a viral or vaccine challenge in space remain intact. It should however be noted that, while we measured the quantity of plasma κ and λ FLC and total Immunoglobulins in crewmembers, we did not characterize the quality of these antibodies. Animal studies have indeed showed that while similar quantities of antibodies could be produced in response to antigenic challenge in space (3), the quality of the produced antibodies remained inferior to those produced on earth (2). Consequently, although the present study shows that B-cell homeostasis appears to be preserved during long duration spaceflight, it could be hypothesized that sub-optimal immune responses could still be observed upon antigenic challenge in space.

One limitation of our study lies in its retrospective nature. These results were obtained from samples collected during two previously completed NASA studies, and all plasma immunoglobulin and FLC analysis were performed on previously archived samples. As such, we were unable to measure the antibody response to a specific vaccine, but rather measured B-cell homeostasis during long duration spaceflight. Furthermore, the changes in B-cell number and phenotype during spaceflight were only characterized on the *Salivary Markers* cohort (n=8). Technical constraints unique to spaceflight research prevented the immediate analysis of the isolated B-cell populations. To mitigate this limitation, we performed validation work in our laboratory prior to this study, and found that B-cell number and phenotype remained unaltered for 48 hours when collected from vacutainers supplemented with an acidified glucose nutrient solution. Finally, we also collected blood samples from ground-based controls to parallel samples collected on the ISS, thus controlling for any potential *ex vivo* aging of the blood samples. Unfortunately however, as the ground-based

control subjects were recruited to ensure assay validity, rather than to serve as paired-controls with each astronauts, they were not age- or sex-matched with the different crewmembers. Future studies should attempt to recruit populations of ground-based controls that closely match the astronaut population to further reduce the effects of various confounding factors (age, sex, fitness level etc...) on the observed changes in immune function during spaceflight.

In conclusion, this is the first study to comprehensively show that long-duration spaceflight in human astronauts has no – or very limited – effect on B-cell number, phenotype and antibody output. These important results suggest that plasma immune competency is maintained in microgravity, and that future *in-flight* vaccine-based countermeasures are likely to be efficient at further protecting astronauts from immune dysregulation and symptomatic latent viral reactivations during prolonged exploration class missions.

References

1. **Aulakh NK, Bansal E, Bose A, Aulakh GS, Aulakh BS, and Singh MR**. Can cystatin C become an easy and reliable tool for anesthesiologists to calculate glomerular filtration rate? *J Anaesthesiol Clin Pharmacol* 31: 44-48, 2015.

2. **Bascove M, Gueguinou N, Schaerlinger B, Gauquelin-Koch G, and Frippiat JP**. Decrease in antibody somatic hypermutation frequency under extreme, extended spaceflight conditions. *FASEB J* 25: 2947-2955, 2011.
3. **Bascove M, Huin-Schohn C, Gueguinou N, Tschirhart E, and Frippiat JP**. Spaceflight-associated changes in immunoglobulin VH gene expression in the amphibian Pleurodeles waltl. *FASEB J* 23: 1607-1615, 2009.
4. **Bellary S, Faint JM, Assi LK, Hutchison CA, Harding SJ, Raymond NT, and Barnett AH**. Elevated serum free light chains predict cardiovascular events in type 2 diabetes. *Diabetes Care* 37: 2028-2030, 2014.
5. **Boxio R, Dournon C, and Frippiat JP**. Effects of a long-term spaceflight on immunoglobulin heavy chains of the urodele amphibian Pleurodeles waltl. *J Appl Physiol (1985)* 98: 905-910, 2005.
6. **Brebner JA, and Stockley RA**. Polyclonal free light chains: a biomarker of inflammatory disease or treatment target? *F1000 Med Rep* 5: 4, 2013.
7. **Campbell J, Heaney J, Gleeson M, He C, Killer S, Svendson S, Taylor I, Phillips AC, and Drayson M**. Salivary immunoglobulin free light chains: reference ranges in younger and older adults and responses to exercise. In: *International Society of Exercise Immunology*. Vienna, Austria: 2015.
8. **Chapes SK, Morrison DR, Guikema JA, Lewis ML, and Spooner BS**. Production and action of cytokines in space. *Adv Space Res* 14: 5-9, 1994.
9. **Covens K, Verbinnen B, Geukens N, Meyts I, Schuit F, Van Lommel L, Jacquemin M, and Bossuyt X**. Characterization of proposed human B-1 cells reveals pre-plasmablast phenotype. *Blood* 121: 5176-5183, 2013.
10. **Crucian B, Johnston S, Mehta S, Stowe R, Uchakin P, Quiriarte H, Pierson D, Laudenslager ML, and Sams C**. A case of persistent skin rash and rhinitis with immune system dysregulation onboard the International Space Station. *J Allergy Clin Immunol Pract* 4: 759-762 e758, 2016.
11. **Crucian B, and Sams C**. Immune system dysregulation during spaceflight: clinical risk for exploration-class missions. *J Leukoc Biol* 86: 1017-1018, 2009.
12. **Crucian B, Stowe RP, Mehta S, Quiriarte H, Pierson D, and Sams C**. Alterations in adaptive immunity persist during long-duration spaceflight. *NPJ Microgravity* 1: 15013, 2015.
13. **Crucian BE, Chouker A, Simpson RJ, Mehta S, Marshall G, Smith SM, Zwart SR, Heer M, Ponomarev S, Whitmire A, Frippiat JP, Douglas GL, Lorenzi H, Buchheim JI, Makedonas G, Ginsburg GS, Ott CM, Pierson DL, Krieger SS, Baecker N, and Sams C**. Immune System Dysregulation During Spaceflight: Potential Countermeasures for Deep Space Exploration Missions. *Front Immunol* 9: 1437, 2018.
14. **Crucian BE, Stowe RP, Pierson DL, and Sams CF**. Immune system dysregulation following short- vs long-duration spaceflight. *Aviat Space Environ Med* 79: 835-843, 2008.
15. **Crucian BE, Zwart SR, Mehta S, Uchakin P, Quiriarte HD, Pierson D, Sams CF, and Smith SM**. Plasma cytokine concentrations indicate that in vivo hormonal regulation of immunity is altered during long-duration spaceflight. *J Interferon Cytokine Res* 34: 778-786, 2014.
16. **Davids MS, Murali MR, and Kuter DJ**. Serum free light chain analysis. *Am J Hematol* 85: 787-790, 2010.
17. **Dispenzieri A, Katzmann JA, Kyle RA, Larson DR, Therneau TM, Colby CL, Clark RJ, Mead GP, Kumar S, Melton LJ, 3rd, and Rajkumar SV**. Use of nonclonal serum immunoglobulin free light chains to predict overall survival in the general population. *Mayo Clin Proc* 87: 517-523, 2012.

18. **Emmons R, Niemiro GM, Owolabi O, and De Lisio M**. Acute exercise mobilizes hematopoietic stem and progenitor cells and alters the mesenchymal stromal cell secretome. *J Appl Physiol (1985)* 120: 624-632, 2016.
19. **Fitzgerald W, Chen S, Walz C, Zimmerberg J, Margolis L, and Grivel JC**. Immune suppression of human lymphoid tissues and cells in rotating suspension culture and onboard the International Space Station. *In Vitro Cell Dev Biol Anim* 45: 622-632, 2009.
20. **Gaignier F, Legrand-Frossi C, Stragier E, Mathiot J, Merlin JL, Cohen-Salmon C, Lanfumey L, and Frippiat JP**. A Model of Chronic Exposure to Unpredictable Mild Socio-Environmental Stressors Replicates Some Spaceflight-Induced Immunological Changes. *Front Physiol* 9: 514, 2018.
21. **Gridley DS, and Pecaut MJ**. Changes in the distribution and function of leukocytes after whole-body iron ion irradiation. *J Radiat Res* 57: 477-491, 2016.
22. **Gridley DS, Slater JM, Luo-Owen X, Rizvi A, Chapes SK, Stodieck LS, Ferguson VL, and Pecaut MJ**. Spaceflight effects on T lymphocyte distribution, function and gene expression. *J Appl Physiol (1985)* 106: 194-202, 2009.
23. **Grove DS, Pishak SA, and Mastro AM**. The effect of a 10-day space flight on the function, phenotype, and adhesion molecule expression of splenocytes and lymph node lymphocytes. *Exp Cell Res* 219: 102-109, 1995.
24. **Gueguinou N, Huin-Schohn C, Bascove M, Bueb JL, Tschirhart E, Legrand-Frossi C, and Frippiat JP**. Could spaceflight-associated immune system weakening preclude the expansion of human presence beyond Earth's orbit? *J Leukoc Biol* 86: 1027-1038, 2009.
25. **Hauschild S, Tauber S, Lauber B, Thiel CS, Layer LE, and Ullrich O**. T cell regulation in microgravity – The current knowledge from in vitro experiments conducted in space, parabolic flights and ground-based facilities. *Acta Astronautica* 104: 365-377, 2014.
26. **Hughes-Fulford M, Chang TT, Martinez EM, and Li CF**. Spaceflight alters expression of microRNA during T-cell activation. *FASEB J* 29: 4893-4900, 2015.
27. **Hutchison CA, Cockwell P, Harding S, Mead GP, Bradwell AR, and Barnett AH**. Quantitative assessment of serum and urinary polyclonal free light chains in patients with type II diabetes: an early marker of diabetic kidney disease? *Expert Opin Ther Targets* 12: 667-676, 2008.
28. **Hutchison CA, and Landgren O**. Polyclonal immunoglobulin free light chains as a potential biomarker of immune stimulation and inflammation. *Clin Chem* 57: 1387-1389, 2011.
29. **Katzmann JA, Clark RJ, Abraham RS, Bryant S, Lymp JF, Bradwell AR, and Kyle RA**. Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. *Clin Chem* 48: 1437-1444, 2002.
30. **Kaur I, Simons ER, Castro VA, Mark Ott C, and Pierson DL**. Changes in neutrophil functions in astronauts. *Brain Behav Immun* 18: 443-450, 2004.
31. **Kaur I, Simons ER, Castro VA, Ott CM, and Pierson DL**. Changes in monocyte functions of astronauts. *Brain Behav Immun* 19: 547-554, 2005.
32. **Lescale C, Schenten V, Djeghloul D, Bennabi M, Gaignier F, Vandamme K, Strazielle C, Kuzniak I, Petite H, Dosquet C, Frippiat JP, and Goodhardt M**. Hind limb unloading, a model of spaceflight conditions, leads to decreased B lymphopoiesis similar to aging. *FASEB J* 29: 455-463, 2015.
33. **Lesnyak AT, Sonnenfeld G, Rykova MP, Meshkov DO, Mastro A, and Konstantinova I**. Immune changes in test animals during spaceflight. *J Leukoc Biol* 54: 214-226, 1993.

34. **Martinez EM, Yoshida MC, Candelario TL, and Hughes-Fulford M**. Spaceflight and simulated microgravity cause a significant reduction of key gene expression in early T-cell activation. *Am J Physiol Regul Integr Comp Physiol* 308: R480-488, 2015.
35. **Mehta SK, Crucian BE, Stowe RP, Simpson RJ, Ott CM, Sams CF, and Pierson DL**. Reactivation of latent viruses is associated with increased plasma cytokines in astronauts. *Cytokine* 61: 205-209, 2013.
36. **Mehta SK, Laudenslager ML, Stowe RP, Crucian BE, Feiveson AH, Sams CF, and Pierson DL**. Latent virus reactivation in astronauts on the international space station. *npj Microgravity* 3: 11, 2017.
37. **Mehta SK, Laudenslager ML, Stowe RP, Crucian BE, Sams CF, and Pierson DL**. Multiple latent viruses reactivate in astronauts during Space Shuttle missions. *Brain Behav Immun* 41: 210-217, 2014.
38. **Mehta SK, Stowe RP, Feiveson AH, Tyring SK, and Pierson DL**. Reactivation and shedding of cytomegalovirus in astronauts during spaceflight. *J Infect Dis* 182: 1761-1764, 2000.
39. **Nakano T, Matsui M, Inoue I, Awata T, Katayama S, and Murakoshi T**. Free immunoglobulin light chain: its biology and implications in diseases. *Clin Chim Acta* 412: 843-849, 2011.
40. **Nash PV, Konstantinova IV, Fuchs BB, Rakhmilevich AL, Lesnyak AT, and Mastro AM**. Effect of spaceflight on lymphocyte proliferation and interleukin-2 production. *J Appl Physiol (1985)* 73: 186S-190S, 1992.
41. **Ortega MT, Pecaut MJ, Gridley DS, Stodieck LS, Ferguson V, and Chapes SK**. Shifts in bone marrow cell phenotypes caused by spaceflight. *J Appl Physiol (1985)* 106: 548-555, 2009.
42. **Plett PA, Frankovitz SM, Abonour R, and Orschell-Traycoff CM**. Proliferation of human hematopoietic bone marrow cells in simulated microgravity. *In Vitro Cell Dev Biol Anim* 37: 73-78, 2001.
43. **Rao M, Lamont JL, Chan J, Concannon TW, Comenzo R, Ratichek SJ, and Avendano EE**. In: *Serum Free Light Chain Analysis for the Diagnosis, Management, and Prognosis of Plasma Cell Dyscrasias: Future Research Needs: Identification of Future Research Needs From Comparative Effectiveness Review No 73*. Rockville (MD): 2012.
44. **Rao M, Yu WW, Chan J, Patel K, Comenzo R, Lamont JL, Ip S, and Lau J**. In: *Serum Free Light Chain Analysis for the Diagnosis, Management, and Prognosis of Plasma Cell Dyscrasias*. Rockville (MD): 2012.
45. **Rosenzweig JA, Abogunde O, Thomas K, Lawal A, Nguyen YU, Sodipe A, and Jejelowo O**. Spaceflight and modeled microgravity effects on microbial growth and virulence. *Appl Microbiol Biotechnol* 85: 885-891, 2010.
46. **Rykova MP, Antropova EN, Larina I, and Morukov BV**. Humoral and Cellular Immunity in cosmonauts after the ISS missions. *Acta Astronaut* 63: 697-705, 2008.
47. **Shlipak MG, Coresh J, and Gansevoort RT**. Cystatin C versus creatinine for kidney function-based risk. *N Engl J Med* 369: 2459, 2013.
48. **Sonnenfeld G**. Animal models for the study of the effects of spaceflight on the immune system. *Adv Space Res* 32: 1473-1476, 2003.
49. **Sonnenfeld G**. Effect of space flight on cytokine production. *Acta Astronautica* 33: 143-147, 1994.
50. **Sonnenfeld G, and Shearer WT**. Immune function during space flight. *Nutrition* 18: 899-903, 2002.
51. **Spielmann G, Laughlin MS, Kunz H, Crucian BE, Quiriarte HD, Mehta SK, Pierson DL, and Simpson RJ**. Latent viral reactivation is associated with changes in plasma antimicrobial protein concentrations during long-duration spaceflight. *Acta Astronautica* 146: 111-116, 2018.

52. **Stowe RP, Sams CF, Mehta SK, Kaur I, Jones ML, Feeback DL, and Pierson DL**. Leukocyte subsets and neutrophil function after short-term spaceflight. *J Leukoc Biol* 65: 179-186, 1999.
53. **Tosi P, Tomassetti S, Merli A, and Polli V**. Serum free light-chain assay for the detection and monitoring of multiple myeloma and related conditions. *Ther Adv Hematol* 4: 37-41, 2013.
54. **Turner JE, Spielmann G, Wadley AJ, Aldred S, Simpson RJ, and Campbell JP**. Exercise-induced B cell mobilisation: Preliminary evidence for an influx of immature cells into the bloodstream. *Physiol Behav* 164: 376-382, 2016.
55. **van Ravenhorst MB, den Hartog G, van der Klis FRM, van Rooijen DM, Sanders EAM, and Berbers GAM**. Induction of salivary antibody levels in Dutch adolescents after immunization with monovalent meningococcal serogroup C or quadrivalent meningococcal serogroup A, C, W and Y conjugate vaccine. *PLoS One* 13: e0191261, 2018.
56. **Voss EW, Jr.** Prolonged weightlessness and humoral immunity. *Science* 225: 214-215, 1984.
57. **Waldmann TA, Strober W, and Mogielnicki RP**. The renal handling of low molecular weight proteins. II. Disorders of serum protein catabolism in patients with tubular proteinuria, the nephrotic syndrome, or uremia. *J Clin Invest* 51: 2162-2174, 1972.
58. **Ward C, Rettig TA, Hlavacek S, Bye BA, Pecaut MJ, and Chapes SK**. Effects of spaceflight on the immunoglobulin repertoire of unimmunized C57BL/6 mice. *Life Sci Space Res (Amst)* 16: 63-75, 2018.
59. **Webster AD**. Laboratory investigations of primary deficiencies of the lymphoid system. *clinical allergy immunology* 5: 447-467, 1985.
60. **Yadav AP, Mishra KP, Ganju L, and Singh SB**. Wintering in Antarctica: impact on immune response of Indian expeditioners. *Neuroimmunomodulation* 19: 327-333, 2012.

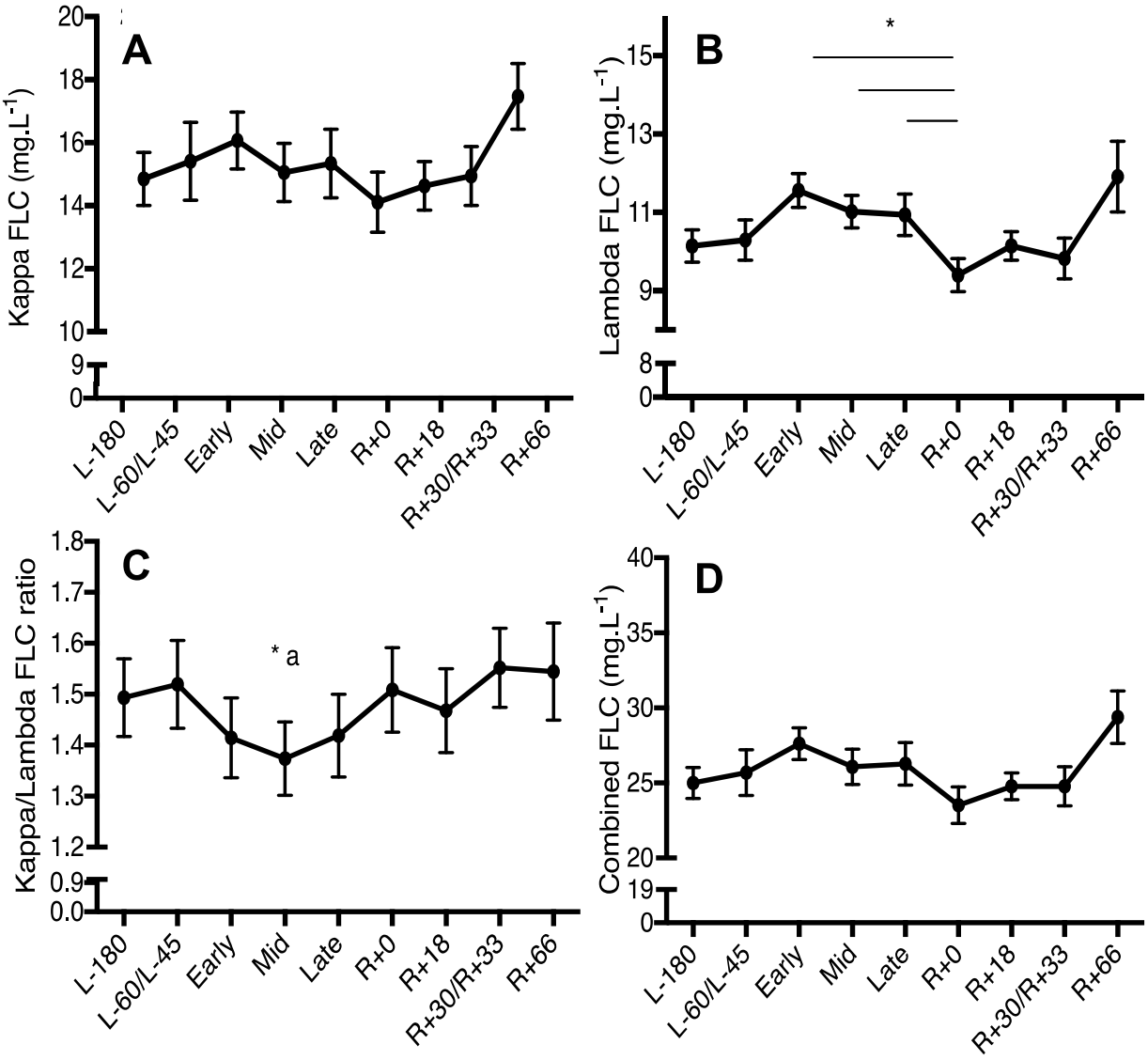

Figure 1. Changes in Kappa κ (A) and Lambda λ (B) Free Light Chains in 6 months ISS astronauts (n=23). The ratio of κ/λ and total Free Light Chains are presented in panel C and D respectively. Mean values are presented ± SEM. Significant differences between immediately post-flight values (R+0) (* $p<0.05$) and baseline values (L-45) (a $p<0.05$)

Table 1. Phenotypic characterization of the different B-cell subsets

Cell Type	Phenotype	Reference
B-cells	CD20+	(Leandro 2013)
Immature B-cells	CD20+/CD43-/CD27-/IgD-	(Inui et al. 2015; Sims et al. 2005; Wei, Jung, and Sanz 2011)
Naïve/Transitional B-cells	CD20+/CD43-/CD27-/IgD+	(Inui et al. 2015; Sims et al. 2005; Wei, Jung, and Sanz 2011)
IgM+ B-cells	CD20+/CD27+/IgM+	(Berkowska et al. 2011)
Memory B-cells	CD20+/CD43+/CD27+/CD38-	(Quach et al. 2016)
IgG+ memory B-cells	CD20+/CD27+/IgG+	(Berkowska et al. 2011)
B1 cells	CD20+/CD43+/CD27+/CD5-	(Griffin, Holodick, and Rothstein 2011)
Regulatory B-cells	CD20+/CD43+/CD27-/CD5+	(Mauri and Menon 2015)
Plasmablasts/Plasma cells	CD20+/CD43+/CD27+/CD38^{hi}	(Quach et al. 2016)

Berkowska, M. A., G. J. Driessen, V. Bikos, C. Grosserichter-Wagener, K. Stamatopoulos, A. Cerutti, B. He, K. Biermann, J. F. Lange, M. van der Burg, J. J. van Dongen, and M. C. van Zelm. 2011. 'Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways', *Blood*, 118: 2150-8.

Griffin, D. O., N. E. Holodick, and T. L. Rothstein. 2011. 'Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70', *J Exp Med*, 208: 67-80.

Inui, M., S. Hirota, K. Hirano, H. Fujii, A. Sugahara-Tobinai, T. Ishii, H. Harigae, and T. Takai. 2015. 'Human CD43+ B cells are closely related not only to memory B cells phenotypically but also to plasmablasts developmentally in healthy individuals', *Int Immunol*, 27: 345-55.

Leandro, M. J. 2013. 'B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies', *Arthritis Res Ther*, 15 Suppl 1: S3.

Mauri, C., and M. Menon. 2015. 'The expanding family of regulatory B cells', *Int Immunol*, 27: 479-86.

Quach, T. D., N. Rodriguez-Zhurbenko, T. J. Hopkins, X. Guo, A. M. Hernandez, W. Li, and T. L. Rothstein. 2016. 'Distinctions among Circulating Antibody-Secreting Cell Populations, Including B-1 Cells, in Human Adult Peripheral Blood', *J Immunol*, 196: 1060-9.

Sims, G. P., R. Ettinger, Y. Shirota, C. H. Yarboro, G. G. Illei, and P. E. Lipsky. 2005. 'Identification and characterization of circulating human transitional B cells', *Blood*, 105: 4390-8.

Wei, C., J. Jung, and I. Sanz. 2011. 'OMIP-003: phenotypic analysis of human memory B cells', *Cytometry A*, 79: 894-6.

Table 2. Changes in the numbers (cells/100µL) of B lymphocyte lineage cells in 6 months ISS crewmembers (n=8). Average values are presented ± SD.

	Sample Timepoint								Main Effect of Time F-statistic (p value)
	L-180	L-60	FD-90/Mid-Flight	R-1/Late-Flight	R+0	R+18	R+33	R+66	
Total B-cells Cells/100µL									
ISS Crewmembers (n=8)	12128 ± 4080	15316 ± 3036	21892 ± 12747	24920 ± 14955	19865 ± 13955	14279 ± 6157	13831 ± 3272	11856 ± 4376	0.248 (0.972)
% Lymphocytes									
ISS Crewmembers (n=8)	10 ± 4	12 ± 3	13 ± 4	11 ± 4	13 ± 7	11 ± 5	11 ± 4	9 ± 4	1.603 (0.142)
Immature B-cells									
ISS Crewmembers (n=8)	482 ± 271	625 ± 568	2618 ± 2936	2508 ± 3785	2322 ± 3534	1022 ± 1233	866 ± 768	602 ± 645	0.665 (0.702)
Naïve/Transitional B-cells									
ISS Crewmembers (n=8)	7091 ± 3751	9787 ± 4250	11083 ± 5605	12896 ± 6237	11196 ± 7842	7855 ± 3706	8045 ± 3292	7044 ± 3447	0.626 (0.733)
IgM+ B-cells									
ISS Crewmembers (n=8)	53 ± 155	11 ± 13	16 ± 22	29 ± 45	34 ± 46	6 ± 9	10 ± 11	11 ± 11	0.702 (0.670)
Memory B-cells									
ISS Crewmembers (n=8)	179 ± 158	208 ± 133	327 ± 243	409 ± 310	381 ± 327	202 ± 141	394 ± 477	196 ± 122	1.878 (0.080)
IgG+ memory B-cells									
ISS Crewmembers (n=8)	74 ± 177	38 ± 29	67 ± 63	41 ± 46	42 ± 43	57 ± 95	61 ± 73	28 ± 21	1.004 (0.432)
B1 cells									
ISS Crewmembers (n=8)	123 ± 117	211 ± 256	225 ± 177	283 ± 357	242 ± 216	211 ± 171	167 ± 221	306 ± 383	0.932 (0.485)
Regulatory B-cells									
ISS Crewmembers (n=8)	129 ± 104	177 ± 64	288 ± 240	269 ± 151	312 ± 494	152 ± 51	252 ± 206	202 ± 124	0.943 (0.476)
Plasmablasts/Plasma cells									
ISS Crewmembers (n=8)	104 ± 86	178 ± 155	169 ± 102	197 ± 177	185 ± 202	161 ± 157	99 ± 93	173 ± 233	0.225 (0.979)

Table 3. Changes in plasma IgA, IgM and IgG in Astronauts before, during and following 6 months in the ISS (n=23). Average values are presented ± SD Significant differences from baseline pre-flight values (L-180 and L-60/L-45) are represented with * ($p<0.05$).

		L-180	L-60/L-45	Early-Flight	FD90/Mid-Flight	R-1/Late-Flight	R+0	R+18	R+30/R+33	R+66
IgA (mg/dL) ± SD	Crewmembers (n=23)	131.51 ± 63.73	112.73 ± 49.64	126.60 ± 58.05 *	136.38 ± 68.12 *	140.58 ±75.08 *	126.64 ± 59.56	103.14 ± 32.67	112.46 ± 55.11	101.73 ± 27.51
IgG (mg/dL) ± SD	Crewmembers (n=23)	1218.41 ± 231.66	1190.41 ± 253.88	1181.47 ± 310.80	1235.41 ± 277.07	1249.98 ± 298.99	1184.38 ± 209.64	1359.33 ± 304.05	1226.67 ± 399.88	1267.50 ± 255.92
IgM (mg/dL) ± SD	Crewmembers (n=23)	373.51 ± 546.67	408.84 ± 530.97	476.08 ± 665. 62	422.57 ± 476.36	410.57 ± 491.51	346.95 ± 360.77	651.04 ± 751.93	295.78 ± 268.07	471.20 ± 324.15