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## **Analysis of femurs from mice embarked on board BION-M1 biosatellite reveals a decrease of immune cell development, including B cells, after one week of recovery on Earth**

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### **Running title**

Bone and marrow proteomics of space flown mice

## Abbreviations

ACN: acetonitrile

AGC: automatic gain control

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

CV: coefficient of variation

DTT: dithiothréitol

EDTA: ethylenediaminetetraacetic acid

FDR: false discovery rate

HCD: higher energy collisional dissociation

HPLC: high-performance liquid chromatography

HSC: hematopoietic stem cells

HU: hindlimb unloading

HGNC: HUGO (Human genome organization) Gene Nomenclature Committee

Ig: immunoglobulin

iRT: indexed retention time

ISS: international space station

KEGG: Kyoto encyclopedia of genes and genomes

LC: liquid chromatography

MaxLFQ: MaxQuant *Label-free Quantitation*

m/z: mass-to-charge ratio

mRNA: messenger ribonucleic acid

MS and MS/MS: mass spectrometry and tandem mass spectrometry

*P. waltl*: *Pleurodeles waltl*

PVDF: polyvinylidene difluoride

QC: quality control

RNA: ribonucleic acid

SDS: sodium dodecyl sulfate

tRNA : transfer ribonucleic acid

TCA: trichloroacetic acid

TCR $\beta$ : T cell receptor  $\beta$

UPLC : ultra performance liquid chromatography

## **Abstract**

Bone loss and immune dysregulation are among the main adverse outcomes of spaceflight challenging astronaut's health and safety. However, consequences on B cell development and responses are still under-investigated. To fill this gap, we used advanced proteomics analysis of femur bone and marrow to compare mice flown for one month on board the BION-M1 biosatellite, followed or not by one week of recovery on Earth, to control mice kept on Earth. Our data revealed an adverse effect on B lymphopoiesis one week after landing. This phenomenon was associated with a 41% reduction of B cells in the spleen. These reductions may contribute to explain increased susceptibility to infection even if our data suggest that flown animals can mount a humoral immune response. Future studies should investigate the quality/efficiency of produced antibodies and whether longer missions worsen these immune alterations.

**Keywords: Lymphopoiesis; Bone; Immunity; Proteomics; Gravity**

## Introduction

Spaceflight expose astronauts to adverse effects, including bone loss, muscle atrophy, metabolic, cardiovascular, hematologic and immunologic deregulations (1). Immune system alterations associated with spaceflight, in conjunction with limited clinical care, represent a risk to crewmembers during deep-space missions. Indeed, the first epidemiological study based on medical data collected on 46 astronauts who spent six months onboard the ISS (International Space Station) showed that 46% of them faced immunological problems; infection being the second most frequent event (2). In the framework of the envisioned deep space missions to Mars, the Moon, or an asteroid that will require very long duration flights, the study of immune system responses to long-term spaceflight is therefore mandatory.

Up to now, most studies addressed the effects of spaceflight on innate immunity and T-cell responses (3, 4), while humoral immunity and B cell development have been less intensively investigated. Using the urodele amphibian *Pleurodeles waltl* as an animal model, it was shown that spaceflight affect antibody production in response to an antigen stimulation (5)(6). Additionally, somatic hypermutation that diversify antibody binding-sites to improve their affinity, was shown to occur in space following immunization but at a frequency two-times lower than on Earth (7). These data show that spaceflight quantitatively and qualitatively affect the amphibian humoral immune response. These results are likely transposable to mammals because the cardinal elements of the adaptive immune system are shared by all gnathostomes (8-10). Furthermore, it was shown that *P. waltl* antibody isotypes have mammalian analogs (11).

Concerning lymphocyte development, it was shown that T lymphopoiesis is sensitive to gravity changes. Indeed, reductions in CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> thymocyte populations were reported when murine fetal thymuses were cultured under clinorotation used to simulate microgravity (12). Furthermore, hypergravity exposure during murine gestation strongly modified pups TCR $\beta$  repertoire created during T cell maturation (13). On the other hand, gravity changes during *P. waltl* development affected the transcription of IgM heavy chains and of the lymphoid-determining transcription factor Ikaros, suggesting a modification in B lymphopoiesis (14). Hindlimb unloading, a rodent ground-based model frequently used to simulate spaceflight conditions, confirmed this decrease of B lymphopoiesis and highlighted a major block at the pro-B to pre-B cell transition (5-10 fold decrease) (15). This observation was

associated to changes in bone micro-architecture, which is not surprising because it is known *i)* that immune-competent lymphocytes are derived from hematopoietic stem cells (HSC) that reside in the bone marrow within specialized niches made up of bone and vascular structures, e.g. bone forming osteoblasts and bone resorbing osteoclasts (16) and *ii)* that interactions between HSC and bone marrow niches control the balance between quiescence, self-renewal and differentiation of HSC (17-19).

The objectives of this study were to determine whether extreme conditions encountered during a real spaceflight affect B cell development. To reach that goal, advanced quantitative proteomics, as well as molecular and cellular technologies, were used to decipher the responses of bone and marrow of mice embarked on board the Bion-M1 biosatellite during a 30-day spaceflight. We did also examine the reversibility of the effects upon return to Earth gravitational conditions. Our data revealed a decrease of the expression of several proteins involved in the development of immune cells, as well as 61% and 41% decreases of B cell number in the bone marrow and spleen, respectively, one week after landing thereby indicating a reduction of immune cell development, including B cells at this stage.

## **Materials and methods**

### **Ethical statement**

The IACUC of the MSU Institute of Mitoengineering (Protocol No. 35) and the Biomedical Ethics Committee of the Institute for Biomedical Problems (IBMP) and Russian Academy of Sciences (Protocol No. 319) approved the experimental design of the Bion-M1 project. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8<sup>th</sup> ed., 2011).

Hypergravity exposure was carried out in accordance with the French Legislation and the Council Directive of the European Communities on the Protection of Animals Used for Experimental and Other Scientific Purposes (2010/63/UE). Furthermore, the protocol was approved by the local ethical committee (authorization 04827).

### **Bion-M1 experiment: space conditions**

Space conditions included a cylindrical module habitat, which approx. volume was 1.7 l (3 mice per habitat, acclimatization during 3 days before launching), and a paste food diet based on standard chow with water and a casein gelling agent. All mice were exposed to 12-h light/dark cycles with mean ( $\pm$ S.D.) temperatures of  $21.1 \pm 0.4$  °C and  $21.3 \pm 0.8$  °C for flown mice and ground control animals, respectively. During their recovery period, mice were housed in standard conditions with a standard chow diet.

### **Bion-M1 experiment: animals and study design**

C57/BL6 male mice (23 week-old) were launched on board the Bion-M1 biosatellite to be exposed to weightlessness during a 30-day spaceflight (~570 km orbit) (20). Proteomics analysis was performed on the right femur of four mice euthanized by cervical dislocation 13-16 h after landing (Flight group), four mice put to death after a one-week recovery period on Earth (Recovery group) and five control mice retained on Earth under space housing and feeding conditions (Ground Control group). These femurs were dissected immediately after sacrifice and frozen at  $-80$  °C until proteome analysis. The right tibia of 4-6 other mice from each of these 3 groups were also dissected after sacrifice and immediately flushed with RPMI-1640 medium to collect bone marrow cells that were directly analysed by flow cytometry. The same analysis was performed on their spleen.

### **Hypergravity experiment: animals and study design**

Eight-week-old C57/BL6 male mice of a mean body mass of 20 g were purchased from Charles River (Bois des Oncins, France). Upon arrival, they were housed by groups of four in standard cages with food and water *ad libitum*, in a quiet room with constant temperature (22 °C), 50% relative humidity, and 12-h light/dark cycles (dark period 8 pm-8 am) during a week for acclimation. Then, half of the cages were placed in the gondolas of the CNES large radius centrifuge for hypergravity exposure at 2 or 3g (n=12 at 2g; n=8 at 3g), according to Guéguinou et al. (21). Mice were supplied with enough food and water for three weeks, so that the centrifuge was operating continuously. Mice were left undisturbed during the three weeks of chronic centrifugation. All environmental variables, except the gravity level, were the same as in standard housing. Half of the cages, containing control mice (n=12 for the control of the 2g experiment; n=8 for the control of the 3g experiment), were placed in the same gondolas in the same room than centrifuged mice, but in a static position. At the end of the 21 days of centrifugation, control and hypergravity mice were put to death by cervical dislocation, and the liver was collected and stored at -80°C until western blotting analysis.

### **Analysis of femur proteome from Bion-M1 mice**

The femurs from Flight (n=4), recovery (n=4) and Ground control (n=5) mice were analysed individually. Frozen mid distal part of right femurs were weighed and grinded using a ball mill (2 × 30 s @ 25 Hz; MM400, Retsch) under liquid nitrogen and proteins were extracted using 10 volumes (i.e. 10 µL/mg of tissue) of extraction buffer (8 M urea, 2 M thiourea, 2% CHAPS, 2% DTT, 50 mM Tris pH 8.8, protease inhibitors). After solubilisation for 30 min at 37 °C, samples were centrifuged (5 min, 4°C, 15000 x g) to remove possible cell/tissue debris. After TCA-precipitation of proteins overnight at 4°C and a centrifugation (10 min, 4°C, 15000 x g), pelleted proteins were washed using cold acetone and re-solubilised in the extraction buffer without reducing agents and detergents. Protein concentration was then determined by a Bradford assay (Bio-Rad, Hercules, CA, USA). At this stage, a sample pool comprising equal amounts of all protein extracts was made, to be repeatedly analysed during the whole duration of nanoLC-MS/MS experiments to allow QC-related measurements. Reproducibility of protein electrophoretic profiles was checked for all protein extracts after dilution of 30 µg of proteins in Laemmli buffer (10mM Tris pH 6.8, 1mM EDTA, 5% β-mercapto-ethanol, 5% SDS,



10% glycerol, bromophenol blue), denaturation at 95°C, and SDS-Page electrophoresis on 12% acrylamide gels.

30 µg of each protein extract were mixed with 4x SDS sample buffer (1x: 50 mM Tris pH 6.8, 1 mM EDTA, 5 % β-mercapto-ethanol, 2.5 % SDS and 10 % glycerol), incubated at 95°C for 5 minutes and run for 15 minutes at 35 V into a 4 % polyacrylamide stacking gel in order to let proteins enter approximately 12 mm into the gel. Electrophoresis was stopped at this point and gels were stained by Colloidal Coomassie Blue. Both the “stacked” protein band and the part of the gel (separated into 3 slices) above this band were excised, with the stacked band being afterwards divided into 4 pieces and the 3 slices into 8 pieces each before in-gel digestion using an automatic pipetting device (MassPrep, Waters, Milford, MA). Following de-staining, reduction (DTT) and alkylation (iodoacetamide), proteins were digested overnight at 37 °C using 300 ng of trypsin for the stacked band and 100 ng for slices coming from the upper part of the gel. Peptides were then extracted in 60 % acetonitrile/0.1 % formic acid in water for one hour then in 100 % acetonitrile (ACN) for 10 minutes at 450 rpm on an orbital shaker, and the three peptide extracts coming from the upper part of the gel were pooled. At this stage, a set of reference peptides (iRT kit; Biognosys AG, Schlieren, Switzerland) was added to each sample to enable following the stability of instrument performances (QC-related measurements). Just before LC-MS/MS analysis, the volume of peptide extracts was reduced in a speed-vac and sample volumes were adjusted to 60 µl with 1% ACN, 0.1 % formic acid in water.

Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive plus, Thermo Scientific, San Jose, CA). Two µl of each sample were concentrated/desalted on a trap column (Symmetry C18, 180 µm x 20 mm, 5 µm; Waters) using 99 % of solvent A (0.1 % formic acid in water)/1 % solvent B (0.1 % formic acid in ACN) at a flow rate of five µl/min for 3 minutes. Afterwards, peptides were transferred and eluted from the separation column (BEH130 C18, 250 mm x 75 µm, 1.7 µm; Waters) maintained at 60 °C using a 150 minutes gradient from 1-40 % of B. All samples were injected using a randomized and blocked injection sequence (one biological replicate of each group plus the pool in each block). To minimize carry-over, a column wash (50% ACN during 20 minutes) was included in between each block in addition to a solvent blank injection, which was performed after each sample.

The Q-Exactive Plus was operated in positive ion mode with source temperature set to 250°C and spray voltage to 1.8 kV. Full scan MS spectra (300-1800 m/z) were acquired at a resolution of 140,000 at m/z 200, a maximum injection time of 50 ms and an AGC target value of  $3 \times 10^6$  charges with the lock-mass option being enabled (445.12002 m/z). Up to 10 most intense precursors per full scan were isolated using a 2 m/z window and fragmented using higher energy collisional dissociation (HCD, normalised collision energy of 27eV) and dynamic exclusion of already fragmented precursors was set to 60 sec. MS/MS spectra were acquired with a resolution of 17,500 at m/z 200, a maximum injection time of 100 ms and an AGC target value of  $1 \times 10^5$ . The system was fully controlled by the XCalibur software (v3.0.63; Thermo Fisher Scientific).

MS raw data were processed using MaxQuant (version 1.5.3.30). Peak lists were created using default parameters and searched using the Andromeda search engine (revert mode) implemented in MaxQuant against a protein database created using the MSDA software suite (22). The database contained mice protein sequences (Swissprot; Taxonomy ID: 10090; 16873 entries), which were downloaded in May 2017. Sequences of common contaminants like keratins and trypsin (247 entries) were finally added to the database (contaminants.fasta included in MaxQuant). The first search was performed using a precursor mass tolerance of 20 ppm, and 4.5 ppm for the main search after recalibration. Fragment ion mass tolerance was set to 20 ppm. Carbamidomethylation of cysteine residues was considered as fixed and oxidation of methionine residues and acetylation of protein N-termini as variable modifications during the search. A maximum number of two missed cleavages and a false discovery rate (FDR) of 1% for both peptide spectrum matches (minimum length of seven amino acids) and proteins was accepted during identification. Regarding quantification, data normalisation and protein abundance estimation was performed using the MaxLFQ (label free quantification) option implemented in MaxQuant (23) using a “minimal ratio count” of one. “Match between runs” was enabled using a 3-minute time window after retention time alignment. Both unmodified and modified (acetylation of protein N-termini and oxidation of methionine residues) peptides were considered for quantification while shared peptides were excluded. All other MaxQuant parameters were set as default. Proteins identified with only one unique peptide were not considered for quantification. Only proteins with at least three of four or five valid values per group as well as the ones “absent” (i.e. 0 valid values) in samples from a given group were kept for further analysis. Relative protein abundances (i.e.

normalized LFQ-values, see above) are expressed as means  $\pm$  SEM of 3-5 determinations per group (arbitrary units; values in the Flight and Recovery groups normalized to those in the Ground control group, which were arbitrarily set to 1). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (24) partner repository with the dataset identifier PXD010114.

QC-related measurements showed that HPLC performance remained very good and stable throughout the whole experiment, with a median coefficient of variation (CV) of 0.7% concerning retention times of all iRT peptides when considering all injections. The reproducibility of quantitative data was also satisfactory as we recorded low median CVs for the raw intensity of iRT peptides in all injections (13%) and for Maxquant-derived LFQ values of all quantified proteins within each of the three experimental groups (18.6%) and in the sample pool of all samples injected repeatedly during the course of MS-based analyses (13%).

#### **Lymphocytes in the bone marrow and spleen from Bion-M1 mice**

Bone marrow was flushed from the right tibia of Flight (n=5), Recovery (n=4) and Ground control (n=6) mice with 1 ml of complete RPMI-1640 medium. Spleens (n=5 for Flight and Recovery mice and n=6 for Ground control animals) were dissociated in the same medium. For immunophenotyping,  $10^5$  cells were incubated for 30 min at 4°C in the dark with fluorescence-labeled monoclonal antibodies (anti-CD19 PE; eBio 1D3), anti-CD3e FITC (145-2C11), anti-CD4 PE (GK1.5), anti-CD8a PerCy5.5 (53-6.7) purchased from eBioscience (San Diego, CA, USA) and Mouse Seroblock FcR reagent (Bio-Rad, Hercules, CA, USA). Then, erythrocytes were lysed using the OptiLyse C solution (Beckman Coulter, Brea, CA, USA). Labelled cells were washed twice with FACS buffer and resuspended in 500  $\mu$ l of 1% paraformaldehyde (in PBS + 0.1% sodium azide). At least  $10^4$  events per tube were acquired using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Analysis was performed using the CellQuest software (Becton Dickinson). Data are presented as mean percentages  $\pm$  SEM of lymphocytes expressing relevant cell surface antigen markers.

#### **Analysis of relative C3 levels in the liver of centrifuged mice**

The liver from centrifuged (n=11 at 2g; n=7 at 3g) and control (n=11 for the control of the 2g experiment; n=7 for the control of the 3g experiment) mice were analysed individually. Proteins were prepared by lysing a piece of mice liver in lysis buffer (15 mM Tris-HCl pH 7.4,

150 mM NaCl, 1 mM EDTA, 1% Triton X-100). 40 µg of total proteins were heated at 95°C for 5 minutes, run on 8% SDS-polyacrylamide gels and electrotransferred to PVDF membranes (Amersham, Buckinghamshire, UK). Membranes were incubated with antibodies against complement C3 alpha chain (GTX101316, Tebubio, Le Perray-en-Yvelines, France), analyzed, stripped with stripping buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and reprobed against  $\alpha$ -tubulin used as a housekeeping protein for C3 level normalization (clone EP1332Y, Abcam, Cambridge, UK) and a horseradish peroxidase-conjugated rat anti-rabbit secondary antibody. Immunodetection was performed using the Pierce ECL western blotting Substrate (Thermo Fisher Scientific, Illkirch, France). Signals were visualized by chemiluminescence (FX7, Vilbert-Lourmat, Marne la Vallée, France) and analyzed by densitometry (ImageJ<sup>®</sup>, NIH, USA). Relative C3 levels (i.e. normalized to  $\alpha$ -tubulin levels) are expressed as means  $\pm$  SEM of 7-11 determinations per group (arbitrary units; values in hypergravity groups normalized to those in the respective control groups, which were arbitrarily set to 1).

#### **Bioinformatics analysis of proteomics data**

Quantified proteins were functionally categorized on the basis of literature examination and their annotations in the Gene Ontology (April 2018), KEGG (April 2018) and HGNC (September 2017) databases. Retrieving of human proteins that are homologous to the murine proteins quantified in the current study was performed using Blast searches against Swiss-Prot-derived *Homo sapiens* protein sequences (TaxID 9606; March 2018) using the FASTA program (v36; downloaded from [http://fasta.bioch.virginia.edu/fasta\\_www2/fasta\\_down.shtml](http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml); only the best hits were retained). On the basis of these human protein identifiers, extraction of 'target tissue expression' information from the neXtProt database (April 2018) was performed using Sparkl requests provided by the neXtProt team.

#### **Statistical analyses**

Statistical analysis of proteomics quantitative data of individual mice was performed under R software environment v3.4.0 (25). Abundance values obtained for the thirteen mice (Flight, n=4; Recovery, n=4; Ground control, n=5) were considered individually, no sample combination was made. Normality of MaxLFQ values distribution and homoscedasticity were checked using Shapiro-Wilk and Bartlett tests ( $P > 0.01$ ), respectively. Changes in protein

abundances among mice groups were tested using 1-way ANOVA with Tukey as post hoc tests, including adjustment of p-values according to Bonferroni-Holm. Significance was set to  $P < 0.05$ .

The StatView software (SAS Institute Inc, Cary, NC, USA) was used to perform statistical analyses of western blotting and flow cytometry data. Homogeneity of variance was determined using the Fisher test (2 groups) or Levene test (3 groups) and the normality of distribution was determined using the Kolmogorov-Smirnov test. When homogenous variances and distributions were observed, t-test (2 groups) or one-way ANOVAs (3 groups) followed by the Tukey-Kramer *post-hoc* test were done. When the variance and distribution were not homogeneous, Mann-Whitney (2 groups) or Kruskal Wallis (3 groups) non-parametric tests were performed. Significance was set to  $P < 0.05$ .

## Results

### Overview of proteomics data

The robust identification of 3254 proteins was achieved, of which 3063 fulfilled the criteria (see the Materials and Methods section) for MS1 intensity-based label-free quantification (Table S1). Statistical analysis of individual mice data indicated that the abundance of 57 and 29 proteins was, respectively, significantly decreased and increased in flown mice versus Ground Control animals (Figure 1, see also Tables S2 and S3). In the Recovery compared to the Flight group, the abundance of 70 and 31 proteins was significantly lower and higher, respectively. Taking a closer look at the effects of the Recovery period, we observed that abundance levels of six proteins downregulated in the Flight group were normalized in Recovery animals. The restoration of abundance levels of nine proteins upregulated in flown mice was also observed in the Recovery group. Additional changes in protein abundance were observed one week after landing despite no change in flown versus control mice, with 26 down-regulated and three up-regulated proteins in Recovery versus Flight animals. Moreover, 150 and 83 proteins were significantly less and more abundant in Recovery than in Ground Control mice, respectively. These proteins notably correspond to 26 proteins downregulated in the Flight condition and 9 proteins upregulated in flown mice versus Ground controls, the abundance of which was not restored by a week of Recovery. The remaining 124 and 74 proteins correspond to proteins not significantly differential between the Flight and Ground control condition. The abundance levels of 35 and 22 of them were significantly lower and higher in Recovery versus Flight mice, respectively, thus indicating that these 57 proteins were specifically regulated during the week of recovery. In addition, for proteins for which significance was not reached in Flight versus Control and Recovery versus Flight comparisons, 89 and 52 of the proteins were down- and up-regulated in Recovery versus Ground control mice, respectively.

Functional annotations (Nextprot) were available for 2425 out of the 3063 quantified proteins, i.e. 79%, of which 2331 were annotated as being detected in the bone marrow and only 94 in the bone only (see Table S1). The proteome we analysed is thus obviously mostly that of bone marrow, and that is the reason why we hereafter discuss our results based on this assumption.

### Proteins involved in the development of immune cells and skeletal system

Among differentially expressed proteins, several are known to be involved in the development of immune cells and skeletal system (Figure 2). Interestingly, they followed very similar regulations, with aminopeptidase N (Anpep), guanine nucleotide-binding protein G(q) alpha (Gnaq), SPARC (or osteonectin), tyrosine-protein kinase SYK, and E3 ubiquitin-protein ligase Trim33 remaining unchanged after the 30-day flight compared to the control situation and then being 1.3 to 2.3 times downregulated after one week of recovery post-flight. The 1.3 to 3.2-fold decrease in Recovery versus Flight mice of abundances of also core-binding factor beta (Cbfb), collagen alpha-1(II) chain (Col2a1), collagen alpha-2(XI) chain (Col11a2), and collagenase 3 (Mmp13) was very close to significance. Finally, the levels of staphylococcal nuclease domain-containing protein 1 (Snd1) were 1.3 times decreased in flown mice and remained lowered in Recovery animals, and those of enhancer of rudimentary homolog (Erh) were non-significantly reduced by the Flight and recovery period in a gradual manner, which resulted in a significant 1.4-fold decrease in Recovery compared to Ground control mice. Taken together, these data suggest that the development of immune cells could be reduced one week after landing. To check this hypothesis, we determined the percentages of B cells in the bone marrow. B and T cell percentages were also determined in the spleen. As shown in Figure 3, the percentage of B cells was not affected at landing in bone marrow, while a small but significant decrease was observed in the spleen. However, 1.8-2.5 reductions were observed in both tissues after one week of recovery. The decrease of splenic B cells observed at that time point was compensated by an increase of T cells (CD3+CD4+; Figure 3).

### **Proteins involved in apoptosis**

Among differential proteins, we identified only 4 apoptosis-related factors (Figure 2; see also Table S1). The levels of Bcl-2 homologous antagonist/killer (Bak1) were significantly decreased (3 times) in flown versus control mice and were not restored after one week of recovery. The abundance of programmed cell death protein 4 (Pcd4) and serine/threonine-protein kinase 24 (Stk4) was not changed by the flight, but was significantly reduced (1.4 times) in the Recovery compared to Flight group. Finally, we recorded a 1.2-fold decrease of the expression levels of RNA-binding protein 25 (Rbm25) in flown mice which was close to but did not reach significance ( $P=0.056$ ).

### **Proteins involved in the coagulation and complement cascades**

The abundance of ten proteins of the coagulation cascade was found to be significantly changed in Bion-M1 mice, with essentially two types of profiles (Figure 4A). The levels of a first set of proteins, including coagulation factors X (F10), IX (F9), and VII (F7), and plasminogen (Plg), remained unchanged in flown versus ground control mice, while they were decreased (1.6-2.1 times) after the recovery period. The levels of a second set of proteins, including antithrombin-III (Serpinc1), alpha-1-antitrypsin 1-2 (Serpina1b) and 1-4 (Serpina1d), and alpha-2-antiplasmin (Serpinf2), were 1.3-1.4 times higher in Flight versus ground control animals and were then restored by the recovery period. Finally, expression levels of beta-2-glycoprotein 1 (ApoH) and vitamin K-dependent protein Z (Proz) tended to be 1.2-1.3 times increased by the flight (non-significant), while they were 1.6-2.1 times decreased during the recovery period.

We also observed changes in the abundance of four factors of the complement cascade (Figure 4B). The levels of C1q subcomponent subunits A (C1qa) and B were significantly 1.6-2.2 times lower in flown versus ground control mice; the levels of C1qa were then fully restored by the recovery period and those of C1qb only partially. Plasma protease C1 inhibitor (Serping1) levels were unchanged by the flight but 1.6 times decreased after one week of recovery at 1g. Finally, a twofold increase of complement C5 (C5) abundance was noted at landing and this change was not restored in Recovery mice. Western blotting experiments revealed a similar overexpression (1.4-1.8 increase) of complement C3 (C3) in mice subjected to increased g force during 21 days (Figure 5), confirming that this cascade is sensitive to gravity changes.

#### **Proteins involved in immune response**

The abundance of several immunoglobulin peptides (Ig kappa chain V-II region 7S34.1, Ig kappa chain V-V region K2, Ig kappa chain C region, and Ig gamma-2A chain C region secreted form) was found to be increased (1.4-2.4 times) in Flight versus Ground Control mice, while it was restored in Recovery animals (Figure 6). We also observed that the levels of Ig gamma-2B chain C region were not affected by the flight while they were 1.6 times decreased in Recovery versus Flight mice (Figure 6; see also Table S1).

Changes in the abundance of other proteins involved in immune response were also recorded (Figure 6). The levels of Bcl-2 homologous antagonist/killer (Bak1), Probable ATP-dependent RNA helicase DDX58, H-2 class I histocompatibility antigen (H2-D1 and/or H2-L), stomatin-like



protein 2 (Stoml2), immunity-related GTPase family M protein 1 (Irgm1), and syntaxin-binding protein 2 (Stxbp2) dropped (1.3-3.0 times) in Flight versus Ground Control mice and no or weak restoration was observed after the recovery period. The levels of aminopeptidase N (Anpep), drebin-like protein (Dbnl1), exosome component 10 (Exosc10), guanine nucleotide-binding protein G(q) alpha (Gnaq), nuclear factor NF-kappa-B p105 subunit (Nfkb1), deoxynucleotide triphosphate triphosphohydrolase (Samhd1), signal transducer and transcription activator 6 (Stat6), tyrosine-protein kinase SYK, and tapasin (Tapbp) only dropped (1.3-1.5 times) in Recovery animals. The abundance of serine/threonine-protein kinase 10 (Stk10) and DNA mismatch repair protein (Msh2) was non-significantly reduced by the Flight and recovery period in a gradual manner, which resulted in significant 1.8- and 1.5-fold decreases in Recovery compared to Ground control mice, respectively. Finally, expression levels of C->U-editing enzyme APOBEC-2 were increased (1.9 times) by the Flight and remained at high levels after one week of recovery, while those of UBX domain-containing protein 1 (Ubx1) were only increased (1.6 times) in Recovery animals.

#### **Proteins involved in protein-synthesis-related processes**

A high number of the differential proteins found here are known to play a role in protein-synthesis-related processes (Figure 7 and Table S1). Changes of few additional proteins that fall into this category were close to significance; they are not described or discussed here but can be seen in Supplementary Table 1.

First about mRNA synthesis and spliceosome, we observed a 1.3-1.4-fold downregulation of signal transducer and transcription activator 6 (Stat6) and transcription elongation factor A protein 1 (Tcea1) in Recovery mice only. The levels of FACT complex subunit SSRP1, pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15, Pre-mRNA-processing factor 19 (Prpf19), spliceosome RNA helicase Ddx39b, ATP-dependent RNA helicase DDX1, 116 kDa U5 small nuclear ribonucleoprotein component (Eftud2), and RNA-binding proteins 25 (Rbm25) and 39 (Rbm39), were reduced (1.3-1.9 times) both in Flight and Recovery mice versus Ground control animals. The downregulation (1.3-1.8-fold) of U4/U6 small nuclear ribonucleoprotein Prpf4, U5 small nuclear ribonucleoprotein 40 kDa protein (Snrnp40), small nuclear ribonucleoprotein F (Snrpf), serine/arginine-rich splicing factor 1 (Srsf1), nuclear cap-binding protein subunit 1 (Ncbp1), ATP-dependent RNA helicase DDX39A, and heterogeneous nuclear ribonucleoproteins U (Hnrnpu) and M (Hnrnpm) in Flight and Recovery mice versus Ground

Control animals was more gradual, reaching significance only after the recovery period. The twofold decrease of the levels of superkiller viralicidic activity 2-like 2 (Skiv2l2) in flown mice was only partially restored after one week of recovery, contrary to the 1.8-fold increase of the levels of zinc finger CCHC domain-containing protein 8 (Zcch8) that fully recovered. In addition, serine/arginine-rich splicing factor 3 (Srsf3) levels were twice higher in Recovery than in control mice, and those of transcription factor BTF3 homolog 4 (Btf3l4) were 1.8-fold higher in both Flight and Recovery mice compared to control animals.

Regarding tRNA biosynthesis, the trend towards lower (1.2 times) levels of cytoplasmic aspartate-tRNA ligase (Dars) in Flight and Recovery versus control mice was close to reach significance ( $p = 0.06-0.07$ ). Among proteins involved in ribosome biogenesis, decreased levels (up to 1.4 times) of nucleolar protein 56 (Nop56), 60S ribosomal protein L15 (Rpl15), casein kinase II subunit alpha (Csnk2a2), and exosome component 10 (Exosc10) were observed especially in Recovery mice. The 1.4-fold drop in the abundance of nucleolar transcription factor 1 (Ubtf) was significant both in Flight and Recovery mice versus Ground control animals, and the mild 1.2-fold reduction in 60S ribosomal protein L3 (Rpl3) levels in flown mice was partially restored at the end of the recovery period. Expression levels of three proteins only, namely 60S ribosomal protein L17 (Rpl17), 40S ribosomal protein S17 (Rps17) and S19 (Rps19), were increased (up to 1.6 times) in the Recovery versus control condition. Of the proteins that are involved in RNA export from the nucleus to the cytoplasm, the levels of GTP-binding nuclear protein Ran, SUMO-conjugating enzyme UBC9 (Ube2i), and nuclear pore complex protein Nup98-Nup96 (Nup98) were essentially reduced (1.2-1.4 times) only after the recovery period.

Concerning factors involved in post-transcriptional events, we observed a significant 1.2-fold reduction in the levels of eukaryotic translation initiation factor 2 subunit 3, X-linked (Eif2s3x) in Recovery compared to Ground Control mice. Main alterations were found with regard to factors involved in protein processing within the endoplasmic reticulum. Among folding proteins, the levels of neutral alpha-glucosidase AB (Ganab), cytoskeleton-associated protein 4 (Ckap4), and dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit (Ddost) were similarly reduced (1.2-1.4 times) in Flight and Recovery mice compared to control animals. The levels of calnexin (Canx), 78 kDa glucose-regulated protein (Hspa5), endoplasmic chaperone (Hsp90b1), and vesicular integral-membrane protein VIP36 (Lman2) were also reduced (1.2-1.4 times) by the flight but then were restored after the recovery period. Most

of the factors that contribute to eliminate misfolded proteins, including Bcl-2 homologous antagonist/killer (Bak1), heat shock protein 105 kDa (Hsph1), heat shock cognate 71 kDa protein (Hspa8), and heat shock protein HSP 90-alpha (Hsp90aa1) and -beta (Hsp90ab1), were downregulated by the flight (up to three times) with all of them except Bak1 being normalized to control values after one week post-flight at 1g. Finally, the abundance of alpha-crystallin B chain (Cryab) was found to be increased (3-4 times) in recovery versus Flight and Ground Control animals.

## Discussion

Our proteomics studies suggested a reduction of immune cell development after one week of recovery post-flight (Figure 2), which was confirmed at the cellular level for B cells. Given that the expression of only four apoptosis-related factors was affected and that no increase of their expression was noted after the flight or recovery period, we do not believe that the decrease of immune cell production in the bone marrow, including B cells, is due to this process. Our flow cytometry data also revealed a decrease of B cell percentage in the spleen after the recovery period and suggested the occurrence of an in-flight infection.

In support of the reduction of immune cell development, it was previously shown that Bion-M1 flight induced a 64% reduction in femur trabecular volume of the same mice that was not restored by the recovery period (26), and that their ankle bone volume loss is aggravated after one week of Earth reambulation (27). From our previous studies showing that 21 days of hindlimb unloading (HU) cause bone remodeling and a concomitant decrease in B lymphocyte maturation in the bone marrow of the same mouse strain (15), we expected that immune cell development would be affected already at landing because it is established that spaceflight induces bone loss (28). However, this was not the case. This could be due to differences in bone alteration kinetics between HU and real spaceflight or the age of animals since HU mice (15) were younger (3 months) than Bion-M1 mice. Impaired B lymphopoiesis could also result from differences in the imbalance between bone formation and resorption as it was shown that bone loss results from reduced bone formation during HU (29) and from increased osteoclastic resorption during spaceflight (26). However, despite this remark, these two studies clearly demonstrate that spaceflight conditions have a negative effect on B lymphopoiesis. This conclusion, combined with studies having shown that myelopoiesis is reduced under spaceflight conditions (30-33) and data about T lymphopoiesis presented in the introduction of this paper, demonstrate that spaceflight negatively affects the development of most immune cells.

Our study also revealed a 41% reduction of B cells content in the spleen. A 59% decrease of B cells was also noted in the spleen of HU mice (34). This stronger reduction could be due to the stronger or more rapid effect of HU by comparison to spaceflight, or to other assumptions made above. The lower number of B cells in the spleen could contribute, at least partially, to

explain the higher susceptibility to infection described in astronauts (2) and mice subjected to HU (35, 36).

Statistical analysis of proteomics data from individual mice highlighted changes in immunoglobulin abundances suggesting the occurrence of an in-flight infection having induced the production of plasma cells secreting IgG2 containing kappa light chains made from two different V $\kappa$  segments. Indeed, peptides 'Ig kappa chain V-II region 7S34.1' and 'Ig kappa chain V-V region K2' correspond to the IGKV2-137 and IGKV12-41 gene segments, respectively. This infection could perhaps result from injuries (bites/cuts) that occurred during this automatized mission as decreases or normalizations of expression levels of 10 proteins of the coagulation cascade were noted one week after landing. Whatever the origin of this infection, this observation confirms that a humoral immune response can be mounted in space, as previously shown in an amphibian species (5-7). However, we could not evaluate its quality because the nature of the antigen(s) that induced this response is unknown. Recovery after landing indicates healing facilitated by normal housing conditions and readaptation to Earth gravity following deconditioning. We believe that this infection is unlikely responsible for changes in immune cells development at the end of the recovery period because the mobilization of these cells in response to infection should be followed by a new synthesis and therefore an increase of lymphopoiesis/myelopoiesis in the bone marrow.

Many other proteins involved in immune response were affected (Figure 6) showing that the negative effects of spaceflight on the immune system persist during at least one week after return to Earth. In the same way, changes in amphibian immune response were still observed 10 days after landing (5-7). Note however that this inhibition was not complete since mice and amphibians were still able to produce antibodies in response to an antigen challenge.

Besides, proteomics data revealed that changes in C5 levels, a major inflammation mediator, were not exclusively linked to the potential in-flight infection. Indeed, we noted similar changes in C3 expression, another mediator of inflammation acting upstream of C5, in response to hypergravity. Taken together these data suggest that hypergravity associated to landing modulates the expression of complement molecules and could explain why Baqai et al. reported that exposure to the spaceflight environment can increase anti-inflammatory mechanisms in mice (37).

Finally, a high number of proteins known to play a role in protein-synthesis-related processes, from mRNA synthesis to the processing of proteins in the endoplasmic reticulum, were

expressed at reduced levels after the recovery. These data are in agreement with previous studies having shown that immune cell signal transduction and transcription are disturbed by spaceflight conditions (38-40) and demonstrate that these processes require more than one week to return to homeostasis.

In conclusion, this study demonstrates that spaceflight negatively affect B cell development in the bone marrow. Combined with previous studies about T cell maturation and myelopoiesis, it thus appears that spaceflight impair the maturation of a vast array of immune cells. This reduction of B cells production, coupled to the 41% reduction of splenic B cells content, could contribute to explain higher susceptibility to infection despite the fact that a humoral immune response can still be mounted in space, as our data suggested a possible in-flight infection. Additionally, we noted that the effects of spaceflight persist during at least one week after return to Earth. In the future, it would be important to determine, through kinetics studies, the time required by the immune system to fully recover. It would also be interesting to determine if immune alterations are worsening as a function of mission duration and ensure that the quality of antibodies produced by mammals in response to an infection is not affected. Finally, this study illustrates the importance of taking into account connections between physiological systems, here the skeletal and immune systems.

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## **Competing interests**

We declare no competing interests.

## **Author contributions**

G. Gauquelin-Koch, L. Vico, J.P. Frippiat. and F. Bertile conceived the study. F. Bertile supervised proteomics analyses. G. Tascher, P. Maes and B. Chazarin conducted mass spectrometry-based analyses. M. Gerbaix critically assisted them in data analysis. S. Ghislin and N. Ouzren-Zarhloul performed western blot studies. E. Antropova and G. Vasslieva performed flow cytometry studies. J.P. Frippiat and F. Bertile interpreted data and drafted the manuscript. All authors made comments and gave final approval for publication.

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## Figure legends

### **Figure 1. Expression profiles of differentially expressed proteins**

From quantitative proteomics data, changes in the abundance of 284 differential proteins are schematized here into ten different expression profiles (see Table S1 for protein details). Numbers of proteins are given in the dark and bright grey boxes, which indicate significant (SIG) and non-significant (NS) changes in Flight and Recovery mice relative to Ground control (GC) animals. \* Indicates significance vs. Flight.

### **Figure 2. Quantification of proteins involved in immune cell and skeletal system development, and in apoptosis using proteomics**

From quantitative proteomics data, changes in the abundance (means  $\pm$  SEM of 3-5 determinations per group) of fifteen proteins known to be involved in immune cell development (panel A), skeletal system development (panel B), and apoptosis (panel C) were found to be significant ( $P < 0.05$  when bars do not share the same superscript letter). P-values are given when changes are close to significance (see Table S1 for protein details). \* indicates proteins also involved in skeletal system development.

### **Figure 3. Percentages of B lymphocytes in the bone marrow, and of B and T cells in the spleen of Bion-M1 mice.**

Results obtained with bone marrow (panel A) and spleen (panel B) are shown as means  $\pm$  SEM. Statistically significant differences between groups, as revealed using an ANOVA test ( $P_{ANOVA} < 0.005$ ) and its associated *post-hoc* test, are indicated by an 'S'. GC, F, and R: Ground Control, Flight, and Recovery mice, respectively.

### **Figure 4. Quantification of proteins involved in the coagulation and complement cascades using proteomics**

From quantitative proteomics data, changes in the abundance (means  $\pm$  SEM of 3-5 determinations per group) of fourteen proteins known to be involved in the coagulation (panel A) and complement (panel B) cascades were found to be significant ( $P < 0.05$  when bars do

not share the same superscript letter). Proteins that were detected but not significantly changed are shown in blue, those not detected in black (see Table S1 for protein details).

**Figure 5. Quantification of complement C3  $\alpha$ -chain in the liver of mice exposed during 21 days to an hypergravity of 2g (A) or 3g (B).**

Complement C3 expression was studied by Western blotting. Expression levels were normalized to  $\alpha$ -tubulin. Values obtained with control mice were set to 1. Results are expressed as means  $\pm$  SEM. "A.U." stands for "arbitrary units". Statically significant differences were revealed using an unpaired t-test in (A) and a Mann-Whitney non-parametric test in (B).

**Figure 6. Quantification of proteins involved in immune response using proteomics**

From quantitative proteomics data, changes in the abundance (means  $\pm$  SEM of 3-5 determinations per group) of five immunoglobulins (panel A) and 18 other proteins known to be involved in immune response (panel B) were found to be significant ( $P < 0.05$  when bars do not share the same superscript letter). P-values are given when changes are close to significance (see Table S1 for protein details). Note: Anpep, Gnaq, and Syk (illustrated in Figure 2) are also involved in immune response.

**Figure 7. Quantification of proteins involved in protein synthesis-related processes using proteomics**

From quantitative proteomics data, changes in the abundance (means  $\pm$  SEM of 3-5 determinations per group) of 54 proteins known to be involved in protein-synthesis-related processes were found to be significant ( $P < 0.05$  when bars do not share the same superscript letter). Only few of these proteins are illustrated here to show the different expression profiles that were observed (see the main text and Table S1 for details on all 54 proteins). ER: endoplasmic reticulum.

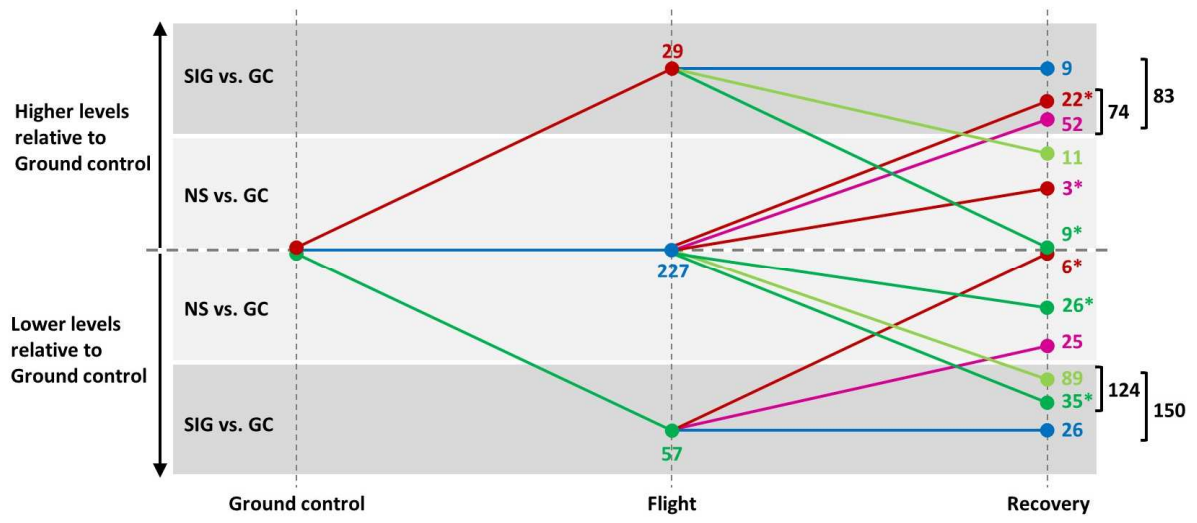
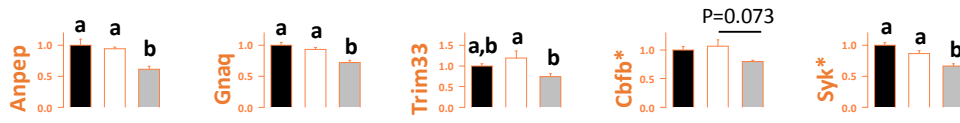
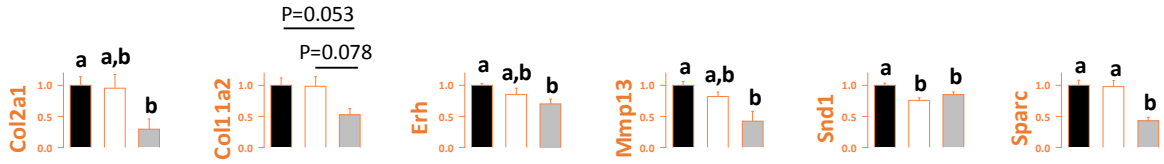


Figure 1.

## A. Immune cell development



## B. Skeletal system development



## C. Apoptosis

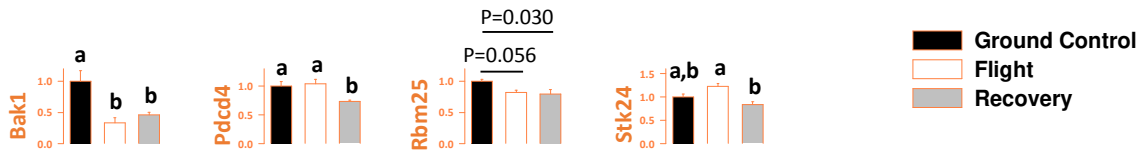
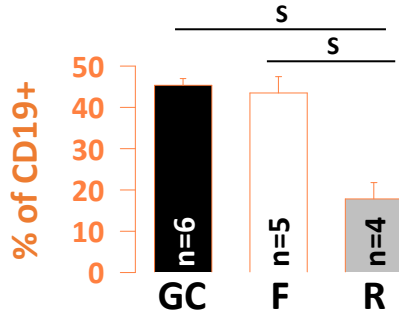


Figure 2.

### A. Bone marrow



### B. Spleen

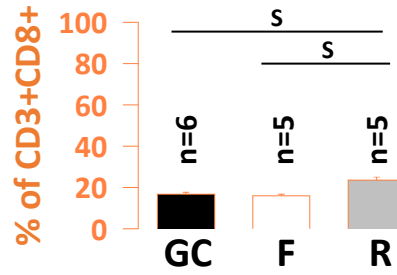
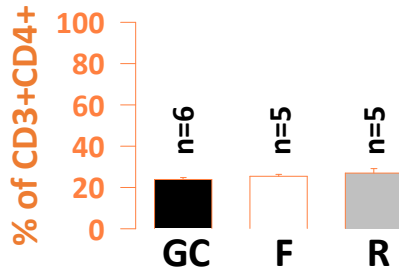
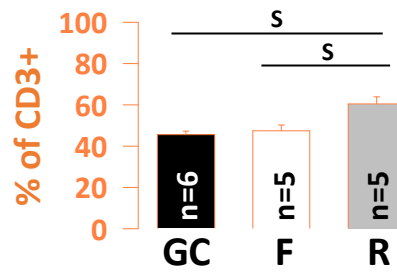
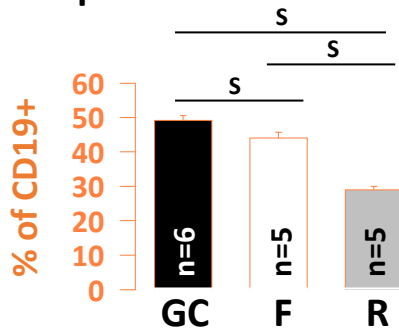
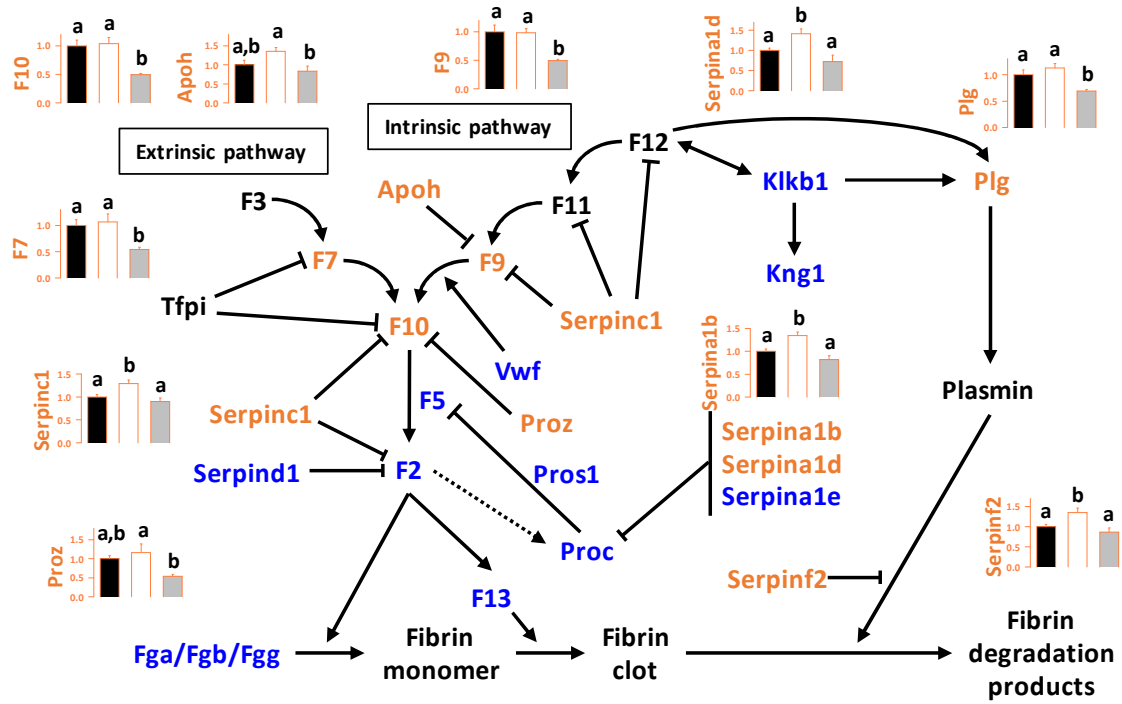


Figure 3.

### A. Coagulation cascade



### B. Complement cascade

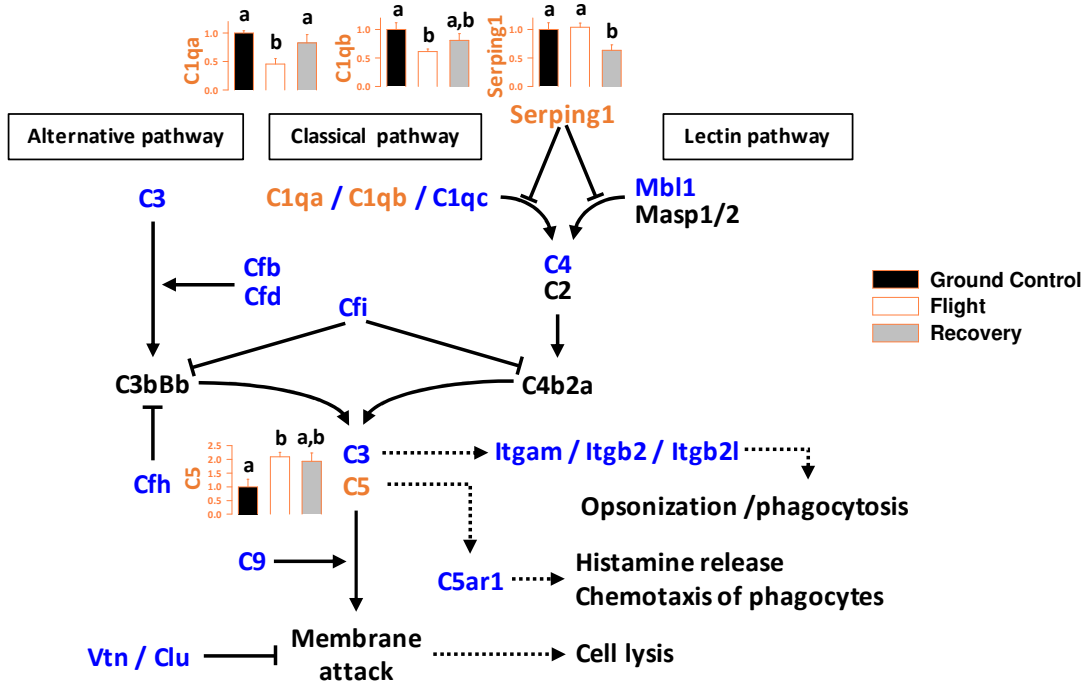


Figure 4.



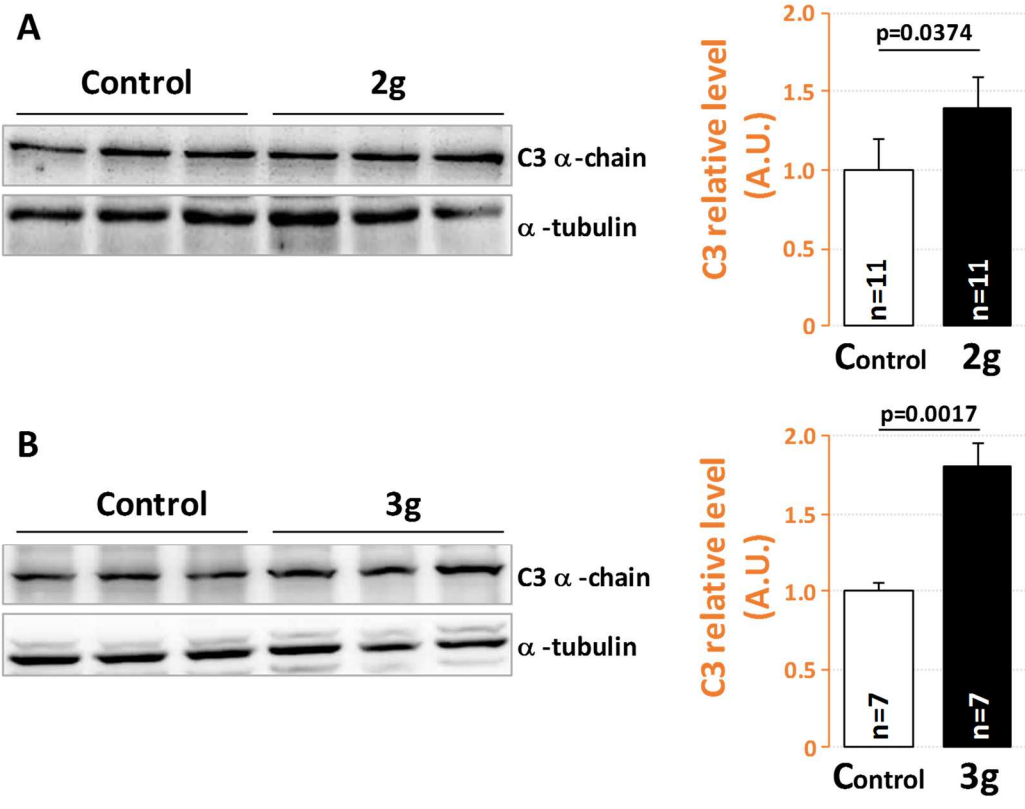
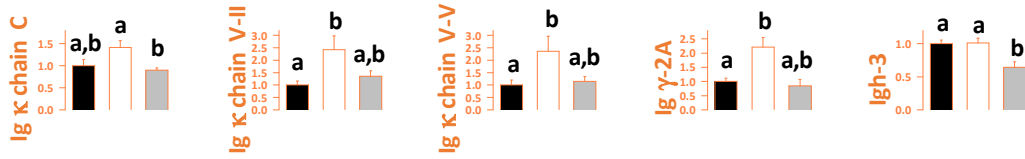


Figure 5.

## A. Immunoglobulins



## B. Other immune-related proteins

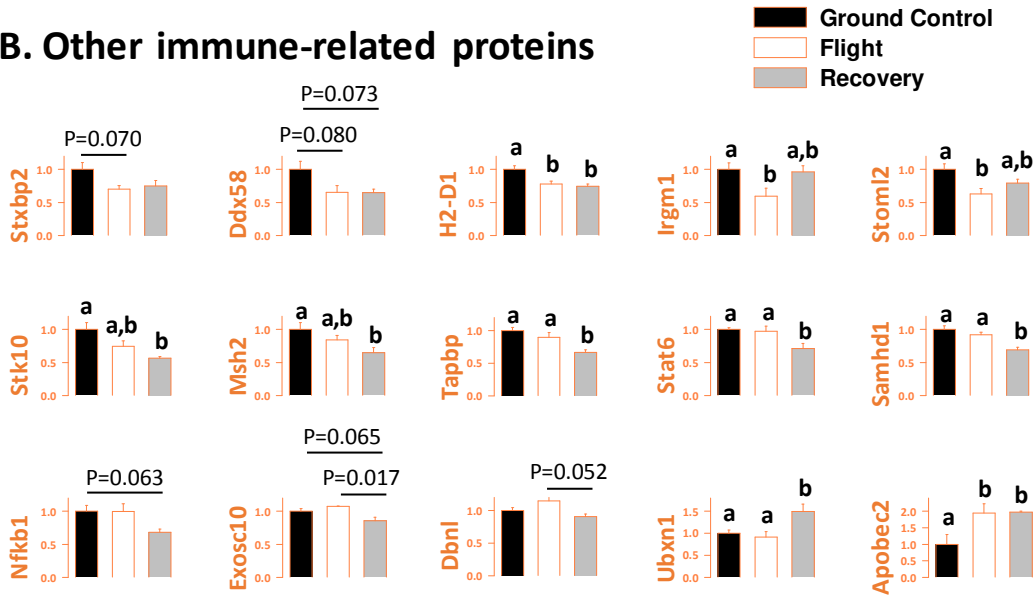


Figure 6

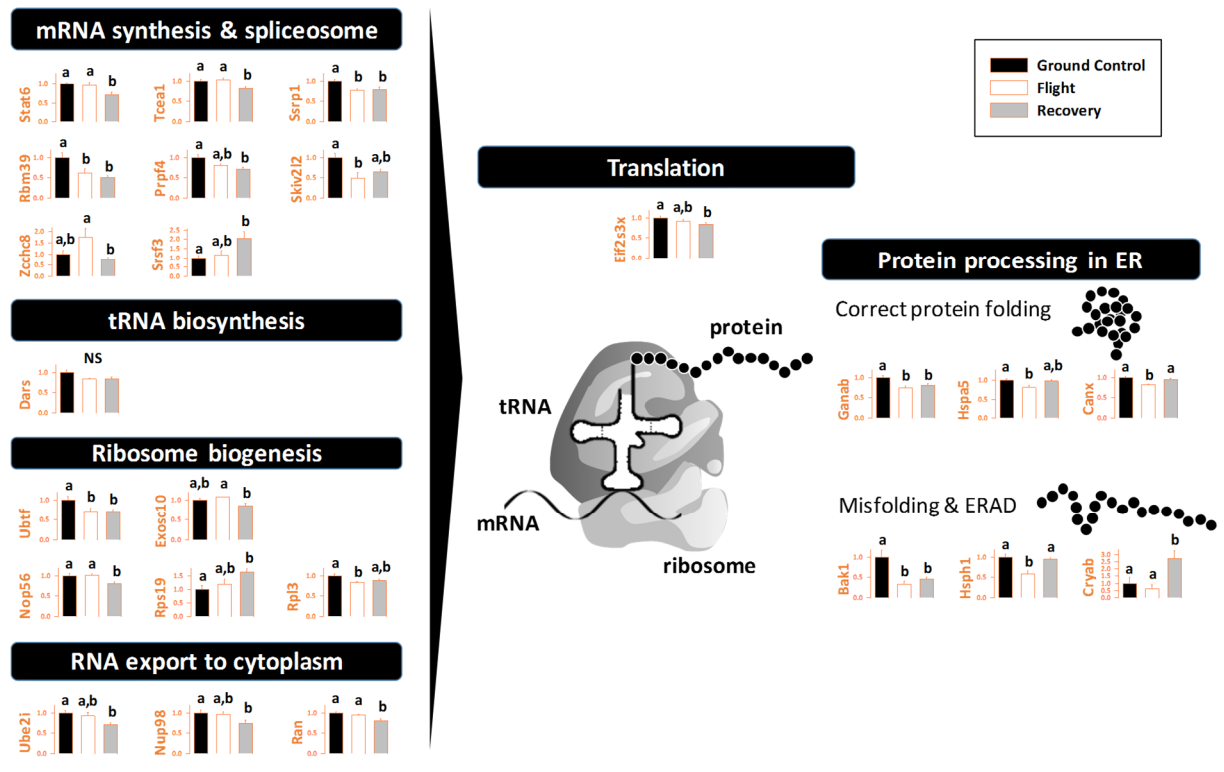


Figure 7.