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Supplemental Information

Beyond Low-Earth Orbit: Characterizing Immune and microRNA Differentials following Simulated Deep Spaceflight Conditions in Mice

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Supplemental Information

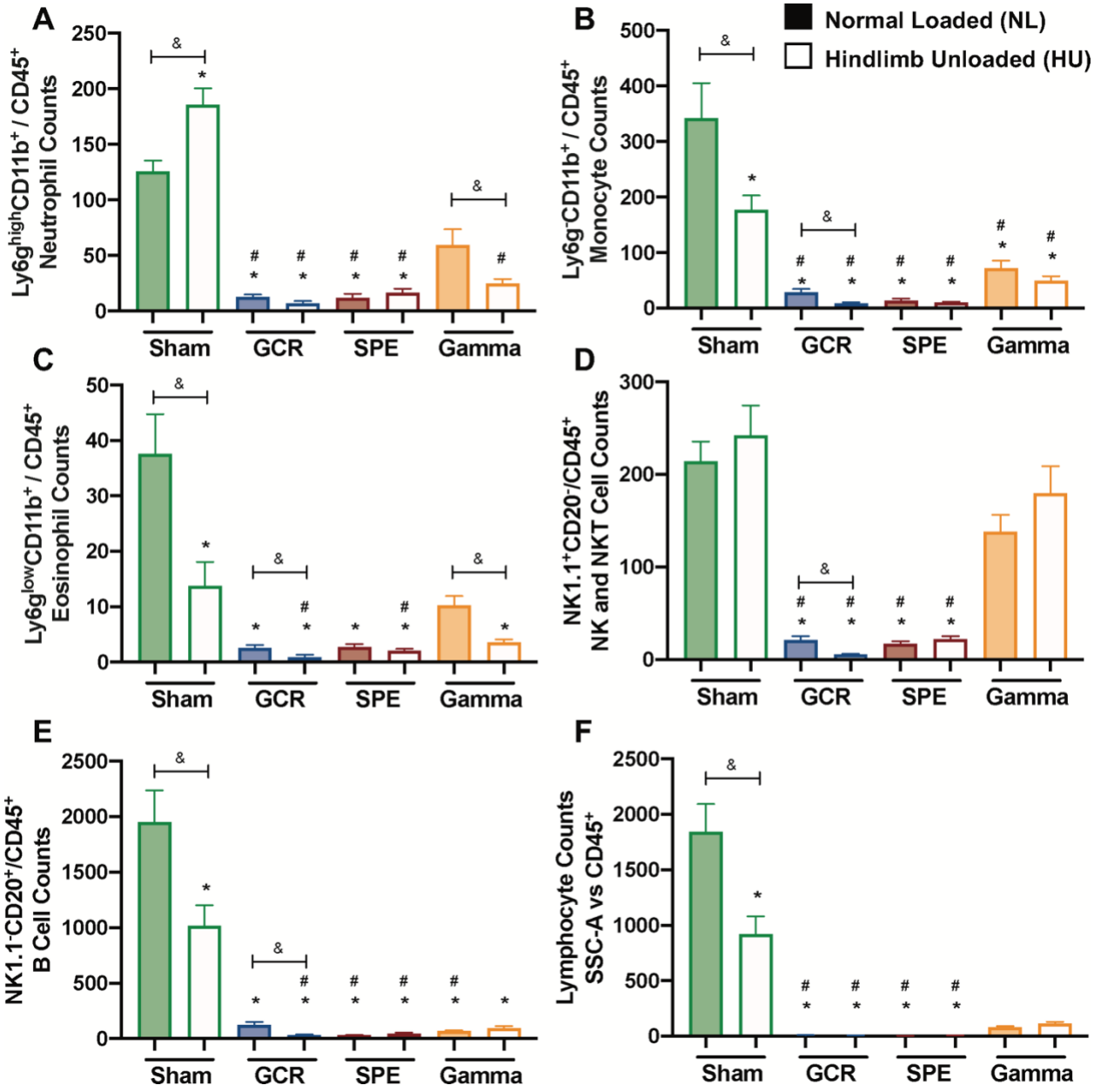

Figure S1. Lymphocyte differentials reveal changes in absolute counts following deep space exposures. Absolute counts of neutrophils ($Ly6g^{high}CD11b^{+}/CD45^{+}$) (A), monocytes ($Ly6g^{low}CD11b^{+}/CD45^{+}$) (B), eosinophils ($Ly6g^{-}CD11b^{+}/CD45^{+}$) (C), NK and NKT cells ($CD20^{-}NK1.1^{+}/CD45^{+}$) (D), B cells ($CD20^{+}NK1.1^{-}/CD45^{+}$) (E) and total lymphocytes (SSC-A vs. $CD45^{+}$) (F) are displayed within total leukocyte populations. (C) Median fluorescence intensity (MFI) of cell surface expression of CD20 on all $CD45^{+}$ cells. (D) Percent (%) of NK/NKT cells ($NK1.1^{+}CD20^{-}/CD45^{+}$). Bar graph data represents means ±SEM ($p < 0.05$, n = 7-10 per group). Statistical tests and labels are the same as Figure 1.

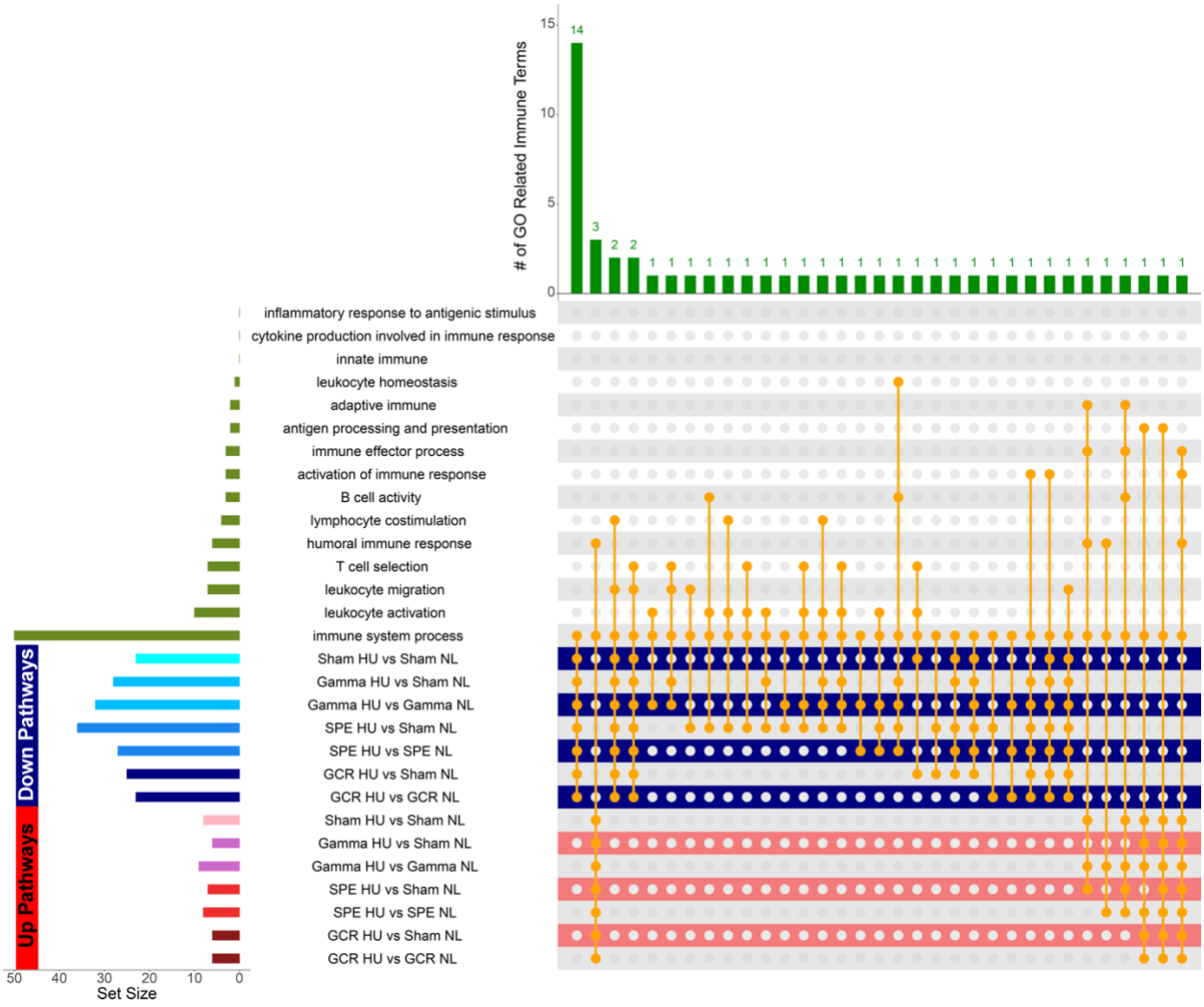

Figure S2. Upset plot displays general up- and down-regulated immune pathways. Gene ontology (GO) terms with an FDR < 0.05 cutoff were considered significant. Specific immune-related GO terms were mapped to the GO Mouse Genome Informatics (MGI) and were plotted with R packages. Upset plot of biological and molecular immune pathways engaged following high and low LET IR exposure, singly or in combination with HU.

Analyte/ Blood	L-45 ‡	L-45 (on gel)	FD15		FD30		FD60		FD120		FD180		R+0		R+30
IGF-1 ng/mL	180.3 ±44.4 n=40	176±42 n=39	220±62 n=40	***	216± 52 n=32	***	219 ±53 n=40	***	215 ±51 n=33	***	219 ±50 n=37	***	202 ±60 n=40	***	164 ±46 n=40
IL-1β pg/mL	1.56±2.17 n=40	1.93±2.5 n=39	2.48 ±4.0 n=47	***	1.44 ±2.10 n=39		2.17 ±3.11 n=47	***	1.87 ±2.98 n=39		2.51 ±3.52 n=38	***	1.41 ±1.80 n=47		1.49 ±1.88 n=46
IL-1α pg/mL	4.34 ±8.49 n=47	6.59 ±11.23 n=42	7.54 ±13.43 n=47	***	5.44 ±10.1 2 n=39		7.86 ±13.87 n=47	***	5.27 ±8.59 n=39		9.16 ±15.19 n=38	***	2.75 ±4.19 n=47		3.91 ±6.50 n=46
IL-1RA pg/mL	364±201 n=44	526 ±392 n=39	567 ±366 n=44	***	483 ±327 n=36		596 ±433 n=44	***	600 ±515 n=36	***	612 ±384 n=37	***	625 ±524 n=44	***	484 ±553 n=43

Table S1. Astronaut Physiological data from blood validate inflammation during spaceflight. Data are mean ± SD. *** P<0.001 compared to L-45. ‡ The column labelled "L-45 (on gel)" represents data from the sample frozen in the tube and stored for batched analysis alongside the flight samples. In most cases, this came from the same draw as the L-45 collection. ‡‡ As described in methods, crewmembers on R+0 are not necessarily fasted prior to blood collection.

Transparent Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Fc Block (CD16/CD32 Monoclonal Antibody (93), eBioscience™)	Thermo Fisher Scientific	14-0161-86
CD45 Monoclonal Antibody (30-F11), FITC, eBioscience™)	Thermo Fisher Scientific	11-0451-85
CD11b Monoclonal Antibody (M1/70), PE-Cyanine7, eBioscience™	Thermo Fisher Scientific	25-0112-82
Ly-6G Monoclonal Antibody (1A8-Ly6g), PE, eBioscience™)	Thermo Fisher Scientific	12-9668-82
NK1.1 Monoclonal Antibody (PK136), APC, eBioscience™	Thermo Fisher Scientific	17-5941-82
CD20 Monoclonal Antibody (AISB12), PE, eBioscience™	Thermo Fisher Scientific	12-0201-82
CD3e Monoclonal Antibody (145-2C11), FITC, eBioscience™	Thermo Fisher Scientific	11-0031-85
CD4 Monoclonal Antibody (GK1.5), PE, eBioscience™	Thermo Fisher Scientific	12-0041-85
CD8a Monoclonal Antibody (53-6.7), FITC, eBioscience™	Thermo Fisher Scientific	11-0081-85
CD127 (IL-7R)Monoclonal Antibody (A7R34), Alexa Fluor 488, eBioscience™	Thermo Fisher Scientific	53-1271-82
Biological Samples		
Mouse Blood	C57BL/6J *Wt* female	n/a
Mouse Plasma	C57BL/6J *Wt* female	n/a
Mouse Spleen	C57BL/6J *Wt* female	n/a
Mouse Thymus	C57BL/6J *Wt* female	n/a
Human Blood (serum, plasma) and Urine	Male and female astronauts	n/a
Chemicals, Peptides, and Recombinant Proteins		
Paraformaldehyde (16%)	Fisher Scientific	AA433689M
1x Phosphate Buffered Saline (pH 7.4)	Thermo Fisher Scientific	10010023
eBioscience™ 1x RBC Lysis Buffer	Thermo Fisher Scientific	00-4333-57
UltraPure™ 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
Critical Commercial Assays		
miRNeasy Mini Kit	QIAgen	217004
iSCRIPT™ cDNA synthesis kit	Bio-Rad	1708890
iQ™ SYBR® Green Supermix	Bio-Rad	170-8880
miRNeasy serum/plasma kit	QIAgen	217184
TruSeq Small RNA Sample Prep Kits	Illumina	RS-200-0012
Deposited Data		
miRNA-sequence mouse plasma dataset	NASA GeneLab (https://genelab.nasa.gov/)	GLDS-336, DOI: 10.26030/qasa-rr29

Experimental Models: Organisms/Strains		
C57BL/6J *Wt* female mice	Jackson Laboratories	000664
Simplified 5-ion GCR Simulation	Protons at 1000 MeV, ^{28}Si at 600 MeV/n, ^{4}He at 250 MeV/n, ^{16}O at 350 MeV/n, ^{56}Fe at 600 MeV/n, and protons at 250 MeV	n/a
Simulated SPE	Protons at 50MeV to 150MeV	n/a
Simulated Gamma	^{137}Cs source (5 Gy)	n/a
Oligonucleotides		
Gapdh FWD 5'-CAGGAGAGTGTTTCCTCGTCC-3' REV 5'-TTCCCATTCTCGGCCTTGAC-3'	IDT	NM_001289726.1
Ifn-γ FWD 5'- AGGAACTGGCAAAAGGATGGT-3' REV 5'-TCATTGAATGCTTGGCGCTG-3'	IDT	NM_008337.4
Il-4 FWD 5'- CCATATCCACGGATGCGACA-3' REV 5'- AAGCCCGAAAGAGTCTCTGC-3'	IDT	NM_021283.2
Il-1β FWD 5'- TGCCACCTTTTGACAGTGATG-3' REV 5'- AAGGTCCACGGGAAAGACAC-3'	IDT	NM_008361.4
Tnf-α FWD 5'- CCCACGTCGTAGCAAACCA-3' REV 5'- ACAAGGTACAACCCATCGGC-3'	IDT	NM_013693.3
iNos FWD 5'- AGGGACTGAGCTGTTAGAGACA-3' REV 5'- GTCATCTTGTATTGTTGGGCTGAG-3'	IDT	NM_010927.4
Il-6 FWD 5'- GCCTTCTTGGGACTGATGCT-3' REV 5'- TGCCATTGCACAACTCTTTTC-3'	IDT	NM_031168.2
Il-10 FWD 5'- GGTTGCCAAGCCTTATCGGA-3' REV 5'- GGGGAGAAATCGATGACAGC-3'	IDT	NM_010548.2
Software and Algorithms		
FlowJo Software v10.5.3	BD	https://www.flowjo.com/
Prism v8.4.2	GraphPad	https://www.graphpad.com/scientific-software/prism/
ACGT101-miR (LC Sciences)	n/a	n/a
miRBase 22.0	(Kozomara et al., 2019)	http://www.mirbase.org/
RNAfold software	n/a	http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi
RBiomirGS v0.2.12 R package	(Zhang and Storey, 2018)	https://github.com/jzhangc/git_RBiomirGS

GO Mouse Genome Informatics (MGI)	(Smith and Eppig, 2009)	http://www.informatics.jax.org/
R packages UpsetR v1.4.0	(Conway et al., 2017)	https://cran.r-project.org/web/packages/UpSetR/index.html
pheatmap v1.0.12	Kolde Rpheatmap: Pretty heatmaps software; 2015	https://cran.r-project.org/web/packages/pheatmap/index.html
R package v3.6.1	n/a	https://www.r-project.org/
Other		
BD FACSMelody™	BD Biosciences	n/a
Bioanalyzer 2100	Agilent	n/a
Illumina Hiseq 2500	LC Sciences	n/a
ABI 7500 Real-Time PCR	Applied Biosystems	n/a

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics Statement

All experiments were approved by Brookhaven National Laboratory's (BNL) Institutional Animal Care and Use Committee (IACUC) (protocol number: 506) and all experiment were performed by trained personnel in AAALAC accredited animal facilities at BNL, while conforming to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All human astronaut data were reviewed and approved by the NASA Institutional Review Board and all subjects provided written informed consent.

Experimental design

15-week +/- 3-day old, C57BL/6J wildtype (*Wt*) female mice were purchased from Jackson Laboratories and housed at BNL. Upon arrival to BNL, mice were quarantined and acclimated to a standard 12:12h light:dark cycle, with controlled temperature/humidity for 1-week prior to cage acclimation. Food and water were given *ad libitum*, and standard bedding was changed once per week. Mice were cage acclimated (n=10 mice per group; 2 mice per cage to maintain social interaction) 3-days prior to HU, followed by 14-days either normally loaded (NL) or hindlimb unloaded (HU, see details below). Irradiation was administered on day 13 and blood tissues were collected at 24-hours post-irradiation and post-euthanasia by CO_2 overdose, followed by cervical dislocation. Blood was collected via the abdominal aorta in EDTA-coated tubes (0.5 M) and plasma was separated by centrifugation at 2,000xg for 15 minutes. Plasma was collected and flash frozen in liquid nitrogen for -80°C storage. A 100 µl aliquot of cellular fraction was flash frozen and stored at -80°C for RNA analyses, while the remaining cellular fraction was lysed with 1x RBC lysis buffer (Thermo Fisher Scientific) followed by flow cytometric preparation and analyses, as describe below. Body weight tracking was performed on days -3, 0, 7 and 14. The experimental timeline is described in *Figure 1A*.

Hindlimb Unloading

Hindlimb unloading was performed using the adjusted Morey-Holton method for social housing (n=2 per cage) (Tahimic et al., 2019). Briefly, mice were suspended from the tail using non-invasive traction tape attached to an adjustable pulley mounted on the top of a standard rodent cage. The adjustable nature of the device allows the user to position the animal in a head-down position (approximately 30° to the horizon) once attached. The crossbar height, lateral crossbar position, and chain length are adjustable. The pulley is free to move along the crossbar that spans the length of the top of the cage enabling the mouse to navigate freely on one half of the cage using its forepaws and to interact with the second mouse in the cage, without enabling ambulation of the limbs. Individual food and water sources are provided to each mouse and replaced daily. Control mice were housed in identical cages with normal ambulation.

Simulated GCR, SPE and gamma irradiation

On day 13, mice were transported on BNL base to the NASA Space Radiation Laboratory (NSRL) facility by animal care staff and were transferred to individual HU boxes to enable whole body irradiation while maintaining hindlimb suspension. The following doses of irradiation were administered; simplified GCR sim (0.5 Gy), SPE (1 Gy), Gamma (5 Gy) and Sham control (0 Gy). To simulate GCR, we used the simplified GCR simulation of ions, energy, and doses determined by a NASA consensus formula that consists of 5 ions: protons at 1000 MeV, ^{28}Si at 600 MeV/n, ^{4}He at 250 MeV/n, ^{16}O at 350 MeV/n, ^{56}Fe at 600 MeV/n, and protons at 250 MeV. This dose of radiation is equivalent to what an astronaut is predicted to receive in deep space during a Mars mission, though it is modeled as a single exposure over 25 minutes instead of the actual chronic exposures over 1.5 years. Further, GCR sim is a mixture of high and low LET ions in a ratio of 15% to 85%, respectfully. To simulate SPE, we used a total dose of 1Gy protons with energy ranges from 50MeV to 150MeV. For all irradiations a 60x60 beam was utilized at NSRL. For radiation dose equivalence and biological reference, we used 5 Gy gamma irradiation with the cesium resource available at BNL, in the absence of HU due to resource limitations. Sham controls were treated similar to GCR/SPE irradiated mice, including HU cage boxes and beam line (without irradiation) for the same duration as GCR simualtion, i.e. 25 minutes.

METHODS DETAILS

Flow cytometry

Collected blood samples were lysed with 1x RBC lysis buffer (Thermo Fisher Scientific) fixed in 4% paraformaldehyde for 15 minutes on ice, washed twice with 1x phosphate buffered saline (PBS, Thermo Fisher Scientific), and stored at 4ºC until staining. Single-cell suspensions were generated for flow cytometry acquisition. Debris was gated off and doublet discrimination was performed. Cells were Fc blocked for 20 minutes and probed with mouse reactive antibodies targeting anti-CD45, anti-CD11b, anti-Ly6G, anti-NK1.1, anti-CD20, anti-CD3, anti-CD4, anti-CD8, and anti-IL-7R. All antibodies and Fc block were purchased from Thermo Fisher Scientific. Unstained and single-color compensation controls were used. Cells were acquired using a BD FACSMelody™ and analyzed using FlowJo™ Software (v10.5.3).

Quantitative (q)PCR

Collected blood samples were flash frozen and total RNA isolation using miRNeasy Mini Kit (QIAgen) following manufactures' recommendations. Concentration and purity were determined using a Bioanalyzer 2100 (Agilent, CA, USA) with RIN number > 8. 1 µg of total RNA was prepared for cDNA synthesis using iSCRIPT cDNA synthesis kits (Bio-Rad). Quantitative PCR was performed using iQ™ SYBR® Green Supermix (Bio-Rad) and primers (IDT) were designed using NCBI design tool. An ABI 7500 Real-Time PCR (Applied Biosystems) was used and threshold cycle values that were ≥ 35 cycles were excluded from the results. Primer sequence for the following genes are listed in the key resource table. Data was analyzed using the ΔΔcT method with *Gapdh* as the normalizer gene, and relative fold change (RFC) is displayed.

miRNA isolation, sequencing, and data analyses

Library construction and sequencing was performed from miRNAs isolated from plasma from the mouse experiments described above. The miRNA extraction was carried out using the QIAgen miRNeasy serum/plasma kit (#217184). The total RNA quality and quantity were analyzed using a Bioanalyzer 2100 (Agilent, CA, USA) with RIN number > 7. Approximately 1 µg of total RNA was used to prepare small RNA library according to protocol of TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA). Single-end sequencing was performed using 50 bp on an Illumina Hiseq 2500 at the LC Sciences (Hangzhou, China) following the vendor's recommended protocol.

Raw reads were subjected to an in-house software program, ACGT101-miR (LC Sciences, Houston, Texas, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18~26 nucleotide were mapped to specific species precursors in miRBase 22.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. Length variation at both 3' and 5' ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to specific species mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known specific species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p- derived miRNA candidates. The remaining sequences were mapped

to other selected species precursors (with the exclusion of specific species) in miRBase 22.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the specific species genomes to determine their genomic locations. The above two we defined as known miRNAs. The unmapped sequences were BLASTed against the specific genomes, and the hairpin RNA structures containing sequences were predicated from the flank 80 nt sequences using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The criteria for secondary structure prediction were: (1) number of nucleotides in one bulge in stem (≤12); (2) number of base pairs in the stem region of the predicted hairpin (≥16); (3) cutoff of free energy (kCal/mol ≤-15); (4) length of hairpin (up and down stems + terminal loop ≥50); (5) length of hairpin loop (≤20); (6) number of nucleotides in one bulge in mature region (≤8); (7) number of biased errors in one bulge in mature region (≤4); (8) number of biased bulges in mature region (≤2); (9) number of errors in mature region (≤7); (10) number of base pairs in the mature region of the predicted hairpin (≥12); and (11) percent of mature in stem (≥80). Differential expression of miRNAs based on normalized deep-sequencing counts was analyzed by selectively using Fisher exact test, Chi-squared 2X2 test, Chi-squared nXn test, Student t test, or ANOVA based on the experimental design. The significance threshold was set to be 0.01 and 0.05 in each test. The plasma miRNA-seq raw fastq files can be found on NASA's GeneLab data repository/platform (https://genelab.nasa.gov/) with the following identifiers: GLDS-336, DOI: 10.26030/qasa-rr29.

To determine gene ontology (GO) and Hallmark pathways being regulated by the miRNAs we performed miRNA gene set analysis utilizing the RBiomirGS v0.2.12 R package (Zhang and Storey, 2018) from the processed miRNA analysis for all conditions in the plasma. From the GO terms we chose an FDR < 0.05 cutoff for significantly regulated GO Terms. We then determined the specific immune related GO terms by mapping to the GO Mouse Genome Informatics (MGI) information for specific GO immune terms (Smith and Eppig, 2009). We plotted the specific GO terms with R packages UpsetR v1.4.0 (Conway et al., 2017) and pheatmap v1.0.12 (Kolde R. pheatmap: Pretty heatmaps).

Human physiological inflammation data

Data are reported from three human subject experiments conducted on the International Space Station: Nutritional Status Assessment (2006-2012), Dietary Intake Can Predict and Protect Against Changes in Bone Metabolism During Space Flight and Recovery (Pro K) (2010-2015), and Biochemical Profile (2013-2018). All protocols were reviewed and approved by the NASA Institutional Review Board and all subjects provided written informed consent. While subsets of some of these data have been published in other papers (Crucian et al., 2018), as a whole, the data provided here have not been previously published. In addition, the inflammation data provided have been reanalyzed from data that were submitted with another paper included in this special NASA Cell issue (Malkani et al., 2020).

Crews from these experiments completed missions of 4-6 months in duration, and these studies included blood collections before, during, and after flight, with analysis of an array of inflammation biochemical markers. Blood samples were collected 2 or 3 times before flight: approximately Launch minus (L-) 45 days. In some cases, a third blood sample was collected (typically along with the L-45 collection), and these tubes were centrifuged and frozen for aliquoting after flight batched with the samples collected inflight. Blood samples were collected inflight, at approximately Flight Day (FD) 15, FD30, FD60, FD120, and FD180. Postflight samples were collected in the first 24-h after landing (designated return+0, R+0) and again 30-d later (R+30). The R+0 samples were not necessarily fasting, given the time of day and nature of return from flight. Of the 59 crewmembers reported: 8 returned on the Space Shuttle, with blood collection 2-4 hours after landing; 51 landed in Kazakhstan, with 7 of them returning to Star City, Russia, with blood collection 8-10 hours after landing; 44 were transported directly back to the Johnson Space Center in Houston, with blood collection approximately 24-h after landing. Pre and postflight collections included two 24-h urine collections, and inflight collections included one 24-h urine collection. These collection techniques have been previously described (Smith et al., 2012; Zwart et al., 2011).

We report here inflammatory markers were analyzed using standard techniques as reported previously (Crucian et al., 2018; Zwart et al., 2016; Zwart et al., 2013; Zwart et al., 2009). As of this writing, data were available for 59 crewmembers (47 males and 12 females). Age at launch was 47.0 ± 5.6 y, body mass at launch was 79.2 ± 11.8 kg (M: 83.3 ± 9.3; F: 63.0 ± 4.5). Body mass index was 25.5 ± 2.9 kg/m2 (M: 26.4 ± 2.6; F: 22.3 ± 1.5). All available data are reported here, although the reported n for any given test or session varies for a number of reasons, including, not all experiments had all analytes included, mission length differences for some crewmembers, schedule or other issues occasionally precluded sample collection, and methods changes over time. Repeated measures analysis of variance was conducted to

test for differences during and after flight compared to preflight, and comparisons among time points were made using a Bonferroni t-test. Multiple comparisons were accounted for, and only those tests with $p^{***} < 0.001$ are reported.

Quantification and Statistical Analysis

For Figures 1-5, a Grubbs test was performed on all datasets followed by testing for normal distribution via a Kolmogorov-Smirnov test. If data were normally distributed, a one-way ANOVA Dunnett test was performed to compared NL and HU controls to all groups and a parametric unpaired t-test with Welsh's correction was performed to compared between similar irradiation groups. If normality was not passed, both a non-parametric Kruskal-Wallis test with a Dunn's posthoc analyses was performed to compare NL and HU controls to all groups and a non-parametric Mann-Whitney U-test compared between similar irradiation groups were performed. All data are means ±SEM ($p < 0.05$, n = 5-10 per group depending on associated figure). "*" denotes significant difference between NL-Sham and associated group, "#" denotes significant difference between HU-Sham associated group, and intergroup "brackets with a &" denotes significant difference between each group. Filled circles, boxes, and bars denotes normal loaded (NL) and non-filled circles, boxes, and bars denotes hindlimb unloaded (HU).

For Figure 7, a repeated measures analysis of variance was conducted to test for differences during and after flight compared to preflight, and comparisons among time points were made using a Bonferroni t-test. Multiple comparisons were accounted for and data are means ±SEM ($p^{***} < 0.001$, n = 59 crewmembers; 47 males, 12 females). GraphPad Prism software was used to analyze all associated figures (v. 8.4.2).

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