Genome and Clonal Hematopoiesis Stability Contrasts with Immune, cfDNA, mitochondrial, and telomere length changes to Short Duration Spaceflight

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Abstract

The I4 mission, the first all-civilian orbital flight mission, investigated the physiological effects of short duration spaceflight through a multi-omic approach. We analyzed telomere length, clonal hematopoiesis of indeterminate potential (CHIP), whole genome stability, cell-free DNA (cfDNA) cell lysis, and immune cell gene expression. Our results revealed telomere length dynamics similar to those observed in the NASA Twins Study and in astronauts spending 6 months on the ISS, with lengthening in space and shortening upon return to Earth. Our cell-type of origin analysis of cfDNA fragments revealed an increased presence of innate and adaptive immune cell signatures that persisted over a month after return to earth. No significant relationship between spaceflight and CHIP-related or whole genome abnormalities were observed. Longitudinal mitochondrial, ribosomal and immune function gene expression changes occurred across both adaptive and innate immune cells, suggesting adaptations to the space environment can extend months after return from spaceflight and alter immune function. Our findings provide valuable insights into the physiological consequences of short duration spaceflight and will serve as a reference point for future space tourism, low Earth-orbit (LEO) missions, and deep-space exploration.

Introduction

Human spaceflight frequency has increased over the last decade, and with it, our grasp of its effects on human physiology. Despite this, there is much to learn about how humans adapt to the unique challenges of the space environment. The space environment includes unique stressors, such as microgravity (e.g. weightlessness), immune system perturbations, and space radiation exposure. Determining the effects of spaceflight on the human genome is imperative to support long-term human presence in space. Additionally, there are a host of physiological adaptations that occur when humans travel into space, ranging from cardiovascular and musculoskeletal deconditioning, vision changes (e.g. spaceflight associated neuroocular syndrome), immune suppression and metabolic changes, among others[1]. Moreover, there is increasing evidence highlighting the systemic immune dysregulation crewmembers experience from spaceflight exposure. Indeed, there appears to be a reduction in T cell frequency, suppressed cytotoxic function, as well as fluctuations in cytokine concentrations, with increased expression of TNF-*α*, interleukin-8, interleukin-1ra, thrombopoietin, VEGF, and various chemokines (CCL2, CCL4, CXCL5, etc) associated with spaceflight[2].

To date, space missions have been led primarily by professional astronauts from government space programs. More recently, however, SpaceX launched a crew of four civilian astronauts on the Inspiration 4 (I4) mission, marking a new era for human space exploration. This present study of the I4 mission aims to understand the impact of short duration spaceflight on civilians, along with the post-flight and long-term biomedical, immunologic, and genetic alterations resulting from spaceflight. This includes rapid telomeric responses, whereby telomere elongation occurs as a function of spaceflight, followed by shortening upon return to Earth[3]. Both unusually short and long telomeres have been associated with adverse health effects involving aging and age-related pathologies such as cardiovascular disease and

cancer. Furthermore, a variety of lifestyle factors and environmental exposures influence telomere length. Previous studies have also shown changes in clonal hematopoiesis, where a small number of hematopoietic stem cells (HSCs) gain a clonal advantage through the acquisition of somatic mutations. Clonal hematopoiesis is associated with cardiovascular disease, the development of acute myeloid leukemia (AML), and increased overall mortality[4,5]. The NASA Twins study shed light on telomeric responses and the chronic inflammatory state resulting from long duration spaceflight and provided the first measures of telomere length and clonal hematopoiesis in astronauts[3,6]. Mutations in epigenetic regulators, such as DNMT3A and TET2, were found at increased rates in both career astronaut twins when compared to their civilian counterparts. A retrospective study of 14 astronauts who flew shuttle missions has also shown an elevated presence of genetic abnormalities in CHIP-driver genes in the astronaut population[3,5,7].

Here we investigate immune adaptations, telomere length dynamics, cell free DNA release, genomic stability, single cell transcriptomic analysis, and biochemical adaptations of the I4 crewmembers traveling into lower earth orbit over the course of 3–4 days, investigating the effects of short duration spaceflight. It is imperative to study these adaptations in this context for future space missions, as prolonged space environment exposure could exponentially increase the rate of mutational burden[8], be it due to the inflammatory milieu resulting from spaceflight or the particular challenges of the space environment, such as microgravity and radiation exposure. Overall, the I4 mission provides a unique opportunity to examine the development of genetic abnormalities in the astronaut population by studying civilians' physiological response to the demands of spaceflight and assessing the feasibility of expanding humans' presence in space.

Results

Telomere Length Measurement (MMqPCR)

We collected serum and whole blood from I4 crew members before (L-92, L-44, L-3), during (FD1, FD2, FD3), and after flight (R + 1, R + 45, R + 82), isolated DNA, and then measured telomere length via multiplexed quantitative PCR (MMqPCR) (Fig. 1A). Telomere lengthening was observed during spaceflight compared with pre- and post-flight normalized means (Fig. 1A). Telomere length returned to pre-flight values in 3 of the 4 astronauts post-flight.

Assessment of spaceflight-associated telomere length dynamics (changes over time) demonstrated rapid telomere elongation during spaceflight as compared to the pre-flight baseline, and telomere shortening upon return to earth in all I4 astronauts. Intriguingly, despite the short duration of the mission (3 days of orbital flight), our results correlated with findings from the NASA Twins Study (One Year Mission astronaut) and with astronauts on ~ 6 month missions aboard the International Space Station (ISS)[3,9].

Cell-free DNA

Cell-free DNA (cfDNA) from the I4 crew's plasma was extracted from Streck tubes and sequenced to compare to pre-flight baseline, post-flight, and recovery period responses. Extraction and pre-processing of plasma were not implemented during the flight, so all samples were collected on Earth to mitigate cfDNA contamination from apoptotic blood cells during sample transportation (Fig S1A&B)[8].

We analyzed plasma mitochondrial cfDNA (cf-mtDNA) relative to the chromosomal cfDNA fraction (Fig. 1B & 1C, Table S3), as a potential biomarker for long duration spaceflight adaptations. In this mission, cf-mtDNA levels did not rise significantly following the return to Earth (R + 1), possibly due to the short flight duration. Cf-mtDNA enrichment was only observed in the twin astronauts on the ISS after months of exposure to the space environment. The marker levels display high between-sample heterogeneity, and the increase in pre-flight measurements may be connected to the I4 crew's pre-flight preparation (e.g. high-altitude training).

Given that cfDNA fragments encompass one nucleosome, we inspected depletion in read coverage distribution around the transcription start sites (TSS) as a measurement of gene expression[6,10]. We applied coverage normalization specific to the genomic neighborhood and completed Fourier Transforms to determine nucleosome footprints based on signal periodicity. The cfDNA fragment tissue and cell-type-of-origin enrichment of fragments were correlated with either tissue-specific expression signatures from the Human Proteome Map (HPM)[11] or individual astronaut expression profiles from peripheral blood[12] (Fig. 1D&E, Fig. 1SC, Table S1&S2). The most represented sequences were of hematopoietic origin, while a small increased presence of cfDNA fragments from both innate and adaptive immune cells was observed post-landing and during the recovery period. Interestingly, there was a significant increase in the cfDNA originating from all immune cells in all astronauts after over a month following their return to Earth (R + 82).

Clonal Hematopoiesis of Indeterminate Potential (CHIP) Assessment

CHIP has been previously studied in astronauts, with the NASA Twins Study assessing the mutational burden of long duration spaceflight in established epigenetic control genes[3]. The contrast between the I4 cohort, a civilian crew that had never undergone orbital flight and the NASA Twins Study provides us with the unique opportunity to study the effects of short duration spaceflight on civilians.

We performed deep targeted sequencing (> 30,000x) of 10 known epigenetic regulators and CHIP-associated genes to assess the mutational burden of short duration spaceflight in the I4 astronaut cohort. The mutational burden of the CHIP-associated genes did not change as a function of spaceflight and remained stable for up to 6 months post spaceflight (Fig. 2A). No known deleterious mutations arose in the I4 cohort post-flight, nor did any pre-existing somatic mutations increase in allele frequency (Fig. 2B & 2D). This remained true across all genes in the panel, regardless of mutational consequence and genie location, with the exception of subject C004 who showed a rare missense variant identified as an

epigenetic regulator gene (DNMT3A) with a variant allele frequency (VAF) of ~ 0.01, chr2:g.25235778C > G, but otherwise stable throughout the study.

We continue to monitor clonal hematopoietic mutational burden in both subjects from the NASA Twins Study years postflight. Subject TW (Long duration spaceflight) still presents mutation TET2.p.Cys1273Tyr in a bulk PBMC sample at a VAF of ~ 0.075 (Fig. 2D). Subject HR (Ground control) presents the mutation DNMT3A.p.Trp698Ter at a VAF of ~ 0.086 and the mutation DNMT3A.p.Asp856Gly not previously found at a VAF of ~ 0.05 (Fig. 2E). Both astronauts present increased clonality when compared to previous timepoints, however, this clonal expansion may not be abnormal given the association between aging and CHIP[13].

Longitudinal Comparison of Whole Genome Mutations Reveals Genomic Stability Months After Short-duration Orbital Flight

To assess the long-term consequences of spaceflight on the genomic stability of astronauts, we conducted whole-genome sequencing (WGS) and variant calling pre- and post-flight. Comparison of single nucleotide variant (SNV) and indel presence between timepoints (L-44 and R + 45) for each astronaut, which indicated no significant changes for genes with the highest mutational burden (Fig. 3A), epigenetic regulator genes (Fig S3), or other gene sets of interest. The analysis of variant annotations (Fig. 3A&C) revealed that most identified variants are within intronic regions and have no significant effects. We also have noted no disproportional mutational load for variants post-flight (Fig. 3D). We performed variant calling in single nucleus RNA-seq (snRNA-seq) libraries and compared it across all timepoints and against WGS for genes with read coverage > 10x (Suppl. Figure 3B&C). Whole genome coverage of the snRNA-seq libraries varied broadly across timepoints, but did not show any significant evidence of genomic instability within our cohort.

Spaceflight induces cell-type-specific changes in gene expression profiles and transcriptional heterogeneity as determined by single-cell transcriptomics

Using snRNA-seq data, we examined gene expression changes across immune cell types due to spaceflight. Cell type-specific genes were used to computationally filter cell types. CD8 + T cells, and CD4 + T cells, CD14 + monocytes, CD16 + monocytes, dendritic cells (DCs) and Natural Killer cells (NK) were filtered from PBMCs at six different timepoints: L-92, L-44, L-3, R + 1, R + 45, and R + 82.

Gene expression of CD8 + T cells at R + 1 and R + 82 showed increased expression of genes related to immune function, ribosomal and mitochondrial activity (e.g., *NKG7, CCL5, HLA-C, S100A4, RPL41, RPS11, MT-CO3, PTMA, B2M*, see Fig. 4B). Similar trends were seen with CD14 + monocytes, DCs, CD4+, FCGR3A + monocytes, NK cells, and CD3D + cells (Suppl. Figure 4C, 4D). Increased gene expression of mitochondrial and ribosomal genes is conserved across cell types for both L-92 vs R + 1 and L-92 vs R + 82 comparisons. PLCG2, MTRNR2L12, VCAN, and MAML2 transcripts associated with immune function,

mitochondrial function, cell adhesion and Notch signaling are consistently downregulated across all cell types (Fig. 4 & Suppl. Figure 4).

Interestingly, the proportion of cells expressing these genes does not necessarily change in parallel with total gene expression levels. *PLCG2* relative, per cell, expression decreased but the total proportion of cells expressing *PLCG2* is increased among CD8 + T cells at R + 1 (0.98) vs L-92 (0.81 +/- 0.07 s.e.) and R + 1 vs R + 82 (0.66 +/- 0.04 s.e.), classical monocytes at R + 1 (0.99) vs L-92 (0.96 +/- 0.01 s.e.) and R + 1 vs R + 82 (0.92 +/- 0.05 s.e.) as well as DCs at R + 1 (0.99) vs L-92 (0.96 +/- 0.01 s.e.) and R + 1 vs R + 82 (0.92 +/- 0.04 s.e.) (Fig. 4A, 4C & 4E). Gene expression of mitochondrial cytochrome c oxidase, particularly subunits 2 and 3 (*MT-CO2* & *MT-CO3*), increased across all cell types, per cell, for R + 1 compared to L-92 and R + 82, with no significant change in the proportion of cells expressing these genes. *MTRNR2L12* expression was consistently decreased across all studied cell types for R + 1 vs L-92 but greatly increased for R + 82 vs L-92 and R + 82 vs R + 1, suggesting changes in gene expression lasting over two months postflight in both adaptive and innate immune cells.

Discussion

The I4 mission is the first study to date to analyze the effect of short duration spaceflight on civilians using a comprehensive multi-omics approach. Our findings, including telomere length dynamics, CHIP-related clonal expansion, WGS genomic stability, cfDNA cell lysis analysis, and immune cell longitudinal gene expression profiling, broadly contextualize the physiological burdens of short duration spaceflight. This study will serve as a reference for future space tourism, short missions around Earth's orbit, and longer-duration missions into deep space.

Telomeric Adaptations as a Function of Spaceflight

The NASA Twins Study was the first to report spaceflight-specific telomere elongation in humans, with astronaut TW experiencing telomere elongation during his one-year mission, rapid telomere shortening upon return to Earth, and telomere length recovery to near pre-flight baseline values over the following months, although many more short telomeres after spaceflight than before were also observed[3]. Luxton et al. reported similar spaceflight associated telomere length dynamics in 3 unrelated astronauts on ~ 6 month missions onboard the ISS[9]. It was hypothesized that such dramatic shifts in telomere length dynamics were associated with chronic exposure to the space radiation environment and represented an adaptive response to chronic oxidative damage specifically to telomeres, whereby the Alternative Lengthening of Telomeres (ALT) pathway is transiently activated in normal somatic cells[9]. Activation of DNA damage responses were reported, and our cfDNA enrichment analyses also suggested increased DNA damage consistent with radiation exposure and increased senescence-associated foci. Similar changes in telomere length dynamics seen in the i4 crew as a function of spaceflight suggest that telomere length alterations are fast-acting and associated with spaceflight irrespective of mission duration.

Circulating cell-free DNA as a biomarker of spaceflight

Circulating cell-free DNA (cfDNA) fragments, originating from various tissues and the immune system, offer a non-invasive method for assessing astronauts' dynamic immune responses to spaceflight-induced physiological stress. As an emerging biomarker, cfDNA concentration and molecular profile yield valuable insights into stress responses in unique spaceflight environments, despite its heterogeneous and sensitive nature.

Our study indicates that the impact of short duration spaceflight on cfDNA concentration in astronaut plasma is subtler than long duration missions, but increased innate and adaptive immune system activity is still apparent. Moreover, cfDNA enrichment from all immune cells persists post-flight, suggesting a delayed response involving immune cell turnover and active DNA repair mechanisms, warranting further investigation.

Effects of Short and Long Duration Spaceflight on CHIP Genomic Alterations in Civilian and Career Astronauts

In the I4 cohort, we found no relationship between spaceflight and increased CHIP-related genetic abnormalities, at least up to three months after spaceflight. With our deep sequencing targeted probe approach, we found a rare mutation present in subject C004's DNMT3A gene chr2:g.25235778C > G at a VAF of ~ 0.012 for all timepoints with spaceflight having no discernible effects up to 3 months postflight. Likewise, somatic mutations in CH-linked genes were not found at disproportionate allele frequencies postflight, nor were *de novo* mutations seen in the postflight samples. This suggests that the expedition did not cause lasting or significant mutagenic effects in the all-civilian astronauts.

Although our findings suggest that no pathological genomic alterations occurred as a function of spaceflight in the Inspiration4 cohort, further longitudinal tracking of the astronauts will help us discern the long-term effects, if any, of short duration missions. Should any of the astronauts return to space in the future, it will provide us with the opportunity to study the compound effects of spaceflight on CH abnormalities. This field has yet to be explored in humans due to previous mission cohorts relying on career astronauts with multiple expeditions of experience prior to participation in the study of CH-related abnormalities.

The space environment is known to be chronically inflammatory as astronauts in previous studies have reported increased inflammation markers for the flight duration[14–16]. In the NASA Twins Study, both career astronauts had increased mutational burden in epigenetic regulators such as DNMT3A and TET2 compared to prostate cancer patient controls. Although only one of the twins was in orbital spaceflight for a year, both twins displayed comparable CHIP-related mutations shortly after flight and for several years of follow-up[6]. We continue to monitor the CHIP-related mutational burden of both astronauts years post-flight. The VAF of mutations found in both subjects has, as expected, increased overtime and a new missense variant has been identified in subject HR in the last 3 year follow-up. This is to be expected as

CHIP-mutational burden is known to increase as a function of aging. Both astronauts, however, presented clonal burdens that preceded their age-matched control for over two decades at the time of the original study. The fact that TW (space subject) did not present a greater mutational burden postflight than HR (ground control), despite the length of his last mission, might indicate that the overall number of flights rather than their duration is a greater extrinsic factor for clonal positive selection. Since both career astronauts had completed many flights prior to the study, understanding the effects of singular spaceflights on clonal dynamics was not possible. The I4 cohort provides a unique opportunity to assess the short and long term physiological effects of a singular spaceflight mission. As civilians, the I4 cohort's physiological findings are likely a better representation of what first-time fliers may expect as we gear up towards an increased human presence in space.

Whole Genome Sequencing Reveals Limited Genome-Wide Mutational Burden in First-Time Civilian Astronauts Months Postflight

The longitudinal comparison of point mutations and indels from whole-genome sequencing data supports the results of the targeted deep-sequencing CHIP panel. There does not seem to be a significant increase in the genome-wide mutational burden for first-flight civilian astronauts, which favorably indicates that short-time spaceflight does not contribute to overall increased genomic instability, on a timescale of months postflight. Extending the analysis to future missions and continuous longitudinal tracking for years postflight could shed more light on the long-term consequences of spaceflight in relation to its altitude and duration, as well as repeated exposure of astronauts to space radiation.

Longitudinal Mitochondrial Adaptations to the Space Environment

Transient increases in mitochondrial gene expression were seen as a response to spaceflight across a broad range of immune cells within the i4 cohort. *MT-CO2, MT-CO3* gene expression was elevated at the postflight R + 1 time point in comparison to preflight L-92 and postflight R + 82 within CD86 + DCs, NCAM + NK cells, CD14+ & FCGR3A + monocytes, and CD3 delta + CD8+ & CD4 + T cells (Fig. 4B, 4D, 4F & Suppl Fig. 4B, 4D, 4F, 4H). *MT-CO2* and *MT-CO3* encode subunits of cytochrome c oxidase, which is involved in reduction of oxygen to water. Decreases in MT-CO2 and MT-CO3 expression cause significant cytochrome c oxidase and mitochondrial complex IV deficiencies which may lead to tissue maladaptations[17,18]. Most mitochondrial genes return to basal levels of expression in our L-92 vs R + 82 comparison, suggesting relief from spaceflight-induced mitochondrial oxidative stress. DCs, monocytes, and T cells showed increased *MT-ND4L* expression over two months after return from flight. The *MT-ND4L* gene encodes for NADH-ubiquinone oxidoreductase chain 4L, a component of the respiratory chain Complex I, a protein required for electron transfer and dehydrogenation from NADH to ubiquinone. Longitudinal elevation in its gene expression may suggest that mitochondrial ATP production is altered long after return to earth. However, whether the increased gene expression is representative of increased activity of the electron

transport chain or if it is a physiological response, seeking to restore mitochondrial function due to the hypofunctional aberrant effects of oxidative stress, remains to be elucidated.

The R + 1 vs R + 82 post-flight comparison indicated *MT-ATP6, MT-CYB,MT-CO2, MT-CO3* were downregulated across all immune cell phenotypes at the R-82 timepoint. *MT-ATP6* encodes for ATP synthase membrane subunit 6, a component of the electron transport chain complex V, which converts ADP into ATP in the presence of a protein gradient between the outside and inside mitochondrial membranes. *MT-CYB* encodes for cytochrome b, a key component of Complex III, the ubiquinol-cytochrome c reductase complex, also part of the respiratory chain while *MT-CO2* and *MT-CO3* are part of the cytochrome c oxidase protein, also known as Complex IV, which transfers electrons to oxygen as part of the electron transport chain. Decreases in expression of genes associated with mitochondrial function in the post-flight comparison suggest a transient response to oxidative stress, a finding that has been previously described. Human induced pluripotent stem cell-derived cardiomyocytes had increased expression of genes related to the mitochondrial electron transport chain, mitochondrial transit peptide, mitochondrial translocation, and mitoribosomes[19]. The NASA Twins Study also revealed transient enrichment of mitochondrial signaling that returned to baseline in the recovery period[20].

Within our cell populations, longitudinal gene expression analyses revealed a conserved trend across both adaptive and innate immune cells that had never been described before in the spaceflight literature. In comparisons between L-92 vs R + 1 all immune cells demonstrated decreased expression of *MTRNRL12*. *PLCG2* expression was decreased in R + 1 relative to L-92 and increased in R + 82 when compared to both the R + 1 and L-92 timepoints. *MTRNRL12* is a pseudogene thought to be involved in negative regulation of the execution phase of apoptosis[21]. As such, decreased *MTRNRL12* expression could indicate increased immune cell apoptosis. Increased immune cell apoptosis was seen in our cfDNA analysis at the R + 82 timepoint, providing support to the gene expression alterations seen. While PLCG2 has pleiotropic functions, it is primarily known for its role in immune cell signaling downstream of surface receptor tyrosine kinases (RTKs) including T and B cell receptors by cleaving PIP2 into IP3 and diacylglycerol (DAG) leading to the generation of key secondary messengers involved in immune activation[22]. In addition to its roles in adaptive immunity, PLCG2 signaling is critical in innate immune cells, such as monocytes, macrophages and NK cells, mediating signaling downstream of the Fc gamma receptor. Thus, PLCG2 expression changes across multiple immune subsets may reflect global alterations in signaling in response to various RTKs modulating antigen responses, as well as cytokine and Toll-like receptor signaling. Furthermore, although the exact physiological pathway is not known, PLCG2 positively regulates mitochondrial respiration[23]. These findings suggest the mitochondrial electron transport chain may sustain functional alterations months after return to earth despite the short duration of the mission.

Immune Adaptations to Short Duration Spaceflight

Immune system dysregulation in crewmembers following spaceflight has been consistently reported, revealing adaptive immune system changes and general shifts towards a Th2 T cell phenotype[24,25]. Space environment exposure leads to suppressed activation and reduction in T cells, along with

fluctuations in cytokine gene expression, such as increased TNF-a, interleukin-8, interleukin-1ra, thrombopoietin, VEGF, and various chemokines[2,25,26].

Our PBMC phenotype analysis offers insight into alterations within immune cell subpopulations, cell state, and genotype associated with short duration orbital flight in an all-civilian crew. T cells, specifically CD8 + and CD4 + cells, exhibit increased *AHR* expression in R + 1 (Fig. 4, Suppl. Figure 4C), a transcription factor known to promote the development of pro-inflammatory Th17 and Th22 cells[27,28]. Thelper (CD4+) populations present increased *IL-32* gene expression (Suppl. Figure 4D) at both R + 1 and R + 82 timepoints relative to preflight L-92, suggesting a lasting pro-inflammatory phenotype shift up to three months postflight.

Immune dysfunction involving cytotoxic T cells is a proposed side effect of space travel[29], associated with an elevated risk of opportunistic infections during spaceflight, such as latent herpes virus reactivation[30]. Short-term exposure to spaceflight conditions may affect innate immune system components, including functional alterations of key immune cell populations like CD14 + monocytes and dendritic cells. Microgravity-induced changes can result in decreased neutrophil and macrophage phagocytic activity, thus affecting the initial response to pathogens. Moreover, microgravity may alter monocyte maturation and function, which play a critical role in antigen presentation and Tcell activation. Kaur et al. (2005) describe that, although the percentage of CD14 + monocytes was not significantly reduced in astronauts following spaceflight, days after landing, the ability of these cells to engulf pathogens, degranulate, and mount an immune response was reduced compared to that of ground controls[31]. Though little is known about the influence of spaceflight on dendritic cell function, their role in inflammation is well characterized as they play key roles in modulating cytokine production, mediating migration, and enhancing antigen capture and processing[32].

Long-term effects of short-duration spaceflight on the immune system, particularly in civilians and first-fliers, is minimally understood, but critical as we expand human presence in orbit and deep space. Previous studies identify several adaptations within CD8 + T cell subsets of space shuttle astronauts post-spaceflight[25]. Long duration spaceflight appears to elicit alterations in CD8 + T cell maturation, along with a general reduction of these cell types post 6-months of spaceflight, with differential responses to various mitogens[26]. A comparison study of short versus long duration spaceflight showed similar changes occurring between different mission types, with one distinction being that ISS crew members demonstrated a statistically significant reduction in early T cell activation potential post-spaceflight, with the percentage of T cells capable of producing IL-2 being reduced relative to Space Shuttle mission astronauts[31]. Moreover, a spaceflight mission of rodents revealed a decrease in CD4 + CD8 + double positive precursor and CD4+, CD8 + single positive mature T cells, consistent with impaired T cell development, highlighting the role of gravity with these cell types[14].

Based on our gene expression analysis from the CD8 + T cell population, we observed increased expression of *CXCR4*, *CCL5*, *JUNB*, *NKG7*, and *SRGN* in R + 1 compared to L-92 and *CCL5, IL32, NKG7*,

and *GNLY* in R + 82 compared to L-92, which may signal Cytotoxic T cell activation(Fig. 4B). We detected gene expression profile changes in innate immune cells across R + 1 and R + 82 timepoints compared to L-92 (Suppl. Tables 3 & 4). CD14 + monocytes had transient upregulation of *CTSD, NFKBIA*, and *S100A8* at R + 1 vs L-92, but not in R + 82. *CTSD* upregulation may enhance lysosomal proteolysis and endocytosis, improving antigen presentation by MHC class II molecules. *NFKBIA* upregulation may modulate NF-kB signaling, affecting proinflammatory gene expression. *S100A8* is a pro-inflammatory chemokine, and its upregulation might enhance Ca2 + signaling, migration, and immune activation, leading to an overactive innate response if uncontrolled. In contrast, CD14 + monocytes showed downregulation of *PLCG2, RIPOR2, ANKRD44* at R + 1 vs L-92. *PLCG2* downregulation could impair second messenger generation and downstream immune signaling, weakening the immune response. *RIPOR2* reduction may affect cell motility and monocyte migration, impacting the overall immune response. *ANKRD44* downregulation might alter monocyte activation and immune response through immune-related transcription factor regulation. These opposing changes may represent physiological countermeasures to the effects of spaceflight in an attempt to maintain and/or restore homeostasis.

While certain genes returned to baseline at R + 82 vs L-92, some long-lasting changes persisted. *B2M, HLA-C*, and *S100A4* were upregulated at R + 1 and R + 82, potentially increasing antigen presentation and immune surveillance. In contrast, *MAML2, CD44*, and *CAMK4* were downregulated at R + 82 vs L-92, which may affect monocyte differentiation, cell adhesion, migration, and signal transduction, leading to a weaker immune response. The combination of these pro-inflammatory and impaired immune function genes is an indication of innate immunity maladaptations that are present for months after short duration orbital flight.

Finally, it is worth noting that Space Shuttle studies revealed decreased lymphocyte response, postflight neutrophil increase, and eosinophil decrease, correlating with inflight stress rather than microgravity[26,33,34]. Latent herpesvirus reactivation and cytomegalovirus shedding were also observed during space shuttle flightst[14]. Similar adaptations occurred in ISS astronauts. ISS crew members experienced mild infectious diseases, atypical allergies, or dermatitis without significant operational impact[14,28]. A 12-year ISS immune data comparison indicated improvements in immunity, stress, and viral reactivation due to operational and biomedical countermeasures onboard ISS such as resupply frequency, improvements in personal communication, exercise equipment and food quality and variety suggesting the potential of improved quality of life on the health of astronauts[35]. Such operational and biomedical considerations from earlier missions, as well as the cellular and molecular data shown here, can help future crews and missions maintain crew health and safety, as well as guide efforts for lifetime health studies of astronauts.

Methods

I4 launched from Kennedy Space Center's Launch Complex 39A and traveled into Low-Earth Orbit across a three-day mission, reaching an orbital altitude of approximately 364 miles and ultimately splashed down into the Atlantic Ocean.

Dried Blood Spot (DBS) Pre-flight, In-flight, and Post-flight Sampling. Crew members warmed and massaged their fingertips to maximize blood flow. Fingertips were sterilized (BZK antiseptic towelette, Dynarex, Reorder No. 1303) and punctured using a contact-activated lancet (BD Biosciences, #366593) or a 21-gauge needle (BD Biosciences, #305167). Whatman 903 Protein Saver DBS cards (Cytiva, #10534612) were used to capture, transfer, and then store capillary blood with a desiccant pack (Cytiva, #10548239) at ambient temperature.

DNA Extraction for qPCR-based assessment of Telomere Length. Three 3 mm circular punches were cut from the Whatman 903 Protein Saver Cards (cat# WHA10534612) containing blood samples using an Integra Miltex Standard Biopsy Punch (cat# 12-460-406) and placed into a 1.5 mL microcentrifuge tube with sterile tweezers. Samples were prepared using the Qiagen QIAamp DNA Investigator Kit (cat# 56504) following the manufacturer's isolation of total DNA from FTA and Guthrie Cards protocol. The quantification of DNA in each sample was determined through fluorometric quantification with the Qubit 4 Fluorometer (Thermo Fisher Scientific, cat# Q33238) and the 1X dsDNA HS Assay Kit (cat# Q33231). DNA samples were sent to Colorado State University for multiplex qPCR analysis.

Multiplex Quantitative PCR Telomere Length Measurement. MMqPCR measurements of telomere length were carried out by preparing 22 µL of master mix using SYBR green GoTaq qPCR master mix (Promega #A6001) combined with the telomere forward primer (TelG; 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), telomere reverse primer (TelC; 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCT AACA-3'), albumin forward primer (AlbU; 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGA AATGCTGCACAGAATCCTTG-3'), albumin reverse primer (AlbD; 5'-GCCCGGCCCGCCG 4 CGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3') at 10 µM per primer (Integrated DNA Technologies), and RNase/DNase free water. 3 µL of DNA at 3.33 ng/uL was added for a final volume of 25 µL, final TelG/C primers concentration of 900 nM, and the AlbU/D primers at 400 nM. A Bio-Rad CFX-96 qPCR machine was used to measure telomere length. The cycle design was as follows: 95°C for 3 min; 94°C for 15 s, 49°C for 15 s, for 2 cycles; 94°C for 15 s, 62°C for 10 s, 74°C for 15 s, 84°C for 10 s, and 88°C for 15 s, for 32 cycles. The melting curve was established by a 72°C to 95°C ramp at 0.5°C/second increase with a 30 second hold. Standard curves were prepared using human genomic DNA (Promega Cat # G3041) with 3-fold dilutions ranging from 50 ng to 0.617 ng in 3 µL per dilution. Negative controls included a no-template TelG/C only and AlbU/D only, and a combined TelG/C and AlbU/D control. Samples were normalized across plates using a human genomic DNA standard. Each sample was run in triplicate on a 96-well plate format and relative telomere length was established using a telomere (T) to albumin (A) ratio.

Whole Genome Extraction and Sequencing

DNA were extracted from the cell pellets of spun down cfDNA blood collection tubes (Streck, #230470) using the QIAamp Blood Maxi Kit (Qiagen #51192), and then shipped to Element Biosciences for library preparation. The extracted DNA was quantified using Thermo Fisher Qubit dsDNA HS Assay Kit (cat#

Q238253) and 8 samples were prepared using the KAPA HyperPrep Kit and KAPA Unique-Dual Indexed Adapter Kit (cat# 8861919702). The DNA libraries were quantified using Thermo Fisher Qubit dsDNA HS Assay Kit (cat# Q32854) and sized using Agilent High Sensitivity DNA Kit (cat# 5067 – 4626).

The 8 DNA libraries generated with the KAPA HyperPrep Kit were processed using Adept Library Compatibility Kit (Element Biosciences, Cat# 830-00003), individually circularized with 0.5pmol (30 ul of 16.67nM) input, and quantified using the kit-provided qPCR standard and primer mix. The libraries were pooled into 4 separate 2-plex pools, each denatured and sequenced on Element AVITI system (Element Biosciences, Part #88 – 00001) using 2x150 paired end reads with indexing. Primary analysis was performed onboard the AVITI sequencing instrument. FASTQ files were analyzed using a secondary analysis pipeline from Sentieon.

cfDNA Extraction and Sequencing

cfDNA was isolated from 500uL aliquots of plasma from cfDNA blood collection tubes (Streck, #230470). cfDNA was extracted from each crew member from all timepoints (4 crew members, 6 timepoints, 24 total extractions). cfDNA was extracted using Qiagen's QIAamp ccf DNA/RNA Kit and eluted in 15 uL Qiagen Elution Buffer per sample. Yield was measured for each sample using Thermo Fisher Qubit 1X dsDNA HS Assay (cat# Q33231).

Entire extract volume was used as input for library preparation using NEBNext Ultra II DNA Library Preparation Kit for cfDNA protocol. Each sample was barcoded using NEBNext Multiplex Oligos for Illumina (Unique Dual Index UMI Adaptors – 96 reactions). Final library was eluted in 30uL and checked for concentration using Thermo Fisher Qubit 1X dsDNA HS Assay (cat# Q33231). Fragment sizes were determined using Agilent's Tapestation 2100 and D1000 reagents and screentape, with resulting average fragment size ~ 380 bp. 0.25 pmol of each sample.

A total of 24 cfDNA libraries generated with the NEBNext Ultra II DNA Library Preparation kit were processed using Adept Library Compatibility Kit (Element Biosciences, Cat# 830-00003). Each library was circularized individually with an input range of 0.2–0.5 pmol (30 ul of 6.67-16.67nM) based on linear library yields. The final circularized libraries were quantified using qPCR standard and primer mix and pooled into 2 separate 4-plex pools. Each 4-plex pool was denatured and sequenced on Element AVITI system (Element Biosciences, Part #88 – 00001) using 2x147 paired reads with 19bp UMI/index 1 and 8 bp index 2. Primary analysis was performed onboard the AVITI sequencing instrument. FASTQ files were analyzed using a secondary analysis pipeline from Sentieon.

Clonal Hematopoiesis Targeted Variant Calling:

Genomic DNA was obtained from purified, granulocyte-depleted Peripheral Blood Mononuclear Cells using the same protocol as NASA Twins Study[6]. All samples from the testing and validation cohort were

sequenced using a custom designed DNA sequencing assay (DB0188, VariantPlex, ArcherDX). This panel captures the nine genes most commonly mutated in solid tumor patients following therapeutic radiation including the full exonic regions of five genes (*DNMT3A, TET2, ASXL1, TP53, CHEK2*) and targeted exonic regions of four genes (*JAK2, SRSF2, SF3B1, PPM1D*). Libraries were prepared from 250 ng gDNA using the VariantPlex protocol (ArcherDx Inc., Boulder, CO, USA) which utilizes Anchored Multiplex PCR (AMP) technology to generate target-enriched sequencing-ready libraries. Following DNA fragmentation ligation with a universal ArcherDx molecular barcode (MBC) adapter is performed, which tags each DNA molecule with a unique molecular index (UMI) and allows for unidirectional amplification of the sample using gene-specific primers. The resulting libraries were sequenced using a NovaSeq 6000 instrument (Illumina), as per manufacturer's instructions. UMI Consensus was built using Sentieon's UMI extract tool, alignment to the GRCh 38 reference genome was performed with BWA MEM (v0.7.15). Sentieon TNscope RNA-seq variant pipeline (v202010) was used for variant calling, filtering of reads based on mapping quality, depth, and strand bias[36]. BCFtools(v1.9) was used to filter by triallelic sites, short tandem repeats, read quality and read position bias. Varient-Effect-Predictor VEP(v107) and SnpEff(v4.3) was used for annotation of variants and further filtering based on predicted impact of mutations. Data wrangling, tidying, and visualizations were performed using R (v4.1.2), RStudio(v2021.09.2) and libraries (Tidyverse, Dplyr, data.table, ggplot2).

Whole Genome / cfDNA Preprocessing

Blood and plasma samples were subjected to whole genome and cfDNA short read sequencing as detailed above. Resultant FASTQ files were validated using FastQC (v0.11.9) and MultiQC (v1.13). Read adapters were trimmed at 3' and 5' ends for low quality using Trim Galore (v0.6.5), lower quality reads were classified and removed, retaining only those reads with length > = 25bp, and ph read quality > = 20. Reads were aligned against the hg38 human reference genome with BWA MEM (v.0.7.15) and subjected to standard QC and deduplication procedures as a part of Sentieon's TNscope (v202010) DNAseq workflow.

Whole Genome / cfDNA / Single cell Variant Calling

Aligned and preprocessed reads were subjected to the TNScope variant calling pipeline. Calls were filtered using Fisher's exact test and subsetted to SNP variants using samtools (v1.16.1), and filtered by triallelic sites, short tandem repeats, read quality, and read position bias using BCFtools (v1.16). Varient-Effect-Predictor VEP (v107) was utilized for annotation of variants and further filtering based on predicted impact of mutations. Resulting coordinates were processed into allele and gene frequency matrices, and visualized in R using the tidyverse (v1.3.2) suite of packages.

cfDNA Fragment Analysis

Fragment size distribution was calculated using the bamPEFragmentSize tool from the deepTools Python package (v3.5.1). Levels of cfDNA (read counts) originating from different chromosomes were normalized by chromosome length and total number of reads in the library generating an Read per

Killobase per Million reads (RPKM) measurement. The fraction of cell-free mtDNA relative to chromosomal cfDNA in plasma was compared and visualized in R using the tidyverse (v1.3.2) suite of packages.

cfDNA Tissue of Origin Deconvolution

The enrichment of cfDNA fragments from various tissues were calculated by read coverage depletion analysis at transcription starting sites (TSSs) to estimate nucleosome positioning and infer gene expression. The pipeline is described in detail in Bezdan et al. 2020[7]. The resulting nucleosome periodicity was correlated with (1) per-tissue gene expression reference matrix retrieved from the Human Proteome Map (HPM; Kim et al. 2014[11]) or (2) individual astronaut pseudo-bulk expression of different cell subpopulations extracted from PBMC scRNAseq dataset. In both cases the tissue/subpopulation-periodicity correlations were ranked by the value of Pearson's correlation coefficient, clustered (utilizing Ward method with Euclidean distances) and visualized in R using the tidyverse (v1.3.2) and ComplexHeatmap (v2.14.0) packages.

Longitudinal Gene Expression Analyses

Longitudinal single cell data was processed in R using the Seurat package (v4.3.0) to normalize, scale and cluster cell populations. Cell identities were determined through computational gating parameters of inclusion based on gene expression of key markers, similar to gating from Fluorescent-Activated Cell Sorting. CD8 + T cells were selected from the PBMC population by filtering CD3D + CD8A + positive cells. CD4 + T cells from CD3D + CD8A + cells, CD14 + monocytes from CCR2 + CD14 + cells, CD16 + monocytes from CD14 + CD16 + cells, NK cells from NCAM1 + CD3- and NCR3 + CD3- cells and DCs from CD86+, CD83 + cells. Seurat package (v4.3.0) was used for normalization, scaling, and differential gene expression analysis of sn-DNA data. Data wrangling, tidying, and visualizations were performed using R (v4.1.2), RStudio(v2021.09.2) and libraries (Tidyverse, Dplyr, data.table, ggplot2).

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Figures

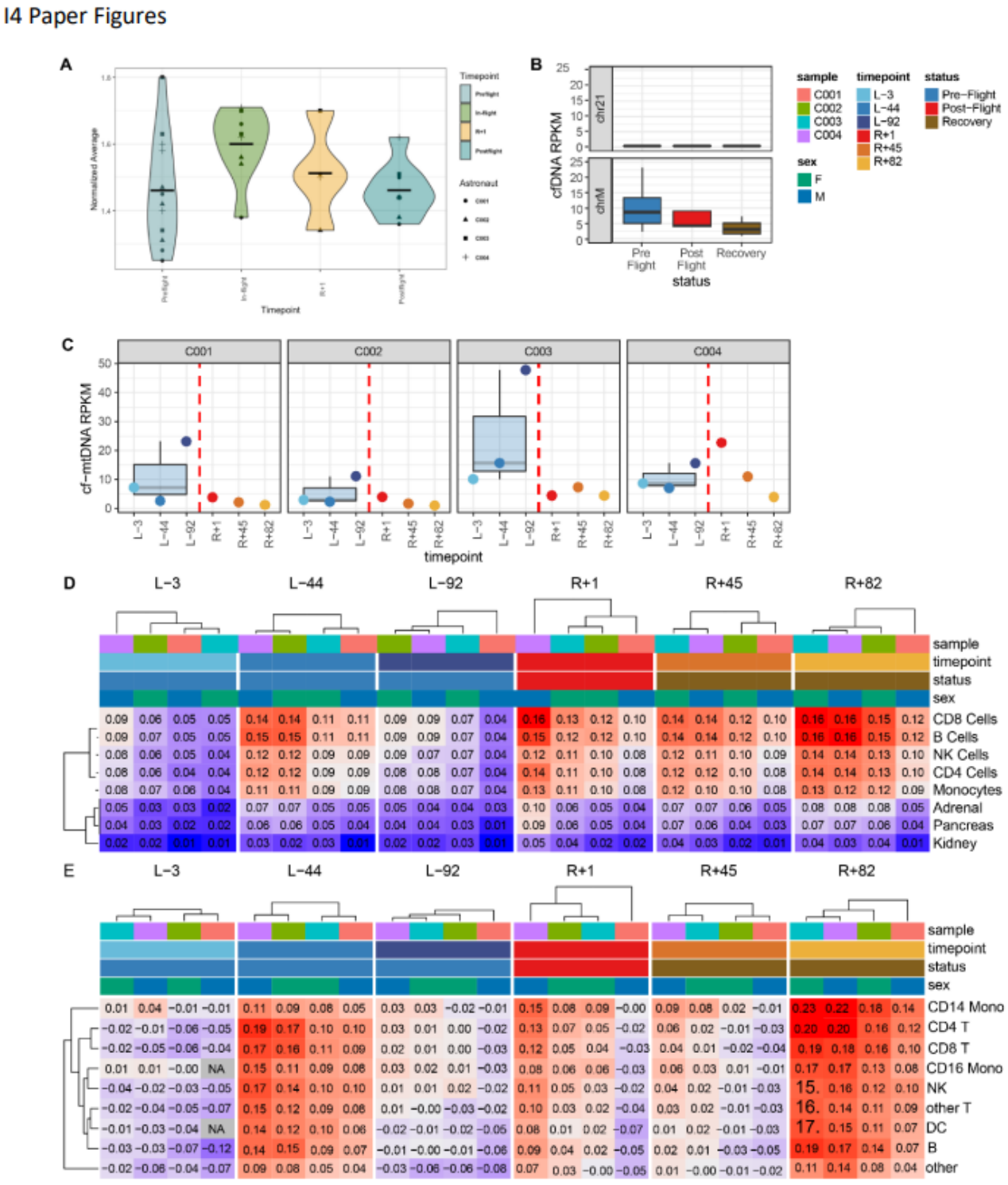

Figure 1

Telomere length dynamics, Mitochondrial, and Cell-Free DNA Cell Lysis Identity as a function of spaceflight. 1A. Telomere length dynamics assessed by MMqPCR in I4 crewmembers. Normalized analysis shows increased average telomere length during orbital flight compared to preflight baseline, and a rapid decrease after landing that continues during post-flight recovery months. 1B&C. Autosomes do not show any spaceflight-related change in RPKM (reads per kilobase per million reads), as exemplified using chr21. The cf-mtDNA, while noticeably more enriched, shows high between-sample heterogeneity. 1D. Tissue of origin deconvolution for circulating cfDNA fragments reveals an increased cfDNA signature of both adaptive and innate immune cells post-landing and during recovery. The enrichment of tissue signatures from the HPM9 was calculated based on inferred gene expression and nucleosomal footprinting of the cfDNA fragments. Average correlation coefficients (multiplied by -1) over technical replicates are depicted for each sample and time point. The heatmap was subsetted to relevant tissue signatures, extended data is available in Fig S1 and Table S1. 1E. Cross-examination of cfDNA origin deconvolution using each of I4 astronaut cell-subpopulation-specific expression markers - derived from peripheral blood (PBMC) dataset - supports the results in 1D. We note an increased presence of innate and adaptive immune cfDNA in R+1 and R+82 when compared to all other timepoints. The extended data is available in Table S2

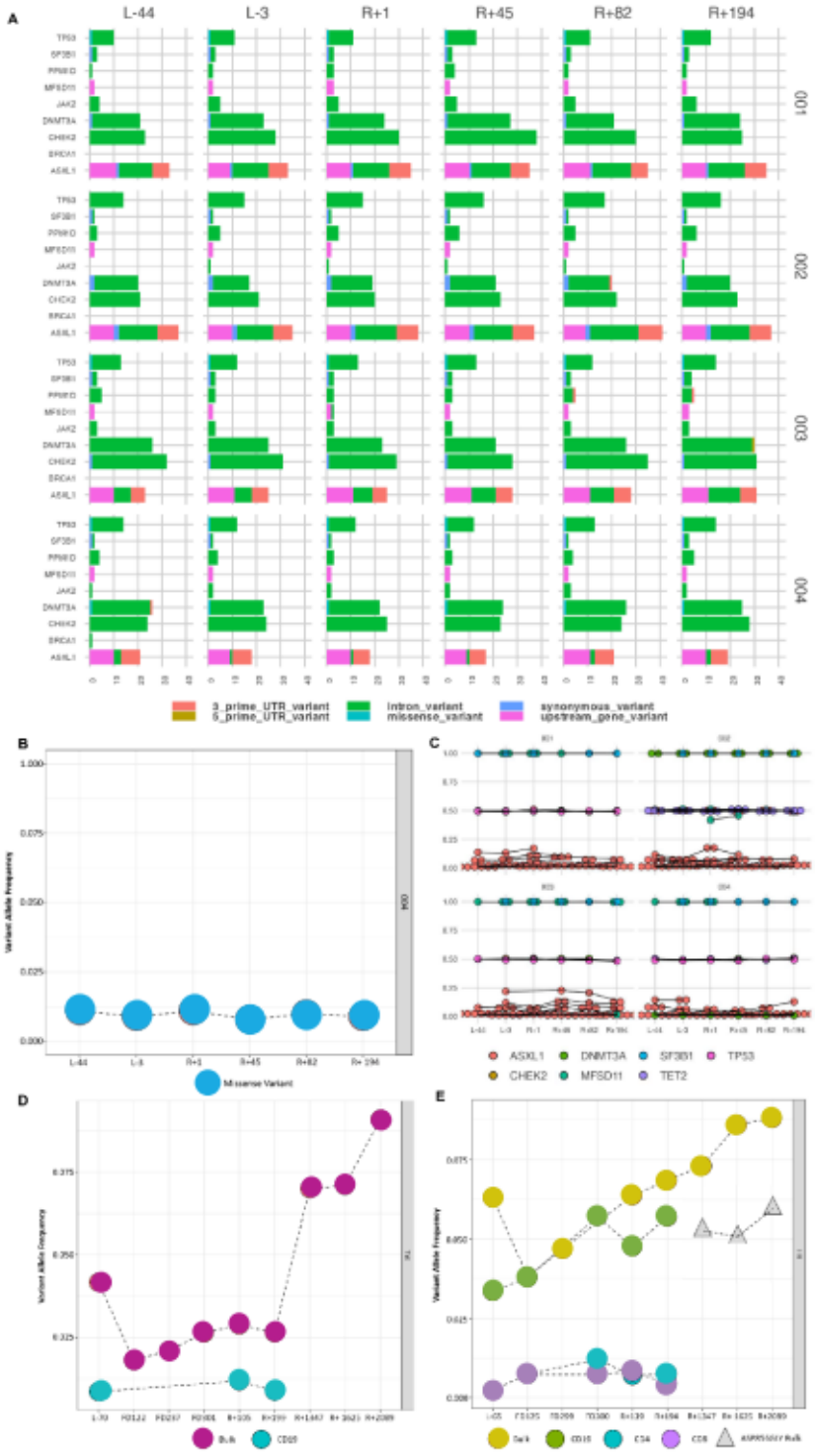

Figure 2

Targeted deep sequencing of CHIP-related genes exhibit genomic stability and comparable mutational burden as a function of spaceflight. 2A. Mutational burden of each gene per astronaut longitudinally shows the proportion of mutations which are non-coding and an increased mutational count of intronic variants for DNMT3A and CHEK2 genes. 2B. Variant allele frequency of coding Single Nucleotide Polymorphisms as a function of time demonstrates overarching genomic stability. 2C. Variant allele

frequency of rare missense variant (~ 0.01, chr2:g.25235778C>G (DNMT3A)) in subject 004 remains comparable both during preflight timepoints and postflight recovery. 2D. Variant allele frequency of TET2.p.Cys1273Tyr mutation in TW (spaceflight) subject of the NASA Twins Study with increased mutational burden throughout 6 years of follow-up. 2E. Variant allele frequency of DNMT3A.p.Trp698Ter and the new mutation DNMT3A.p.Asp856Gly in HR (ground subject) show increased variant allele frequency through a 6 year follow-up period

Figure 3

Whole genome sequencing and variant calling reveals genomic stability postflight. 3A. Comparison of variant consequences before and after spaceflight of genes with the greatest mutational burden demonstrates no gene is disproportionately mutated postflight. We report unique counts of (variant, effect annotation) pairs per gene of interest. 3B. De novo mutational comparison elucidates comparable mutational burdens at a genome-wide scale. Here we count variants as unique changes of reference to alternative alleles at a given position regardless of variant annotation. 3C. Variant effect annotation for

variants called in both timepoints shows most mutations are in non-coding regions. Variant annotations were ranked according to the severity of the variant effect estimated by Ensembl12. We report unique counts of (variant, effect annotation) pairs across the whole genome. 3D. Timepoint comparison of variant effect annotations across the whole genome shows no disproportional mutational load for variants called uniquely post-flight. Here we show the distribution for moderate to high-severity variants

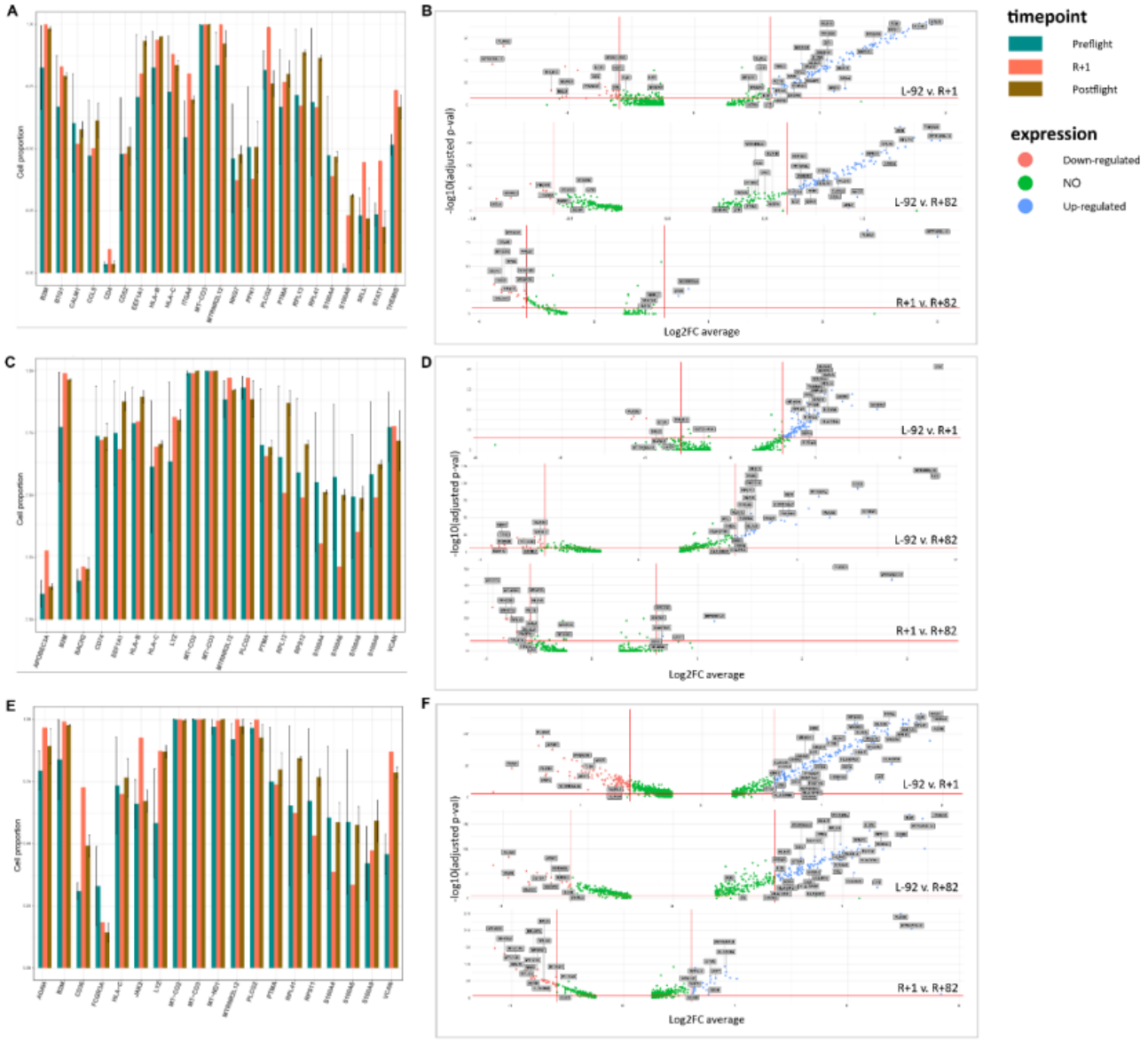

Figure 4

Differential gene expression and cell population proportion of expression analyses reveals, conserved, long and short-term adaptations across adaptive and innate immune cells. 4A. Proportion of CD8 T cells expressing genes 4B.CD8 T cells volcano plot comparison of differential gene expression in L-92 vs R+1, L-92 vs R+82 & R+1 vs R+82. 4C. Proportion of Classical monocytes expressing genes. 4D. Classical

monocytes longitudinal differential expression 4E. Proportion of Dendritic cells expressing genes. 4E. Dendritic cells longitudinal differential expression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- SupplementalFigures.pdf