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Alterations in adaptive immunity persist during long-duration spaceflight

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BACKGROUND: It is currently unknown whether immune system alterations persist during long-duration spaceflight. In this study various adaptive immune parameters were assessed in astronauts at three intervals during 6-month spaceflight on board the International Space Station (ISS).
AIMS: To assess phenotypic and functional immune system alterations in astronauts participating in 6-month orbital spaceflight.
METHODS: Blood was collected before, during, and after flight from 23 astronauts participating in 6-month ISS expeditions. In-flight samples were returned to Earth within 48 h of collection for immediate analysis. Assays included peripheral leukocyte distribution, T-cell function, virus-specific immunity, and mitogen-stimulated cytokine production profiles.
RESULTS: Redistribution of leukocyte subsets occurred during flight, including an elevated white blood cell (WBC) count and alterations in $CD8^+$ T-cell maturation. A reduction in general T-cell function (both $CD4^+$ and $CD8^+$) persisted for the duration of the 6-month spaceflights, with differential responses between mitogens suggesting an activation threshold shift. The percentage of $CD4^+$ T cells capable of producing IL-2 was depressed after landing. Significant reductions in mitogen-stimulated production of IFNγ, IL-10, IL-5, TNFα, and IL-6 persisted during spaceflight. Following lipopolysaccharide (LPS) stimulation, production of IL-10 was reduced, whereas IL-8 production was increased during flight.
CONCLUSIONS: The data indicated that immune alterations persist during long-duration spaceflight. This phenomenon, in the absence of appropriate countermeasures, has the potential to increase specific clinical risks for crewmembers during exploration-class deep space missions.

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INTRODUCTION

Immune system alterations have been previously characterized as essentially a postflight phenomenon, in animal studies or using terrestrial modeled microgravity or in-flight cell culture. It generally consists of altered cytokine production patterns,[1–5] natural killer (NK)-cell function,[6–9] leukocyte distribution,[10,11] monocyte function,[12] granulocyte function,[11,13] T-cell intracellular signaling,[14–16] neuroendocrine responses,[17,18] and leukocyte proliferation following activation.[19,20] The capability of lymphocytes to migrate through tissues was inhibited in modeled microgravity conditions.[21] Little in-flight information has been generated regarding immunocompetence of astronauts during spaceflight. Taylor *et al.* found that delayed-type hypersensitivity reactions to recall antigens were blunted during short-duration spaceflight.[22] Recently, immune alterations were characterized in astronaut subjects participating in short-duration Space Shuttle missions,[23] confirming the presence of an in-flight phenomenon, as opposed to merely a stress-response related to landing and readaptation. The reactivation of latent herpesviruses, including Epstein–Barr virus (EBV), cytomegalovirus (CMV), and varicella zoster virus (VZV), has been well documented in astronauts participating in short-duration spaceflight.[24–28] The reactivation of latent herpesviruses in astronauts was recently found to correlate with altered immunity.[29] It remains unknown, however, whether the phenomenon observed during short-duration flights would persist during long-duration spaceflight or resolve, having merely been owing to the stressors of launch and early adaptation to spaceflight. A paucity of data exists regarding human immunity during long-duration spaceflight. In a very limited number of subjects, it was reported that cell-mediated immunity was depressed during flights on board the Russian Mir Space Station.[30] Should immune alterations persist for the duration of a human Mars mission, it could elevate specific clinical risks to crewmembers including infectious disease, allergies and hypersensitivities, autoimmunity, altered wound healing, and the consequences of persistent latent herpes virus reactivation. Certainly in synergy with increased radiation exposure, there would be an increased concern for the development of malignancy. The final construction of the International Space Station (ISS), with its unique laboratory support facilities and six-person crew, afforded an excellent opportunity to perform a comprehensive assessment of immune parameters as they equilibrate during long-duration spaceflight. In this study we performed an integrated assessment of immune function, cytokine profiles, and viral immunity during flight on board the ISS. Blood samples were collected at three intervals during flight, always shortly before undock of a visiting vehicle, and were immediately returned (ambient) for analysis. This analysis strategy was preferred, since as laboratory analysis capability on board ISS is currently limited, terrestrial analysis of samples collected on-orbit afforded a wider array of analytical capability. As microgravity cell culture has been found to inhibit T-cell activation, terrestrial

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Table 1. Fluorescent antibody matrix for flow cytometry analysis of astronaut peripheral leukocyte distribution

Cell population	*Marker combination/analysis method*
WBC	Hematology analyzer
Neutrophils	$CD45^+/CD14^-$ (scatter characteristics)
Lymphocytes	$CD45^+/CD14^-$ (scatter characteristics)
Monocytes	$CD45^+/CD14^+$ (scatter characteristics)
T cells	$CD45^+/CD3^+$
B cells	$CD45^+/CD19^+$
NK cells	$CD45^+/CD16^+CD56^+$
$CD4^+$ T-cell subset	$CD45^+/CD3^+/CD4^+$
$CD8^+$ T-cell subset	$CD45^+/CD3^+/CD8^+$
Bulk memory CD4	$CD3^+/CD4^+/CD45RO^+$
Bulk memory CD8	$CD3^+/CD8^+/CD45RO^+$
Undifferentiated CD8	$CD3^+/CD8^+/CD244^-/CD28^+$
Active cytotoxic CD8	$CD3^+/CD8^+/CD244^+/CD28^+$
Senescent CD8	$CD3^+/CD8^+/CD244^+/CD28^-$
CD8 true naive	$CD3^+/CD8^+/CD45RA^+/CD62L^+$
CD8 central memory	$CD3^+/CD8^+/CD45RA^-/CD62L^+$
CD8 effector memory	$CD3^+/CD8^+/CD45RA^-/CD62L^-$
CD8 terminally differentiated	$CD3^+/CD8^+/CD45RA^+/CD62L^-$
Activated CD4	$CD3^+/CD4^+/HLA-DR^+$
Activated CD8	$CD3^+/CD8^+/HLA-DR^+$
EBV viral peptide-specific CD8	$CD3^+/CD8^+/$EBV Tetramer$^+$
CMV viral peptide-specific CD8	$CD3^+/CD8^+/$CMV Tetramer$^+$

Abbreviations: CMV, cytomegalovirus; EBV, Epstein–Barr virus; WBC, white blood cell.
The gating strategy involved resolution of mixed populations of interest from red blood cells and artifactual debris via a gating antibody (usually CD45 or CD3). Relevant subpopulations of interest were then quantified via plotting the subsequent subpopulation-specific markers as appropriate. Populations were quantified as relative % of the parent population, i.e., lymphocyte subsets from all leukocytes, or T-cell subsets from all T cells.

(unit gravity) cell culture would afford an assessment of crew immunocompetence without this potential confounding variable.

MATERIALS AND METHODS

Subjects and missions

Twenty-three ISS astronauts participated in this study. To determine sample size, existing subject incidence rates for detectable immune system changes were used for power calculations (based on previous spaceflight experiments). Sixteen of the subjects flew on the Russian 'Soyuz' vehicle, and mission durations were nearly 6 months. The remaining seven subjects flew on the US Space Shuttle. For the Shuttle-ISS astronauts, five had mission durations of >100 days, still considered 'long-duration' spaceflight. The remaining two astronauts had mission durations of <60 days. Of the 23 subjects, 18 were male and 5 were female, and their mean age was 53 years. Two of the assays required 'compatibility' of subjects. The MHC-tetramer staining for viral peptide-specific T cells requires subjects to be HLA-A2 positive. For this assay, 8 subjects were compatible. Also, for reasons less understood, certain individuals do not respond well to T-cell stimulation with soluble antibodies to CD3 and CD28.[31] For this study, 14 astronauts were compatible with anti-CD3 mitogenic stimulation, employed in both the 24 h blastogenesis, and the 48 h cytokine production assays. Institutional review board approval was obtained from the Committee for the Protection of Human Subjects at the Johnson Space Center (JSC), Houston, TX, USA. Informed consent was obtained from all subjects who participated in the study.

Blood samples

For this study a sample consisted of 8.5 ml acid–citrate–dextrose (ACD) anticoagulated and 10.0 ml ethylenediaminetetraacetic acid (EDTA) anticoagulated peripheral blood. Blood samples were collected at the following time points: 180 and 45 days before flight (L-180 and L-45, respectively), during flight at approximately mission day 14 (early), between mission months 2 and 4 (mid-mission), and about 6 months (late in the mission), 3 h after landing (R+0), and 30 days after landing (R+30). For only the two 'shorter duration' subjects with mission durations of <60 days, only two in-flight samples were collected. The samples from these two shorter duration crews were termed 'early' and 'mid-mission' respectively. In-flight and R+0 samples were processed at the Kennedy Space Center, Florida, or Edwards Air Force Base, CA, USA if returned on the Shuttle, or in Star City, Russia, if returned on Soyuz, where blood cells or culture supernatants were cultured, stained and/or stabilized and transported to JSC for analysis.

All samples, both ground and flight, were processed immediately upon receipt in the laboratory. Owing to the time required to transport the samples from ISS to the laboratory, the effect of a processing delay of up to 72 h on the cell viability, leukocyte distribution, and cellular functional assays was assessed prior to the initiation of the study. This was conducted by performing the cellular analysis assays on ACD blood samples collected from healthy Test Subjects. ACD blood collection tubes (commonly used in blood banking) contain both anticoagulant and nutrients designed to maintain cellular viability. Samples were assessed upon collection, and after a room temperature processing delay of 24, 48, and 72 h. The data demonstrated that the ACD blood collection tube maintained leukocyte distribution and functional integrity for all assays to at least 72 h with minimal variation (data not shown). The WBC parameter was similarly validated from an EDTA blood sample, since blood collection in the ACD tube results in a degree of sample dilution, skewing absolute cell count measurements. The observed time delay from sample collection on orbit to terrestrial sample processing averaged 37 h over the course of the experiment.

Immunophenotype analysis

A white blood cell (WBC) count was determined using a standard clinical hematology analyzer (Beckman-Coulter Unicel DxH 800, Miami, FL, USA). A comprehensive five-color flow cytometry antibody matrix was formulated for peripheral blood immunophenotype analysis (Table 1). Fluorescent-labeled antibodies were obtained from Beckman-Coulter (Miami, FL, USA). The specific fluorescent antibody matrix setup, as well as blood sample staining, lysis and flow cytometry analysis were set up and performed as previously described.[23] This panel assessed the major leukocyte and lymphocyte subsets, T-cell subsets, memory/naive, and central memory T-cell subsets, and constitutively activated T-cell percentages. Whole blood, without any artificial cellular enhancement or purification, was stained for analysis.

T-cell function

The expression of T-cell activation antigens following mitogenic stimulus was used to assess T-cell functional capability, or monitor progression through the early stages of T-cell activation. This concept has been previously applied to spaceflight investigations.[32] All live cell culture assays were performed using peripheral blood mononuclear cells (PBMCs), which were purified by Ficoll density-gradient centrifugation. All cultures were performed using 1×10^6 PBMCs in 1.0 ml RPMI medium. T-cell function was assessed by culturing in the presence of 0.125 μg/ml anti-CD3 and 0.25 μg/ml anti-CD28 soluble antibodies (both Becton Dickinson, Franklin Lakes, NJ, USA), or 10 μg each of *Staphylococcus* enterotoxin A (SEA) and B (SEB) (Sigma-Aldrich, St Louis, MO, USA). Cultures were incubated for 24 h at 37 °C. Following incubation, 800 μl of supernatant was removed and discarded from the cell pellet. The cell pellet was then resuspended in the remaining medium. A four-color staining of cell surface markers (anti-CD25/FITC, CD69/PE, CD8/ECD, and either CD4 or CD3/PC5) was performed as described previously.[23] Fluorescent-labeled antibodies were obtained from Beckman-Coulter (Miami, FL, USA). Flow cytometry was performed on a Beckman-Coulter XL flow cytometer. The gating strategy consisted of T-cell resolution and separation into CD4 and CD8 subsets, followed by enumeration of total $CD69^+$ and $CD69^+/CD25^+$ dual-positive events. T-cell function is defined as expression of either CD69 (early function) or co-expression of CD69 and CD25 (late function) following mitogenic stimulation as described.

Intracellular cytokine analysis

Cytokine production was assessed for specific T-cell subsets at the single-cell level using intracellular flow cytometry. PBMCs (1×10^6) were cultured in 1.0 ml culture media containing 10 ng/ml PMA, 1.0 μg/ml ionomycin and 3 μM monensin (Sigma-Aldrich). Cultures were incubated overnight (6 h) at

4 °C. Following incubation, the supernatants were removed and the cells were fixed in 4.0% paraformaldehyde for 10 min. To detect intracellular production of IFNγ or IL-2 (following surface marker staining), the fixed PBMCs were resuspended in 200 µl of permeabilization buffer (5.0% nonfat dry milk and 0.5% saponin in phosphate-buffered saline (PBS)) to which 0.5 µg of labeled mouse antibody to human IL-2 (FITC), IFNγ (PE), CD8 (ECD), and CD3 (PC5) was added. Fluorescent-labeled antibodies were obtained from Beckman-Coulter (Miami, FL, USA). The cells were incubated at 4°C for 16 h and then washed in PBS-containing saponin. The cells were then resuspended in 1.0% paraformaldehyde and analyzed on a Beckman Coulter XL flow cytometer. The gating strategy consisted of T-cell identification, resolution of CD4 and CD8 subsets, followed by resolution and enumeration of cells producing IFNγ and IL-2.

Secreted cytokine cytometric bead array

For analysis of secreted Th1/Th2 cytokine profiles, 1×10^6 PBMCs were cultured in 1.0 ml RPMI media. Mitogenic stimulation consisted of culture in the presence of 0.125 µg/ml anti-CD3 and 0.25 µg/ml anti-CD28 (both from Becton Dickinson) to activate T cells only via the T-cell receptor (TCR), 10 ng/ml PMA+2 µg/ml ionomycin (both Sigma-Aldrich) as a broader pharmacologic stimulus, or 10 µg/ml lipopolysaccharide (LPS) *Escherichia coli* serotype 026:B6 (Sigma-Aldrich) for monocyte activation. Cultures were incubated for 48 h. Supernatants were then removed and frozen until analysis. On CD3/CD28 and PMA-I cultures, a Th1/Th2 cytometric bead array assessment was performed according to the manufacturer's instructions (Becton Dickinson). This array simultaneously analyzes secreted IL-17A, IFNγ, TNFα, IL-10, IL-5, IL-4 and IL-2 using distinct bead populations that fluoresce to varying degrees along a single emission wavelength. For LPS-stimulated cultures, an inflammatory cytometric bead array (CBA) assay was performed, which assessed secreted TNFα, IL-10, IL-6, IL-1β and IL-8. For analysis, a Beckman-Coulter flow cytometer was configured to resolve all six-bead populations and allow increases in cytokine concentration to be detected. Both CBA assay kits were obtained from Becton Dickinson. As all crewmember samples were batch-analyzed, data were recorded as mean fluorescence intensity (MFI) and converted to pg/ml concentration to show subject-relative cytokine production alterations throughout the mission. For this assay, the MFIs directly relate to supernatant concentration of each cytokine. Conversion was performed by plotting subject MFI data against the MFI data derived for the standard curve. The lone exception is IFNγ, for which the MFI data are presented. For this cytokine only, a defective standard reagent precluded the conversion of MFI data to concentration.

Tetramer assay

HLA-A*0201-restricted tetramers (EBV BMLF; CMV pp65) were obtained from Beckman Coulter (San Diego, CA). PBMCs were incubated with PE-labeled viral-peptide-specific tetramer complexes that bind to the major histocompatibility molecule (MHC), along with CD8-PerCP (BD Biosciences), in Dulbecco's phosphate-buffered saline (dPBS) with 2% fetal calf serum for 30 min at room temperature. Cells were then washed and fixed in 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer using CellQuest software for data collection and analysis.

Peptide stimulation

Intracellular cytokine staining assays were performed as previously described.[33] PBMCs were isolated from heparinized whole blood by density-gradient centrifugation and washed three times in dPBS prior to use in functional studies. PBMCs were used immediately and stimulated with HLA-A*0201-restricted epitopic peptides (10 µg/ml/peptide) or controls (PBS). Peptides used in this study were CMV pp65 (residues 495–503; NLVPMVATV) and EBV BMLF (residues 280–288; GLCTLVAML). Costimulatory monoclonal antibodies (mAbs)—CD28 and CD49d mAbs (1 µg/ml each) (BD Immunocytometry Systems, San Jose, CA, USA)—were added to each tube. The tubes were vortexed and incubated for 6 h at 37 °C with 5% CO_2, with the addition of brefeldin A (10 µg/ml; Sigma, St. Louis, MO, USA) for the last 5 h. Following stimulation, cells were washed in PBS, incubated for 5 min at 37 °C in 0.02% EDTA and washed in dPBS. Cells were then sequentially incubated for 10 min in FACSLyse and FACSPerm solutions (both from Becton-Dickinson Biosciences), washed, and stained with anti-IFNγ (PE), anti-CD8 (PerCP), and anti-CD69 (APC) antibodies. Samples were then fixed in 1% paraformaldehyde and analyzed by four-color flow cytometry using a FACSCalibur cytometer and CellQuest software (both BD Biosciences). Flow cytometry data were analyzed and presented using Flow Jo software (Tree Star, San Carlos, CA, USA); 25,000 events were analyzed for each sample.

Statistical analysis

This was a longitudinal study of human subjects participating in spaceflight. The L-180 sample was considered the best baseline, as samples collected closer to launch may be influenced by premission stress. Statistical significance for each assay was evaluated using Student's paired *t*-test. For each assay, the data were first confirmed as a normal distribution using the Shapiro–Wilk Normality Test. Significance was then determined by comparing the mean value for the baseline L-180 timepoint to all in-flight and post-landing values. The mean differences between time points were considered significant if $P < 0.05$ and are indicated as such (*) on each data figure. Where normality failed, data were examined for outlier values, and significance was reanalyzed with outlier values removed. In only one instance, indicated in the text, this altered significance for an individual timepoint.

RESULTS

Peripheral leukocyte distribution

Among the bulk leukocyte subsets (WBC, differential, lymphocyte subsets), significant differences were observed during spaceflight. Relative to L-180 baseline data, both the absolute WBC and granulocyte levels increased during flight, whereas levels of lymphocytes and monocytes were unaltered during flight (Figure 1a). Levels of T cells, CD4 and CD8 subsets, and memory ($CD45RO^+$) T cells were also unaltered during spaceflight (Figure 1b, c). The level of NK cells was elevated at the late in-flight timepoint only, whereas B-cell levels were unaltered during flight (Figure 1b). In-flight alterations were observed among specific subsets of $CD8^+$ T cells. The absolute level of active cytotoxic ($CD28^+/CD244^+$) $CD8^+$ T cells was significantly increased only at the early in-flight time point, whereas the corresponding late senescent ($CD28^-/CD244^+$) T-cell population were significantly decreased during flight at the mid-mission timepoint (Figure 1d). For both subsets, nonsignificant in-flight data time points had mean data trending in a similar manner, and the mean levels of undifferentiated (true naive) $CD8^+$ T cells ($CD28^+/CD244^-$) trended downward through all in-flight data time points (Figure 1d). Central memory-effector T-cell subsets were also evaluated; however, because of large standard errors, significant in-flight differences were observed only for the central memory subset of cells ($CD62L^-/CD45RA^+$), which decreased early in-flight but trended similarly throughout flight (Figure 1e). Levels of early senescent ($CD57^+$) T-cell subsets (both $CD4^+$ and $CD8^+$) trended negatively during flight; however, the decrease reached significance for only the $CD4^+/CD57^+$ subset immediately after landing (Figure 1f). Interestingly, among constitutively activated T-cell subsets disparity was observed between early-activated ($CD69^+$) and late-activated ($HLA\text{-}DR^+$) subsets. For both CD4 and $CD8^+$ T-cell subsets, early-activated T cells trended to be elevated, but reached significance only at R+0 for the $CD8^+$ subset (Figure 1g). In contrast, levels of late-activated T cells (both subsets) trended downward from baseline during flight, reaching significance for the CD8 population at the mid-mission timepoint and for the CD4 population at R+0 (Figure 1h). Levels of T cells specific for viral peptides from EBV and CMV were unaltered during flight (Figure 1i).

Intracellular cytokine profiles

After 6 h of stimulation in the presence of PMA+ionomycin and monensin as described, the percentage of T-cell subsets capable of being stimulated to produce cytokine was assessed. The percentage of $CD8^+$ T cells capable of being stimulated to produce IFNγ was unchanged during spaceflight (Figure 2). The percentage of $CD4^+$ T cells capable of being stimulated to produce IL-2 was also unchanged during spaceflight, but it was significantly reduced immediately after landing (Figure 2).

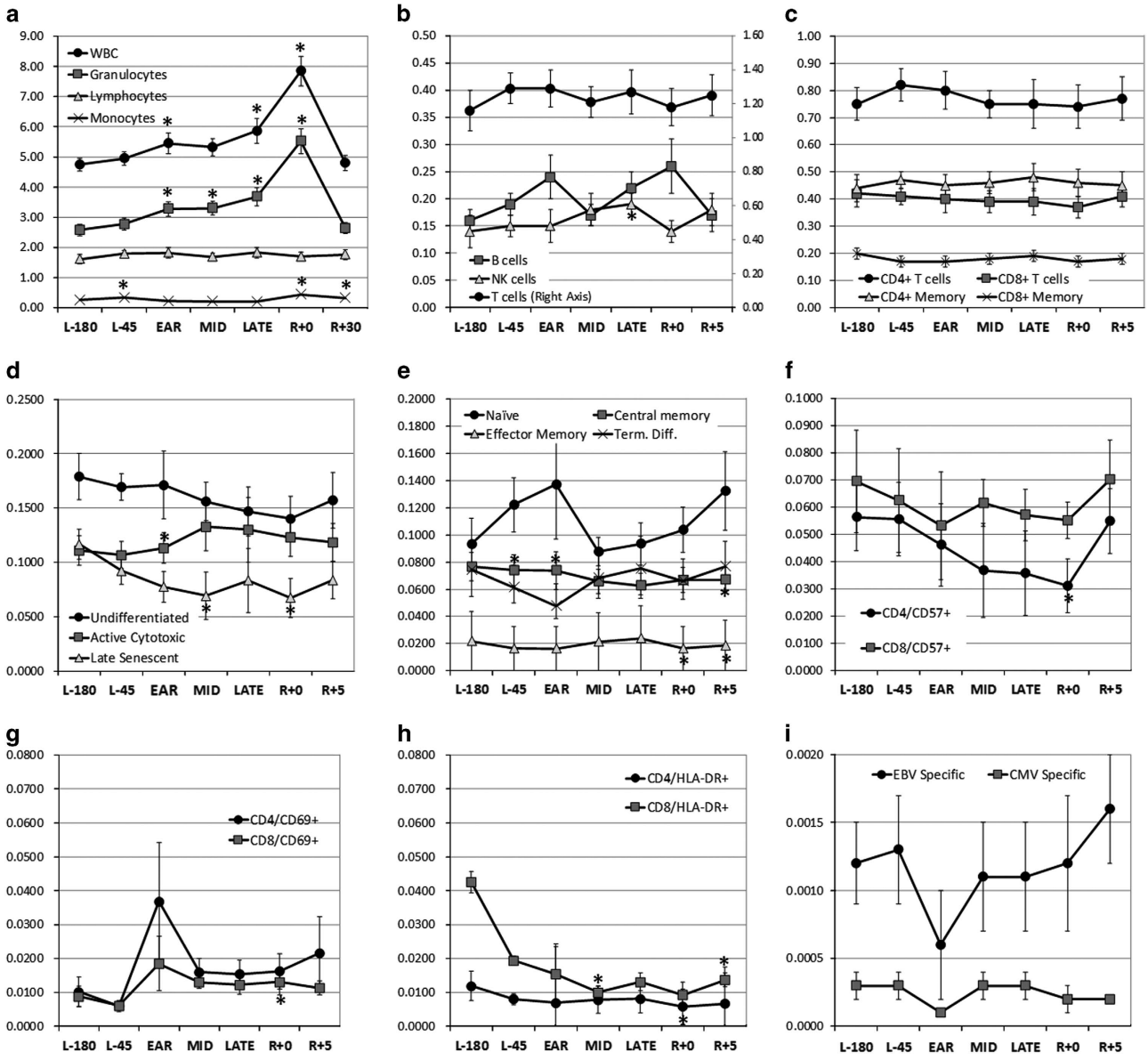

Figure 1. Absolute peripheral blood levels (×1000 cells/μl) for the indicated leukocyte, lymphocyte, and T-cell subpopulations before, during (early, mid-mission, and late) and following spaceflight. (**a**) Leukocyte subsets; (**b**) lymphocyte subsets; (**c**) T cell subsets; (**d**) cytotoxic $CD8^+$ T cell subsets; (**e**) central memory $CD8^+$ T cell subsets; (**f**) senescent T cell subsets; (**g** and **h**) constitutively activated T cell subsets; (**i**) viral peptide-specific T cell subsets. Data are presented as mean ± s.e. Significance was evaluated via a Student's Paired *t*-test, by comparing all other data points to L-180 baseline data. Significant differences ($P \leqslant 0.05$) are indicated (*). Sample size for all data is 23 astronaut subjects, except for EBV- and CMV-specific T cells, where the assay is restricted to HLA-A2-positive subjects ($n = 8$). CMV, cytomegalovirus; EBV, Epstein–Barr virus.

Early T-cell function

After 24 h of T-cell stimulation in the presence of SEA+SEB, the percentage of $CD69^+$ and $CD69^+/CD25^+$ cells decreased during flight for both $CD4^+$ and $CD8^+$ T-cell subsets (Figure 3a). All four parameters were significantly decreased at the early timepoint. Although there seemed to be a general trend towards recovery through the mid-mission and late in-flight time points, all values remained significantly depressed except for the $CD8^+$ subset, which recovered function by the late timepoint (Figure 3a). When T cells were stimulated directly using antibodies to CD3 and CD28, no in-flight alteration was observed among either early-activated subset ($CD69^+$), but the $CD4^+$ subset reflecting full activation ($CD69^+/CD25^+$) was significantly decreased at the early timepoint only (Figure 3b).

Virus-specific T-cell function

Function of both EBV- – and CMV-specific T cells was determined by peptide stimulation followed by intracellular determination of IFNγ. For both subsets, quantity was determined by the MHC-tetramer method. The functional percentage of both EBV and CMV peptide-specific T cells was calculated as described above. The absolute levels of both EBV and CMV peptide-specific T cells were not significantly altered at any phase of flight, nor post flight (Figure 4).

Mitogen-stimulated cytokine profiles

Secreted cytokine profiles were determined in culture supernatants following mitogen stimulation for 48 h. After T-cell-specific stimulation (anti-CD3 and anti-CD28 antibodies), production of

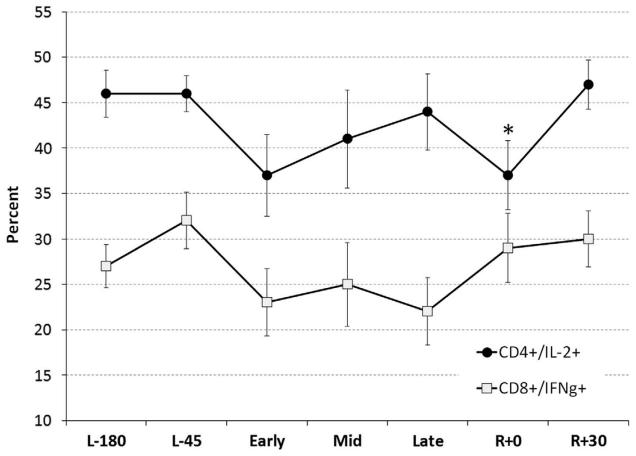

Figure 2. Intracellular cytokine analysis; mean percentage of T cells in samples collected before, during (early, mid-mission, and late) and after spaceflight that were capable of being stimulated to produce either IL-2 ($CD4^+$) or IFNγ ($CD8^+$) following mitogenic stimulation. Purified peripheral mononuclear cells were stimulated with PMA +ionomycin for 4 h; data are expressed as percentage of T cells that were positive. Data are presented as mean ± s.e. Significance was evaluated via a Student's paired *t*-test by comparing all other data points to L-180 baseline data. Significant differences ($P \leqslant 0.05$) are indicated (*). Sample size for all data is 23 ISS astronaut subjects.

IFNγ, IL-4, IL-5, IL-10, and IL-17A were all significantly reduced during flight at all three in-flight time points (Figure 5a). Following culture in the presence of PMA and ionomycin, a stronger mitogenic stimulus which bypasses some early intracellular signaling events and may activate most leukocyte populations, production of IFNγ, IL-4, IL-5, IL-10, IL-17A, TNFα, and IL-6 was significantly decreased at all three in-flight time points (Figure 5b). Following stimulation with LPS, production of both IL-1b and IL-10 was depressed at least one time point during flight, whereas production of IL-8 was actually elevated during flight at the early and mid-mission time points (Figure 5c).

DISCUSSION

Investigations of immunity immediately after spaceflight may be confounded by the high-G reentry and stressors of readaptation to terrestrial gravity following prolonged deconditioning. Some studies have indicated that the function of various immune subpopulations may be depressed either following or during short-duration spaceflight.[2,10–13,18,19,23] Immune studies associated with short-duration spaceflight, typically the ~7- to 14-day durations of a Space Shuttle mission, are essentially conducted during what is perceived to be the 'early adaptation' phase of spaceflight. Space Shuttle missions are typically extremely busy and stressful, more so than a typical work day on board ISS, where crew schedules are managed relative to the longer mission duration. Because of these conditions, short-duration data may not reflect how immunity may function during long-duration spaceflight. With limited exceptions, the status of the human immune system during long-duration spaceflight has remained uncharacterized. On board, the Russia Mir space station, the 'Multitest' assay of cell-mediated immunity was performed on three long-duration subjects, and the data indicated that cell-mediated immunity was depressed during spaceflight.[30] Otherwise, no comprehensive immune investigation has been conducted on human subjects during long-duration spaceflight.

The advent of ISS has afforded researchers an excellent opportunity to address our persisting knowledge gaps related to human physiology during space travel. In the study reported here

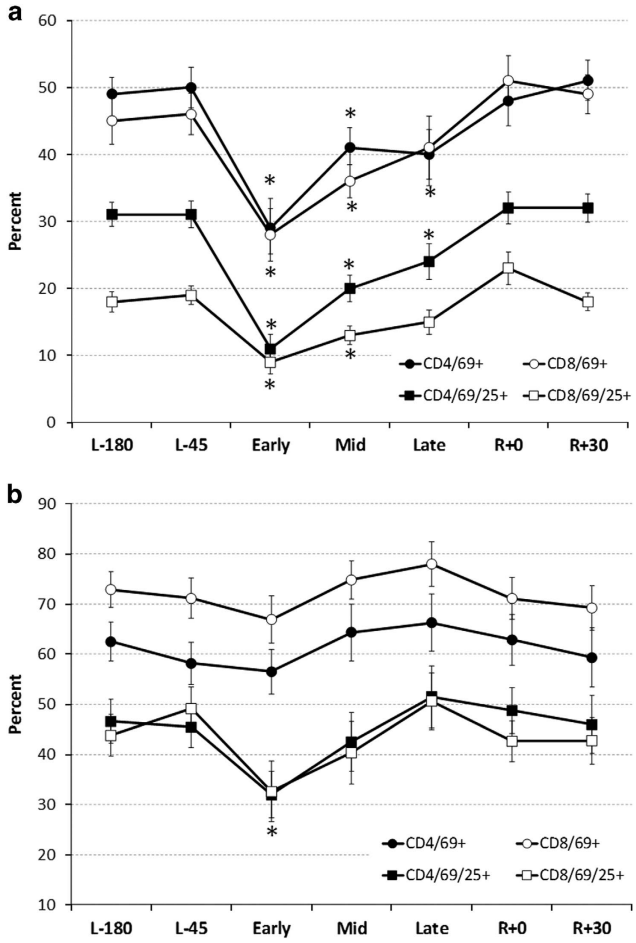

Figure 3. T-cell function (early blastogenesis) data: expression of either CD69 or CD69/CD25 following 24-h culture in the presence of (**a**) staphylococcal enterotoxin (**a** and **b**); or (**b**) antibodies to CD3 and CD28. Data are presented as mean ± standard error. Significance was evaluated via a Student's paired *t*-test by comparing all other data points to L-180 baseline data. Significant differences ($P \leqslant 0.05$) are indicated (*). Sample size for all data is 23 ISS astronaut subjects. ISS, International Space Station.

we assessed a variety of adaptive immune parameters at three points (at ~2 weeks, between 2 and 4 months, and at 6 months) during long-duration orbital spaceflight. Given a lack of analytical equipment supporting immunology or hematology parameters on board ISS, the study was designed to collect samples on board ISS shortly before a vehicle undocking, so that ambient viable blood samples could be returned for terrestrial analysis. Blood collected in nutrient- and anticoagulant-containing ACD tubes was validated in the laboratory to maintain viability for the relevant cell populations and culture assays for up to 48–72 h, which was the nominal delay until processing and analysis (data not shown). For each subject, in-flight data were compared with baseline data collected 180 days before launch.

Operationally the study was conducted without incident. Crews were instructed in phlebotomy prior to mission start, and most flight samples were collected as instructed. Samples were returned within the timeframes designated, and were either recovered at the Kennedy Space Center or Edwards Air Force Base if returned on the Shuttle, or in Star City, Russia, if returned on Soyuz. Cellular purification, culture setup and post-culture processing, as well as immunophenotype staining, were all performed on location. Stained cell products or culture

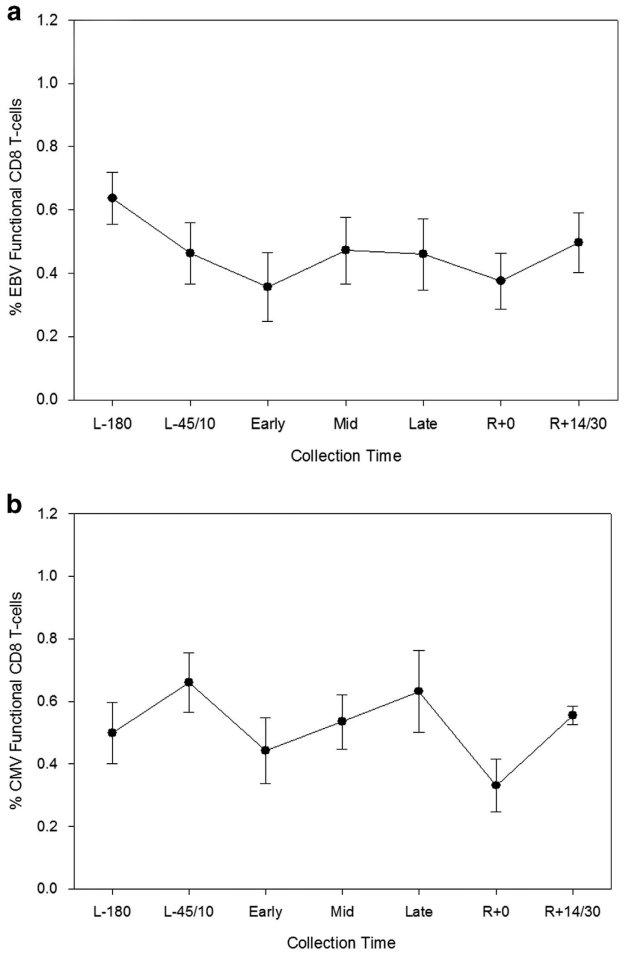

Figure 4. Quantification of virus-specific T-cell function. The frequency of $CD69^+/IFN\gamma^+$ positive (functional) $CD8^+$ T cells following culture in the presence of A*0201-restricted viral peptides was evaluated by flow cytometry. The absolute and functional numbers of viral peptide-specific T cells were used to derive a functional percentage for (**a**) EBV-specific and (**b**) CMV-specific $CD8^+$ T cells during spaceflight. Significance was evaluated via a Student's paired *t*-test by comparing all other data points to L-180 baseline data. Significant differences ($P \leqslant 0.05$) are indicated (*). This assay is restricted to HLA-A2-positive subjects ($n = 8$). CMV, cytomegalovirus; EBV, Epstein–Barr virus.

supernatants were stabilized as appropriate (fixed or frozen) for transport to the Johnson Space Center for analysis.

The data revealed that with respect to the peripheral leukocyte distribution, the WBC count increases during spaceflight. A previously observed surge in the WBC count after landing was confirmed, but this finding has been purported to be solely a postflight effect mediated by stress-induced demargination of neutrophils.[11] Terrestrial space analog studies have suggested that altered leukocyte distribution in astronauts may result from stress and not microgravity conditions.[34] For ISS crews the WBC count was significantly elevated during missions at both the early and late time points, relative to the L-180 baseline data. No in-flight alterations were observed among the bulk lymphocyte, T and T-memory subsets, with the exception of an increased NK-cell concentration at the late in-flight timepoint. Previous studies have actually reported decreased percentages of NK cells[7,35] or decreases in NK-cell functional capabilities[7,8,35] associated with spaceflight. Other studies indicate no decrement to NK function during flight.[6] A recent investigation of Shuttle astronauts found no difference in NK concentration during flight, but a significant decrease following flight.[23] Although the literature may vary, the common finding among the various terrestrial analogs and human flight investigations appears to be unaltered or decreased numbers of NK cells, and reduced (although sometimes target-selective) NK-cell function. Although NK-cell function was not evaluated for the current study, primarly due to sample volumes considerations, our finding of an actual increase in NK-cell levels late during ISS missions is discordant with some of these previous reports. Although the reason for the contrary NK finding is unknown, it may relate to the wide disparity between the experimental platforms, terrestrial analogs, short-duration post-flight and in-flight, and even long-duration flight but on different vehicles. A follow up investigation of both NK-cell number and function on ISS astronauts, focusing on other uninvestigated innate immune parameters may be warranted.

'Fine' T-cell subsets were assessed using a variety of markers. An in-flight increase was observed for the 'cytotoxic' $CD8^+$ subset ($CD28^+/CD244^+$), whereas a decrease was observed for the central memory subset ($CD62L^+/CD45RA^-$). Several markers of senescence were assessed. A decrease in senescent $CD8^+$ T cells was observed during flight by using both the $CD244^+/CD28^-$ phenotype and expression of CD57 as senescence markers. CD69 and HLA-DR were used to assess constitutively activated T cells. Although it trended upwards relative to preflight baseline data, the number of $CD69^+$ T cells did not increase during flight for either the $CD4^+$ or $CD8^+$ subset. However, the number of $CD8^+/HLA\text{-}DR^+$ T cells decreased during flight, reaching significance at the mid-mission timepoint. No alteration in the level of EBV- or CMV-specific T cells was observed during flight, although being HLA restricted, this assay was performed on only 10 crewmembers.

This leukocyte distribution pattern suggests that some *in vivo* immunological processes are occurring and persisting during long-duration spaceflight, including an elevated WBC count, largely because of an increased number of neutrophils and a shift within the $CD8^+$ T-cell compartment toward a more active phenotype. It is possible that these alterations represent innate immunity compensating for diminished adaptive immune capacity. It is also possible that maturation shifts within $CD8^+$ T cells may parallel persistent latent herpes virus reactivation.

Functional analyses were also performed as part of this flight investigation. CD69, preformed in the cytoplasm, is expressed about 1 h after activation, whereas CD25 requires new mRNA synthesis and is expressed closer to 24 h after activation. Therefore, following 24 h of mitogenic stimulation, the assessment of CD69 and CD25 constitutes a measurement of T-cell ability to progress through early activation events. Assessment of these two markers as a measure of T-cell activation potential as been previously established.[31] Two T-cell mitogens were used separately, a combination of SEA+SEB or soluble antibodies to both the TCR (CD3) and CD28. In the former, costimulation is mediated by antigen presenting cell (APC) binding, whereas in the latter costimulation is delivered by direct CD28 triggering. These mitogens by nature trigger different percentages of responding cells; enterotoxins a specific Vβ T-cell subset, whereas soluble antibodies to CD3 theoretically may stimulate all available T cells. However, as previously postulated,[23] these mitogens may also be delivering differing strengths of signal to the nucleus. It is possible that enterotoxins may deliver a weaker 'physiological' signal (SEA +SEB) by triggering only at the cell:cell interface, whereas anti-CD3/CD28 may deliver a more powerful signal via binding of all available ligands on the cell surface.

Similar to a previous assessment during short-duration spaceflight,[23] during ISS missions disparate results were observed between the two mitogens. During flight, activation responses for both the CD4+ and $CD8^+$ T-cell subsets following SEA+SEB stimulation were significantly reduced, a finding which, although it trended toward baseline, remained largely significant for the duration of the 6-month spaceflight (Figure 3a). Conversely, in assessment of total $CD69^+$, no reduction in T-cell function was

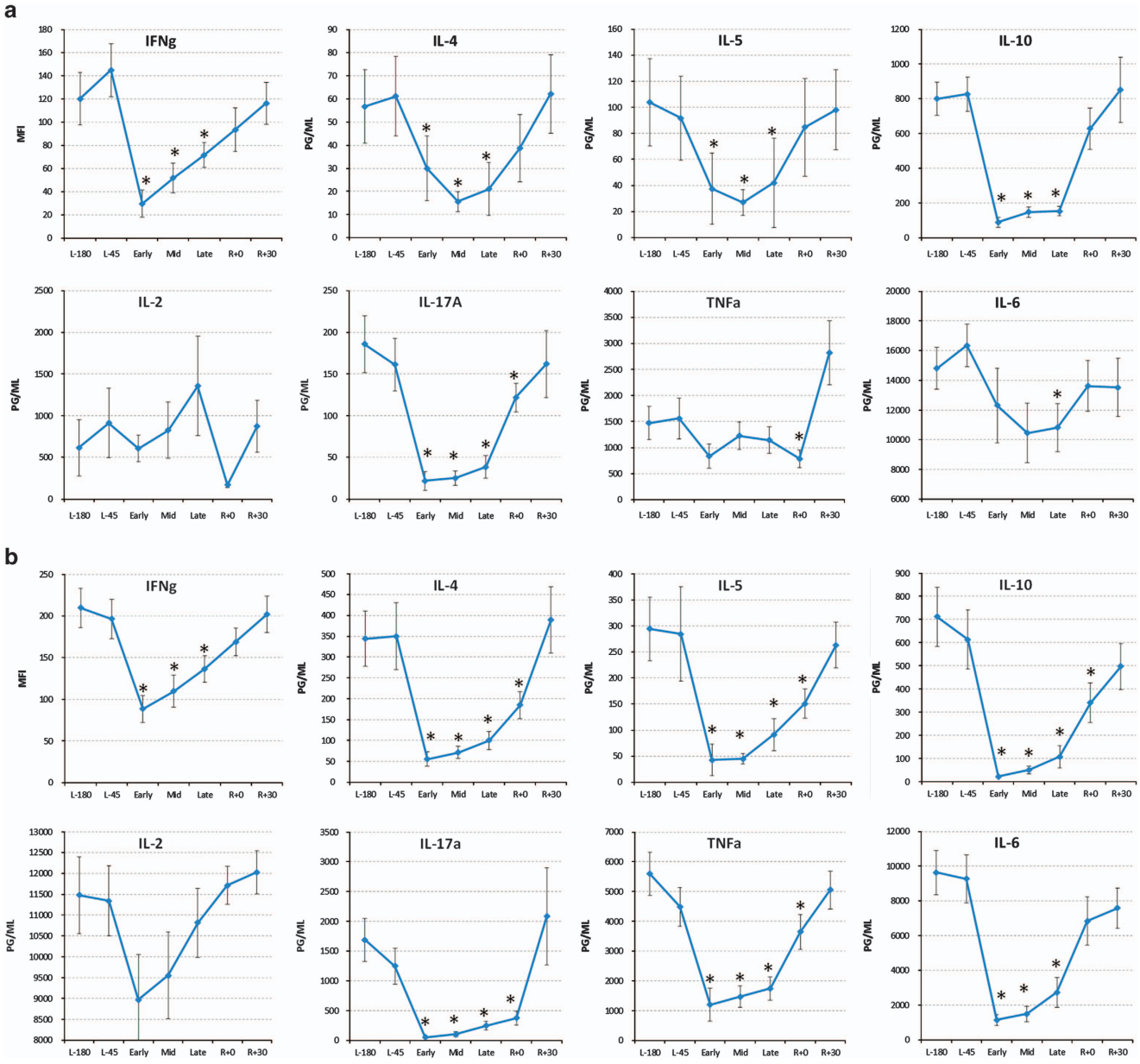

Figure 5. Mean secreted cytokine levels following mitogenic stimulation. For secreted cytokine production, the mitogen was (**a**) antibodies to both CD3 and CD28, (**b**) PMA+ionomycin or (**c**) LPS. Cytokine concentration data are expressed mean concentration in pg/ml ± s.e.m. The lone exception is IFNγ, for which data is presented as mean fluorescence intensity (MFI), which corresponds to concentration. Significance was evaluated via a Student's paired *t*-test by comparing all other data points to L-180 baseline data. Significant differences ($P \leqslant 0.05$) are indicated (*). Sample size for all data is 23 ISS astronaut subjects. For IL-4 (**a**), late time point only, one outlier value removed prior to statistical analysis. ISS, International Space Station; LPS, lipopolysaccharide.

observed during spaceflight following CD3 and CD28 stimulation (Figure 3b). When $CD25^{+}$ was assessed, however, a significant decrease was observed at the early in-flight timepoint only. The concept of an activation threshold shift has been established previously.[36] We suggest the possibility that T cells from astronauts participating in long-duration spaceflight may, as was suggested previously based on similar short-duration findings, display an activation 'threshold shift' during spaceflight.[23] Functional lymphocyte alterations observed during microgravity cell culture have been found to persist well after return to terrestrial unit gravity conditions.[21] We believe that our data can be explained as observation of an activation threshold shift, via the delivery of a 'physiological strength' signal by SEA+SEB, and a more potent activation signal by anti-CD3/CD28 antibodies. Terrestrial modeled microgravity mechanistic studies may be necessary to ascertain whether a threshold shift is associated with microgravity, which could explain many of the terrestrial analog (modeled microgravity) findings regarding a depression in T-cell activation associated with altered gravity fields.[14,32] Alternative explanations are possible, in particular that the APC population, necessary only for the enterotoxin stimulation, may be more sensitive to the effects of spaceflight. However the LPS induced production of IL-8, a product of innate immune cells, was actually elevated during spaceflight. Although further studies of APCs would be necessary, this finding suggests APCs are viable and functional in our culture system.

It is interesting that the findings of immune system alterations in astronauts would be observed using blood samples collected

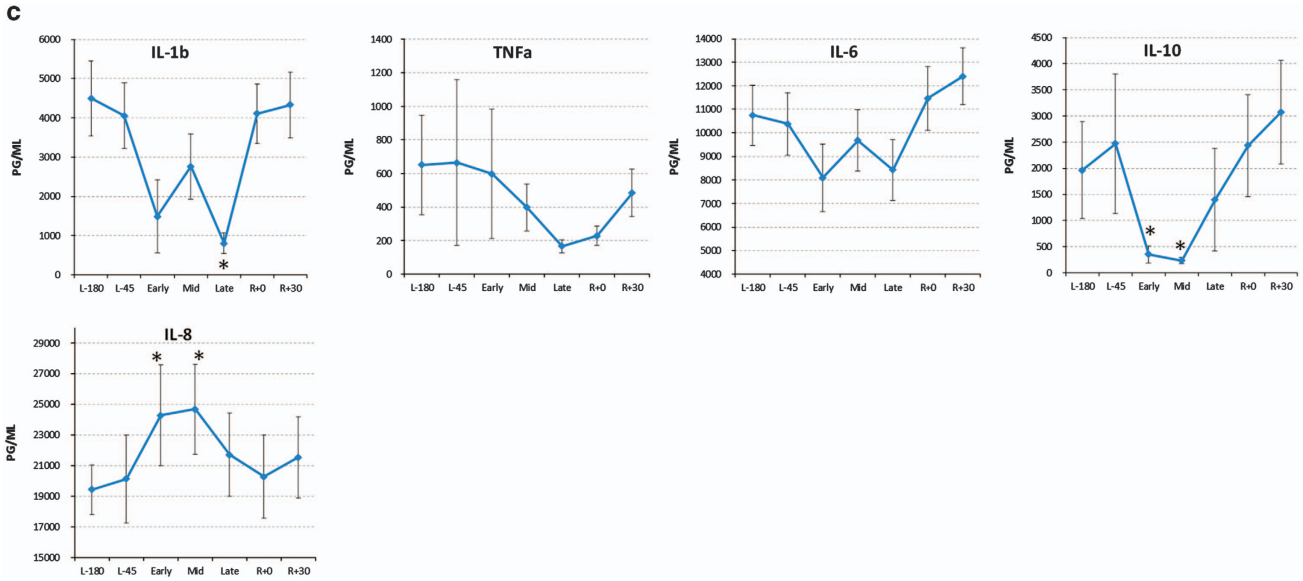

Figure 5. Continued.

during flight, yet processed using 1*g* terrestrial cell culture conditions. Alternative to a microgravity-related cause, it is also possible that other factors such as physiological stressors could at least partially explain the observations. Astronauts do work in an environment with multiple stressors, which may vary in duration in intensity. Assessments of urine and plasma cortisol have been assessed during flight, but reported alterations have varied considerably during studies, reviewed by Pierson *et al*.[37] Generally, urine cortisol appears commonly elevated early during flight, but returns to baseline following adaptation. It appears that longer flight may manifest greater activation of the HPA axis than shorter flights.[18]

Secreted cytokine production represents a more downstream measure of cellular function. For this flight investigation cultures were maintained for 48 h, and supernatants were then assessed for cytokine concentrations. The mitogens used were as follows: anti- CD3/CD28 to stimulate T cells; PMA+ionomycin as a broader pharmacological stimulus (bypassing several gravi-sensitive signal transduction pathways); and LPS to stimulate innate immune cells. Following T-cell stimulation, production of IFNγ, IL-10, IL-5, and IL-17A were all significantly decreased at all three in-flight time points. Following stimulation by PMA-ionomycin, a mitogen which was thought would overcome depressions in in-flight cellular function, a remarkable pattern of suppression was observed. Concentrations of IFNγ, IL-4, IL-5, IL-10, IL-17A, TNFα, and IL-6 were all significantly decreased at all three in-flight time points. Following stimulation with LPS, no significant decreases were observed during flight, but the concentration of IL-8 was actually increased at all three in-flight time points (Figure 5c).

Intracellular cytokine detection by flow cytometry, in positively identified cell subsets, has the capacity to measure the numbers of cells capable of being stimulated to produce cytokine, as opposed to bulk production levels. It is well understood terrestrially that it is typically only PMA+ionomycin stimulation that generates concentrations high enough so that, after secretion is blocked, cytokine levels will accumulate within the cell to detectable levels. For this study, the percentages of T-cell subsets capable of being stimulated to produce IL-2 and IFNγ were determined. During flight, no significant alteration was observed in the percentage of $CD4^+$ T cells capable of being stimulated to produce IL-2, nor in the percentage of $CD8^+$ T cells capable of being stimulated to produce IFNγ. We suggest that these findings indicate that the decreases in secreted cytokines during flight are therefore due to diminished bulk production capability, as opposed to the exit of cytokine-secreting T cells from the peripheral circulation.

These summary functional changes indicate that crewmembers on board ISS do possess significant functional immune alterations that persist for the duration of a 6-month orbital spaceflight. These alterations may partly explain, from a mechanistic perspective, the root cause of the persistent reactivation of latent herpesviruses reported to occur during spaceflight. Although an attempt was made to investigate changes specific to various adaptive immune biases (Th1, Th2, Th17, and so on), no 'bias shifts' were observed. Instead, the functional depressions seem to be across the various aspects of adaptive T-cell immunity. Although this study did not investigate innate immunity in a comprehensive fashion, the lack of cytokine suppression following LPS stimulation may indicate that innate parameters are unaffected. In fact, the increase in IL-8, coupled with other reports of a mild 'pro-inflammatory' state persisting during ISS missions,[38] may indicate compensatory increases in innate immune function. We also interpret that the increase in IL-8 production supports the acceptability of the sampling architecture, including delays in processing, used for this study.

These data indicate that immune system alterations, a phenomenon previously established to occur post flight and during short-duration spaceflight, in fact persists during long-duration missions. In an applied longitudinal survey of astronauts, it is of course difficult to ascertain the specific causal factors. Potential causes include microgravity exposure, confinement, disrupted circadian rhythms or the physiological stress associated with spaceflight itself. Also, the study was conducted in a fashion perceived to be the best for science, but within operational constraints. For example, the time of blood sample collection could vary within a day due to other mission-associated impacts to the daily schedule. As immune parameters are sensitive to circadian issues, inability to collect all samples in the morning may be a confounding factor. However, in the end any flight study must operate within certain operational constraints. It may also be postulated that the simple act of collecting and storing samples in microgravity may introduce some confounding variable. Unfortunately this is impossible to control, and as discussed earlier the sample-return terrestrial analysis strategy is believed to in fact be more relevant than the alternative microgravity cell culture and

analysis. Nevertheless we consider it unlikely that microgravity culture would alter the samples in any appreciable way. As with any in-vitro assay, interpretation to the in vivo situation carries certain caveats, but the viral reactivation astronaut data[24–28] and the MIR-18 crew delayed-type hypersensitivity data[30] would seem to support the current interpretation.

Exploration-class deep space missions will consist of elevated radiation exposure beyond the Van Allen belt, limited clinical care capability based on that of the projected Orion vehicle (plus any 'transit phase' module), and other exploration-specific variables (toxins, planetary exploration risks). We suggest that based on the data presented herein, persistent immune system alterations during these missions of exploration could increase specific clinical risks during exploration missions. At this point, as various aspects of immunity during spaceflight remain uninvestigated, further characterization of the in-flight phenomenon would be necessary prior to assessing specific health risks. The complete characterization would need to be interpreted in the context of terrestrial disease findings. It is possible that the immune changes in astronauts may be physiological indicators which may precede specific disease. If so, the development of immune-specific countermeasures, similar to those being developed for other physiological aspects of flight (bone loss, renal stone risk, and so on) would be necessary to ensure the health of astronauts.

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CONTRIBUTIONS

BC: study operational support, flow cytometry assays, manuscript preparation. RPS: virus-specific immunity assays. SM: Virus reactivation assays. HQ: Study operational support, flow cytometry assays. DP: virus reactivation assays. CS: principal investigator.

COMPETING INTERESTS

DP is a NASA Virologist and CS and BC are NASA Immunologists. All remaining authors possess positions (contractor scientist) at, or are funded by, NASA. The remaining authors declare no conflict of interest.

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