Plasma Cytokine Concentrations Indicate In-vivo Hormonal Regulation of Immunity is Altered During Long-Duration Spaceflight

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Plasma cytokine concentrations during spaceflight

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ABSTRACT

Background: Aspects of immune system dysregulation associated with long-duration spaceflight have yet to be fully characterized, and may represent a clinical risk to crewmembers during deep space missions. Plasma cytokine concentration may serve as an indicator of *in vivo* physiological changes or immune system mobilization. ***Methods:*** The plasma concentrations of 22 cytokines were monitored in 28 astronauts during long-duration spaceflight onboard the International Space Station. Blood samples were collected three times before flight, 3-5 times during flight (depending on mission duration), at landing and 30 days post-landing. Analysis was performed by bead array immunoassay. ***Results:*** With few exceptions, minimal detectable mean plasma levels (<10 pg/ml) were observed at baseline (launch minus 180) for innate inflammatory cytokines or adaptive regulatory cytokines, however IL-1ra and several chemokines were constitutively present. An increase in the plasma concentration IL-8, IL-1ra, Tpo, CCL4, CXCL5, TNFα, GM-CSF and VEGF was observed associated with spaceflight. Significant post-flight increases were observed for IL-6 and CCL2. No significant alterations were observed during or following spaceflight for adaptive/T-regulatory cytokines (IL-2, IFNγ, IL-17, IL4, IL-5, IL-10). ***Conclusions:*** This pattern of cytokine dysregulation suggests multiple physiological adaptations persist during flight, including inflammation, leukocyte recruitment, angiogenesis and thrombocyte regulation.

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INTRODUCTION

Immune system dysregulation has long been a recognized phenomenon immediately following short and long-duration spaceflight, consisting of altered leukocyte distribution, diminished function of specific immunocyte populations and altered cytokine production profiles following mitogenic stimuli **(Gueguinou 2009)**. Recent data have confirmed that immune dysregulation also occurs during short-duration spaceflight, indicating an in-flight phenomenon, not merely a post-flight landing effect **(Crucian 2012)**. The reactivation of latent herpesviruses, likely as a result of diminished immune control, has also been documented to occur during spaceflight **(Mehta 2000, Pierson 2005)**. Post-flight alterations in cytokine profiles following short-duration spaceflight were recently found to positively correlate with astronaut reactivation of latent herpesviruses **(Mehta 2012)**.

Plasma cytokine concentration may serve as an indicator of *in-vivo* physiological changes or immune system mobilization **(Kroemer 1991)**. Increases in plasma concentrations may reflect large magnitude localized reactions, or the manifestation of systemic reactions. Being very diverse group of immune regulatory mediators, cytokines may be subdivided into several categories, allowing monitoring of different types of biological responses. These include innate immunity (e.g. TNF-a, IL-1b, IL-6, IL-12, IFN-a, TGF-b), adaptive (e.g. IFN-g, IL-4, -10, -17, -21), growth factors (e.g. G-, M-, GM-CGF, Tpo, EPO, FGFb) that regulate hematopoiesis and chemokines which orchestrate chemotactic trafficking of immunocompetent cells (e.g. IL-8, CCL….). Furthermore, the very same cytokines can be subdivided into 2 major groups based on their physiological functions: pro- vs anti-inflammatory (e.g. TNFα, IL-1b, IL-6, IL-12 on the one side and IL-10, TGF-b, IL-1ra on the other side). Therefore, assessment of many cytokines, and the determination of specific pattern shifts, can indicate the presence of specific disease types. For example, it is well

established that rheumatoid arthritis and multiple sclerosis are 'Th1' diseases, whereas systemic autoimmune diseases and allergies are 'Th2' diseases **(Kasakura 1998)**.

Aside from autocrine and paracrine effects many cytokines can act in endocrine manner following exogenous (e.g. pathogens) or endogenous (e.g. other cytokines, autoantigens) stimulation and produce systemic outcomes such as cytokine storm (rev in **Tisoncik et al 2012, Harrison 2010**). Most cytokines possess a short half-life and are locally acting; therefore the plasma level for most cytokines is generally low. Exceptions include some chemokines, which due to their role in leukocyte trafficking and recruitment must leave a localized site of inflammation to recruit specific cells types from the general circulation.

Plasma cytokine assessment has been demonstrated to have clinical utility as a biomarker for various specific immunologic diseases or other disruptions in physiological homeostasis. Elevated levels of IL-6, IL-7, IL-10 and IFNγ have been detected in the plasma of HIV-infected patients and correlate well with prognosis **(Chuenchitra 2012)**. Plasma levels of cytokines have also been found to correlate with disease presence or prognosis in rheumatoid arthritis **(Khan 2009)**, myelofibrosis **(Tefferi 2011)**, Sjogrens syndrome **(Szodoray 2004)**, COPD **(Bon 2010)** and pelvic inflammatory disease **(Chen 2008)**.
It has been suggested that persistent immune dysregulation may increase specific clinical risks for astronaut crewmembers participating in exploration-class deep space missions **(Crucian 2009)**. A broad human survey of human immunity during long-duration spaceflight has not yet been performed. To determine in-vivo immune homeostasis during long-duration orbital spaceflight, we investigated astronaut plasma cytokine levels as a biomarker of immune status during missions to the International Space Station (ISS).

MATERIALS AND METHODS

Subjects and missions. Twenty-eight International Space Station astronaut crewmembers participated in this study. Mission durations were approximately 6 months, which is considered long-duration spaceflight. Of the 28 subjects, 21 were male and 7 were female, and their mean age was 49±4 years. Approval was obtained from the *Intuitional Review Board* at the NASA Johnson Space Center; Houston, TX. Informed consent was obtained from all subjects prior to participation.

Plasma samples. Whole blood samples (5.0 ml) were collected at the following timepoints: 180, 45 or 10 days before flight (L-180, L-45, L-10, respectively), at five timepoints during spaceflight (flight days 15, 30, 60, 120 and 180), within 24 hours following landing (R+0), and 30 days after landing (R+30). All sample collection dates are approximations, and could vary somewhat based on operational constraints. Samples were collected in EDTA tubes containing plasma-separation gel. Following collection, samples were centrifuged for 30 minutes to allow plasma separation. All samples (pre, in and postflight) were frozen until batch analysis after the in-flight samples were returned to Earth onboard either the US Space Shuttle or SpaceX Dragon capsule.

Plasma cytokine concentration. The concentrations for 22 plasma cytokines representing five broad categories of function **(Table 1)** were determined simultaneously in duplicate using a commercially available multiplex bead immunoassay (R&D Systems). Samples were processed according to the manufacturer's instructions. Briefly, 50 ul plasma were incubated with beads bound to a cytokine capture antibody. The 22 bead populations vary by fluorescence intensity so that they may be resolved for

individual analysis. Bead cytokine concentrations were then washed and incubated with a fluorescent secondary antibody, specific for each cytokine, but fluorescing along a single channel distinct from the bead populations. The assay was performed in a 96 well plate, and analysis was performed using a Luminex 100 instrument (Luminex, Inc., Austin, Texas).

Statistical analysis. A repeated-measures one-way ANOVA was performed to determine the effect of spaceflight on plasma cytokine levels. If there was a significant main effect observed for spaceflight, then an additional post hoc Bonferroni t-test was performed comparing each time point to the L-180 time point. Statistical analyses were performed using Sigma Stat 3.11 (Systat Software, San Jose, CA, USA).

RESULTS

Inflammatory/anti-inflammatory. Mean L-180 baseline levels of IL-1α, IL-1β, TNFα and IL-6 were all below ≤2.0 pg/ml **(Table 2)**. There was no statistically significant in-flight alteration for IL-1α, IL-1β or IL-6. The concentration of TNFα was increased during spaceflight (main effect $p<0.01$), however the more conservative post hoc analysis did not indicate significant increases for any specific in-flight timepoints. A main-effect increase in IL-8 was observed during spaceflight ($p<0.001$), with several in-flight timepoints achieving individual statistical significance when compared to L-180 **(Table 2)**. Although no in-flight increase was observed for plasma IL-6, there was a significantly elevated concentration immediately post-flight ($p<0.001$). There was a fairly stable pre-flight baseline concentration of IL-1ra between L-180 and L-45 (378 and 363 pg/ml respectively). By L-10, levels were trending upward and were higher than baseline (L-180) for the duration of the in-flight and post-flight timepoints, resulting in a significant main-effect difference ($p<0.001$; **Table 2)**. Individual analysis of the IL-1ra increase achieved significance by FD120 and R+0 ($p<0.001$).

Adaptive immunity cytokines. Mean L-180 baseline levels of IFNγ, IL-2, IL-17, IL-4, IL-5 and IL-10 were all below 2.0 pg/ml **(Table 2)**. There were no statistically significant differencesin any adaptive immunity cytokines at any measured in-flight or post-flight timepoints.

Growth factors. The mean baseline concentration of the cytokines identified as 'growth factors' varied from <1.0 pg/ml (GM-CSF) to 141 pg/ml for Tpo **(Table 2)**. During spaceflight, there were no significant alterations in the plasma concentration of G-CSF or FGF basic. GM-CSF concentration was increased during spaceflight (main effect, $p<0.05$). In-flight increases were detected in the plasma concentration of both Tpo and VEGF ($p<0.001$), with Tpo significantly elevated at all five in-flight timepoints, and VEGF elevated at the FD30 timepoint. In-flight alterations for all growth factors returned to baseline immediately following landing.

Chemokines. Generally, the baseline levels for chemokines were much higher than for the other measured cytokines. The mean baseline concentration for chemokines ranged from 9.4 pg/ml (CCL3) to 3427 pg/ml (CCL5) **(Table 2)**. During spaceflight, a main-effect increase was observed for both CXCL5 and CCL4 ($p<0.001$), with single point significance detected for CXCL5 at all five in-flight points, and on FD120 for CCL4. A main effect increase was also observed for CCL2, however no in-flight increases were observed but a post-flight significant increase was detected **(Table 2)**. No deviations from baseline were observed for CCL3 or CCL5.

DISCUSSION

Alterations in plasma cytokine levels are an established biomarker for many diseases. Newer array technology with simultaneous multiple analyte detection increases assay efficiency. Multiple types of cytokines may now be measured rapidly in a single, small volume (50ul) sample. As the dysregulation of the immune system is an established post flight phenomenon **(Gueguinou 2009)**, and persistent

dysregulation may be a clinical risk to crewmembers **(Crucian 2009)**, it is appropriate to survey plasma cytokine levels in astronauts during long duration spaceflight. For this study, 22 plasma cytokines were assessed at 5 timepoints during 6-month flight onboard ISS. Timepoints were spaced to include the early adaptation phase (FD 15, 30), and subsequent long-adaptation points through the entire 6 month mission (FD60, 120, 180). This approach allowed the kinetics of the entire flight to be assessed, while freezing all samples for batch analysis eliminated any inter-assay variability concerns. All in-flight data were compared to the baseline sample collected ~180 days before launch. The L-180 sampling is considered early enough to avoid any pre-launch or training stresses, thus establishing a legitimate baseline value. A second pre-launch sample (L-45) allows another correlative pre-launch sampling, and a general measure of intra-subject variability. A third pre-flight sample was available at 10 days before launch, however it has been established that within the period 10 days before launch, immune parameters are visibly influenced by pre-mission stress **(Stowe 2000)**, and therefore a sub-optimal baseline sample. Although the in-flight data is most relevant for long-duration exploration class spaceflight, samples were also collected immediately following landing (R+0). Sample collection after landing assesses post-flight physiological changes that are associated with a high-G re-entry following prolonged microgravity-associated deconditioning. Such data is highly relevant for the immediate post-landing phase of interplanetary exploration, such as a Mars landing. The 'recovery' sample, collected 30 days post-landing was included to ensure that any flight or landing-associated alterations would be trending towards baseline values. This is relevant to establishing how quickly crews recover from the adverse effects of spaceflight.

Individual subject data analysis indicated that where statistically significant alterations occurred, crewmember data was remarkably consistent. Single subject analysis of CXCL5 and VEGF shows that all crewmembers manifested a positive alteration, with no obvious negative outliers **(Figure 1a, b)**. Plotting

the individual data also clearly resolves in-flight effects from obvious post-flight effects such as the increase in plasma IL-6 present for most crewmembers after landing **(Figure 1c)**. When tracking individual crew data, occasional outlier crewmembers may be observed who show, usually at a few specific timepoints only, relatively high concentrations of certain cytokines. Examples are shown in **Figure 3d-f**, where clear single-subject outliers are visible for IL-1β, TNFα and IL-4. Although these alterations were not frequent enough to influence statistical significance, on a case by case basis these shifts may be clinically relevant. Crewmembers do occasionally experience adverse medical events during spaceflight, including infectious disease or hypersensitivity responses. It is unknown if these particular outliers correlated with adverse medical events, however, such a correlation would appear possible even if the underlying mechanistic cause was subclinical. It is therefore feasible that monitoring crewmember plasma cytokine concentration may have routine use for either clinical monitoring or augmenting primary diagnostic/prognostic laboratory measures.

Inflammatory cytokines recruit leukocytes to a localized immune reaction, promote fever, vascular changes, cellular activation and acute phase responses. With rare exceptions, minimal detectable mean plasma levels (<10 pg/ml) were observed at baseline (L-180) for innate inflammatory cytokines (IL-1α, IL-1β, TNFα, IL-6), and adaptive regulatory cytokines (IL-2, IFNγ, IL-17, IL-4, IL-5, IL-10), however IL-1RA and several chemokines were constitutively present. This pattern is consistent with generally healthy astronauts free of infectious disease or inflammatory processes during pre-mission training.

During spaceflight there was a significant increase in plasma IL-8 at multiple in-flight timepoints, whereas TNFα demonstrated a significant main-effect increase associated with spaceflight **(Table 1)**. Although the levels of other inflammatory cytokines (IL-1a, IL-1b, IL-6) were not elevated during spaceflight, the increase in IL-8 and TNFα is indicative of mild inflammation, and would be consistent with sensitized innate immunocyte function resulting in persistent low level inflammation during spaceflight. Persistent

inflammation during flight could reflect flight-associated alterations in the gut microbiome or the consistent exposure to increased environmental radiation. It is interesting that plasma levels of IL-1ra, an inhibitor of the pro-inflammatory effects of IL-1, was also consistently elevated during spaceflight. IL-1ra demonstrated both a main-effect increase associated with flight, and also a specific increase at the FD120 timepoint **(Table 2)**. Assuming that spaceflight factors induce persistent low-grade systemic or localized inflammation, elevations in circulating IL-1ra may represent an adaptive physiological response to the inflammatory stress **(Suzuki 2002)**. In fact, similar stimuli induce cells to secrete both IL-1 and IL-1ra, but plasma concentrations of IL-1ra are consistently about 100-fold higher than those of IL-1 **(Burger 2000)**, which would support the current observation of levels of IL-1ra in astronauts during spaceflight being 300 to 600-fold higher than those of IL-1a or IL-1b **(Table 2)**. It is also noteworthy that systemic plasma IL-1a or IL-1b did not increase during spaceflight. It is therefore possible that the in-flight cytokine profile does reflect some localized inflammation (i.e., gut) whereas the major systemic observation is the significant increase in IL-1ra. In fact, it has been previously demonstrated that localized inflammation does result in a primarily anti-inflammatory systemic response **(Rivera-Chavez 2003)**, and that increased systemic levels of IL-1ra may serve as a good prognosis indicator for a localized inflammatory disease **(John 2008)**.

Further supporting an inflammatory state during flight are the observed elevations in CCL4 and CXCL5 during spaceflight **(Table 2)**. Many cytokines have pleotropic redundant or overlapping functions. Although classified separately in this article as 'chemokines', these molecules are also directly involved in the process of inflammation. CXCL5 (ENA-78), a potent neutrophil chemoattractant, is produced by cells subsequent to stimulation with inflammatory cytokines such as IL-1 or TNFα. CCL4 (MIP-1b) is a pro-inflammatory molecule produced by macrophages, and an attractant for monocytes, NK cells and other immunocytes. CCL4 (and CCL3) induce expression of IL-1, IL-6 and TNFα, further upregulating the process of inflammation. Increased plasma levels of CCL4 have been found to correlate with clinical diseases such

as multiple meyeloma **(Terpos 2005)** and hepatitis C **(Zeremski 2007)**. Increased levels of CXCL5 have been found to correlate with inflammatory diseases such as rheumatoid arthritis **(Walz 1997)**. Although it is unclear why only these two chemokines displayed consistent in-flight increases, their elevation also supports some degree of a chronic pro-inflammatory state during spaceflight. Levels of CXCL5 increased by nearly 10-fold during flight, remained elevated for an entire 6-month spaceflight, and return to baseline almost immediately upon landing **(Table 2)**.

Normal human levels of adaptive immunity cytokines are very low in plasma, as adaptive immune processes should end to protect the host from the damaging effects of a continuous uncontrolled immune activation. In this study, no increases were observed for any Th1, Th17 or Th2 cytokines during spaceflight **(Table 2)**. Astronauts onboard ISS are essentially in a well maintained isolation chamber, and unlikely to be exposed to various transmissible pathogens, and therefore may mount fewer adaptive immune responses. The plasma cytokine data may suggest the astronauts were simply free from infectious disease; however there may be an alternative explanation. Numerous microgravity or microgravity-analog cell culture experiments have demonstrated that T cells do not activate normally during reduced gravity conditions (**Hashemi 1999, Boonyaratanakornkit 2005**). There have also been previous reports of diminished T cell function in astronauts associated with spaceflight. In-flight and post-flight mitogen-stimulated cell cultures from astronauts produce greatly reduced levels of cytokines than pre-flight baseline cultures **(Crucian 2008)**. This has been interpreted as a generalized reduction in T cell function during spaceflight. Consequently, astronauts display persistent in-flight reactivation of various latent herpesviruses **(Mehta 2000, Pierson 2005)**, known to associated with reduced cytotoxic T cell function. Likely causes for adaptive immunosuppression during flight include physiological stress, circadian misalignment, isolation and confinement, or a microgravity-associated defect in T cell intracellular signal transduction **(Boonyaratanakornkit 2005)**. Therefore, the absence of increased levels

of adaptive cytokine during spaceflight could also result from, to some degree, crewmember inability to mount adaptive responses during flight. Further complicating the astronaut risk scenario, the recently described alterations in microbial virulence may yet alter susceptibility for infectious diseases during spaceflight **(Wilson 2007)**.

The observed increases in growth factors and chemokines may indicate other types of adaptation, such as enhanced innate immune parameters, or attempts to overcome diminished immunocyte function. Increased Tpo and VEGF were previously reported in a single subject participating in a 21 day short duration spaceflight. Tpo was found to be elevated throughout the flight, but VEGF elevated only during the early stages of flight and then returned to baseline **(Gunga 1999)**. The increase in VEGF was suggested to be related to intravascular fluid shifts. However, Gunsilius et al have reported a striking correlation between platelet levels and VEGF, with VEGF substantially lower in thrombocytopenic patients **(Gunsilius 1999)**. They suggested that platelets are a predominant source of VEGF in serum, therefore when Tpo levels are elevated (and platelet levels would be correspondingly low), VEGF levels would be expected to be low. Gunsilius suggested that the rapid decrease in VEGF in the single subject may correlate with thrombocyte depletion. Thrombocytopenia is known to occur during spaceflight **(Kalandarova 1991, Davis 1996)** and would be supported by elevated plasma Tpo levels observed in the same single subject. Our data however, indicates that both Tpo and VEGF remain elevated throughout a 6 month orbital spaceflight. Tpo was significantly elevated at all five in-flight timepoints, whereas levels of VEGF tended to be elevated at all inflight points, with a significant point-specific increase on FD30 **(Table 2)**. These data suggest that the rapid in-flight return to baseline in VEGF previously reported **(Gunga 1999)** may not be reflective of astronauts in general. Tpo stimulates platelet production but is regulated via a negative feedback loop. Tpo is bound onto the surface of platelets via the CD110 surface receptor, therefore removing it from circulation. When platelet levels decrease, Tpo therefore increases and

stimulates new platelet production. It would seem logical therefore that since thrombopenia is associated with spaceflight, Tpo levels would be elevated. It is unclear if the rise in VEGF may therefore be associated with a platelet decrease (destruction and release into plasma), or if a VEGF rise may be associated with other physiological processes (fluid shifts, angiogenesis). It is noteworthy that the chemokine CXCL5, also consistently elevated during spaceflight, also displays angiogenic properties and supports tumor formation **(Li 2011)**. Although activated platelets are a rich source of several chemokines **(Gleissner 2008, Gear 2003)**, in this study other platelet associated chemokines (RANTES, MIP-1a) were not elevated during spaceflight. It is therefore questionable if the elevations in CXCL5 result from platelet activation, even though levels of Tpo increased during spaceflight.

It is noteworthy that in this study plasma samples were generally collected during relatively lower-stress mission phases away from vehicle dockings, extravehicular activities (spacewalks) or docked vehicle operations. Docked vehicle operations, such as the arrival of a visiting cargo vehicle, are periods of intensive work usually accompanied by a circadian shift. This was the preferred sampling option, since samples could be frozen on-orbit for storage, and later returned for analysis. Other studies onboard ISS, which sample crewmembers and return ambient blood for analysis, will define the immunological changes associated with periods of elevated stress. To determine clinical risks for exploration class deep space missions, a thorough understanding of immune system dysregulation is necessary for all mission phases. This includes the post-launch space adaptation phase, the 'space normal' transit phase equilibration, and the stressful post-landing phase following prolonged deconditioning.

From the data described here, it is clear that a pattern of persistent physiological adaptations occur during spaceflight that include shifts in immune/hormonal regulation. It has yet to be determined if these adaptations increase crew risk for adverse medical events during spaceflight. The pattern of cytokine

elevations observed during spaceflight is somewhat surprising, in that within categories there are differential increases: some inflammatory cytokines/chemokines are elevated, whereas others are not. This may be explained by different plasma half-life among the cytokines, different kinetics/magnitude of expression versus resorption by target cells, or the location/nature of the pro-inflammatory stimuli that may affect crewmembers during spaceflight. Further investigations will be required to precisely define the mechanistic causes of in-flight immune dysregulation. It is clear however, that immunity is dysregulated during spaceflight, including the previously described alterations in cellular distribution and function **(Crucian 2012)**, and now evidence of *in vivo* immunoregulatory alterations. Even if this phenomenon is subclinical during orbital flight, clinical risk to crewmembers could be elevated for deep space missions. As future studies continue to characterize in-flight immune alterations, the development of countermeasures to enable exploration missions to be conducted safely may be warranted.

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Figure Legends

Figure 1: Representative individual crewmember plasma cytokine data normalizing baseline concentrations (L-180) and plotting deviations from baseline for all other timepoints. Statically significant alterations in raw mean values are indicated (*) where P≤0.05; n=28.

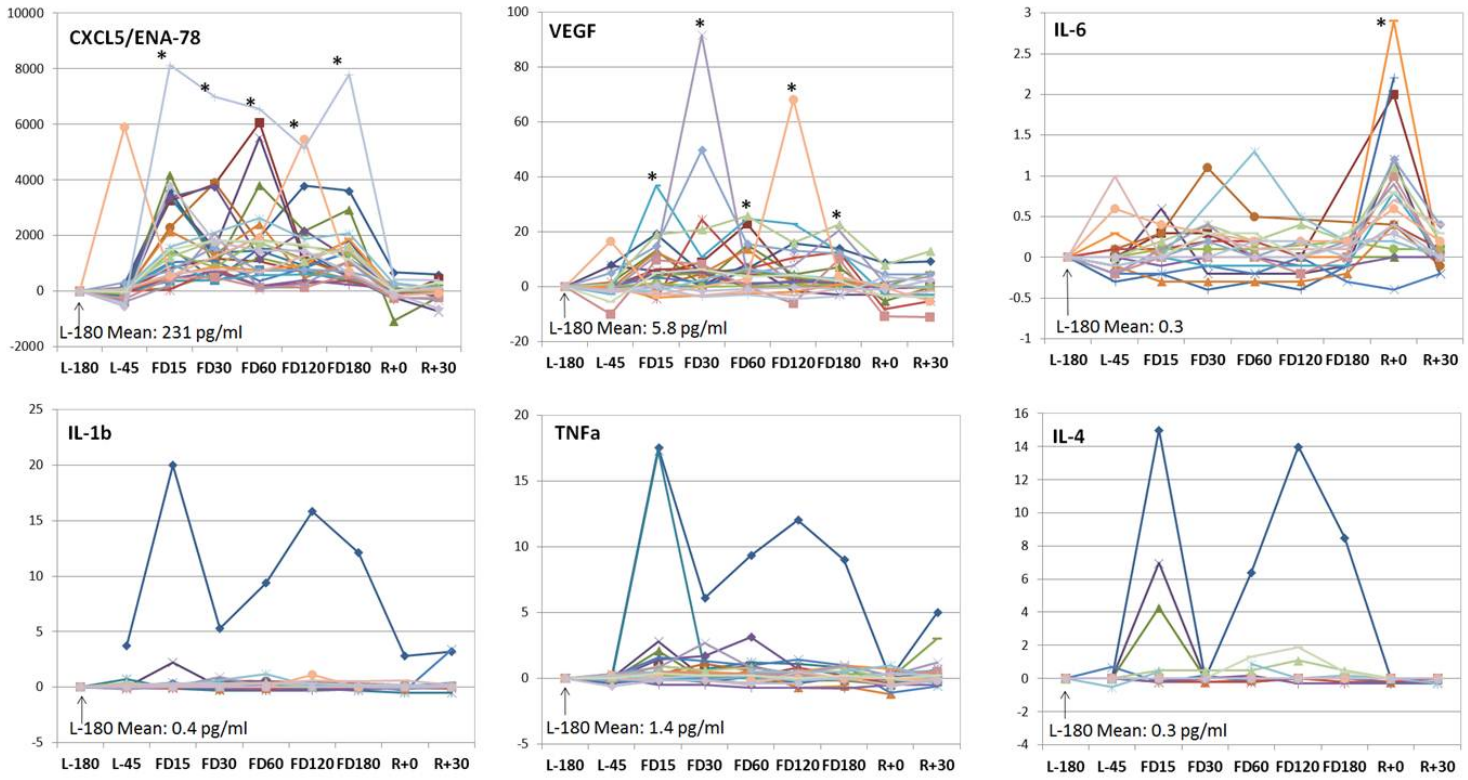

Table 1: Twenty two cytokines for analysis by category

Inflammatory	Anti-Inflammatory	Adaptive/ Regulatory	Growth Factors	Chemokines
IL-1α	IL-1ra	IFNγ	G-CSF	CCL2/MCP-1
IL-1β		IL-2	GM-CSF	CCL3/MIP-1 alpha
TNFα		IL-17	FGF basic	CCL4/MIP-1 beta
IL-6		IL-4	Tpo	CCL5/RANTES
IL-8		IL-5	VEGF	CXCL5/ENA-78
		IL-10		

Table 2: Mean plasma cytokine levels for ISS astronauts before, during, and following spaceflight. Data are expressed as mean concentration pg/ml ± SEM. Main effects were determined by performing a repeated-measures one-way ANOVA analysis compared to the baseline L-180 sample, resulting in the indicated 'P' values. Individual timepoint significance was determined by a post hoc Bonferroni t-test, with significant differences indicated by '*'. Overall 'n' was 28 subjects, the specific 'n' for each timepoint/cytokine indicated.

					Spaceflight						
Cytokine (pg/mL)	**P value**	**L-180**	**L-45**	**L-10**	**FD15**	**FD30**	**FD60**	**FD120**	**FD180**	**R+0**	**R+30**
n:		26	28	24	28	26	28	24	21	28	27
IL-1a		0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
IL-1b		0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	1.1 ± 0.7	0.5 ± 0.2	0.6 ± 0.3	0.9 ± 0.7	0.8 ± 0.6	0.2 ± 0.1	0.2 ± 0.1
TNFα	P<0.01	1.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.2	2.1 ± 0.6	1.6 ± 0.3	1.7 ± 0.3	1.9 ± 0.5	1.7 ± 0.4	1.0 ± 0.1	1.2 ± 0.2
IL-6	P<0.001	0.2 ± 0.0 (n=23)	0.2 ± 0.1 (n=25)	0.2 ± 0.0 (n=21)	0.4 ± 0.2 (n=25)	0.2 ± 0.1 (n=23)	0.2 ± 0.1 (n=25)	0.2 ± 0.1 (n=21)	0.2 ± 0.1 (n=20)	**0.9* ± 0.2 (n=25)**	0.2 ± 0 (n=24)
IL-8	P<0.001	1.7 ± 0.3	1.8 ± 0.3	2.7 ± 0.4	**7.6* ± 2.2**	6.2 ± 1.4	**7.0* ± 1.7**	6.7 ± 2.0	**6.7* ± 2.3**	1.8 ± 0.3	2.0 ± 0.4
IL-1ra	P<0.01	379 ± 37 (n=23)	363 ± 35 (n=25)	505 ± 61 (n=21)	556 ± 61 (n=25)	558 ± 77 (n=23)	635 ± 99 (n=25)	**722* ± 128 (n=21)**	631 ± 81.8 (n=20)	**687* ± 117 (n=25)**	576 ± 144 (n=24)
INFg		0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
IL-2		1.0 ± 0.4	0.8 ± 0.3	1.2 ± 0.4	0.7 ± 0.3	0.8 ± 0.3	1.0 ± 0.4	1.3 ± 0.4	1 .0± 0.4	0.8 ± 0.3	0.8 ± 0.3
IL-17		0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
IL-4		0.1 ± 0.0 (n=23)	0.1 ± 0.1 (n=25)	0.1 ± 0.0 (n=21)	1.1 ± 0.6 (n=25)	0.1 ± 0.1 (n=23)	0.4 ± 0.3 (n=25)	0.8 ± 0.6 (n=21)	0.5 ± 0.4 (n=20)	0.0 ± 0.0 (n=25)	0.0 ± 0.0 (n=24)
IL-5		0.1 ± 0.0 (n=23)	0.1 ± 0.0 (n=25)	0.1 ± 0.0 (n=21)	0.1 ± 0.0 (n=25)	0.1 ± 0.0 (n=23)	0.1 ± 0.0 (n=25)	0.1 ± 0.0 (n=21)	0.0 ± 0.0 (n=20)	0.1 ± 0.0 (n=25)	0.1 ± 0.0 (n=24)
IL-10		0.1 ± 0.0 (n=23)	0.1 ± 0.0 (n=25)	0.2 ± 0.1 (n=21)	0.3 ± 0.2 (n=25)	0.1 ± 0.0 (n=23)	0.1 ± 0.0 (n=25)	0.3 ± 0.2 (n=21)	0.1 ± 0.0 (n=20)	0.2 ± 0.1 (n=25)	0.1 ± 0.0 (n=24)
G-CSF		5.4 ± 1.6	4.7 ± 1.2	6.2 ± 1.6	5.0 ± 1.3	3.1 ± 0.6	5.3 ± 1.5	10.7 ± 5.9	6.6 ± 2.3	7.2 ± 1.6	4.3 ± 1.0
GM-CSF	p<0.05	0.3 ± 0.1	0.1 ± 0.0	0.4 ± 0.2	1.7 ± 1.0	1.2 ± 0.6	1.4 ± 0.7	1.4 ± 1.1	1.5 ± 1.1	0.3 ± 0.2	0.3 ± 0.2
FGFb		7.7 ± 3.4	6.8 ± 2.8	8.9 ± 3.3	6.2 ± 1.9	11.4 ± 3.5	11.6 ± 3.3	7.7 ± 2.7	6.8 ± 2.2	5.6 ± 2.2	6.4 ± 2.4
Tpo	P<0.001	141 ± 16	139 ± 17	165 ± 22	**183 ± 18***	**186* ± 28**	**184* ± 20.5**	**194* ± 27**	**215* ± 22**	138 ± 16 (n=27)	129 ± 15
VEGF	P<0.001	3.7 ± 0.8 (n=23)	5.1 ± 1.4 (n=25)	6.7 ± 2.2 (n=21)	10.2 ± 1.9 (n=25)	**14.3* ± 4.5 (n=23)**	10.5 ± 1.7 (n=25)	11.5 ± 2.9 (n=21)	10.6 ± 1.8 (n=20)	3.5 ± 0.8 (n=25)	3.7 ± 0.9 (n=23)
CCL2/MCP-1	P<0.001	72.4 ± 6.4	77.7 ± 7.6	69.4 ± 7.6	70.7 ± 5.4	66.6 ± 5.5	77.6 ± 6.6	85.4 ± 7.1	86.6 ± 7.5	**122.5* ± 17.4**	90.9 ± 7

CCL3/MIP-1a		9.4 ± 3 (n=23)	7.6 ± 3.1 (n=25)	8.6 ± 3.2 (n=21)	10.2 ± 4 (n=25)	5.2 ± 2.2 (n=23)	7.3 ± 3 (n=25)	9.8 ± 3.9 (n=21)	7.9 ± 4.1 (n=20)	7.1 ± 2.9 (n=25)	6.6 ± 2.8 (n=24)
CCL4/MIP1b	p<0.05	16.2 ± 2.1	16.5 ± 2.7	17.2 ± 3.0	22.1 ± 2.8	20.1 ± 2.4	21.8 ± 2.8	**24.3* ± 5**	21.1 ± 3.2	17 ± 2.2	18.7 ± 3.8
CCL5/RANTES		3427 ± 306 (n=23)	3247 ± 242 (n=25)	3986± 180 (n=21)	3609 ± 190 (n=25)	3741 ± 197 (n=23)	3546 ± 182 (n=25)	3720 ± 232 (n=21)	4003 ± 199 (n=20)	3366 ± 262 (n=25)	3562 ± 216 (n=24)
CXCL5/ENA-78	P<0.001	239 ± 60	348 ± 203	**898* ± 251**	**2020* ± 363**	**1882* ± 319**	**1933* ± 330**	**1741* ± 309**	**1841* ± 385**	189 ± 48	204 ± 53