IMMUNE DYSREGULATION FOLLOWING SHORT VERSUS LONG DURATION SPACE FLIGHT

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ABSTRACT

Immune system dysregulation has been demonstrated to occur during spaceflight and has the potential to cause serious health risks to crewmembers participating in exploration-class missions. A comprehensive immune assessment was recently performed on 13 short duration Space Shuttle crewmembers and 8 long duration International Space Station (ISS) crewmembers. Statistically significant post-flight phenotype alterations (as compared to pre-flight baseline) for the Shuttle crewmembers included: granulocytosis, increased percentage of B cells, reduced percentage of NK cells, elevated CD4/CD8 ratio, elevated levels of memory CD4+ T cells, and a CD8+ T cell shift to a less differentiated state. For the Shuttle crewmembers, T cell function was surprisingly *elevated* post-flight, among both the CD4+ and CD8+ subsets. This is likely an acute stress response in less-deconditioned crewmembers. The percentage of CD4+/IL-2+, CD4+/IFNg+ and CD8+/IFNg+ T cells were all decreased at landing. Culture secreted IFNg production was significantly decreased at landing, whereas production of Th2 cytokines was largely unchanged. It was found that the IFNg:IL-10 ratio was obviously declined in the Shuttle crewmembers immediately post-flight. A similar pattern of alterations were observed for the long duration ISS crewmembers. In contrast to Shuttle crewmembers, the ISS crewmembers demonstrated a dramatic *reduction* in T cell function immediately post-flight. This may be related to the effect of acute landing stress in conjunction with prolonged deconditioning associated with extended flight. The reduction in IFNg:IL-10 ratio (Th2 shift) was also observed post-flight in the ISS crewmembers to a much higher degree. These data indicate consistent peripheral phenotype changes and altered cytokine production profiles occur following space travel of both short and long duration.

Background

Dysregulation of the immune system has been shown to be associated with spaceflight, and there have been several excellent reviews published recently regarding this subject **[1,32, 33, 37]**. Specific immune system alterations that have been observed following spaceflight (immediate postflight testing) include altered: cytokine production patterns **[3, 8, 12, 13, 18, 23, 31, 34, 35, 36]**; NK cell function **[2, 17, 22]**; leukocyte distribution **[8, 39]**; monocyte function **[16, 20]**; neutrophil function **[15, 39]**; T cell intracellular signaling **[5, 6, 19, 27, 28]**; neuorendocrine responses **[40]**; leukocyte proliferation following activation **[14, 24]**. Due to the complexities associated with in-flight experiments, there have been comparatively few *in-flight* studies of immune function. Those that have been performed have found reactivation of latent herpes viruses during short duration flight **[21, 25, 26, 38]** and altered cell mediated immunity during long duration flight **(4, 11)**. As a whole, these data strongly suggest that immune dysregulation is associated with spaceflight, regardless of duration. However, the precise in-flight nature of the dysregulation, especially as equilibrates over longer duration flights (distinct from launch/landing stresses) is not known.

The goal of NASA and the space life science community is to determine the clinical risks associated with all flight effects on human physiology, so that countermeasures may be developed prior to the initiation of exploration-class space missions. This need has been heightened by the impending lunar/Mars program, which are scheduled be initiated by NASA in the next decade.

The data presented in this article outline the results of a comprehensive assessment of immunity, consisting of peripheral immune phenotype, cytokine production profiles (intracellular and secreted), as well as T cell function. This study was performed pre- and post-flight on both short-duration (Shuttle) and long-duration International Space Station (ISS) crewmembers as part of the *Epstein Barr* flight study (E129, DSO-500). Epstein Barr assesses immunity, physiological stress and latent viral reactivation following spaceflight. NASA will follow this post-flight study with a related in-flight study (*Integrated Immue*, SMO-015, SDBI-1900) that will commence in late 2007. *Integrated Immune* will distinguish what immune dysregulation persists during flight from post-flight assessments that have the potential to be influenced by landing and readaptation.

Materials and Methods

Subjects/missions. 13 short duration Space Shuttle crewmembers and 8 long duration ISS crewmembers participated in this study. The Shuttle astronauts flew on Space Shuttle missions STS-114, STS-121, STS-115 and STS-116. The ISS astronauts (or Russian cosmonauts) flew on ISS Expeditions 11, 12, 13 and 14. Institutional review board approval was obtained from the *Committee for the Protection of Human Subjects* at the Johnson Space Center; Houston, TX. Informed consent was obtained from all subjects who participated in the study.

Whole blood samples. For this study the sample consisted of 1.0 ml heparin or 0.5 ml EDTA anticoagulated whole blood. Whole blood samples were collected by standard phlebotomy techniques according to the schedule shown in **figure 1**. 'L-_' indicates the

number of days prior to launch a sample was collected and 'R+_' indicates the number of days following landing a sample is collected. The precise date of sampling could vary due to crew training schedules and other logistical considerations (especially true for ISS crewmembers), and not every sample was collected on each subject. However, for all subjects a landing day (R+0) sample was collected, in addition to at least two pre-mission baseline samples and a post-mission recovery timepoint. For immediate post-flight samples processed remotely at either the Kennedy Space Center (Shuttle landing) or Star City, Russia (Soyuz landing), cells or culture supernatants were stained and stabilized as previously described **(7)** and transported to JSC for analysis.

Immunophenotype analysis. A comprehensive 5-color flow cytometry antibody matrix was created that assessed all the major leukocyte/lymphocyte subsets, as well as various activated, cytotoxic/effector and memory/naïve T cell subsets. Cell surface markers were stained first diluting the 500ul whole EDTA whole blood sample with 500 ul of PBS. One hundred microliters of the diluted EDTA whole blood was then combined with 10 μl of each appropriate labeled monoclonal antibodies. Staining was performed by incubating the mixture at room temperature for a minimum of 20 minutes. Red blood cells were then lysed using the Beckman-Coulter Optilyse reagent as described by the manufacturer, and the cells were washed in PBS. The stained leukocytes were then fixed in 1.0% paraformaldehyde in PBS for 10 minutes and analyzed on a Beckman-Coulter Epics XL flow cytometer. The cytometry panel is outlined in **table 1**.

T cell function. Immune suppression may consist of a reduced capacity for lymphocytes to respond to stimulus, even though the relative distribution of immune cells is unchanged. For this study the functional response of T cells was measured by activating whole blood cultures in the presence of T cell mitogenic stimuli, followed by measurement of surface activation marker expression on T cell subsets. Culture in the presence of anti-CD3 and anti-CD28 antibodies was used for T cell stimulation. These antibodies activate T cells by triggering T cell surface molecules, requiring the full compliment of intracellular signaling to be utilized (as opposed to phorbol ester or ionomycin). T cell progression through a full activation cycle may be monitored by culturing cells after 24 hours by determining the expression of CD69 (early activation) and CD25 (mid-activation, receptor for IL-2 that requires new gene synthesis). It is noteworthy that some individuals are incompatible with T cell activation via soluble CD3/CD28 triggering for reasons that are unclear **(29, 30)**. For this study 8 of 13 Shuttle and 6 of 8 ISS astronauts were 'responders' and compatible with the T cell function assessment. Only data from 'responders' are presented.

***Intracellular cytokine analysis*.** Lymphocyte and monocyte cytokine production will be assessed for specific cell subsets at the single-cell level utilizing intracellular flow cytometry. The unique advantage of intracellular flow cytometry is the ability to assess the production of multiple cytokines simultaneously in positively identified cell sub-populations using multi-color flow cytometry. Whole blood cultures will be set up by adding 100 ul heparin whole blood to 1.0 culture media containing 10 ng/ml PMA, 1.0 ug/ml ionomycin and 3 uM monensin. Cultures were incubated for 4.0 hours at 37 degrees C. Following incubation, the supernatants were removed, the RBCs lysed as noted above, and the remaining WBCs were fixed in 4.0% paraformaldehyde for 10 minutes. To detect intracellular production of IFNγ or IL-2 (following surface marker staining), the fixed PBMC's were resuspended in 200 μl of

permeabilization buffer, consisting of 5.0% non-fat dry milk and 0.5% saponin in PBS to which 0.5 μg of labeled mouse antibody to either IFNγ or IL-2 (or both) was added. The cells were incubated at room temperature for 25 minutes and then washed in PBS containing saponin. The cells were then resuspended in 1.0% paraformaldehyde for analysis.

***Cytometric Bead Array Analysis*.** For analysis of secreted Th1/Th2 cytokine profiles, whole blood cultures were set up as described above. Mitogenic stimulation consisted of either anti-CD3/CD28 or PMA+ionomycin for 48 hours. Following culture, supernatants were removed and frozen until analysis. The Th1/Th2 cytometric bead array assessment was performed according to the manufacturer's instructions (Becton Dickinson). Since all crewmember assessments were batch-analyzed, data are presented as mean fluorescence intensity (MFI) to show relative cytokine changes throughout the duration of the mission. The MFI directly relates to supernatant cytokine concentration.

Statistical Analysis. Statistical significance was determined using the Student's Paired T Test. Unless otherwise indicated, statistical significance was determined by comparing the mean value for last pre-mission timepoint to R+0 landing day sample. In the event a particular subject missed the last pre-mission timepoint, the missing data was imputed from the other baseline timepoint,so that in all cases R+0 data were compared to pre-flight baseline values. The differences between timepoints were considered significant if $P < 0.05$ and are indicated as such (*) on each data figure.

Results

Immunophenotype. Alterations were observed in the distribution of the peripheral leukocyte subsets immediately following landing for both the Shuttle crewmembers and the ISS crewmembers. To assess significance, landing day data was compared to pre-flight baseline data (usually the L-10 values). Data are presented in **tables 2 and 3** for both Shuttle and ISS crewmembers, respectively.

For the Shuttle crewmembers, statistically significant subset changes upon landing (as compared to pre-flight baseline values) included granulocytosis, decreased lymphocyte percentage, elevated B cells and decreased NK cells. The percentage of 'bulk' memory CD4+ /CD45RO+ T cells was significantly elevated after flight, but the level of 'bulk' memory CD8+/CD45RO+ T cells was unchanged. Regarding the 'fine' memory CD8+ T cell subsets, there was a significant shift observed from the late senescent CD28-/CD244+ subset to the non-differentiated CD28+/CD244- subset. There was also a significant reduction in the percentage of circulating early senescent CD8+/CD57+ T cells. Central memory CD8+ T cell subsets were assessed, and a significant shift from the terminally differentiated CD62L-/CD45RA+ subset to the true naïve CD62L+/CD45RA+ subset was observed. All of these analyses indicate the phenotype of the peripheral CD8+ T cells was significantly less differentiated (more naïve) following the short-duration space flight. There were no observed increases after landing in any of the early activated (CD69+) or late activated (HLA-DR+) CD4+ or CD8+ T cell subsets.

The ISS crewmembers shared some, but not all of the observed post-flight peripheral phenotype changes with the Shuttle crewmembers. For the long duration ISS crewmembers,

statistically significant changes upon landing included granulocytosis, decreased lymphocyte percentage, elevated B cells and decreased NK cells. The CD4:CD8 ration was unchanged, however there was an elevation in both the CD4 and the CD8 'bulk' memory T cell subsets (CD45RO+). No significant changes were observed in the ISS crewmembers regarding central memory, cytotoxic/senescent and activated T cell subsets.

T cell function. The functional capabilities of the circulating T cell subsets were assessed by performing whole blood cultures for 24 hours in the presence of antibodies to CD3 and CD28. Following culture, expression of the early activation surface marker CD69 and later activation marker CD25 on T cell subsets were then assessed by multiparameter flow cytometry. Representative flow cytometry scatter plots from one long-duration ISS crewmember demonstrating resolution of the activated CD4+ T cell subsets is presented in **figure 2**. For short duration Space Shuttle crewmembers the mean percentage of both T cell subsets that could be stimulated to co-express both activation markers was consistent throughout all pre-flight assessments (L-180, L-65, L-10; **figure 3A**). Following landing day (R+0) assessments, a significant *increase* in the percentage of T cell subsets that could be activated to co-express CD69/CD25 was observed, that resolved to baseline levels by R+14 **(figure 3A)**.

For ISS crewmembers, the mean percentage of both T cell subsets that could be stimulated to full activation (CD69+/CD25+) was also stable between both pre-flight timepoints **(figure 3B)**. However, landing day assessments for the ISS crewmembers revealed a significant *decrease* in percentage of both CD4+ and CD8+ T cells that could be stimulated to co-express CD69/CD25 **(figure 3B)**. It is noteworthy that the total percentage of T cell subsets expressing CD69 alone was reduced, but not significantly different, on landing day. Thus, expression of the early activation marker is relatively unchanged, but the expression of CD25 (requiring new mRNA expression) was significantly decreased. Values for the ISS crewmembers for this assay trended to resolution at R+3 and had fully returned to baseline values by R+30 **(figure 3B)**.

Intracellular cytokine analysis. The percentage of T cell subsets (CD4 and CD8) capable of being stimulated to produce either IL-2 or IFNg were determined by intracellular flow cytometry following whole blood culture for 5 hours in the presence of PMA, ionomycin and monensin **(figure 4A, 4B)**. For the short duration Shuttle crewmembers, there was a significant decrease in the percentage of CD4+/IL-2+ T cells at R+0 as compared to the pre-mission baseline values **(figure 4C)**. The percentage of CD8+/IL-2+ T cells was reduced on landing day, but not in a significant fashion. In addition, there was an observable trend towards a decrease in both the CD8+/IFNg+ and CD4+/IFNg+ T cell subsets that also did not reach significance **(figure 4C)**. All the observed R+0 intracellular cytokine alterations appeared to fully resolve by the R+14 timepoint.

For ISS crewmembers, there a significant decrease in the percentage of CD4+/IL-2+ T cells at R+0 as compared to the pre-mission baseline values **(figure 4D)**. Similarly to the Shuttle crewmembers, there was also no significant decrease in the percentage of CD8+/IL-2+, CD4+/IFNg+ or CD8+/IFNg+. In contrast to the Shuttle crewmembers however, we observed no trend towards a decrease in the ISS subject for these subsets **(figure 4D)**. Due to the low 'n', these data were especially vulnerable to masking individual subject variations. In particular, when individual ISS crewmembers data were analyzed, two of the individual long

duration crewmembers demonstrated a dramatic reduction in CD8+/IFNg+ subset **(data not shown)**. No other noteworthy individual data were observed.

Secreted Th1/Th2 cytokine analysis. In an effort to gauge the crewmembers Th1/Th2 cytokine bias, cytokines secreted during whole blood culture activated with either anti-CD3/CD28 or PMA+ionomycin were detected by cytometric bead array. A representative flow cytometry scatter plot demonstrating this analysis technique is presented in **figure 5A**. Cytokine expression data are generated as mean fluorescence intensity, and since six cytokines are assayed simultaneously, relative comparisons may be made between the cytokines. Also, since all samples were frozen and assayed simultaneously per mission, relative comparisons (per crewmember) across the data points are valid. **Figures 5B and 5C** show average expression for all six cytokines following whole blood CD3/CD28 stimulation for Shuttle and ISS crewmembers respectively. For the shuttle crewmembers, expression of IFNg far surpassed all other cytokines, yet there was a statistically significant drop in IFNg expression on R+0 that trended to resolve by R+14 **(figure 5B)**. Interestingly, relative expression of IFNg was lower pre-flight for the ISS crewmembers, but also did drop on landing day whereas other cytokines appeared unchanged **(figure 5C)**. Post-flight recovery data for the ISS crewmembers revealed IFNg expression that approached the Shuttle crewmembers in intensity **(figure 5C)**. Interestingly, expression of IL-10 among the ISS crewmembers was actually elevated at R+0, and trended downwards to baseline values by R+30 **(Figure 5C)**.

Average expression for all six cytokines following whole blood PMA and ionomycin stimulation are presented for Shuttle and ISS crewmembers in **figures 5D and 5E** respectively. A differing pattern of expression was obtained following PMA+ionomycin stimulation as compared to CD3/CD28 stimulation. Extremely high levels of IL-2 and IFNg predominated, with levels of all four other cytokines minimal by comparison. This is supported by the fact that following PMA+ionomycin stimulation, only IFNg and IL-2 are readily detectable by intracellular cytokine assays. For the Shuttle crewmembers, the greatest elevation of IFNg and IL-2 expression was seen at the L-10 and R+3 data points **(figure 5D)**. No other consistent pattern of altered production was observed in the Shuttle crewmembers. For the ISS crewmembers, levels of IFNg and IL-2 expression were also highest at R+3. Interestingly, levels of TNFa, which were minimal at all pre-flight points for the ISS crewmembers, was also significantly elevated at R+3 **(figure 5E)**. These elevations were generally trending to resolve by R+30. Given that an IFNg:IL-10 ratio may be an indicator of Th1:Th2 balance, such a ratio was calculated using the CD3/CD28 culture data for all crewmembers. Individual subject IFNg:IL-10 ratios are presented for Shuttle and ISS subjects in **figures 5F and 5G** respectively. For most Shuttle crewmembers, a relatively depressed ration is observed at R+0, as compared to both pre-flight baseline values and post-flight recovery data **(figure 5F)**. For 4 of 5 ISS crewmembers tested, a similar shift was detected, but to an even more dramatic level: R+0 IFNg:IL-10 rations were dramatically reduced compared to both pre-flight and post-flight recovery data **(figure 5G)**. To check for inter subject and assay variability, four healthy control subjects were evaluated and relatively similar timepoints. The individual control subject data re presented in **figure 5H**, and show that IFNg:IL-10 rations were relatively stable over time, with no Th1:Th2 shifts observed.

Discussion

Determining the effect of space travel on the human immune system has proven to be extremely challenging. Limited opportunities for flight studies, varying mission durations, technical and logistical obstacles, low subject numbers and a broad range of potential assays have contributed to this problem. Also, the inherent complexity of the immune system, with the vast array of cell populations, sub-populations, diverse regulatory molecules and broad interactions with other physiological systems makes determining just what to measure extremely complicated. In addition, the perspective of the flight surgeon must be considered in determining the clinical significance of immune alterations (relating to a real in-flight clinical risk). The effect of this problem may be observed by scanning publications dealing with immunity and space flight, which initiated during the 1970s. Although an admirable body of work, the literature to date suffers from widely varying sampling methods, assay techniques, low subject counts and disparate focus on narrow aspects of immunity. This emphasizes the need for an integrated comprehensive approach to determining the effect of immunity during space flight.

This study attempted to survey crewmember immune status following spaceflight of both short and long duration. An attempt was made to utilized assays that would have the must value in determining clinical risk. This is difficult since in the sense of broad utility, limited phenotyping and viral antibody titers are the only immunology assays used in clinical medicine. For this study a comprehensive phenotype analysis, T cell function and cytokine profiles were selected. It was believed that the phenotype assessment would provide direct evidence for in-vivo immune activation/pathology, whereas the functional assessments would determine immunity as relates to SAID(spell out and define-looks like done below) and deconditioning. The granulocytosis observed at landing is almost certainly a transient response to the stress of landing, and does not reflect persistent immune alterations. It is possible that at least some of the other phenotypic changes (reduced NK cells, increased memory T cells, increased levels of immature CD8+ T cells) are not related to landing effects. Of these, the significant reduction in NK cell percentages may represent a risk. NK cells are responsible for tumor surveillance, which would be especially important in the high-energy radiation environment of deep space.

T cell function was assessed using CD3/CD28 antibody stimulation and whole blood culture. This assay was developed to provide as close as possible a simulation of in-vivo functional capabilities. Whole blood culture retains all soluble plasma factors and does not artificially purify cells, retaining any cell-cell interactions that may be required. Also, CD3/CD28 activation triggers via the TCR, requiring the full compliment of intracellular signaling to be utilized. A marked *reduction* in T cell function was observed on landing day for the ISS crewmembers **(figure 3B)**, whereas landing day T cell function was actually *increased* in the Shuttle crewmembers **(figure 3A)**. This dysregulation was observed for both the CD4+ and CD8+ T cell subsets. It is not unexpected that the acute landing-day stress would be immunostimulatory in the less-deconditioned Shuttle crewmembers. Dhabhar et al. have shown that acute stress may enhance immunity, whereas chronic stress may be immunosuppressive **(9, 10)**. For the ISS crewmembers it is likely that the longer flight duration and more severe deconditioning contributed to the reduction in T cell function. Whether the altered function was already present during flight, or was caused by solely landing cannot be determined. Persistently reduced T cell function would almost certainly

pose a risk during flight, but even if this is a landing-day only effect it could pose a clinical risk for crewmembers landing on Mars. The initial days on Mars could bring exposure to a variety of unique immunological challenges.

Alterations in cytokine production were observed both at the intracellular (reduced numbers of cells producing) and the secreted (bulk production, irrespective of source) levels. The reduction in the numbers of T cells capable of being secreted to produce IL-2 were significant for both the Shuttle and the ISS crewmembers **(figure 4)**. The number of T cells capable of being secreted to produce IFNg were also reduced, but did not achieve statistical significance. In general reduced cytokine production, as compared to baseline, may be considered as a reduction in immune function. To assess the specific Th1:Th2 balance, the secreted cytokine bead array was used. This assay allows the direct comparison of the secretion of multiple cytokines via simultaneous assessment **(figure 5A)**. For this assay, direct comparison of the mean fluorescence intensity (relates to expression level) was utilized for data interpretation. For mean expression among Shuttle crewmembers, following CD3/CD28 stimulation there was an obvious mission-associated reduction in IFNg secretion that quickly resolved **(figure 5B)**. Levels of the other cytokines were minimally reduced. Interestingly, among the ISS crewmembers a similar reduction if IFNg secretion was observed, although with lower baseline values **(figure 5C)**. It cannot be determined, however this may related to prolonged overseas training which the Shuttle crewmembers do not experience. However, levels of IL-10 production in the ISS crewmembers were actually elevated postflight **(figure 5C)**. Reduced IFNg production and elevated IL-10 production almost certainly indicates a Th1:Th2 shift has occurred. As a positive control, culture activation was also performed with the pharmacologic agents PMA and ionomycin. This activation is known to produce exceedingly elevated levels of cytokine production. For the Shuttle and ISS crewmembers, mean production of individual cytokines following PMA and ionomycin are presented in **figures 5D and 5E** respectively. In general, PMA+ionomycin stimulation resulted in extremely high levels of IFNg and IL-2 production, yet surprisingly minimal levels of the other four cytokines (actually below CD3/CD28 in some cases). This likely explains why PMA+ionomycin are excellent for intracellular IFNg and IL-2, yet poor for detection of any other cytokine via that method. For both Shuttle and ISS, although R+0 levels were lower compared to post-flight recovery points, it could not be said that mission associated changes occurred due to the lower pre-flight baseline values **(figures 5D and 5E)**. It is noteworthy that following PMA+ionomycin stimulation, for the ISS crewmembers levels of TNFa were elevated at R+0, and much more elevated at R+3, with values resolved by R+30 **(figure 5E)**. Since this change was not observed in the Shuttle crewmembers, this must also be related to longer flight duration, and (since levels were higher at R+3) may represent readaptation stress following severe deconditioning.

To better illustrate the Th1:Th2 shift in subjects, individual crewmember IFNg:IL-10 ratios were plotted for both Shuttle and ISS **(figures 5F and 5G respectively)**. Although individual Shuttle crewmembers varied, in general most demonstrated a Th2 shift at landing that resolved postflight **(figure 5F)**. Among the ISS crewmembers, 4 of 5 demonstrated a dramatic Th2 shift, with much higher IL-10 levels (compared to IFNg levels) on R+0 **(figure 5G)**. One outlier ISS subject did not follow this trend. If this Th2 shift persisted during long-duration flight, and was not a landing day effect, it could represent a significant exploration clinical risk for Th2 autoimmune diseases and inflammatory infections. Data derived from 4 healthy controls over a similar time span demonstrated that such Th2 shifts are not caused

by normal subject variation. All four control subjects had positive ratios with little fluctuation over the mission duration **(figure 5H)**.

[Th2 shift more susceptible to viral reactivation, relates to in-flight reactivation data?]Spaceflight-associated immune dysfunction (SAID) may be caused by factors uniquely associated with space travel (microgravity, radiation) or mission-associated factors not uniquely associated with space travel (prolonged confinement, isolation, physiologic stress, altered nutrition, altered circadian rhythms or an altered microbial environment). In fact, it is likely that several of these factors synergize during longer flight, resulting in a final common (unknown) effect on human immunity. Although immune dysfunction has been observed during even short duration missions, it is not believed that there is a clinical risk associated with transient shorter-duration SAID. In fact, any dysregulation observed during short duration flights would be different than the dysfunction that would persist during longer duration flight. The nature of short and long duration flight is dramatically different with respect to human physiology. Shorter flights (primarily Shuttle) are associated with less deconditioning and much busier work schedules, whereas longer duration flights (ISS, ~6 months in duration) are associated with greater deconditioning and prolonged exposure to microgravity and radiation.

The data presented here indicate that several interesting immune changes are detectable immediately following space flight, including dramatic reductions in T cell function and a Th2 shift following long-duration flight. It cannot yet be determined if there is a clinical risk associated with immune dysregulation for exploration-class (lunar and Mars) space missions. The situation experienced by a crewmember during exploration-class flight will be dramatically different from short or long duration orbital flight. This is primarily due to a lack of return option, years of microgravity exposure and deconditioning, significantly higher isolation and exposure to higher energy radiation than orbital flight. A subsequent integrated study with in-flight sampling is required to accurately determine the status of the immune system *during* long duration spaceflight and correctly assess the clinical risk for exploration-class space missions.

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FIGURE LEGENDS

Table 1: Phenotype of peripheral leukocyte subsets identified for Shuttle and ISS astronauts by multi-color cytometry.

	Marker combination used to identify cell subset			
Granulocytes	CD45+	CD14-	(identified by scatter)	
Lymphocytes	CD45+	CD14-	(identified by scatter)	
Monocytes	CD45+	CD14+	(identified by scatter)	
T cells	CD45+	CD3+		
B cells	CD45+	CD19+		
NK cells	CD45+	CD16/56+		
CD4+ T cells	CD3+	CD4+		
CD8+ T cells	CD3+	CD8+		
CD8+ Non-differentiated	CD3+	CD8+	CD28+	CD244-
CD8+ Active cytotoxic	CD3+	CD8+	CD28+	CD244+
CD8+ late senescent	CD3+	CD8+	CD28-	CD244+
CD8+ early senescent	CD3+	CD8+	CD57+	
CD4+ Bulk memory	CD3+	CD4+	CD45RA-	CD45RO+
CD8+ Bulk memory	CD3+	CD8+	CD45RA-	CD45RO+
CD8+ True naïve	CD3+	CD8+	CD62L+	CD45RA+
CD8+ Central memory	CD3+	CD8+	CD62L+	CD45RA-
CD8+ Effector memory	CD3+	CD8+	CD62L-	CD45RA-
CD8+ Teminally differentiated	CD3+	CD8+	CD62L-	CD45RA+
T cells, CD4+early activated	CD3+	CD4+	CD69+	
T cells, CD4+ late activated	CD3+	CD4+	HLA-DR+	
T cells, CD8+ early activated	CD3+	CD8+	CD69+	
T cells, CD8+ late activated	CD3+	CD8+	HLA-DR+	

Table 2: Short duration Shuttle astronauts (n=13), mean peripheral phenotype data expressed in relative percentages.

	Timepoint				
	L-180	**L-65**	**L-10**	**R+0**	**R+14**
Granulocytes	56	51	56	**76***	53
Lymphocytes	36	41	36	**17***	39
Monocytes	7	8	7	5	7
T cells	70	69	72	74	71
B cells	11	11	11	**16***	11
NK cells	11	12	9	**3***	10
CD4+ T cells	63	63	65	70	65
CD8+ T cells	31	32	29	26	30
CD4+ Bulk memory	59	59	58	**63***	58
CD8+ Bulk memory	47	43	48	46	46
CD8+ True naïve	38	42	38	**44***	37
CD8+ Central memory	14	11	15	16	16
CD8+ Effector memory	27	22	29	27	31
CD8+ Teminally differentiated	21	25	17	**13***	17
CD8+ Non-differentiated	41	40	47	**56***	45
CD8+ Active cytotoxic	20	15	21	20	19
CD8+ late senescent	28	35	24	**16***	27
CD8+ early senescent	9	13	12	**5***	11
T cells, CD4+early activated	1	2	1	1	2
T cells, CD4+ late activated	6	7	6	8	6
T cells, CD8+ early activated	4	4	4	3	3
T cells, CD8+ late activated	12	14	12	12	12

*=statistically significant difference from pre-mission values (p,0.05)

Table 3: Mean peripheral leukocyte subset data expressed in relative percent, long duration ISS astronauts (n=8),

	Timepoint				
	L-180	**L-65**	**R+0**	**R+3**	**R+30**
Granulocytes	56	56	**71***	53	57
Lymphocytes	36	35	**20***	36	34
Monocytes	7	7	8	8	8
T cells	70	70	66	73	71
B cells	10	9	**14***	11	9
NK cells	13	12	**8***	7	11
CD4+ T cells	61	67	63	67	65
CD8+ T cells	32	27	29	27	28
CD4+ Bulk memory	67	64	**73***	63	66
CD8+ Bulk memory	51	48	**56***	50	50
CD8+ True naïve	34	37	30	43	35
CD8+ Central memory	33	25	25	18	22
CD8+ Effector memory	23	24	30	26	22
CD8+ Teminally differentiated	10	14	15	13	20
CD8+ Non-differentiated	51	54	49	54	44
CD8+ Active cytotoxic	13	17	20	15	18
CD8+ late senescent	24	24	27	27	38
CD8+ early senescent	10	11	8	6	11
T cells, CD4+early activated	1	1	1	1	1
T cells, CD4+ late activated	8	2	2	4	8
T cells, CD8+ early activated	2	2	2	2	2
T cells, CD8+ late activated	9	8	8	7	12

*=statistically significant difference from pre-mission values ($p<0.05$)

Figure 1: Blood collection timepoints for Shuttle and ISS crewmembers.

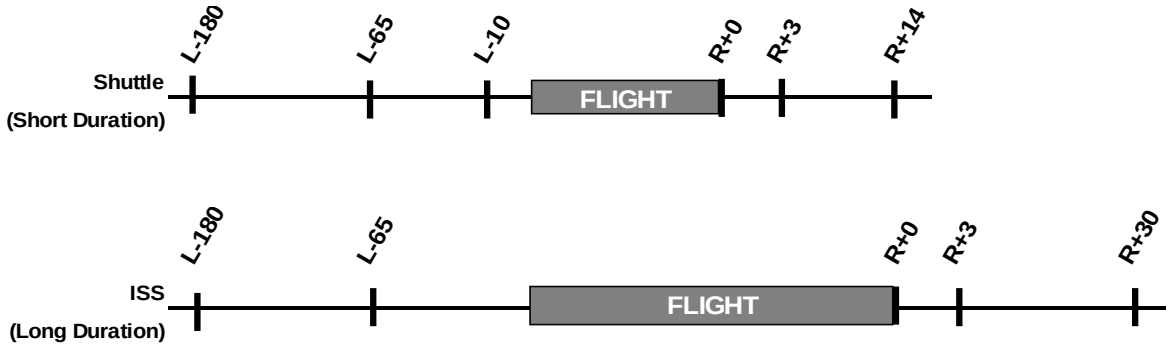


FIGURE 1

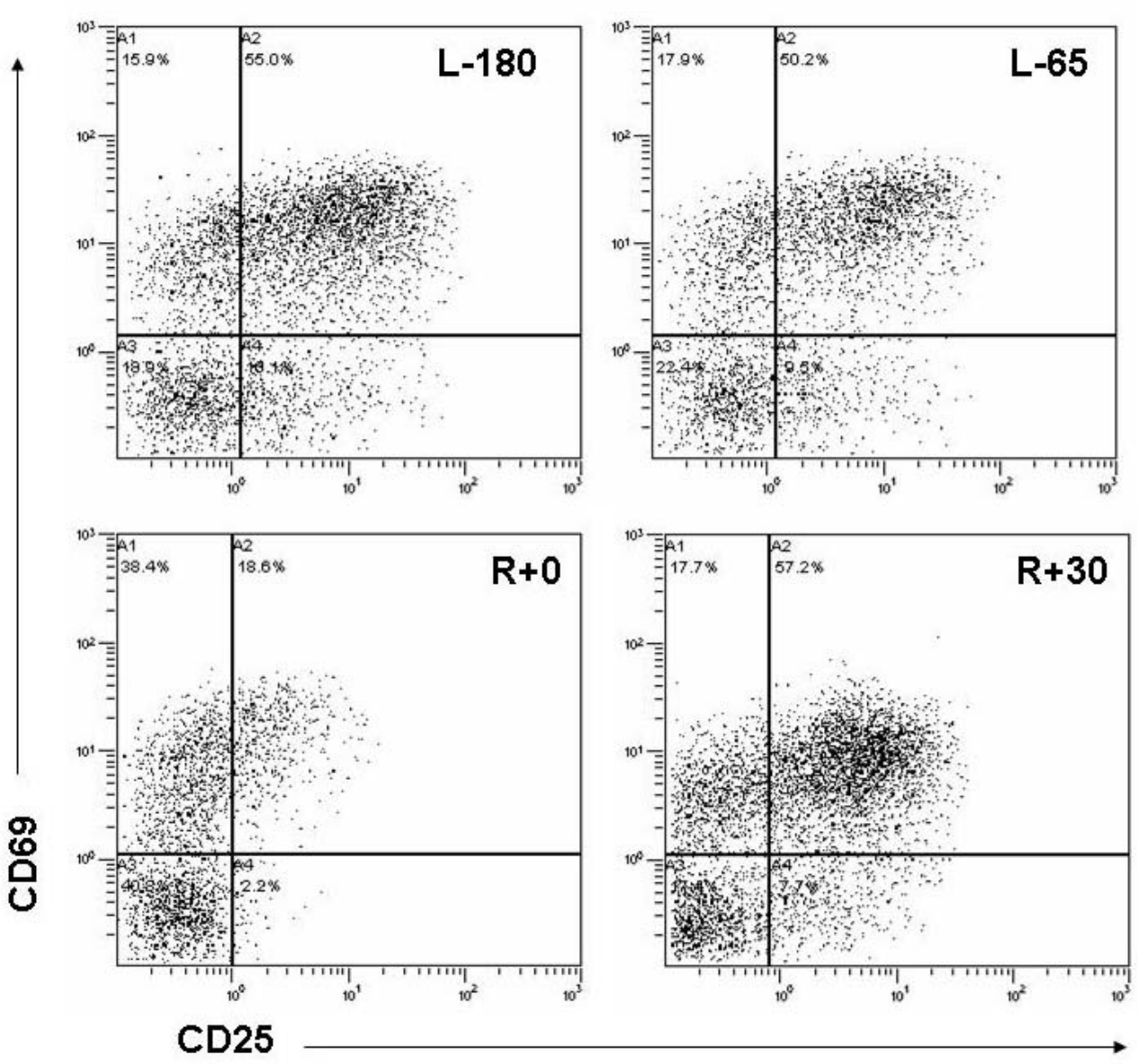
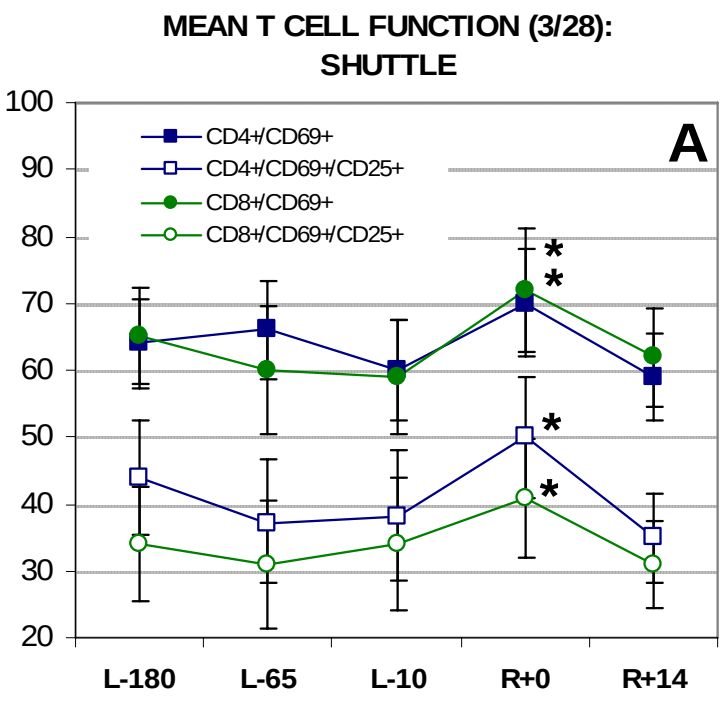

L-180
A1
15.9%
A2
55.0%
A3
18.9%
A4
10.1%
L-65
A1
17.9%
A2
50.2%
A3
22.4%
A4
9.5%
R+0
A1
38.4%
A2
18.6%
A3
40.8%
A4
2.2%
R+30
A1
17.7%
A2
57.2%
A3
17.4%
A4
7.7%
CD69
CD25

FIGURE 2


MEAN T CELL FUNCTION (3/28): SHUTTLE
A
CD4+/CD69+
CD4+/CD69+/CD25+
CD8+/CD69+
CD8+/CD69+/CD25+
100
90
80
70
60
50
40
30
20
L-180
L-65
L-10
R+0
R+14
*
*
*
*

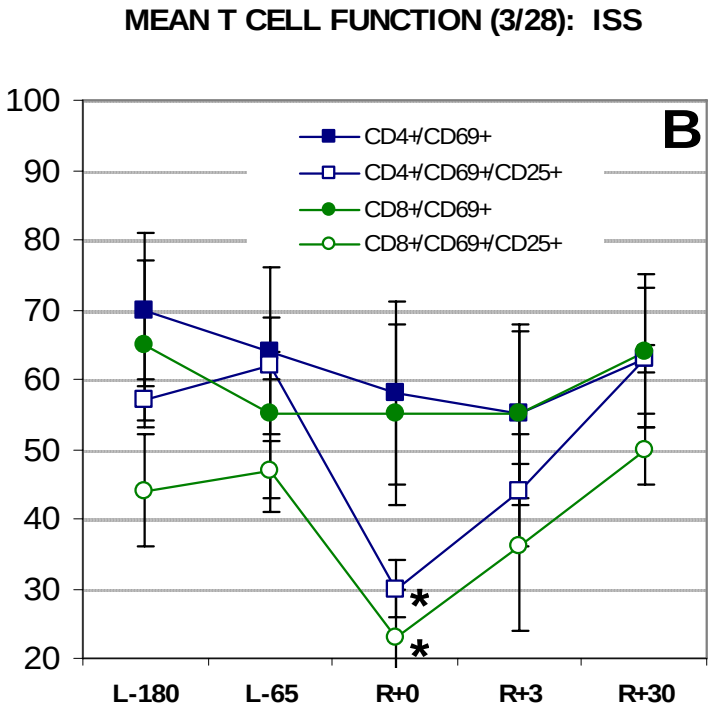

MEAN T CELL FUNCTION (3/28): ISS
B
CD4+/CD69+
CD4+/CD69+/CD25+
CD8+/CD69+
CD8+/CD69+/CD25+
100
90
80
70
60
50
40
30
20
L-180
L-65
R+0
R+3
R+30
*
*

FIGURE 3

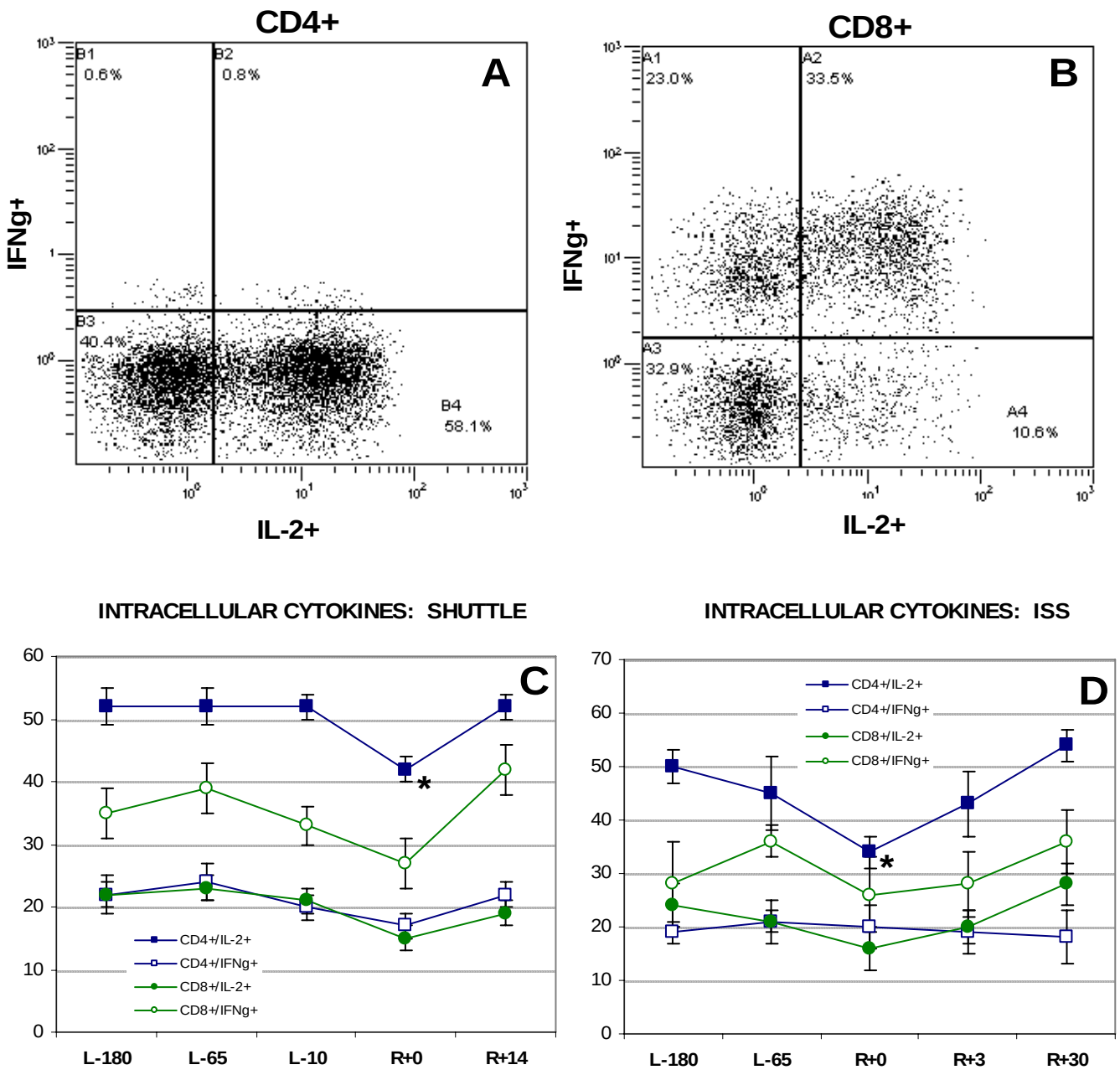

CD4+
A
B1
0.6%
B2
0.8%
B3
40.4%
B4
58.1%
IFNg+
IL-2+
CD8+
B
A1
23.0%
A2
33.5%
A3
32.9%
A4
10.6%
IFNg+
IL-2+
INTRACELLULAR CYTOKINES: SHUTTLE
C
CD4+/IL-2+
CD4+/IFNg+
CD8+/IL-2+
CD8+/IFNg+
L-180
L-65
L-10
R+0
R+14
INTRACELLULAR CYTOKINES: ISS
D
CD4+/IL-2+
CD4+/IFNg+
CD8+/IL-2+
CD8+/IFNg+
L-180
L-65
R+0
R+3
R+30

FIGURE 4

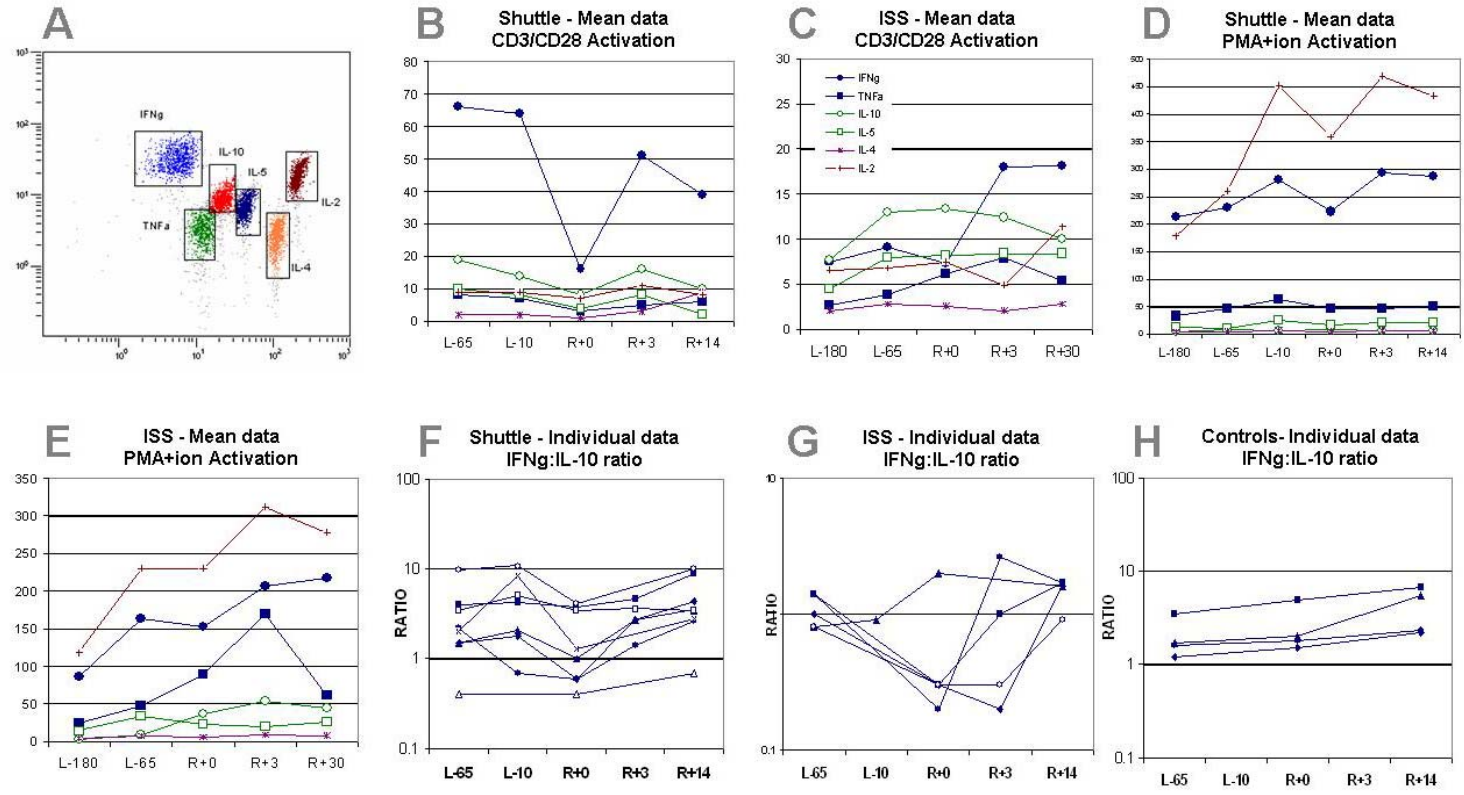

A
IFNg
IL-10
IL-5
IL-2
TNFa
IL-4
B
Shuttle - Mean data
CD3/CD28 Activation
L-65
L-10
R+0
R+3
R+14
C
ISS - Mean data
CD3/CD28 Activation
IFNg
TNFa
IL-10
IL-5
IL-4
IL-2
L-180
L-65
R+0
R+3
R+30
D
Shuttle - Mean data
PMA+ion Activation
L-180
L-65
L-10
R+0
R+3
R+14
E
ISS - Mean data
PMA+ion Activation
L-180
L-65
R+0
R+3
R+30
F
Shuttle - Individual data
IFNg:IL-10 ratio
RATIO
L-65
L-10
R+0
R+3
R+14
G
ISS - Individual data
IFNg:IL-10 ratio
RATIO
L-65
L-10
R+0
R+3
R+14
H
Controls- Individual data
IFNg:IL-10 ratio
RATIO
L-65
L-10
R+0
R+3
R+14

FIGURE 5