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Evaluation of *in vitro* macrophage differentiation during space flight

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Abstract

We differentiated mouse bone marrow cells in the presence of recombinant macrophage colony stimulating (rM-CSF) factor for 14 days during the flight of space shuttle Space Transportation System (STS)-126. We tested the hypothesis that the receptor expression for M-CSF, c-Fms was reduced. We used flow cytometry to assess molecules on cells that were preserved during flight to define the differentiation state of the developing bone marrow macrophages; including CD11b, CD31, CD44, Ly6C, Ly6G, F4/80, Mac2, c-Fos as well as c-Fms. In addition, RNA was preserved during the flight and was used to perform a gene microarray. We found that there were significant differences in the number of macrophages that developed in space compared to controls maintained on Earth. We found that there were significant changes in the distribution of cells that expressed CD11b, CD31, F4/80, Mac2, Ly6C and c-Fos. However, there were no changes in c-Fms expression and no consistent pattern of advanced or retarded differentiation during space flight. We also found a pattern of transcript levels that would be consistent with a relatively normal differentiation outcome but increased proliferation by the bone marrow macrophages that were assayed after 14 days of space flight. There also was a surprising pattern of space flight influence on genes of the coagulation pathway. These data confirm that a space flight can have an impact on the *in vitro* development of macrophages from mouse bone marrow cells.

Introduction

Although the value of the space shuttle has been controversial (Charles, 2011), one of the accomplishments of the space-shuttle era has been to establish that there are profound physiological changes during space flight (Chapes, 2004, Charles, 2011, Fagette et al. , 1999, Harris et al. , 2000, Ronca and Alberts, 2000, Stowe et al. , 2003, Suda, 1998). In particular, we have evidence that space flight suppresses hematopoietic differentiation of macrophages and other blood cells (Ichiki et al. , 1996, Sonnenfeld et al. , 1992, Sonnenfeld et al. , 1990, Vacek et al. , 1983). Space flight has been found to decrease blood monocytes in circulation (Taylor et al. , 1986), induce monocytes lacking insulin growth factor receptors (Meehan et al. , 1992), and it changes leukocyte subpopulations in the bone marrow and spleen (Baqai et al. , 2009, Gridley et al. , 2009, Ortega et al. , 2009, Pecaut et al. , 2003). Decreases in the expression of the GM-CSF receptor may explain some of the *in vivo* physiological changes in macrophages that have been observed (Kaur et al. , 2005).

Space flight experiments with rodents also have revealed a diminution in the percentage and number of early blast cells (CFU-GM) in bone marrow (Sonnenfeld, Mandel, 1992, Sonnenfeld, Mandel, 1990). There were also increases in the number of $CD34^{+}$ cells in the bone marrow of mice assessed after the flight of STS-108 (Pecaut, Nelson, 2003). Skeletal unloading, using antiorthostatic suspension, simulates some of the physiological changes associated with space flight (Chapes et al. , 1993, Morey-Holton and Globus, 1998, 2002) also diminishes the number of macrophage progenitor cells in the bone marrow and affects hematopoiesis (Armstrong et al. , 1994, Armstrong et al. , 1995a, Armstrong et al. , 1993, Dunn et al. , 1983, Dunn et al. , 1985,

Sonnenfeld, Mandel, 1992). Therefore, there are important health issues that might arise from space flight impacts on hematopoiesis.

Space flight affects cells by inducing broad physiological changes and/or it can have direct gravitational impacts on the cells themselves (Todd, 1989). We previously addressed the direct impact of space flight on macrophage differentiation at the cellular level on three different space shuttle flights (Space Transportation System (STS)-57, 60 and 62). There was increased mouse bone marrow macrophage proliferation and inhibited differentiation based on changes in expression of MHCII and Mac2 surface molecules (Armstrong et al. , 1995b). Because these studies were done at less than optimal physiological temperatures (22.5° C to 27.0° C), there were some questions about the impact of these conditions on the outcome.

During the flight of the space shuttle Endeavour, STS-126, we had an opportunity to re-examine macrophage growth and differentiation from stem cells at optimal physiological temperatures (37° C). This experiment allowed us to assess bone marrow differentiation *in vitro* in the absence of the complex *in vivo* environment. In particular, we tested the hypothesis that changes in the receptor for macrophage colony stimulating factor (M-CSF) may have been responsible for the effects of space flight on bone marrow macrophage differentiation. We also had an opportunity to assess global changes in transcript levels to provide insights about biochemical processes that may have been perturbed during the differentiation process.

Materials and Methods

Antibodies

Fluorescein isothiocyanate conjugated- (FITC-) anti Ly6C (Clone AL-21), FITC-anti IgM (Clone RA-22), Phycoerythrin conjugated- (PE-) anti CD31 (Clone MEC13.3), PE- anti IgG2a (Clone R35-95), PE- anti CD44 (Clone IM7), PE- anti IgG2b (Clone A95-1), Allophycocyanin conjugated- (APC-) anti CD3 (Clone 145-2C11), and APC- anti IgG1 were purchased from BD Pharmingen (San Jose California, CA). Alexa Fluor 647 conjugated-(AF647)– anti Mac2 (Clone eBioM3/38), AF647- anti IgG2a (Clone eBR2a), PE- anti CD11b (Clone M1-70), PE- anti IgG2b (Clone eB149/0H5), PE- anti Ly6G (Clone RB6-8C5), PE- anti IgG2b (Clone eB149/0H5), APC- anti F4/80 (Clone BM8), and APC- anti IgG2a (Clone eBR2a) were purchased from eBioscience Inc. (San Diego, CA.). Purified- anti c-Fms (Clone 20), purified – anti IgG, PE- anti IgG, PE- anti c-Fos (Clone 4) and PE- anti IgG2b (Clone not categorized) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA.).

Bone marrow cells and assay set up. Bone marrow cells were harvested from humeri, femora, and tibiae of adult C57BL/6 mice (>8-week old; n=21) originally obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at Kansas State University (KSU) (Armstrong, Nelson, 1993). All animal experiments were approved by Kansas State University Institutional Animal Care and Use Committee. Briefly, the bones were recovered and cleaned of all non-osseous tissue. The marrow cavity was flushed with a sterile PBS solution. The red blood cells were lysed by incubating in ammonium chloride lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.3) for 5 min at 4° C. Pooled cells were centrifuged (300 x

g, 5 min) and washed two times with Dulbecco's Modified Minimal Essentials Medium (Hyclone Laboratories, Inc., Logan, UT) containing 1% fetal bovine serum, 1% Nu serum (BD, Bedford, MA), Glutamine plus (2mM, Atlanta Biologicals, Atlanta, GA), 0.1 M HEPES and 10% Opti-MEM (Invitrogen, Grand Island, N.Y.)($DMEM_2$). Primary bone marrow cells were suspended in $DMEM_2$ supplemented with recombinant mouse macrophage colony stimulating factor (rmM-CSF; 1.5 ug/ml, R & D Systems, Minneapolis, MN) in preparation for culture in Fluid Processing Apparatus hardware (FPAs; Figure 1B)(Armstrong, Gerren, 1995b, Hoehn et al. , 2004, Luttges, 1992, Wilson et al. , 2007). Briefly, FPAs are 11.70 cm long and 1.35 cm diameter (1.31 mm glass thickness) glass barrels. The FPAs have a bypass which allows for the transfer of media from one chamber to another. The FPAs were siliconized with Rain-X (Blue Coral-Slick 50, Ltd; Cleveland, OH) and fitted with a previously siliconized rubber septum, 1.2 cm from the distal end of the barrel. Bacti-caps (16-mm diam.; Oxford Labware, St. Louis, MO) were placed on the proximal end of the FPAs before sterilization.

The bone marrow cells were loaded into the primary chamber of 48 FPAs (1 x 10^7 cells per 3 ml $DMEM_2$ supplemented with rmM-CSF). A second chamber was formed by sliding a sterile, siliconized septum parallel to the first septum. Excess air was evacuated through a 26GA needle. $DMEM_2$ supplemented rmM-CSF was loaded into the second chamber of all 48 FPAs. A third chamber was formed by adding an additional septum similarly to the second. Thirty-two FPAs were loaded with 8% formalin. Eight FPAs were loaded with 6.0 M guanidinium isothiocyanate (GITC) (Woods and Chapes, 1994) and 8 FPAs were loaded with $DMEM_2$ plus rmM-CSF

(returned as viable cultures). The third chambers were sealed with septa as described above. The FPAs were transported (day -2 of spaceflight, Figure 1A) at 4^{o}C to the National Aeronautics and Space Administration (NASA) Space Life Sciences Laboratory Facility (SLSL) at Kennedy Space Center (KSC)**.** The FPAs were loaded into 6 Group Activation Packs (GAPs) (Hoehn, Klaus, 2004), 8 FPAs/GAP. GAPs were placed into the Commercial Generic Bioprocessing Apparatus (CGBA) (Hoehn, Klaus, 2004, Woods and Chapes, 1994)) at 37^{o} C to start incubation. Parallel temperature and activation profile conditions were maintained on GAP's kept at the SLSL. The incubation was started before the launch of space shuttle (Endeavour) flight Space Transportation System (STS)-126 (day -1 of spaceflight, Figure 1A). On day 6 of cell differentiation (day 5 of spaceflight, Figure 1), 1.5 ml of $DMEM_2$ supplemented with rmM-CSF was added to the cell suspension by mixing chambers 1 and 2 of the FPAs through the bypass (Hoehn, Klaus, 2004). On day 15 of cell differentiation (day 14 of spaceflight) the content of the FPA's third chamber was mixed with the cell suspension in the previously merged chambers. STS-126 landing occurred in California 17 days after the start of 37° C cell culture incubation (day 16 of spaceflight). Samples were placed at 4° C and transported to SLSL in Florida. The FPAs were unloaded from GAPs, inspected, and the cells in medium were collected from 8 FPAs and viable cells (trypan blue exclusion) were counted on a hemacytometer. Cell-free media were collected from these FPA and were frozen and sent to KSU. Glucose content was measured in each sample using a digital glucometer (Home Diagnostics, Inc., Ft. Lauderdale, FL). FPAs prepared to fix cells in formalin or GITC were transported to KSU at 4° C and were

processed 19 days after the cells were place at 37° C to begin differentiation. Total cell counts were done on formalin-fixed cells,

Flow cytometry

At KSU, bone marrow cells fixed in formalin were washed in Hank's Buffered Salt Solution (HBSS) and counted and cell concentrations were adjusted to 1×10^7 cells per ml. Phenotypic analysis of bone marrow-derived cells was performed by fluorescence-activated cell sorting as has been described previously by our group (Ortega, Pecaut, 2009, Potts et al. , 2008). Five hundred thousand bone marrow cells were blocked with PBS:goat serum (50:50; 50 µl) at 4^o C for 0.5 h. AF647- anti Mac2 or anti IgG2a (0.5 µg), FITC- anti Ly6C or anti IgM (0.5 µg), PE- anti CD11b or anti IgG2b (0.1 µg) , APC- anti CD31 or anti IgG2a (0.5 µg), APC- anti CD3 or anti IgG1 (1 µg), PE- anti Ly6G or anti IgG2b (0.1 µg), APC- anti F4/80 or anti IgG2a (1.4 µg), PE- anti CD44 or anti IgG2b (1 µg), purified- anti c-Fms or anti IgG (0.25 µg), and PE- anti c-Fos or anti IgG2b (3.8 µg) were added to the cell suspensions and incubated at 4^oC for 1 h. In some instances, multiplexing of antibodies with compatible flurochromes was done (*e.g.* c-Fos and CD31 or Ly6C and CD31). The cells were then washed twice in HBSS and resuspended in HBSS containing 1% formalin. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickson. Rockville, MD) and a minimum of 20,000 events were collected for each sample.

Microarray analysis.

At KSU, bone marrow cells preserved in GITC were combined in pools of 2 FPAs per sample to provide four independent flight and four ground samples for assessment. RNA was phenol extracted and purified in the aqueous phase using phase-lock tubes (5-prime, Gaithersburg, MD) centrifuged at 10,000 g for 10 min. RNA was ethanol precipitated and DNase treated (Qiagen RNeasy). Frozen RNA was sent to the University of Kansas microarray facility at the University of Kansas Medical Center (Kansas City, KS). At the facility, RNA concentrations and qualities were analyzed with an Agilent 2100 Bioanalyzer and RNA 6000 pico assay (Agilent Technologies, Santa Clara, CA). Based on RNA quality, the four best RNA samples were selected (Flight A 21.2 ng, RNA Integrity Value (RIN), 3; Flight D 25ng, RIN, 3; Ground B 12.5ng, RIN, 3; Ground D 24.1 ng, RIN, 3). All available RNA was concentrated and processed for target labeling using the 2x IVT labeling protocol. Briefly, the two round RNA amplification and labeling procedure was performed using the Affymetrix Small Sample Labeling Protocol vII (2xIVT) as follows: 50ng of total RNA was primed with T7-oligo(dT) promoter primer (Affymetrix, Santa Clara, CA) and reverse transcribed using SuperScript Reverse Transcriptase vII kit (Invitrogen). The first round of RNA amplification was performed on the cDNA using the MEGAScript T7 Invitro-Transciption kit (Ambion). Amplified RNA (aRNA) was reverse transcribed using random primers and SuperScript Reverse Transcriptase vII kit. The second round RNA amplification and biotin labeling was conducted using the GeneChip 3' IVT labeling kit (Affymetrix). Biotin labeled aRNA was fragmented and hybridized to the GeneChip Mouse Genome 430 2.0, 3' expression array (Affymetrix) according to manufacturer's instructions. Array washing and staining was conducted using the GeneChip Fluidics

Station 450 followed by a 1x scan with the GeneChip 3000 Scanner 7G with Autoloader. GeneChip processing and data collection was performed using GeneChip Operating System v1.4 (GCOS). Probe intensities were consistent amongst the 4 samples and correlation coefficients among the samples were all greater than 0.95.

Affymetrix Mouse Genome 430 2.0 CHP files were imported into GeneSpring GX11 and were transformed to $\log_2$ based to create a Flight *vs*. Ground data comparison. We performed gene-level analysis with Advanced Workflow with GeneSpring software. A 50 percentile shift normalization algorithm (Yang et al., 2002) was applied to the samples. The correlation coefficients within each treatment group were between 0.9759831 and 0.9926981. The box plot (Figure 2A) of Flight and Ground groups shows some extreme values above the maximum value in both data sets. From the Principal Component Analysis plot (Figure 2B), we confirmed some sample-to-sample variation but there were additional variable differences between Flight and Ground treatments. ANOVA without Multiple Testing Correction was applied and 1678 significant genes out of 28,972 total genes were selected at a p-value < 0.05 with up or down gene-level fold changes greater than 1.5 (Figure 2C)(Dudoit et al. , 2002). To reduce the Type I Error, we performed ANOVA again with the Benjamini Hochberg FDR of Multiple Testing Correction method with cut-off 0.05 on 1678 genes (Benjamini and Hochberg, 1995). We obtained 1678 genes with corrected p-values between 0.00165 and 0.04998.

The data sets that were uploaded into Ingenuity pathway analysis (IPA) 9.0 software were: 1,678 significant genes with corrected p values < 0.05 and fold changes > 1.5; 28,972 “all” genes from the Affymetrix array, and 137 genes which were related to

cell growth and proliferation. All input data sets consisted of three data columns, "Affymetrix Mouse Genome 430 2.0 Array probe ID, *p*-value, and Fold Change". While running a "Core Analysis" to the dataset, the "Filters and General Settings" were set up for the analysis. "Direct" and "Indirect Relationships" were included as interactions and "endogenous chemicals (metabolites)" in the network analysis, all the data sources were selected, and "Mouse" as the species. The IPA result panel included: "Summary, Networks, Functions, Canonical Pathways, Lists, My Pathways, Molecules, Network Explorer, and Overlapping Networks". We focused on "Networks, Functions, and Canonical Pathways".

Microarray data and the sample-quality data are publicly accessible by creating an account and logging into www.bioinformatics.kumc.edu/mdms/login.php. Thereafter the "Share Data with Users/Groups" link may be used, followed by "Browse through the shares". The raw data and analyzed data sets may be accessed using the "Bone Marrow Macrophage Array".

Statistical analysis.

Data were evaluated by Student's *t*-test or by Chi-Square (χ^2) test (Statmost, Detaxiom Software Inc, Los Angeles, CA). *P*-values of <0.05 were selected to indicate significance. The data are presented as the mean ± standard deviation (Sd) of the replicate number.

Results

Cell growth studies

In previous work, we found that macrophage growth was enhanced by space flight. However, the flight conditions necessitated that the cells differentiated at less than optimal physiological temperatures (Armstrong, Gerren, 1995b). To determine if temperature would impact macrophage growth or differentiation during the STS-126 space flight, we determined cell proliferation in two ways. Viable cell numbers were determined at day 17 in the FPA set that was kept in medium throughout the entire mission and total cell numbers were counted in FPAs that were fixed in formalin at day 14 (Figure 1) of the STS-126 mission. When we counted cells kept in medium for the entire mission (nonpreserved), we found that we had more viable cells in the flight FPAs ($3.0 \pm 0.6 \times 10^7$; mean ± Sd; n=7) compared to the ground ($1.7 \pm 0.4 \times 10^7$; mean ± Sd; n=4; *t*-test; $p<0.01$). We also found more cells in the formalin-fixed flight FPAs ($5.3 \pm 0.6 \times 10^6$; mean ± Sd; n=28) compared to cell numbers in the FPAs of the ground controls ($4.4 \pm 0.6 \times 10^6$; mean ± Sd; n=32; *t*-test; $p<0.01$). Although we could not determine the viability of the fixed cells using the trypan blue exclusion test, the increase in cell number from the time the cells were fixed on day 14 to recovery on at day 17 (Figure 1A), suggests that the cells were viable at the time of fixation. There also was a similar amount of RNA collected per cell from each of the treatment groups; 4.1×10^{-6} ng/cell. Therefore, the fixed-cell estimates appear to be accurate and there appears to be more cell proliferation of the differentiated macrophages in space than on the ground. Flight cell numbers increased an average of 5.7 fold and ground cell numbers increased an average of 3.9 fold from day 14 to day 17. We also measured glucose utilization by the cells in the unfixed FPA's. We found significantly less ($p<0.05$, *t*-test) glucose usage by flight cells (121±4 mg/dl) compared to cells grown on the

ground (159±3 mg/dl). These data suggest that the cells required less energy to proliferate more in space.

Assessment of macrophage phenotype after space flight

We found that there was a decrease in MHCII and Mac2 cell surface molecule expression on bone marrow cells differentiated into macrophages during space flight (Armstrong, Gerren, 1995b). In those studies, cells were differentiated at temperatures ranging from 22.5° C-27.0° C. We wanted to confirm that the space-flight differences were not due to the culture temperatures and we wanted to obtain a more comprehensive phenotypic analysis of the bone marrow-derived, M-CSF-dependent macrophages that emerged. We examined the phenotype of the cells using flow cytometry. We assigned 4 subpopulations of cells based on size (forward scatter, FSC-H) and granularity (side scatter, SSC-H) (Figure 3A and B)**.** Region (R) 1 identified the largest, most granular cells. R4 represented the smallest, least granular cells in the differentiated cell population. The macrophages in R1 and R2 had the highest expression level of c-Fms, and F4/80 macrophage markers compared to R3 and R4 (*e.g.* R1+R2 *vs*. R3+R4: c-Fms, 105.3% *vs.* 44.7%; F4/80, 6.4% *vs.* 0.1%; Table 1). Space flight did not affect these distributions (Table 1).

When we compared the distribution of cells in R1-R4 between space flight samples and ground controls we had significant differences in the expression in Ly6C, CD11b, CD31, F4/80, Mac2 and c-Fos ($p<0.05$, X^2; Table 1). There was an overall decrease in Ly6C, CD11b, and c-Fos expression on cells differentiated in flight compared to those differentiated on the ground while there was an overall increase in

CD31, F4/80 and Mac2 in flight cells compared to those differentiated on the ground (Table 1).

We anticipated that the developing cells would have a macrophage phenotype after 15 days because the bone marrow cells were differentiated in the presence of rmM-CSF (Metcalf, 1989). Therefore, we examined the cells for the concurrent expression of c-Fms and c-Fos with Mac2 and CD44, Ly6C and Ly6G (Gr-1) with F4/80 to help establish specific macrophage differentiation stages (Table 2). We found a significant increase in the overall expression of $Mac2^{+}$c-Fms^{+} cells and $Mac2^{+}$c-Fos^{+} cells differentiated in space compared to ground controls ($p<0.05$) and a significant change in the distribution between the two treatment groups ($p<0.05$, χ^2 analysis; Table 2). However, we did not see a difference in the distribution of $F4/80^{+}CD44^{+}$ cells , $F4/80^{+}Ly6C^{+}$ cells or $F4/80^{+}Gr\text{-}1^{+}$ cells (Table 2) between space-flight samples and ground controls.

Microarray analysis

To address possible mechanisms of how spaceflight affects macrophage proliferation and differentiation, we compared the transcriptional profile of bone marrow cells differentiated during space flight compared to cells differentiated on Earth. After 14 days of differentiation during space flight, the macrophages were preserved in GITC and RNA was hybridized to the Affymetrix Mouse Genome 430 2.0 array as described in the Materials and Methods. We found that 607 genes had gene transcript levels >1.5 fold higher for flight samples than ground controls. In contrast, we found that 1071 genes had gene transcript levels >1.5 fold lower than ground controls ($p<0.05$). The

genes were sorted into biological function categories using IPA software (Table 3). These included genes involved in Carbohydrate metabolism, Cellular development, Hematopoiesis, Cellullar growth and proliferation, Lipid metabolism and many other functions (Table 3).

Since the cells were stimulated with rmM-CSF, we were particularly interested in transcriptional regulation of genes that are involved in cell division and development (Assigned to the Cell Death, Hematopoiesis, Cellular Development, Cellular Growth and Proliferation categories in Table 3). Using IPA software (www.ingenuity.com), we found 607 unique genes that had significantly higher transcript levels and 1071 unique genes that had significantly lower transcript levels during space flight compared to ground controls within the Cell Death, Cellular Growth and Proliferation, Cellular Development and Hematopoiesis subsets (Table 3). These genes were further classified based on gene ontology (GO) annotations (Supplement 1). In particular, the genes with down regulated transcripts encoded enzymes (*Lfng*, LFNG O-fucosylpeptide 3-beta-N acetylglucosaminyltransferase; *Lipe*, lipase, hormone-sensitive; *Mettl8*, methyltransferase like 8; and *Adcy7* adenylate cyclase 7), growth factors (*Pgf*, placental growth factor; *Igf1*, insulin-like growth factor 1 (somatomedin C); *Angpt1*, angiopoietin 1; and *Nrg2*, neuregulin 2), Transcription factor binding (*Meox2*, mesenchyme homeobox 2; *Mylb1*, v-myb myeloblastosis viral oncogene homolog (avian)-like 1; *Nab1*, NGFI-A binding protein 1 or EGR1 binding protein 1). The genes that had significantly up regulated transcripts encoded cytokines (*Ccl5*; chemokine ligand 5), enzymes (*Ido1*, indoleamine 2,3-dioxygenase 1; *Nlgn1*, neuroligin 1; *Hs6st2*, heparin sulfate 6-O-sulfotransferase 2); G-protein coupled receptors (*Agtr1b*, angiotensin II receptor;

Bdkrb2, bradykinin receptor B2) transporters (Slc7a5, soute carrier family 7; Hba-a1, hemoglobin, alpha 1, Lrp2, low density lipoprotein receptor-related protein 2). Molecules assigned to “other" molecule function were further classified according to MGI GO assignments (Supplement 1).

Analysis of the effect of space flight on the M-CSF signaling pathway revealed that gene transcripts encoding for the proteins *Csf1r*, *Etv3*, *Fos*, *Rbl-1, Gata1, Gata2, Myc, Runx1,* and *E2f4* were downregulated greater than 1.5 fold in cells grown in space compared to ground controls. Alternatively, gene transcripts encoding proteins *Egr1, Hoxb4* and *Myb* were higher in cells grown in space compared to cells maintained on Earth (Table 4). However, there were only significant differences ($p<0.05$) in transcripts fold change for the genes *Csf1r, E2f4, Rbl1, Egr1, Hoxb4, Gata2, Myc and Runx1*.

We also classified the gene microarray data into global canonical pathways and we selected pathways which were associated with genes whose transcription was significantly affected by the space flight. Canonical pathways were ranked based on the –log(*p* value) scores ($p<0.05$). We found that the coagulation system (Table 5), Fc-gamma receptor-mediated phagocytosis in macrophages and monocytes, endoplasmic reticulum stress, and growth hormone signaling were pathways containing genes which had transcript levels that were significantly lower in flight samples compared to ground controls (1.5 fold change, $p<0.05$; Supplement 2). The coagulation system had the highest –log(*p* value) score of any canonical pathway in this analysis. There were 6 genes which had significantly lower transcript levels in spaceflight samples compared to ground controls ($p<0.05$; Table 5). In addition, 8 genes showed a trend where transcript levels were lower than ground controls even though they were not statistically different.

The insulin receptor signaling pathway also had a high –log(*p* value) score amongst canonical signaling pathways identified by the IPA analysis. Six genes had significantly lower transcript levels in comparison to ground controls ($p<0.05$; Table 6). When we examined other signaling pathways that are relevant to macrophage function (Oda et al. , 2004, Raza et al. , 2008), we found that several other macrophage signaling pathways also had genes that had lower transcript levels in space flight samples compared to ground controls ($p<0.05$). These included Fcγ receptor mediated phagocytosis in macrophages and monocytes, mTOR, CCR5 signaling, p38 map kinase, and FLT3 signaling in hematopoietic progenitor cells (Supplement 2).

Discussion

We reexamined the impact of space flight on macrophage growth and development during the space flight of STS-126 to test whether changes in the expression of the receptor for M-CSF were affected by space flight. We found that bone marrow-derived macrophages proliferated faster during space flight compared to ground controls to reaffirm previous findings (Armstrong, Gerren, 1995b) even though there were significant temperature differences between these experiments and those older experiments. The data also support observations that show that bacteria (Benoit and Klaus, 2007), plant (Matia et al. , 2010) and mammalian cells (Slentz et al. , 2001, Tobin et al. , 2001) grow faster in space or clinorotation. The increased proliferation was not associated with a concomitant increase in glucose use to mediate that growth and supports the hypothesis that cells do not have to work as hard to grow in space. However, not all cell types respond in this same manner during space flight.

Osteoblasts grew less and used less glucose than comparable ground controls on STS-56 (Hughes-Fulford and Lewis, 1996) and U937 cells depleted glucose rapidly when grown in space hardware (Hatton et al. , 1999). Therefore, glucose utilization during growth may be cell-type specific.

Our primary hypothesis was that the changes in growth and differentiation during space flight were due to decreased expression of the receptor for M-CSF (c-Fms); the growth factor used to induce the differentiation of macrophages from bone marrow stem cells. We found that the transcript level of *Csfr* was lower in space flight samples compared to ground controls. However, when we examined the level of c-Fms molecules on the surface of the cells by flow cytometry, we found an increase in the receptor. This conundrum could be explained by increased protein translation efficiency during space flight. Factors such as protein concentration affect translational efficiency (Morgan et al. , 1971), and the lack of convection in space could affect local concentrations of M-CSF as well as other cytokines. Signal transduction can also be altered by space flight (Akiyama et al. , 1999, Cogoli, 1997, De Groot et al. , 1991, Hatton, Gaubert, 1999, Nickerson et al. , 2000, Schwarzenberg, 1999) and the efficiency of protein synthesis can be altered by cytokines and growth factors and their coordinating pathways (Hornberger and Esser, 2004). However, Etheridge *et al*. found that the components that control the RNAi process that regulate translation and the inhibitory effects of RNAi were unaffected by space flight (Etheridge et al. , 2011). Therefore, questioning the efficiency of translation may not be appropriate. Alternatively, the discrepancy between the transcript level and protein level could have resulted from when we measured these processes. *Csfr* transcripts are stabilized

during macrophage differentiation (Stone et al. , 1990) and c-Fms plasma membrane levels are generally stable and dependent on the concentration of M-CSF present (Rettenmier et al. , 1987). Therefore, the macrophages in our space culture FPAs may have had more c-Fms molecules present because there was less rmM-CSF remaining in those FPAs. Unfortunately, we did not assay for M-CSF in the cultures from this experiment. We found very low levels of all the cytokines we did perform assays for (GM-CSF, IL-1, TNF, IL-6, data not shown). By 15 days of culture, the supernatants were generally devoid of labile cytokines. We also did not have enough RNA left over after the gene array to validate the *Csfr* transcript concentrations. We note, however, that *Runx1* transcript levels were also significantly lower in the space-flown cells. There is a strong correlation in expression between *Csfr* and *Runx1*, (Himes et al. , 2005) which helps to increase our confidence in these data. Nevertheless, additional work will be needed to resolve this issue.

The increased number $Mac2^{+}c\text{-}Fms^{+}$ cells and the generally increased expression of F4/80 on the macrophages differentiated in space suggests that there is a more differentiated population of cells present after space flight. F4/80 (Caminschi et al. , 2001, Hume et al. , 2002) and Mac2 (Dong and Hughes, 1997, Ho and Springer, 1982) are macrophage-specific markers that tend to increase as macrophages mature (Leenen et al. , 1994, Leenen et al. , 1990). A decrease in Ly6C in the total, R1 and R2 populations in the cells we identified would also support this conclusion since Ly6C identifies myeloid cells at an intermediate stages of differentiation (Leenen, de Bruijn, 1994). However, other data related to the differentiation state of the macrophages in our space cultures were not consistent with this conclusion. We did not see a decrease

in the Ly6G or CD31 expression on the macrophages flown in space. Ly6G is expressed on macrophages early in differentiation, granulocytes and other cells (Ammon et al. , 2000, Ferret-Bernard et al. , 2004, Leenen, Melis, 1990) and CD31 is expressed on macrophages early in differentiation and decreases as they mature (de Bruijn et al. , 1994, de Bruijn et al. , 1998, Watt et al. , 1993). We also did not see an increase in CD11b or in the F4/80^{+}Ly6C^{-} or the F4/80^{+}Ly6G^{-} populations. These latter two populations should have increased as macrophages became more differentiated. Therefore, it might be more appropriate to say that the macrophages that develop in space are different from those that develop on the Earth even though we can not necessarily characterize them as more or less differentiated from each other. This conclusion would not contradict data from STS-57, STS-60 and STS-62 (Armstrong, Gerren, 1995b). Moreover, when we assessed the relative levels of transcripts of transcription factors (*Sfpi1*, *Egr1*, *Myc*, *Stat3*, *Tnf*, *Hoxb7*, *Cebpb*, *Runx1*, *Chrac1*, *Egr1*, *Irf1*, *Jun*, and *Fos*) that are necessary for the differentiation of M-CFU into macrophages (Valledor et al. , 1998), we found that most of these were not significantly up or down regulated (>1.5 fold) in the space-flight samples compared to ground controls. This suggests that space flight was not affecting the differentiation of the macrophages.

There were significant changes in transcript levels of 1678 genes in macrophages that develop in space compared to those that develop on Earth. These data suggest that space flight has an effect on M-CSF-stimulated macrophages *in vitro*. The array of gene transcripts that were altered suggests that there were global impacts on the cells; not just on specific molecular signaling pathways. Nevertheless, when we

examined pathways particularly relevant to bone marrow macrophage growth and differentiation, *i.e.* the M-CSF signaling pathway, there were transcript changes that were consistent with increased cellular proliferation. For example, the down regulation of *Rbl-1* (encodes p107) in the M-CSF pathway in the flight cells is consistent with the increased proliferation of myeloid cells in p107 knock-out mice (LeCouter et al. , 1998). Similarly, the significant down regulation of *E2f4* would also be consistent with increased proliferation. *E2f4* regulates the cell cycle and inhibits cell proliferation (Attwooll et al. , 2004). Mutations in *E2f4* lead to hematological cancers (Komatsu et al. , 2000) because of its role in regulating cell fate (Enos et al. , 2008). Furthermore, if one examines the transcript levels of transcription factors that are activated during the differentiation of stem cells into M-CFU macrophage progenitors (Valledor, Borras, 1998), only *Hoxb3* and *Hoxb4* were up regulated and only *Hoxb4* was significant (>1.5 fold increase; $p<0.05$). Hoxb3 and Hoxb4 are necessary for myeloid cell proliferation (Bjornsson et al. , 2003, Sauvageau et al. , 1997) but are not needed for lineage commitment (Bjornsson, Larsson, 2003). In contrast, two of the three transcription factors that are significantly down regulated during early macrophage differentiation in space flight samples (*Gata2* and *Runx1*) are either not needed for macrophage terminal differentiation (*Gata2*) (Tsai and Orkin, 1997) or serve as an inhibitor of proliferation (*Runx1*) (Himes, Cronau, 2005). Interestingly, *Myc* was also down regulated and macrophage proliferation is also driven by c-Myc protein (Wickstrom et al. , 1988, Yu et al. , 2005). This inconsistency might be because c-Myc is needed early in macrophage proliferative response during differentiation (Valledor, Borras, 1998) and we were already 15 days into the differentiation process. This hypothesis is supported by data

showing that transcripts of other key inducers of proliferation such as cyclophilin A (*Ppia*) and cyclin-dependent kinase (*Cdk2*) also trended lower in space-flight samples (*Ppia*, -1.11 fold change; *Cdk2*, -1.26 fold change) and cyclin-dependent kinase inhibitor (*Cdkn1a*), an inhibitor of cellular proliferation trended with higher transcript levels in space-flight cells (*Cdkn2a*, -1.32 fold change). It appears that the proliferative phase was in the process of changing by the 15th day of culture.

The most interesting revelation of the IPA analysis of the transcriptional array of bone marrow macrophage differentiation in space was the broad impact of space flight on the coagulation pathway. Sixteen genes were down regulated *in toto*. Genes encoding proteins involved in both the intrinsic and extrinsic pathways were affected; indicating a broad impact. Kimzey *et al.* suggested that there may be a "hypercoagulative condition" after the flight of the Skylab astronauts (Kimzey et al. , 1975b). However, after closer examination of the data from Skylab missions (Kimzey, 1977, Kimzey et al. , 1975a, Kimzey et al. , 1976, Kimzey, Ritzmann, 1975b), it appears that this hypothesis was based on observations of platelets and not coagulation-pathway proteins. If there are alterations in the ability of space travelers to coagulate blood, this could have ramifications on astronaut recovery from injury from bleeding, angiogenesis and inflammation. For example, fragments of plasminogen (encoded by *Plg*), which had significantly lower transcript concentrations in flight samples compared to ground controls, inhibit angiogenesis (O'Reilly et al. , 1994) and are involved in regulating macrophage migration (Gong et al. , 2008). Additional examination of these systems *in vivo* is justified.

Conclusion

We have confirmed that space flight has a significant impact on murine bone marrow macrophages *in vitro*. We see significant increases in cell proliferation and changes in the pattern of expression of cell-surface differentiation antigens. Differences in gene expression in 1,678 genes in the differentiating macrophages during space flight are consistent with this observation. Importantly, these changes do not appear to be from decreases in the surface expression of the receptor for M-CSF. We recently found that there are changes in bone marrow subpopulations in mice after they are subjected to space flight (Ortega, Pecaut, 2009). The data from STS-126 indicates that there can also be direct gravitational effects on those bone marrow cells. The long-term effects of these changes have yet to be determined and should be the focus of appropriate studies on the International Space Station.

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Figure Legends

Figure 1. A. Time-line of activities for bone marrow macrophage differentiation on STS-126. B. Fluid Processing Apparatus (FPA). 3.0 ml of cells were placed in a chamber 1 separated by two rubber septa. The FPA is engineered with a bypass so that when the internal assembly of septa and biological samples are pushed to the left the medium in the second chamber will mix with the material in chamber 1 and septa 2 and 3 will compress. Septa 3 and 4 also compress with additional movement to mix the contents of chamber 3 with the contents previously mixed.

Figure 2. Analyses of data from microarray. (A) The box plot of gene expression Flight and Ground control groups. (B) Principal component analysis of Flight and Ground treatments. (C) Volcano plot of Flight sample transcript levels that are significantly different from Ground control samples. Dark dots represent genes that have >1.5 fold-change (x-axis) as well as high statistical significance cut off with a *p*-value < 0.05 (y-axis). The gray dots represent genes that are not significantly different.

Figure 3. Flow cytometric analysis of Flight (left column) and Ground control (right column) samples. (A and B) Representative plots of forward *vs*. side scatter to establish Regions 1-4 for further analysis. Histograms of total cells (C and D), Region 1 (E and F), Region 2 (G and H), Region 3 (I and J), Region 4 (K and L) for c-Fms expression.

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Table

Table 1. Effect of spaceflight on M-CSF differentiated bone marrow-derived cell phenotypic markers.

Cell marker	Subpopulation	Spaceflight	Ground control
Ly6C*	Total cells	5.8[a]	9.2
	R1	20.0	24.7
	R2	2.9	4.5
	R3	0.5	7.1
	R4	1.1	4.7
c-Fms	Total cells	34.8	30.3
	R1	77.4	67.9
	R2	56.2	37.4
	R3	45.0	35.3
	R4	10.5	9.4
CD11b*	Total cells	0.1	6.7
	R1	1.2	1.4
	R2	0	7.8
	R3	0	57.3
	R4	0	49.9
CD44	Total cells	45.4	45.7
	R1	62.7	67.0
	R2	23.9	31.8
	R3	16.3	16.8
	R4	6.0	3.4
CD31 (PECAM)*	Total cells	4.8	3.2
	R1	3.8	2.8
	R2	14.2	4.1
	R3	19.0	4.5
	R4	13.3	15.1
Ly6G (Gr-1)	Total cells	32.6	30.0
	R1	39.7	41.0
	R2	11.5	8.4
	R3	6.6	8.9
	R4	4.3	8.4
CD3	Total cells	0.4	0
	R1	0.1	0
	R2	2.2	0
	R3	0.8	0
	R4	1.5	0
F4/80*	Total cells	5.0	2.7
	R1	11.6	3.7
	R2	10.4	5.2
	R3	1.4	0.1
	R4	1.0	0.0
Mac2*	Total cells	5.5	4.5
	R1	8.5	0.5
	R2	8.6	0
	R3	6.2	0.4
	R4	4.7	0.6
c-Fos*	Total cells	2.5	3.3
	R1	3.7	4.0
	R2	8.0	3.6

R3	10.6	0
R4	7.7	0

Numbers indicate % of cells above isotype control staining; 20,000 cells analyzed per sample. R1, region 1; R2, region 2; R3 region 3; R4, region 4. * Indicates flight sample is distributed differently from ground control as assessed by χ^2 analysis.

Table

Table 2. Effect of spaceflight on expression of double positive cell surface markers of differentiated bone marrow derived cells.

Cell marker	Subpopulation [a]	Spaceflight [b]	Ground control [b]
$Mac2^+$, c-Fms^+ [c]	Total cells	15.9	1.7
	R1	12.7	3.6
	R2	27.0	0
	R3	19.3	1.3
	R4	12.3	1.3
$Mac2^+$, c-Fos^+ [c]	Total cells	6.8	0
	R1	8.4	0.1
	R2	24.9	0
	R3	13.4	0
	R4	1.5	0
$F4/80^+$, $CD44^+$	Total cells	3.1	3.2
	R1	5.8	5.7
	R2	3.8	0
	R3	0.5	1.3
	R4	0.7	0.1
$F4/80^+$, $Ly6C^+$	Total cells	0.3	0.1
	R1	0.4	5.0
	R2	0.5	1.4
	R3	0.2	0.5
	R4	0.1	0.3
$F4/80^+$, $Gr1^+$	Total cells	0.5	0
	R1	0.8	3.2
	R2	0.4	0.9
	R3	0.1	0.1
	R4	0	0

[a] Subpopulations established with forward *vs*. side scatter dot plots. R1, region 1; R2, region 2; R3 region 3; R4, region 4

[b] Numbers indicate % cells above isotype control staining; 20,000 cells analyzed per sample.

[c] Indicates flight sample is distributed differently from ground control as assessed by χ^2 analysis.

Table

Table 3. Biologic function classification of bone marrow derived macrophages gene regulation due to spaceflight

Biological function categories[a]	Lower p-value[b] ($\times 10^{-6}$)	Upper p-value[b] ($\times 10^{-6}$)	Number of genes	Number of genes upregulated[c]	Number of genes downregulated[c]
Inflammatory Response	0.34	46300	100	32	68
Carbohydrate Metabolism	46.8	44300	44	6	38
Molecular Transport	56.7	44300	82	19	63
Small Molecule Biochemistry	56.7	44300	102	21	81
Cell Death	64.5	47300	81	21	60
Hematological System Development and Function	87.9	47700	118	38	80
Hematopoiesis	87.9	47900	76	23	53
Organismal Development	87.9	43200	72	22	50
Tissue Development	87.9	46300	108	37	71
Cellular Compromise	212	46100	43	14	29
Cardiovascular System Development and Function	239	43200	79	25	54
Cellular Development	245	47900	141	39	103
Cellular Growth and Proliferation	378	47300	137	45	92
Humoral Immune Response	378	39600	43	11	32
Organismal Survival	385	7710	56	18	38
Developmental Disorder	591	21300	17	1	16
Genetic Disorder	591	21300	34	8	26
Metabolic Disease	591	23200	16	1	15
Cell-To-Cell Signaling and Interaction	654	46100	60	17	43
Gastrointestinal Disease	671	28000	20	6	14
Inflammatory Disease	671	35700	17	7	10
Cellular Movement	701	47700	96	27	69

Immune Cell Trafficking	701	47700	65	18	47
Embryonic Development	824	37600	39	9	30
Lipid Metabolism	824	44300	72	12	60
Cell Cycle	949	42500	23	8	15
Antimicrobial Response	959	21400	12	3	9
Hematological Disease	1170	34800	32	11	21
Organ Development	1240	39600	46	14	32
Cellular Assembly and Organization	1410	42500	66	22	44
Cellular Function and Maintenance	1410	47800	95	25	70
Tissue Morphology	1850	39600	110	36	74
Organismal Injury and Abnormalities	2000	44300	35	9	26
Antigen Presentation	2260	46300	36	9	27
Cell-mediated Immune Response	2260	21800	24	6	18
Lymphoid Tissue Structure and Development	2260	46300	54	10	44
Cell Morphology	3960	35700	33	7	26
Connective Tissue Development and Function	3960	39600	41	10	31
Connective Tissue Disorders	3960	34800	6	0	6
Digestive System Development and Function	3960	11400	4	2	2
Drug Metabolism	3960	34800	9	2	7
Endocrine System Development and Function	3960	11400	4	2	2
Hepatic System Development and Function	3960	11400	7	2	5
Hypersensitivity Response	3960	41800	7	2	5
Nervous System Development and Function	3960	44300	23	8	15
Neurological Disease	3960	28700	11	4	7

Nucleic Acid Metabolism	3960	34800	4	1	3
Organ Morphology	3960	44300	40	10	30
Skeletal and Muscular Disorders	3960	21800	14	0	14
Skeletal and Muscular System Development and Function	3960	34800	34	14	20
Cancer	4310	47900	68	20	48
Respiratory Disease	4770	44300	12	4	8
Visual System Development and Function	5140	39600	12	2	10
Free Radical Scavenging	7060	46300	19	5	14
Dermatological Diseases and Conditions	7190	43900	21	8	13
Immunological Disease	7190	34800	23	7	16
Reproductive System Development and Function	7780	44300	18	7	11
Cardiovascular Disease	8570	47900	33	8	25
Endocrine System Disorders	9400	47900	14	1	13
Organismal Functions	10000	32900	7	2	5
Hair and Skin Development and Function	11400	11400	2	1	1
Hepatic System Disease	11400	11400	2	1	1
Reproductive System Disease	11400	28000	9	1	8
Infectious Disease	12300	35700	22	6	16
Protein Synthesis	12400	21800	18	2	16
Cell Signaling	15700	33500	10	1	9
Amino Acid Metabolism	21400	33700	11	4	7
Gene Expression	21500	21500	54	24	30
Auditory Disease	21800	28700	7	3	4
Ophthalmic Disease	21800	21800	2	0	2
Protein Trafficking	21800	21800	2	1	1

Nutritional Disease	33500	33500	9	2	7
Post-Translational Modification	33500	33500	9	1	8
Behavior	33700	33700	6	1	5
DNA Replication, Recombination, and Repair	34800	42500	9	6	3
Renal and Urological System Development and Function	34800	34800	2	0	2
Vitamin and Mineral Metabolism	34800	34800	2	0	2

[a] Total number of unique genes with transcript levels at significantly higher concentration than ground control samples (FDR value at 0.05 and 1.5 fold change). The input for the IPA analysis was upregulated genes 607 and downregulated genes 1071 genes.
[b] Gene expression data and IPA analysis was subjected to FDR (Benjamini-Holchberg) correction. P-value ranges reflect the chance genes have been randomly assigned to a specific biological function category using IPA software.
[c] Genes may have been assigned to more than one category based on biological function classification. The unique upregulated genes were 130 and downregulated genes were 267 which were classified in the biological functional categories.

Table

Table 4. M-CSF pathway of bone marrow derived macrophages gene regulation due to spaceflight

Gene symbol	Entrez gene ID	Gene name	*p*-value[a]	Fold change
Csf1r	12978	Colony stimulating factor 1 receptor	0.04*	-1.6
Hras1	15461	Harvey rat sarcoma virus oncogene 1	0.24	-1.3
Ets1	23871	E26 avian leukemia oncogene 1, 5' domain	0.59	1.3
Ets2	23872	E26 avian leukemia oncogene 2, 3' domain	0.79	1.1
Etv3	27049	ETS-domain transcriptional repressor, METS, Pe1	0.32	-2.0
Ddx20	53975	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20, Dp103.	0.83	-1.1
Jun	16476	Jun oncogene	0.62	1.3
Fos	14281	FBJ osteosarcoma oncogene	0.14	-1.9
Sin3	20467	Transcriptional regulator, SIN3B (yeast)	0.89	-1.0
Hdac2	15182	Histone deacetylase 2	0.92	1.0
Ncord2	20602	Nuclear receptor co-repressor 2	0.37	-1.2
E2f4	104394	E2F transcription factor 4	0.04*	-1.6
Rbl-1	19650	Retinoblastoma-like 1 (p107)	0.02*	-4.4
Rbl-2	19651	Retinoblastoma-like 2	0.35	-1.4
Hoxb7	15415	Homeobox B7	0.08	1.1
Egr1	13653	Early growth response 1	0.03	2.1
Irf1	16362	Interferon regulatory factor 1	0.79	-1.0
Chrac1	93696	Chromatin accessibility complex 1	0.50	-1.1
Hoxb4	15412	Homeobox B4	0.01	1.7
Hoxb3	15410	homeobox B3	0.03	1.2
Cebpa	12606	CCAAT/enhancer binding protein (C/EBP), alpha	0.10	-1.0
Cebpb	12608	CCAAT/enhancer binding protein (C/EBP), beta	0.09	1.1
Cebpg	12611	CCAAT/enhancer binding protein (C/EBP), gamma	0.69	-1.1
Gata1	14460	GATA binding protein 1	0.37	-1.5
Gata2	14461	GATA binding protein 2	0.04*	-2.1
Scly	50880	Selenocysteine lyase	0.38	1.1
Myb	17863	Myeloblastosis oncogene	0.19	1.8
Myc	17869	Myelocytomatosis oncogene	0.05	-1.7
Runx1	12394	Runt related transcription factor 1	0.04*	-1.6
Tnf	21926	Tumor necrosis factor	0.66	-1.1
Stat3	20848	signal transducer and activator of transcription 3	0.64	-1.1

Sfpi1	20375	SFFV proviral integration 1	0.56	1.1
Zbtb16	235320	Zinc finger and BTB domain containing 16	0.21	1.2

[a] Gene expression data and IPA analysis was subjected to FDR (Benjamini-Holchberg) correction with a cut-off value at 0.05. * indicates genes below the cut off.

Table

Table 5. Transcriptional changes in coagulation system components

Fold change	*p*-value[a]	Coagulation components and regulators[b]
-1.1	NS	Factor II, Prothrombin: C
-1.8	0.04	Factor III, Thromboplastin (Tissue Factor): E
1.8	NS	Factor V, Labile factor (accelerator globulin, accelerin): C
-1.9	NS	Factor VII, Serum (or tissue) prothrombin: E
-2.5	NS	Factor VIII, Anti-hemophilic factor A: I
-2.7	0.04	Factor IX, Christmas factor:
-1.5	NS	Factor X, Stuart Power factor: C
1.5	NS	Factor XI, Plasma thromboplastin antecedent (PTA): I
-4.6	NS	Factor XII, Hageman factor (contact factor): I
-1.8	NS	Factor XIII, Fibrin-stabilizing factor: C
-2.9	NS	Protein C: I and C
-2.3	0.04	Protein S: I and C
-2.1	NS	Thrombomodulin: I and C
-5.0	0.04	Kallekrein B1: I
-2.4	0.04	Tissue factor pathway inhibitor: E
-6.5	0.04	Plasmin: F
-1.5	NS	Von Willebrand factor: I and E
-1.5	NS	Plasminogen activator, urokinase: F

[a] Gene expression data and IPA analysis was subjected to FDR (Benjamini-Holchberg) correction. No significant difference (NS), FDR value at 0.05.
[b] Components involved in intrinsic (I), extrinsic (E), or common (C) coagulation pathways or in fibrinolysis (F).

Table

Table 6. Insulin receptor signaling pathway of bone marrow derived macrophages gene regulation due to spaceflight.

Gene symbol	Entrez gene ID	Gene name	*p-value*	Fold change
Insr	16337	insulin receptor	0.044*	-2.01
Cbl	12402	Casitas B-lineage lymphoma	0.008*	-1.90
Rhoq	104215	ras homolog gene family, member Q	0.008	-1.35
Tsc2	22084	tuberous sclerosis 2	0.044*	-2.50
Rapgef1	107746	Rap guanine nucleotide exchange factor (GEF) 1	0.045*	-1.61
Pten	19211	phosphatase and tensin homolog	0.045*	-1.83
Lipe	16890	lipase, hormone sensitive	0.041*	-3.61
Nck1	17973	non-catalytic region of tyrosine kinase adaptor protein 1	0.048*	-1.70
Gab1	14388	growth factor receptor bound protein 2-associated protein 1	0.048*	-2.53
Ptpn11	19247	protein tyrosine phosphatase, non-receptor type 11	0.076	-1.44
Foxo4	54601	forkhead box O4	0.101	-3.22
Sgk1	20393	serum/glucocorticoid regulated kinase 1	0.127	-1.99
Socs3	12702	suppressor of cytokine signaling 3	0.138	2.41
Stx4a	20909	syntaxin 4A (placental)	0.139	1.09
Mtor	56717	mechanistic target of rapamycin (serine/threonine kinase)	0.155	-1.31
Rptor	74370	regulatory associated protein of MTOR, complex 1	0.174	-1.66
Eif4ebp1	13685	eukaryotic translation initiation factor 4E binding protein 1	0.185	-1.25
Raf1	110157	v-raf-leukemia viral oncogene 1	0.191	1.67
Irs1	16367	insulin receptor substrate 1	0.198	-1.55

Mapk8	26419	mitogen-activated protein kinase 8	0.257	-1.47
Ptpn1	19246	protein tyrosine phosphatase, non-receptor type 1	0.289	-1.26
Foxo3	56484	forkhead box O3	0.298	1.28
Ptprf	19268	protein tyrosine phosphatase, receptor type, F	0.340	2.08
Pde3b	18576	phosphodiesterase 3B, cGMP-inhibited	0.357	1.32
Stxbp4	20913	syntaxin binding protein 4	0.409	-1.59
Slc2a4	20528	solute carrier family 2 (facilitated glucose transporter), member 4	0.445	1.81
Vamp2	22318	vesicle-associated membrane protein 2	0.450	1.24
Tsc1	64930	tuberous sclerosis 1	0.461	-1.43
Pdpk1	18607	3-phosphoinositide dependent protein kinase 1	0.520	-1.30
Eif4e	13684	eukaryotic translation initiation factor 4E	0.555	-1.33
Bad	12015	BCL2-associated agonist of cell death	0.558	1.20
Grb2	14784	growth factor receptor bound protein 2	0.672	-1.10
Fyn	14360	Fyn proto-oncogene	0.704	-1.13
Shc1	20416	src homology 2 domain-containing transforming protein C1	0.756	1.15
Acly	104112	ATP citrate lyase	0.889	-1.04
Grb10	14783	growth factor receptor bound protein 10	0.917	-1.08
Trip10	106628	thyroid hormone receptor interactor 10	0.960	1.02
Akt1	11651	thymoma viral proto-oncogene 1	0.432	-1.64
Mapk1	26413	mitogen-activated protein kinase 1	0.422	-1.65
Foxo1	56458	forkhead box O1	0.381	-1.15

Gsk3b	56637	glycogen synthase kinase 3 beta	0.105	-1.57
Gys1	14936	glycogen synthase 1, muscle	0.834	1.13
Irs1	16367	insulin receptor substrate 1	0.198	-1.54
Jak1	16451	Janus kinase 1	0.075	-1.62
Map2k1	26395	mitogen-activated protein kinase kinase 1	0.236	-1.75
Rps6kb1	72508	ribosomal protein S6 kinase, polypeptide 1	0.220	-1.72
Pik3r1	18708	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	0.051	-1.84
Prkaca	18747	protein kinase, cAMP dependent, catalytic, alpha	0.657	1.05
Prkcz	18762	protein kinase C, zeta	0.772	-1.22
Ppp1cc	19047	protein phosphatase 1, catalytic subunit, gamma isoform	0.096	-1.15
Rasa1	218397	RAS p21 protein activator 1	0.239	-1.57
Inpp5d	16331	inositol polyphosphate-5-phosphatase D	0.149	-1.27
Kcnj8	16523	potassium inwardly-rectifying channel, subfamily J, member 8	0.298	1.36

[a] Gene expression data and IPA analysis were subjected to FDR (Benjamini-Holchberg) correction. * indicates genes below the 0.05 cut off.

Figure

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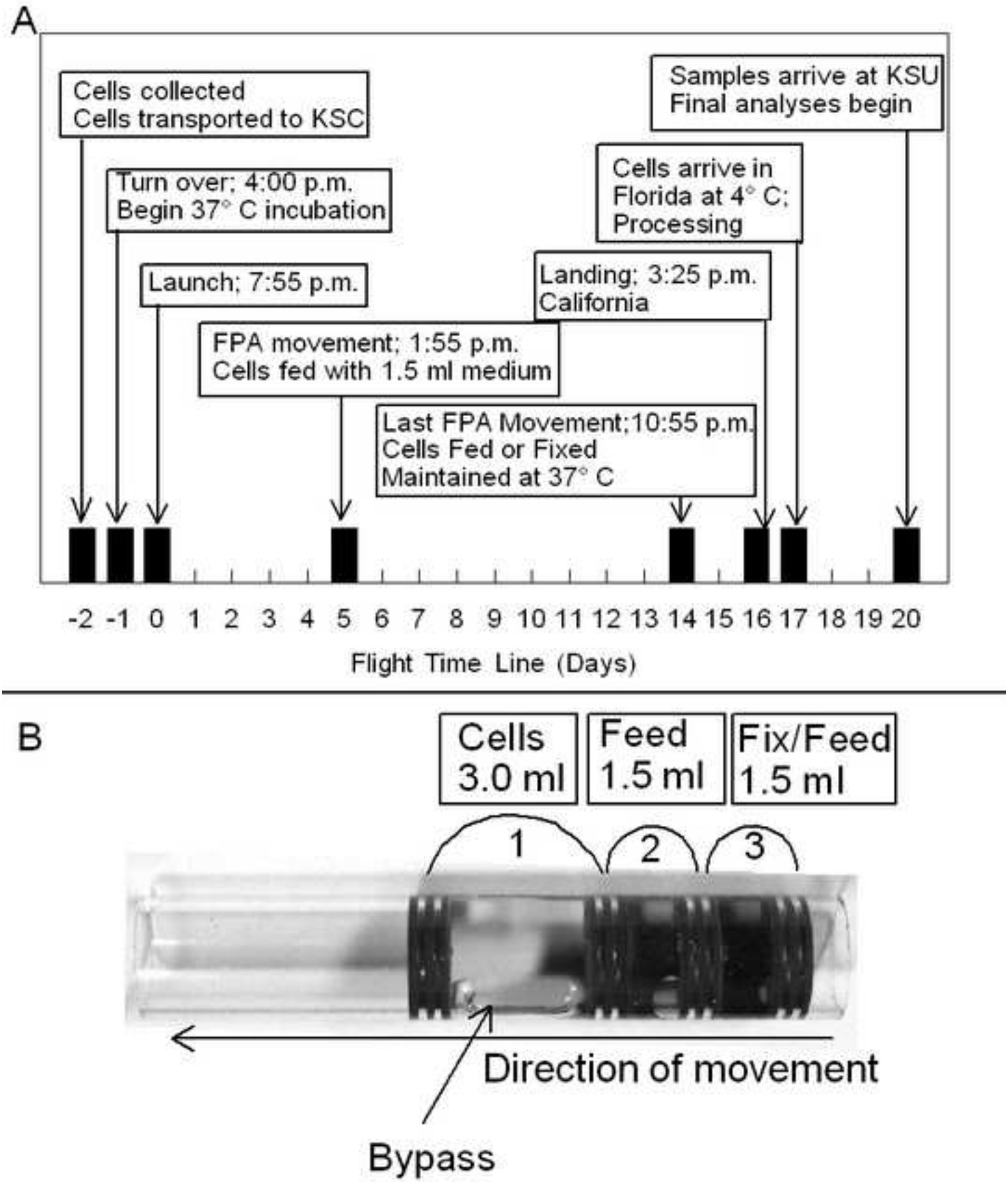

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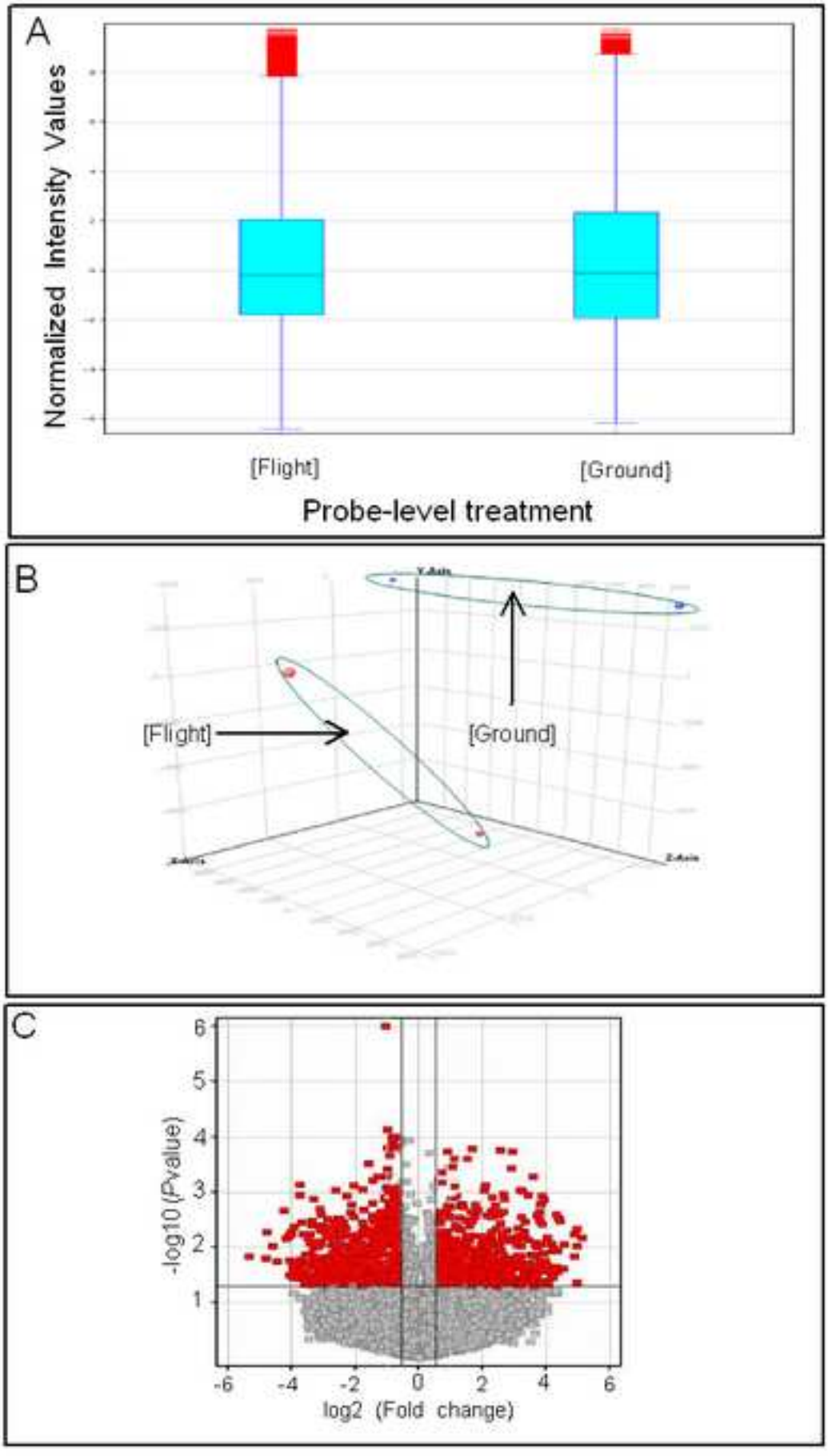

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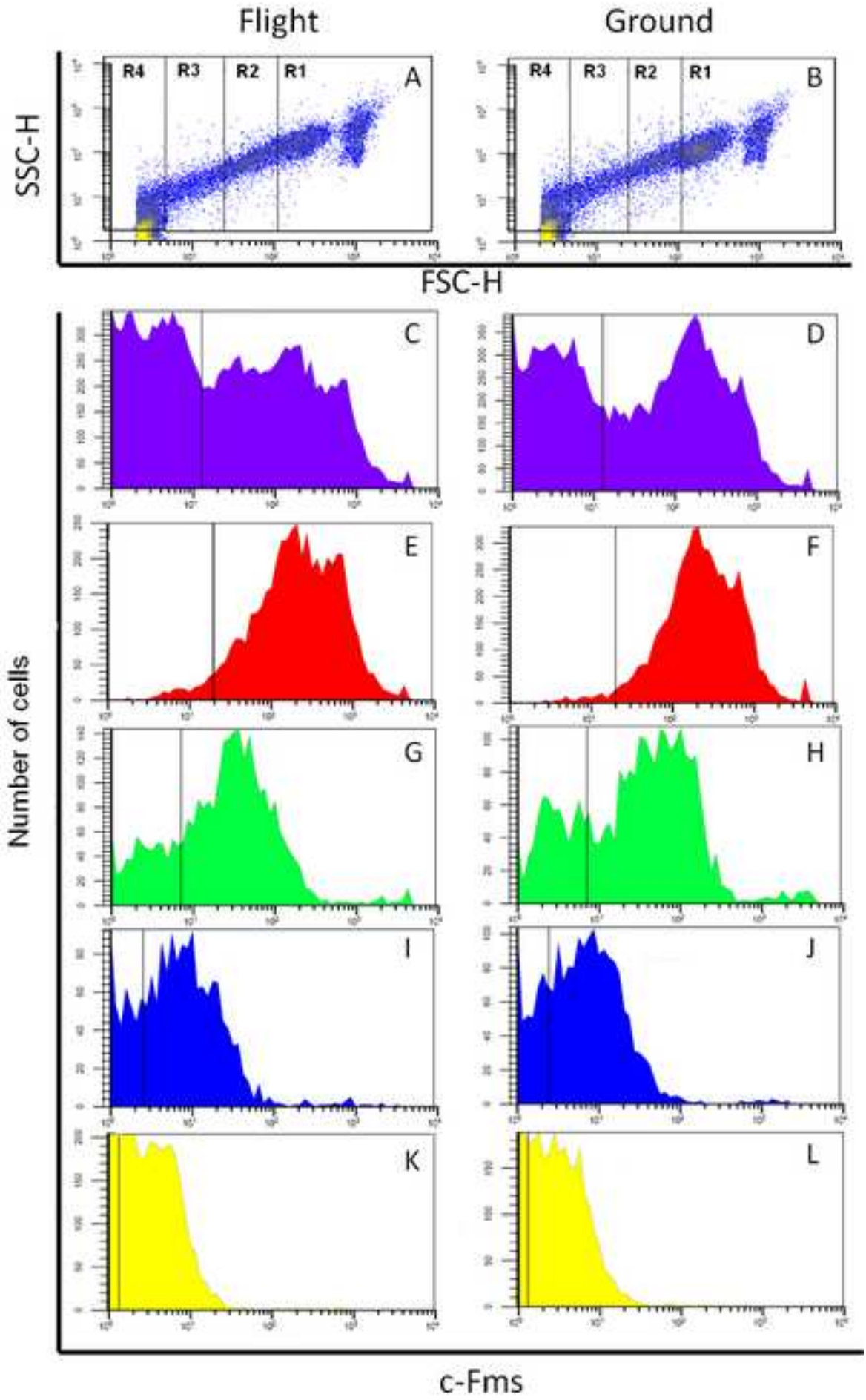

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