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Microarray Analysis of Spaceflown Murine Thymus Tissue Reveals Changes in Gene Expression Regulating Stress and Glucocorticoid Receptors

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ABSTRACT

The detrimental effects of spaceflight and simulated microgravity on the immune system have been extensively documented. We report here microarray gene expression analysis, in concert with quantitative RT-PCR, in young adult C57BL/6NTac mice at 8 weeks of age after exposure to spaceflight aboard the space shuttle (STS-118) for a period of 13 days. Upon conclusion of the mission, thymus lobes were extracted from space flown mice (FLT) as well as age- and sex-matched ground control mice similarly housed in animal enclosure modules (AEM). mRNA was extracted and an automated array analysis for gene expression was performed. Examination of the microarray data revealed 970 individual probes that had a 1.5-fold or greater change. When these data were averaged ($n = 4$), we identified 12 genes that were significantly up- or down-regulated by at least 1.5-fold after spaceflight ($P \leq 0.05$). The genes that significantly differed from the AEM controls and that were also confirmed via QRT-PCR were as follows: *Rbm3* (up-regulated) and *Hsph110*, *Hsp90aa1*, *Cxcl10*, *Stip1*, *Fkbp4* (down-regulated). QRT-PCR confirmed the microarray results and demonstrated additional gene expression alteration in other T cell related genes, including: *Ctla-4*, *IFN-α2a* (up-regulated) and *CD44* (down-regulated). Together, these data demonstrate that spaceflight induces significant changes in the thymic mRNA expression of genes that regulate stress, glucocorticoid receptor metabolism, and T cell signaling activity. These data explain, in part, the reported systemic compromise of the immune system after exposure to the microgravity of space. J. Cell. Biochem. 110: 372–381, 2010.

KEY WORDS: THYMUS; MICROGRAVITY; SPACEFLIGHT; GENE EXPRESSION; MICROARRAY; T CELLS; STRESS

Spaceflight has been reported to affect immune function in mammals [Sonnenfeld et al., 1990, 1992; Nash and Mastro, 1992; Konstantinova et al., 1993; Lesnyak et al., 1993; Taylor, 1993; Grove et al., 1995; Chapes et al., 1999; Crucian et al., 2008]. Space travel also has been shown to cause significant immunological effects due to factors such as radiation and increased levels of stress-hormones, both of which are known to lower immune responsiveness as well as to destroy sensitive immune cells [Tipton et al., 1996; Stowe et al., 2001; Fong, 2004]. Such deficiencies in immune function have also been reported to diminish the responsiveness of lymphoid cells to appropriate stimuli, and to decrease cellular interactions among immune cells resulting in the loss of regulatory events [Grove et al., 1995; Taylor et al., 1997; Cooper et al., 2001; Sonnenfeld, 2005]. Previous studies documented a decrease in

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immune function expressed as subnormal cell activation and proliferation [Sonnenfeld et al., 1992; Konstantinova et al., 1993; Crucian et al., 2008]. Decreases in the content and phytohemagglutinin reactivity of T cells, T cell cytotoxicity, and T cell helper activity have also been similarly reported [Lesnyak et al., 1993; Cooper and Pellis, 1998; Chapes et al., 1999]. This spectrum of immunological changes have also been confirmed in ground-based simulations of microgravity [Armstrong et al., 1993; Chapes et al., 1993; Cooper et al., 2001; Woods et al., 2003, 2005; Boonyaratanakornkit et al., 2005; Ward et al., 2006; Wang et al., 2009].

We have previously examined T cell development and immune interaction using both in vivo as well as in vitro models [Woods et al., 2003, 2005; Lebsack et al., 2005]. In order to examine how microgravity exposure affects the thymus and T cell development we designed and tested the efficacy of a microgravity organ culture dish system (MOCDS) that can support thymic cell culture in a microgravity environment [Woods et al., 2003, 2005]. Our previous work using a clinostat, to produce a vector-averaged simulation of microgravity, suggests that T cell development is significantly hindered by microgravity. We found that T cells were blocked at the immature single positive stage (ISP). CD44/CD25 analysis of the triple negative (TN) lymphocyte population, after 3 days of clinorotation, showed that this microgravitational-environment-induced arrest occurred after the CD4−CD8− double negative (DN) stage but just before the CD4+CD8+ double positive (DP) stage. We also found that addition of anti-CD3ε-specific monoclonal antibody to clinorotated cells rescued development to the $CD3^-$ DP cell stage [Woods et al., 2005]. These results support the idea that exposure to microgravity results in a signal dampening effect on developing thymocytes, which prevents successful β-selection, and thus the normal production of DP T cells. With these data in mind, the question arises as to the cause of this signal dampening effect, which ultimately results in the loss of DP T cells.

Microarray studies have been used by others to examine changes in gene expression in space flown tissues such as liver, T cells, osteoblasts, renal cells, and muscle cells [Hammond et al., 2000; Allen et al., 2009; Baqai et al., 2009; Capulli et al., 2009]. For example, a cDNA microarray of leukemic T cells flown in space for 24 h revealed an 11.7-fold increase of a tumor suppressor gene [Lewis et al., 2001]. The potential benefit of discovering significant changes in previously considered or unconsidered genes makes microarray analysis a valuable tool in such research.

We report here the first use of microarray technology to investigate gene expression in thymus tissue of space-flown mice. The focus of this study was to identify changes in gene expression in the thymus, specifically those genes that had the potential to influence phenotype and development.

MATERIALS AND METHODS

MICE, HOUSING, AND SAMPLE COLLECTION

C57BL/6NTac female mice (n = 36; Taconic Farms, Inc., Germantown, NY) were shipped directly to the National Aeronautics and Space Administration (NASA) Space Life Sciences Laboratory (SLSL) at Kennedy Space Center at approximately 7 weeks of age. Animal enclosure modules (AEM) with food bars and water were used to house flight mice (FLT, n = 12) and ground controls (AEM, n = 12). Mice at 8 weeks of age were acclimated to the enclosures for a period of 1 week prior to spaceflight. The FLT mice were flown on board the Space Shuttle Endeavour (STS-118) for 13 days. Average FLT temperature for 13 days aboard the shuttle was 81.5°F (±0.47) and the average AEM ground control temperature was 82.4°F (±0.12). Mice were euthanized with 100% CO_2 within 3–5 h after landing. Organs, including spleen and thymus, were prepared at the SLSL and shipped frozen on dry ice to Loma Linda University (LLU) where a portion of the thymus lobes were then shipped frozen at −80°C to our lab at The University of Arizona.

RNA ISOLATION

Total RNA from thymus tissue was isolated using Qiagen Rneasy© reagents and protocols, including the tissue disruption and homogenization involving the use of a mortar and pestle in liquid nitrogen (LN2) and spinning with Qiagen QIAshredder© tubes. Quality and purity of RNA preparations were assessed by electrophoresis and via spectrophotometric determination of the ratio of absorbance at 260/280 nm using the ND-1000 spectrophometer (NanoDrop Tech.). Isolated RNA samples were labeled and placed in −80°C until they were delivered to the University of Arizona Genomics Core Microarray facility for quality control and microarray analysis, as well as preparation of cDNA for RT-PCR.

MICROARRAY STUDIES

After RNA extraction and isolation, the samples were kept separate and not pooled. Quality of each potential sample was assessed by the University of Arizona Genomics Core Microarray facility in order to determine RNA integrity and quantization prior to selecting the optimal samples for microarray analysis. The Affymetrix GeneChip® Mouse Gene 1.0 ST Array (The Affymetrix GeneChip® Mouse Gene 1.0 ST Array analyzes over 20,000 different genes) analysis was performed by Genomics Core Microarray using the qualified RNA from 4 spaceflown and 4 ground control samples (n = 4). Microarray data were analyzed at The University of Arizona Genomics Shared Service (GSS). Raw microarray data are publicly available within the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database under the following accession numbers: GSM458594, GSM458595, GSM458596, GSM458597, GSM458598, GSM458599, GSM458600, and GSM458601.

GENE EXPRESSION PROFILES

Selected gene lists were uploaded to the database website along with their respective *Z* score. In order to be selected for the final gene list, the expression value of a particular gene had to be at least 1.5 times up- or down-regulated from the control (AEM). Differences were considered statistically significant only if they had an adjusted *P*-value ≤ 0.05. Using distance based gene selection, gene expression profiles were created and a heat map and database were generated.

QUANTITATIVE PCR

Quantitative RT-PCR (QRT-PCR) was used to confirm expression changes observed in the microarray analysis. RNA was extracted using the TRIzol procedure. First strand cDNA was prepared from total RNA (5 μg) using Superscript III cDNA synthesis kit

(Invitrogen, Carlsbad, CA). cDNA preparations were then diluted and used in reactions with SYBER green QPCR master mix. Reactions were amplified using an ABI Prism 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Each cDNA sample was assayed in triplicate using the primer sequences listed in Table III and β-actin as a control. Thermal cycle conditions consisted of an initial denaturation step at 95°C for 600 s, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. Fluorescence was measured at the beginning of each annealing/extension step. To check specificity of each primer the predicted Amplicon melting temperature was confirmed via dissociation curve analysis. PCR products were denatured and gradually re-annealed. Amplicon size was also determined by electrophoresis on an agarose gel (2% w/v). Relative expression changes in genes were then calculated using the $\Delta\Delta C_t$ quantification method. In some instances the $\Delta\Delta C_t$ value was averaged and normalized for the FLT group compared to the AEM group.

STATISTICAL ANALYSIS

All experiments were repeated four times, using four arrays for space flown mice, and four control arrays. To determine the significance of various gene expression changes between FLT and AEM, the *P* values were determined using paired Student's *t*-test with equal variance. Arrays were normalized using R programming (http://www.R-project.org) and Robust Multichip Average (RMA methodology) from the Oligo library [Bolstad et al., 2003; Irizarry et al., 2003] from the BioConductor project (http://www.BioConductor.org). We calculated the statistical significance of the expression level changes between space and control conditions, using the Affy and Limma Bioconductor libraries. For each probe set, the variance of mean signal intensities was estimated, improved by an empirical Bayes method for combining variances of probes showing similar variability, and the significance of the difference between the means was evaluated with a *t*-test to obtain a *P*-value [McCarthy and Smyth, 2009]. *P* values were adjusted for multiple hypothesis testing using the Benjamini and Hochberg method to control the false discovery rate [Benjamini et al., 2001]. False discovery rates are given as *B* statistic values, the log-odds that the probe set is differentially expressed between FLT and AEM controls.

RESULTS

MICROARRAY ANALYSIS

Figure 1 shows results of DNA microarray studies for spaceflown (STS-118) murine thymic lobes. Nine hundred and seventy individual probes (out of >20,000), showed a greater than 1.5-fold change in FLT versus the AEM group. Six hundred forty four, of these probes, were up-regulated; and 326 were down-regulated. To better evaluate these differential expressions, hierarchical clustering of probes showing a greater than 2.0-fold averaged change was examined. Heat map analysis revealed 47 probe sets (43 genes) that revealed a greater than 2.0-fold averaged change. Of the 43 genes with the greatest average differential expression, it is noted that 7 genes (9 probes) were up-regulated, and 36 genes (38 probes) were down-regulated.

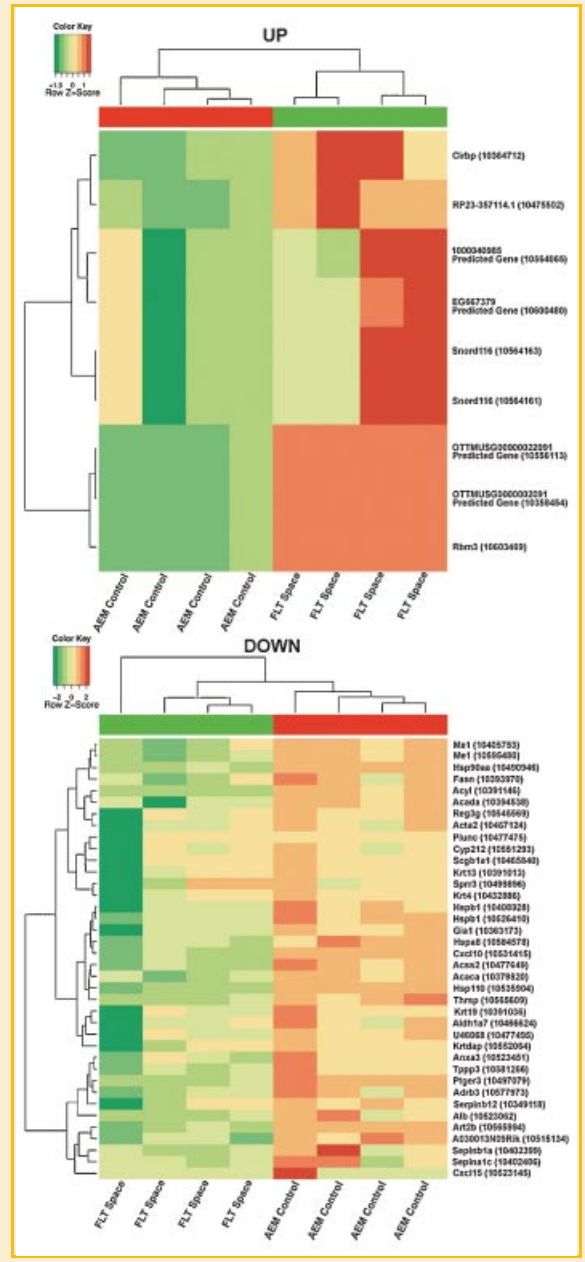

Fig. 1. Hierarchical clustering of the 47 probe sets (43 genes) that have the greatest fold change in up- or down-regulation in both AEM control (AEM) and space flight (FLT) mice (n = 4). Each row corresponds to a single probe set, and each column corresponds to a single array. Gene symbols and probe ID numbers are indicated to the right of the map. Gene expression signal strength levels are indicated by color as shown by the Row *Z* score color key. The top dendrogram shows genes with up-regulated expression due to space flight and the lower dendrogram shows genes that have down-regulated expression due to space flight. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These data were further refined by selecting for differential expression changes that were of at least 1.5-fold or greater in FLT versus AEM and had an adjusted *P*-value ≤0.05. Data for the top 25 up-regulated genes based on descending order significance is presented in Table I. Data for the top 25 down-regulated genes based

TABLE I. Top 25 Up-Regulated Genes With >1.5-Fold Change and in Space Flown Murine Thymus (FLT)

Genbank ID	Gene symbol	Description	FC	*P*-Value
XM_001480197	0TTMUSG00000022091	Predicted gene	2.60	0.001
NM_016809	Rbm3	RNA binding motif protein 3	2.46	0.001
NM_001085518	RP23-357114.1	Novel protein similar to (Slc28a2)	2.27	0.050
NM_007705	Cirbp	Cold inducible RNA binding protein	2.41	0.081
NM_146787	Olfr920	Olfactory receptor 920	1.55	0.117
NM_009843	CTLA-4	Cytotoxic T-lymphocyte protein 4	1.61	0.194
NM_007799	Ctse	Cathepsin E	1.63	0.196
NM_028705	Herc3	Hect domain and RLD 3	1.54	0.211
NM_013602	Mt1	Metallothionein 1	1.68	0.243
NM_030739	V1rd4	Vomeronasal 1 receptor, D4	1.61	0.252
NM_177912	Gsdmc2	Gasdermin C2	1.51	0.263
NM_207230	Olfr320	Olfactory receptor 320	1.74	0.314
NM_178404	Zc3h6	Zinc finger CCCH type containing 6	1.53	0.348
NM_001039554	Angptl7	Angiopoietin-like 7	1.80	0.348
NM_008630	Mt2	Metallothionein 2	1.96	0.361
NM_147033	Olfr714	Olfactory receptor 714	1.59	0.391
NM_207146	Olfr670	Olfactory receptor 670	1.60	0.393
NM_146392	Olfr720	Olfactory receptor 720	1.54	0.416
NM_016710	Nsbp1	Nucleosome binding protein 1	1.51	0.427
NM_183370	Sytl3	Synaptotagmin-like 3	1.71	0.432
NM_013495	Cpt1a	Carnitine palmitoyltransferase 1a	1.56	0.433
NM_001011531	Olfr329	Olfactory receptor 329	1.72	0.437
NM_199064	Ssxb10	Synovial sarcoma, X member B, breakpoint 10	1.75	0.444
NM_010766	Marco	Macrophage receptor with collagenous structure	1.58	0.444
NM_146369	Olfr434	Olfactory receptor 434	1.52	0.444

Detection of top 25 up-regulated genes based on significance of adjusted *P*-value (n = 4 FLT; n = 4 AEM). Table shows the results of micro array fold change (FC) for 25 genes differing at least 1.5-fold or greater in space flight (FLT) versus control (AEM) mice.

on descending order significance is presented in Table II. In total, there were 3 up-regulated genes and 9 down-regulated genes showing an average fold change >1.5 and $P \leq 0.05$.

mRNA AND QRT-PCR VALIDATION OF DIFFERENTIAL EXPRESSION

To validate the observed microarray expression results for selected genes, QRT-PCR analysis was performed. Seven of the 12 significant genes ($P \leq 0.05$) were chosen randomly. We also selected 4 other T cell related genes that showed a >1.5-fold change. Primer pairs for selected genes are listed in Table III.

Overall the QRT-PCR results closely matched the microarray data in terms of fold change, direction, and magnitude. Of the seven genes analyzed via QRT-PCR, six were found to have a significant and similar change in differential expression when compared to the microarray data. A confirmed increase was observed for RBM3 (Table IV). Confirmation was also observed in down-regulated genes as well. STIP1, HSP90AA1, CXCL10, HSP110, and FKBP4 were all

TABLE II. Top 25 Down-Regulated Genes With >1.5-Fold Change and in Space Flown Murine Thymus (FLT)

Genbank ID	Gene symbol	Description	FC	*P*-Value
NM_013559	Hsph110	Heat shock protein 110	−3.72	0.002
NM_010480	Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class A	−2.03	0.011
NM_146254	Wdr78	WD repeat domain 78	−1.93	0.011
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	−2.83	0.011
NM_134037	Acly	ATP citrate lyase	−2.35	0.014
NM_011196	Ptger3	Prostaglandin E receptor 3 (subtype EP3)	−2.38	0.017
NM_016737	Stip1	Stress-induced phosphoprotein 1	−1.84	0.042
NM_010219	Fkbp4	FK506 binding protein 4	−1.91	0.043
NM_009825	Serpinh1	Serine (or cysteine) peptidase inhibitor, clade H	−1.83	0.050
NM_009388	Tkt	Transketolase	−1.68	0.060
NM_031165	Hspa8	Heat shock protein 8	−2.01	0.077
NM_009381	Thrsp	Thyroid hormone responsive SPOT14 homolog	−2.31	0.077
NM_173427	Klhdc7a	Kelch domain containing 7A	−1.69	0.077
NM_001081324	Neto2	Neuropilin (NRP) and tolloid (TLL)-like 2	−1.62	0.077
NM_019915	Art2b	ADP-ribosyltransferase 2b	−2.23	0.081
NM_009272	Srm	Spermidine synthase	−1.73	0.109
NM_025844	Chordc1	Cysteine and histidine-rich domain (CHORD)	−1.72	0.117
NM_009786	Cacybp	Calcyclin binding protein	−1.84	0.117
NM_153526	Insig1	Insulin induced gene 1	−1.80	0.117
NM_146036	Ahsa1	AHA1, activator of heat shock protein ATPase	−1.65	0.117
NM_026178	Mmd	Monocyte to macrophage differentiation	−1.98	0.117
NM_001024474	Diras2	DIRAS family, GTP-binding RAS-like 2	−1.52	0.117
NM_008615	Me1	Malic enzyme 1, NADP(+)-dependent, cytosolic	−2.47	0.117
NM_019811	Acss2	Acyl-CoA synthetase short-chain member 2	−2.07	0.117
NM_001081322	Myo5c	Myosin VC	−1.59	0.117

Detection of top 25 down-regulated genes based on significance of adjusted *P*-value (n = 4 FLT; n = 4 AEM). Table shows the results of micro array fold change (FC) for 25 genes differing at least 1.5-fold or greater in space flight (FLT) versus control (AEM) mice.

TABLE III. Primer Pairs Used for Real-Time (RT-PCR)

Gene	Forward	Reverse
Rbm3	5′-TCT GCC GTC CTC TGA CTT TT-3′	5′-TGG GTT TGT GAA GGT GAT GA-3′
Cxcl10	5′-CCC ACG TGT TGA GAT CAT TG-3′	5′-CAC TGG GTA AAG GGG AGT GA-3′
Hsp110	5′-CAC AGC CCC AGG TAC AAA CT-3′	5′-TTT GCT TTG TCA GCA TCT GG-3′
Hsp90aa	5′-GTG TGC AAC AGC TGA AGG AA-3′	5′-CTC TCC ATG TTT GCT GTC CA-3′
Stip1	5′-AGC AGC TGT GCA CTT TGA GA-3′	5′-TGG CAC TTC TTG AGC ACA TC-3′
Ptger3	5′-TAC CTG TTT CCC TGG GTC TG-3′	5′-CAA AGG TTC TGA GGC TGG AG-3′
Fkbp4	5′-GAG GAA ATG CAA AAG GTC CA-3′	5′-CTT CTC GTT GTT GCT GTC CA-3′
Ctla4	5′-CAG GTG ACC CAA CCT TCA GT-3′	5′-CAG TCC TTG GAT GGT GAG GT-3′
CD44	5′-TGG ATC CGA ATT AGC TGG AC-3′	5′-AGC TTT TTC TTC TGC CCA CA-3′
IL-7	5′-ATC CTT GTT CTG CTG CCT GT-3′	5′-TGG TTC ATT ATT CGG GCA AT-3′
IFN-α2a	5′-TCT GTG CTT TCC TCG TGA TG-3′	5′-TTG AGC CTT CTG GAT CTG CT-3′

Primers shown were used in real time quantitative PCR. RT-PCR conditions were as follows: Stage 1, 1 cycle at 50°C for 120 s. Stage 2, 1 cycle at 95°C for 600 s. Stage 3, 40 cycles at 95°C for 15 s, 60°C for 60 s. Stage 4 dissociation stage 1 cycle at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s.

observed with a similar pattern of regulation to that of the microarray, and only PTGER3 differed (Table IV).

EVALUATION OF STRESS AND T CELL GENES VIA QRT-PCR

Once identified to be significantly influenced by spaceflight, the genes were further separated into two classes: (1) genes that have known association with T cell function and development, and (2) genes that are related to the glucocorticoid receptor pathway or stress-related functions. The cDNA was compared using QRT-PCR and normalized versus β-actin. The relative expression of FLT vs. AEM was then calculated for the mean ± SEM of four independent experiments.

Normalized expression differences associated with T cell related functions in FLT and AEM by QRT-PCR are shown in Figure 2. Gels from semi-quantitative RT-PCR are also shown for each of the T cell genes analyzed. QRT-PCR validated the down-regulation observed in the microarray of CXCL10 (Fig. 2A), as well as up-regulation of CTLA-4 (Fig. 2C). Like CXCL10, the relative expression of CD44 was also found to be significantly down-regulated via QRT-PCR in the thymus of FLT mice (Fig. 2B). This down-regulation of CD44 was also observed in the microarray results, although not statistically significant when all four FLT mice were averaged. The microarray previously yielded a −1.56-fold decrease of CD44 ($P = 0.37$, data not shown). IFN-α2a was found to have a 5.16-fold increase in relative expression ($P < 0.05$) via QRT-PCR (Fig. 2D). This result was in agreement with the microarray results, which yielded a 1.30-fold increase ($P < 0.05$, data not shown). The QRT-PCR results of IL-7 were inconclusive and not statistically significant (Fig. 2E).

Normalized expression changes in the genes associated with stress responses are shown in Figure 3. The QRT-PCR data were not only in agreement with the microarray results, but also yielded significant changes in relative expression in five of the six stress-related genes examined. A statistically significant decrease in relative expression was observed in HSP110, HSP90AA, STIP1, and FKBP4. Likewise there was an increase in the relative expression evidenced in RBM3. Of the six genes tested, only the PTGER3 results were not statistically significant.

DISCUSSION

DNA microarrays provide a powerful and efficient tool by which to compare differential expression of a large number of genes in a single reaction. The results of the microarray combined with QRT-PCR not only provide quantitative information on genomic variation, but also allow for the deduction of cellular function and response in the thymus of space-flown mice. Most previous genetic studies looking at the immunological effects of spaceflight and ground-based microgravity models showed that microgravity alters genes associated with multiple cellular functions such as structure, proliferation, differentiation, maturation, and cell survival [Lewis et al., 2001; Sonnenfeld, 2005; Nichols et al., 2006]. Among these studies are several that relate to the effects of (simulated) microgravity and spaceflight on T cells and the thymus in general [Gridley et al., 2003, 2009; Boonyaratanakornkit et al., 2005]. These findings both support and conflict with the previous in vitro and in vivo microgravity model work from our lab using the

TABLE IV. Quantitative RT-PCR Analysis/Confirmation of Up-/Down-Regulated Genes in Space Flown Murine Thymus (FLT)

Genbank ID	Gene symbol	Description	FC	*P*-Value
NM_016737	Stip1	Stress-induced phosphoprotein 1	−2.01	0.0003
NM_010480	Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class A	−2.86	0.0004
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	−5.51	0.0071
NM_016809	Rbm3	RNA binding motif protein 3	3.51	0.0089
NM_013559	Hsph110	Heat shock protein 110	−4.23	0.0114
NM_010219	Fkbp4	FK506 binding protein 4	−1.95	0.0165
NM_011196	Ptger3	Prostaglandin E receptor 3 (subtype EP3)	−0.71	0.6028

Quantitative RT-PCR analysis of up-/down-regulated genes in space flown murine thymus (FLT). QRT-PCR was performed on the seven genes found to be most significant via gene array. Fold change (FC) is listed along with *P*-value of selected target genes. RT-PCR was performed on the exact same murine RNA used for micro array in order to confirm or rebut gene array results, control mice (n = 4, AEM) and space flown (n = 4, FLT).

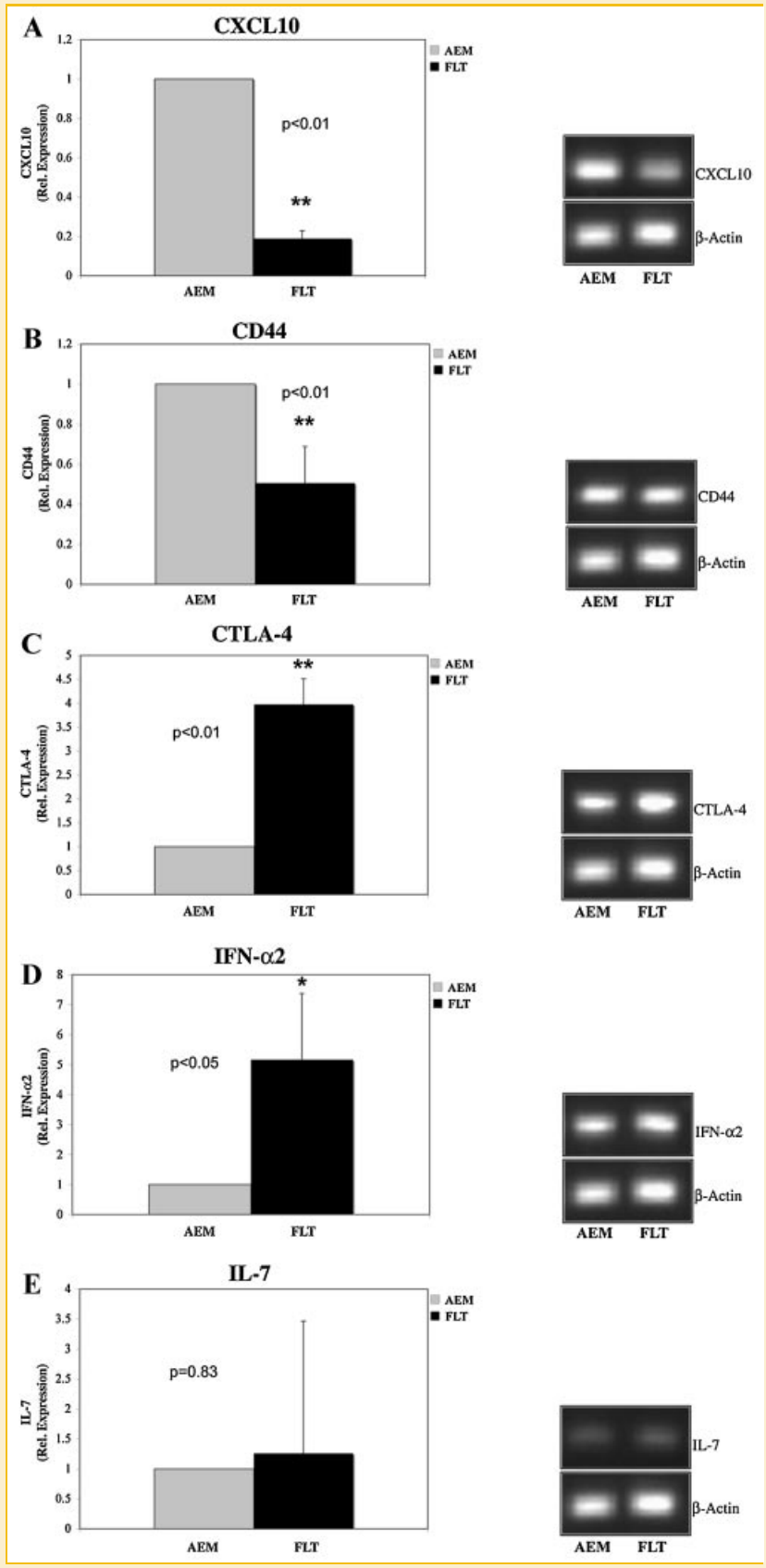

Fig. 2. T cell related gene expression in AEM and FLT murine thymus. Cells were collected and RNA extracted, and reverse transcribed. cDNA was subjected to comparative real-time QPCR using primer pairs specific for (A) CXCL10, (B) CD44, (C) CTLA-4, (D) IFN-a2, and (E) IL-7. Data shown is the relative expression normalized versus the β-actin for both AEM and FLT groups ($n = 4$ where $^{*}P < 0.05$ and $^{**}P < 0.01$). Confirmation of changes was also observed using semi-quantitative RT-PCR (shown at right of each graph).

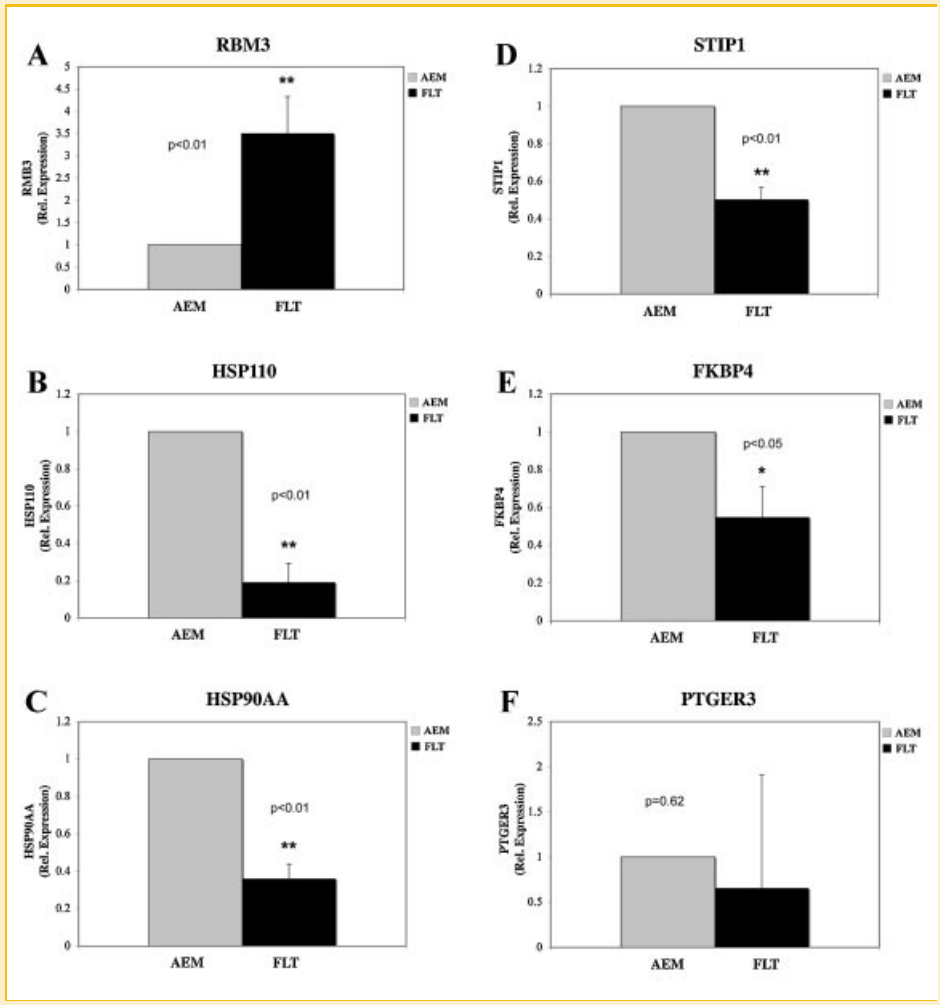

Fig. 3. Stress related gene expression in AEM and FLT murine thymus. Cells were collected and RNA extracted, and reverse transcribed. cDNA was subjected to comparative real-time QPCR using primer pairs specific for (A) RBM3, (B) HSP110, (C) HSP90AA, (D) STIP1, (E) FKBP4, and (F) PTGER3. Data shown is the relative expression normalized versus the β-actin for both AEM and FLT groups (n = 4 where $^{*}P < 0.05$ and $^{**}P < 0.01$).

clinostat and antiorthotic suspension [Woods et al., 2003, 2005; Lebsack et al., 2005]. One particular study [Lewis et al., 2001] implicates the disruption of cytoskeletal gene expression during spaceflight in the arrested growth of T cells. These data can be extrapolated to explain the developmental block of T cells previously reported by our lab, since the DN (CD4−CD8−) to DP (CD4+CD8+) transition also correlates with a period of rapid cell proliferation. The observed arrest of T cell development, in our previous in vitro studies [Woods et al., 2003], was characterized by a loss of DP T cells and increased frequency of the immature single positive (ISP) intermediate populations. The presence of these ISP cells at a higher frequency indicates successful β-chain rearrangement but an inability to trigger sufficient CD8 and CD4 expression, which is normally achieved via successful pre-TCR signaling [Miosge and Zamoyska, 2007]. Taken together, our previous work [Woods et al., 2005] and the work of others [Lewis et al., 2001; Boonyaratanakornkit et al., 2005; Hughes-Fulford et al., 2005] support the hypothesis that exposure to microgravity results in a disruptive effect on signaling which prevents the full manifestation of successful β-selection, and therefore blunts the normal production of DP T cells.

Many of the genes that were found to have significant changes in expression in the FLT mice were related to the stress response. The heat shock or stress response is one of the most highly conserved adaptive responses in nature. Heat shock proteins (HSPs) belong to a group of stress proteins comprising the glucocorticoid receptor (GR) heterocomplex. HSPs serve as cellular chaperones and the central role of these stress proteins in the transfer of peptides through the cell account for their importance in immune modulation, regulation, and development [Fuchs et al., 1996; Feder and Hofmann, 1999]. Two HSP's in particular, HSP110 and HSP90, have been directly implicated in the apoptosis pathway via the GR [Beere et al., 2000; Arya et al., 2007]. Interestingly we found both HSP110 and HSP90aa, were prominently down-regulated (−3.72 and −2.73 respectively) in FLT thymus. HSPa8 was also down-regulated, but just missed significance at $P = 0.077$ (Table II). Down-regulation of these HSP's was also confirmed via QRT-PCR (Table IV). Furthermore, the down-regulation of HSPs 90 and 110 that we observed in

FLT animals is consistent with previous findings using murine space-flown thymus as well as rat osteoblasts and heart cells [Liu et al., 2000; Kumei et al., 2002; Gridley et al., 2009].

Similar alterations in gene expression were also observed in other stress-related genes involving the GR heterocomplex. STIP1, which has been shown to inhibit apoptosis [Zanata et al., 2002], was down regulated in FLT mice by −1.84-fold in the array and −2.01-fold via QRT-PCR (Table I and Fig. 3). In addition to STIP1, the GR heterocomplex consists of several related chaperone proteins, including HSP90, HSP70, and the immunophilins FKBP51 and FKBP52. Interestingly, FKBP4 was also found to be significantly down-regulated −1.91-fold in the thymus of FLT mice (Table I and Fig. 3). The primary role of FKBP4 is to code for the FK506 binding protein 4, more commonly known as FKPB52, which is a vital component of the GR heterocomplex (along with HSP90 and STIP1) [Daikoku et al., 2005]. Additionally, this protein is associated with an ever-increasing array of biological functions from sub-cellular receptor transport, via dynein interaction, to the anti-viral activity of binding single stranded DNA [Chambraud et al., 2007; Jayandharan et al., 2008].

Of all the significant stress-response related genes only the RNA binding motif 3 (RBM3) and the cold inducible binding protein (CIRBP) were shown to increase in expression via microarray while the rest of the ''stress'' genes experienced a decrease in expression. RBM3 experienced a 2.46-fold increase and CIRBP saw a 2.41-fold increase via spaceflight (Table I). Additionally CIRBP has also been found to be significantly up-regulated in murine skeletal muscle cells due to spaceflight [Allen et al., 2009]. Our results would seem to confirm that CIRBP expression is not only altered in the skeletal muscle tissue, but that it is also up-regulated in thymus as well.

The variations we observed in GR associated genes would significantly affect the corticosteroid response in the thymus of FLT mice as well as T cell signaling, regulation and development. Previous work has documented elevated corticosteroid levels resulting from spaceflight [Chapes et al., 1999; Stowe et al., 2003]. There is also evidence that glucocorticoids are involved in not only stress related apoptosis, but also play a role in T cell differentiation and death via the TCR trigger [Gruber et al., 1994; Penninger and Kroemer, 1998]. The cells that are most susceptible to undergo apoptosis in the thymus are the double positive CD4+CD8+ cells, and many of these DP T cells naturally undergo apoptosis in the thymus during positive selection [Vacchio et al., 1999; De Boosscher et al., 2003]. Glucocorticoids, however, are thought to increase apoptosis and modulate gene expression of these cell types by inhibiting NF-κB and AP-1 [Auphan et al., 1995; Wang et al., 1998; Ashwell et al., 2000; De Bosscher et al., 2000]. Thusly the down-regulation in expression of the GR related genes we observed in the thymus of FLT animals has the potential to greatly affect the maturation of thymocytes and could very well account for the changes in T cell phenotype and signaling dysfunction that result due to exposure to microgravity and spaceflight.

Many of the most significantly up- and down-regulated chemokines and epitopes are highly relevant to T cell development. The specific T cell related genes we examined (CD44, CXCL10, IFN-α2a, and CTLA-4), in particular, have specific effects on various T cell functions. Some of these functions include regulation of immune response as well as inflammation and homing. One of these specialized T cell genes that we found to be altered due to spaceflight was CD44. The CD44 cell protein participates in a wide variety of cellular functions including lymphocyte activation, recirculation, homing, hematopoiesis, and tumor metastasis [Nagano and Saya, 2004; Rajasagi et al., 2009]. While CD44 is a well-known marker for various T cell intermediates in the thymus due to its role in migration, a recent study revealed that the CD44s isoform has a major impact on progenitor cell homing into the thymus and lymph nodes, while other isoforms, CD44v6 and CD44v7, do not [Rajasagi et al., 2009]. Furthermore there is evidence of a shift in the population of certain CD44 cells in the bone marrow of space-flown mice. It was shown that spaceflight causes a significant decrease in the largest, most granular $CD44^{high}$ cells [Ortega et al., 2009]. Our data are consistent with these findings in that we observed a significant decrease in relative expression of CD44 in the thymus of spaceflown mice (Fig. 2).

Another significant gene found to be altered in FLT mice with very specific and specialized T cell related functions is CXCL10. CXCL10 (also known as IP-10) is a member of a family of small proinflammatory chemotactic polypeptides. CXCL10 is known to promote the production of Th1-cytokines and suppress Th2 responses [Romagnani et al., 2005], and is also found in epithelial cells mainly localized to the medulla of postnatal human thymus, which is attributed to the homing of thymocyte precursors [Ying et al., 2005]. CXCL10 not only impacts thymic migration of T cells, but the altered expression of CXCL10 due to spaceflight also relates back to the changes in expression that we observed in the GR heterocomplex and stress-related genes. Recent work has suggested that CXCL10 is down-regulated in splenic mRNA due to stress via restraint [Curtin et al., 2009], as well as in the mRNA of T cells and osteoblasts cultured in a vector less gravity bioreactor [Hughes-Fulford et al., 2005; Capulli et al., 2009]. Similar to many of the previously mentioned genes that were found to be altered by spaceflight, this down-regulation observed in times of stress most likely occurs due to changes in STAT1 and the NF-κB pathway. The CXCL10 promoter contains one IFN-stimulated response element and two NF-κB elements [Majumder et al., 1998]. It is also known that IFN-γ stimulates the activation of NF-κB via the JAK/STAT1 pathway thus inducing CXCL10 production. As we have shown in this study, HSPs, the GR and thusly NF-κB become down-regulated in FLT animals. It is likely that the disregulation up-stream of the HSPs and NF-κB lead to the down-stream down-regulation in CXCL10. Furthermore that this suppressive effect on CXCL10 expression could then potentially negatively impact the immune system and thymic cell regulation.

Interestingly, yet another gene with ties to both NF-κB and T cell regulation was found to have altered gene expression due to spaceflight. Microarray analysis of the CTLA-4 gene revealed a 1.61-fold increase ($P = 0.19$), while QRT-PCR revealed a 3.97-fold increase ($P < 0.001$) (Fig. 2). CTLA-4, which is expressed mainly on CD4+ T cells and has well documented T cell inhibitory functions and plays a key role in regulating and controlling T cell proliferation and activation [Fraser et al., 1999; Rudd and Schneider, 2003; Mao et al., 2004]. Interestingly, ligation of CTLA-4 has been shown to cause a reduction in transcription mediated by both AP-1 and NF-κB

[Fraser et al., 1999]. Thusly, up-regulation of CTLA-4 (as we observed due to spaceflight) can in turn reduce expression of genes regulated by NF-κB (something we also observed), which can inhibit T cell differentiation and attenuate signals generated by the T cell receptor complex.

In conclusion, this study underscores the varying effects that spaceflight has on the expression of genes associated with general stress responses as well as specific immunological functions and T cell signaling pathways. The results of this study provide insight into how spaceflight affects stress-related gene expression in addition to influencing genes associated with specific immunological processes in the thymus itself. The results also show the connection between many of the genes that we found to be altered by spaceflight via their relation to the GR heterocomplex.

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