Articles in PresS. J Appl Physiol (December 29, 2005). doi:10.1152/japplphysiol.00887.2005

Final accepted version: JAP-00887-2005.R1

Dynamic Culture in a Rotating-Wall Vessel Bioreactor Differentially Inhibits Murine T Lymphocyte Activation by Mitogenic Stimuli Upon Return to Static Conditions in a Time-Dependent Manner

Simons, D.M.[1]; Gardner, E.M.[2]; Lelkes, P.I.[1]

[1]Drexel University, School of Biomedical Engineering, Science, and Health Systems; [2]Drexel University, Department of Bioscience and Biotechnology

Running head: Inhibitory kinetics of RWV culture on T cell activation

Corresponding Author: Dr. Peter I. Lelkes

Drexel University School of Biomedical Engineering, Science, and Health Systems, Commonwealth Hall 7-721, 3142 Chestnut Street, Philadelphia, PA 19104
Phone: 215-895-2219
Fax: 215-895-4982
E-mail: pilelkes@drexel.edu

Abstract

Depressed immune function is a well-documented effect of spaceflight. Both in-flight studies and ground-based studies using microgravity analogs, such as Rotating Wall Vessel (RWV) bioreactors, have demonstrated that mitogen-stimulated T lymphocytes exhibit decreased proliferation, IL-2 secretion, and activation marker expression in true microgravity and the dynamic RWV-culture environment. This study investigates the kinetics of RWV-induced T lymphocyte inhibition by monitoring the ability of Balb/c mouse splenocytes to become activated under static culture conditions following concanavalin A (Con A) stimulation in an RWV. Splenocytes were stimulated with Con A and cultured for up to 24-hrs in the RWV before being allowed to "recover" under static culture conditions in the continued presence of Con A. The T lymphocyte fraction of splenocytes was assayed during the recovery period for IL-2 secretion, expansion of the T lymphocyte population, and expression of the activation marker CD25. Our results indicate that CD25 expression was not affected by any duration of RWV-exposure. In contrast, proliferation and IL-2 secretion were inhibited by greater than 8- and 12-hrs of exposure respectively. Culture in the RWV for 24-hrs resulted in a near-complete loss of cellular viability during the recovery period, which was not seen in cells maintained in the RWV for 16-hrs or less. Taken together, these results indicate that for up to 8-hrs of RWV culture activation is not significantly impaired upon return to static conditions; longer duration RWV culture results in a gradual loss of activation during the recovery period most likely due to decreased T cell viability and/or IL-2 production.

Keywords: T lymphocyte, activation, microgravity, RWV, immune response

Introduction

During spaceflight astronauts experience multiple physiological changes including alterations of the immune response (2,5,31,32). *In vivo* studies using a delayed-type hypersensitivity skin test to common recall antigens first demonstrated a depressed cell-mediated immune response during spaceflight (6). Subsequent *ex vivo* studies showed that peripheral blood drawn from astronauts and immune cells isolated from space-flown animals exhibited decreased proliferation and cytokine secretion in response to mitogenic lectins (10,11,29), and altered distributions of immune cell populations following spaceflight (12,21). Both environmental factors, such as the microgravity encountered during orbital flight and human factors, such as acute stress due to launch and flight conditions have been implicated as contributors to altered immunity in astronauts (19,25,28,30). While it is likely that immune inhibition observed *in vivo* is due to the complex interplay between these two factors, direct evidence for the inhibitory affect of microgravity on T lymphocyte activation has been demonstrated by *in vitro* culture of lymphocytes during spaceflight (3,4,13,22,23). In these studies human peripheral blood mononuclear cells (PBMC) cultured during orbital-flight failed to proliferate, express activation markers, or secrete interleukin-2 (IL-2) in response to T cell-specific mitogens. In contrast, PBMC cultured under artificial 1-g conditions produced by an in-flight centrifuge responded normally to mitogenic stimulation.

Due to the limited opportunities for in-flight experimentation, studies to confirm and expand on these results have utilized rotating-wall vessel (RWV) bioreactors and other systems as ground-based analogs to microgravity culture. While it is impossible to eliminate the force of gravity from any ground-based culture system, several model systems such as the fast-rotating clinostat, the random positioning machine, and the RWV have been developed that mimic certain aspects of *in vitro* culture in spaceflight.

Specifically, RWV bioreactors are fluid-filled three-dimensional suspension-culture systems that utilize solid-body clinostat rotation to place cells in a low-shear freefall environment (15,35). In this system, the gravitational vector is randomized over time, averaging approximately 10^{-2}xg (37). Importantly, numerous experiments using RWVs as a ground-based microgravity analog (*"modeled microgravity"*, MMG[1]) have recapitulated critical aspects of the impaired T cell response seen in spaceflight (7,26,36) and have played an integral role in providing direction for in-flight studies of T cell activation in true microgravity. For example, several studies using the RWV as a ground-based microgravity analog provided the first mechanistic insights into microgravity induced inhibition of T cell activation (8,13,33,34). These studies linked inhibition to a specific lesion in signal transduction upstream of protein kinase C (PKC), and were subsequently confirmed by in-flight experiments (13).

In this study, we have used the RWV as a ground-based model of *in vitro* microgravity culture to test the hypothesis that the process of inhibition of the T cell response in the RWV depends on the duration of exposure to the MMG environment. To test this hypothesis we stimulated murine splenocytes with concanavalin A (Con A) and cultured them for increasing durations in High Aspect Ratio Vessel (HARV)-type RWV bioreactors. We then measured the ability of T lymphocytes in these cultures to recover activation when allowed to recuperate under static culture conditions. T lymphocyte activation was assessed by IL-2 secretion and by proliferation using thymidine-incorporation. Flow cytometry was used to specifically examine the activation of $CD3^+$ T cells within the mixed culture as assessed by expression of the activation marker CD25. Our results suggest that for up to 8-hrs in RWV culture the

[1]We are cognizant of the complexity of the RWV culture environment, which includes cell suspension in constant freefall in a low-shear environment with a randomized gravitational vector component amongst other factors. At present there is a lack of consensus on the terminology for describing what has been termed "simulated microgravity", "modeled microgravity", "microgravity analog", or most recently "vectorless gravity". While cognizant that it is impossible to fully recapitulate microgravity here on earth, we operationally define this environment as modeled microgravity (MMG), in line with numerous previous publications, and refer to it as such henceforth in the manuscript.

T cell response to Con A is analogous to that of synchronous delayed controls. In contrast, for exposure times greater than 8-hrs, the ability of Con A-activated T cells to proliferate during the recovery period decreased with increasing exposure to MMG. Culture in the RWV for 24-hrs or more resulted in a near-complete loss of T-cell viability during the recovery period. We conclude that in spite of Con A binding to the cell surface the lack of T cell activation during the first 8-hrs of culture in the RWV is tantamount to the cells not receiving an activating signal. For durations between 8- and 24-hrs additional mechanisms become inhibited that prevent the full activation of T lymphocytes when returned to static culture conditions. Exposure to 24-hrs or more of MMG induces processes that lead to cell death during the recovery period.

Materials and Methods

Animals: All experiments were performed using cells isolated from the spleens of adult male Balb/c mice (Jackson Laboratories), which were housed in a biological safety level-2 facility at the Drexel University College of Medicine. Prior to experimentation, mice were allowed 1-week to acclimate to the animal facility following delivery from the supplier.

Splenocyte isolation and culture: Each of the experiments presented here were performed using pooled cells from the spleens of at least five mice. Immune cells were isolated from each spleen according to an established protocol following procedures approved by the Drexel University Institutional Animal Care and Use Committee. In brief: just prior to experimentation, mice were euthanized by asphyxiation with CO_2. Spleens were aseptically removed, and placed in Roswell Park Memorial Institute (RPMI) 1640 medium (Biowhittaker, Walkersville, MD). The intact tissue was then disrupted using a sterile tissue homogenizer; and the resulting cell suspensions were layered over 5-ml of Histopaque-1083 (Sigma Diagnostics, Pittsburg, PA) in 15-ml centrifuge tubes. Splenic leukocytes were concentrated at the RPMI-histopaque interface by centrifugation at 1,300xg for 20-minutes. These cells were then collected, transferred to a new 15-ml centrifuge-tube using a glass Pasteur pipette and washed 3 times by centrifugation at 400xg for 10-minutes followed by resuspension of the pellet in RPMI. Following the last wash, cells were suspended in RPMI 1640 supplemented with 10-% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, $5x10^{-5}$ M β-mercaptoethanol, and 50 mM gentamicin. Viability was determined by trypan blue exclusion. T lymphocytes in the splenocyte cultures were activated by addition of 1.25-ug/ml of concanavalin A (Sigma diagnostics, Pittsburg, PA). In all of the experiments presented here, splenocytes were cultured at a density of $2.5x10^6$/ml in either RWVs (Synthecon,

Houston, TX) rotated at 14-rpm, T-25 flasks (Nunc, Rochester, NY), or 96-well plates (MP Biomedicals, Irvine, CA) in a humidified incubator at 37 °C/5-% CO_2. The optimal cell density, concentration of Con A, and RWV rotational speed were determined in preliminary studies (data not shown).

Proliferation assay: Proliferation was measured by the cells' ability to incorporate [methyl ^{3}H] thymidine into newly synthesized DNA. Triplicate aliquots of each experimental and control culture were assayed for proliferation 48- or 72-hrs after the initiation of each experiment. During the last 4-hrs of incubation cells were pulsed with 1.0-mCi [methyl ^{3}H] thymidine (48-Ci/mmol; Amersham Biosciences, Piskataway, NJ), and then harvested onto glass-fiber filters using a Unifilter-96 automated cell harvester (PerkinElmer, Torrance, CA). [^{3}H] thymidine incorporation was measured as counts per minute (CPM) using a TopCount NXT liquid scintillation counter (PerkinElmer, Torrance, CA). For comparison between experiments proliferation data are presented as the percentage-incorporation of stimulated splenocytes cultured in parallel under static culture conditions in a 96-well plate for the duration of the experiment.

IL-2 Assay: Measurement of IL-2 secretion into the culture supernatant by T cells in response to mitogenic stimulation was measured by ELISA (ebioscience, San Diego, CA). Cell-free supernatants were collected from all MMG-exposed and delayed activation samples after 24-, 48-, and 72-hrs total culture time and stored at -20 °C until analysis. Samples were then thawed, diluted 1:5, and the ELISA was performed following the manufacturer's protocol. To quantify the amount of secreted IL-2, the OD of each sample at 450-nm was measured using a Fluoroskan Ascent cytofluorimeter (Thermo Electron,

Milford, MA), and the concentration of IL-2 was calculated based on the OD readings of a recombinant IL-2 standard curve.

Antibody staining: At each time point, 10^6 cells were double-stained with fluorescently labeled antibodies (ebioscience, San Diego, CA) using an Allophycocyanin (APC)-conjugated CD3 as a pan-specific T cell marker, and a R-Phycoerythrin (PE)-conjugated antibody against the IL-2 receptor high-affinity α-subunit (CD25). In brief, cells were washed 3 times with ice-cold Hanks buffered saline solution + 1-% FBS (wash buffer) and incubated on ice for 30-minutes with the above antibodies. Cells were washed 3 more times with ice-cold wash buffer and stored in 1-% paraformaldehyde at 4 °C until analysis by flow cytometry (less than a week for all experiments).

Flow cytometry: For analysis by flow cytometry 50,000 events were acquired on a FACSCanto (Becton-Dickenson, San Jose, CA) flow cytometer using FACS Diva™ software (Becton-Dickenson, San Jose, CA). Evaluation of flow data was performed using FlowJo (Tree Star, Ashland, OR). Live cells were determined using a live-cell gate that was previously validated based on the light scattering properties of ethidium-stained samples of live and dead cells (data not shown). Comparisons of the number of T cells in each culture were made by gating on APC positive cells that fell within the live-cell gate. The percentage of the total cell population (live and dead) was used for all statistical calculations. Using this procedure, we found that each of our splenocyte isolations contained between 10-15-% T lymphocytes. Gates for $CD25^+$ cells were established independently for each experiment based on the geometric mean fluorescence intensity (gMFI) of CD25 on freshly isolated splenocytes falling within the $CD3^+$ gate.

Experimental Design and RWV Culture: To investigate the effect of RWV culture on T lymphocyte activation, mouse splenocytes were cultured in the presence of Con A for a total of 72-hrs, of which up to 24-hrs were spent in a 10-ml HARV-type RWV rotated at 14-rpm. At the end of each exposure period, the cells were transferred to static conditions (96-well plate) and were allowed to recover for up to 64-hrs in the continued presence of Con A (**Figure 1**). During the recovery period, the cells were monitored for the ability to become activated as assessed by IL-2 secretion, expression of the IL-2R α-chain (CD25), and the ability to expand the $CD3^+$ population of lymphocytes (proliferation). These parameters were measured immediately upon exit from the RWV culture venue (CD3 and CD25 only), and again after 24-, 48-, and 72-hrs of total culture time. Thus, the total culture time was held constant for all samples while the exposure time was gradually increased. As a result, cells exposed to the longest duration of MMG had the shortest amount of time to recover. To account for this, control splenocytes were cultured under static conditions in the absence of mitogen for a duration equivalent to MMG exposure, and Con A was added to these cells coincident with transfer of each experimental sample from the RWV to static culture (synchronous delayed controls). These samples were analyzed identically to and concomitant with the MMG-exposed samples. All culture and sampling times are given as the total time in culture and designated as time post stimulation (p.s.) of the MMG-exposed samples.

Statistical Analysis: Comparisons between MMG-exposed and delayed activation samples were made using a Student's t-test. A one-way ANOVA was used to compare the percent $CD3^+$ cells in samples from each treatment period with the initial population of cells. A Dunnett post-test was then used to detect differences between individual samples and the initial population of cells. P values of less than

0.05 were considered statistically significant. All calculations were made using the InStat statistics program (GraphPad Software, San Diego, CA).

Results

Cell Proliferation: The ultimate response of splenocytes to Con A stimulation is the proliferation of T lymphocytes. To gauge the effect of MMG-culture on T lymphocyte proliferation we used a thymidine incorporation assay to compare the rate of DNA synthesis by cells in the S phase of the cell cycle after 48- and 72-hrs of total culture time. As seen in **Figure 2,** T lymphocytes fully retain the ability to proliferate in response to mitogenic stimulation only for MMG exposure periods less than 8-hrs. In this case thymidine incorporation by MMG-exposed splenocytes was equivalent to the synchronous delayed control. Following 12- and 16-hrs of MMG-exposure, splenocytes' incorporated 33- and 47-% less thymidine respectively than synchronous delayed controls ($p<0.05$) as determined after 48-hrs in culture. This trend was also observed after 72-hrs in culture (**Figure 2, inset**). When cultured for 24-hrs in the RWV or left unstimulated for 24-hrs, the cells failed to incorporate detectable amounts of thymidine after 48-hrs in culture. For the MMG-exposed cells this inhibition appeared to be permanent, as culturing the cells under static conditions for an additional 24-hrs (i.e. until 72-hrs p.s.) was sufficient for detectable thymidine incorporation by the synchronous delayed control cells, but not by the MMG-exposed cells (**Figure 2, inset**).

IL-2 secretion: Secretion of the autocrine cytokine interleukin-2 by T cells in response to mitogenic stimulus provides the driving force behind the proliferative response. Measurements of IL-2 in the supernatants of Con A treated cells stimulated under normal conditions showed large amounts of IL-2 secreted into the supernatant (~400-pg/ml) by 24-hrs. This IL-2 was subsequently bound and metabolized by the cells resulting in decreasing levels at 48- and 72-hrs p.s. (**Figure 3**). In both MMG-exposed and synchronous delayed stimulation samples, secretion of IL-2 was greatly decreased compared to this control. In addition, IL-2 in these supernatants reached a maximum by 48-hrs and was

not noticeably decreased by 72-hrs p.s. Statistically significant differences in supernatant IL-2 levels of MMG and synchronous delay samples were only found for exposure times of 16 and 24-hrs (**Figure 3**). In both cases, T cells exposed to MMG secreted significantly less IL-2 than the corresponding delayed stimulation samples (16-hrs, $p<0.05$; 24-hrs, $p<0.01$). For the 24-hrs MMG-exposed sample IL-2 secretion was indistinguishable from background.

Splenocytes viability: The viability of mouse splenocytes stimulated with Con A under normal, static conditions for 72-hrs was 40-%. Similarly splenocytes cultured in the RWV for 8-hrs or delayed in their stimulation for up to 12-hrs also contained 40-% viable cells after 72-hrs of total culture time. Exposure to MMG for 12- or 16-hrs or a delay in activation of 12-hrs or more, however, decreased the number of viable cells at 72-hrs p.s. to 20-% (**Figure 4**). Splenocytes cultured in the RWV for 24-hrs experienced a near-complete loss of viable cells during the recovery period.

Expansion of the $CD3^+$ population of splenocytes: Con A stimulation of splenocytes under normal static conditions for 72-hrs resulted in a doubling of the $CD3^+$ population. A similar increase in the percentage of $CD3^+$ cells was seen in Con A stimulated splenocytes exposed to MMG for 8-hrs or delayed for up to 12-hrs. Each of these samples contained approximately 20-% viable $CD3^+$ cells at 72-hrs p.s. representing a nearly 2-fold increase in T cell content compared to the initial splenocyte population, which contained 12-% $CD3^+$ cells ($p<0.05$; **Figure 5**). Culture of splenocytes in MMG for 12- and 16-hrs, or a synchronous delay in activation for more than 12-hrs abolished any expansion of the $CD3^+$ population of cells. At these later times the percentage of $CD3^+$ cells was indistinguishable from that at t = 0 ($p >0.05$, **Figure 5**). In line with our results for splenocyte viability, culture in the RWV for 24-hrs

resulted in a near-complete loss of viable T cells during the recovery period (1-% viable CD3+ cells; $p<0.01$; Figure 5).

Expression and upregulation of CD25: Naïve T cells predominantly express only the low-affinity β- and γ-subunits of the IL-2 receptor. Upon activation with Con A, T cells rapidly upregulate the high-affinity α-subunit (CD25) of this receptor. In comparison to synchronous delayed controls, CD25 expression by viable T cells was not significantly affected following any duration of MMG exposure. Measurements made both after 48- and 72-hrs total culture time showed that the cultures exposed to MMG contained equal percentages of $CD3^+/CD25^+$ cells as the time-matched controls and that there was also no difference in the mean fluorescence intensity (MFI) of CD25 staining by these cells (data not shown).

Discussion

The purpose of this study was to test the hypothesis that the process of inhibition of the T cell response in the RWV depends on the duration of exposure to the MMG environment. Specifically, we focused on the reversibility of this inhibition as assessed by both inductive (CD25 expression, IL-2 secretion) and responsive (proliferation) mechanisms of T cell activation. One important finding of this study is that for up to 8-hrs in RWV culture the T cell response to Con A is analogous to that of synchronous delayed controls. In other words, in spite of the presence of Con A in the RWV the cells behave as though no stimulus was present. This failure to respond is not due to a lack of Con A binding in the RWV, as we verified by flow cytometric analysis of cells stimulated by FITC labeled Con A. Indeed, the binding of FITC-Con A to splenocytes was identical under static and MMG conditions (data not shown). When removed within the first 8-hrs of RWV-culture and plated under conventional culture conditions, the cells recover just like the synchronous delayed controls. This result indicates that inhibition during the first 8-hrs of culture in the RWV is tantamount to a lack of stimulation and does not extend beyond the culture time in the RWV. These data are in line with previous findings by others implicating a signal transduction defect in the inhibition of T cell responses in both true- and modeled-microgravity (8,13,14,34). Another important finding of this study is that beyond 8-hrs of culture in the RWV the situation becomes more complex. Prolonged culture in the RWV or in the absence of a stimulant leads to significant impairment of subsequent activation. Ultimately, the prolonged absence of stimulation activates processes that lead to cell death, and these processes become activated more rapidly in the RWV than in the synchronous delayed controls. While no definitive conclusion about the nature of these processes can be drawn from our data, they suggest that permanent MMG-induced alterations directly affect the ability of murine splenocytes to proliferate and/or survive following prolonged culture in the RWV.

The data on expansion of the T lymphocyte population reported in this study likely reflect a balance between cell death and cell proliferation. The extent of cell death and its effect on this expansion following culture in the RWV for 12- and 16-hrs, therefore, is difficult to determine since apoptosis was not directly measured in this study. Reports in the literature are ambiguous about the role of apoptosis in lymphocyte culture in the RWV. For example, Risin and Pellis reported that spontaneous apoptosis was not observed in purified human T lymphocytes stimulated with CD3 antibody and exogenous IL-2 after culture in the RWV for 18-24-hrs (24). In addition they found that induction of apoptosis by re-engagement of the T cell receptor or γ-irradiation was inhibited by culture in the RWV bioreactor. These results are in contrast to a recent report that apoptosis is increased in human PBMC cultured in the RWV (17). While these results are directly contradictory, several differences in experimental conditions may account for the differences in results, most notably the use of PBMC vs. purified T lymphocytes. A previous report by Bakos et al. (2001) that apoptosis is increased in B lymphocytes but not T lymphocytes in PBMC culture in the RWV would be consistent with these differences (1). Similar to Bakos et al., we used a mixed population of cells, murine splenocytes in this case, and also used flow cytometry to specifically analyze the responses of $CD3^+$ T lymphocytes. For up to 16-hrs in the RWV our findings are in agreement with Bakos et al. The near-complete loss of T lymphocytes in our system following 24-hrs of exposure, however, is contradictory to their findings, but may be species specific, as results in our lab have shown that this loss of T cell viability is not encountered when human PBMC are cultured under identical conditions for 24-hrs (Ritz et al.,FASEB J., in press; Simons, Gardner and Lelkes, unpublished observations).

To date, this is the first documented investigation of the time-dependence of T lymphocyte responses to mitogen following activation in RWV culture. Our data indicate a window during the first 8-hrs of RWV culture when no permanent inhibition of the cells has occurred and the affect of MMG may be limited to inhibition of intracellular signaling cascades. This window offers a convenient time-span for specific examination of the affects of MMG on signal transduction in the absence of confounding factors that may come into play during more prolonged culture periods. The results presented here provide a framework for more detailed investigations of the affected molecular mechanisms that may be responsible for the inhibition of T cell activation in a MMG environment here on earth and, eventually, in real microgravity during space-flight. One caveat, however, is that impaired immune responses in spaceflight are likely due to a combination of the microgravity environment and the psychological and physical stresses of spaceflight.

Acknowledgements: We are grateful to Ms. Jessica Battisto who provided valuable help in validation of the live cell gate used in these experiments to determine viability by flow cytometry. This study was supported in part by NASA grants NAG-1436, NNJ04HC81G-01, and NCC9-130 (PIL) and a NASA GSRP Fellowship (DMS, Award #: NNG04GO19H)

References

1. **Bakos A, Varkonyi A, Minarovits J and Batkai L**. Effect of simulated microgravity on human lymphocytes. *J Gravit Physiol* 8: 69-70, 2001.

2. **Borchers AT, Keen CL and Gershwin ME**. Microgravity and immune responsiveness: implications for space travel. *Nutrition* 18: 889-898, 2002.

3. **Cogoli-Greuter M, Sciola L, Spano A, Meloni MA, Pippia P and Cogoli A**. Lymphocyte movements and interactions in microgravity. *J Gravit Physiol* 2: 117-118, 1995.

4. **Cogoli A, Tschopp A and Fuchs-Bislin P**. Cell sensitivity to gravity. *Science* 225: 228-230, 1984.

5. **Cogoli A**. The effect of space flight on human cellular immunity. *Environ Med* 37: 107-116, 1993.

6. **Cogoli A**. Gravitational physiology of human immune cells: a review of in vivo, ex vivo and in vitro studies. *J Gravit Physiol* 3: 1-9, 1996.

7. **Cogoli A and Cogoli-Greuter M**. Activation and Proliferation of Lymphocytes and Other Mammalian Cells in Microgravity. In: Advances in Space Biology and Medicine, edited by Bonting SL. Greenwich, CT: JAI Press, 1997, p. 33-79.

8. **Cooper D and Pellis NR**. Suppressed PHA activation of T lymphocytes in simulated microgravity is restored by direct activation of protein kinase C. *J Leukoc Biol* 63: 550-562, 1998.

9. **Cooper D, Pride MW, Brown EL, Risin D and Pellis NR**. Suppression of antigen-specific lymphocyte activation in modeled microgravity. *In Vitro Cell Dev Biol Anim* 37: 63-65, 2001.

10. **Crucian BE, Cubbage ML and Sams CF**. Altered cytokine production by specific human peripheral blood cell subsets immediately following space flight. *J Interferon Cytokine Res* 20: 547-556, 2000.

11. **Gridley DS, Nelson GA, Peters LL, Kostenuik PJ, Bateman TA, Morony S, Stodieck LS, Lacey DL, Simske SJ and Pecaut MJ**. Genetic models in applied physiology: selected contribution: effects of spaceflight on immunity in the C57BL/6 mouse. II. Activation, cytokines, erythrocytes, and platelets. *J Appl Physiol* 94: 2095-2103, 2003.

12. **Grove DS, Pishak SA and Mastro AM**. The effect of a 10-day space flight on the function, phenotype, and adhesion molecule expression of splenocytes and lymph node lymphocytes. *Exp Cell Res* 219: 102-109, 1995.

13. **Hashemi BB, Penkala JE, Vens C, Huls H, Cubbage M and Sams CF**. T cell activation responses are differentially regulated during clinorotation and in spaceflight. *FASEB J* 13: 2071-2082, 1999.

14. **Hatton JP, Gaubert F, Cazenave JP and Schmitt D**. Microgravity modifies protein kinase C isoform translocation in the human monocytic cell line U937 and human peripheral blood T-cells. *J Cell Biochem* 87: 39-50, 2002.

15. **Klaus DM**. Clinostats and bioreactors. *Gravit Space Biol Bull* 14: 55-64, 2001.

16. **Lewis ML, Reynolds JL, Cubano LA, Hatton JP, Lawless BD and Piepmeier EH**. Spaceflight alters microtubules and increases apoptosis in human lymphocytes (Jurkat). *FASEB J* 12: 1007-1018, 1998.

17. **Maccarrone M, Battista N, Meloni M, Bari M, Galleri G, Pippia P, Cogoli A and Finazzi-Agro A**. Creating conditions similar to those that occur during exposure of cells to microgravity induces apoptosis in human lymphocytes by 5-lipoxygenase-mediated mitochondrial uncoupling and cytochrome c release. *J Leukoc Biol* 73: 472-481, 2003.

18. **McPhee JC and White RJ**. Physiology, medicine, long-duration space flight and the NSBRI. *Acta Astronaut* 53: 239-248, 2003.

19. **Mehta SK, Kaur I, Grimm EA, Smid C, Feeback DL and Pierson DL**. Decreased non-MHC-restricted (CD56+) killer cell cytotoxicity after spaceflight. *J Appl Physiol* 91: 1814-1818, 2001.

20. **Papaseit C, Pochon N and Tabony J**. Microtubule self-organization is gravity-dependent. *Proc Natl Acad Sci U S A* 97: 8364-8368, 2000.

21. **Pecaut MJ, Nelson GA, Peters LL, Kostenuik PJ, Bateman TA, Morony S, Stodieck LS, Lacey DL, Simske SJ and Gridley DS**. Genetic models in applied physiology: selected contribution: effects of spaceflight on immunity in the C57BL/6 mouse. I. Immune population distributions. *J Appl Physiol* 94: 2085-2094, 2003.

22. **Pellis NR, Goodwin TJ, Risin D, McIntyre BW, Pizzini RP, Cooper D, Baker TL and Spaulding GF**. Changes in gravity inhibit lymphocyte locomotion through type I collagen. *In Vitro Cell Dev Biol Anim* 33: 398-405, 1997.

23. **Pippia P, Sciola L, Cogoli-Greuter M, Meloni MA, Spano A and Cogoli A**. Activation signals of T lymphocytes in microgravity. *J Biotechnol* 47: 215-222, 1996.

24. **Risin D and Pellis NR**. Modeled microgravity inhibits apoptosis in peripheral blood lymphocytes. *In Vitro Cell Dev Biol Anim* 37: 66-72, 2001.

25. **Sams C, D'Aunno D and Feeback DL**. The Influence of Environmental Stress on Cell-Mediated Immune Function. In: Isolation: NASA Experiments in Closed-Environment Living, edited by Lane HW, Sauer RL and Feeback DL. San Diego, CA: Univelt, Inc., 2004, p. 357-368.

26. **Sastry KJ, Nehete PN and Savary CA**. Impairment of antigen-specific cellular immune responses under simulated microgravity conditions. *In Vitro Cell Dev Biol Anim* 37: 203-208, 2001.

27. **Schatten H, Lewis ML and Chakrabarti A**. Spaceflight and clinorotation cause cytoskeleton and mitochondria changes and increases in apoptosis in cultured cells. *Acta Astronaut* 49: 399-418, 2001.

28. **Sheridan JF, Dobbs C, Jung J, Chu X, Konstantinos A, Padgett D and Glaser R**. Stress-induced neuroendocrine modulation of viral pathogenesis and immunity. *Ann N Y Acad Sci* 840:803-8.: 803-808, 1998.

29. **Sonnenfeld G, Foster M, Morton D, Bailliard F, Fowler NA, Hakenewerth AM, Bates R and Miller ES, Jr.** Spaceflight and development of immune responses. *J Appl Physiol* 85: 1429-1433, 1998.

30. **Sonnenfeld G**. Space flight, microgravity, stress, and immune responses. *Adv Space Res* 23: 1945-1953, 1999.

31. **Sonnenfeld G and Shearer WT**. Immune function during space flight. *Nutrition* 18: 899-903, 2002.

32. **Sonnenfeld G**. The immune system in space and microgravity. *Med Sci Sports Exerc* 34: 2021-2027, 2002.

33. **Sundaresan A, Risin D and Pellis NR**. Loss of signal transduction and inhibition of lymphocyte locomotion in a ground-based model of microgravity. *In Vitro Cell Dev Biol Anim* 38: 118-122, 2002.

34. **Sundaresan A, Risin D and Pellis NR**. Modeled microgravity-induced protein kinase C isoform expression in human lymphocytes. *J Appl Physiol* 96: 2028-2033, 2004.

35. **Unsworth BR and Lelkes PI**. Growing tissues in microgravity. *Nat Med* 4: 901-907, 1998.

36. **Walther I, Pippia P, Meloni MA, Turrini F, Mannu F and Cogoli A**. Simulated microgravity inhibits the genetic expression of interleukin-2 and its receptor in mitogen-activated T lymphocytes. *FEBS Lett* 436: 115-118, 1998.

37. **Walther I**. Space bioreactors: their use, their future. *J Gravit Physiol* 8: 13-16, 2001.

FIGURE LEGENDS

Figure 1. Experimental design for exposure of T lymphocytes to modeled microgravity followed by recovery under static conditions. In order to assess the effects of modeled microgravity (MMG) on the functional mechanisms of T cell activation, mouse splenocytes were stimulated with the T cell mitogen concanavalin A (Con A), cultured for increasing durations in RWV bioreactors rotated at 14-rpm to produce MMG, and then allowed to recover under static conditions. As a means of comparison, control cells were left unstimulated for an equivalent duration in static culture, and then stimulated with Con A coincident to transfer of the experimental samples from MMG to static culture. All time points are measured post stimulation (p.s.) of the MMG-exposed samples.

Figure 2. Thymidine incorporation by splenocytes following increasing durations of MMG. Splenocytes were stimulated and cultured in MMG (⊠) or left unstimulated under static conditions (□) for the indicated period of time. At each time point cells were removed from the RWV and allowed to recover in static culture. Control samples were stimulated at the beginning of the recovery period. After 48- or 72-hrs (inset) total culture time cells were pulsed for 4-hrs with [^{3}H-methyl] thymidine and then assayed for incorporation. The control (■) sample was stimulated with Con A under static conditions for the duration of the experiment. Control at 48-hrs = 214,000-cpm; Control at 72-hrs = 200,000-cpm Data are the mean 8-independent experiments. Error bars are SEM. *p< 0.05 MMG vs. delayed stimulation; †approaches significance, p = .073

Figure 3. IL-2 secretion by splenocytes following culture in MMG. Splenocytes were stimulated and cultured in MMG or left unstimulated in T flasks for the indicated period of time. At each time point cells were removed from the RWV and allowed to recover under static conditions. Control samples were stimulated at the beginning of the recovery period. At 24- (□), 48- (■) and 72-hrs p.s. (⊡) cell-free supernatants were collected from the cultures and assayed for IL-2 by ELISA. The control sample (ctl) was stimulated with Con A in static culture for the duration of the experiment. Data are the mean of 3-independent experiments. Error bars are SEM. *p< 0.05 MMG vs. delayed activation. **p<0.001 vs. delayed activation.

Figure 4. Viability of splenocytes following MMG culture. Splenocytes were stimulated and cultured in MMG (⊠) or left unstimulated under static conditions (□) for the indicated period of time. At each time point cells were removed from the RWV and allowed to recover in static culture. Delayed samples were stimulated at the beginning of the recovery period. After 72-hrs total culture time the percentage of viable cells was determined by flow cytometry. The control sample (■) was stimulated with Con A under static conditions for the duration of the experiment. Data are the mean of 3-independent experiments. 50,000 events were collected for all samples. Error bars are SEM. *p<0.05 vs. delay, **p< 0.01 vs. delayed.

Figure 5. Expansion of CD3$^+$ cells following MMG culture. Splenocytes were stimulated and cultured in MMG (⊠) or left unstimulated under static condtions (□) for the indicated period of time. At each time point cells were removed from the RWV and allowed to recover in static culture. Delayed samples were stimulated at the beginning of the recovery period. After 72-hrs total culture time the percentage of viable CD3$^+$ cells was determined by flow cytometry. The control sample (■) was stimulated with Con A under static conditions for the duration of the experiment. Data are the mean of 3-independent experiments. 50,000 events were collected for all samples. Error bars are SEM. *p<0.05 vs. initial, **p< 0.01 vs. initial.

Figure 1

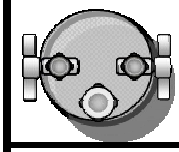
	Initial	Exposure	Recovery	Analysis
	Con A, MMG	Rotate in RWV at 14-rpm for **MMG exposure**	Transfer to 96-well plate for recovery in static culture	Assay cells for thymidine incorporation Flow cytometry for $CD3^+$ cells, and CD25 expression Assay supernatants for IL-2 secretion
	No Con A, static	Culture unstimulated in flask for static control	Stimulate with Con A, transfer to 96-well plate for **delayed activation**	
Time	t = 0	t = 8-24-hrs p.s.	Until t = 48 and 72-hrs p.s.	t = 24-, 48- and 72-hrs p.s.

Figure 2

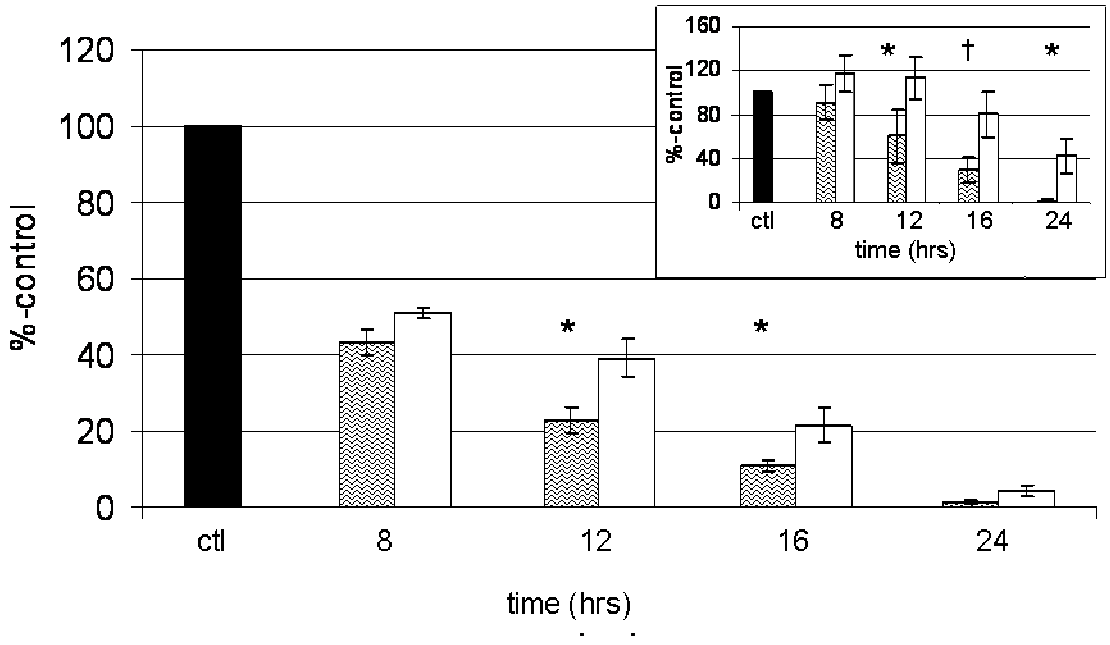


Figure 3

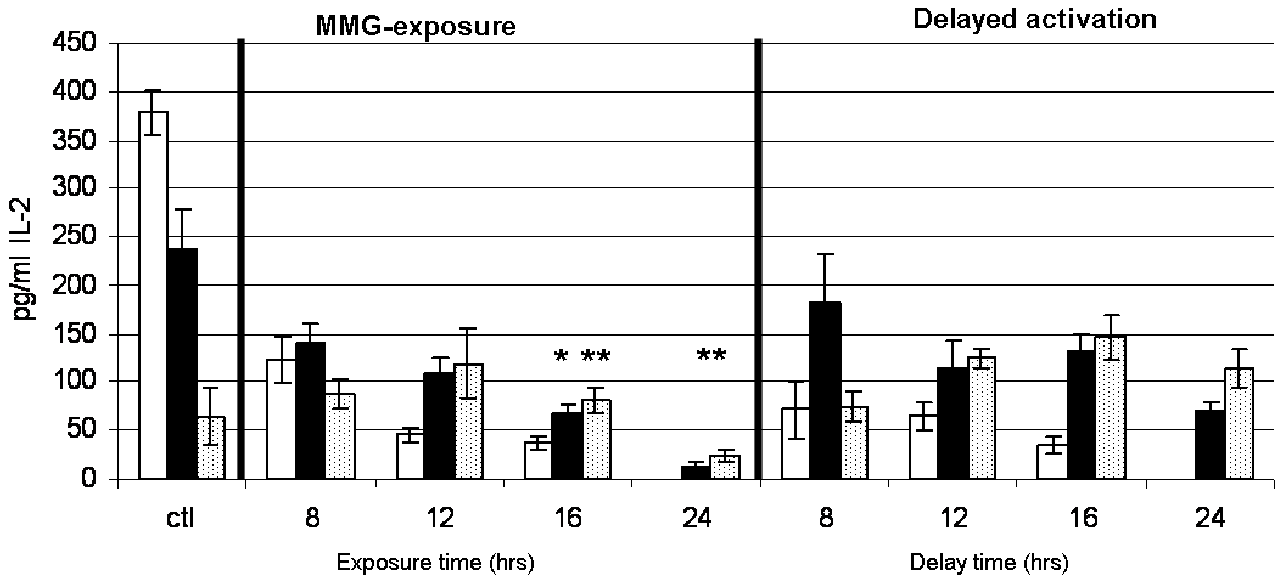


Figure 4

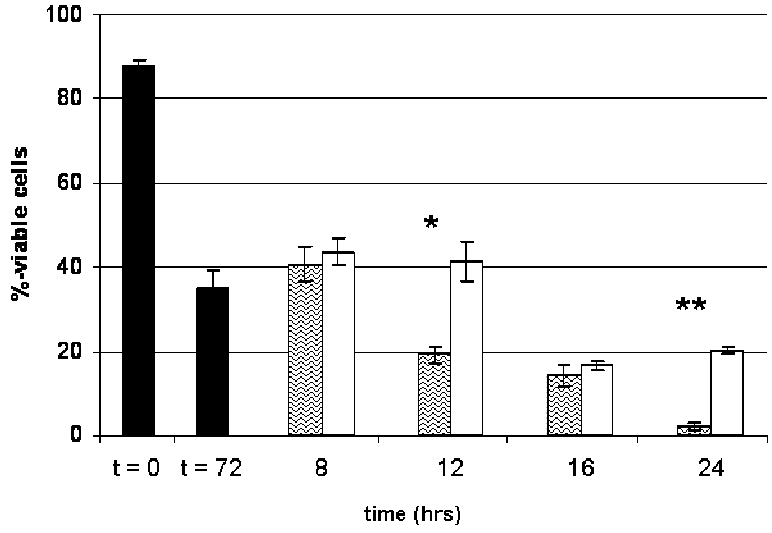


Figure 5