Functional recovery of peripheral blood mononuclear cells in modeled microgravity

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ABSTRACT

Immune function is suppressed in space flight, demonstrated by reduced mitogen-stimulated proliferation of postflight astronaut peripheral blood mononuclear cells (PBMCs). While flight studies are limited, the development of rotating wall vessel (RWV) bioreactors, such as the high aspect ratio vessel (HARV), has facilitated ground-based studies of the effects of modeled microgravity (MMG) on cell-mediated immunity. Astronauts regain immune function 3 days postflight, but this recovery has not yet been demonstrated following MMG. MMG eliminated phytohemagglutinin (PHA)-stimulated proliferation of PBMCs. Upon removal from HARV, full recovery was gradually achieved over a 72 h period, in agreement with postflight studies of astronauts. Recovery from MMG delayed, but did not reduce, the maximal proliferative response compared with PHA-activated stationary cultures. Likewise, peak expression of T cell surface activation markers CD69 and CD25 was delayed upon stimulation following exposure to MMG. MMG and recovery from MMG differentially affected the detection of IL-2 and IFN in supernatants. Further development of this model of immune recovery is important for investigating the mechanisms of immune suppression and recovery in space flight, as well as possible countermeasures to prevent immunosuppression or enhance recovery. Given the analogous immune suppression observed in microgravity, MMG, and aging, further investigation may also lead to advances in anti-aging medicine.

Key words: T cell • cytokine • human • lymphocyte • space flight

Preparation for multiyear, manned space flight missions, as necessary to meet stated goals of extended lunar stays and a manned flight to Mars, requires increased consideration of the medical status of the flight personnel compared with the relatively short-duration shuttle missions of the past (1, 2). To date, short flights and space station activity for up to approximately 1 year have provided some limited understanding of the profound physiological changes endured by astronauts in space, including loss of calcium and minerals from bone, decreased skeletal muscle mass, altered absorption of nutrients, disturbed fine motor control, anemia, and depressed immune function (3). It is now clear that humans can survive in space for periods as long as a year despite these challenges, and there is some indication that most
physiological changes, including decreased immune function, are reversible upon return to Earth (3–5). However, longer-term space flight may be associated with some elevated or specific risks, and there exists the potential for chronic detrimental effects on astronaut health. Hence, a thoughtful investigation of the effects of space flight conditions, such as microgravity, on astronaut health, as well as the development of appropriate countermeasures, are necessary before subjecting astronauts to these long-duration missions.

One of the central health issues that must be considered is the effect of space flight on immune function. It was recognized early in the space program that space flight is associated with an increased proclivity to infectious disease (6). However, limited contact with astronauts before flight and the maintenance of a clean flight cabin have been effective (6, 7), and astronauts have not required intensive immunotherapy postflight (8). Whether these preventive measures can be maintained in long-duration flights is not known. Human in vivo studies of immune function in microgravity in orbit have been limited to the demonstration of reduced delayed-type hypersensitivity (DTH) skin prick tests, which are easily completed in flight (7, 9). Alternatively, most research has relied on ex vivo analyses of astronaut peripheral blood mononuclear cells (PBMCs) immediately postflight as compared with preflight results. Such studies have clearly demonstrated a decrease in lymphocyte proliferation and altered cytokine production (6, 10–13). Humoral immunity, including B cell reactivity and the production of total and specific immunoglobulins (Igs), appears to remain unchanged in response to space flight (6, 8, 13).

Since space flight studies are limited, contemporary research must rely on validated ground-based models of microgravity (MMG), which are also recommended as tools for planning efficient future flight studies (14). Rotating wall vessel (RWV) bioreactors were developed more than a decade ago at Johnson Space Center and have been optimized as low shear stress cell culture venues, allowing the culture of shear-sensitive mammalian cells (15, 16). By eliminating head space, RWVs create a rotating solid fluid body that can suspend cells in sustained free fall at terminal velocity (14). RWVs can achieve a time averaged gravity vector of $10^{-2}$ g, which is considered a useful paradigm for studying some aspects of microgravity, although near-Earth free fall orbit is $10^{-4}$ to $10^{-6}$ g (17). Clinostats (vector-averaged gravity) and RWVs have become the most widely used ground-based models in studying the effects of microgravity on lymphocyte function. While important experiments were lost aboard STS-107 (Space Shuttle Discovery), comparisons of RWVs with culture systems on board the International Space Station continue to provide insight into the reliability of RWVs as tools for studying proliferation, antibody production, and cytokine response in microgravity (18).

Ex vivo astronaut studies (10, 11), in-flight cell culture (19–22), and ground-based RWV studies (14, 23–25) have consistently demonstrated a decrease in mitogen-induced lymphocyte proliferation in microgravity or MMG. Both decreased proliferation and suppressed or altered cytokine secretion are transient in that they return to pre-flight values by three days postflight (10, 13, 19). However, while the recovery of proliferative response to stimulus has been demonstrated following space flight, ground-based models have not been applied to the study of immune recovery. We hypothesized that the MMG environment in RWV is suitable for modeling not only the immunosuppression during space flight, as previously suggested, but also the recovery of immune function that is observed after space flight. In this study, we describe that the PHA-stimulated proliferation of human PBMCs is completely eliminated by exposure to MMG, but gradually and fully recovers over a 72 h period following removal from MMG. In
addition, our experiments evaluate the expression of activation markers during recovery and demonstrate the recovered production of functional IL-2 by 24 h following removal from MMG, which preceded proliferation and the accumulation of functional IFN. The observation of full recovery of PHA-stimulated proliferation of PBMCs by 72 h following MMG exposure parallels postflight data and suggests that RWVs may be used in the future to study both the suppression and recovery of cell-mediated immune processes. Further, RWVs may be suitable for designing and testing countermeasures that may either prevent immunosuppression during microgravity exposure or accelerate its recovery.

MATERIALS AND METHODS

Isolation of PBMCs

Whole blood was obtained from young (ages 18–39) volunteers and collected into EDTA-coated vacutainer tubes. Following centrifugation, the buffy coat, containing PBMCs (lymphocytes, macrophages, and other white cells) was removed, and PBMCs were isolated on Ficoll Histopaque-1077 (Sigma, St. Louis, MO) gradient by centrifugation (25). Cells were counted and cell viability determined by trypan blue exclusion. Following two washes, cells were suspended at 1 × 10^6 cells/ml in media.

Cell culture media

Cell culture media consisted of 10% fetal bovine serum (FBS; Sigma), 1% L-glutamine (Sigma), 0.1% gentamicin (Sigma), and 0.1% β-mercaptoethanol (Sigma) in RPMI-1640 (Mediatech, Herndon, VA).

Mitogen-stimulated proliferation assay

T cell proliferation of PBMCs was assessed by stimulation with 8 μg/ml phytohemagglutinin (PHA, Sigma). PHA-stimulated and control cultures were incubated in parallel at 37°C, 5% CO₂ (Fisher Isotemp) in 10 ml T-flasks or 96-well plates to represent stationary conditions. Triplicate samples in 96-well plates were pulsed with 1 μCi [3H]-thymidine (tritium; Amersham, Piscataway, NJ), according to established procedures (26). Tritium-labeled samples were incubated for 4 h, harvested (Packard Filtermate 196; Perkin-Elmer, Boston, MA), and dried overnight. Proliferation was measured on a Top Count Counter (Packard). Where appropriate, proliferation is reported as net counts per minute (CPM), such that CPM = mean CPM with PHA – mean CPM with media alone.

MMG

PHA-stimulated PBMCs were cultured in disposable 10 ml high aspect ratio vessel (HARV) units (Synthecon, Houston, TX). HARVs were rinsed with RPMI, loaded through a 10 ml syringe, and mounted onto a four-position rotator base (Synthecon) located in the incubator (37°C and 5% CO₂). Rotational speed was set to 8.5 rpm, based on preliminary experiments.
Cell viability

In addition to determining cell viability by trypan blue exclusion, $1 \times 10^6$ PBMCs were suspended in PBS containing 1% FBS and 0.625 μg/ml ethidium monoazide (EMA, Molecular Probes, Eugene, OR) and crosslinked under fluorescent light for 15 min. Following multiple washes, cells were then labeled with APC-conjugated antibody to CD3 (pan T cell marker), and live and dead cells were distinguished by gating on CD3+/EMA(FL-2)– and CD3+/EMA+ populations, respectively. Heat-treated PBMCs (30 min at 65°C) were used as a positive control for dead cells.

Cytokine analysis by ELISA

Supernatants were collected and stored at −70°C until analysis. Both IL-2 and IFN-γ were analyzed in aliquots of these supernatants using commercial ELISA kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Concentrations (pg/ml) of cytokines were quantitated using a recombinant IL-2 or IFN-γ standard.

IL-2 bioassay

The bioassay to assess IL-2 activity has previously been described in detail (26). Briefly, supernatant samples and recombinant IL-2 standards (Genzyme, Cambridge, MA) were serially diluted in 96-well plates and incubated for 24 h with the IL-2-dependent cytotoxic T cell line, CTLL. Plates were labeled for the final 4 h with 1 μCi tritium, harvested, and counted as per proliferation assay described above. One unit of IL-2 activity was defined as the amount of supernatant that supported 50% of the maximum proliferative response.

IFN-α/β bioassay

The titer of IFN-α/β was determined using human IFN standard (WHO International) and human foreskin fibroblast cells, as described previously (26). One unit of IFN activity was defined as the inverse of the dilution that gave 50% protection against the cytopathic effect of encephalomyocarditis virus.

Recovery following modeled microgravity

As depicted in Fig. 1, PHA-stimulated PBMCs were cultured in stationary T-flasks or 10 ml HARV units (Synthecon). After 48 h, viable cells were identified by trypan blue exclusion and counted, and samples were resuspended at an equal concentration of $1 \times 10^6$ cells/ml. The media of stimulated samples were replenished with 8 μg/ml PHA, while unstimulated controls were left unstimulated, and all samples were incubated under stationary conditions for up to 5 additional days to assess the time course of recovery following exposure to MMG. At various time intervals during the stationary recovery period, samples were labeled for analysis by flow cytometry and/or loaded in triplicate into 96-well plates for a 4 h proliferation assay. Supernatants were analyzed for cytokine content (IL-2 and IFN-α/β and -γ), as described above.
Immunophenotyping by flow cytometry

Following multiple washes, $5 \times 10^5$ PBMCs from each sample of interest were resuspended in PBS containing fluorochrome-conjugated antibodies (eBioscience) to CD4 (Cyc), CD8 (PE), CD69 (APC), and CD25 (Fitc) and incubated on ice in the dark for 30 min. Samples were then washed, fixed in 1% paraformaldehyde (PFA) solution, and stored at 4°C until analysis on FACSCanto using FACSDiva software (BD Biosciences, San Jose, CA).

Statistical analysis

All original data represent samples in triplicate. The number of independent repetitions (e.g., individual blood donor samples) per assay ($n$) is indicated where appropriate, and the error bars depict SEM. The statistical significance of the comparisons between stationary and MMG conditions was evaluated using one-way ANOVA and Tukey’s post hoc test for multiple comparisons. The data in Fig. 3 were analyzed by two-tailed paired t test. Significance for all data was set at the 95% confidence level, such that $P < 0.05$ was required for statistical significance. Individual $P$ values are listed for each figure.

RESULTS

Suppressed proliferation of PBMCs in modeled microgravity

PBMCs from young human donors were isolated, stimulated with PHA, and cultured in HARV for 48 h. MMG by HARV-type RWV completely eliminated PHA-induced proliferation to the level of unstimulated stationary control (Fig. 2A) ($P<0.001$). A similar complete suppression of PBMC proliferative response in HARV was demonstrated at 24 and 72 h and further verified by 24, 48, and 72 h cultures of mouse splenocytes stimulated with concanavalin A (ConA, Sigma), a nonspecific mitogen similar to PHA (data not shown).

To further characterize the kinetics of the suppressed proliferative response in MMG, samples were taken from stimulated stationary and stimulated HARV cultures at 2, 4, 6, and 24 h and assayed for PBMC proliferation. As shown in Fig. 2B, MMG inhibited PHA-stimulated proliferation of PBMCs by 50% at 6 h ($P<0.05$) and completely by 24 h ($P<0.001$).

To determine if the loss in PHA-induced proliferation of PBMCs in MMG was due to cell damage in the HARV resulting in a decrease in cell viability, samples of PBMCs that had been subjected to stimulated stationary or stimulated HARV culture conditions for 48 h were first counted by trypan blue staining. We consistently observed that cell losses were similar, yielding no statistically significant difference between conditions (data not shown). Next, to quantify the percentage of dead T cells, $1 \times 10^6$ cells from each group were labeled with anti-CD3 and EMA, which will not permeate the intact membranes of live cells, and analyzed by flow cytometry. Neither MFI nor percent of T cells positive for EMA was significantly different following 48 h in stimulated stationary or stimulated HARV conditions (data not shown), definitively indicating that reduced proliferation is not due to an increase in cellular damage or death in the HARV-treated samples compared with stimulated stationary PBMCs.
Recovery of proliferation following space flight

To investigate the recovery of suppressed mitogen-stimulated proliferation following exposure to MMG, we first quantitated the recovery of cell-mediated immunity following space flight from shuttle data published by Taylor and Dardano in 1983 (10). Preflight data were collected 2, 10, and 30 days before launch date and are presented as the mean of these values. As seen in Fig. 3, PHA-stimulated lymphocyte proliferation immediately postflight was decreased compared with preflight and 3- to 5-day postflight responses \((P<0.05)\). Three- to 5-day postflight proliferation response did not differ statistically from preflight values.

Recovery of proliferation following exposure to modeled microgravity

After culturing PBMCs in HARVs or stationary conditions for 48 h, the cells were resuspended at an equal concentration of \(1 \times 10^6\) cells/ml, and recovered under stationary conditions, as described in Materials and Methods. At 24 h of recovery (Fig. 4A) the PHA-stimulated proliferation of HARV cultures was 8% of stimulated stationary controls, which was not statistically different from that of unstimulated controls. By 48 h (Fig. 4B), the proliferative response of PBMCs previously exposed to MMG increased to 75% of stimulated stationary controls \((P<0.05)\), indicating 75% (i.e., partial) recovery. Full recovery of the proliferative response, depicted in Fig. 4C, was not observed until 72 h, in agreement with published data from flight studies in which full immune recovery occurred only 3 days after landing. At 72 h of recovery, stationary controls and stimulated HARV samples did not statistically differ in their extent of proliferative response, and both were elevated above unstimulated controls \((P<0.001)\).

In further characterizing the time course of the proliferative recovery, we investigated PBMC proliferation from 0–24 h as well as 72–120 h following exposure to 48 h MMG. PHA-stimulated stationary controls reached maximum proliferation at 48 h, while the PHA-stimulated proliferation of cells recovered after 48 h in HARV peaked at 72 h (Fig. 5). Importantly, these results indicate a delay, but no statistically significant reduction, in the mitogen-stimulated proliferation of PBMCs following exposure to MMG.

Recovery of activation marker expression following exposure to MMG

Recovered PBMCs, as described above, were sampled at various time points during the recovery period and stained with fluochrome-conjugated antibodies for CD4, CD8, CD69, and CD25 for kinetic analysis by flow cytometry. Following stimulation with PHA, T cells not exposed to MMG maximally expressed CD69 at 6 h and CD25 at 24 h (Fig. 6A). In contrast, T cells previously exposed to 48 h in MMG began CD69 expression at 6 h, but maintained expression until a maximum point at 24 h. Maximal expression of CD25 then followed at 48 h poststimulation (Fig. 6B).

Flow cytometry confirmed that recovered cells did not differ in the distribution of T cell subtypes \((CD4+\ vs.\ CD8+)\) compared with stimulated stationary controls. Both unstimulated PBMCs and stimulated PBMCs exposed to MMG demonstrated a CD4+:CD8+ ration of ~3:1, while that of stimulated stationary PBMCs was 1.5:1, indicating either an increase in the CD8+ subset or a decrease in CD4+ T cells. PBMCs stimulated with PHA following 48 h exposure to
MMG appeared similar to stimulated stationary cultures, with a CD4+:CD8+ ratio of 1.5:1 first observed at 6 h poststimulation.

**Recovery of cytokine production following exposure to modeled microgravity**

IL-2 in cell culture supernatants, as detected by ELISA, was reduced by ~80% in samples from HARVs cultured for 24 h (Fig. 7A) and was not detectable in any samples cultured for 48 h or more. The detection of IFN-γ in MMG cultures, while not significantly reduced at 24 h, was indistinguishable from stationary controls at 48 h (Fig. 7B).

Following 48 h exposure to MMG, IL-2 in the supernatants increased from 0 to 24 h of recovery (Fig. 8). Interestingly, IL-2 in supernatants from recovered PHA-stimulated HARV samples was significantly greater than from stimulated stationary samples at 8, 12, and 20 h of recovery ($P<0.05$, Fig. 8A). IFN-γ detected in supernatants was nearly constant from 0-120 h of recovery and did not differ between stimulated stationary and HARV cultures (data not shown). The detection of functional IL-2 and IFN-α/β, as assessed by bioassays, was similar to stimulated stationary cultures by 24 h after exposure to MMG. Functional IL-2 was greatest in supernatant at 24 h of recovery ($P<0.05$, Fig. 8B). IFN-α/β appeared to increase from 24 to 72 h of recovery, although this was not significant (Fig. 8C).

**DISCUSSION**

Exposure to MMG in the HARV-type RWV bioreactor inhibited the PHA-stimulated proliferation of PBMCs, followed by full recovery after 72 h in stationary conditions, clearly recapitulating important changes in cell-mediated immunity also observed during space flight and postflight recovery on the ground. The experimental focus on PBMC cultures, either flown in space or exposed to MMG, eliminates in vivo confounders, such as neuroendocrine signals that result from the physical and psychological stress of space flight (23, 27–29), suggesting that RWV cell culture is perhaps a suitable means for assessing the direct effects of microgravity on cellular immune function. Early studies by Cogoli et al. demonstrated both the inhibitory effect of hypogravity (clinorotation) and the stimulatory effect of hypergravity (centrifuge) on lymphocyte proliferation (7, 22). However, changes in shear stress and fluid dynamics in those systems can also interfere with cellular immune reactivity and must be considered. Improved low-shear culture venues for providing an MMG environment, such as HARV-type bioreactors, are used because some cellular immune changes do appear to be directly influenced by microgravity, independent of culture conditions (6, 24). Our results using nuclear staining with EMA, which cannot permeate an intact cell membrane, confirm that the decreased proliferation response to mitogen in the HARV-type RWV is not due to a loss in cellular viability relative to stimulated stationary controls at a total incubation time of 48 h.

While previous studies have suggested that MMG does not permanently eliminate the ability of resting ($G_0$) cells to respond to a stimulus (25, 28), the recovery of proliferation following exposure to MMG had not been previously characterized. We demonstrate here that the proliferation of PBMCs, completely eliminated by 48 h exposure to MMG, gradually recovered when resuspended at equal concentrations in PHA-replenished media and returned to stationary ground conditions. Recovery was partial at 48 h (75%) and full at 72 h, as compared with stimulated stationary controls. When expressed as a percent of maximal response, the recovery
of the proliferative response following exposure to MMG was delayed, but not reduced, as summarized in Fig. 9. This finding is important in that it further validates RWV bioreactors as high-fidelity analog systems that model certain cell-innate responses to microgravity observed during, as well as following, space flight.

The expression kinetics of T cell activation markers CD69 (very early activation) and CD25 (late activation, IL-2 receptor) can provide additional information on lymphocyte function (30). Studies in MMG have demonstrated a reduction in gene expression of both IL-2 and the IL-2 receptor α-chain (27), and surface expression of CD69 and CD25 have been reported to be decreased in both MMG and flown cell culture (20, 31). The decrease in CD69 expression in flown culture was reported to be less dramatic than the loss of CD25 expression, suggesting that lymphocytes retain some ability to express CD69 in true microgravity, possibly because CD69 is stored intracellularly and requires no new RNA or protein for expression (20). In contrast, CD25 expression, which requires gene transcription in response to activation, was eliminated along with the proliferation response to mitogen (20, 31). IL-2 receptor expression is an absolute prerequisite to T cell proliferation in response to mitogen, while the actual role of CD69 remains poorly understood (32). We demonstrate here an alteration in activation marker expression of stimulated PBMCs following exposure to MMG that is consistent with a relative delay in IL-2 uptake and the proliferation response to mitogen. While stimulated stationary cultures maximally expressed CD69 and CD25 at 6 and 24 h, respectively, PBMCs recovered following 48 h exposure to MMG demonstrated an elongated expression of CD69, beginning at 6 h and reaching a maximum expression at 24 h. CD25 expression reached its maximum at 48 h. In both cases, CD25 expression peaked 24 h before maximum proliferation (Fig. 9A).

Cytokine data from flight- or ground-based cell culture studies are rather limited, although they are important to understanding the cellular immune response to (modeled) microgravity. Production of IL-2 and IFN-α/β and -γ in RWV studies are generally reported as being decreased (21, 22, 24), although there is some controversy with respect to postflight data (19, 33). We report a significant decrease in the detection of IL-2 at 24 h of exposure to MMG and a nonsignificant drop in IFN-γ, as assessed by ELISA. Cooper and Pellis (14) reported an initial suppression of IFN-α/β that returned to normal by day three of culture in the RWV. The data presented here support this finding, suggesting that IFN production overcomes the effects of MMG. No studies, however, have reported on the recovery of cytokine production or function following exposure to MMG. Functional IL-2 in supernatants was biphasic in both stimulated stationary and HARV cultures, reaching a maximum within 24 h of both MMG exposure and recovery and dropping off significantly by 48 h. IL-2 detection in supernatants at 24 or 48 h of recovery did not differ between HARV and stimulated stationary cultures; however, the detection of IL-2 in the early stages following exposure to MMG, that is, <24 h, exceeded that of stimulated stationary cultures. This may likely represent a delay in IL-2 consumption in those cultures recovered from MMG as compared with stimulated stationary controls, which is corroborated by the observed delay in IL-2 receptor expression in these cells. Delayed IL-2 receptor expression, then, appears to predestine the delay in maximal proliferative response observed in PBMCs recovered from 48 h exposure to MMG (Fig. 9A, 9B). IL-2 was not detectable by ELISA in supernatants from any cultures stimulated for 48 h or greater, and the bioassay revealed no functional IL-2 in the supernatants from most subjects at 72 h of recovery.
Taken together, these results suggest that, following early accumulation associated with a relative delay in signaling events leading to receptor expression, IL-2 may have become bound to the IL-2 receptors, thus contributing to the observed gradual recovery of proliferation over a 72 h time period following exposure to MMG. Conversely, there was no difference in IFN-γ in cell culture supernatants, as detected by ELISA, for up to 120 h of recovery. Functional IFN-α/β appeared to possibly increase from 24 to 72 h of recovery, similar to stationary controls. In short, we conclude that the IL-2 that was being produced in cultures recovered from exposure to MMG was being consumed by the proliferating T cells, while IFN was being produced but not consumed. The IL-2 produced by proliferating T cells is also required for proliferation (autocrine), while IFN is not (paracrine), such that the detection of functional IFN in the supernatants of activated T cells may be expected to increase as IL-2 decreases (Fig. 9B).

Our studies extend for the first time the realm of an RWV-based investigation beyond the duration of acute MMG exposure and into the recovery phase. Given the similarities between our findings and those observed during recovery after space flight, our results confirm and broaden the previous notion that RWVs, such as the HARV, may be suitable microgravity analogs for studying the cellular effects of (modeled) microgravity. It is unknown at this time what effects a longer-duration sojourn in space, such as is anticipated within the next few decades, will have on immune function during or following space flight. There exists some limited indication that the severity of immune dysfunction in space is increased with increased flight duration (6, 8). Increased incidence of infectious diseases remains a concern following return from space (5, 6), and the effects of increased flight duration on immune recovery and the possible associated clinical consequences remain entirely unknown. An emerging concern is the unknown effect of long-term microgravity exposure and/or stress-induced suppression of cell-mediated immunity on the reactivation of latent viral infections (34). As a result, continued research must strive to identify prophylactic countermeasures for microgravity- and stress-induced immune suppression in flight, design interventions to bolster immunity in space, and develop agents to enhance postflight immune recovery. Such initiatives are of paramount interest to NASA as delineated in the current Bioastronautics Critical Path Roadmap (35). Further, the notable similarities between microgravity-induced cellular immune suppression and that experienced in aging (36, 37) have led to the suggestion that (modeled) microgravity may serve as a condensed model for the study of long-term age-related immune degradation (5, 38, 39). Insights gained in the investigation of mechanisms of immune recovery following exposure to actual gravity or MMG, as well as the identification of possible nutritional, pharmaceutical, or other such countermeasures to enhance or expedite immune recovery, may lead to advances in anti-aging medicine (40). Our studies reinforce previously published data suggesting that MMG is an innovative, valuable tool for studying multiple aspects of cellular immune function.

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REFERENCES


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Fig. 1

**Figure 1.** Overview of methodology. PMBCs, peripheral blood mononuclear cells; HARV, high-aspect ratio vessel; PHA, phytohemagglutinin.
Figure 2. Suppressed PHA-stimulated proliferation of PBMCs in MMG. **A**) 48 h, n = 8, *P* < 0.001. **B**) Kinetics of suppressed PHA-stimulated proliferation of PBMCs in MMG, 2–24 h, n = 4, individual data points shown, *P* < 0.05, **P** < 0.001. Error bars indicate SEM; CPM, counts per minute.
**Figure 3.** PHA-stimulated lymphocyte proliferation of shuttle astronauts preflight, postflight day of landing, and 3–5 days postflight recovery. *n* = 8 individual data points shown, *P* < 0.05. Horizontal bars indicate means; CPM, counts per minute.
Figure 4. Twenty-four to 72 h recovery of PHA-stimulated PBMCs following 48 h exposure to MMG. A) At 24 h, the proliferative response of stimulated HARV samples (c) was not significantly different from unstimulated controls (a) and was significantly different from stimulated stationary controls (b); n = 12, P < 0.001. B) At 48 h, the proliferative response of stimulated HARV samples (c) remained significantly different from stimulated stationary controls (b); n = 10, P < 0.05. C) By 72 h, stimulated HARV samples (c) demonstrated a proliferative response similar to stimulated stationary controls (b); n = 4. Error bars indicate SEM; CPM, counts per minute.
Figure 5. Four to 120 h recovery of PHA-stimulated PBMCs following 48 h exposure to MMG. Stimulated stationary cultures reached maximum proliferation at 48 h, while HARV cultures peaked at 72 h; $n \geq 6$ for 24–72 h, $n = 3$ for other time points. Error bars indicate SEM; CPM, counts per minute.
Figure 6. Recovery of PHA-stimulated activation marker expression following exposure to MMG. Figure represents average values for a repeated experiment showing CD69 and CD25 expression kinetics for PHA-stimulated stationary condition (A) and PHA-stimulated recovery following 48-hour exposure to MMG (B). Solid lines represent the CD4+ T cell subset, and dashed lines represent the CD8+ subset. MFI, mean fluorescence intensity.
Figure 7. IL-2 and IFN in MMG. A) IL-2 was reduced in supernatant in MMG culture. ELISA, 24 h, n = 4, *P < 0.05. B) Differences in IFN accumulation in supernatants were not significant. ELISA, 24 and 48 h, n = 3. Error bars indicate SEM.
Figure 8. Recovery of IL-2 and IFN following exposure to MMG. A) Zero to 24 h recovery of IL-2 following 48 h exposure to MMG. ELISA, n = 3, *P < 0.05. B) Twenty-four and 48 h recovery of IL-2 following 48 h exposure to MMG. Bioassay, n = 4, *P < 0.05. C) Twenty-four, 48, and 72 h recovery of IFN-α/β following 48 h exposure to MMG. Bioassay, n = 10 for 24–48 h, n = 4 for 72 h. Error bars indicate SEM.
Figure 9. Overview. A) Kinetics of CD69 and CD25 expression in MMG (HARV) relative to stimulated stationary control. Maximal proliferation in HARV was delayed but not reduced (ns). Maximal CD25 (IL-2 receptor) expression, indicated by arrows, preceded peak proliferation by 24 h in both conditions. B) Schematic diagram summarizing the elimination of T-lymphocyte proliferation in 48 h MMG and subsequent functional recovery. Changes in cytokine accumulation in MMG (↑,↓,=) are relative to stimulated stationary culture at the same time points. IL-2 receptor (IL-2R) expression and maximal proliferation response to PHA were delayed in MMG.