Short communication

Sub-mitogenic phorbol myristate acetate co-stimulation rescues the PHA-induced activation of both naïve and memory T cells cultured in the rotating-wall vessel bioreactor

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Abstract

T lymphocytes are unresponsive to T cell receptor (TCR) stimulation during culture in spaceflight or ground-based microgravity analogs such as the rotating-wall vessel (RWV) bioreactor. The TCR-induced activation of a subset of T cells can be rescued in the RWV by co-stimulation with sub-mitogenic doses of phorbol ester (PMA). We report that PMA co-stimulation of primary human T cells cultured in the RWV rescues the phytohemagglutinin (PHA)-induced activation of the CD8$^+$ and CD4$^+$ T cell subsets as well as naïve and memory CD4$^+$ T cells. Importantly, T cells activated in the RWV by PHA + PMA contained these subsets in proportions strikingly similar to control cultures activated with PHA alone. The data indicate that rescuing T cell activation with PMA co-stimulation does not significantly perturb the heterogeneity of the responding cells, and represent an important proof of principle for the design of immune-boosting agents for use in spaceflight.
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1. Introduction

Astronauts experience a decline in T lymphocyte-mediated immunity during spaceflight (Taylor and Dardano, 1983;Taylor and Janney, 1992; Crucian et al., 2000). Microgravity is thought to play a direct role in this decline since T cells are unresponsive to T cell receptor (TCR) stimulation when cultured *in vitro* during orbital-flight and responsiveness can be restored by culture in a 1-g in-flight centrifuge (Cogoli et al.,

1984). Similar results are seen when T cells are cultured in ground-based microgravity analogs such as the rotating-wall vessel (RWV) bioreactor including a complete loss of proliferation, cytokine secretion, and activation-marker expression in response to TCR agonists (Simons et al., 2006; Ritz et al., 2006; Sastry et al., 2001). In both spaceflight and the RWV, T cells remain at least partially responsive to direct activation by diacyl glycerol (DAG) and calcium signaling downstream of the TCR (Hashemi et al., 1999). Ground-based experiments have shown that this result depends primarily upon enhancement of DAG signaling since sub-mitogenic doses of the DAG-mimetic phorbol myristate acetate (PMA), but not inducers of calcium signaling, can rescue TCR-induced proliferation and activation-marker expression by a subset of T cells during culture in the RWV (Cooper and Pellis, 1998).

The human T cell compartment is comprised of CD4$^+$ helper and CD8$^+$ cytotoxic T cells, and each of these populations can be further divided into naïve and memory cells.

These subsets are morphologically and functionally distinct, and have intrinsic differences in their capacity to respond to mitogenic stimuli. It is unclear, therefore, whether the "PMA-responsive" subset of T cells that can become activated in the RWV contains all of these cell-types or is enriched for a single subset. We have now addressed this issue by measuring the responsiveness of the major functional and effector phenotypes of human T cells to concomitant stimulation with PHA and a sub-mitogenic dose of PMA during culture in the modeled microgravity environment of the RWV.

2. Materials and methods

2.1. The rotating-wall vessel bioreactor

Simulated microgravity conditions were generated by culturing cells in HARV-type RWVs (high aspect ratio vessel; Synthecon, Houston, TX, USA) rotated at 14 rpm (Simons et al., 2006). The RWV is a 3-dimensional suspension culture system that utilizes solid-body clinostat rotation to suspend cells in a state of constant free-fall and thereby reduce the effective gravitational force experienced by cultured cells to $0.01 \times g$ (Unsworth and Lelkes, 1998).

2.2. Human PBMC isolation and culture

Human buffy coats were purchased from Biological Specialty Corporation (Colmar, PA, USA) and PBMC were isolated by gradient centrifugation over histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). In all experiments cells were cultured at 10^6/ml in RPMI supplemented with 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin (Mediatech, Herndon, VA, USA), and 10% heat-inactivated FBS (HyClone, Logan, UT, USA). T cells within the PBMC were stimulated with 5 μg/ml phytohemagglutinin (Sigma) with or without 0.5 ng/ml phorbol myristate acetate (Sigma), and cultured for 48 h in tissue culture flasks (static conditions) or an RWV. To measure cell proliferation, 20 μg/ml BrdU (Invitrogen, Carlsbad, CA, USA) was included in the culture media for the duration of the experiment. All experimental procedures were approved by the Drexel University IRB and were in compliance with HIPPA guidelines.

2.3. Immunostaining

At the end of each experiment cells were removed from the culture venues, counted, and immunostained with fluorescently conjugated antibodies against CD3 and/or CD4, and in some experiments for CD25, CD45RA and CD45RO (eBioscience, San Diego, CA, USA). For analysis of proliferation, surface-stained cells were fixed overnight at 4 °C in 1% paraformaldehyde and immunostained with a BrdU-specific antibody (Invitrogen) as follows. Briefly, the cells were washed with PBS and permeabilized with PBS + 0.1% Triton X-100. Genomic DNA was subsequently denatured by incubation in 50 kunitz units of DNAse I (Sigma) per 10^6 cells for 30 min at 37 °C. The cells were washed, incubated for 30–60 min on ice

in blocking buffer (PBS + 0.1% Triton + 0.5% BSA). The cells were then immunostained for 30 min on ice, washed, and analyzed immediately by flow cytometry.

2.4. Flow cytometry

Flow cytometric data were collected for analysis on a BD FACSCanto flow cyometer using FACSDiva software. Flow data were analyzed using FlowJo (Treestar, Ashland, OR, USA). Naïve and memory cells were identified by their differential expression of the RA and RO isoforms of CD45 (Gray, 1993). Memory T cells express high levels of CD45RO but relatively low levels of CD45RA, and were therefore gated as CD45RA^{low}RO^{high}. This expression pattern is reversed on resting naïve T cells. Naïve T were gated as CD45RA^{high}CD45RO$^{low \rightarrow high}$, however, to account for the rapid upregulation of CD45RO that occurs in response to activating stimuli such as PHA (see Fig. 2d) (Picker et al., 1993).

2.5. Statistical analysis

Statistical differences between samples were detected by a Student's T test or by a one-way ANOVA with Tukey or Dunnett post-tests as appropriate. Statistical calculations were made using the InStat software package (Graphpad Software, San Diego, CA, USA).

3. Results

3.1. Proliferation of CD4$^+$ and CD8$^+$ T cells in response to PHA + PMA stimulation in the RWV

We first measured the capacity of sub-mitogenic PMA to confer PHA responsiveness upon the CD4$^+$ and CD8$^+$ T cell subsets (identified here as CD3$^+$CD4$^+$ and CD3$^+$CD4$^-$, respectively) during culture in the RWV. Under static conditions PHA stimulation induced 36% of CD4$^+$ and 43% CD8$^+$ T cells to enter the cell cycle (Fig. 1). The addition of sub-mitogenic PMA (PHA + PMA) significantly increased the fraction of proliferating cells to 81 and 78% respectively ($p < 0.001$; $n = 4$). Stimulation with PHA alone failed to induce a proliferative response in the RWV (data not shown). By contrast, stimulation with PHA + PMA in the RWV resulted in BrdU incorporation by 24% of CD4$^+$ and 31% of CD8$^+$ T cells after 48 h in culture. The activation of neither subset appeared to be favored by PMA co-stimulation in the bioreactor since the ratio of CD4$^+$/CD8$^+$ T cells within the CD3$^+$BrdU$^+$ population was not significantly changed compared to static controls (2.25 ± 0.54, static vs. 1.91 ± 0.06, RWV; $p > 0.05$, $N = 4$)

3.2. CD25 expression by naïve and memory CD4$^+$ T cells in response to PHA + PMA stimulation in the RWV

We next compared the ability of PMA to rescue the activation of naïve and memory CD4$^+$ T cells in the RWV. The high-affinity IL-2 receptor α-chain (CD25) is not expressed by
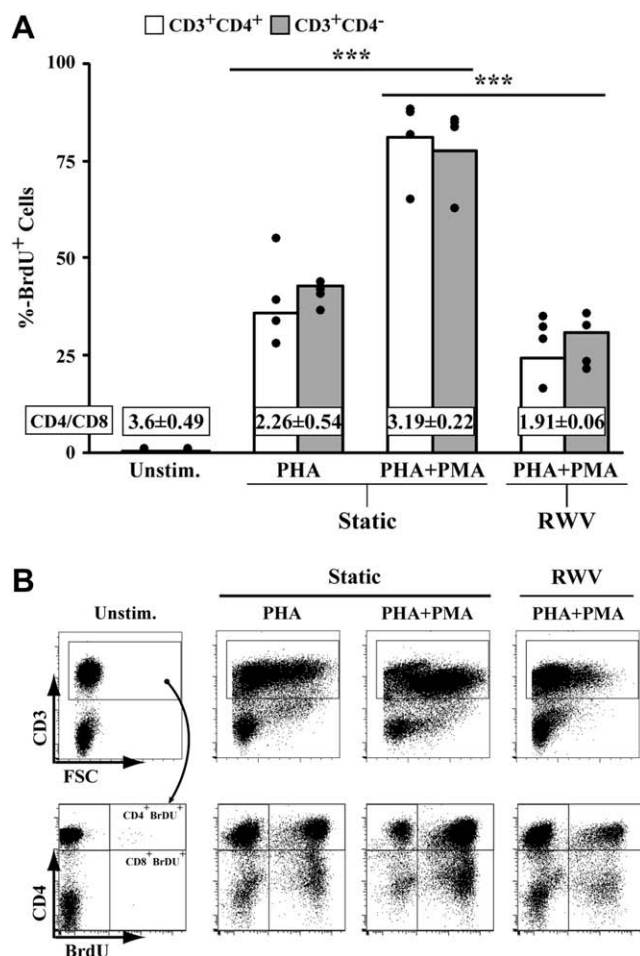
Fig. 1. BrdU incorporation by CD4$^+$ and CD8$^+$ T cells in response to PHA + PMA stimulation in the RWV. Human PBMC were stimulated with PHA or PHA + PMA in the RWV or static conditions. PBMC left unstimulated under static conditions were included as a control. After 48 h, cells were removed from the culture venue and processed for flow cytometry with surface staining for CD3 and CD4 and intracellular staining for BrdU. A. The percentage of CD3$^+$CD4$^+$ (open bars) or CD3$^+$CD4$^-$ (i.e. CD8$^+$; filled bars) PBMC that were BrdU$^+$. The CD4$^+$/CD8$^+$ ratio (mean ± SD) of BrdU$^+$ T cells (stim. samples) or of resting T cells (unstim. sample) is overlayed on each sample. Data from individual experiments are shown as dots; each bar represents the mean of 4 independent experiments. ***$p < 0.001$. B. Representative flow data from a single experiment. Dot plots in the upper row show the CD3$^+$ gate used to limit analysis to T cells. BrdU incorporation and CD4 expression by cells falling within this gate are shown in the lower panels.

resting conventional T cells, but becomes rapidly upregulated in response to mitogens such as PHA. Stimulation of PBMC with PHA for 48 h under static conditions resulted in the upregulation of CD25 on 85% of both naïve and memory CD4$^+$ T cells, and this fraction was increased to 97% of each subset by co-stimulation with PMA (Table 1). In contrast, stimulation with PHA + PMA in the RWV induced CD25 upregulation on 73% of memory T cells but only 54% of naïve cells. Direct comparisons between memory and naïve T cells were made by calculating the relative fraction of each population (T$_{mem}$/T$_{naïve}$) within CD4$^+$CD25$^+$ PBMC (or CD4$^+$ PBMC in unstimulated conditions). On average, unstimulated CD4$^+$ PBMC contained more than twice as many memory as

naïve T cells (T$_{mem}$/T$_{naïve}$ = 2.35; Fig. 2a). Stimulation with PHA or PHA + PMA in static conditions resulted in an inversion of the T$_{mem}$/T$_{naïve}$ ratio within the activated population of T cells (T$_{mem}$/T$_{naïve}$ ~ 0.6 for both PHA and PHA + PMA). A similar, albeit less pronounced, enrichment of naïve cells within the CD4$^+$CD25$^+$ population was also seen in response to PHA + PMA stimulation during culture in the RWV (T$_{mem}$/T$_{naïve}$ = 1.4).

To better understand this result we estimated the sensitivity of each subset to stimulation in the RWV by using the median fluorescence intensity (MFI) of staining to compare levels of CD25 expression (Fig. 2c and d). Under static conditions PHA and PHA + PMA induced naïve and memory T cells to express high levels of CD25 with only slightly higher CD25 expression on memory T cells. The bulk of memory T cells activated in the RWV also expressed high levels of CD25. The MFI of this expression, however, was markedly reduced compared to cells activated with PHA or PHA + PMA in static conditions. By contrast surface expression of CD25 by naïve T cells in the RWV was heterogeneous with only a fraction of cells expressing high surface levels of CD25.

3.3. Proliferation of naïve and memory CD4$^+$ T cells in response to PHA + PMA stimulation in the RWV

Expression of CD25 occurs early in the sequence of events that lead to full T cell activation, and confers responsiveness to the autocrine cytokine IL-2, which drives the proliferation of activated T cells. Decreased expression of this receptor on PHA + PMA stimulated T cells in the RWV raised the possibility that many of these cells were not competent to progress further down the activation pathway. We therefore used BrdU incorporation to examine proliferation by naïve and memory T cells activated with PHA + PMA in the RWV. In static conditions, PHA stimulation resulted in roughly equal fractions (58 and 54%) of naïve and memory CD4$^+$ T cells incorporating BrdU into their DNA (Table 1). Introducing sub-mitogenic PMA as co-stimulus induced an additional 23% of cells within each subset to incorporate BrdU in static conditions. PHA + PMA stimulation in the RWV generated less than half the number of activated cells as stimulation with PHA alone in static conditions (25 vs. 57%), but in contrast to our CD25 data, this reduction was seen to equally affect naïve and memory CD4$^+$ T cells (~25% of each subset incorporated BrdU). When directly comparing naïve and memory cells by calculating the T$_{mem/naïve}$ ratio, it was found that in static conditions the CD4$^+$BrdU$^+$ subset was enriched in naïve T cells compared to CD4$^+$ cells left unstimulated for the duration of the experiment (T$_{mem}$/T$_{naïve}$ = 0.58 vs. 2.33 respectively; Fig. 2b). A similar enrichment of naïve T cells was also observed within CD4$^+$BrdU$^+$ cells activated by PHA + PMA in the RWV (T$_{mem}$/T$_{naïve}$ = 0.77).

4. Discussion

We have phenotypically characterized the subset of T cells that respond to PHA stimulation in the RWV when

Table 1
Percentage[a] of T cell subsets activated in the RWV by PHA or PHA + PMA.

Venue	Stimulus	%-CD25+			%-BrdU+		
		CD4+	Naïve	Memory	CD4+	Naïve	Memory
Static	Unstim	3.1 ± 2.1	1.1 ± 0.7	1.7 ± 0.7	0.2 ± 0.2	0.5 ± 0.6	0.1 ± 0.1
Static	PHA	87.2 ± 2.2	84.8 ± 1.5	85.2 ± 2.1	57 ± 5.0	58.2 ± 6.3	54.4 ± 10.4
Static	PHA + PMA	97.8 ± 1.3	96.4 ± 1.2	97.5 ± 0.5	84.4 ± 1.2	86.7 ± 1.8	78.8 ± 1.7
RWV	PHA + PMA	65.1 ± 16.4	53.6 ± 21.4	72.8 ± 3.8	25.3 ± 2.5	25.3 ± 1.9	25.6 ± 7.1

[a] Data are the percentage of CD25+ or BrdU+ cells within the indicated populations, and are the means ± SD of three independent experiments.

sub-mitogenic PMA is used as co-stimulus. The key finding of this study is that PMA co-stimulation induces the activation of both helper and cytotoxic T cells in the RWV, and that the responding population contains naïve and memory T cells in proportions similar to static controls. Previous reports of rescued T cell activation in the RWV measured proliferation or CD25 expression by bulk T cells, and given the natural heterogeneity of this population the functional identity of the responding cells was unclear (Cooper and Pellis, 1998; Hashemi et al., 1999).

We set out by testing the hypothesis that PMA co-stimulation favors the activation of CD8+ T cells in the RWV, since this subset has a greater intrinsic capacity for proliferation than CD4+ T cells (Seder and Ahmed, 2003; Foulds et al., 2002). Contrariwise, however, we found that neither CD4+ nor CD8+ T cells were preferentially induced to proliferate by PMA co-stimulation in the RWV. Memory T cells have a lower threshold of TCR-induced activation than their naïve counterparts; and a second possibility considered here is that PMA co-stimulation activated memory but not naïve T cells in the RWV (Gray, 1993). In support of this hypothesis, a greater fraction of the memory T cell population expressed CD25 in response to PHA + PMA in the RWV, and these cells expressed higher levels of CD25 than their naïve counterparts (see Fig. 2d, lower panels). However, when we compared the proportion of naïve to memory cells within the CD4+CD25+
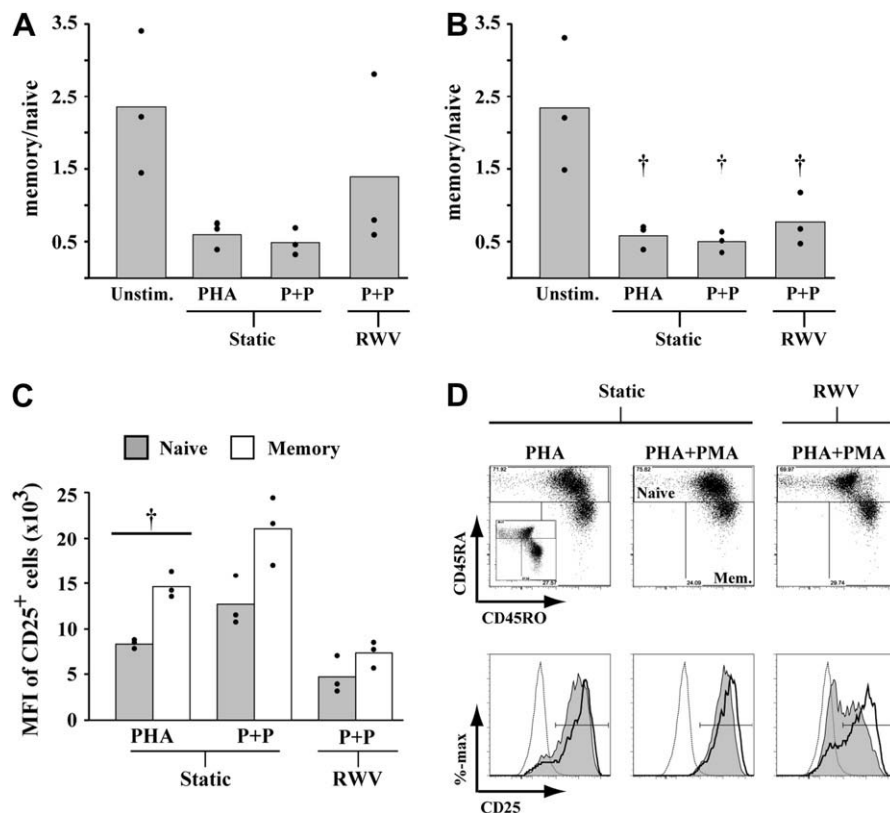

Fig. 2. Activation of naïve and memory CD4+ T cells in response to PHA + PMA stimulation in the RWV. Human PBMC were stimulated with PHA or PHA + PMA in the RWV or static conditions. PBMC left unstimulated under static conditions were included as a control. After 48 h, cells were removed from the culture venue and processed for flow cytometry with immunostaining for CD4, CD45RA, CD45RO, and CD25 or BrdU. **A & B.** The ratio of memory to naïve T cells within A. CD4+CD25+ or B. CD4+BrdU+ PBMC or within CD4+ PBMC left unstimulated for 48 h (unstim.). C. The MFI of CD25 staining by naïve and memory T cells. D. Representative flow data for CD25 staining from a single experiment. Dot plots in the upper row are gated on CD4+CD25+ cells and show the gates used to identify naïve and memory CD4+CD25+ T cells (similar gating was used for CD4+BrdU+ T cells); the inset shows unstimulated cells. The bottom panels are histograms of the intensity of staining for CD25 by naïve (filled histogram), memory (bold line), and unstimulated CD4+ (open histogram) T cells. In **A**, **B**, and **C**, data from individual experiments are shown as dots; bars are the means of 3 independent experiments. †$p < 0.05$. P + P = PHA + PMA stimulation.

population, naïve T cells predominated in both the RWV and static conditions. This difference between the apparent sensitivity and relative responsiveness of naïve and memory T cells is likely to be due to enhanced proliferation of naïve cells rather than a selective loss of memory cells. This conclusion is supported by the finding that naïve T cells were enriched within the $CD4^+BrdU^+$ subset in both static conditions and the RWV, and is in line with observations that T cell proliferation is inversely correlated with maturational stage, so that naïve T cells are capable of undergoing more rounds of division than memory cells (Lanzavecchia and Sallusto, 2005).

Our data therefore do not support the hypothesis that PHA + PMA preferentially activates memory T cells in the RWV, since any advantage conferred upon memory T cells by an enhanced sensitivity to mitogenic stimulus is counterbalanced by the greater proliferative capacity of naïve T cells. Ultimately, therefore, any bias towards memory cell activation is insufficient to significantly perturb the heterogeneity of the activated $CD4^+$ T cell subset.

These findings are of particular relevance to the development of nutritional or pharmacological supplements that counteract immune senescence during spaceflight. Spaceflight presents a number of risks to astronaut health including the reactivation of latent viral infections, enrichment of environmental pathogens (due to air-recycling), and an increased tumor risk from exposure to cosmic radiation, which necessitate fully functional, if not heightened immune surveillance (Mehta et al., 2004; NASA, 2005). The heterogeneity of the T cell compartment reflects the ability of these cells to respond to a variety of pathogens and environmental insults, and its maintenance is essential to immune surveillance. It is therefore critical that immunological countermeasures designed for use in spaceflight act on both T cell subsets and boost not only established immunity in the form of memory T cells, but also naïve T cells capable of responding to novel pathogens. Our data represent an important proof of concept demonstrating that a uniform enhancement of T cell responsiveness can be achieved in simulated microgravity by manipulation of lipid signaling.

Acknowledgments

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