Cell Biology International 33 (2009) 882–886

Cell Biology International

www.elsevier.com/locate/cellbi

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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Received 16 November 2008; accepted 29 April 2009

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.

Keywords: Microgravity; Modeled microgravity; Immune response; Countermeasure

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.

Abbreviations: TCR, T cell receptor; RWV, rotating-wall vessel; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; DAG, diacyl glycerol; PBMC, peripheral blood mononuclear cells.

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doi:10.1016/j.cellbi.2009.04.024

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 ×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