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Intact T Cell Receptor Signaling by CD4$^+$ T Cells Cultured in the Rotating Wall-Vessel Bioreactor

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ABSTRACT

T lymphocytes fail to proliferate or secrete cytokines in response to T cell receptor (TCR) agonists during culture in spaceflight or ground-based microgravity analogs such as rotating wall-vessel (RWV) bioreactors. In RWVs, these responses can be rescued by co-stimulation with sub-mitogenic doses of the diacyl glycerol (DAG) mimetic phorbol myristate acetate. Based on this result we hypothesized that TCR activation is abrogated in the RWV due to impaired DAG signaling downstream of the TCR. To test this hypothesis we compared TCR-induced signal transduction by primary, human, CD4$^+$ T cells in RWV, and static culture. Surprisingly, we found little evidence of impaired DAG signaling in the RWV. Upstream of DAG, the tyrosine phosphorylation of several key components of the TCR-proximal signal was not affected by culture in the RWV. Similarly, the phosphorylation and compartmentalization of ERK and the degradation of IκB were unchanged by culture in the RWV indicating that RAS- and PKC-mediated signaling downstream of DAG are also unaffected by simulated microgravity. We conclude from these data that TCR signaling through DAG remains intact during culture in the RWV, and that the loss of functional T cell activation in this venue derives from the affect of simulated microgravity on cellular processes that are independent of the canonical TCR pathway. J. Cell. Biochem. 109: 1201–1209, 2010.

KEY WORDS: MICROGRAVITY; LYMPHOCYTE; SIGNAL TRANSDUCTION; ACTIVATION; SPACEFLIGHT

Spaceflight has profound effects on astronaut physiology including decreased immunocompetence [Taylor and Dardano, 1983; Taylor et al., 1986; Committee on the Longitudinal Study of Astronaut Health, 2004]. Astronauts exhibit impaired responses in both ex vivo and in vivo assays of T cell function during and immediately following orbital flight suggesting that decreased immune function stems from a defect in the T cell compartment [Taylor and Janney, 1992; Crucian et al., 2000]. In support of this notion, T cells do not proliferate or secrete cytokines when stimulated with T cell receptor (TCR)-agonists in vitro during spaceflight [Cogoli et al., 1984; Pippia et al., 1996; Hashemi et al., 1999]. These responses are rescued by culturing the cells in a 1-g in-flight centrifuge indicating that this inhibition is largely due to microgravity. Accordingly, T cells are unresponsive to mitogenic lectins and antibodies during culture in ground-based microgravity analogs such as the rotating wall-vessel (RWV) bioreactor [Cooper et al., 2001; Sastry et al., 2001; Ritz et al., 2006; Simons et al., 2006]. Preliminary experiments in the RWV have provided the first hints of a mechanism by which microgravity impairs T cell activation. Specifically, the activation of T cells by TCR agonists can be rescued in the RWV by co-stimulation with sub-mitogenic doses of the diacyl glycerol (DAG)-mimetic PMA, implying a crucial role for DAG signaling in conferring T cell sensitivity to microgravity [Cooper and Pellis, 1998; Hashemi et al., 1999; Simons et al., 2009].

The primary source of DAG downstream of the TCR is phospholipase C-γ1 (PLCγ1). TCR-induced activation of PLCγ1 requires the phosphotyrosine-mediated assembly of an ad hoc signaling network at the plasma membrane [summarized in Fig. 1, and reviewed in Simeoni et al., 2005]. This process is initiated by the phosphorylation of immune receptor tyrosine-based activation motifs (ITAMs) within the intracellular domains of the TCR complex allowing the recruitment and activation of the syk-family kinase ZAP-70 (ζ-chain-associated protein, 70 kDa). Subsequent phosphorylation of the adaptor proteins LAT (linker for activation in

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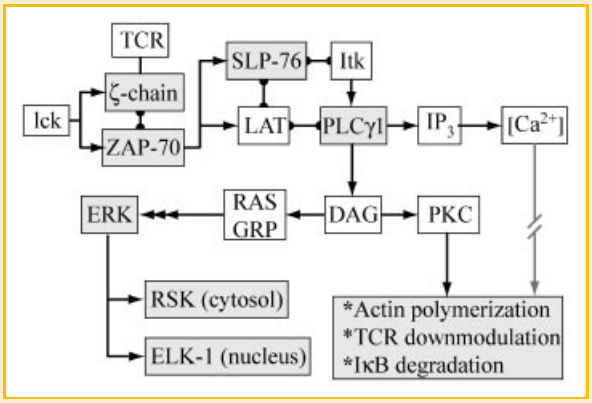

Fig. 1. TCR signaling through DAG. TCR-triggering induces the phosphorylation of the TCR ζ-chains and ZAP-70 by the tyrosine kinase lck. ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76, which form the backbone of a multimeric signaling complex containing PLCγ1 and Itk. Upon phosphorylation by Itk, PLCγ1 cleaves PIP_2 to release DAG and IP_3. The major downstream targets of DAG are PKCθ and RASGRP. PKCθ plays a role in many cellular processes including signaling to the nucleus via NFκB. RASGRP initiates RAS signaling to ERK. ERK has multiple substrates that are essential to T cell activation including ELK-1 in the nucleus and RSK in the cytosol. Components of these pathways that were examined in this study are shaded.

T cells) and SLP-76 (SH2-containing lymphocyte protein, 76 kDa) by ZAP-70 nucleates the assembly of a multimeric signaling complex that recruits and activates PLCγ1 to generate DAG. The DAG produced by this pathway couples the TCR to ERK- and NFκB-signaling via RAS and PKCθ, respectively, and is essential for initiating the genetic program of T cell activation [Mor and Philips, 2006; Hayashi and Altman, 2007].

Ultimately, signals emanating from the TCR drive the expression of activation markers, secretion of cytokines, and proliferative response that characterizes T cell activation. The inability of T cells to execute this program during culture in spaceflight or the RWV support the hypothesis that key aspects of these signals require the presence of gravity for their function. The ability of PMA to synergize with TCR agonists during simulated microgravity culture in the RWV suggests that this gravitational sensitivity lies within the processes that initiate or respond to DAG signaling. In the present study we have tested this hypothesis directly by measuring TCR-induced DAG signaling in primary, human, $CD4^+$ T cells cultured in the RWV. Surprisingly we found little difference in the TCR-driven signals generated by T cells maintained in the RWV compared to those stimulated under static conditions. We conclude from these data that antigen-receptor signaling by T cells cultured in the RWV is intact, and that functional readouts of T cell activation such as proliferation become decoupled from the TCR signal due to the affect of simulated microgravity on processes that are independent of canonical TCR signaling.

MATERIALS AND METHODS

CHEMICAL REAGENTS AND ANTIBODIES

T cells were activated by receptor cross-linking with mouse monoclonal antibodies against CD3 and CD28 (eBioscience, San Diego, CA), and a goat $F(ab')_2$ against mouse IgG (Upstate, Charlottesville, VA). Immunofluorescent staining of T cell surface markers utilized antibodies against CD3 and CD4 from eBioscience. BrdU incorporation assay was performed using BrdU and an anti-BrdU monoclonal antibody both from Invitrogen/Molecular Probes (Carlsbad, CA). For intracellular staining of site-specific phosphorylation of tyrosine (pY), threonine (pT), and serine (pS), antibodies recognizing pY242 of the TCR ζ-chains, pY319 of ZAP-70, pY128 of SLP-76, and pY783 of PLCγ1 were purchased from BD Biosciences (San Jose, CA), and antibodies against dually phosphorylated Erk-1/2 (pT202/pY204; clone 197G2), pS383 of Elk-1, and pT573 of p90RSK were purchased from Cell Signaling Technology (Danvers, MA). Antibody against IκBα (Cell Signaling Technology) was also used for intracellular staining. Alexafluor 488-conjugated goat anti-rabbit $F(ab')_2$ (Invitrogen) was used for secondary detection of unconjugated antibodies. Western blots used antibodies against pY319 of ZAP-70 (BD), pY783 of PLCγ1 (Invitrogen/Biosource) or pan-specific antibodies against ZAP-70 and PLCγ1 from Cell Signaling Technology and Sigma–Aldrich (St. Louis, MO), respectively. For measurement of F-actin TRITC-conjugated phalloidin was purchased from Sigma–Aldrich.

CELL ISOLATION AND CULTURE

Buffy coats from healthy human donors were purchased from Biological Specialty Corporation (Colmar, PA) and PBMC were purified by gradient centrifugation over histopaque 1077 (Sigma–Aldrich). PBMC were cultured in RPMI supplemented with 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin (Mediatech, Herndon, VA), and 10% heat-inactivated FBS (HyClone, Logan, UT). Untouched $CD4^+$ T cells were purified from PBMC by magnetic depletion of unwanted cells using the $CD4^+$ T cell isolation kit II and an AutoMACSpro cell sorter from Miltenyi Biotec (Auburn, CA), or were purchased directly from the human immunology core facility of the University of Pennsylvania. The purified cell suspensions used in these experiments contained >90% $CD4^+$ T cells. The blood cells purchased for each experiment described in this report were purified from unique donors. During the course of this study cells were isolated from >20 anonymous donors, and no donor contributed blood to more than one experiment. All experiments were approved by the Drexel University Internal Review Board, and conducted in accordance to HIPAA guidelines.

T CELL STIMULATION IN THE RWV

The RWV is a zero-headspace suspension culture system. In this system the culture media and vessel rotate as a solid-body, suspending the cells in a state of constant freefall while randomizing their gravitational vector. The effective gravitational force experienced by the cells cultured in the RWV is ~1/100th that of normal gravity (a state often referred to as simulated microgravity) [Unsworth and Lelkes, 1998]. Tyrosine phosphorylation of TCR signaling components occurs within seconds of receptor triggering. Accordingly our experiments were designed so that the cells could be stimulated directly within the dynamic culture environment of the RWV. To accomplish this, T cells were first labeled with mouse monoclonal antibodies against CD3 and CD28 on ice. A portion of

the labeled cells were then transferred to a 15 ml centrifuge tube for the static control and the remainder of the cell suspension was used to load a 10 ml HARV-type (High Aspect Ratio Vessel, Synthecon, Houston, TX) RWV to capacity under slightly negative pressure. Both samples were incubated briefly at 37°C to allow the cell suspension to warm prior to stimulation. The RWV was rotated at 14 rpm during this period [Simons et al., 2006]. The cells were subsequently stimulated by direct addition of 10× anti-mouse IgG. This was achieved without stopping rotation of the HARV by simultaneously injecting 0.5 ml of antibody solution into each of the RWV's two syringe ports. The stimulated cells were incubated at 37°C for 5–90 min and then processed for flow cytometry or Western blot as described below. The final concentration of antibodies in stimulated samples was 2 μg/ml each of anti-CD3 and -CD28, and 10 μg/ml goat anti-mouse IgG (CD3 × CD28). An unstimulated control was included in all experiments to measure basal tyrosine phosphorylation.

IMMUNOSTAINING OF CELL SURFACE MARKERS FOR FLOW CYTOMETRY

T cells were immunostained for surface markers as follows. The cells were washed with PBS containing 1% FBS (FACSwash), stained for 30 min on ice with antibodies against surface markers diluted in FACSwash, washed, and stored in 1% paraformaldehyde (PFA) until analysis.

BrdU INCORPORATION ASSAY

Human PBMC were stimulated as described above and cultured for 48 h in the presence of 10 μg/ml BrdU in an RWV or in a tissue culture flask. Cells were surface-stained as indicated above, fixed in 1% PFA and BrdU incorporation was assessed by intracellular staining using 0.1% Triton X-100 to permeabilize the cells and 50 U/ml DNAse I to denature cellular DNA.

INTRACELLULAR IMMUNOSTAINING

T cell stimulation was terminated by the direct addition of pre-warmed, 4% PFA to each sample at a ratio of 1:1. For cells stimulated in the RWV, this was accomplished by withdrawing the cell suspension into a 10 cm^3 syringe half-filled with 4% PFA without stopping rotation of the bioreactor. In experiments where several time points were measured a 1.5×10^6 cell aliquot was removed from one syringe port of the RWV as above while an equal volume of media was simultaneously injected into the opposite syringe port. Cells were then processed for intracellular staining as published previously [Perez and Nolan, 2002]. Briefly, cells were fixed in 2% PFA for 10 min at 37°C and permeabilized for at least 30 min (and no longer than overnight) in 95% methanol at 4°C. Cells were then washed and blocked for 30 min at room temperature in PBS containing 15% normal mouse serum (Sigma). Aliquots from each sample were subsequently stained for 60 min on ice with antibodies against IκBα or phospho-specific epitopes of the ζ-chains, ZAP-70, SLP-76, PLCγ1, ERK, RSK, or Elk-1. Cells were then stained with an alexafluor 488-conjugated secondary antibody as necessary and analyzed immediately by flow cytometry.

F-ACTIN STAINING

Experiments that analyzed actin polymerization were terminated as described above except the cells were stored overnight at 4°C in PFA following fixation at 37°C. Cells were subsequently washed with PBS and permeabilized by incubation for 5 min with 0.1% Triton X-100 in PBS. Excess Triton was washed from the cells and they were then incubated for 30 min in TRITC-conjugated phalloidin at 0.5 μg/ml, washed and analyzed by flow cytometry.

SAMPLE PROCESSING FOR WESTERN BLOT

T cell stimulation was terminated by rapidly cooling the samples to 4°C in the presence of a phosphotyrosine phosphatase inhibitor. For the static control samples this was accomplished by adding an excess of ice-cold 50 μM Na_3VO_4 in PBS (vanadate buffer) and immediately placing the sample on ice. Cells cultured in the HARV

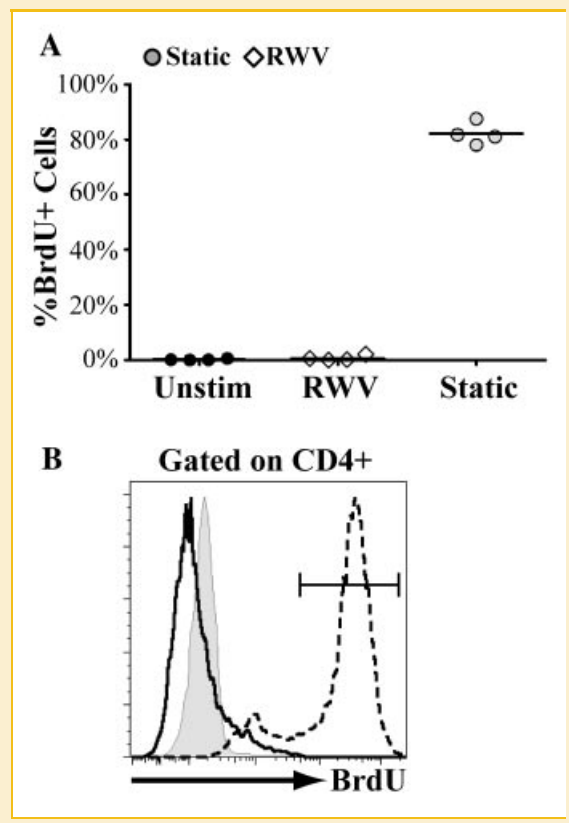

Fig. 2. BrdU incorporation by CD3 × CD28 stimulated $CD4^+$ PBMC cultured in the RWV or static conditions. Human PBMC were stimulated by CD3 × CD28 and cultured with BrdU in an RWV or under static conditions. All antibody concentrations were identical to those used for measurement of TCR signaling. After 48-h cells were removed from the culture venue, immunostained for CD4 and BrdU, and analyzed by flow cytometry. A: The percentage of $CD4^+$ T cells that were $BrdU^+$. Data are from four independent experiments. Each experiment used cells purified from the blood of a unique donor. B: A representative histogram from a single experiment showing the fluorescence intensity of BrdU staining by $CD4^+$ cells stimulated in the RWV (bold line), under static conditions (dashed line), or left unstimulated for the duration of the experiment (shaded histogram).

were rapidly removed from the RWV using a 10 cm^3 syringe and transferred to a 15 ml tube containing 5 ml of vanadate buffer on ice. The cells were then pelleted and lysed by suspension in buffer consisting of 10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM $Na_2P_4O_7$, 1 mM Na_3VO_4, 1 mM PMSF, 1% NP-40, and protease inhibitor cocktail (Sigma). Lysates were incubated for 30 min on ice, insoluble material was pelleted and supernatants were stored at −80°C. Total protein within the lysates was quantified using a BCA assay kit according to the manufacturer's protocol (Pierce, Rockford, IL).

SDS–PAGE AND WESTERN BLOTS

Five to 20 μg of total protein were separated by SDS–PAGE on 5–15% polyacrylamide gels (Biorad, Hercules, CA), and electro-transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were probed with antibodies against phosphorylated ZAP-70 or PLCγ1, and detected with HRP-conjugated secondary antibodies and enhanced chemiluminescence (Pierce). Blots were then stripped using stripping buffer (Pierce) according to the manufacturer's protocol and reprobed with pan-specific antibodies against ZAP-70 or PLCγ1.

FLOW CYTOMETRY

Flow cytometric data were collected for analysis on a BD FACSCanto flow cytometer using FACSDiva software. Flow data were analyzed using FlowJo (Treestar, Ashland, OR).

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. All statistical calculations were made using the InStat software package (Graphpad Software, San Diego, CA). A *P*-value of <0.05 was considered statistically significant. The number of statistically independent measurements (N) reported for each experiment refers to experimental runs performed on different days using blood cells isolated from unique donors. No donor contributed blood to more than one experiment.

RESULTS

TCR SIGNALING UPSTREAM OF PLCγ1

In line with previous observations, we found that $CD4^+$ T cells failed to proliferate in response to antibody-mediated cross-linking of CD3 and CD28 (CD3 × CD28) during culture in the RWV bioreactor [Fig. 2; see also Cooper and Pellis, 1998; Hashemi et al., 1999]. We hypothesized that this defect was due to impaired DAG signaling during culture in the RWV. Induction of tyrosine kinase activity at the TCR is essential for the generation of DAG in response to agonist ligands. To determine the effect of simulated microgravity on this pathway we used flow cytometry to measure the tyrosine phosphorylation the ζ-chains, ZAP-70, SLP-76, and PLCγ1. Stimulation of $CD4^+$ T cells with CD3 × CD28 for 5 min under static conditions significantly increased the tyrosine phosphorylation of these molecules relative to unstimulated controls; an identical increase in phosphorylation was observed in cells cultured in the RWV ($P < 0.05$, vs. unstim., RWV and static, n = 5; Fig. 3A). Increased phosphorylation was most evident for the ζ-chains and SLP-76; which exhibited 1.8- and 2.3-fold increases in staining intensity, respectively. Similarly, the staining for pZAP-70 was increased 1.5-fold by CD3 × CD28 stimulation regardless of culture venue. A less pronounced (1.1-fold increase), but statistically discernible increase in PLCγ1 phosphorylation was detected in both static and RWV culture. In support of this finding we observed an identical pattern of PLCγ1 phosphorylation in parallel experiments that measured pZAP-70 and pPLCγ1 in cell lysates (Fig. 3C). We conclude from these data that the tyrosine kinase cascade linking the TCR to PLCγ1 is not inhibited by culture in the RWV.

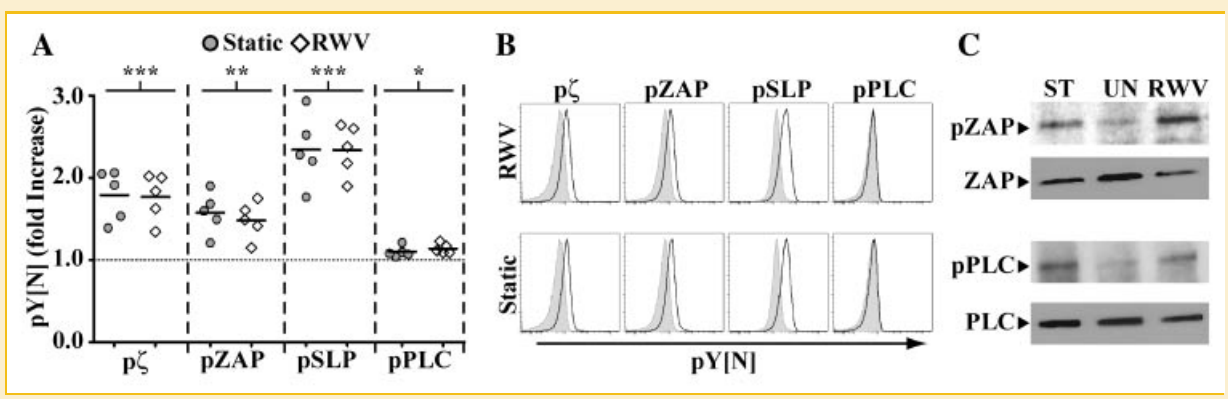

Fig. 3. TCR-proximal signal transduction in the RWV. Purified $CD4^+$ T cells were stimulated by CD3 × CD28 for 5 min during culture in the RWV or under static conditions, and then processed for analysis by flow cytometry or Western blot. A: The median fluorescence intensity (MFI) of immunostaining with the phospho-specific antibodies indicated was assessed by flow cytometry. Data are means ± SD of five independent experiments using cells isolated from the blood of unique individuals for each experiment. Data are presented as the fold increase over unstimulated cells. $^*P < 0.05$ versus unstim.; $^{**}P < 0.01$ versus unstim.; $^{***}P < 0.001$ versus unstim. B: Representative flow data from a single experiment. Filled histograms represent unstimulated cells. Open histograms are cells stimulated in the RWV or static conditions. C: In parallel experiments cells were lysed and phosphorylation of ZAP-70 and PLCγ1 was visualized by Western blot. Representative data from one of three experiments are shown.

TCR SIGNALING DOWNSTREAM OF PLCγ1—PKC TO NFκB

Induction of PKC activity. Members of the PKC family of serine/threonine kinases are direct downstream targets of DAG. Of the PKC isoforms expressed by T cells, PKCθ is the isoform most strongly associated with the earliest stages of T cell activation [Hayashi and Altman, 2007]. T cells lacking PKCθ are unable to engage the actin cytoskeleton in response to CD3 × CD28 stimulation [Sasahara et al., 2002]. Similarly downregulation of the TCR in response to CD3 × CD28 stimulation is impaired in the absence of PKCθ [von Essen et al., 2006]. Actin polymerization and TCR downregulation are therefore early functional markers of T cell activation that strongly correlate with activation of PKCθ and were used as such here. Engagement of the actin cytoskeleton was determined by flow cytometry using a fluorescent conjugate of phalloidin to stain filamentous actin (F-actin) within purified T cells. We noted a significant increase in the F-actin content of $CD4^+$ T cells following 5 min of stimulation in both RWV and static conditions relative to unstimulated controls ($P < 0.05$, $n = 4$; Fig. 4A). We did not, however, detect any significant difference between cells stimulated in the RWV or static conditions. TCR downregulation was determined by measuring CD3 expression by $CD4^+$ PBMC following 48 h of culture in an RWV or static conditions (Fig. 4B). $CD4^+$ cells that were left unstimulated for the duration of the experiment in either venue maintained high expression levels of the TCR. Receptor cross-linking in both culture venues resulted in a significant reduction in the percentage of $CD4^+$ PBMC that expressed high levels of the TCR on their surface ($P < 0.001$, vs. unstim., $n = 4$; RWV and static). Taken together these data indicate that PKC activity, and in particular the activity of PKCθ, downstream of the TCR is not impaired by culture in the RWV.

Induction of NFκB signaling in the RWV. PKCθ promotes signaling to the nucleus via the NFκB family of transcription factors [Sun et al., 2000]. A key step in this pathway is the proteosomal degradation of the IκB proteins, and we used flow cytometry to monitor this process as an indicator of NFκB signaling to the nucleus. TCR cross-linking in both static and RWV conditions resulted in a time-dependent decrease in the percentage of cells that expressed high levels of IκBα (Fig. 5A,B). This reduction was discernable as early as 30 min following stimulation, and by 60 min <75% of cells in both the RWV and static conditions remained $I\kappa B\alpha^{high}$. IκBα degradation appeared to be slightly accelerated in the

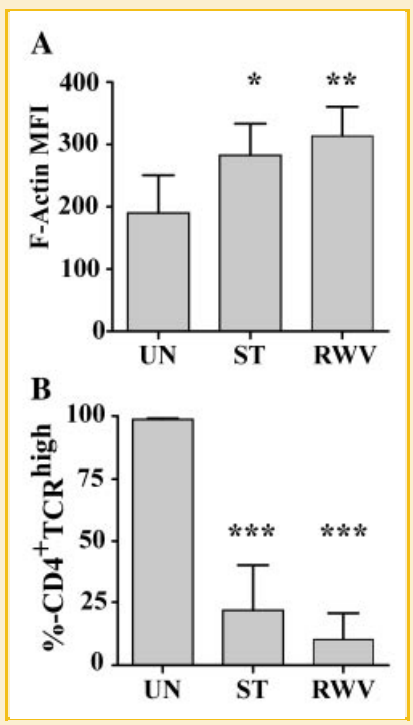

Fig. 4. PKC activity during culture in the RWV. Purified $CD4^+$ T cells (A) or PBMC (B) were stimulated by CD3 × CD28 during culture in an RWV or under static conditions. A: The median fluorescence intensity of F-actin staining after 5 min of stimulation. B: TCR expression by $CD4^+$ T cells was assessed by staining for CD3 after 48 h in culture. Data are means ± SD of four independent experiments. $^*P < 0.05$ versus unstim.; $^{**}P < 0.01$ versus unstim.; $^{***}P < 0.001$ versus unstim.

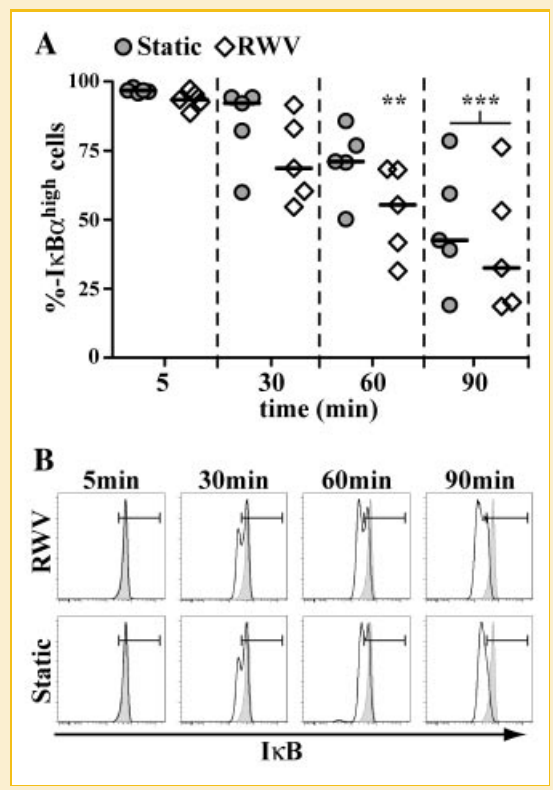

Fig. 5. Induction of NFκB signaling during culture in the RWV. Purified $CD4^+$ T cells were stimulated by CD3 × CD28 in an RWV or under static conditions. A: Cells were removed from the culture venue at the indicated time point and processed for flow cytometry with intracellular staining for IκBα. Data indicate the percentage of cells that were $I\kappa B\alpha^{high}$ as assessed by flow cytometry. Data from individual experiments are shown as symbols, and the median values are indicated by horizontal lines. $^{**}P < 0.01$ versus initial; $^{***}P < 0.001$ versus initial. B: Histograms of IκBα staining intensity by purified $CD4^+$ T cells as assessed by flow cytometry. Representative data from one of five experiments are shown. Filled histograms are of cells left unstimulated until t = 30 min; open histograms are cells stimulated in the RWV or static conditions. Gates used to determine the percentage of $IkB\alpha^{high}$ cells are shown on each plot.

RWV, however, the significance of this observation is unclear, since by 90 min a similar fraction of cells in both culture venues expressed reduced levels of IκB, with $IκB^{high}$ cells typically comprising <50% of the total cell population in both static and RWV conditions ($P < 0.001$ vs. initial, n = 5; RWV and static). We conclude from these data that IκBα is degraded and that NFκB-family transcription factors are capable of reaching their nuclear targets during culture in the RWV.

TCR SIGNALING DOWNSTREAM OF PLCγ1—RASGRP TO ERK

We determined the sensitivity of the RAS/MAPK pathway to simulated microgravity by comparing TCR-induced ERK activation during RWV- and static-culture. In both conditions peak ERK phosphorylation was observed after 5 min of stimulation (Fig. 6A). At this time point, >50% of $CD4^+$ T cells expressed phosphorylated ERK compared to <3% in unstimulated cells ($P < 0.01$ vs. unstim., n = 3, for both RWV and static). Thereafter the frequency of $pERK^+$ cells rapidly declined, so that after 30 min of stimulation their percentage was less than half of peak levels (typically between 10% and 20% for both static and RWV). After 60 min of stimulation the fraction of $pERK^+$ cells was just above baseline and remained at this level for the remainder of the experiment.

To account for possible disturbances in the compartmentalization of ERK during RWV-culture, we next measured the phosphorylation of cytosolic and nuclear ERK-substrates in response to TCR ligation. RSK is a prominent cytosolic target of activated ERK, and phosphorylation of this enzyme on threonine 573 is a critical step in its activation [Dalby et al., 1998; Frodin and Gammeltoft, 1999]. In static conditions and in the RWV we found that the kinetics of RSK phosphorylation at this residue closely followed that of pERK (Fig. 7A). In both venues, peak phosphorylation of RSK occurred

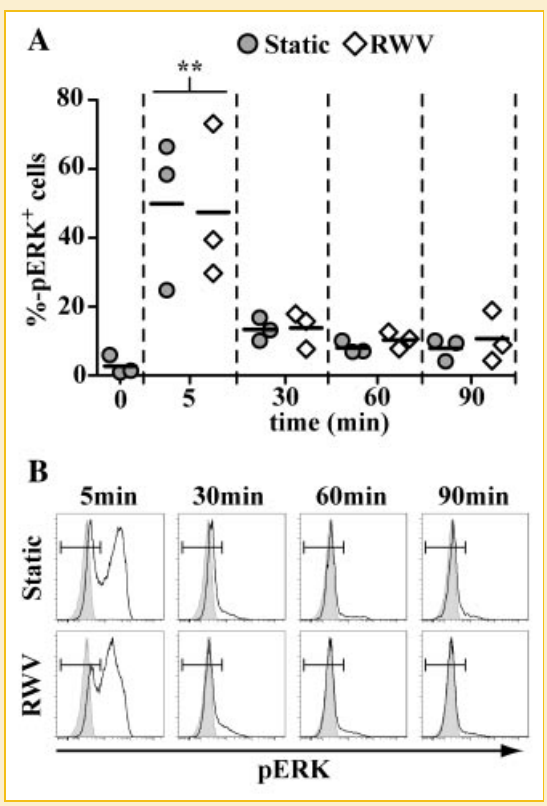

Fig. 6. Activation of ERK during culture in the RWV. Purified $CD4^+$ T cells were stimulated by CD3 × CD28 during culture in an RWV or under static conditions. Cells were removed from the culture venues at each time point and processed for flow cytometry with immunostaining for phosphorylated ERK (pERK). A: Percentage of $pERK^+$ cells at each time point. Data from individual experiments are shown as symbols, and the mean values are indicated by horizontal lines. B: Representative data from one of three experiments. Filled histograms are unstimulated cells. Open histograms are cells stimulated in the RWV or static conditions. Gates used to determine the percentage of $pERK^+$ cells are shown on each plot.

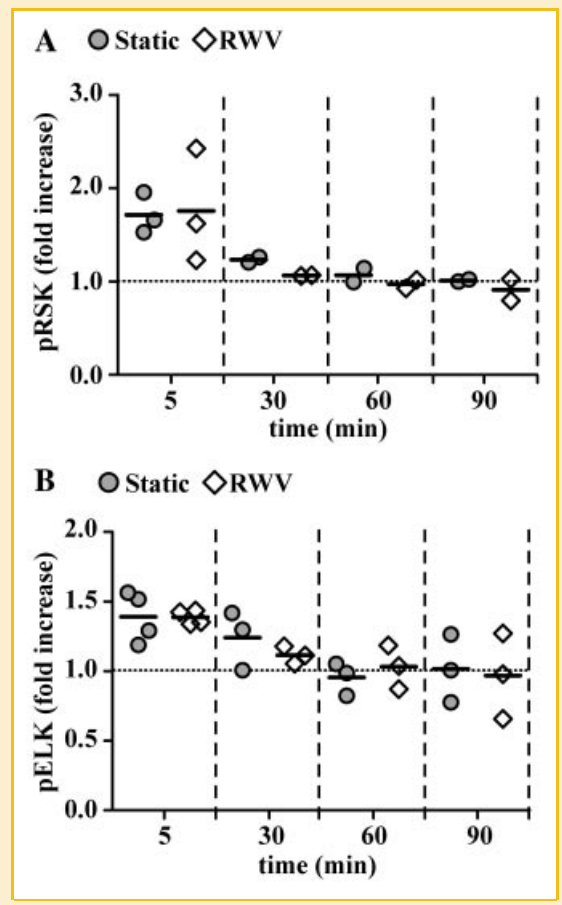

Fig. 7. ERK-induced phosphorylation of cytosolic (RSK) and nuclear (ELK-1) targets during culture in the RWV. Purified $CD4^+$ T cells were stimulated by CD3 × CD28 during culture in an RWV or under static conditions. At the indicated time points, cells were removed from each culture venue and processed for flow cytometry with immunostaining for phosphorylated RSK or ELK-1, and the MFI of staining for each molecule determined by flow cytometry. A: The fold increase in pRSK staining intensity over unstimulated cells. B: The fold increase in pELK-1 staining intensity over unstimulated cells. Data from individual experiments are shown as symbols, and the mean values are indicated by horizontal lines.

5 min after stimulation and declined steadily to baseline by 60 min. Phosphorylation of Serine 383 of the transcription factor ELK-1 requires activated ERK to be present in the nucleus [Brunet et al., 1999; Raman et al., 2007]. Similar to pRSK, our measurements of this molecule's phosphorylation paralleled the kinetic data for pERK (Fig. 7B). ELK-1 phosphorylation peaked after 5 min of stimulation, was only slightly elevated at 30 min, and by 60 min had returned to baseline where it remained for the rest of the experiment. We did not observe a significant difference in the kinetics or magnitude of pELK-1 phosphorylation between the culture venues. As a whole, these data indicate that neither the kinetics nor the localization of RAS/MAPK signaling downstream of the TCR are significantly altered by culture in the RWV.

DISCUSSION

Spaceflight presents a number of environmental hazards including exposure to cosmic radiation and the concentration of environmental pathogens due to air recycling that require intact if not enhanced immune surveillance. Counteracting spaceflight-induced immunosenescence is therefore of utmost importance for maintaining crew health during long-term manned space missions. The use of nutritional or pharmacological supplements (countermeasures) has been proposed for this purpose, but the design of such countermeasures is hampered by our limited understanding of how the spaceflight environment impinges upon immune function [NASA, 2005]. Microgravity is thought to play a key role since it can independently inhibit T cell activation by TCR agonists [Pippia et al., 1996; Hashemi et al., 1999]. A mechanistic explanation of this inhibition has remained elusive, but the ability of PMA to rescue T cell activation in simulated microgravity suggests a role for DAG-responsive processes [Cooper and Pellis, 1998; Simons et al., 2009]. To investigate this possibility we analyzed TCR-induced DAG signaling during culture in a ground-based microgravity analog, the RWV bioreactor. Surprisingly, we found that signals immediately upstream of DAG were not inhibited by the RWV, nor were NFκB induction and RAS/MAPK signaling downstream of DAG. This finding contrasts sharply with the complete lack of proliferation, cytokine secretion, and activation marker expression by T cells stimulated with TCR agonists in the RWV, and suggests that T cell function is decoupled from upstream TCR signaling during culture in a simulated microgravity environment.

The earliest point at which changes in TCR signaling can lead to changes in cellular function is at the TCR itself. For example, T cells undergoing selection in the thymus generate unique TCR signals (e.g., differential levels of ζ-chain and ZAP-70 phosphorylation) in response to strong and weak TCR agonists that result in opposing functional outcomes (e.g., survival vs. deletion) [Daniels et al., 2006]. Our use of flow cytometry to make precise measurements of tyrosine phosphorylation allowed us to uniquely address the possibility that T cells generate quantitatively distinct TCR signals during culture in the RWV. Contrary to our initial hypothesis we found that the ζ-chains, ZAP-70 and SLP-76 were phosphorylated to the same degree in both static- and RWV-culture (see Fig. 3). Although it remains a technical possibility that culture in the RWV affects the TCR signal at later time points, we find such a scenario is unlikely. Our data are consistent with observations that LAT phosphorylation in response to TCR cross-linking is maintained at levels equivalent to static controls for up to 30 min by T cells cultured in another (non-RWV) ground-based model of microgravity [Boonyaratanakornkit et al., 2005]. Further support for this conclusion can be found in studies comparing TCR signaling in response to agonist, partial agonist, and antagonistic TCR ligands. Such studies have shown that the efficacy of TCR stimulation is reflected in the magnitude rather than in the kinetics of TCR-proximal tyrosine phosphorylation, but that both the kinetics and magnitude of ERK phosphorylation are modulated in response to altered TCR-stimulating ligands [Smyth et al., 1998; Mirshahidi et al., 2004; Schade and Levine, 2004]. Thus, our data showing that T cells stimulated in the RWV generate signals at the TCR and downstream to ERK that are quantitatively identical to those generated in static conditions do not support the notion that culture in simulated microgravity prevents T cell activation by acting directly on the TCR-proximal signal.

The induction of second-messenger signaling and recruitment of activated transcription factors to the nucleus is critical for initiating the genetic program of T cell activation. There is evidence, however, that T cells fail to fully initiate this program during culture in simulated microgravity. Using acridine orange staining, Hashemi et al. [1999] were unable to detect any increase in the mRNA content of purified T cells stimulated by cross-linking CD3 and CD28 in an RWV. Similarly, Walther et al. [1998] reported that the transcription of IL-2 and CD25 mRNA by T cells stimulated with concanavalin A was impaired by simulated microgravity. The transcription of CD25 and IL-2 requires the concerted action of the NFAT, AP-1, and NFκB families of transcription factors. The rescue of T cell activation in the RWV by PMA does not require, nor is it enhanced by, a calcium ionophore, which implies that calcium signaling to NFAT is intact during culture in simulated microgravity [Cooper and Pellis, 1998; Hashemi et al., 1999]. We report here that IκBα is degraded to the same extent in the RWV and static conditions indicating that NFκB signaling to the nucleus is also present in simulated microgravity. The status of AP-1 in the RWV is less clearly inferred from our data. AP-1 is downstream of RAS/MAPK signaling and its activation requires (1) the de novo synthesis of c-fos (in response to ELK-1 activation), and (2) that activated ERK remain in the nucleus for sufficient time to phosphorylate the mature c-fos protein [Raman et al., 2007; Shaul and Seger, 2007]. Our data show that activated ERK enters the nucleus and phosphorylates ELK-1 indicating that the former of these requirements is met in the RWV. We also did not observe any changes in the kinetics of ERK activation during culture, which implies that the latter condition is also satisfied. Although we cannot explicitly rule out the possibility that activated ERK was excluded from the nucleus at later time points, our data provide limited evidence that signaling from the TCR to AP-1 is not impaired by culture in the RWV. We therefore find it unlikely that simulated microgravity prevents T cell activation by abrogating the signals required for the induction of NFκB and AP-1.

In conclusion, we demonstrate that DAG signaling downstream of the TCR is not impaired by culture in the RWV. Significantly, our data imply that T cells stimulated in the RWV appear to be poised on

the brink of activation, but held in check at this point by some unique aspect of the simulated microgravity environment. It would appear, then, that simulated microgravity prevents T cell activation by modulating the cellular response to the TCR signal rather than by abrogating or limiting the signal itself. How this modulation is achieved remains an open question, but we would propose that future studies must consider the affect of simulated microgravity on TCR-independent sources of DAG such as outside-in signaling via the integrin LFA-1 [Mor et al., 2007]. From the perspective of countermeasure-design these results are encouraging since they imply that the cellular mechanisms required for antigen specificity are unaffected by microgravity, and that cellular function can be restored by targeting pathways that, in the absence of canonical TCR-signaling, would not have the undesired side-effect of polyclonal T cell activation.

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