Differential Phosphorylation of Akt and signaling in CD4+ T Cells in Pathogenic and Apathogenic SIV Infection

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ABSTRACT

Increased CD4+ T cell apoptosis and activation induced cell death (AICD) as a result of HIV infection in humans and SIV infection in Rhesus macaques (RM) is indicative of disease. Some non-human primate species naturally infected by SIV, such as African sooty mangabeys (SM), do not succumb to SIV despite high viral loads. Previously, we showed that mRNA levels of GSK-3β a kinase involved in T cell signaling, are significantly decreased in SIV+ RM compared to SIV+ SM. The current study confirms that expression of GSK-3β is decreased at the protein level in SIV+ RM. In addition, CD4+ T cells from SIV+ RM, but not other animals show an increase in both total Akt, a kinase directly interacting with GSK-3β and p-AktThr308 in response to stimulation via CD3/CD28, which is associated with an increase in apoptosis. Furthermore, the differences between the uninfected and pathogenically or non-pathogenically infected animals are not only species specific, but also T cell subset specific and that these trends correlate with AICD. This is one of few studies indicating the activity of Akt can be specific to only one phosphorylation site and may be linked to the differences in AICD and resistance to the lentivirus induced disease.

KEYWORDS:

T cells – AIDS – Nonhuman primate – Akt – intracellular signaling

SOUHRN

Stephenson S. T., Bostik V., Bostik P.: Rozdíly ve fosforylaci Akt v CD4 lymfocytech u patogenní a nepatogenní infekce SIV

HIV infekce u lidí a SIV infekce u makaků Rhesus (RM) se vyznačuje zvýšenou apoptózou a aktivací vyvolanou buněčnou smrtí (AICD) CD4 lymfocytů. Avšak některé druhy primátů, jako například afričtí mangabejové (SM), jsou přirozeně infikováni SIV a nerozvine se u nich symptomatické onemocnění. Tato studie ukazuje, že lymfocyty RM infikovaných SIV vykazují sníženou vnitrobuněčnou expresi GSK3beta a současně zvýšenou expresi a fosforylaci (Thr308) kinázy Akt spojenou se stimulací receptorů CD3 a CD28. Tyto rozdíly jsou specificky měřitelné pouze v některých definovaných subpopulacích CD4 lymfocytů. Je to jeden z mála příkladů monofosforylace Akt a tato signalizace může představovat jeden z mechanismů vedoucích k rozdílům v AICD a případně i rezistenci k infekci SIV.

KLÍČOVÁ SLOVA:

T lymfocyty – AIDS – primate – Akt – vnitrobuněčná signalizace

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INTRODUCTION

The gradual loss of CD4+ T cells accompanied by a loss of optimal CD4+ T cell activation and function in the remaining CD4+ T cells is the hallmark of pathogenic HIV-1 infection of humans and SIV infection of select Asian species of nonhuman primates (NHP) such as rhesus macaques [1–3]. SIV infection in these animals is the only currently available model of human AIDS. The precise mechanism(s) that lead to such loss of CD4+ T cells and/or their function has yet to be defined. Previously, our laboratory has documented the fact that such massive CD4+ T cell loss and dysfunction does not occur in African sooty mangabeys (SM) following natural and/or experimental SIV infection despite showing levels of plasma and cellular viral loads that normally lead to disease and death in Asian macaques [4–6].

Physiologic T cell activation and response requires a proper sequence of a signaling cascade which consists of engagement of not only the TCR by its cognate ligand but also the ligation of appropriate co-stimulatory molecules (e.g. co-stimulation). These cell membrane events are followed by intracellular signaling, that leads to the expression of effector genes and the activation of effector mechanisms [7]. Studies have shown that pathogenic HIV/SIV infection affects multiple intracellular signaling pathways at various stages of the signaling cascade within lymphoid cells [8, 9]. Indeed, our previous studies showed marked differences in CD4+ T cell signaling between SIV infected SM and rhesus macaques (RM) in a number of regulatory pathways involved in T cell responses and function

[10–12] including the GSK-3 pathway. The core of this pathway consists of the PI3K-Akt-GSK3 cascade. Akt is a serine-threonine protein kinase, which lies at the hub of a number of different cell signaling pathways (reviewed in [13]), including those involved in the activation and apoptosis of $CD4^+$ T cells. The activation of Akt is generally considered to be pro-proliferative and over-expression or up-regulation of Akt has been shown to increase survival of T lymphocytes [14]. However, the biology of Akt is relatively complex and its potential role(s) within the cell continues to be defined. GSK-3, which is inhibited by Akt, has also been shown to regulate a number of cellular processes including apoptosis and T cell proliferation [15, 16].

Our results provide evidence for distinct differences in the intracellular signaling along the Akt-GSK3 pathway within highly enriched population of $CD4^+$ T cells isolated from SIV sero-positive and sero-negative RM in comparison with SM. Some of these individual differences between pathogenic SIV infection of RM and apathogenic infection of SM are even more pronounced in the phenotypically and functionally defined $CD4^+$ T cell sub-populations and specifically dysregulated in either the central memory, effector memory or naïve cells. Additionally, the dysregulation along the Akt-GSK pathway correlates with increased levels of activation induced cell death (AICD) in $CD4^+$ T cells of SIV infected RM. These data contribute to further our understanding of one of the potential mechanisms of $CD4^+$ T cell dysfunction in SIV pathogenesis.

MATERIALS AND METHODS

Experimental samples. Peripheral blood samples were obtained from normal healthy adult rhesus macaques (*Macaca mulatta*) denoted as SIV-RM, rhesus macaques experimentally infected with SIVmac251 during the chronic stage denoted as SIV+RM, SIV naïve sooty mangabeys (*Cercocebus atys*) denoted as SIV-SM and naturally SIV infected sooty mangabeys denoted as SIV+SM. Each cohort included at least six animals. All monkeys were maintained at the Yerkes Regional Primate Research Center of Emory University according to the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council and the Health and Human Services guidelines "Guide for the Care and Use of Laboratory Animals." PBMC were isolated using Lymphoprep lymphocyte separation medium (AXIS-SHIELD PoC AS, Oslo, Norway). $CD4^+$ T cells were isolated from PBMC using the Dynabeads CD4 Positive Isolation Kit (Invitrogen, Carlsbad, CA) and an aliquot of these cells analyzed for the efficiency of isolation and found to be > 92% $CD4^+$.

In vitro cell cultures. Purified $CD4^+$ T cells were cultured *in vitro* for either 12 or 36 hours as noted in RPMI 1640 media supplemented with 10% FBS, 2 mM L-glutamine, 50 µg/ml gentamicin and 20U/ml IL-2. To prepare activated $CD4^+$ T cells, the cell cultures were incubated with Dynabeads M-450 (Invitrogen) conjugated with anti-CD3 and anti-CD28 (both from Biosource, Camarillo, CA) antibodies at the previously determined optimum ratio of 1:4 of the 2 antibodies. For each indicated cell culture, 2 beads per cell were added to the media for the specified period of time. In some experiments, the cell cultures were treated with a pre-determined optimum concentration of the inhibitors LY294002 (25 µM) and LiCl (10 mM; both Sigma-Aldrich, St. Louis, MO) as indicated.

Flow cytometry. For flow cytometric analyses, the cells were cultured *in vitro* with anti-CD3/CD28 beads in the presence of media or the inhibitors for 12 hours, washed twice in PBS and surface stained using FITC mouse anti-human CD95 (BD Biosciences, San Jose, CA), PE conjugated anti-CD28 (BD Biosciences). Cells were then washed twice, fixed in 2% formaldehyde, permeabilized with 90% methanol and blocked in PBS containing 0.5% BSA. Aliquots of these cells were then incubated with the manufacturer's recommended dilutions of rabbit antibodies specific for Akt, phosphorylated *p-Akt_{Ser473}, *p-Akt_{Thr308} and Cox2 (Cell Signaling, Austin, TX). After 1 hour incubation at room temperature, cells were washed twice in blocking solution and then incubated with an APC conjugated goat anti-rabbit IgG (R&D Systems, Minneapolis, MN) developing antibody. Cells were incubated for 30 minutes, washed twice with blocking solution and re-suspended in 2% paraformaldehyde. The samples were subjected to flow cytometric analysis using a FACS Calibur (BD Biosciences) and the data on a minimum of 10,000 cells analyzed using FlowJo software.

Immunoblotting. Cells were cultured *in vitro* with a previously defined optimum concentration of anti-CD3/CD28 beads in the presence of media or the inhibitors for 36 hours and washed twice in PBS before being lysed. Whole cell lysates from control and experimental samples (30 µg per sample) were separated on SDS-PAGE using a 4–20% gradient ReadyGel (Bio-Rad, Hercules, CA), transferred to nitrocellulose membrane (BioRad) and the membrane blocked with 5% non-fat milk in T-TBS prior to incubation with either a rabbit anti-GSK-3β or a rabbit anti-*p-GSK-3β (Cell Signaling). The secondary antibody used was a donkey anti-rabbit IgG (Amersham Biosciences, Uppsala, Sweden). All bands were visualized using the ECL detection system (Amersham Biosciences). Equal loading of the samples was determined using anti-β actin antibodies with each analysis (Sigma).

Annexin V staining. Cells for the analysis of apoptosis were incubated for 36 hrs with anti-CD3/CD28 beads and then stained with PE-conjugated Annexin V (Southern Biotech, Birmingham, AL) according to the manufacturer's instructions. Briefly, aliquots of 0.5 million cells in 500 µl of binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% BSA) were incubated at 4C for 15 min with or without (control) 10 µl of Annexin V-PE. The cells were then washed, re-suspended in binding buffer and subjected to flow cytometric analysis using a FACS Calibur (BD Biosciences) and the data analyzed using FlowJo software.

Data processing. The data were analyzed by calculating the means for each individual group (n > 5). For the statistical significance the p values were calculated using t-test.

RESULTS

Pathogenic SIV infection leads to a decrease of GSK-3β expression and phosphorylation and is associated with increased apoptosis. Previous studies by our laboratory have documented a number of differences in the intracellular signaling pathways involved in T cell

responses and function within CD4+ T cells from RM and SM [5, 6, 11]. One such study indicated that SIV infection results in a marked decrease in the levels of GSK-3 mRNA within CD4+ T cells from SIV infected RM but not SM, especially following anti-CD3/CD28 activation [12]. The Akt-GSK3 pathway is important in executing normal physiological function and activation of T cells [17, 18]. It has been also shown that it plays an important role in the regulation of apoptosis [19] and our previous findings indicated that pathogenic SIV infection of RM is characterized by an increase in the level of apoptosis of CD4+ T cells [20]. To further elucidate the potential role of this pathway in the SIV induced apoptosis (and pathogenesis of the disease) in RM we first confirmed that the differences in the levels of GSK-3 message corresponded to differences in GSK-3 at the protein level by measuring the expression of the common GSK-3 isoform GSK-3β in CD4+ T cells from SIV positive and negative RM and SM. The results in Figure 1 (a) indeed showed that normal non-activated RM CD4+ T cells along with CD4+ T cells from SIV- and SIV+SM (lanes 1, 4 and 10) have readily detectable levels of GSK3 as compared with CD4+ T cells form SIV+RM (lane 7). We simultaneously activated an aliquot of the cells with anti-CD3/CD28, which would induce AICD in susceptible cells. This did not change the level of GSK3 in SIV-SM, SIV+SM and SIV-RM (lanes 2, 5 and 11), but induced a slight increase in expression in SIV+ RM (lane 8). These differences confirm on the protein level, that pathogenic SIV infection leads to decreases in levels of GSK3ß, which can be rescued only partially by the stimulation.

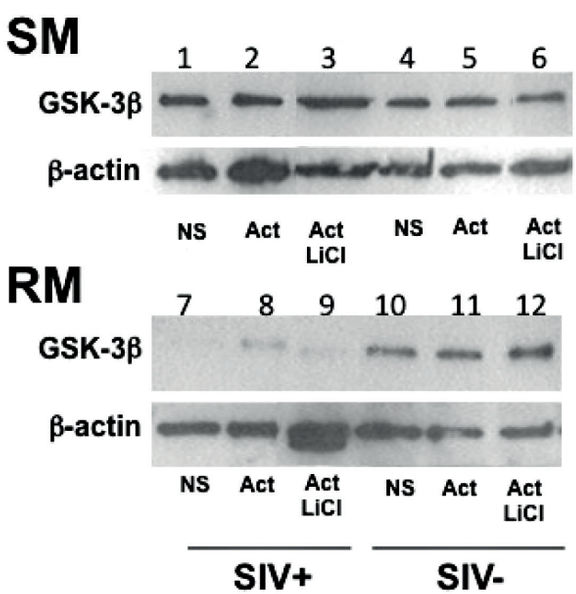

Figure 1. Decrease of GSK-3ß expression and phosphorylation in CD4+ T cells from SIV infected RM
Levels of (a) expression of GSK-3ß were assessed in CD4+ T cells from both SIV+ and SIV- RM and SM by Western blot. Cells were cultivated in media alone (NS), activated with anti-CD3/28 beads (Act), or activated in the presence of LiCl. Representative data are shown with ß-actin as control.

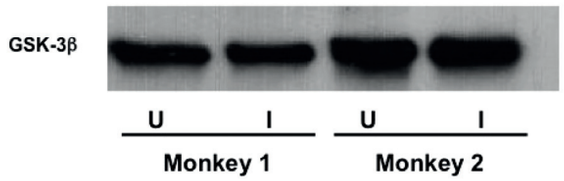

Figure 2. GSK-3ß levels in *in-vitro* SIV infected cells
CD4+ T cells from SIV negative RM and SM were infected with SIV *in vitro* and levels of GSK-3ß expression were analyzed by Western blot in uninfected (U) and infected (I) samples in parallel. Representative data from each species are shown.

These data suggest that SIV infection markedly inhibits the synthesis of GSK-3ß in RM but not SM.
To further determine if the changes seen in SIV+RM are a result solely of the SIV infection of CD4+ T cells or require other factors only available *in vivo*, we infected CD8+ depleted PBMCs from SIV-RM using spin inoculation as previously described [21]. Once active infection was confirmed via p27 ELISA, CD4+ T cells were isolated and the levels of GSK-3ß were determined by immunoblot. Surprisingly, the levels of GSK-3ß remained consistent in both infected and uninfected controls (Figure 2). Equal loading of samples was confirmed by ß-actin analysis (data not shown). This suggests that other factors induced by SIV in pathogenically infected organism must play an active role in the down regulation of GSK-3ß in SIV+RM. Taken together, these data confirmed previous mRNA data indicating that SIV+RM have a substantial decrease in GSK-3β both at the mRNA and protein level. Additionally, the inhibition of GSK-3β by LiCl results in an increase in GSK-3β phosphorylation, indicating the efficacy of the treatment and functionality of GSK-3β. This corresponded to an increase in apoptosis of CD4+ T cells in pathogenic SIV infection shown previously.

SIV infection differentially affects Akt expression and activation in CD4+ T cells from pathogenically infected RM and apathogenically infected SM. Since the results above showed that SIV infection has a marked effect on GSK-3ß expression in CD4+ T cells from RM but not SM, further studies were conducted to assess the potential differential effects of SIV infection *in vivo* on the regulation of Akt, an upstream regulator of GSK-3 and a key member of several intracellular signaling pathways. Akt is activated by dual phosphorylation on Ser_{473} and Thr_{308} [22]. In order to determine the potential role of Akt in SIV induced CD4+ T cell dysfunction, it was also important to establish, whether the changes, if any, in the Akt-GSK3 signaling pathway were affecting the entire CD4+ T population or if some/all of these effects were specific to phenotypically and functionally defined subsets of CD4+ T cells such as the central memory ($CD28^+CD95^+$), effector memory ($CD28^-CD95^+$) or naïve ($CD28^+CD95^-$) CD4+ T cells.
The intracellular staining followed by the flow-cytometry analysis showed, that there were no detectable differences in the frequency of total Akt expressing CD4+ T cells (Figure 3(a) between SIV-RM and SIV-SM. When $^*p\text{-}Akt_{Ser\ 473}$ was examined, the frequencies of positive cells in RM were similar to those expressing total Akt.

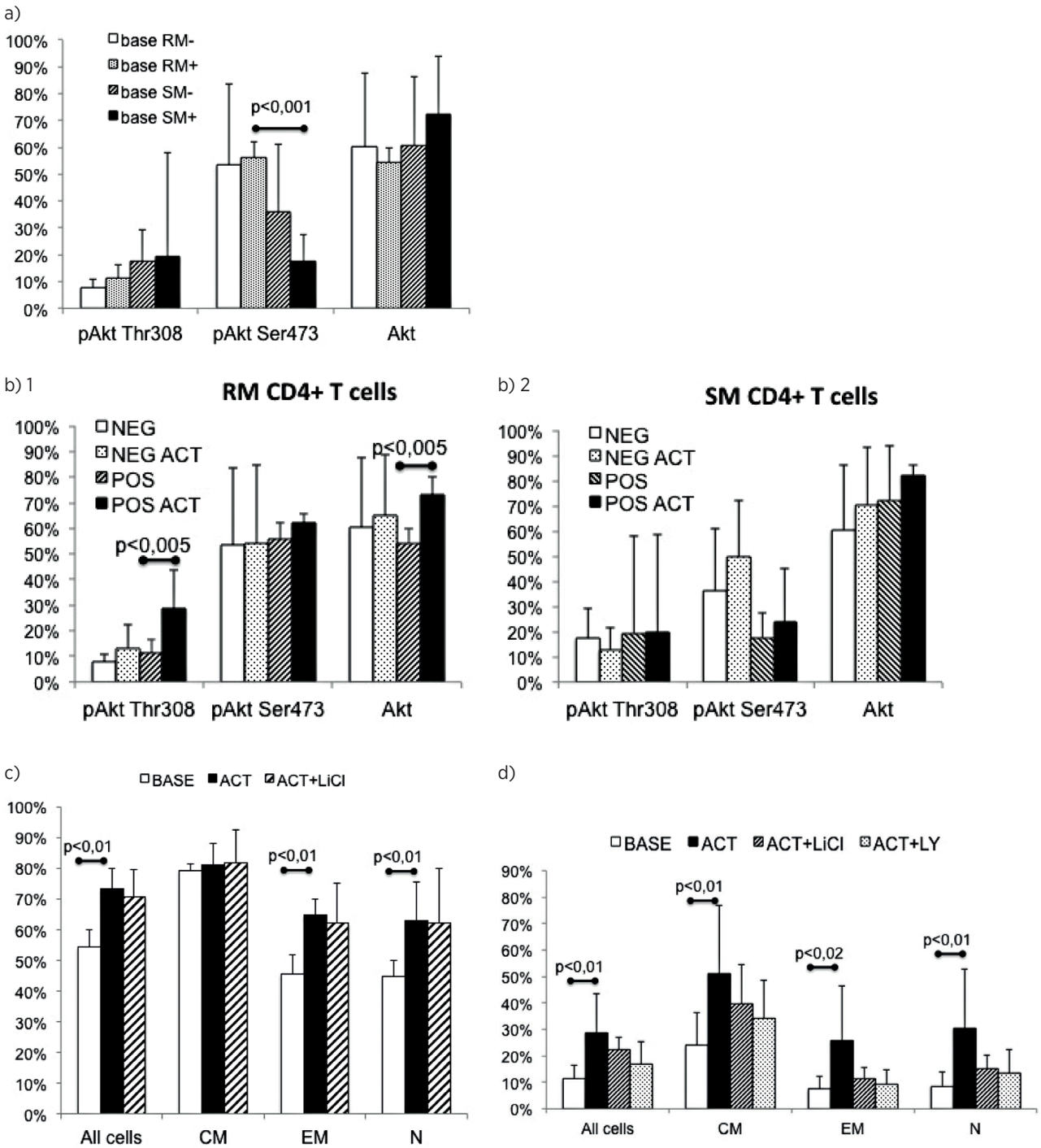

Figure 3. Expression and phosphorylation of Akt in CD4⁺ T cells from RM and SM
(a) Baseline frequencies of Akt, p-AktThr$_{308}$ and p-AktSer473 expressing CD4⁺ T cells from SIV positive animals (RM+, SM+) and SIV negative animals (RM-, SM-).
(b) Frequencies of Akt, p-AktThr$_{308}$ and p-AktSer$_{473}$ expressing CD4⁺ T cells from RM (left pannel) and SM (right pannel). Cells from SIV positive (POS) or negative (NEG) animals were cultured in media alone, or activated by CD3/CD28 beads (NEG ACT, POS ACT).
(c) Frequencies of Akt expressing or (d) p-AktThr$_{308}$ positive CD4⁺ T cells from SIV positive RM were analyzed in unfractionated population (All cells), central memory (CM), effector memory (EM) and naive cells cultivated in media alone (BASE), stimulated (ACT), or stimulated with anti CD3/28 beads in the presence of either LiCl (ACT+LiCl), or LY294002 (ACT+LY) All analyses were performed on groups with at least 6 samples by flow cytometry and mean values with significant differences are shown. P values were calculated using t-test.

However, in the SIV-SM the frequencies of *p-Akt$_{Ser473}$ positive cells were markedly lower than the total Akt positive cells and were further diminished in SIV + SM. This trend was consistent across all 3 CD4$^+$ T cell sub-categories: central memory, effector memory and naïve (data not shown). When *p-Akt$_{Thr\ 308}$ was examined, the frequencies of *p-Akt$_{Thr\ 308}$ expressing cells were markedly lower than those expressing *p-Akt$_{Ser\ 473}$ or total Akt positive cells. Although there were no significant differences in the Akt$_{Thr\ 308}$ phosphorylation, it is important to note, that the levels in both the SIV- and SIV+ cells from SM were notably higher than those found in the corresponding cells from RM.

These data indicate that the CD4$^+$ T cells in SM clearly function even when fewer cells exhibit Akt$_{Ser473}$ phosphorylation. Although activation of Akt is generally considered to involve both phosphorylation sites, the major functional difference is ascribed to the phosphorylation on Thr$_{308}$ with the Ser$_{473}$ having a supportive role directing the downstream substrate specificity [23].

Since the Akt signaling pathway plays an important role in both T cell activation and apoptosis, experiments were carried out to determine whether the activation of CD4$^+$ T cells will further enhance/diminish the effects of SIV, possibly indicating a role of this pathway in CD4$^+$ T cell dysfunction in pathogenic infection. Therefore, enriched population of CD4$^+$ T cells were anti-CD3/CD28 activated and the IC staining was performed as above. The data showed almost no differences in both Akt expression and phosphorylation in both SIV positive and negative SM, as well as SIV negative RM (see Fig. 3(b). However there was clear increase in both the total Akt expression and Thr$_{308}$ phosphorylation in SIV+ RM, which was highly significant ($p < 0.005$). Further analysis of cell subpopulations (see Figure 3(c, d) showed that while the levels of total Akt in activated naïve and effector memory cells increased upon activation, the levels in central memory cells remained constant (see Figure 3c). However, the increase of phosphorylation of Thr$_{308}$ upon activation was noted in all CD4$^+$ T cell subpopulations, although it was less significant in effector memory cells (see Figure 3d). This could be due to a lack of activation, and therefore signaling, within these cells.

Inhibition of PI3K or GSK3 does not affect the phosphorylation pattern of Akt in CD4$^+$ T cells from SIV+RM. The differences in *p-Akt$_{Thr308}$ and *p-Akt$_{Ser473}$ in SIV + RM versus SIV-RM and SM prompted us to examine if inhibition of the Akt-GSK3 pathway at different levels leads to different phenotypes of Akt phosphorylation within our cohorts. First, anti-CD3/CD28 activated CD4$^+$ T cells were treated overnight with either LY294002, a selective inhibitor of upstream PI-3K. This inhibition resulted in a notable decrease in *p-Akt$_{Thr\ 308}$ in all cell populations compared to stimulated controls, although this decrease was not statistically significant (see Figure 3d).

Next, the cells were treated with selective GSK-3ß inhibitor LiCl and anti-CD3/CD28 stimulated CD4$^+$ T cells. Similar to the results obtained with PI3K inhibition, cells treated overnight with LiCl did not show a significant change in Akt phosphorylation when compared to stimulated cells of the same, although a decreasing trend was, again, notable (see Figure 3c).

Taken together, these findings again point to different roles for the two Akt phosphorylation sites in the signaling within CD4$^+$ T cells. Additionally, they indicate signaling pathway differences in CD4$^+$ T cells in pathogenically SIV infected RM CD4$^+$ T cells versus CD4$^+$ T cells from SIV sero-positive SM.

Increased apoptosis in stimulated CD4$^+$ T cells (AICD) from SIV+RM is mitigated by inhibition of the Akt signaling pathway. The fact that the Akt-GSK3ß pathway has been shown to play an important role in the regulation of apoptosis [19] combined with our previous findings that pathogenic SIV infection of RM is characterized by an increase in the level of apoptosis of CD4$^+$ T cells [20] and data shown above (see Figure 2), led to subsequent experiments aimed at determining the relationship between differences in the Akt signaling and the susceptibility of the cells to apoptosis.

The baseline levels of apoptosis (frequency of Annexin V positive cells at 36 hrs incubation in media alone) were significantly higher in CD4$^+$ T cells from SIV+RM but lower and similar in CD4$^+$ T cells from SIV-RM, SIV-SM and SIV+SM (Figure 4). When activation induced cell death (AICD) was measured in the corresponding cultures of anti-CD3/CD28 stimulated cells *in vitro* (see Figure 4), the CD4$^+$ T cells from the SIV+RM showed significantly higher levels of apoptosis ($p < 0,05$) than the cells from SIV+SM. When the anti-CD3/CD28 activated CD4$^+$ T cells were treated with GSK-3β inhibitor LiCl, the AICD in CD4$^+$ T cells from SIV + RM increased 3.3-fold. In contrast, activation of CD4$^+$ T cells from SIV-RM or SIV- or + SM in the presence of LiCl did not have statistically significant effects.

Thus, for the SIV induced apoptosis in the cells from RM the Akt-GSK-3β signaling pathway is important and the inhibition of this pathway has an effect on the AICD, but the AICD does not occur solely through this pathway. On the other hand, the importance of this pathway for AICD in SM cells appears to be less significant. This could offer CD4$^+$ T cells in SM a protective effect against cell death.

SIV particles without active infection do not cause changes in Akt pathway. As shown above, SIV infection led to marked increases in *p-Akt$_{Thr308}$ in all three T cells subsets in RM. To further determine whether this was due to active virus replication or secondary to the contact with virus proteins we measured the Akt phosphorylation in the cell populations as above cultured *in vitro* in the presence/absence of varying concentrations of AT-2 (2,2-dithiodipyridine) treated SIV. This treatment failed to show any detectable effects on levels of total, *p-Akt$_{Thr308\ or}$ *p-Akt$_{472}$ (data not shown) suggesting that active infection is a requirement for influencing the Akt-GSK pathway.

DISCUSSION

Pathogenic HIV-1 infection in humans and SIV infection of select Asian species of nonhuman primates (NHP) results in the dysfunction of CD4$^+$ T cells. This dysfunction manifests itself as unresponsiveness of T cells to proper stimulation, the development of aberrant activation patterns, and increased susceptibility to apoptosis [1–3, 24–26]. Despite active on-going investigation, the de-

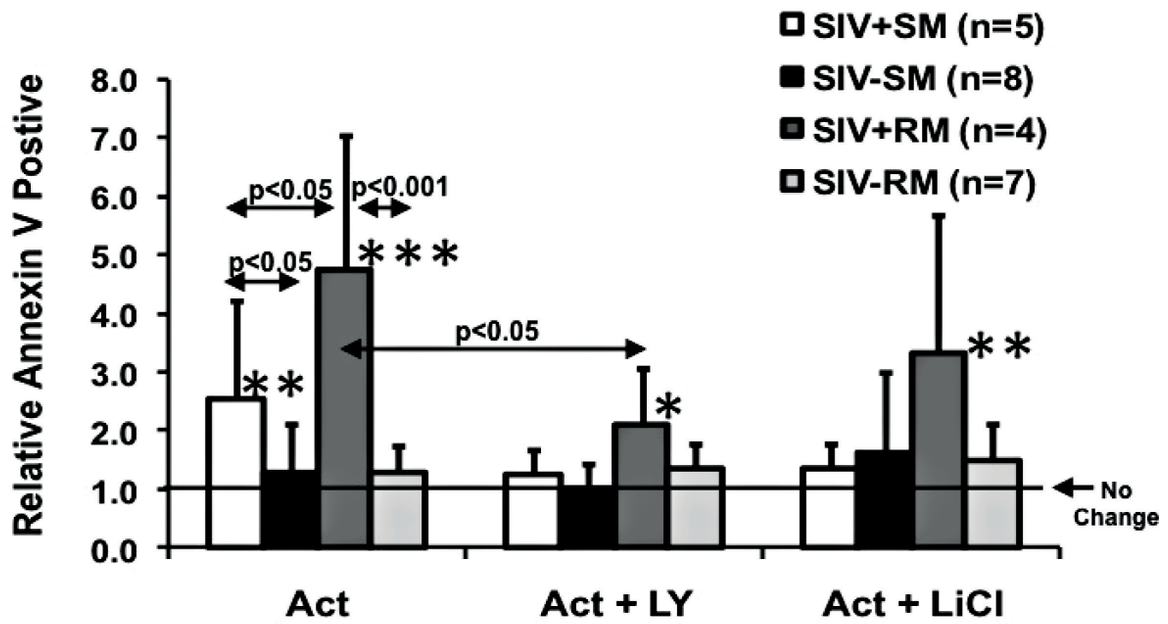

Figure 4. AICD in CD4⁺ T cells from RM and SM
Frequencies of Annexin+ CD4⁺ T cells from both SIV positive (SIV+RM and SM) and SIV negative (SIV-RM and SM) animals were measured by flow cytometry. Frequencies of cells from individual animals stimulated with anti-CD3/28 beads (Act), activated in the presence of either LiCl (ACT+LiCl), or LY294002 (ACT+LY) were measured and compared to the frequencies of cells from the individual animals cultured in media alone and expressed as relative ratios of treated/untreated. Numbers of animals in each group (n) and significant differences are shown (p values were calculated using t-test). Ratio of 1 indicates no change in treated vs untreated cells.

tailed mechanism(s) responsible for this dysfunction remain ill-defined.

The focus of current studies was to define the potential mechanisms that contribute to the increased susceptibility of CD4⁺ T cells from SIV infected RM to undergo AICD with an emphasis on the role of the Akt-GSK3 pathway. The results of these studies led to three significant findings. First, the data confirmed the earlier finding that GSK-3 transcription was markedly reduced not only at the message level but also at the level of protein expression in CD4⁺ T cells from SIV+RM compared to SIV-RM and SIV-/+SM (see Figure 1). Secondly, while the baseline numbers of Akt and p-Akt_{Thr308} expressing cells are comparable among the cells from all four animal cohorts, the numbers of cells positive for phosphorylated $Akt_{Ser\ 473}$ are significantly lower in SIV+ SM compared to the SIV+ RM. Thirdly, the stimulation of CD4⁺ T cells leads to a marked increase in both total Akt and *p-Akt_{Thr308} in the SIV+RM but not the other 3 cohorts (SIV-RM, SIV-/+SM) studied (see Figure 3) and these differences correlated with increased susceptibility for AICD (see Figure 4). Finally, the data clearly show differences in the phosphorylation of Akt at Ser_{473} and Thr_{308} in anti-CD3/CD28 activated CD4⁺ T cells and that these differences are both species and CD4⁺ T cell sub-population specific (see Figure 3).

The finding that GSK-3β is substantially decreased in SIV+RM presents interesting questions regarding the mechanisms resulting in the lack of mRNA/protein expression. In 1999, Lau et al. characterized the human GSK-3β promoter [27]. A possible repressor sequence at position -1421 to -1363 could be engaged, by an as yet unknown mechanism, as a result of SIV infection in RM but not SM. It is also potentially possible that this region is absent or mutated in the GSK-3β promoter of SM which could result in the lack of repression upon infection. Conversely, an activator sequence found at -427 to -384 may be silenced through mutation or repression, which affects transcription rates as a result of SIV infection in RM but not SM. Of course, a combination of these factors or epigenetic effects occurring post-translationally could also be contributing factors. Clearly, further investigation of the promoter region in all four cohorts is needed to parse out the mechanisms involved in disruption of GSK-3β expression following SIV infection in RM. Similar to the current work, a previous study [28] found that although GSK-3β expression is present in the alveolar macrophages of asymptomatic HIV+ persons, the kinase was constitutively phosphorylated at Ser_9 resulting in inhibition of activity. This further indicates the involvement of GSK-3β inhibition in HIV/SIV infection and the importance of continued investigation regarding the mechanisms and consequences of this inactivity within cells of the immune system.

In the current study, the finding that levels of *p-Akt_{Thr308} increase upon anti-CD3/CD28 activation of CD4⁺ T cells from SIV+RM, but not the other three cohorts further points to SIV infection resulting in differences between the two species along the Akt-GSK3

pathway (see Figure 4). The Akt pathway is important in that it is involved in the regulation of transcription of multiple genes necessary for proper T cell function and includes PI3K-PDK1-Akt-GSK3-NFATc signaling. Activated Akt phosphorylates/inactivates GSK-3β which allows NFATc to be dephosphorylated/activated by calcineurin (Ca^{2+}). In addition, activation of Akt also leads to the degradation of IkB and the activation of NFkB – another important pathway of transcriptional regulation, including SIV transcription. The role of Akt in cell signaling is complex, involving other signaling pathways and intermediates such as Ft1, FOXO3, cell cycle regulators, and others [29–34].

The activation of different signaling pathways, which involve alternative Akt roles, may be involved in the protection from pathogenic SIV disease in SM. Indeed, we have previously shown that $CD4^+$ T cells from SM exhibit an increased resistance to the development of anergy *in vitro* [12]. This study suggested that, regardless of SIV status, the $CD4^+$ T cells from SM synthesize IL-2 upon stimulation of CD3/TCR (signal 1) alone, which prevents anergy induction. Anergic cells have an inability to proliferate and express IL-2 following TCR specific stimulation by their cognate antigen in the presence of adequate co-stimulation [35, 36]. It is possible that the differences in the Akt activation pattern between SM and RM identified in the studies reported herein represent one of the contributory mechanisms underlying the ability of $CD4^+$ T cells from SM to produce IL-2 upon CD3/TCR stimulation alone. This, in turn, may lead to the protective effects on infected T cell populations.

Since this study has shown highly significant increases in numbers of cells positive for Akt phosphorylation at Thr_{308} in all $CD4^+$ T cell subpopulations from SIV+RM, one may infer the possible involvement of SIV protein(s) in the interference of signaling along the axis of T cell activation (CD28/CD3) and Akt signaling in $CD4^+$ T cells. These findings are further underlined by increases in phosphorylation at Thr_{308} in all $CD4^+$ T cell subpopulations from SIV-RM when PI3K is inhibited (see Figure 3d). Nef, a viral protein encoded by both HIV and SIV, has been shown, in addition to other functions, to down-regulate several cell surface proteins including both CD3, CD28 and MHC-I [37, 38]. The Nef protein consists of a PxxP domain [39], and this domain is a consensus sequence for binding of SH3 domain containing proteins [40, 41], such as PI3K [42]. The effects of Nef on CD28 could thus result in the increase in *p-$Akt_{Thr\ 308}$ seen in CD28/CD3 stimulated $CD4^+$ T cells from SIV+RM. Since our previous data have shown that CD28 stimulation is not required for proliferation and IL-2 production in SM $CD4^+$ T cells [12], one can speculate that the pathogenic effects of Nef are not subsequently seen, or are diminished in these cells. Contrary to this hypothesis, Nef has been shown to activate PI3K [43]. These studies, however, were conducted using various transfected cell lines, and thus, the biology of these signaling pathways is likely different from our system utilizing primary cells.

The inhibition of either PI3K or GSK-3β alone was not sufficient to induce increases in apoptosis in cells from SIV-RM (see Figure 4). It is reasoned that disruptions in the signaling pathway, both upstream and downstream of Akt, may be required for apoptosis induction. Additionally, the inhibition of the upstream PI3K and downstream GSK-3 decreased but did not completely abolish AICD seen in SIV+RM. This indicates that pathways including these proteins are involved, but not solely responsible for AICD in SIV infected RM $CD4^+$ T cells. This could further protect SM from the pathogenic effects of SIV, since the inhibition of the pathway at PI3K had no effect on downstream Akt signaling in $CD4^+$ T cells from SIV sero-positive SM.

Akt is known to have two phosphorylation sites, one at Ser_{473} and one at Thr_{308}. Originally, phosphorylation of both sites was thought to be necessary for proper activation. While it has been known since 1996 that PDK-1 phosphorylates Akt at Thr_{308} [22], the kinase, which phosphorylates Akt at Ser_{473} has only recently been identified [44]. Furthermore, it has been shown that major increase in Akt activity is mediated by the phosphorylation at Thr_{308} [22], but the full activation requires phosphorylation at both sites. Further studies indicated that the two phosphorylation sites can be regulated independently and that the phosphorylation of Ser_{473} dictated downstream substrate specificity [45, 46]. In addition to species and T cell subset specific differences in Akt signaling, the increases in phosphorylation of Akt at Thr_{308}, but not Ser_{473}, in only the SIV infected RM cohort, also suggests the activity of Akt, and thus the signaling pathways in which it participates, may be dependent on the phosphorylation status of these two sites. These findings are supported by results of the study conducted by Jacinto et al [46]. In those studies, cells that were deficient in *p-$Akt_{Ser\ 473,}$ still showed Akt being phosphorylated at Thr_{308}. Additionally, *p-Akt_{Thr308} was still capable of phosphorylating TSC2 and GSK-3. The study also showed that phosphorylation of another Akt target, Fox01/3a, at its Thr24/Thr32 phosphorylation site was greatly affected by the lack of *p-$Akt_{Ser\ 473}$. Data in the current study clearly show that phosphorylation of Akt at Ser_{473} and Thr_{308} are not dependent on each other and are differentially affected by not only SIV infection status but also involve species specific regulation.

CONCLUSIONS

In summary, our data point to significantly different effects of anti-CD3/CD28 stimulation on Akt signaling in $CD4^+$ T cells derived from SIV sero-positive sooty mangabeys and rhesus macaques. These differences correlate with the relative resistance of $CD4^+$ T cells from sooty mangabeys to undergo AICD. We hypothesize that differences in CD3(TCR)/CD28 induced T cell signaling between the two species involving the Akt-GSK-3β pathway offers inherent protection of mangabeys from the pathogenic effects of SIV. Additionally, some of the effects were observed globally in all $CD4^+$ T cells, while others were specific for central memory, effector memory or naïve cells. This indicates that the effects of SIV are not only species but also T cell sub-population specific. The pathways involved in T cell signaling and their relevance is an area of active research, as is the role of Akt in the biology of the cell. Our data suggest that the two phosphorylation sites of Akt play different roles in these mechanisms and are only beginning to be understood. Further research is needed to fully understand the effect HIV and SIV on this rapidly evolving and complex field.

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