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Age-related Changes in Lck-Vav Signaling Pathways in Mouse CD4 T Cells

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Abstract

Activation of lck-fyn kinases during T cell receptor signaling leads to Vav phosphorylation, activation of downstream targets including Rac1, and a transient decline in ezrin and moesin phosphorylation. We have shown that age increases Rac1 activity and lowers ezrin and moesin phosphorylation in resting mouse CD4 cells, changes that could be the results of alterations in lck-Vav signaling. Analysis of Vav in CD4 cells from old mice shows increases in the phosphorylation of two key regulatory residues, Tyr160 and Tyr174, suggesting enhancement of Vav GTPase activity. In addition, analysis of lck status also shows age-related increases in phosphorylation of two key residues, Tyr394 and Tyr505, which have opposite effects on lck function. These changes in lck-Vav signals in resting CD4 cells may contribute in turn to age-related increases in Rac1 activity and declines in phosphorylation of cytoskeletal proteins including Ezrin and Moesin.

Keywords

T Lymphocytes; Aging; Cytoskeleton; Tyrosine Kinases; Signal Transduction; Cellular Activation; TCR signaling

Introduction

Signaling through the T cell receptor (TCR) leads to the translocation of multiple signaling proteins to the area of interaction between T cells and antigen presenting cells. These translocations involve an active mechanism mediated by ATP dependent reorganization of the cytoskeleton [1–3]. The process requires the activation of TCR associated tyrosine kinases, including lck and fyn [4–6], and the subsequent phosphorylation of downstream targets, like Vav, known to regulate downstream signaling events critical for cytoskeleton reorganization [2;3]. Activation of lck and fyn is regulated by two phosphotyrosine residues. For lck, phosphorylation of Tyr394 (p394) enhances enzymatic kinase activity, while phosphorylation of Tyr505 (p505) decreases activity. In addition, the lack of phosphorylation in either site can result in partial activation of lck [7–10]. The balance among these three pools of differentially phosphorylated lck is thought to determine the general level of enzymatic activity of lck in resting T cells or during TCR signaling ([11]).

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The Vav family of GTPases are important downstream substrates of lck and fyn enzymatic activity [12–14]. Experimental data suggests that during TCR stimulation, lck and fyn can activate Vav GTPase function by phosphorylation of Tyr160 (p160) and probably Tyr174 (p174) of Vav [15–18]. In addition, TCR engagement leads to a transient dephosphorylation of the ezrin and moesin cytoskeletal proteins (ERM) that regulate a series of protein translocation events including exclusion of CD43 from the immunosynapse [19–21]. It has been suggested that Vav can control the status of ERM phosphorylation by regulating the activity of the RhoA family of small GTPases, including RhoA and Rac1 [22–25]. However, details of the lck-Vav-Rho signaling pathway dynamics and how they regulate ERM phosphorylation are not well understood.

We have shown previously that CD4 T cells from old mice do not form an efficient immunosynapse and do not exclude CD43 from the area of APC T cell interactions [26–29]. Those changes are accompanied by a decline in ERM phosphorylation and changes in the association of at least three proteins, Ezrin-binding protein-50 (EBP50), and the surface proteins CD43 and CD44, to the ERM[30]. We hypothesize that these defects in ERM signaling may be, at least in part, responsible for the lack of CD43 exclusion and lack of immunosynapse formation. In addition, we have shown age-related alterations signaling of the Rho family of small GTPases known to regulate ERM phosphorylation, including increases in Rac1 in CD4 T cells from old mice. These results suggested the hypothesis that pathways upstream of ERM/Rac1 could also be altered by age. In this paper we present evidence that age increases Vav activity and alters lck phosphorylation status, changes that could explain some of the increases in Rac1 activity and declines in the ERM phosphorylation of resting CD4 T lymphocytes from old mice.

Material and Methods

Animals and cell culture

Specific-pathogen free male [BALB/c x C57BL/6]F1 (CB6F1) mice were purchased from the National Institute of Aging contract colonies at the Charles River Laboratories (Kingston, NJ) and at Harlan (Indianapolis, IN). Mice were given free access to food and water. Sentinel animals were examined quarterly for serological evidence of viral infection; all such tests were negative during the course of these studies. Mice that were found to have splenomegaly or macroscopically visible tumors at the time of sacrifice were not used for experiments. CB6F1 mice were used at 6–8 (young) or 22–24 (old) months of age.

Antibodies, reagents and cell preparations

Rabbit anti-lck, anti-fyn, anti-phosphoTyr416 of Src tyrosine kinase (crossreacting with p394 of lck and fyn), anti-p505lck, anti-ERM, anti-Rac1 were purchased from Cell Signaling (www.cellsignal.com). Rabbit anti-Vav and anti-p174 Vav were purchased from Santa Cruz (www.scbt.com), while the rabbit anti-p160Vav1 was purchased from GeneTex (www.genetex.com). Rabbit anti-EBP50 was obtained from Affinity Bioreagents (www.bioreagents.com). Antibody specific for the CD3 ϵ chain of the TCR was purchased from Dako (www.dako.com). The CD4⁺ T cells were obtained by negative selection methods previously described in [31] and [32]. Flow-cytometric analysis of a typical preparation showed it to be 90–95 % positive for both CD3 and CD4.

Membrane preparations, Immunoprecipitations and western blot assays

Approximately 10×10^6 CD4⁺ T cells were stimulated for 5 minutes by crosslinking CD3 ϵ with CD4 and CD28 as described previously [27;29]; for the controls the antibodies were omit it from the incubation. For membrane preparation we used the Mem-Per Mammalian Membrane fractionation kit from Pierce (www.pierce.com) using the manufacturer's

instructions. Briefly, 10×10^6 CD4 T cells were lysed with 250 μ l of lysis buffer (buffer A) for 10 minutes at room temperature, and after 10,000xg centrifugation the supernatants were mixed with 600 μ l of phase separation buffer (buffer B and C mix). The suspension was incubated for 30 min at 4°C with rotation and then for 20 min at 37°C for phase separation. A final spin for 5 minute 10,000xg centrifugation was used to further define both membrane and non-membrane (cytosol) fractions. Each fraction was collected and stored at -20 °C for analysis.

For immunoprecipitations, the membrane fractionations from 10×10^6 stimulated or unstimulated CD4 T cells from young and old mice were resuspended in 1 ml of 1% Brij-58, PBS, pH 7.4, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate for 30 minutes at 4°C. The lysates were centrifuged at 10,000xg for 15 minutes at 4°C. The supernatant was used for analysis, or subjected to immunoprecipitation for 6 hours with specific antibodies pre-coupled to protein G Sepharose as previously described [27]. Samples were fractionated using polyacrylamide gels, transferred to PVDF, and incubated with specific antibodies as described in the text; the bands were visualized by chemifluorescence and quantified as previously described [27;30].

Statistical analyses

Unless otherwise indicated, results are presented as means $\pm$ SEM. Statistical significance was assessed using a Mann-Whitney test at $p = 0.05$.

Results

Age increases the level of Vav phosphorylation

During TCR signaling, cytoskeletal reorganization and Rac1 activation are dependent on the enhancement of p174Vav phosphorylation, leading to the activation of Vav1 GTPase nucleotide exchange factor [16;17;25]. Other Vav residues are also phosphorylated during the TCR signaling, including p160Vav [17;25], but the significance of these events for Vav activation, and their effects on downstream signaling are not well understood. We recently demonstrated [30] age-related increases in Rac1 activity in CD4 T cells, suggesting that this increase may be the result of alteration in the Vav activity in CD4 T cells from old mice. To test this hypothesis, we purified CD4 T cells from young and old mice and stimulated the cells by crosslinking the TCR with CD4 and CD28 as previously described [27]. We then measured the relative levels of p160Vav and p174Vav in Vav immunoprecipitates using phosphospecific antibodies and western blots. Figure 1 shows a typical analysis of p160 and p174Vav levels, in addition to levels of Vav, in samples of resting and activated CD4 T cells from young and old mice. The experiment illustrates an increase in the levels of both p160 and p174Vav in samples of resting CD4 T from old mice. TCR stimulation induced an increase in both forms of phosphorylated Vav in the young mice, as expected [15;18;33;34], but had no effect on Vav phosphorylation in samples from old mice. Figure 1 also shows the mean and SEM of 5 experiments, with p160 and p174 values normalized to the amount of Vav immunoprecipitated from each sample. We found no significant differences in the amount of Vav immunoprecipitated between samples, but resting CD4 from old mice a significant two-fold increase in p160Vav ($p=0.0079$) and p174Vav ($p=0.005$) when compared to resting CD4 from young mice. In addition, TCR stimulation in CD4 T cells from young mice shows a significant two-fold increase in p160 ($p=0.012$) and p174Vav ($p=0.035$), compared to resting cells from the young mice. In contrast, samples from old mice show no significant changes as a result of TCR stimulation. These results suggest that resting CD4 T cells from old mice have higher baseline activity of Vav GTPase. However, TCR stimulation does not increase Vav phosphorylation or activity in CD4 T cells from old mice, suggesting defects in the upstream signaling pathways leading to Vav phosphorylation.

Age increases the levels of phosphorylated Vav associated to the membrane

It has been suggested that translocation of Vav to the plasma membrane is important in the regulation and activation of key downstream signaling proteins [3;35]. Because of the age-related changes in Vav phosphorylation shown in Figure 1, we hypothesized that age might alter association of Vav, or its phosphorylated forms to the cellular membrane fraction. To test this idea, we purified resting CD4 T cells from young and old mice and separated the membrane and non-membrane (i.e. cytosol) fractions using a method based on separation of hydrophobic and hydrophilic proteins [36]. As shown in Figure 2, the CD3 ϵ chain of the TCR serves as a marker of efficiency in the membrane isolation and, as expected, is only found in the membrane fraction. Vav was immunoprecipitated from membrane and cytosolic fractions, and p174Vav, p160Vav and Vav protein measured by western blots. In addition, samples of membrane fractions of resting CD4 from young and old mice were also analyzed for the levels of ERM, EBP50, and Rac1 using western blots. Figure 2 (top panel) shows a typical digital image of p174 and p160Vav, CD3 ϵ chain and Vav proteins in the membrane and the cytosolic fractions from two old and two young mice. The image suggests that levels of both p174 and p160Vav are higher in CD4 T cells from the old mice in both cytosol and membrane fractions, consistent with the data of Figure 1. The statistical analysis of 5 independent experiments, presented in the bar graph of Figure 2, showed a small (25%), but statistically significant, age-related increase in the amount of both p174Vav and p160Vav in the cytosolic fraction ($p=0.02$ for each phosphoprotein). The levels of p174Vav and p160Vav also increase significantly with age in cytosol and membrane fractions ($p = 0.05$ and $p = 0.03$, respectively); the increase with age is about two-fold in each fraction. No significant differences were found in the amount of Vav immunoprecipitated between any studied groups. The overall results suggest that the age-related increases in Vav phosphorylation and activity affect cytosolic and membrane fractions, but may be more dramatic at the membrane level.

We have recently shown age-related declines in the phosphorylation of the cytoskeletal proteins of the ezrin-radixin-moesin family (ERM proteins), accompanied by declines in the ERM-associated molecules CD44 and EBP50 [30]. Current models of ERM function [1;37–40] suggest that declines in ERM phosphorylation should be accompanied by declines in association of ERM to the plasma membrane. To test this hypothesis, we isolated the membrane fraction of resting CD4 T cells from young and old mice and analyzed ERM, EBP50, Vav and Rac1 levels using western blots. Figure 3 shows an analysis of two young and two old mice for each of these proteins, as well as the CD3 ϵ chain of the TCR, used as an internal control to normalize for variations among samples. As expected, there are no age-dependent differences in CD3 ϵ in the membrane fractions. There were, however, age associated declines in membrane levels for ERM, EBP50, and Rac1. Age had no effect on membrane content of Vav protein, in good agreement with the immunoprecipitation data of Figure 2. Figure 3 also shows means and SEM from 5 experiments, documenting a significant 50% decline in membrane-associated ERM ($p=0.009$) and EBP50 ($p=0.0004$) in CD4 T cells from old mice compared to samples from young mice. The changes in ERM and EBP50 are in agreement with our initial hypothesis and the current model of ERM function [39]. We also found significant age-related declines in Rac1 membrane association ($p=0.0008$) whose implications for TCR function will require further study.

Age increases the level of pTyr505 and pTry394 of lck, but not fyn

Two phosphorylation sites in lck regulate its kinase activity. Phosphorylation at Tyr394 (p394) increases lck enzymatic activity, and phosphorylation at Tyr505 (p505) inhibited activity [7–10]. A similar balance regulate fyn activity [7;8;41]. During TCR signaling, there is an enhancement of lck phosphorylation in p394 that result in increases in lck kinase activity, increases that may be directly responsible for p160 and p174 phosphorylation in Vav [15–18]. However, other kinases involved in TCR signal cascades, including Zap70, may also be

implicated in the phosphorylation of Vav [42]. To investigate the possible role of lck and fyn in age-related increases in Vav phosphorylation shown Figure 1 and Figure 2, we measured the level of p394 and p505lck in lck immunoprecipitates from resting or activated CD4 T cells of young and aged mice, as shown in Figure 4. Resting CD4 T cells from old mice show increased levels of both p505 and p395, compared with resting CD4 T cells from young mice. TCR stimulation increases p394 and p505lck phosphorylation in CD4 samples from young mice, but has no such effect in CD4 T cells from old mice. Figure 4 also shows mean and SEM of 5 experiments: age leads to a significant 75% fold increase in p394 ($p=0.011$) and a 50% fold increase in p505 ($p=0.036$). TCR stimulation leads to significant 50% increases in p394 ($p=0.047$) and p505 ($p=0.05$) in lck samples from young mice, but does not enhance phosphorylation in samples from old mice. There were no changes in the levels of lck protein between age or stimulation group of samples. Because fyn can also participate in the activation of Vav, we evaluated the effects of age and activation on p394fyn status, but found no differences between young and old T cells either before or after stimulation (data not shown). Because of the lack of specific antibodies, we were unable to analyze the fyn residue that serves as the equivalent of p505lck.

Discussion

During TCR activation, activation of tyrosine kinases including lck, fyn and Zap-70 leads to the activation of Vav, a necessary event in reorganization of the T cell cytoskeleton and translocation of proteins to the immunosynapse [42]. In addition, Vav activity regulates some of the members of the Rho family of small GTPases, including Rac1 [15–18], which in turn control ERM signaling [1;22]. We have recently shown that age can increase the level of Rac1 activity, accompanied by declines in ERM phosphorylation and declines in the ERM association signaling molecules, such as EBP50 and CD44 [30]. We hypothesized that changes in ERM and Rac1 may be linked to changes in upstream signaling pathways, including signals through Vav and lck. Analysis of Vav phosphorylation status in Vav immunoprecipitates, shown in Figure 1, suggests that age increases the level of p174 and p160 Vav, and thus presumably Vav activity, in resting CD4 T cells. These results are in agreement with the hypothesis that increases in Vav activity may be responsible for the age-related increase in Rac1 activity [30], but changes in other signaling pathways controlling Rac1 may also contribute to this phenomena. In addition, Figure 1 suggests that TCR stimulation can further increase Vav activity in CD4 from young mice, in agreement with the reported literature [2; 17;33]. However, CD4 samples from old mice show no effect of stimulation, suggesting age-related defects in the upstream signaling of Vav activation. It has been suggested that association of Vav and signaling molecules such as ERM and EBP50 to the membrane is necessary to deliver an efficient TCR signaling to downstream targets such as Rac1. Age-related alterations in the association of these proteins to the membrane could have important implications in the decline of T cell function. Analysis of the relative levels of Vav and phosphoVav associated with membranes (Fig. 2) shows that significant portions of both p164 and p170Vav of CD4 T cells from old mice are localized in the membrane fraction; age does not seem to alter the level of Vav associated to the membrane, but only its phosphorylation status. These results suggest that upstream regulators of Vav are also preferentially altered at the level of the membrane. We have shown that Zap-70 tyrosine kinase increases its association with the TCR in CD4 T cells from old mice [31]. It is possible that the increases in Zap70 membrane association to the TCR may be responsible for the high level of Vav phosphorylation in CD4 T cells from old mice. In addition, we cannot exclude the possibility that changes in tyrosine phosphatases involved in the TCR signaling can also responsible for the Vav changes. Those changes in phosphorylation status of Vav are in contrast with the data shown in Figure 3 suggesting a significant age-related decline in the membrane association of Rac1, ezrin, moesin (ERM) and EBP50. The age-related declines in membrane association of ezrin and moesin are in agreement with the current model of ERM function where declines in ERM

phosphorylation should lead to declines in membrane association and declines in EBP50 membrane colocalization [30]. As expected, there were no significant changes in the expression or membrane association of CD3e chain, making this protein suitable for normalization across experiments. Interestingly, we also found significant declines in Rac1 membrane association. We know that Rac1 activity increases with age, and it would be interesting to test if those changes happen at the membrane level or the cytosolic fraction. However, the effects of membrane purification on GTPase function make it impossible to assess directly the nucleotide loading of membrane-associated Rac1.

Vav phosphorylation of CD4 T cells from old mice suggests that upstream regulators of Vav activity may also be affected by age. In addition to Zap-70, other tyrosine kinases including lck and fyn can also regulate Vav phosphorylation [30;42]. Published studies of the effect of age on lck are contradictory, with some reports suggesting age-related declines in lck activity or expression [43–47], and others suggesting increases in phosphorylation, or no changes in lck activity [48–52]. Our results (Fig. 4) suggest no age-related changes in lck protein expression, but analysis of the p394 and p505lck by western blots suggests significant age-related increases in phosphorylation at both sites. Because phosphorylation of p394 and p505 sites have opposite effects on lck activity, we cannot draw any clear inferences about age-associated changes in lck function from these data. To address this problem would require additional information about age effects on the different pools of phosphorylated and unphosphorylated lck molecules, and their localization to membrane, cytoskeleton, and other signaling complexes [11;53;54]. It has been reported that changes in the isoforms and glycosylation of CD45 affects the phosphorylation and activity of lck associated to CD4 [53; 54]; it would be interesting to test if age-dependent changes in the glycosylation of CD45 contribute to changes in lck phosphorylation in CD4 T cells from old mice [55].

We found no evidence for an age effect on phosphorylation of fyn at site p394, but were unable to evaluate the fyn site corresponding to p505 of lck. Interestingly, it has been reported that exposure of resting T cells to oxidative stress, such as H₂O₂, can lead to increases in p394lck and p505lck [56–60] and potentially alter the Vav phosphorylation status. If aging leads to oxidative damage to T cell membranes, it is possible that altered lck function in that compartment might lead to changes in membrane-associated Vav, and through this way produce changes in cytoskeleton reorganization, including changes in the Rho family of GTPases and declines in ERM phosphorylation.

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Figure 1. Western blot analysis reveals age-related increases in Vav phosphorylation

CD4 T cells from young (Y) and old (O) mice were stimulated (+) or left unstimulated (-), and Vav was immunoprecipitated. The phosphorylation status of the p160 and p174Vav (pVav) and total Vav was measured by western blots using specific antibodies. The top panel shows a typical digital image of the western blot results. The graph shows the mean and SEM of 5 independent experiments involving 8 young and 6 old mice, where the pVav value was normalized against the Vav immunoprecipitated for each sample. The groups indicated by * are different at a significance level of $p < 0.05$ respect to unstimulated cells from young mice.

Figure 2. Age increases phosphorylation of membrane-associated Vav
 CD4 T cells from young (Y) and old (O) mice were lysed and membrane (Mem) or non-membrane fractions (cytosol) were enriched as described in the Material and Methods section. Samples of each fraction were analyzed for the presence of CD3 ϵ protein using western blots. Vav was immunoprecipitated from each fraction and p160Vav, p174Vav and Vav expression was analyzed by western blots. The digital image represents a typical experiment, and the graph represents the mean and SEM of 5 experiments using 5 young and 5 old mice, where the values of pVav were normalized to Vav from each sample. The groups indicated by * are different at a significance level of $p < 0.05$ with respect to T cells of young mice.

Figure 3. Age induces a decline in membrane associated ERM, Rac1, and EBP50

CD4 T cells from young (Y) and old (O) mice were lysed, and membrane (Mem) or non-membrane fractions (cytosol) were enriched as described in the Material and Methods section. The digital image show western blots of ERM, EBP50, CD3 ϵ , and Rac1 in enriched membranes of CD4 T cells from young and old mice. The bar graph represents the mean and SEM of 6 independent experiments using 15 young and 9 old mice; relative values were normalized using the level of CD3 ϵ from each sample. The * represents a statistically significant difference between young and old at $p < 0.05$.

Figure 4. Western blot analysis reveals age-related increases in lck phosphorylation

CD4 T cells from young (Y) and old (O) mice were stimulated (+) or not stimulated (-), and lck was immunoprecipitated. Phosphorylation status of p394, p505, and the amount of total lck protein were measured by western blots using specific antibodies. The top panel shows a typical digital image of the western blot results. The graph represents the mean and SEM of 5 independent experiments using 5 young and 5 old mice, where the pLck value was normalized against the lck immunoprecipitated for each sample. The groups indicated by * are different at a significance level of $p < 0.05$.