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Impairment of Non-Muscle Myosin IIA in human CD4+ T cells contributes to functional deficits in the elderly

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Abstract

Physiological aging imposes significant alterations in T cell repertoire and functions. Although several studies have reported defects upon antigen-induced activation of T cells during aging, the molecular mechanisms that control T cell receptor (TCR) down-modulation remain to be fully defined. While previous, studies have assessed the role of F-actin in regulating activation-induced TCR internalization, few of these have delineated the role of motor proteins, such as non-muscle myosin IIA (NMMIIA). In this study, we describe a series of experiments supporting the hypothesis that effective TCR down modulation requires not only efficient reorganization of the actin cytoskeleton, but also functional NMMIIA. For the first time, we show that $CD4^+$ T cells from elderly human donors have dysfunctional NMMIIA that contributes to delay activation-induced TCR internalization and lower calcium mobilization. Additionally, our studies demonstrate that chemical inhibition of NMMIIA in $CD4^+$ T cells from young donors also result in complete abrogation of TCR internalization, strongly supporting the fundamental role for NMMIIA in modulating this event. Recent observations that the generation of an efficient T cell response requires migration, prompted us to delineate whether NMMIIA also played a regulatory role in $CD4^+$ T cell migration. We now show that chemical inhibition of NMMIIA down-modulates chemotactic migration in $CD4^+$ T cells from both young and elderly donors. Together, these data demonstrate a significant contribution of dysfunctional NMMIIA in TCR-mediated functional defects during aging.

Keywords

chemotaxis; Hsp90; immune senescence; NMMIIA; TCR internalization

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Introduction

Aging is a complex process which is accompanied by a decline in various physiological processes.[1] In particular, immune senescence that often accompanies aging of the immune system is characterized by alterations in both innate and adaptive immunity, as well as, by the occurrence of chronic inflammatory processes.[2–4] The increased incidence and severity of infectious diseases in elderly population and their reduced ability to mount an appropriate immune response upon immunization[5, 6] correlate well with relatively weak and non specific $CD4^+$ T cell activation, resulting in a dramatic decrease in the efficacy of response to vaccination in the elderly.[6] Clinically, this is important since the elderly are highly encouraged to get vaccinated for infectious diseases such as influenza and pneumococcal pneumonia.[1, 5] While, on the one hand minimal alterations in the total number of $CD4^+$ T cells have been noted with age, on the other, substantial alterations in their signal transduction,[3] proliferative responses and migration[7–9] have been documented in murine models. Elegant studies, employing a mouse model for aging, have shown that $CD4^+$ T cells from aged TCR transgenic mice fail to form efficient immunological synapse (IS) with antigen presenting cells (APC) as seen in $CD4^+$ T cells from young mice.[10] Additionally, these data demonstrate that aging in mice leads to decreased recruitment of TCR-associated proteins, such as Lck, Vav, Moesin/Ezrin to the IS.[9, 11] This age-related reduction in recruitment of the signaling molecules in $CD4^+$ T cells from older mice is approximately 50% of that observed in the young cohorts. Furthermore, other studies have shown that $CD4^+$ T cells from aged mice have significant changes in cytoskeletal rearrangement, irrespective of the activation status, which leads to alteration in TCR-rich contact zones and T cell trafficking.[12] Although, the role of microcluster formation in the initiation of T cell activation is well established, in murine models and cell lines, the molecular mechanisms that underlie termination of T cell activation, such as TCR internalization[13] in primary human T cells remain poorly defined.

The initial contact of $CD4^+$ T cells with APC[14–17] leads to a series of cytoskeleton reorganization, including molecular segregation of T cell signaling molecules, polymerization and depolymerization of F-actin[16, 21] and development of lamellipodia[18, 19], as posited by the kinetic segregation model of T cell activation. These events, which are tightly controlled in terms of duration and strength of the response, are followed by termination events, primarily consisting of down-modulation of the cell surface TCR expression due to a combination of increased internalization, decreased recycling and increased degradation.

Emerging studies, primarily employing cancer cells, have demonstrated the fundamental role of Hsp90 in regulating microtubules/intermediate filaments of the cytoskeleton.[25–27] However, limited information is available about the role of Hsp90 in cell morphology and migration of T cells and in activation-dependent early events, we therefore assessed the contribution of Hsp90 in early events of T cell activation and termination.

Data generated previously from our laboratory clearly demonstrated that aging is accompanied by a decline in both the amount and function of Hsp90, which may contribute to the inability of $CD4^+$ T cells from elderly humans to undergo activation-induced

proliferation, as well as IL-2 secretion. Given that key tyrosine kinases involved in T cell signaling are Hsp90 client proteins[27], we were interested in determining if immune senescence impacted the pattern of Hsp90 interacting proteins in $CD4^+$ T cells. We now show, that NMMIIA,[28–30] a motor protein composed of six polypeptide chains, is differentially co-precipitated with Hsp90 in lysates obtained from $CD4^+$ T cells from the elderly but not from young donors. NMMIIA is composed of two identical heavy chains (myosin IIA), two essential light chains (ELC) and two regulatory light chains (RLC or MLC). It has been previously reported that activation of NMMIIA requires the phosphorylation of Thr18 and Ser19 residues on the RLCs primarily by myosin light chain kinase (MLCK) and Rho-associated coiled-coil containing kinase (ROCK). In the context of T cell biology, recent work done by Dustin and colleagues,[28] clearly demonstrated that phosphorylation of RLCs is necessary for the proper assembly of the T cell signalosome and for the stability of the IS.

Given the emerging role of NMMIIA in regulating many aspects of T cell biology, we have now provide evidence that outlines the contribution of NMMIIA to the observed defects in primary human $CD4^+$ T cells during aging. Our results for the first time demonstrate that altered functional NMMIIA occurs in T cells from the elderly and this alteration in NMMIIA may contribute to defects in early signaling events including Ca^{2+} mobilization, TCR internalization, as well as, chemotactic migration towards SDF-1α.

Results

Non-muscle myosin IIA is an abundant protein that co-precipitates with Hsp90 in $CD4^+$ T cells from elderly donors

As immune senescence has been demonstrated to be accompanied by lowered levels and reduced chaperone activity of Hsp90 in T cells,[1] we were interested in profiling and identifying Hsp90-associated proteins in T cells during aging. As previous studies employing murine T cells have demonstrated significant change in the ratio of memory to naïve T cells with advancing age, with a preponderance of memory population, we initiated analyses of naïve and memory subsets in our T cell pools. However, our studies employing flow cytometry demonstrated only a minimal increase in memory population with advancing age. In fact, $CD4^+CD45RO^+$ memory T cells, negatively selected using specific antibodies directed against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCRγ/δ, glycophorin A and CD45RA, were present at levels similar to that observed in samples from young donors. We therefore used entire population of $CD4^+$ T cells in our assays to evaluate the impact of aging. We initially immuno-precipitated Hsp90 from $CD4^+$ T cell lysates obtained from young and elderly donors and resolved them using SDS-PAGE, followed by staining with Biosafe Coommassie. One of the abundant proteins obtained following immunoprecipitation derived from $CD4^+$ T cells from elderly donors, appearing at 225kDa, as demonstrated in fig. 1A, was excised and subjected to in-gel trypsin digestion. Tryptic peptides were subjected to LC-MS/MS analysis and proteins were identified from MS/MS spectra by database searching using Mascot search engine.

As shown in fig. 1B the protein band detected in lysates from the elderly, resolving at about 225kDa was identified as NMMIIA. About 412 unique peptides were identified that

matched with NMMIIA, from a total of 311 spectra, 180 were found to be unique to the matched protein. Overall, these peptides covered 58% of the full length NMMIIA protein. The presence of NMMIIA was further confirmed by immunoprecipitation followed by immunoblotting (data not shown). While this is the first demonstration of abundant NMMIIA in Hsp90 immunoprecipitates from $CD4^+$ T cells from elderly donors, NMMIIA has previously been identified in immunoprecipitates of Hsp90, and as an Hsp90 binding partner in proteomic studies in other cell types such as fibroblasts.[46] NMMIIA, a motor protein, is a member of non-muscle motor protein family, and has recently been described to play a vital role in cell polarization, migration, adhesion and cytokinesis.[18, 20, 28]

In fact, the requirement of NMMIIA in the formation and stability of the immunological synapse in human T cells was recently demonstrated.[28] Additionally, studies have also demonstrated the requirement of NMMIIA in mediating contractility in T cell crawling.[30, 31] Thus, NMMIIA appears to be a central mediator of early signaling events in T cells.

Non-muscle myosin IIA expression remains unaltered in $CD4^+$ T cells during aging

Given the observation that increased association of NMMIIA with Hsp90 occurs in T cells obtained from elderly donors when compared to those from young donors, we next assessed both the constitutive and induced protein levels and mRNA expression of NMMIIA in $CD4^+$ T cells obtained from both young and elderly donors. $CD4^+$ T cells were either left untreated or treated with plate bound anti-CD3 for 10min. At the end of the incubation cells were lysed and about 25μg of proteins were resolved by SDS-PAGE followed by western blotting with antibody to NMMIIA. As shown in fig. 2A and 2B we observed a subtle, but consistent, increase in constitutive NMMIIA protein expression in $CD4^+$ T cells from elderly donors when compared to those from young donors. Additionally, treatment with anti-CD3 failed to induce any further change in the expression of NMMIIA above constitutive levels, in cells from both young and old donors, alike. Interestingly, while reactivity of NMMIIA with the antibody resulted in distinct bands at about 250kDa in the lysates of $CD4^+$ T cells from young donors, in contrast, $CD4^+$ T cell lysates obtained from the elderly demonstrated significant reactivity with several lower molecular weight proteins occurring below 250kDa which was further accentuated following anti-CD3 treatment. While it is not clear if these are degradation products or lower molecular weight isoforms, preliminary studies employing phospho-myosin-IIA (data not shown) appear to indicate folding intermediates, future studies will determine the precise nature of these intermediates and the underlying basis for these anti-myosin IIA reactive bands in the cells from the elderly. It is important to note that non-muscle myosin IIB was not detected in primary CD4+ T cells from young or elderly donors, alike.

To determine whether alterations in transcription of non-muscle myosin IIA occur with age, we next examined mRNA expression of NMMIIA employing qRT-PCR in $CD4^+$ T cells from young and elderly human donors. Results presented in fig. 2C demonstrate that mRNA expression levels of NMMIIA in $CD4^+$ T cells are not affected by the age of the donor. Employing Jurkat T cell line and actinomycin D, we next evaluated the half life of NMMIIA mRNA. Our studies indicate a relatively high stability of NMMIIA mRNA, with a turnover rate greater than 72h (data not shown). Thus, increased association of NMMIIA with Hsp90

observed upon immunoprecipitation in $CD4^+$ T cells from the elderly could not be attributed to an overall increase in protein or mRNA expression of NMMIIA.

Myosin regulatory light chain (RLC) is constitutively phosphorylated in $CD4^+$ T cells obtained from elderly donors

As recent studies have demonstrated a critical role for the phosphorylation of RLCs in the assembly of T cell signalosome[21, 28] during the initiation of T cell signaling,[13, 18, 32] we next analyzed the phosphorylation status of RLC in $CD4^+$ T cells from young and elderly donors, prior to and following activation with immobilized anti-CD3 for 10 min. Cell lysates resolved by SDS-PAGE, were immunoblotted using antibody specific to RLC and pRLC (phosphorylated RLC). As shown in fig. 3A and 3B, overall levels of RLC were similar in $CD4^+$ T cells obtained from young and elderly donors, prior to and following activation with anti-CD3. In contrast, pRLC was detected only upon treatment with anti-CD3 in $CD4^+$ T cells from young donors, while they were already detectable under basal conditions in lysates obtained from the elderly. Activation with anti-CD3 resulted in no further increase in pRLC levels in cells from the elderly. Thus, RLC appears to be constitutively phosphorylated in $CD4^+$ T cells from elderly donors and is unaffected by treatment with anti-CD3. To our knowledge, this is the first report of altered activation of NMMIIA in $CD4^+$ T cells from elderly. As pRLC is important in T cell signaling, our data for the first time demonstrates altered regulation of pRLC in $CD4^+$ T cells from the elderly, and we believe that this may underlie reported defects in T cell activation during aging.

Altered distribution of non-muscle myosin IIA but not TCRα/β in T cells accompanies aging

Recent reports in the literature have demonstrated that IS are initiated by signaling in discrete TCR microclusters and that these microclusters play an important role in T cell functional differentiation and effector functions.[18, 33, 34] Further, microcluster movement and assembly have been demonstrated to be associated with centripetal actin flow in coordination with NMMIIA activation.[20, 21, 35] The importance of NMMIIA in T cell signaling and inherent alterations in NMMIIA activation status during aging prompted us to study the cellular localization of NMMIIA in $CD4^+$ T cells from young and elderly donors employing confocal microscopy. $CD4^+$ T cells from young and elderly donors were either left untreated or pretreated with blebbistatin, a NMMIIA specific inhibitor. $CD4^+$ T cells were then stained with either antibody to NMMIIA or TCRα/β, followed by appropriate fluorescent secondary antibody. As shown in fig. 4, NMMIIA appeared to be strongly associated with the plasma membrane in $CD4^+$ T cells from the young however, in $CD4^+$ T cells from the elderly, NMMIIA showed a more diffuse and punctuate cytosolic localization, with minimal membrane localization. Interestingly, pretreatment of the cells with blebbistatin induced a punctuate-diffuse distribution of NMMIIA in $CD4^+$ T cells from young donors mimicking the distribution seen constitutively in $CD4^+$ T cells from elderly donors (fig. 4A, B). As blebbistatin is a chemical inhibitor of functional NMMIIA activity, it appears that inhibition of NMMIIA results in the loss of plasma membrane localization of NMMIIA, as seen in T cells from the elderly. This altered NMMIIA localization is in clear contrast to the pattern of TCR localization observed in $CD4^+$ T cells which was predominantly localized to the plasma membrane both under basal and blebbistatin treated

conditions, irrespective of the age of the T cell donor (fig. 5). Thus, our studies, for the first time demonstrate that inhibition of NMMIIA by blebbistatin results in punctuate, cytosolic rather than plasma membrane distribution of NMMIIA in $CD4^+$ T cells from young donors, mimicking the observation in untreated $CD4^+$ T cells from elderly with little or no impact on the cell surface distribution of TCR α/β.

Non-functional non-muscle myosin IIA negatively impacts F-actin polymerization in $CD4^+$ T cells

It has been extensively reported that one of the earliest event during $CD4^+$ T cell activation is the rapid and transient conversion of monomeric actin, G-actin, into filamentous actin, F-actin. Interaction between actin and NMMIIA has been described extensively,[33] with the acto-myosin complex playing an important role not only in the modification of cell shape but also in crawling and migration in T cells. Although defects in F-actin polymerization have been reported in T cells from murine models of aging,[9] limited data exists with regards to actin dynamics in activated human T cells during aging, and the role of NMMIIA in this mix, remains unknown. Consequently, employing primary $CD4^+$ T cells obtained from young and elderly donors, we evaluated the changes in F-actin polymerization during anti-CD3 induced activation. As shown in fig.6A, anti-CD3 treatment of $CD4^+$ T cells induced an increase in F-actin levels that were significantly more pronounced in $CD4^+$ T cells obtained from the young than those seen in similarly treated cells from the elderly. In addition, we also observed a significant age-related difference in the kinetics of F-actin polymerization. Specifically, in $CD4^+$ T cells from young donors, levels of F-actin peaked as early as 2 min and decreased gradually, returning to near basal levels by 10 min of anti-CD3 activation. However, in $CD4^+$ T cells from elderly donors, the levels of F-actin did not change significantly from basal levels, irrespective of the time of anti-CD3 activation, indicating a defect in activation-induced F-actin polymerization. To determine if the observed defect in F-actin polymerization is due to non functional NMMIIA, we pretreated $CD4^+$ T cells with blebbistatin, and subsequently activated them with anti-CD3 for 2, 5, and 10 min. As shown in fig. 6B, chemical inhibition of NMMIIA resulted in partial inhibition of F-actin polymerization, which was more pronounced in the $CD4^+$ T cells obtained from young donors than those from the elderly. Thus, we conclude that functional inhibition of NMMIIA negatively impacts F-actin polymerization, which may potentially underlie the inability of T cells from elderly to mediate early cytoskeletal events, including microcluster formation in the initiation of T cells activation and TCR internalization in the termination of activation.

Functional non-muscle myosin IIA is necessary for activation-induced internalization of TCRα/β

T cell activation by a cognate antigen or anti-CD3 antibodies results in the rapid internalization of TCR molecules, resulting in fewer TCR molecules at the surface.[13, 17, 36] This down-modulation of TCR-CD3 complex has been demonstrated to play an important role in fine-tuning the avidity and strength of MHC/TCR interaction, and in attenuating TCR signaling. Although recent reports clearly demonstrate a role for NMMIIA on microcluster migration and stability of the IS, a role for NMMIIA in mediating internalization of TCRα/β and receptor down modulation has not been investigated. Consequently, to elucidate the role

of NMMIIA on the internalization of TCRα/β receptors, we performed flow cytometry on $CD4^+$ T cells from young and elderly donors, before and after treatment with anti-CD3, for defined periods of time, in the presence and absence of blebbistatin. As shown in fig. 7, surface expression of TCRα/β receptors were similar in untreated T cells from young and elderly donors, demonstrating no change in TCR expression with age of the donor population. Upon treatment with immobilized anti-CD3, in $CD4^+$ T cell from young donors demonstrated a rapid decrease in the surface expression of TCRα/β as early as 10 min accounting for about 60% loss in expression, which attained a 80% loss in expression by 40 min indicating internalization. In contrast, $CD4^+$ T cells from elderly donors demonstrated decreased internalization, with an average of 30% decrease in TCRα/β surface expression at 10 min following treatment with anti-CD3, approaching a maximum of 50% decrease in expression even after an extended 40 min of treatment with anti-CD3. To evaluate the contribution of functional activity of NMMIIA in this process, we next pretreated $CD4^+$ T cells from young and elderly donors with blebbistatin and then either left them untreated or activated with immobilized anti-CD3 antibody for 10 min and 40 min. Our results (fig. 7 and 8) demonstrate that treatment with blebbistatin per se, does not impact the basal levels of TCRα/β expression, irrespective of the age of the T cell donor. However, treatment with blebbistatin significantly inhibited anti-CD3 mediated internalization of TCRα/β receptors at both 10 min and 40 min, in $CD4^+$ T cells obtained from young donors, resulting in an average internalization of about 20% at 10 min and 30% at 40 min. In contrast, similarly treated cells from the elderly, failed to show a significant decline upon treatment with blebbistatin (fig. 7 and 8). Based on these results, we now conclude that inhibition of the functional activity of NMMIIA negatively impacts anti-CD3 mediated TCRα/β internalization/down-modulation and hence cell surface expression in $CD4^+$ T cells from young donors thus mimicking the overall phenotype observed in anti-CD3 activated, but blebbistatin-untreated $CD4^+$ T cells from the elderly.

Non-muscle myosin IIA negatively regulates intracellular Ca^{2+} release upon TCR engagement in $CD4^+$ T cells

In $CD4^+$ T cells, calcium ions (Ca^{2+}) function as critical second messengers. Ca^{2+} signals regulate the activation of lymphocytes, their differentiation and multiple effector functions, as well as, induction of a variety of transcriptional programs.[13] In lymphocytes, studies have demonstrated that sustained Ca^{2+} entry is necessary for complete activation of calcineurin dependent nuclear factor of activated T cells (NFAT) pathways. It has been previously reported that immune senescence is accompanied by a reduction in calcium release from intracellular stores upon lymphocyte activation. Since a defect in TCRα/β internalization is observed in $CD4^+$ T cells from the elderly, as well as upon pretreatment with blebbistatin in $CD4^+$ T cells from young donors, we next evaluated the impact of inhibition of NMMIIA on activation-induced calcium release from intracellular stores. $CD4^+$ T cells obtained from both young and elderly donors were either pretreated with blebbistatin or left untreated and subsequently incubated with super-antigen pulsed antigen presenting cells (APC) at a ratio of 1:1 [Raji B cells pulsed with Staphylococcus Enterotoxin A (SEA)]. Microfluorescent imaging of intracellular calcium influx was monitored by loading $CD4^+$ T cells with a calcium indicator dye Fura-2AM, as described. As has been reported previously in murine studies, our studies confirmed that $CD4^+$ T cells obtained from elderly human donors,

activated by incubation with pulsed APC, demonstrated lower and sustained calcium release from intracellular stores when compared to similarly treated cells from young donors (fig. 9A, B, C), where the levels of calcium demonstrated a rapid significant peak response. Pretreatment of $CD4^+$ T cells with NMMIIA inhibitor, blebbistatin completely abrogated the release of intracellular calcium upon TCRα/β engagement by antigen-pulsed APC, irrespective of the age of the donor. Interestingly, blebbistatin treatment however, failed to impact ionomycin mediated calcium release from $CD4^+$ T cells from both young and elderly donors alike, implicating a role for NMMIIA function in early events of TCRα/β signaling. Thus, a fully functional NMMIIA appears to be an active and necessary participant in calcium release from intracellular stores following TCR engagement, underscoring its role in regulating key steps in early signaling events activated by effective TCR engagement.

Chemotactic migration of $CD4^+$ T cells in response to SDF1α is enhanced in $CD4^+$ T cells from the elderly

Continuous recirculation of $CD4^+$ T cells and their eventual migration to tissues upon activation is important for protective immunity against invading pathogens.[37, 38] In migrating cells, actin organizes into two basic structures, the lamellipodium and the uropod. It has been previously reported that functional NMMIIA is required not only in the generation of the driving force, but also in the rate of acto-myosin complex formation and F-actin polymerization,[20, 21] therefore, we evaluated the role of NMMIIA in chemotactic migration during aging. Migration[39, 40] was evaluated utilizing a chemotactic gradient employing hSDF-1α. $CD4^+$ T cells from young and elderly donors were allowed to migrate for 4h in transwell plates that were either left uncoated (PBS-BSA) or coated with 100nM of hSDF-1α. As shown in fig. 10A, $CD4^+$ T cells from both young and elderly donors migrated minimally in the absence of hSDF-1α. However, in response to hSDF-1α, $CD4^+$ T cells from both young and elderly donors demonstrated robust migration. We found significantly higher numbers of $CD4^+$ T cells from elderly donors migrated towards hSDF-1α than those from young donors. This observation is in agreement to data previously reported for $CD4^+$ splenic T cells in aged mice, where an increase in the percentage of transmigration was reported in response to SDF-1α.[47] Additionally, increased levels of CXCR4 expression has been demonstrated in T cells from mice during aging, which may account for increased migration towards hSDF-1α[47] observed in our samples. Inhibition of NMMIIA activity by pretreatment with blebbistatin resulted in a profound inhibition of the migration towards hSDF-1α in $CD4^+$ T cells from both young and elderly donors, alike (fig. 10B). Taken together these results suggest that decreased functional NMMIIA in the elderly has a positive impact on the migration of $CD4^+$ T cells. Since complete inhibition of NMMIIA negatively impacts chemotaxis towards hSDF-1α, we believe that increased migration observed in the elderly may be suggestive of differential complexes recruited by functional and non-functional NMMIIA in $CD4^+$ T cell migration. Additionally, as previous studies in other cell types have demonstrated both an increase and a decrease in migration of cells, upon blebbistatin treated cells[31], it is clear that graded NMMIIA levels may regulate migration with partial versus complete inhibition impacting migration differentially. Future studies will dissect the precise role of NMMIIA in T cell migration during aging.

Discussion

In this study we present evidence suggesting a novel role for NMMIIA in T cell functional deficits observed during aging in humans. Previous reports from our laboratory and those of others have shown that $CD4^+$ T cells from healthy elderly human subjects and aged mice exhibit multiple defects in signal transduction events following TCR ligation.[12] These include alterations in tyrosine phosphorylation of TCR-associated ζ chains,[41] calcium release from intracellular stores, polymerization of F-actin[11] and induction of nuclear translocation of transcription factors.[3, 4] As dynamic properties of $CD4^+$ T cells such as migration, adhesion and cell division depend on actin polymerization and NMMIIA-dependent compression and retraction, alterations in the activation status of NMMIA will likely have a profound effect not only on migration and adhesion, but also on $CD4^+$ T cell antigen receptor microcluster formation and immunological synapse stabilization. Studies presented here for the first time demonstrate a central role for NMMIIA dysregulation in age-associated functional defects observed in $CD4^+$ T cells. These defects range from alterations in actin polymerization, TCR internalization to T cell migration towards chemotactic gradient. NMMIIA, that is abundantly associated with Hsp90, is constitutively activated by phosphorylation of regulatory light chain (RLC) in $CD4^+$ T cells obtained from elderly donors. This basal phosphorylation of RLC might explain the inability of these cells to undergo any further stimulation upon treatment with common polyclonal T cell activators. Interestingly, we now provide evidence that pretreatment of $CD4^+$ T cells from young donors with a NMMIIA specific inhibitor, blebbistatin, alters NMMIIA distribution in these cells, and partially induces phosphorylation of RLC, mimicking the observation in T cells from the elderly. Taken together, these results suggest that inhibition of NMMIIA may contribute to immune dysfunction in the elderly.

The dynamics of TCR-down modulation can directly impinge not only on the termination of TCR signaling and T cell activation, but also on events dictated by co-receptors such as CTLA-4 and PD-1.[13, 42, 43] As several studies have now attributed the decrease in number of TCR on the cell surface to a combination of processes involving internalization, recycling and degradation, our studies for the first time show the requirement and involvement of functional NMMIIA in activation-induced TCR internalization. Additionally, we now show that the functional inhibition of NMMIIA in $CD4^+$ T cells from young donors results in an almost complete abrogation of TCR internalization induced upon treatment with anti-CD3 antibody. This block in TCR internalization is similar to that observed upon anti-CD3 treatment in $CD4^+$ T cells from the elderly. Thus, implicating altered NMMIIA in defective TCR internalization in the elderly.

As c-Cbl and Cbl-b, ubiquitin ligases play essential roles in the dynamics of TCR down modulation by regulating vesicle sorting and endosome to lysosome trafficking,[42–45] future studies will be directed at delineating c-Cbl and Cbl-b status in T cells from the elderly.

Contrary to our expectations, defects in NMMIIA function in T cells from the elderly did not drastically impair chemotactic migration towards SDF-1α. In fact, $CD4^+$ T cells from the elderly demonstrated a significant increase in migration index when compared to T cells obtained from young donors. This increased migration towards SDF1 supports data obtained

in murine splenocytes, and is indicative of a reciprocal regulation of NMMIIA and chemotactic activity. As treatment of both $CD4^+$ T cells from young and elderly donors, with blebbistatin completely abrogated chemotactic migration, we believe that complete inhibition of functional activity of NMMIIA interferes with migration, abrogating it in $CD4^+$ T cells from both young and elderly donors. This seemingly paradoxical observation of increased chemotactic migration in $CD4^+$ T cells from the elderly in the context of altered NMMIIA levels may be attributable to a partial but not complete loss in activity of NMMIIA in the elderly. Future studies employing graded amounts of NMMIIA may be able to resolve the magnitude of response to chemokines and the expression of functional NMMIIA. In fact, studies in adherent cells have demonstrated either a reduction or an enhancement in migration upon myosin IIA inhibition. Additionally, alteration in CXCR4 expression, previously reported to be increased, acto-myosin complex formation and F-actin polymerization in $CD4^+$ T cells from elderly may well contribute to overall increase in migration observed in the elderly. As CXCR4 expression on the surface of T cells has been demonstrated to be regulated by NMMIIA, changes in functional NMMIIA in $CD4^+$ T cells from the elderly may indirectly regulate chemotactic migration.

Overall, our studies have uncovered an important contribution of non-muscle myosin IIA in T cell functional responses during immune senescence ranging from its role in activation-mediated TCR internalization, intracellular calcium mobilization to chemotactic migration.

Materials and Methods

Abs and reagents

Antibody to Myosin IIA, Myosin Light Chain 2, and Myosin Light Chain 2 phosphorylated at Ser19 were from Cell Signaling Technology (Danvers, MA). Antibodies to Hsp90, TCRα/β chains and horseradish peroxidase-conjugated goat anti-mouse were from BD Biosciences (San Jose, CA). Antibody to β-actin was from Santa Cruz biotechnology (Santa Cruz, CA). Alexa Fluor 488-conjugated mouse anti-rabbit, Alexa Fluor 555-conjugated goat anti-mouse secondary antibodies and Fura-2AM, were from Invitrogen (Carlsbad, CA). Mouse anti-human CD3 was purified from an OKT3 hybridoma supernatant. Horseradish peroxidase-conjugated goat anti-rabbit was from Thermo Fisher Scientific (Rockford, IL). All fine chemicals, unless otherwise mentioned, were obtained from Sigma-Aldrich (Saint Louis, MO). Rhodamine-phalloidin was obtained from Cytoskeleton (Denver, CO). SDF-1α was from ProSpec-Tany Technogene (Rehovot, Israel). Electrophoresis supplies were from BioRad (Hercules, CA).

Human subjects

Peripheral blood was obtained by venipuncture from healthy young (21–30 years) and elderly (65–89 years) adults enrolled from the greater Little Rock area. Immuno-compromised subjects were excluded from the study, including individuals with asthma and those taking immune modulating drugs. Subjects on antibiotics or who self reported symptoms of recent infection (<3 weeks before enrollment) were also excluded. All protocols involving human subjects were approved by the UAMS Institutional Review Board and appropriate informed consents were obtained. Blood draw was performed at the

Clinical Research Center (CRC) at UAMS. The demographics of the volunteers recruited for the study were as follows: young donor population consisted of 62% males, 38% females with an average age of 25.5 years. 86% of these individuals were Caucasians, 10% Asians and the rest were minorities. Elderly donor population consisted of 43% males and 57% females with an average age of 75 years, and 93% of these individuals were Caucasians and the rest were minorities.

T lymphocyte isolation

$CD4^+$ T cells were negatively selected from blood using the EasySep $CD4^+$ T-cell Enrichment kit adopting the manufacturer's recommended protocol (StemCell Tech, Vancouver, Canada). Purity of isolated $CD4^+$ T cells was determined by flow cytometry and was consistently found to be 90–95% pure. Lymphocytes were isolated from PBMC following F/H gradient centrifugation of whole blood. In our young donor population, T cells represented 80–82% of PBMC, with an average of 85% being $CD4^+$ T cells. In the elderly group, T cells represented 81–83% of PBMC with 84% being $CD4^+$ T cells. Of the $CD4^+$ T lymphocytes, $CD45RA^+$, $CCR7^+$, $CD45RO^-$ represented an average of 54% and $CD45RO^+$,$CD45RA^-$ represented an average of 46% in young donors, and 48% and 52% respectively, in the elderly donor group. T regs, identified as $CD4^+$, $FOXP3^+$, $CD25^+$ represented a mean of 4.82% of the CD4 population in the young donors, and a mean of 5.87% of the CD4 population in the elderly donors.

Flow Cytometry

For surface staining, $CD4^+$ T cells were incubated with antibody directed to TCRα/β (BD Biosciences, San Jose, CA) followed by incubation with an antibody conjugated to Alexa Fluor-555 for 20 min at 4°C. At the end of incubation, cells were washed with ice cold PBS containing 0.1% BSA and fixed with 2% paraformaldehyde. For intracellular staining of F-actin, cells were fixed (2% paraformaldehyde), permeabilized (PBS buffer +0.1% Saponin), and stained with Rhodamine phalloidin based on the manufacturer's protocol (Cytoskeleton, Denver, CO).

RT-qPCR

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). Briefly, 4μg of total RNA for each sample was reverse transcribed and the resultant cDNA was amplified using BioRad iCycler PCR system. Reactions were performed in 96-well PCR reaction plates using 12.5 μL of iQ SYBR Green Supermix (BioRad, Hercules, CA), forward and reverse primers (0.3 nmol each), and cDNA (3 μL) in a final volume of 25 μL. Amplification parameters were denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 30 s and 60 °C for 70 s. Samples were analyzed in duplicate for the expression levels of *myosin IIA*, and human *β-actin* and *GAPDH* were used as a housekeeping genes. Fold induction was calculated after normalization to the housekeeping genes using the $\Delta\Delta C_T$ method. Dissociation curves indicated that each reaction consisted of a single reaction product. Gene-specific primers were designed by Primer3 software employing human sequences obtained from GenBank. Primer sequences will be provided upon request.

Confocal Microscopy

Freshly isolated $CD4^+$ T cells were stained for TCRα/β (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Briefly, cells were incubated with antibody directed to TCRα/β for 20 min and followed by an anti-rabbit Alexa Fluor 555. The cells were then washed and fixed with 2% paraformaldehyde, prior to mounting on poly-L-lysine coated coverslips. For intracellular protein staining of non-muscle myosin IIA, $CD4^+$ T cells were fixed, permeabilized (PBS buffer + 0.1% Saponin), and stained with anti-myosin IIA antibody. Anti-rabbit Alexa Fluor 488 was used as a detection reagent. Series of fluorescence images were captured by a Zeiss confocal LSM 510 META (Carl Zeiss, Thornwood, NY) using a Plan-Apochromat 63/1.4 oil differential interference contrast objective. Images were processed and analyzed with LSM510 and ImageJ software.

Calcium Imaging

Intracellular calcium concentrations were measured with Ca^{2+} indicator dye, Fura-2AM. $CD4^+$ T cells were stimulated with Staphylococcus Enterotoxin A (SEA)-loaded Raji B cells (10ng/mL) or ionomycin (50ng/ml). Briefly, $CD4^+$ T cells were loaded with the dye by incubation with 5μM of Fura-2AM at 37°C for 30 min in complete media, followed by a 30 min chase. Imaging was performed with an InCa dual-wavelength system (Intracellular Imaging, Cincinnati OH) with the Ca^{2+} concentration calculated as the relationship between the ratio of emission at 505 nm to excitation at 340 nm and 380 nm. Each experimental data point represents the average of Ca^{2+} concentration calculated from at least 20 individually measured $CD4^+$ T cells from 3 different fields and 6 independent donor pairs.

Chemotactic Migration assay

$CD4^+$ T cells ($3x10^5$) either left untreated or pretreated with blebbistatin (50μM, 1h) were resuspended in 100μl of media containing 0.2% BSA and placed into the upper chamber of a transwell migration plate (5μm pore, Costar). In the lower chamber 600μl of media containing 0.2%BSA with or without 100nM of SDF-1α was added and cells were allowed to migrate for 4h at 37°C. The number of migrated cells was counted following Trypan Blue staining. Percent migrating cells and migration index were derived from these data.

Western Blotting and Co-immunoprecipitation

$CD4^+$T cell lysates, equalized for protein, were resolved using SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted with specific antibody/s and detected using chemiluminescence.

For co-immunoprecipitation studies, pre-cleared total cell lysates (250μg protein) were incubated with antibody to Hsp90 and protein A/G agarose beads overnight at 4°C with gentle rocking. Protein A/G beads containing the adsorbed immunoprecipitated complex were washed with RIPA buffer, resuspended in 30μl of 2X SDS-Sample buffer and heated in a boiling water-bath for 5 min. Protein complexes were resolved by SDS-PAGE and subjected to western blot analyses, as detailed above.

LC-MS/MS methods

SDS-PAGE gel bands were excised and subjected to in-gel trypsin digestion as follows. Protein-containing gel slices were destained in 50% methanol, 100mM ammonium bicarbonate, followed by reduction in 10mM Tris [2-carboxyethyl]phosphine and alkylation in 50mM iodoacetamide. Gel slices were then dehydrated in acetonitrile, followed by addition of 100 ng sequencing grade porcine trypsin in 100 mM ammonium bicarbonate (Sigma-Aldrich) and incubation at 37°C for 12–16 hours. Peptide products were then acidified in 0.1% formic acid. Tryptic peptides were separated by reverse phase HPLC on a 10cm C18 column using a NanoLC 2D system (Eksigent, Dublin, CA) and ionized by electrospray upon elution, followed by MS/MS analysis using an LTQ XL mass spectrometer (Thermo Scientific, Waltham, MA). Proteins were identified from MS/MS spectra by database searching using the Mascot search engine (Matrix Science).

F-actin polymerization assay

Briefly, primary $CD4^+$ T cells were either left untreated or activated with plate bound αCD3 for 2, 5 and 10 min. At the end of treatment, the cells were subjected to intracellular staining using Rhodamine- phalloidin per manufacturer's protocol, employing flow cytometry. Data were analyzed using Cell-quest-Pro software (BD Biosciences, San Jose, CA).

Statistical analyses

Differences between means of the data generated in the study were analyzed using Student's *t*-test. Differences were considered significant, if $p < 0.05$.

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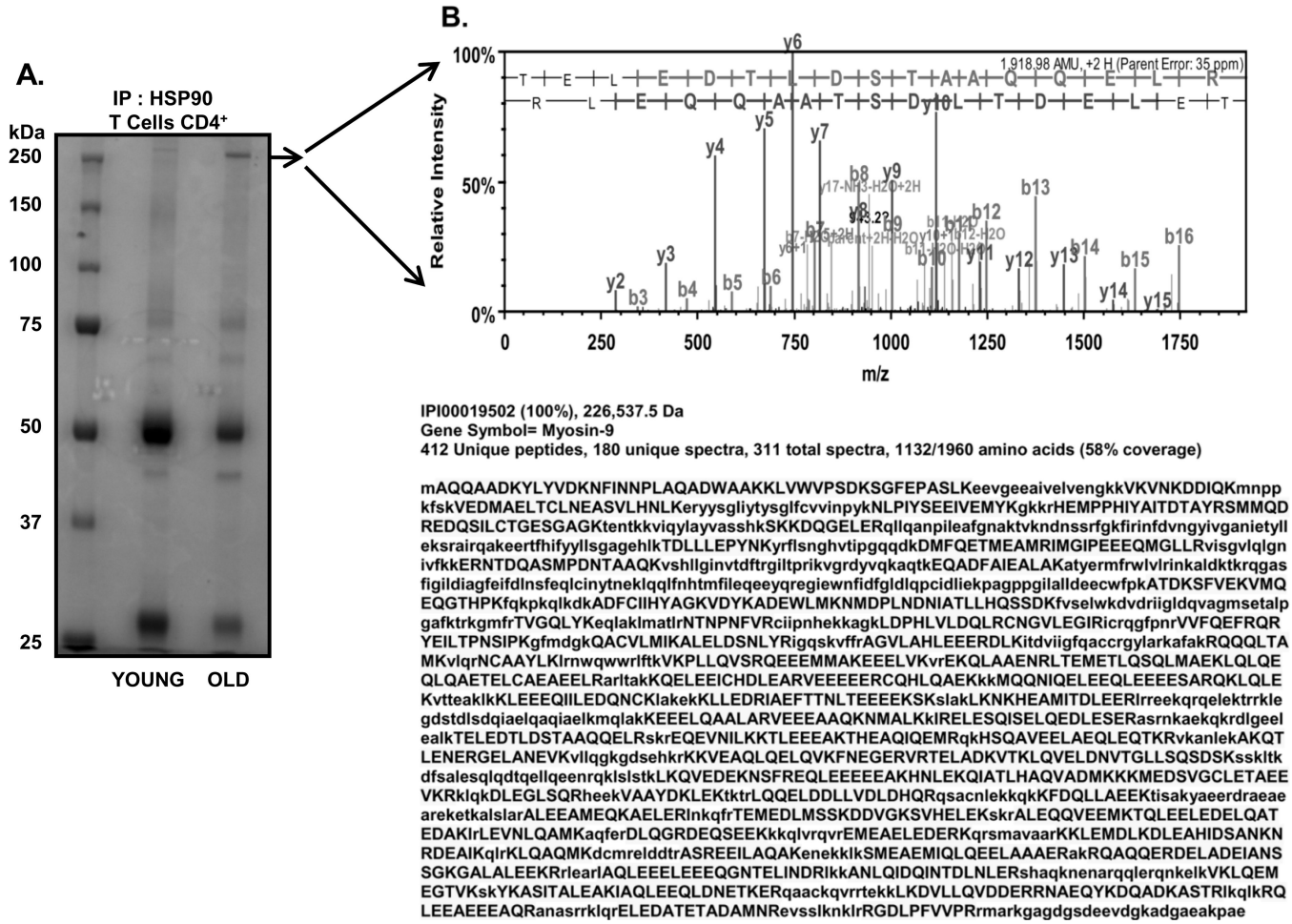

FIGURE 1.

Hsp90 associated proteins detected by co-immunoprecipitation in CD4$^+$ T cell lysates from the elderly. *A*, Hsp90 co-immunoprecipitated proteins obtained from CD4$^+$ T cells from 5 independent young and elderly donor pairs were resolved by SDS-PAGE, and stained with Bio-Safe Coomassie. The predominant band at 225kDa (higher in the elderly) was excised and analyzed by LC-MS/MS. *B*, Proteomic profile (spectrum and amino acid sequence) of peptides that match with myosin IIA protein sequence obtained from the excised band

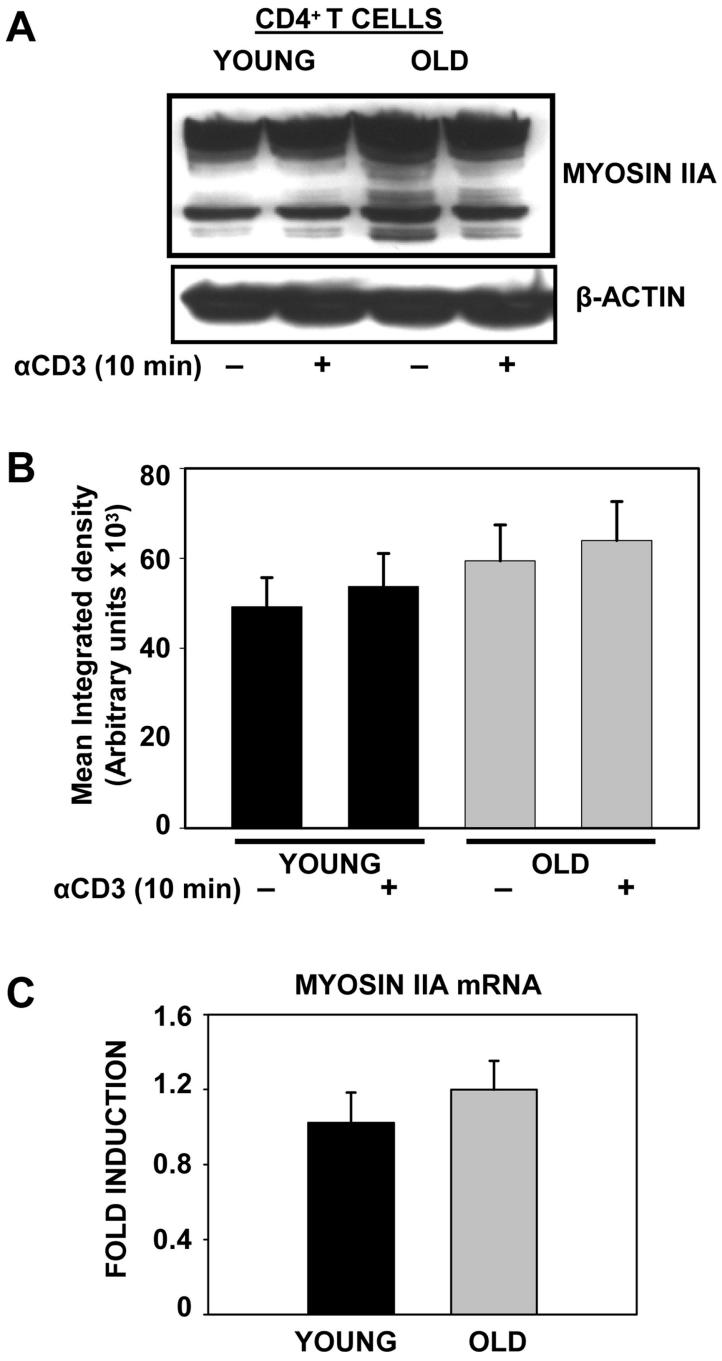

FIGURE 2.

Expression of Non-muscle Myosin IIA in $CD4^+$ T cells from young and elderly donors: *A*, Western blot of NMMIIA levels in $CD4^+$ T cells from young and elderly donors either left untreated or activated with anti-CD3 beads for 10 min. β-actin was used as a control for equal protein loading. Representative data from one donor pair out of 8 pairs tested are provided. *B*, Quantitation of the specific band for myosin IIA protein was carried out by densitometry. Values represent mean integrated density ± SE obtained from a minimum of 8 independent donor pairs. *C*, qRT-PCR analysis of myosin IIA mRNA from total RNA

collected from $CD4^+$ T cells. Average data from ten donor pairs are provided. β-actin and GAPDH were used as reference genes and for normalization. Data are presented as fold induction.

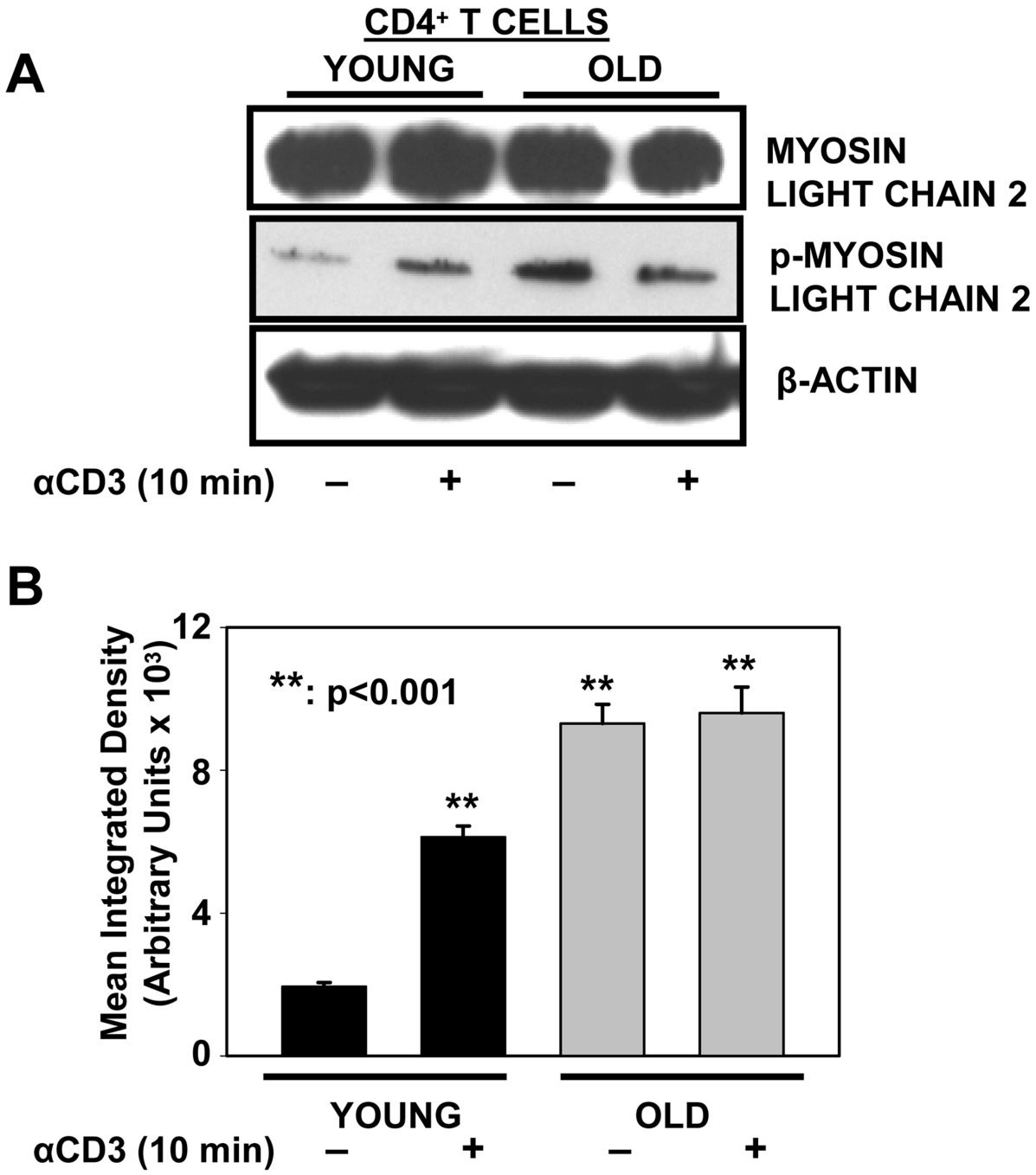

FIGURE 3.

Constitutively activated non-muscle myosin IIA is detected in T cells from the elderly. *A,* Western blot of MLC2 and pMLC2 in $CD4^+$ T cells from young and elderly donors either left untreated or activated with anti-CD3 beads for 10 min. β-actin was used as a control for equal loading. Representative data from one donor pair out of 8 pairs tested are provided. *B,* Quantitation of the specific band for pMLC2 protein was carried out by densitometry. Values represent mean integrated density ± SE obtained from a minimum of 8 independent donor pairs. ** denotes statistical significance at $p<0.001$.

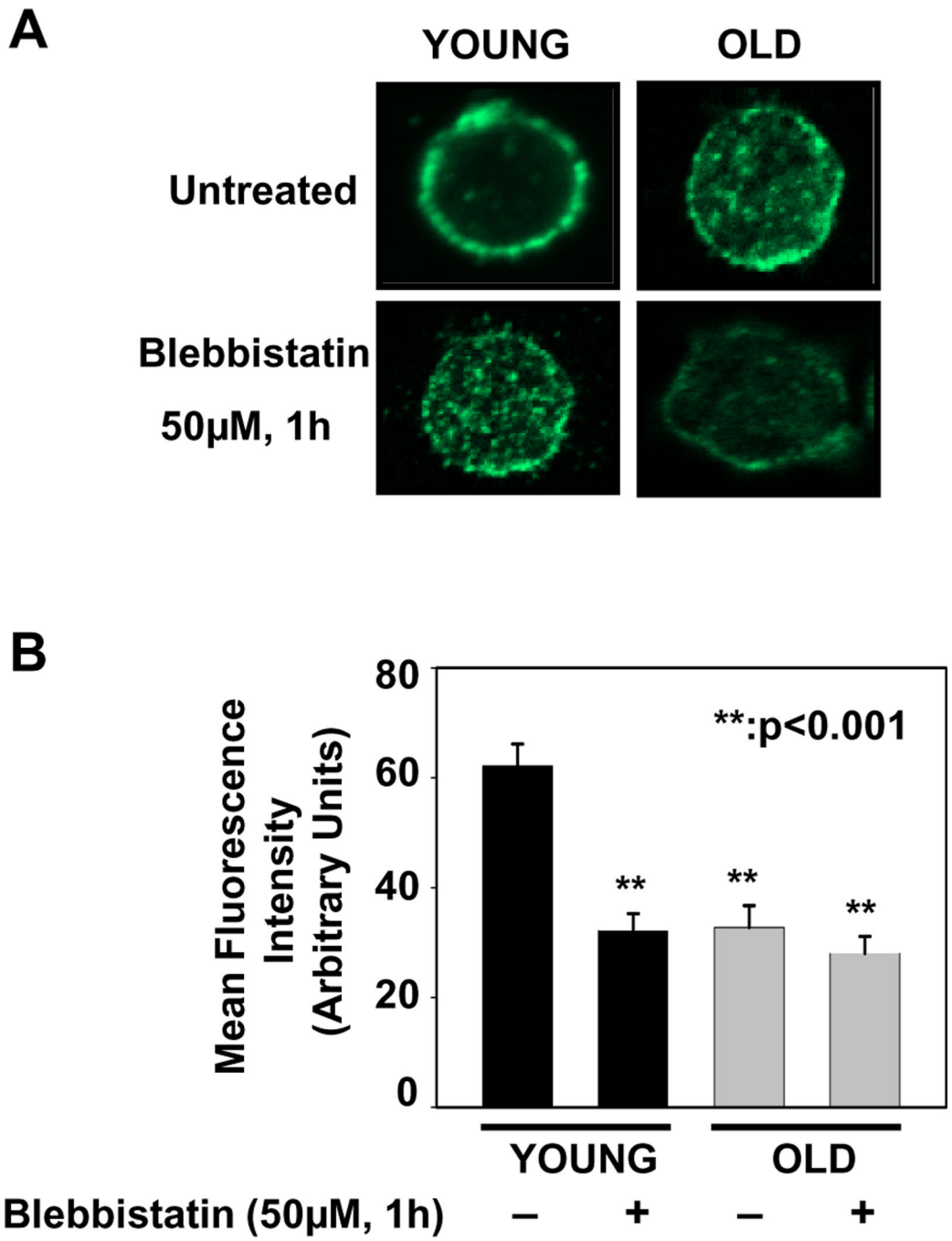

FIGURE 4.

Localization and distribution of myosin IIA in $CD4^+$ T cells from young and elderly donors. *A*, Confocal microscopy of myosin IIA distribution in $CD4^+$ T cells from young and elderly donors that were either left untreated (vehicle control) or pretreated with blebbistatin. Representative data from one donor pair out of a minimum of 10 pairs tested are provided. *B*, Cumulative data of intracellular myosin IIA fluorescence intensity in $CD4^+$ T cells from 10 independent donor pairs. Mean fluorescence intensity of myosin IIA localized on the inner face of the plasma membrane is provided. Quantification of the fluorescence signal of

myosin IIA in the inner face of the plasma membrane was performed on the complete 3D confocal stack for n=30 cells each obtained from 5 different fields from a minimum of 10 independent experiments. Data are represented as the mean plasma membrane associated fluorescence intensity ± SE; ** denotes statistical significance at p<0.001.

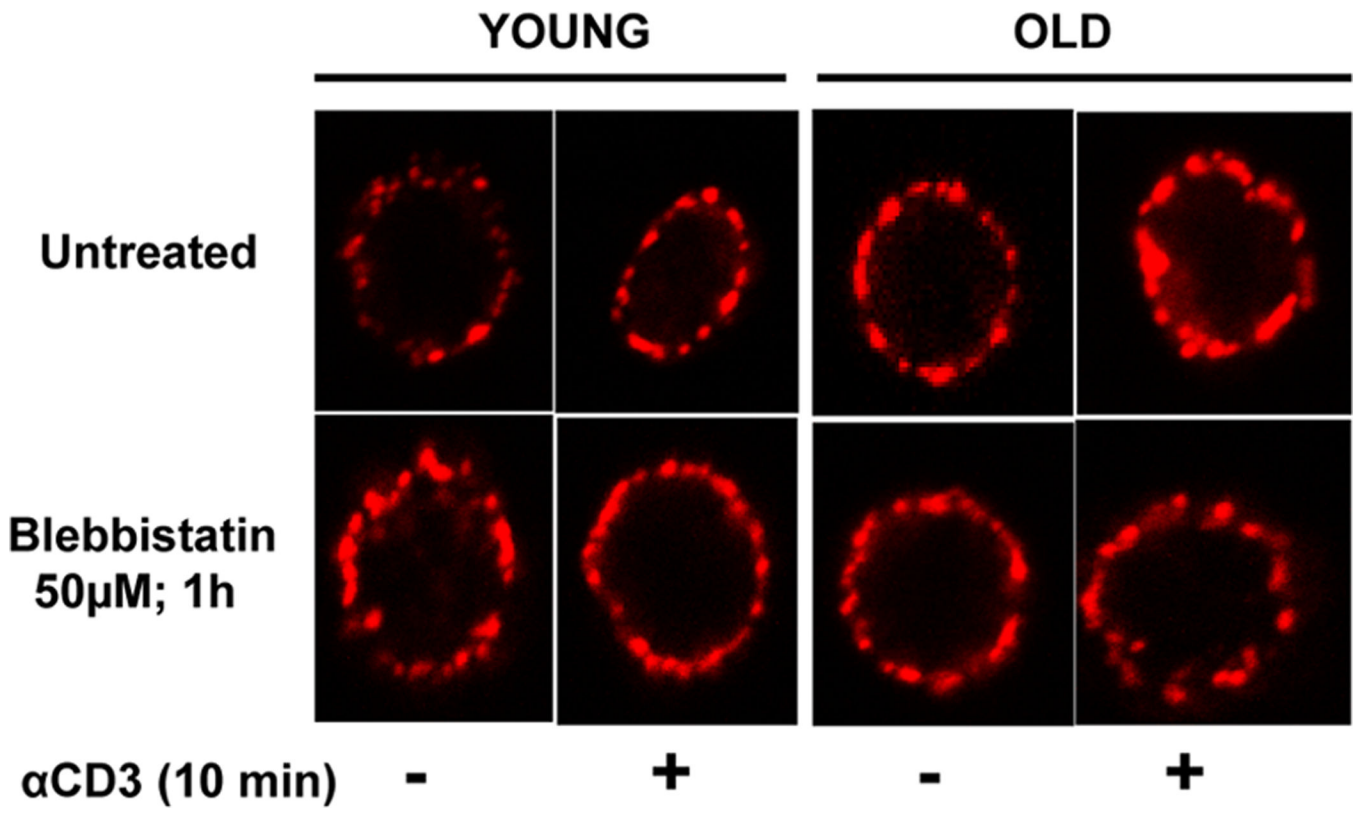

FIGURE 5.

Cell surface TCRα/β expression in $CD4^+$ T cells is not affected by age. Confocal microscopy of TCRα/β expression in $CD4^+$ T cells from young and elderly donors that were either left untreated (vehicle control) or pretreated with blebbistatin. Representative data are from one donor pair out of 10 pairs tested. Fluorescence intensity of TCRα/β in the plasma membrane was performed on the complete 3D confocal stack for *n*=30 cells obtained from a minimum of 5 different fields from a minimum of 10 independent experiments.

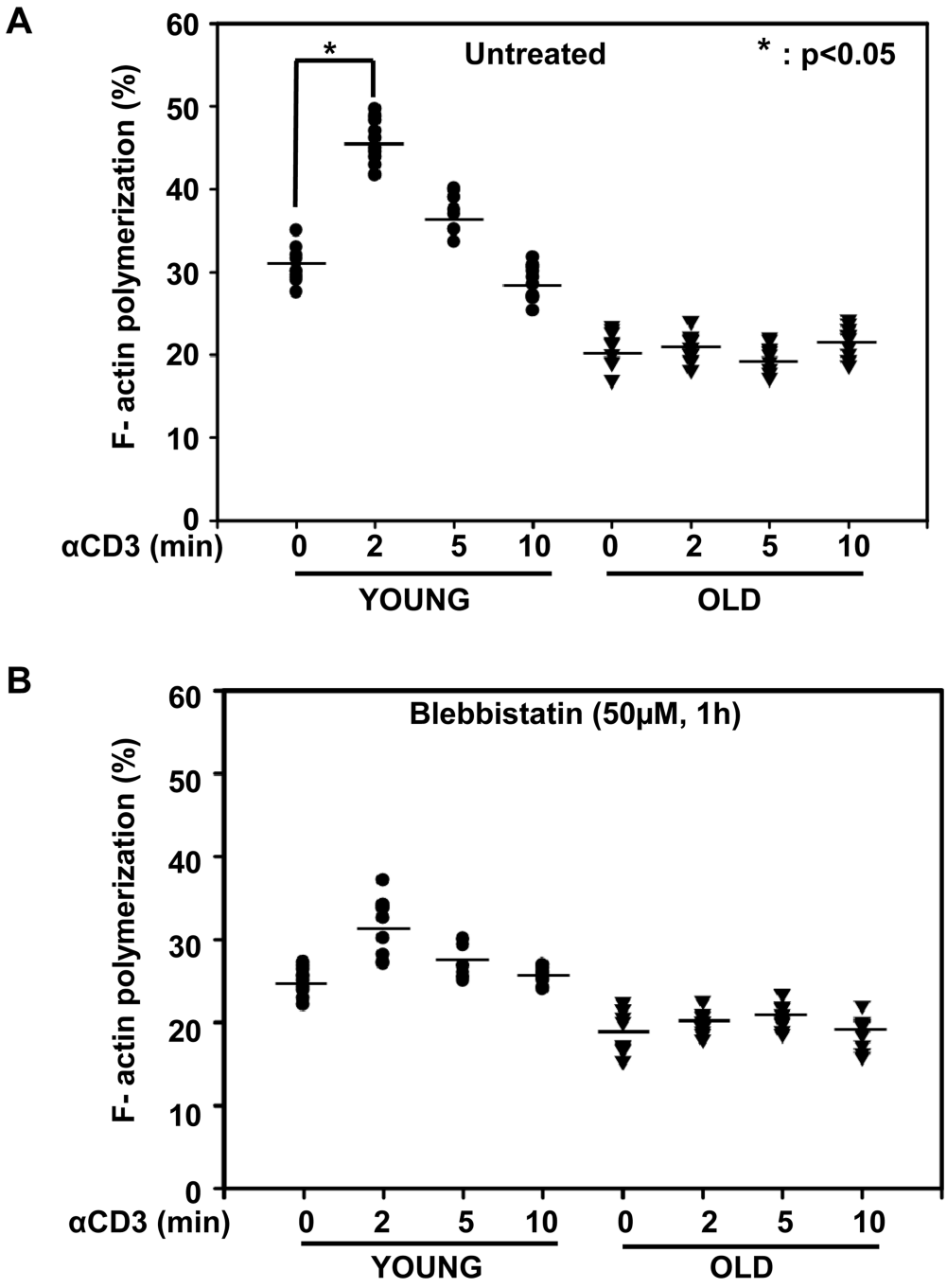

FIGURE 6.

Anti-CD3-induced F-actin polymerization in $CD4^+$ T cells during aging. *A*, $CD4^+$ T cells were either left untreated or activated with plate bound anti-CD3 for 2, 5 and 10 min. At the end of activation cells were fixed, stained with Rhodamine phalloidin and analyzed by flow cytometry. Data represent mean ± SE derived from 10 independent donor pairs. *B*, $CD4^+$ T cells were either pretreated with blebbistatin or vehicle control, and were then activated with plate bound anti-CD3 for 2, 5 and 10 min. F-actin polymerization was determined by flow

cytometry as described. Data represent mean ± SE obtained from 10 independent donor pairs. * denotes statistical significance at $p<0.05$.

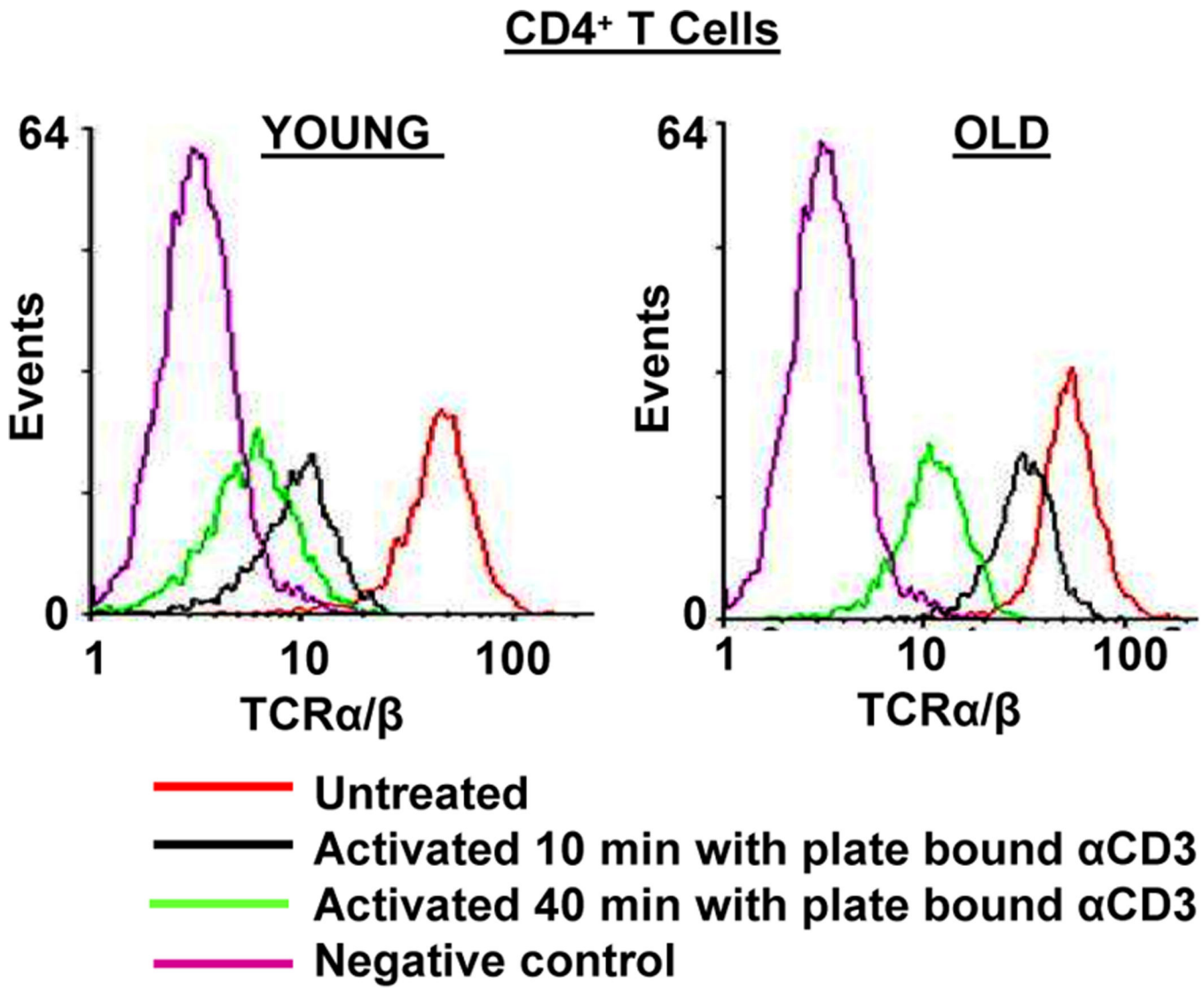

FIGURE 7.

Activation-induced internalization of TCRα/β is delayed in $CD4^+$ T cells from elderly donors. $CD4^+$ T cells obtained from young and elderly donors were either left untreated or activated with 5μg/well plate bound anti-CD3 for 10 and 40 min. The cells were then stained using antibody to TCRα/β, followed by a Alexa Fluor 555-conjugated secondary antibody and then fixed with 2% PFA. Representative data are from one donor pair out of 5 pairs tested. Surface expression of TCR was detected by flow cytometry. Representative data from one donor pair is depicted in the histogram. Cumulative data obtained from a minimum of 5 independent donor pairs are provided.

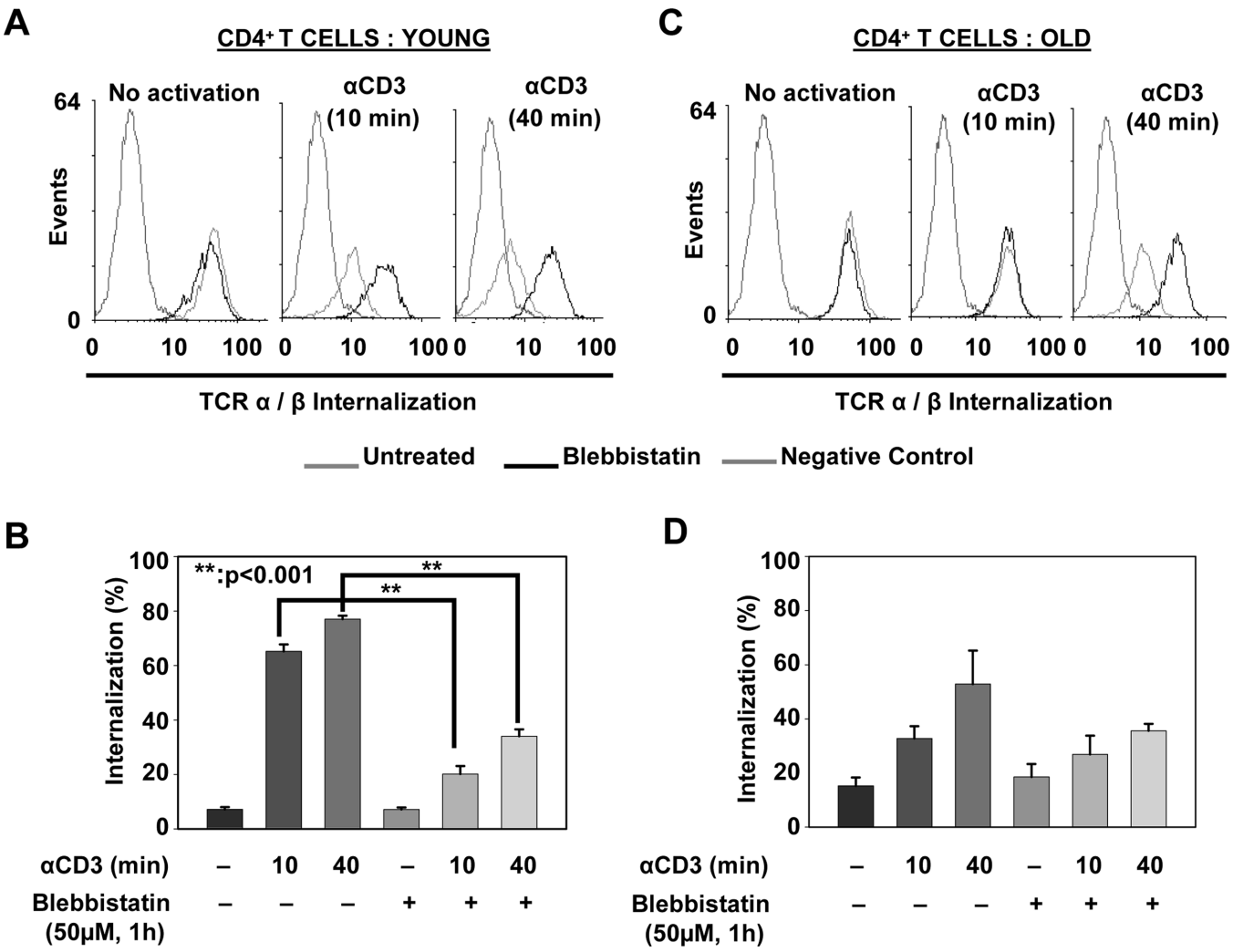

FIGURE 8.

Inhibition of non-muscle myosin IIA results in delayed internalization of cell surface TCRα/β. *A and C*, $CD4^+$ T cells obtained from young and elderly donors were either left untreated or pretreated with blebbistatin then activated with 5μg/well plate bound anti-CD3 for 10 and 40 min. The cells were then stained using antibody to TCRα/β, followed by Alexa Fluor 555-conjugated secondary antibody and fixed with 2% PFA. Surface expression of TCR was detected by flow cytometry. Representative data from one donor pair is shown. *B and D*, Cumulative data from a minimum of 5 independent pairs of young and elderly donors, respectively. Percentage of internalization was calculated based on mean fluorescence intensity. Data represent MFI ± SE. ** denotes statistical significance at $p<0.001$.

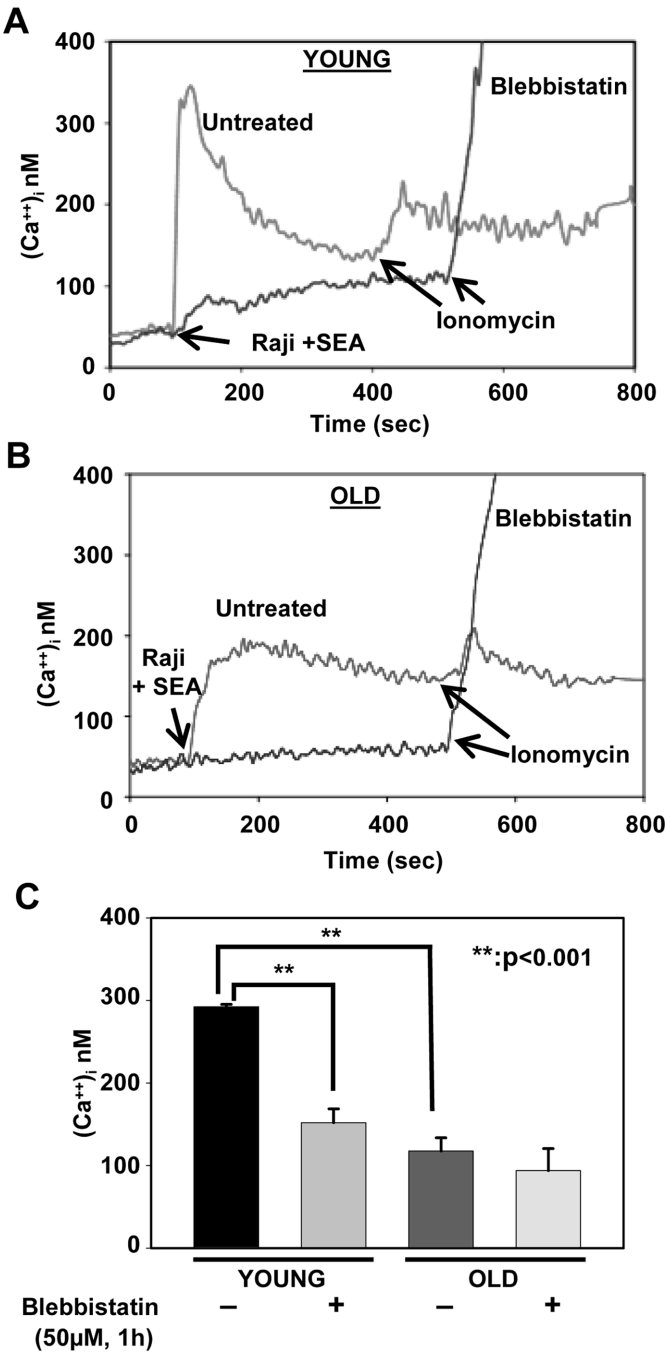

FIGURE 9.

Activation-induced intracellular Ca^{2+} flux is decreased in $CD4^+$ T cells from elderly donors. *A*, Microfluorescent imaging of intracellular calcium flux in $CD4^+$ T cells obtained from one representative young donor. *B*, Microfluorescent imaging of intracellular calcium flux in $CD4^+$ T cells obtained from a representative elderly donor. $CD4^+$ T cells from both young and elderly donors were either pretreated with blebbistatin or vehicle control, and were then incubated with Raji cells pulsed with SEA. Ionomycin was used as a positive control. *C*,

Mean intracellular concentration of Ca^{2+} ± SE from six independent experiments are presented. ** denotes statistical significance at $p<0.001$.

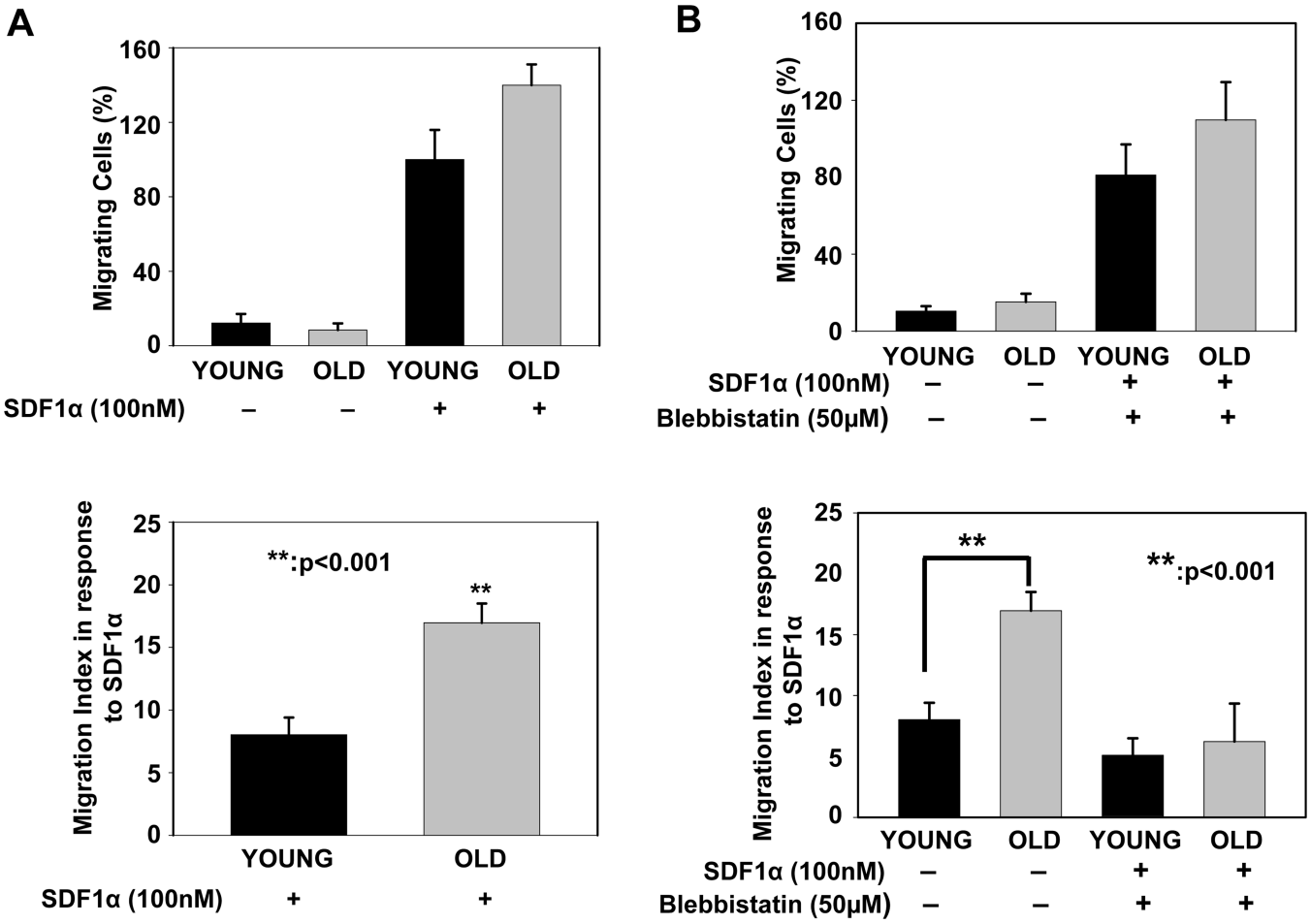

FIGURE 10.

Effect of aging and inhibition of non-muscle myosin IIA on chemotaxis to SDF-1α gradient in CD4+ T cells. *A*, Percent migration in response to SDF-1α gradient, in untreated $CD4^+$ T cells from young and elderly donors. *B*, Percent migration of blebbistatin pretreated $CD4^+$ T cells from young and elderly donors in response to SDF-1α gradient. Data are representative of 12 independent donor pairs. Migration index was derived by calculating migration of $CD4^+$ T cells subjected to a gradient generated by SDF1 in a transwell system normalized to vehicle treated controls. ** denotes statistical significance at $p<0.001$.