Human Subjects Research Protocol

Title: Immune Function and Muscle Adaptations to Resistance Exercise in Older Adults

Funded by: VA Rehabilitation Research and Development

Administered by: Central Arkansas Veterans Healthcare System

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Lay Summary: The loss of muscle mass and strength due to aging leads to serious health problems for older adults. Muscle health can be improved by exercise training, but some people improve their strength substantially, whereas others improve little. The reason for this variation is unknown. This study will investigate whether function of the immune system influences how well people respond to exercise. Older veterans who participate will have their muscle size, strength, and function measured periodically for almost a year. Participants will drink a nutritional supplement or placebo daily and complete a 36 session strength training program. Participants will be vaccinated for tetanus and donate small amounts of blood and muscle tissue during the study so that immune function can be compared to muscle outcomes during training and during a long-term follow-up. The study results should increase our understanding of the negative effects of aging on muscle and will possibly lead to better strategies for muscle maintenance and rehabilitation for older adults.

Key Points of the Supplementation and Exercise Protocol

- **Rationale:** Exercise is clearly able to affect immune function. However, the proposed study will attempt to modulate immune function by nutritional supplementation and determine the effects on exercise training outcomes in older adults.
- **Importance:** Targeting the immune system with nutritional supplementation may be the advantage needed for an older veteran to successfully use exercise training to maintain or restore the muscle mass, strength, and function that is necessary for personal independence.
- **Double-blind randomized placebo-controlled trial:** groups consuming nutritional supplement (Muscle Armor) or placebo (Kool-Aid) will be compared to answer four key questions:
    - Do 2-weeks of supplementation improve immune function?
    - Does supplementation during 12-weeks of progressive high-intensity resistance training boost adaptations to exercise at the whole body or cellular level?
    - Does continued supplementation for 26-weeks after completion of exercise training promote the retention of the gains in muscle size, strength, and function?
    - Do measures of immune function correlate with cellular and whole-body measures of muscle health after exercise training and detraining?
- **Procedures to be completed by veterans (age 60-80, N=50)**
    - Telephone pre-screening with required waiver of HIPAA and permission for verbal consent
    - Enrollment screening after written consent and HIPAA authorization
    - Approximately 50-60 research visits over approximately one year
    - Data collection from CPRS problems and medication lists
    - Orange-flavored supplement or placebo consumption mixed in water twice daily (~42 weeks)
    - Supervised High-Intensity Resistance Exercise (two sessions prior to training to measure response to unaccustomed exercise)
    - Supervised High-Intensity Resistance Exercise Training (36 sessions in ≥ 12 weeks)
    - Post-training follow-up (~26 weeks)
    - Blood draw (9x)
    - Muscle biopsy (5x)
    - Gait and balance testing (4x)
    - TDAP Vaccination (1x)
    - Thigh CT Scan (3x)
    - Compensation (~$1500): $20 per visit, completion bonus for exercise training ($100) and entire study ($300). $20 bonus if veteran refers another eligible subject to participate.

ABSTRACT

Objective: The study will examine the influence of immune function in older adults on improvement of muscle mass, strength, and function by resistance training. The maintenance of those benefits during long term follow-up will also be examined. This objective will be accomplished by a double-blind randomized placebo-controlled trial of a nutritional supplement (Muscle Armor) which evidence suggests can improve immune function, promote muscle growth, and counteract muscle loss. The study premise is that aging results in decreased ability of the immune system to respond to stimuli such as exercise. The study proposes that the supplement will improve muscle health by promoting a shift in immune function of older adults from a pro-inflammatory state towards a state which supports muscle growth and maintenance.

Research Plan: The study will randomize veterans (age 60-80, N=50) to participate in the supplement or placebo groups in a three phase study. The phases of participation correspond to the three specific aims. Aim 1 will determine if 2-weeks of supplementation improves immune function. Humoral immune function will be assessed as the response to vaccination. Innate immune function will be measured as systemic and cellular responses to acute resistance exercise that our previous studies indicate are affected by aging. Aim 2 will determine if supplementation during 36 sessions of progressive high-intensity resistance training boosts improvement in muscle size (CT scan), strength, and function (gait and balance). Muscle adaptations at the cellular levels will also be measured. Aim 3 will determine if continued supplementation for 26-weeks after completion of exercise training promotes the retention of the gains in muscle size, strength, and function. Multivariable testing will then be used to compare the results between Aims 1, 2, and 3 to determine whether or not immune function is correlated with muscle adaptation to training or detraining.

Methods: Participants will undergo nine blood draws and five muscle biopsies of the vastus lateralis over the course of the study so that the effects of the supplement on immune function and cellular adaptations to training can be measured. Three of the blood draws will be used to assess the antibody response to the tetanus, pertussis, and diphtheria vaccine. Muscle and blood will be collected before and after a bout of exercise conducted before and after the 2-weeks of supplementation prior to training. Immune function will be measured using the blood based on pro- and anti-inflammatory cytokine levels, the balance between specific T-cell subpopulations, and the proliferative capacity of mononuclear cells. Immune function will be measured in muscle based on macrophage content of specific cytokines and growth factors. Our previous study showed that these muscle measures strongly correlate with size and strength gain after training. Key signaling pathways including nuclear factor-k B and PI3 kinase will also be measured. The fifth biopsy will be collected post-training to measure adaptation at the cellular level based on changes in number of satellite cells and myonuclei and fiber size. Hypotheses related to these measures will be tested with 80% power to detect at least 0.8 standard deviations difference in means between the supplement and placebo groups.

Clinical Relevance: Exercise is clearly able to affect immune function. However, the proposed study will attempt to modulate immune function and determine the effects on exercise outcomes. The study will also examine detraining, an important issue for older adults, that is usually omitted from training studies. Thus, the study will potentially advance our understanding of the mechanisms of muscle gain and loss in older adults, but more importantly, the study will evaluate a nutritional intervention as a complement to exercise for supporting muscle health during aging. Targeting the immune system may be the advantage needed for an older Veteran to successfully maintain or restore the muscle mass, strength, and function that is necessary for personal independence.

SPECIFIC AIMS

Age-related decline of the immune and musculoskeletal systems leads to serious health problems for older adults. The bodily systems are often co-dependent during health and dysfunction, but the role of immune function in maintaining or improving muscle health during aging is unknown. The relationship between these two systems will be examined through the conduct of a randomized trial using a nutritional supplement (Muscle Armor, Abbott Labs) that is proposed to have beneficial effects on both immune function and skeletal muscle. The trial will determine if the supplement is able to improve immune function, boost muscle adaptation to resistance training, and support retention of the exercise-derived benefits (Figure 1). The supplement ingredients, methylbutyrate, arginine, and glutamine (HMB, Arg, and Gln), are used by weight-lifting enthusiasts to boost gains from resistance training and clinically to counteract muscle wasting. Thus, an exercise trial evaluating Muscle Armor could identify a means to make a significant impact on muscle function, health, and quality of life for older adults. However, the mechanisms by which this supplement improves muscle health are unknown. Thus, in addition to the potential for direct benefit to aging Veterans, conduct of the proposed trial will allow the mechanistic relationships between immune function and adaptation to exercise training and detraining to be investigated.

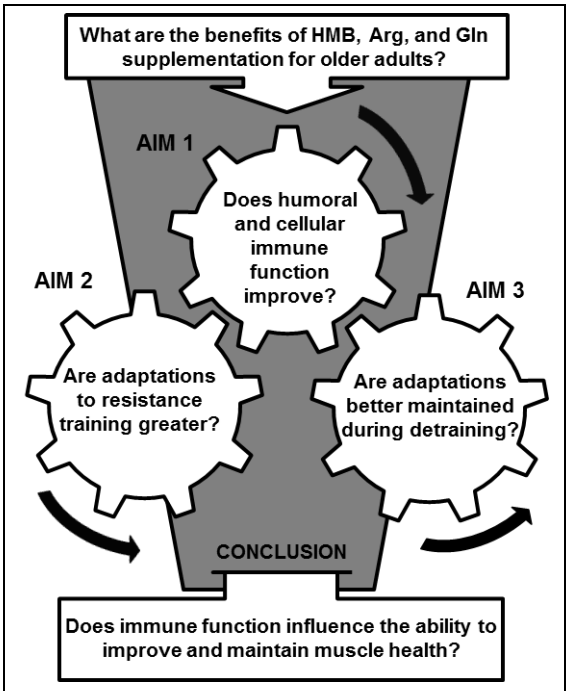

Figure 1. Randomized trial of a nutritional supplement to determine if immune function influences muscle health in older adults.

1. **Determine the effects of Muscle Armor consumption by older adults on the humoral response to vaccination and specific cellular immune responses to an exercise stimulus.** Our findings indicate that various measures of immune function decrease with aging [1;2]. Aim 1 will determine if these measures are improved by 2-weeks of nutritional supplementation. Humoral function will be assessed using the tetanus, diphtheria, pertussis vaccine. Cellular function will be assessed using muscle and blood collected before and after a bout of exercise. Immune cell proliferation and phenotype, and levels of cytokines and growth factors and their signaling actions will be measured.
2. **Determine if Muscle Armor consumption affects the adaptive responses to resistance exercise training at the whole body or cellular level.** Our studies have shown that muscle adaption to training varies greatly between older adults [3]. Thus, a nutritional supplement that boosts the response could be quite valuable to certain individuals. Aim 2 will have subjects continue supplementation while completing 36 sessions of high-intensity resistance training. Whole body measures of adaptation to resistance training will include changes in muscle strength, cross-sectional area, and function. Cellular measures of muscle adaptation will include changes in satellite cell and myonuclear numbers, and fiber size.
3. **Determine if Muscle Armor consumption affects the retention of benefits of exercise during long-term follow-up.** Training studies of older adults rarely measure post-training maintenance of muscle cross-sectional area, strength, and function. However, this information is highly important. The supplement chosen here has shown evidence of ameliorating muscle wasting in chronic disease and is being tested for slowing sarcopenia [4;5]. Aim 3 will determine if continuing supplementation during a post-training follow-up period (i.e. detraining, 26-weeks) promotes the retention of benefits from exercise in older adults.

BACKGROUND AND SIGNIFICANCE

1. **Muscle loss due to aging leads to serious health problems for older adults.** Effective strategies for rehabilitation are critical to the health of older adults since muscle loss decreases functional ability and increases risk for falls and fractures, disability, and mortality [6;7].

2. **Resistance training improves muscle mass, strength, and function in older adults but the response varies between individuals.** Resistance training can be safely performed with benefit to even chronic heart failure and nursing home patients [8], but the magnitude of benefit varies [9]. This suggests that older adults with less adaptive potential will have greater muscle loss due to aging and that rehabilitation will be less effective. Thus, a better understanding of the effects of aging on muscle health may be needed to design interventions that complement resistance training.

3. **The ability of older adults to maintain or improve muscle health may be associated with systemic indicators of the effects of aging on immune function.** Age-related decline in functions of the immune system results in a weaker response to vaccination [10], diminished ability of immune cells to proliferate, and problems that specifically affect muscle. Acute increases in cytokines after exercise initiate muscle repair [11], but with aging inflammation becomes chronic and detrimental. For example, inflammation induces muscle catabolism in rats [12] and is associated with sarcopenia and functional decline in humans [13]. Thus, systemic differences in immune parameters between older adults may reflect ability to improve or maintain muscle health.

4. **Cells of the immune system residing in muscle play a central role in the developing model of muscle adaptation to exercise.** Macrophages transition between three functional phases in response to exercise. Phase 1 is an inflammatory response mediated by cytokines which initiate muscle repair and clear debris from damaged myofibers [14]. After this initial cleanup, Phase 2 is a down-regulation of inflammation by anti-inflammatory cytokines so that secondary damage does not occur. This allows the muscle to become conducive to growth [15]. In the regenerative Phase 3, macrophage arginine metabolism increases and promotes cell growth [16] and growth factors are produced to further stimulate myogenesis [17]. As muscle adapts to training, macrophage function may then become dedicated to producing growth factors required for hypertrophy.

5. **Evidence suggests that the immune system and macrophages influence muscle mass and strength during health and aging.** In animals, extermination of muscle macrophages decreases the ability of muscle to hypertrophy and recover from atrophy [18-20]. The effects were related to decreased macrophage provision of growth factors, myogenesis, and muscle fiber growth [18;20]. In humans, muscle mass and strength gain are also associated with markers of myogenesis [21] that are diminished in older adults [22]. Inflammation may have contributed to those findings. In a training study of older adults, individuals consuming anti-inflammatory drugs gained significantly more muscle mass and strength than those consuming placebo [23]. Our studies also indicate that immune function influences the response to exercise. Muscle size and strength gained by older adults from resistance training were strongly correlated with muscle expression of specific cytokines and growth factors [3]. Muscle macrophage content in those older adults was also strongly correlated with muscle adaptation and growth factor levels. These findings support the idea that consumption of a nutritional supplement that either dampens a negative or boosts a positive effect of inflammation and immune function could benefit older adults during exercise training.

6. **Three signaling pathways may be involved in the regulation of macrophage function, production of cytokines and growth factors and their effects on muscle cells.** In response to pro-inflammatory cytokines and NFkB signaling [24], macrophages may produce cytokines which block signaling that increases transcription of ubiquitin ligases which control protein degradation and lead to muscle atrophy [25]. However, in response to anti-inflammatory cytokines and STAT3 signaling, macrophages [24], macrophages produce factors that support tissue growth and block

atrophy [26]. Examination of these pathways in human muscle is limited and thus warrants further investigation to define their role in the context of aging, exercise, and nutritional supplementation.

7. **A nutritional supplement that affects immune function may improve the ability for older adults to benefit from resistance training.** Muscle Armor contains arginine, glutamine, and methylbutyrate. These ingredients slow cachexia and increase muscle mass in both cancer and AIDS patients [5;27]. They are also used to increase strength in combination with exercise for sports performance [28-30]. Despite this success little is known about the mechanism of action. Methylbutyrate alters protein synthesis, but it also enhances immune function in animals [31-33]. Glutamine and arginine are considered immuno-nutrients that affect immune cell phenotype, proliferation, and production of cytokines [34-37]. The study will determine if the effects of the supplement on muscle health are mechanistically related to its ability to modulate immune function.
8. **Significance: Nutritional intervention that targets the immune system may complement resistance exercise in helping older adults improve and maintain muscle health.** Exercise is clearly affects immune function [38]. However, it is unknown if immune function affects individual benefit from exercise because studies have not altered immune function and determined the effects on training outcomes. The proposed study will attempt to do this using a supplement that supports muscle health. The study will also examine detraining, an important issue, that is often omitted from exercise studies [39]. Thus, the study has the potential to advance our understanding of the mechanisms of muscle gain and loss in older adults, but more importantly, the study will evaluate a nutritional intervention as a complement to exercise for improving muscle health in older adults.

PRELIMINARY STUDIES

The study will determine the relationship between immune function and adaptation to training and nutritional supplementation in older adults. The expectation that improving and maintaining muscle size, strength, and function will be correlated with immune function is based on the following findings.

1. Muscle of older adults, as compared to young adults, has increased expression of cytokines at baseline and lacks cytokine induction in response to acute resistance exercise [2].
2. Muscle of older adults contains fewer macrophages than muscle of younger adults. The number of macrophages that display markers of inflammation or anti-inflammation and growth increase for young but not older adults in response to exercise. Interestingly, macrophages that display the marker of anti-inflammation and growth outnumber macrophages displaying the marker of inflammation by approximately eight to one [2].
3. Muscle of older adults, compared to younger adults, has lower expression of genes associated with tissue growth and remodeling at rest and in response to a bout of resistance exercise [40].
4. Muscle gene expression associated with inflammation and growth is strongly correlated with muscle size and strength gained from training [3]. The number of macrophages in muscle is also correlated with size and strength gain, and gene expression suggesting that macrophage provision of cytokines, and growth and remodeling factors contributes to muscle adaptation to training.
5. The effects of aging on muscle macrophages are consistent with the immunosenescence of immune cells outside of muscle. For example, aging decreases the ability of peripheral blood mononuclear cells and T-cells to proliferate and respond to pro-inflammatory stimuli [1].
6. Pilot study findings suggest that the nutritional supplement can dampen systemic markers of inflammation and boost the antibody response to the pertussis antigen in older adults.

Finding 1: Muscle of older adults, as compared to young adults, has increased expression of cytokines at baseline and also lacks cytokine induction in response to acute resistance exercise [2].

Study: Pro- and anti-inflammatory cytokine mRNA levels were measured in muscle of young (22-41 yrs) and older (66-80 yrs) males (N=17 each) at rest and 72 hrs after completion of a single session of high-intensity resistance exercise targeting the thigh muscles. At rest, muscle of the older adults possessed higher (3.3-fold, P=0.001) the pro-inflammatory cytokine, IL1β, than muscle from the younger adults (Figure 2A). Anti-inflammatory cytokines, IL1RA (P=0.003) and IL10 (P=0.03) were also higher in the old than young (not shown). After exercise, levels of the IL1β increased (~2-fold, P=0.006) in the young muscle, but did not change in the old (Figure 2B). IL10 (P=0.02) also increased after exercise but only for the young subjects.

Conclusions: Pro- and anti-inflammatory muscle cytokine expression is regulated differently in older adults at baseline and in response to exercise. These results were found for older adults who were quite healthy and only ~20% weaker than the young group. Thus, the overt effects on muscle of this altered cytokine expression were not obvious though the results implied that muscle of older adults may contain immune cells (i.e. macrophages) in a state of chronic low-grade inflammation. If this is true, then it is possible that the chronic inflammation could adversely affect levels of growth factors that control muscle hypertrophy which could hinder the adaptive response to resistance exercise training. These possibilities were investigated and the results are presented here in Findings 2 through 4.

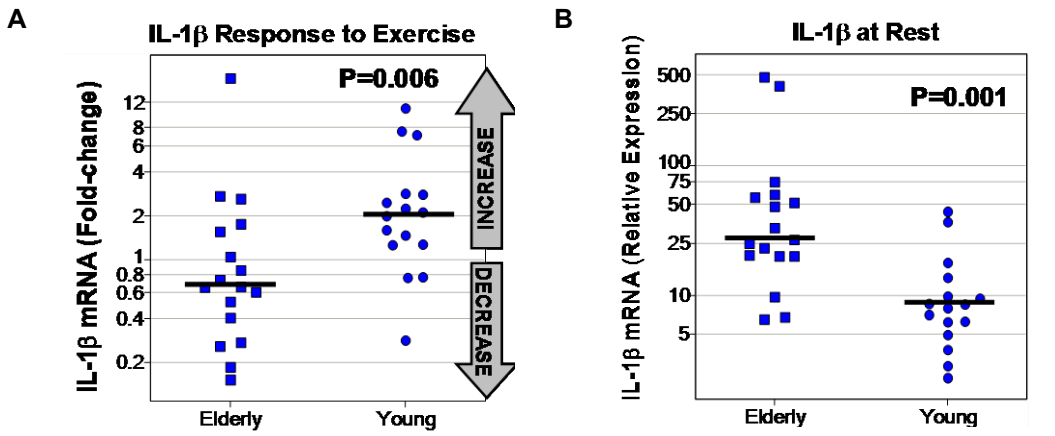

Figure 2. IL-1β at rest and in response to exercise is different in muscle from old and young adults. Data for each subject (dots) and medians (bar) are shown.

Finding 2: Muscle of older adults contains fewer macrophages than muscle of younger adults; and in both age groups, at baseline and in response to exercise, the majority of macrophages display a phenotype consistent with anti-inflammation and growth rather than inflammation [2].

Study: Macrophages in muscle from the young and older males of Finding 1 were counted based on markers of inflammation (CD11b) or anti-inflammation and growth (CD163) [16;41;42]. At rest, muscle of the older adults contained approximately half as many CD11b+ cells (P=0.003, Figure 3A) as younger muscle and tended (P=0.07, Figure 3B) to contain fewer CD163+ cells. 72 hrs after a bout of exercise, the numbers of macrophages did not increase in the older adults, but both CD11b+ (55%, P=0.04) and CD163+ cells increased in younger adults. Interestingly, muscle contained 8-times as many CD163+ cells as CD11b+ cells (P<0.0001).

Conclusions: Muscle of older adults did not contain more macrophages with an inflamed phenotype than muscle of young adults. Muscle macrophages predominantly showed the anti-inflammation and

growth phenotype and like cytokines, their numbers only increased after exercise in the young. Additional analysis was performed in an attempt to identify factors associated with healing and growth which may also impact muscle adaptation in older adults.

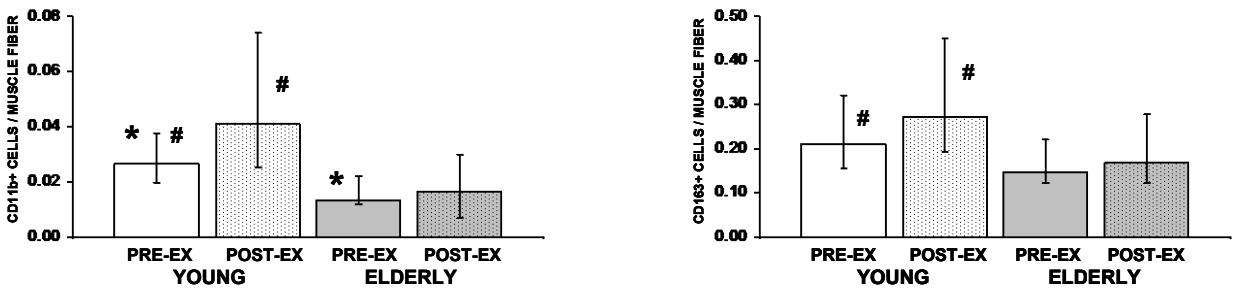

Figure 3: Muscle of older adults contains fewer CD11b+ macrophages and the number does not increase after exercise as it does for young adults. Results for the far more abundant (see y-axes scales) CD163+ macrophages were similar though the age-associated difference was only a trend. Median and inter-quartile range are shown. Significance ($P<0.05$) between (*) and within (#) groups is shown.

Finding 3: Aging alters muscle expression of genes which function in tissue growth and remodeling both at rest and in response to a bout of resistance exercise [40].

Study: The muscle of the young and old adults of Findings 1 and 2 was further analyzed using quantitative real-time PCR to measure expression of 100 select mRNAs. The mRNAs included genes that function in cellular stress, signaling, tissue remodeling or growth. Four mRNAs (IGF1, CNTF, MMP2, and IGFBP5) were significantly greater in younger muscle than older muscle both before and after exercise ($P<0.05$). The median differences ranged from 60% to 230% (shown for IGF1 in Figure 4A). Two mRNAs showed a significant ($P<0.05$) response to exercise. However, the response was found to be significant in only younger adults after statistical correction for multiple comparisons was applied. In younger adults, median TIMP1 levels increased 200% (Figure 4B) and GDF8 levels decreased by 50% after exercise (not shown).

Conclusions: Gene expression profiling identified a number of muscle mRNAs whose levels are affected by aging and acute exercise. The results included the two most commonly studied regulators of muscle mass, IGF1 and GDF8 (also known as myostatin) [43;44] as well as other mRNAs about which little is known in the contexts of exercise, aging, and human muscle. Thus, in addition to the factors discussed here in Findings 1 through 3, growth factors such as CNTF and factors that may participate in tissue remodeling, namely the metalloproteinase MMP2 and its inhibitor TIMP1, are also candidates that require investigation as regulators of muscle mass. Finding 4 will present the results of a training study for older adults that sought to determine the relationship between the molecular and cellular variables discussed and gains in muscle size and strength.

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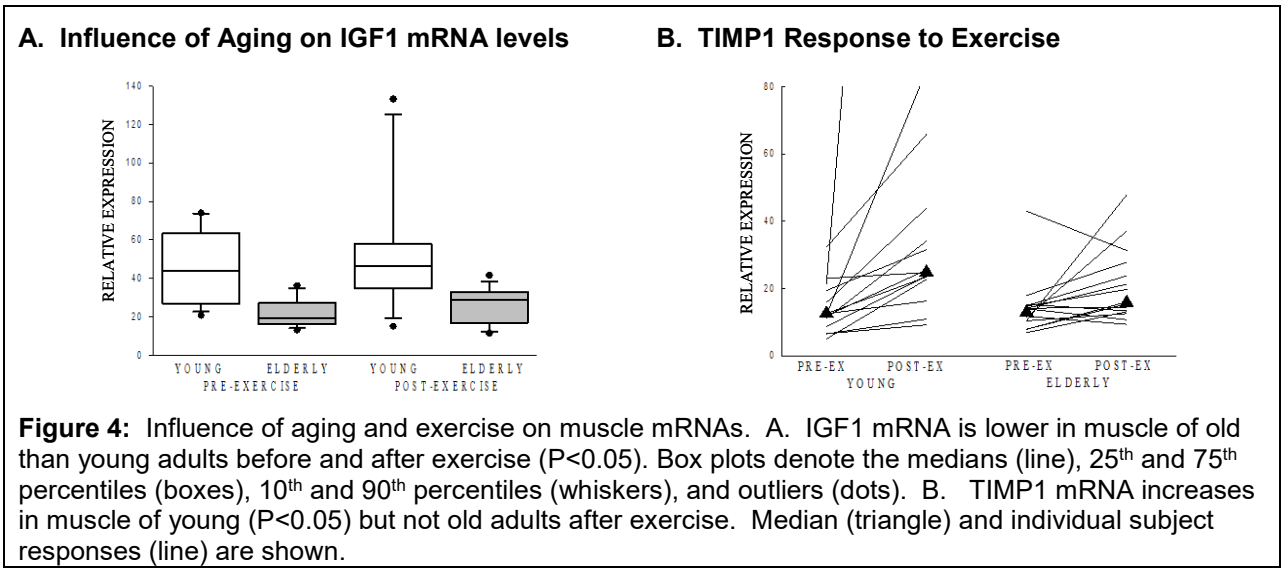

Figure 4: Influence of aging and exercise on muscle mRNAs. A. IGF1 mRNA is lower in muscle of old than young adults before and after exercise (P<0.05). Box plots denote the medians (line), 25th and 75th percentiles (boxes), 10th and 90th percentiles (whiskers), and outliers (dots). B. TIMP1 mRNA increases in muscle of young (P<0.05) but not old adults after exercise. Median (triangle) and individual subject responses (line) are shown.

Finding 4: Muscle expression of genes associated with inflammation, growth, and remodeling is strongly correlated in older adults with muscle size and strength gained from resistance training [3].

Study: Older (61-78 yrs) men (N=3) and women (N=5) completed 12-weeks of high-intensity progressive resistance training of the thigh muscles. Biopsies were collected prior to training and analyzed by real-time PCR and immunohistochemistry. Muscle size change range from -1% to +18% while strength increase ranged from +8% to +65%. Strength gain correlated ($R > 0.7$, $P < 0.05$) with expression of mRNAs encoding cytokines (IL1β, IL10, IL1RA), growth (CNTF, GDF8, IGF1, IGFBP5) and remodeling factors (MMP2, TIMP1). The result is shown for IGF1 in Figure 5A. Strength gain also correlated with the number of CD163+ macrophages in muscle with the exception of one outlier (Figure 5B). Similar results were found for muscle size.

Conclusions: Macrophages and mRNAs associated with inflammation, growth, and remodeling appear to be involved with adaptation to exercise in older adults. However, these variables could be covariates affected by an unknown determinant of muscle adaptation. The proposed study will allow investigation into whether macrophage function or immune function in general influences adaptation to resistance training in older adults.

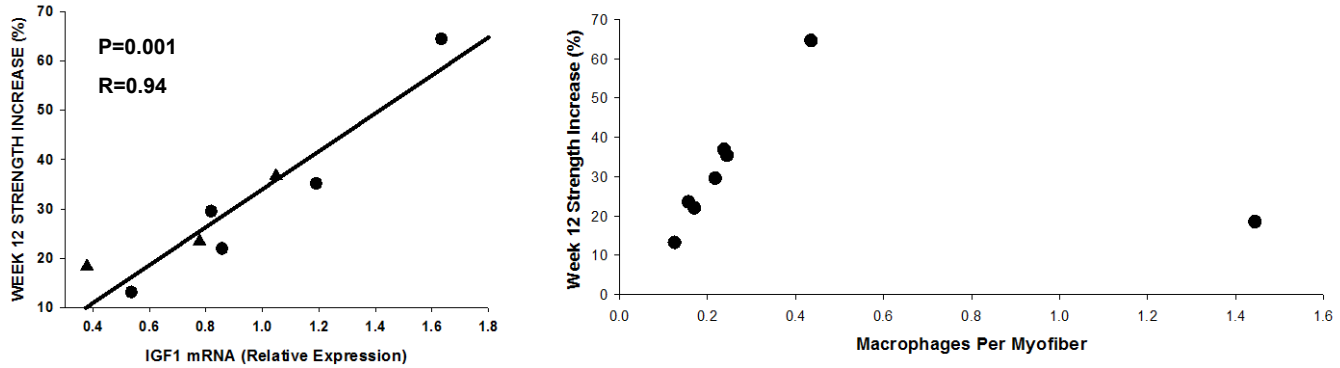

Figure 5. Relationship between baseline molecular and cellular measures for muscle of older adults and percent strength gain for knee extension after 12-weeks of training. A. Pearson correlation for IGF1 mRNA and strength gain (triangles = males, circles = females). B. Relationship between number of muscle macrophages and strength gain.

Findings 5: Aging decreases the ability of immune cells to respond to stimuli that cause cell proliferation and inflammation [1].

Study: The effects of aging on immune cells were examined in two experiments. Experiment 1 compared the T-Cell response to a pro-inflammatory stimulus using blood of young (N=5, 21-30 yrs) and old (N=5, 65-80 yrs) donors [45]. Cells were treated with TNFα and NFkB binding to its promoter was measured. Binding was lower ($P<0.05$, 250% versus 340%) in older than younger adults (Figure 6A). Experiment 2 examined the ability of peripheral blood mononuclear cells (PBMC) to proliferate in response to a phorbol ester and calcium ionophore. After 5 and 8 days of treatment respectively, 84% and 92% of young cells had undergone at least one cell division (Figure 6B); whereas only 52% and 63% of older cells had divided (not shown).

Conclusions: Immunosenescence appears to universally affect mononuclear cells, T-cells, and muscle macrophages by diminishing ability to proliferate and respond to pro-inflammatory and exercise stimuli. The proposed study will determine the extent to which immune function external to muscle reflects the ability of muscle macrophages to respond to exercise or the ability of older adults to benefit from exercise training.

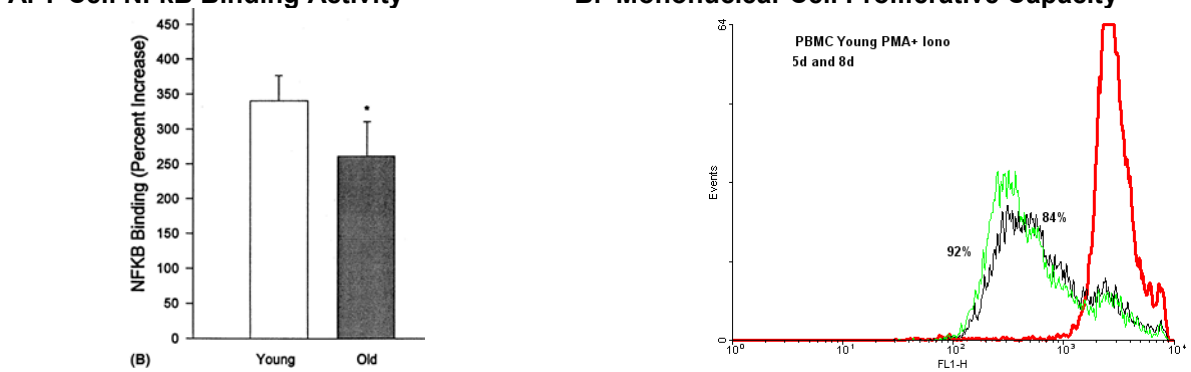

Figure 6. Effects of aging on immune cell function. A. The NFkB promoter binding assay was used to compare the response to a pro-inflammatory stimulus between old and young T-Cells. B. Peripheral blood mononuclear cells from older adults show less of an ability to proliferate than cells from younger adults in response to a pro-inflammatory stimulus.

Findings 6 and 7: Pilot study results support the idea that the nutritional supplement, Muscle Armor, is able to decrease systemic inflammation and improve the response to vaccination in older adults.

Pilot Study: A double-blind placebo-controlled pilot study investigated whether the nutritional supplement, Muscle Armor, affects immune function. Since inflammation is associated with loss of muscle during aging and wasting disease, it was hypothesized that Muscle Armor functions, in part, by exerting anti-inflammatory effects. It was also hypothesized that the supplement would boost the response to vaccination, the gold standard measure of immune function. Subjects were randomized to consume Muscle Armor (N=6) or placebo (N=6) for 4-weeks. The tetanus, diphtheria, and pertussis (TDAP) vaccine was administered two weeks after product consumption began. Plasma was collected prior to the start of consumption, immediately prior to vaccination, and 1- and 2-weeks post-vaccination. Muscle tissue was obtained before and after consumption of supplement or placebo though analyses of these specimens are not yet complete. Plasma C-reactive protein (CRP), tumor necrosis factor-α (TNFα), and interleukin-6 (IL6) were measured as systemic markers of inflammation.

Results and Conclusions for Inflammation: The results for CRP are shown in Figure 7. After 4-weeks of intervention, CRP levels decreased in the supplement group but not in the placebo group

(mean ± SEM: -1.37 ± 1.06 versus 0.42 ± 0.50 mg/L). Based on analysis of log-transformed data, the difference between groups was not significant statistically (P=0.16). Although the sample size was relatively small (N=12), these data provide a reasonable estimate of the expected effect size which was calculated to be 0.85 standard deviations. This effect size is similar to the estimated effect size given for muscle macrophage content under Aim 1. Thus, the power for Aim 1 has been updated to include this new calculation. The proposed sample size (N=50 with 20% dropout) gives 80% power to detect an effect size of 0.85 or greater at a 5% significance level. The results for TNFα (not shown) also suggested that the supplement functions, in part, by decreasing systemic inflammation though a change in IL6 due to the supplement was not detected.

Results and Conclusions for Vaccination: The results for the antibody response to the pertussis antigen are shown in Figure 8. Median levels of antibody increased in both groups after vaccination. The increase from pre-vaccination was greater in the supplement than the placebo group at both 1-week (37.8 v. 19.6) and 2-weeks (131.0 v. 70.7) post-vaccination. Based on a Mann-Whitney test, the difference between groups in the 2-week change in pertussis antibody titers was not statistically significant (P=0.13). Thus, it is unknown whether the proposed sample size (N=50 with 20% dropout) will allow detection of differences between supplement and placebo groups though the nonparametric test result based on only N=12 is suggestive that it may. Regardless, the measure remains valuable to the study because it is a good general measure of immune function that is quite variable for older adults. This variability is amenable to planned within group analyses to determine the correlation between immune function and muscle adaptation to training and detraining. This issue was previously addressed in the pitfalls section of the proposal is reiterated here due to its importance. The aims were purposefully designed to be independent so that conclusions can still be drawn for the relationships between immune function and training outcomes as well as supplementation and training outcomes even if immune function is not altered by supplementation.

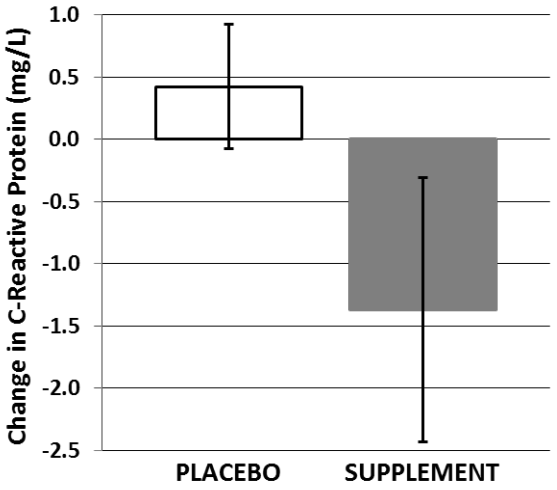

Figure 7. Change in CRP levels after 4-weeks of consumption of Muscle Armor or placebo. Mean and standard error of change are shown.

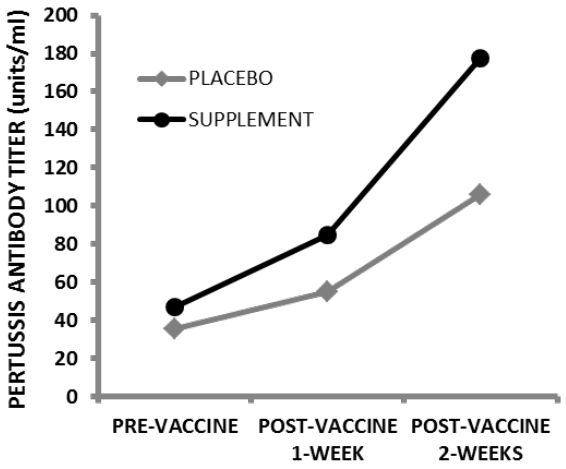

Figure 8. Effects of Muscle Armor or placebo consumption on median response to the pertussis vaccine at 1- and 2-weeks post-vaccination.

RESEARCH DESIGN & METHODS

Study Summary (Figure 9): Veterans (age 60-80 yrs, N=50) will participate in a randomized double-blind placebo-controlled trial. The trial will evaluate the effects of a nutritional supplement (Muscle Armor) on immune function, muscle adaptation to resistance training, and retention of exercise benefits after training is halted. Individual participation will last approximately one year (≥ 47 weeks). This time will include periods for screening and introduction to exercise, supplementation prior to training (2 weeks), supplementation plus supervised resistance training (36 sessions), and supplementation during post-training follow-up (26 weeks). Subjects will undergo four muscle biopsies and blood draws to measure the effects of supplementation on the immune response to a bout of exercise. A fifth biopsy will be collected to measure cellular adaptations in muscle to resistance training. Five additional blood draws will occur to assess eligibility, safety, markers of inflammation, and the response to vaccination for tetanus, diphtheria, and pertussis (TDAP). Strength will be measured bi-weekly, mass and function will be measured at baseline, and after the training and post-training/detraining periods.

Figure 9: Approximate timeline of subject participation and summary of trial protocol.

Week of Participation:	1	2	5	8	9	10	21	37	47
Physical Exam	X								
Intro to Exercise & Function Testing	X	X							
Blinded Randomization	X								
CT Scan			X				X		X
Strength & Function Testing			X				X	X	X
Single Exercise Session			X	X			X		
Blood Draw	X		X/X	X/X	X	X	X		X
Muscle Biopsy			X/X	X/X			X		
Vaccination				X					
Product Consumption (Twice Daily)			1						42
Resistance Training					1		12		
Detraining Period							1		26
Weekly Reminders	1								47

X/X denotes assessment of response to a single bout of exercise by collecting tissue before and after exercise for blood (1 hour) and muscle (72 hours). The above time-frame is optimal though only an approximation because of scheduling issues related to federal holidays, subject availability, etc.

Key procedures to be completed by veterans (age 60-80, N=50):

- o Pre-screening with required waivers and permissions
- o Enrollment screening after written consent and HIPAA
- o Approximately 50-60 research visits over ~1-year
- o Data collection from CPRS problems and medication lists
- o Orange-flavored supplement or placebo consumption mixed in water twice daily (~42 weeks)
- o High-Intensity Resistance Exercise (38x over >12 weeks)
- o Post-training follow-up (~26 weeks)
- o Blood draw (9x)
- o Muscle biopsy (5x)
- o Gait and balance testing (4x)
- o TDAP Vaccination (1x)
- o Thigh CT Scan (3x)
- o Compensation (~$1500): $20 per visit plus bonuses

HUMAN SUBJECT ASPECTS OF THE STUDY

1. **Recruitment (Target Population):** Veterans of age 60-80 years will be recruited.
   - **a.** Accruals Requested: brief pre-screening with required waivers (N=8000), sign consent and undergo screening (N=200), participate in study (N=100), complete all aspects of study considering possible dropout (N=40 to 50). These number will encompass all approved recruitment methods.
   - **b.** Permission is requested for N=50 to complete the exercise and supplementation protocol after consent and screening. If withdrawal or dropout occurs, no additional data will be collected but specimens or data already collected from the subjects will be used unless the subject requests otherwise.
   - **c.** Recruitment will not target vulnerable populations. Pregnant women and fetuses, prisoners, and children are excluded from research due to both VA policy and by the purpose of the study. Should a subject become incarcerated, he or she will be withdrawn from the study by the investigator. If the incarceration is only temporary, does not interfere with subject's ability to participate, and the event is discovered by the investigators after the fact, the IRB will be consulted as to whether the subject must be withdrawn.
   - **d.** Other vulnerable populations (economically and educationally disadvantaged persons, employees and students, and homeless persons) will not be targeted for recruitment but are not specifically excluded. An older veteran may respond to recruitment who by coincidence also happens to be a member of one of these other vulnerable populations. These individuals will undergo the same informed consent process and will be afforded the same research protections as any other participant.
   - **e.** Individuals with diminished cognitive capacity may possibly respond to the recruitment since the study is targeting older (60-80 yrs) adults. However, individuals lacking the cognitive capacity to consent, as judged by a study physician, will be excluded either during the pre-screening or screening phase. The PI will exclude any individuals who appear unable to understand the consent process.
   - **f.** Male and female veterans are eligible.
   - **g.** Minorities are expected to respond to recruitment to the extent of their representation in the local VA population.
2. **Recruitment (Advertising):** Recruitment will occur from flyers (e.g. post-cards or electronic) placed within the CAVHS LR and NLR campuses and by other methods described below. All advertising materials will have been approved by the IRB and the CAVHS Public Affairs office.
   - **a.** Newspaper advertisement may be used but not necessarily due to the high cost of the local newspapers (Democrat Gazette) including military specific publications such as the Combat Airlifter and North Pulaski Leader. Aging Monthly may also be used.
   - **b.** Word-of-mouth may also be used as a recruitment method based on recruit referral from physicians, our subjects, and other individuals.
   - **c.** Flyers and post-cards may be posted within the community at military specific locations such as the American Legion and the VFW. Non-military specific locations in the community, particularly areas frequented by older adults, may also be used.
   - **d.** These recruitment methods most always lead to the initial contact with the veteran being when a recruit calls the study. However, it is possible that the contact could also be in

person. Regardless, “cold-contacts” will not be used. Contact will not involve procedures or data elements except as permitted by the approved protocol.

e. Two new methods of recruitment are requested with the 07-25-2016 modification. The study will recruit by mailings based on search of patient records and also using a staffed information table in concourse areas of the CAVHS hospitals.

i. **Need:** The study needs to mail an advertisement letter (included in submission) in order to recruit participants. This recruitment method is necessary since the use of paper flyers has been banned on the CAVHS campuses. The study is currently using post-cards displayed around campus but they are not effective compared to flyers. The electronic message boards are supposedly available but the study has been unsuccessful in posting on the boards despite requesting access for the past three months. The study also needs to place staff at an information table in concourse areas (e.g. off to the side in concourse outside hallway to primary care).

ii. **Justification:** From 08/2014 to 04/2016 the study received an average of 21 phone calls per month from interested Veterans based on flyer postings and placement of four newspaper ads at a cost of ~$1000 for three days each. Since the 04/2016 banning of flyers, the call rate has averaged only 10 participants per month though this number was helped by the placement of one newspaper ad. More importantly, the use of post cards rather than flyers has skewed the recruitment response towards less healthy individuals. This claim is based on the success rate of individuals making it past the initial eligibility screening by phone which changed from more than four per month to less than one per month. The presumed reason is that flyers are the advertisement method that Veterans are familiar with and accept whereas post-cards are only likely to be noticed by patients who spend much more time at CAVHS.

iii. **Method 1:** The study will mail an advertisement letter (opt-in) to individuals identified through screening primary care appointment lists available in CPRS. These lists are available to anyone with CPRS access. The main benefit of this method is efficiency since these Veterans should have an up-to-date health assessment from the primary care visit. A checklist (included with submission) will be used to determine if individuals meet basic general eligibility criteria. Records will not be accessed if marked “sensitive” or if the person does not meet the age requirement. If all criteria aside from the latest PC visit are met, then the person’s name, last four of SSN, and address will be recorded on a checklist. Then later (e.g. next day) the primary care assessment will be reviewed and if no concerns exist, then an advertisement letter will be mailed. Individuals will be contacted and further recruited only if they respond to the letter. Those who respond will continue recruitment by the already approved method. Non-responders will be placed on the recruitment/enrollment log (name only) so that they are not contacted repeatedly. In order to perform this process waivers of HIPAA and informed consent (see Supplement L) are requested.

iv. **Extension of Method 1:** The package 28 modification requests permission to extend the CPRS appointment list screening of recruits to all CAVHS clinics not just primary care. Permission is also requested to extend the CPRS screening and the mailing of opt-in advertisement letters to past participants in our studies 204327 and 391351 (Waiver of HIPAA and Consent has been requested to access name and last four from those study charts). The already approved process and protections for

screening and mailing of the opt-in letters of invitation also applies to these extensions.

v. **Method 2:** The study will also place staff members at information tables available to Veterans. The most likely area is in the concourse between the elevators and the hall to primary care in NLR (similar to the Million Veterans Program). Staff will offer verbal explanation or an already approved post-card, or conduct the already approved initial contact discussion (see pre-screening process below) if the Veteran is interested. The IRB can be confident that this face-to-face encounter will not include a coercive "sales pitch" as drop out severely hurts enrollment. Participants join the study due to personal interests in exercise and nutritional supplementation and the willingness to attend approximately 50 visits over the course of a year. Stated another way, the study never attempts to "talk someone into" this substantial commitment.

vi. **Additional Protections:** Any Veteran information collected by the processes described above will be protected using the same VA confidentiality and privacy and protocol information security mandates already adhered to by the study. Every effort will be made to ensure that these processes are seen as non-intrusive and respectful of privacy from Veterans or the process will be modified if needed. Appointment lists will not be printed. As with all other study records, paper documents are stored behind a least two locks in the GRECC in the office of the PI or temporarily with the coordinator and electronic records are stored in the PI's folders on the GRECC server.

3. **Pre-Screening:** Pre-screening for eligibility will be performed when a recruit responds to recruitment, is interested in learning more about the study, and is interested in being considered for participation. A request for waivers of HIPAA and written consent is limited to this specific minimal risk activity. Recruits who appear eligible will come to the NLR VA for a research visit to continue the informed consent process, including written consent, HIPAA authorization and further screening for eligibility. Thus, the main purpose of the pre-screening is to minimize the chance of a recruit who does not meet even the basic eligibility criteria from unnecessarily attending a research visit.

   a. **Verbal Informed Consent:** Verbal informed consent will be obtained by telephone to conduct the pre-screening. Recruits must answer affirmatively to having an interest in being considered for the study for the pre-screening to continue. Recruits are told that they may refuse to answer any question. This activity presents minimal risk to the recruit and all information will be protected as confidential. Justifications are included in the IRB-required supplement included with this submission.

   b. **Waiver of HIPAA:** Permission is requested to for a waiver of HIPPA in order to collect pre-screening information from the recruit and from CPRS. Pre-screening information will be documented on the "Initial Contact Form" and "Medical History and Physical Exam Form" as applicable. Justifications are included in the IRB-required supplement included with this submission.

   c. **Initial Contact and Pre-Screening:** Documentation of the pre-screening (partial list of eligibility criteria) will begin on the "Initial Contact Form". The form contains a scripted explanation of the study to be used by a member of the study team who has been delegated this responsibility and trained by the PI. The brief explanation describes study procedures, benefits, and risks. The form contains the last 4 digits of the SSN which will be used to access the recruit's CPRS record if verbal consent to pre-screening is given.

i. If the recruit gives permission for CPRS pre-screening, then the CPRS Problems List for the recruit will be reviewed by the study physician in order to assess for exclusion criteria. This review will be documented on the "Medical History and Physical Exam Form". This screen of the CPRS Problems List includes among the criteria, a check for evidence for lack of cognitive capacity to consent for the study.

ii. Recruits who appear eligible will be contacted to schedule a research visit in which written consent will be obtained and the screening process will be continued by the study physician. It is not a necessity but recruits are asked to bring the following information to their visit: list of current medications; names and telephone numbers of two emergency contacts; name and number of personal physician.

iii. Consent to the CPRS pre-screening process is not a requirement to be considered for the study. Individuals who wish to be pre-screened after written consent will be allowed to do so during a research visit.

iv. Obviously CPRS pre-screening does not apply to recruits who have not previously received VA healthcare. These recruits will be informed that a DD214 is necessary and that a VA medical record must be created for them prior to written consent.

4. **Informed Consent Process and Written Consent:** Recruits passing the pre-screening will attend a research visit to consent the consent process, provide written consent and continue eligibility screening. The goal of the informed consent process is to ensure complete disclosure and voluntary participation for the subjects.

   a. **Informed Consent Process:** Informed consent will be an ongoing process that begins when the recruit first learns of the study and will continue as an ongoing exchange of information between the research team and the subjects throughout the course of the study.

   b. **Written Informed Consent:** Written informed consent will be obtained from recruits by the PI or a trained member of the study team who has been delegated this responsibility. The current IRB-approved and stamped version of VA Form 10-1086 will be used. Written consent will be obtained prior to the continuation of the screening for eligibility.

      i. The process will be conducted in the English language.

      ii. The informed consent form will have been mailed to the participant after the pre-screening if practical. If mailing the consent is not practical such as if the study appointment is the next day, then the recruit will be given as much time as needed at the research visit to read and understand the consent form.

      iii. The elements of the consent form will be discussed with the recruit in an area where privacy can be maintained. The recruit will be told that participation is completely voluntary and that refusal to participate will not influence his healthcare or have negative consequence.

      iv. Recruits will be allowed to ask questions and express concerns. If the recruit feels that he or she has not had sufficient opportunity to consider whether or not to participate in the research, he or she will be allowed to leave and return at a later date. Alteration of the appointment schedule to ensure subject consent, safety, convenience, etc will not be considered a protocol deviation.

      v. If during the consent process, the person obtaining consent believes that the recruit does not understand the procedures and risks of the study or the consequences of not participating, then written consent will not be obtained and the person will be excluded from the study. The process or the reason for not obtaining consent will be documented in the consent process note.

vi. Subject will be given a copy of the signed consent form and the original will be kept in the subject file.

vii. Consent will also be documented in a consent process note placed in CPRS and the subject file within 24 hours of obtaining consent. All subjects participating in the greater than minimal risk aspects the study will have their CPRS record flagged with a Category II patient record flag. The flag will be removed once participation has completed or the subject has been withdrawn and 30+ day monitoring is complete.

viii. The PI will sign the consent document and the document will be hand delivered for scanning within the IRB required time frame of the subject signature. The 10-9012s, HIPAA, and scanning request form will also be included.

5. Screening for Eligibility: Subjects who provide written informed consent will undergo a screening for eligibility by the study physician using the Medical History and Physical Exam form. The inclusion and exclusion criteria are based on the study design and health suitability to undergo exercise training, supplementation, blood draws, biopsy, CT scan, and vaccination. If exclusion criteria are temporary, recruits may participate in the study (or specific procedure related to criteria) once condition is no longer exclusionary. The criteria and method for collection of this information are listed:

a. **Inclusion Criteria: information collected from subject self-report**

(1) Veteran

(2) Age 60-80 years

(3) Body Mass Index of 18.5 - 29.9 kg/m^2

b. **Exclusion Criteria: information collected from subject self-report**

(1) Currently participating in any other research study involving an intervention

(2) Smokes tobacco products (subject word to halt smoking is also sufficient)

(3) Tetanus or TDAP vaccine in previous two years

(4) Allergic to vaccination

(5) Seizure in past 3 months

(6) Guillain-Barre Syndrome in past 3 months

(7) Takes the medications heparin, plavix / clopidigrel, or coumadin / warfarin

(8) Allergic to lidocaine

(9) Significant problem with fainting

(10) Problems walking or exercising with both legs

(11) Participated in a weight-lifting program targeting the thighs in last 3 months

(12) Pains, tightness or pressure in chest during physical activity such as walking, climbing stairs, household chores, or similar activities

c. **Absolute Exclusion Criteria: information collected from CPRS review and/or subject self-report.** The medical history will include a discussion between the subject and the study physician of the exclusion criteria that may have been reviewed during the pre-screening of the CPRS Problem List. The physician will assess the available information and document whether or not the subject is excluded.

(1) Enrolled in another interventional study (CPRS Category II Flag)
(2) Metastatic cancer or undergoing chemotherapy
(3) Cerebral aneurysm or intracranial bleed in past year
(4) End-stage congestive heart failure (NYHA Stage IV)
(5) Unstable abdominal or thoracic aortic aneurysm (>4cm)
(6) Renal disease requiring dialysis
(7) Allergic to lidocaine or vaccination
(8) Acute retinal hemorrhage or ophthalmologic surgery in past 3 months
(9) Bone fractures in the pelvis, legs, or feet in the last 3 months
(10) Hernia that causes pain during physical activity
(11) Myocardial infarction or cardiac surgery in past 3 months
(12) Pulmonary embolism or deep venous thrombosis in past 3 months
(13) Proliferative diabetic retinopathy or severe nonproliferative retinopathy
(14) Active suicidality or suicidal ideation
(15) Systemic bacterial infection
(16) Taking heparin, plavix / clopidigrel, or coumadin / warfarin
(17) Taking ASA (in any form) and unable/unwilling to discontinue at least 10 days prior to muscle biopsy
(18) Unwilling to halt concurrent use of amino acid or protein supplements
(19) Unwilling to halt new use of nutritional supplements
(20) Unwilling to maintain current normal diet
(21) Guillain-Barre Syndrome in past 3 months
(22) Tetanus vaccination in past 2 years
(23) History of allergic reaction to vaccination
(24) Encephalopathy in past 7 days
(25) Active oral or genital herpes
(26) Current use of appetite stimulants
(27) Significant history of seizures or seizures in past 3 months
(28) Current treatment for mania or bipolar disorder or taking lithium.

d. **Potential Exclusion Criteria: evidence for potential exclusion criteria will be collected from CPRS review and/or subject self-report.** *Certain issues may also be assessed by the physical exam.

(1) Diagnosis of a cognitive deficit
(2) Untreated severe aortic stenosis: if there is evidence in CPRS Problem List or subject self-report of untreated aortic stenosis, then physician judgment for inclusion must be

based on an available echocardiogram, or evidence a valve area of < 1cm^2, or a history in the past 3 months of syncope, heart failure, or angina.

(3) *Uncontrolled diabetes mellitus (HbA1C>10)

(4) *Uncontrolled hypertension or hypotension (>160/100, <100 systolic)

(5) *Uncontrolled malignant cardiac arrhythmia (sustained ventricular tachycardia > 3 beats, complete heart block, atrial flutter, symptomatic bradycardia)

(6) Unstable angina (at rest or increased pattern in past month)

(7) Allergic to latex or tape

(8) Bleeding or clotting disorders, family history of bleeding disorders, problems with abnormal bleeding

(9) Taking any non-ASA NSAID and unable or unwilling to discontinue use for 3 days prior to the muscle biopsy procedure

(10) Taking Fish Oil, Gingko, Garlic, Saw Palmetto, Turmeric, or Vitamin E and unable or unwilling to discontinue use for 10 days prior to the muscle biopsy procedure. Multivitamin containing vitamin E is acceptable and does not need to be halted.

(11) Significant history of fainting

(12) Significant problems with chronic pain

(13) Uncontrolled asthma or allergies

(14) Taking lactulose, nitrates plus hypertension medications or Viagra

(15) Liver cirrhosis or other severe liver disease

(16) History of peripheral artery disease

(17) Steroid or androgen use in past 3 months that physician feels may confound study

(18) Other physician judgment

e. **Other Exclusion Criteria: information collected during the physical exam by the study physician or qualified designee.** Blood will be drawn (less than or equal to 60ml or 2 ounces) for lab analyses by the hospital clinical lab at study entry, after completion of the exercise training program, and upon completion of the study for tests related to eligibility, safety, and research data.

(1) Blood Pressure greater than 160/100 or <100 systolic.

(2) HbA1C>10

(3) CBC or PT/PTT/INR results that the study physician determines indicate a risk for excessive bleeding.

(4) Clinical labs performed for safety monitoring including AST, ALT, creatinine, and BUN

(5) Other tests that performed to measure immune function include HbA1C, CBC, CRP, ESR, cholesterol HDL and LDL, and blood differential.

f. **Exclusion Criteria Based on Other Physician Judgments:** older veteran subjects often possess numerous comorbidities that result in a complex and unique set of health issues. Thus, in addition to the specific exclusion criteria named above, the physician must also use a considerable amount of judgment in determining whether a recruit should be excluded even though the recruit meets the named inclusion criteria and does not meet the named exclusion

criteria. The physician also makes such judgments to determine if a subject should be withdrawn once participation has begun. The physician may obtain any additional unforeseen medical information that may be needed at enrollment or during participation. As explained in the consent and HIPAA documents, this information may be collected through CPRS, additional examinations or tests, or by consultation with the subject's healthcare providers from inside or outside the VA. As appropriate, the physician will document and use this information to judge whether the subject can safely participate, should be excluded or withdrawn, or should be referred for treatment.

6. **Scheduling:** A scheduling calendar will be used by the study staff and study visit scheduling cards will be given to the subjects to help ensure compliance with study visits and procedures.

   a. A subject scheduling calendar will be maintained in the form of an Excel spreadsheet. The calendar is needed in order for the study team to coordinate study procedures and data collection particularly when multiple subjects are scheduled for any one day. Thus, the calendar will improve study efficiency, subject safety, and care. The calendar will contain dates, subject ID #, and procedures for each visit. It will be stored in the PI's secure folder containing electronic logs.

   b. The study will also give patients visit cards that will include subject name, visit date and time for up to three visits, procedures, and estimated time required. The calendar and cards will help keep the study team and subjects on track with the study requirements for each visit.

   c. The scheduling cards will typically be hand given to subjects at an appointment when their next appointment time and date is already known. However, scheduling cards may also be mailed, as needed, for instances when an appointment must be scheduled over the phone and the subject desires a written reminder in addition to the reminder telephone calls that are already commonly used. Obviously a card would not be mailed, for example, if a subject is contacted by phone and states that a card is not needed or if the appointment is scheduled for so soon as to not allow time for mail to be received.

7. **Payment of Subjects:** This research is not integrated or related to patient care. Subjects will be compensated for their time and inconvenience and as incentive to participate in compliance with the requirements of the study as explained in informed consent.

   a. Payments will be made in the form of Kroger gift cards.

   b. Participation involves approximately 50-60 research visits over the course of approximately one year. Subjects will receive $20 for each visit attended. However, approximately 10 of the visits (e.g. once every four weeks) will require that the subject return a dosing calendar and the canisters containing any unused portion of supplement or placebo. This process is required to monitor subject compliance with product consumption. At these visits, subjects will receive the $20 if the dosing calendar and canisters are returned. If a subject forgets to bring these items, they can be returned at the next visit and the payment will then be given. If these items are never returned, the subject will not receive that one payment.

   c. The two visits in which the subject undergoes blood draw, biopsy, exercise, and blood draw require approximately 4 hours. For each of these two visits the subject will receive an additional $20 gift card (i.e. $40 for the visit).

d. Subjects will be given two completion bonuses. Subjects will receive $100 after completion of the resistance training program and $300 after completion of the entire study. The total amount earned for completion of all study visits will total approximately $1,500.

e. The compensation is normal compared to similar studies conducted at other institutions. The amount is not considered coercive considering that subjects will have more than 50 visits over a one year span that require a high level of conscientious active participation (exercise training and accurate supplement/placebo consumption, multiple biopsies and blood draws).

f. The consent form will inform the subject of that the VA may report participation payments as income to the IRS.

g. Finder's fees and recruitment bonuses will not paid to VA or study staff. If a subject refers another veteran to the study who signs consent to participate, then a $20 gratuity will be given to the referring subject.

8. Randomization: After medical clearance to participate, subjects will be randomized to either of two parallel groups, treatment with Muscle Armor or placebo. The double-blind randomization will be performed by a research pharmacist. Assignments will be unknown to investigators and subjects. The pharmacist will review all study material prior to approval, and can break the blind if needed for safety purposes, but is otherwise uninvolved in the study. The pharmacist will dispense the products in sealed and blinded packaging. The study will appoint a study monitor (specific un-blinded member of the study team) to review and be involved in pharmacy and subject compliance. This person will not be involved in subject assessments or specimen analysis.

9. Supplementation: Muscle Armor was chosen from a scientific standpoint because of the ability of its ingredients to modulate muscle health and immune function. Muscle Armor is a good choice for practical purposes because it's ingredients are safe to consume [46;47] and it is available to individuals by retail.

a. Subjects in the supplement group will consume orange-flavored Muscle Armor according to the manufacturer's directions: one serving (approximately 30g, i.e. one scoop provided with the product by its manufacturer), twice daily (optimally morning and evening), mixed with 12 ounces (oz) of water. Subjects in the placebo group will similarly consume one serving (approximately 13g, one scoop provided to pharmacy by the study) of orange-flavored Kool-Aid mixed with 12 ounces of water twice daily.

b. Quality control weighing of single doses of supplement and placebo indicates that exact dose will vary based on whether a level, sub-level, or heaping scoop is used and this is acceptable. Dose range for the supplement appears to be 30-35 grams and dose range for the placebo appears to be 11-15 grams. This is less than the actual single serving size listed on the instructions for Kool-Aid but is deemed acceptable due the sugar content of the recommended serving size since diabetics will likely be enrolled and older adults may dislike the taste due to it being too sweet.

c. Supplementation will typically begin the day of the second muscle biopsy (typically Visit 5) and continue for the remainder of the participant's study involvement (optimally approximately 42 weeks). The total dosing time will likely be longer than the optimal time because scheduling of the exercise training and other appointments must be performed based on VA holidays, patient and physician availability, etc. And specific starting date or intermittent dosing lengths of time can be altered without protocol deviation in order to maintain proper spacing of the more time dependent clinical procedures, e.g. blood draw, biopsy, and vaccination.

d. Compliance will be monitored using subject self-report and both the number of doses consumed and weight of product consumed. Each canister will contain 14 doses (7 day supply) though

this number is approximate due to the inexact measurement of one scoop. Canisters will contain 421 grams of supplement or 188 grams of placebo. Subjects will return empty containers. Containers will only be weighed if unused supplement or placebo is returned. Self-report will be based on completion and return of the dosing calendar. This information will be collected and reviewed by a study monitor, i.e. member of the study team who is not blinded. The monitor is planned to review dosing and consumption for compliance with the subject approximately every four weeks by phone or in person or more often as needed. Subject diet data will not be collected but to be involved subjects must agree to maintain their normal diet during participation.

e. Subjects will be given a gym bag to transport their canisters of supplement/placebo and consumption calendar between home and study visits. They will also be given two drink mixer bottles of the type commonly used to mix powder shakes. These products contain the GRECC name and phone number of the study coordinator. Subjects will be allowed to keep the bag and bottles upon completion of the study. The approximate value of the products per subject is ~$28. These gifts are justified due to making it easier for the subject to transport materials to and from study visits which should increase subject compliance with dosing and monitoring requirements.

f. 06/12/2017 Modification. The study has learned that Abbott Nutrition is no longer manufacturing Muscle Armor and will only sell a similar product, Juven, in the future. The study will switch to using Juven with approval of this modification though remaining stores of Muscle Armor will be used until expiration date. The study had originally planned to use Juven but did not use Juven after learning that it is only available in single serve packets which would make the blinding process laborious. Use of Juven will require the study team member who packs the placebo in the pharmacy to pack the supplement Juven in similar fashion, i.e. 14 doses (packets) will be emptied into the usual blinded supplement canisters. Other pertinent details of the switch include the following:

    i. Juven and Muscle Armor are essentially the same product but were being marketed by the company under two different names. They taste, smell, and look identical. Thus, the blind will not be in jeopardy due to the switch.

    ii. The key ingredients of interest (Arginine, Glutamine, Methylbutyrate) are present in the same amounts in Juven and Muscle Armor. Juven lacks the 3g Taurine that is in Muscle Armor and Juven contains phenylalanine, and FD&C Yellow #6 instead of Red #40, and aspartame instead of sucralose.

    iii. The product identity and ingredients were not mentioned in the consent form or other information given to subjects.

    iv. Since Taurine is missing in Juven, the dose size is slightly smaller than Muscle Armor (24g versus 30 g). However, Juven was measured in the same scoop size used for Muscle Armor and the difference was negligible.

    v. Switching to a slightly different product during the study is not optimal but is necessary. The switch is not expected to affect the results. However, the study will track when the switch was made for each subject to determine during statistical analysis if a bias was introduced.

g. **Benefit:** The subjects may or may not have improved muscle health and immune function due to the supplement. The supplement may improve the response to the vaccine. However, it is unknown whether this improvement will convey increased protection from disease.

h. **Risk:** There is little evidence from controlled studies of risk associated with the ingredients of the supplement. However, adverse events have occurred in which the relation to underlying disease or the supplement was not discerned. Thus, this study will take a conservative approach and consider that the ingredients may exacerbate the following conditions: allergies or asthma, herpes**,** and low blood pressure. The supplement may cause side effects such as abdominal pain, bloating, diarrhea, gout, blood abnormalities, sensitivity similar to monosodium glutamate (MSG; headache, flushing, tingling, weakness). Caution should be used when taking the supplement and lactulose. The supplement should not be taken by individuals undergoing chemotherapy, or those having a recent heart attack, liver disease, seizures, mania or bipolar disorder and taking lithium.

i.

Table 2. *Comparison of nutrient information between Muscle Armor supplement and Kool-Aid placebo control for one serving mixed with 12 oz water. Juven ingredients listed in parentheses.

MUSCLE ARMOR	PER SERVING		KOOL-AID	PER SERVING	
AMINO ACIDS			**AMINO ACIDS**		
L-ARGININE	7 (7)	g	L-ARGININE	0	G
L-GLUTAMINE	7 (7)	g	L-GLUTAMINE	0	G
TAURINE	3 (0)	g	TAURING	0	g
CARBOHYDRATE	10 (8)	g	**CARBOHYDRATE**	16	g
SUGARS	5 (2)	g	SUGARS	16	g
MINERALS			**MINERALS**		
CALCIUM	200 (200)	mg	CALCIUM	0	mg
Calcium β-hydroxy-β-methylbutyrate	1.5 (1.5)	g	Calcium β-hydroxy-β-methylbutyrate	0	g
VITAMIN C	0 (0)	mg	VITAMIN C	9	mg
ENERGY	110 (80)	kcal	**ENERGY**	60	kcal

*Juven supplement has the same composition as Muscle Armor except it lacks Taurine.

j. **Minimization of risks:** The risks of participation will be minimized by cautious consideration of the potential adverse effects of Muscle Armor by the study physician during enrollment and monitoring. Individuals who should not take the supplement will be excluded. Subjects will be informed of the potential side-effects and advised to report any symptoms. Symptoms related to the supplement and other study procedures will be monitored for, as applicable, at each study appointment and by attempted telephone contacts weekly. Safety monitoring will also include blood draws for assessment of liver and kidney function at baseline, after completion of the training program and at the end of the study.

9. Vaccination: The International Society of Exercise and Immunology considers response to vaccination the gold-standard for assessment of immune function in the context of exercise [38]. Multiple vaccine options were considered for use in the proposed study. Vaccination with antigen more likely to be naïve to subjects is preferable. However, the risk to benefit ratio of using the keyhole limpet protein or the herpes zoster live-attenuated virus was considered unacceptable. The influenza vaccine was considered since the epitope may change yearly, but this option could limit recruitment outside of flu season by availability of the vaccine and during flu season by availability of unvaccinated veterans. The tetanus, diphtheria, and pertussis (TDAP) vaccine has the advantages of benefit to the subject, low risk, low cost, and available test kits to measure response.

a. **Procedure:** The vaccine will be administered by a study physician to each subject after 2-weeks of supplementation. Blood will be collected at baseline and 1 and 2-weeks post-immunization to determine the effects of the supplement on the humoral response. Two time-points were chosen in case the response is delayed in older adults relative to the response in

younger adults [48]. If needed to accommodate scheduling, the supplementation period may be extended. The vaccination and second biopsy may occur up to 5 days late and the two post-vaccination blood draws may be delayed by one day if necessary.

For each study procedure a qualified study staff member will perform the procedure, i.e. a study physician will administer the vaccine and the study coordinator will draw blood. However, non-study staff such as the GRECC nurse or a CAVHS phlebotomist may also be utilized as needed, without being added as study staff, if the activity falls within their CAVHS job duties.

b. **Benefit:** The TDAP vaccine was chosen because of the direct benefit it will provide to older subjects [49]. Tetanus, for example, is uncommon in the United States, but 70% of cases involve older adults and individuals over 60 years of age experience greater than a 50% mortality rate [50;51]. However, problems with these diseases are avoidable because the vaccine is safe for use in older adults.

c. **Risk:** The most common problems in adults are mild or moderate: pain at injection site (mild 2 in 3, moderate 1 in 100); redness or swelling at site (mild 1 in 5, moderate 1 in 25, severe 3 in 100); fever (mild 1 in 100, higher 1 in 250); headache (mild 3 in 10, moderate 1 in 300); tiredness (mild 1 in 4), and nausea, vomiting, diarrhea, or stomach ache (mild 1 in 10, moderate 1 in 100). Chills, body aches, sore joints, rash, or swollen glands are uncommon. A severe allergic reaction could occur after any vaccine (rare, less than once in a million doses). Individuals who are allergic or have experienced unexplained problems (coma, seizures) after vaccination, or has had Guillian-Barre Syndrome should not be vaccinated.

d. **Minimization of risk:** The most common problems can be safely managed with symptomatic treatment. Individuals who should not be vaccinated will be excluded. The procedure will be postponed until recovery for subjects with mild illness or fever. All subjects will be informed of the risks and advised to call the study physician or staff immediately should a severe reaction occur.

10. **Resistance Exercise Training Program:** The study will use a resistance exercise routine that we have previously used to elicit cellular and molecular responses in muscle associated with immune function and to improve muscle mass and strength of the thigh for older adults [2;3;40;52].

a. **Procedure:** The resistance exercise program will use three exercises to strengthen the thigh muscles including bilateral knee extension, knee curl, and leg press. Exercises are completed on air-driven exercise equipment in a seated position. Exercise sessions are always supervised by a study team member.

b. **Strength Testing:** Strength will be determined using the 1-repetition maximum test [52]. A brief warm-up period will include approximately 10 minutes of mild aerobic activity followed by approximately 6 repetitions of lifting warm up at for a resistance exercise at a “somewhat heavy” load. The load is then gradually (~5-10%) increased such that the maximum capability for lifting is reached in approximately 10 total repetitions. Subjects will rest 60 seconds between repetitions after the warm up. Subjects rest five minutes prior to the next test completed in the order of press, curl, and extension.

c. **Unaccustomed Exercise Sessions (2x):** Prior to exercise training, the study will measure the response to single bouts of the training exercises before and after two weeks of nutritional supplementation. The response to exercise will be measured using both muscle and blood. The pre-exercise time points will be prior to exercise, possibly same say or a different day based on scheduling and/or the judgment of the study physician. The post-exercise time points for collection are 72 hrs for muscle and 1 hr for blood. Our studies have previously shown that 72 hrs is a better than 24 hours for measuring the immune variables of interest in muscle [52].

The 1 hr time point for blood collection is based on other studies that have determined the effects of exercise on systemic measures of immune function [11;53].

d. **Exercise Training Sessions (36x):** The training program plans to have subjects perform supervised exercise at the NLR VA for approximately an hour optimally 3 times per week on non-consecutive days. The program consists of 36 exercise sessions that will optimally take 12-weeks though the total time will likely be longer due to rescheduling based on the needs of the subject or the study team.

e. The exercise load will be based on how much the subject is capable of lifting one time. After strength testing, subjects will perform exercises at loads ranging from 60% to 80% (e.g. sets at 60, 70, 75, and 80% of capability) of their measured capability to the best of their ability. Strength is retested at approximately every 6th session to account for strength gain in maintaining the work load. On days of testing, the strength test replaces one of the four exercise sets. Strength is also tested twice prior to training, at the final training bout, and twice during the post-training follow-up period.

f. Training sessions consist of three sets of 10 repetitions for each exercise and a 4^{th} set completed at voluntary failure to complete a repetition. Subjects will rest approximately two minutes between each set and approximately 5 minutes between exercises.

g. Exercise data will be recorded for each subject on the Exercise Data Form.

h. Exceptions to the plan for each subject to complete 36 exercise training sessions may be necessary on a case-by-case basis at the discretion of the PI in order to ensure that the final muscle biopsy of the protocol occurs 72 hours (3 days) after the final exercise training session. Addition or subtraction of a limited number (optimally 1-2) of exercise sessions may be necessary to meet this time frame due to scheduling difficulties for the biopsy. Scheduling obstacles include physician or subject availability or more commonly the fact that the final exercise session cannot fall on a Wednesday or Thursday unless plans are made to perform the biopsy on Saturday or Sunday which is not preferable. Maintaining this time frame for biopsy is the priority as it has a greater chance of influencing the cellular measurements to be made using the biopsy than would a small difference in the total number of exercise sessions for influencing strength gain over the long training period.

i. **Benefit:** Regular resistance exercise is an effective method for improving many aspects of health and well-being (13). Subjects will likely improve the size (CT scan), strength (strength testing), and function (see below) for their thigh muscles. The assessments of muscle function include:

   (1) Berg Balance Scale (BBS): BBS is used to detect balance impairments in older adults while the individual is static or performing various movements [53]. The test consists of 14 tasks performed in standardized order and the results have been shown to have good inter-rater reliability of 0.98.

   (2) Six Minute Walk Test (6MWT): The test is used to measure mobility performance in older adults and individuals with limitations [54]. The subjects will perform a supervised timed walk on a 200 foot track at a pace without encouragement and which allows speech without shortness of breath.

   (3) Gait Speed: The test measures habitual walking speed [55]. The subjects will walk at their normal preferred pace for 14 meters though only the middle 10 meters are timed. The result is an average of three replicates.

(4) Timed Up and Go: The test is indicative of balance and mobility by a measuring the time required for the subject to rise from a chair, walk three meters, turn around, walk back to the chair, and sit down [55].

(5) Functional assessment data will be collected at four times during the protocol: pre-training, post-training, during post-training follow-up, and at end of study.

j. **Risk:** In all but limited contraindicating circumstances (our exclusion criteria), the benefits of regular exercise exceed the risks. Exercise of the proposed intensity has been conducted safely in even frail elderly and adults in 80-90 years of age (8, 18, 23). However, serious events are possible:

(1) Exercise may cause (estimated at 1 of 100 cases or 1:100) shortness of breath, chest pain, or dizziness; and ligament, tendon, or muscle sprain.

(2) Heart problems are a rare but serious risk of strenuous exercise and may result in sudden death (1:18,000 adults per year). However, even in patients undergoing heart rehabilitation, heart failure (1:100,000), heart attack (1:200,000), and death (1:750,000) are rare events relative to the number of hours spent exercising.

k. **Minimization of Risk:** the risks of exercise will be minimized for the subjects as follows:

(1) Each subject will have undergone a thorough medical history and physical exam including an electrocardiogram (resting and during exercise stress) performed by the study physician prior to participation. Details and physician clearance to participate are documented on the Medical History and Physical Exam Form.

(2) Subjects will not be allowed to begin exercise if their blood pressure is greater than 160/100. After training, if blood pressure is elevated, the subject will remain at the GRECC until it returns to normal. If it does not return to normal the subject will be evaluated by a study physician.

(3) Training will be supervised at all times during exercise by a member of the study team who is certified in basic life support to ensure subject safety and compliance. If the subject cannot complete the exercise with proper form then the work load will be decreased or the set will be halted. The member of the study team will know the emergency response procedures and they will be posted on the wall of the exercise room.

(4) The exercises will be performed using air-driven equipment in a seated position. Thus, there is no risk of the subjects falling, dropping weights, or having weights fall on them.

(5) Subjects will complete an introductory exercise session in order to learn proper exercise form while lifting relatively light loads.

(6) Each training session will be preceded by a warm-up period on a stationary bicycle, treadmill, and/or the exercise equipment. Stretching will be performed as needed during and after the training session.

l. **Emergency Response:** If there is a problem or concern for continued exercise, the person supervising the exercise will immediately halt the exercise. This emergency response also applies to any potential problem occurring on-site. If the problem is a potential emergency, the hospital medical response team will be called immediately at 6-911. This number is posted on the telephone in the exercise training room. The medical response team will arrive within minutes. During this time, basic life support will be used if warranted. If there is a medical

problem that is not an emergency, the study medical staff and/or the GRECC nurse will be contacted to come evaluate the symptoms and determine a course of action.

(1) **Modification to Emergency Response Plan 09-16-2016:**

(2) This greater than minimal risk protocol is modified to inform the IRB that the emergency response procedure for an on-site serious adverse event has changed but there is no significant change in risk to the subjects. Beginning the evening of 09-15-2016, the NLR campus will deploy a "medical response team" (MRT) instead of a "code team" in response to emergencies.

(3) In response to email notification of this change on 09-13-16, Dr. Dennis Sullivan convened a 90-minute meeting (09-14-16) of the physicians and principle investigators of the GRECC. Dr. Sheila Sullivan obtained further clarification on the details of the change from the Quality Management Coordinator. We also reviewed the medication list to be included in the Stat Pack meds of the MRT. We concluded that the risk posed to subjects participating in the ongoing clinical trials at GRECC and specifically this protocol, are not increased based on the following details of the change and our preparedness measures.

(4) This opinion that risk to participants has not changed is consistent with the conclusion of research findings indicating that the provision of pre-hospital ACLS after cardiac arrest did not change the survival rate to hospital discharge [54].

(5) Under the new guidelines for emergency response, the medical officer of the day (MOD) will continue to respond as team leader of the MRT. MRT will provide BLS and other care determined by MOD. The patient will be transferred to an acute care facility, if needed, via MEMS (determined by MOD), as is similar to the prior practice.

(6) Crash carts, including the crash cart on our unit, will no longer house medications. However, Stat Pack medications will be carried to each emergency site by a MRT nurse. The medications included are the same as those previously supplied on the crash cart with the exception of anti-arrhythmics and atropine. However, our unit will stock atropine on site along with the items necessary for administration of these medications (e.g. IV lines and normal saline) so long as unavailable from the MRT.

(7) Additionally, the MRT will not have access to intubation though use of this procedure was previously discouraged by the code teams anyway. The MRT will still have capacity to support patient respiration (Ambu Bag) until MEMS arrives, if necessary.

(8) The likelihood of serious adverse events with the protocol are low though the possibilities of concern include: acute myocardial infarction (MI) due to strenuous exercise, vasovagal response with syncope and possible bradycardia during muscle biopsy, and anaphylaxis due to lidocaine sensitivity during muscle biopsy. Baby aspirin and nitroglycerin are available in the Stat Pack in case of chest pain or other symptoms suggestive of MI. As stated above, atropine and the means of administration will be kept on-hand in case of bradycardia. Benadryl is available in the Stat Pack in case of sensitivity to lidocaine though epinephrine is also available from the MRT for serious reactions.

11. **Post-Training Follow-up Period:** After completion of the training program, subjects will remain in the study for at least 26 additional weeks so that the retention of muscle mass, strength, and function can be examined relative to continued daily consumption of supplement or placebo. Subjects will be required to refrain from strength training of the thighs (i.e. weight lifting, body weight exercise, elastic bands) during this period. It is acceptable for subjects to participate in other forms of exercise such as walking or resistance training with the upper body. Compliance with these and study dietary requirements will be monitored by subject query at monthly visits during the follow-up period. These visits are also required for dispensing of product from the pharmacy and monitoring of consumption compliance.

    a. The study did consider the ethical issue of disallowing resistance training of the thighs during the post-training period since the benefits of the exercise training will be partially lost without it. We concluded that this criterion was acceptable because it eliminates a strong confounder (i.e. strength training) for Aim 3 (Does the supplement promote retention of the benefits of exercise?) of the study; older adults are unlikely to participate in high-intensity training of the thighs anyway; and other options in exercise more commonly practiced by older adults are acceptable as an alternative.

12. **Muscle Biopsy:** Muscle biopsy is a procedure commonly performed in physiology studies. The studies involving this PI have collected several hundred biopsies from the thigh. Only three problems have occurred which involved resumption of bleeding that required a pressure bandage, a mild reaction to the lidocaine injection which resulted in halting of the procedure, and fainting which led to withdrawal of the subject from the study.

    a. **Procedure:** Muscle tissue will be obtained from each subject by needle biopsy of the vastus lateralis (outer thigh) by a study physician. Each subject will undergo the procedure five times. The first four will occur before and 72 hrs after a single bout of exercise completed before and after approximately 2-weeks of consuming the supplement or placebo. The final biopsy will occur 72 hrs after completion of the exercise training program. The procedure is performed after local anesthetic (lidocaine) is used to numb the site. A small incision will be made in the skin and a Bergstrom needle will be inserted briefly into the muscle to remove several pea-sized pieces of muscle (7). Direct pressure will be applied to stop the bleeding. The wound is closed with a band-aid type bandage and covered with a pressure bandage. The procedure will be performed according to an approved Muscle Biopsy SOP. The procedure details and outcomes will be documented using the Subject Procedure Note. Pre-exercise biopsies will be taken from the dominant leg while post-exercise biopsies will be taken from the non-dominant leg.

    b. **Benefit:** The procedure is of no benefit to the subject. The study will investigate the effects of the nutritional supplement on muscle and the response to exercise training and detraining so that this information can be used in the future to potentially improve muscle rehabilitation strategies. This information cannot be obtained by alternative procedures.

    c. **Risk:** The biopsy procedure may cause discomfort. Injection of the local anesthetic may sting. The discomfort of the biopsy needle insertion varies from painless to uncomfortable or painful. The site will be bruised and sore for a few days and will likely cause a small scar. Temporary numbness of the skin at the biopsy site can occur. Infection of the wound is rare. Other risks of the biopsy include an allergic reaction to the local anesthetic, feeling light-headed or fainting during the procedure, or that the wound resumes bleeding later in the day (1:100 cases each).

    d. **Minimization of Risk:** The medical history and physical screening process will be used by the study physician to exclude subjects that appear to be at increased risk for excessive bleeding or have a history of allergic reaction to lidocaine or have had significant problems with fainting. *The protocol standard recommendations are that prior to the muscle biopsies subjects should refrain from using certain products that can influence bleeding. Aspirin products and certain

supplements should not be used for 10 days prior to the procedure. Non-aspirin non-steroidal anti-inflammatory drugs such as ibuprofen should not be taken for 3 days. After biopsy, subjects will be instructed to refrain from use of these products for 24 hrs though Tylenol may be taken if needed. Subjects will be given a reminder list of the time-frame and products from which to refrain. Subjects will be given verbal and written instructions for caring for the wound and extra bandages. Subjects will be contacted later in the day of the procedure to confirm wellness. If the subject is not reachable, then a message will be left if possible and a second attempt will be made later. The written instructions will contain multiple 24-hr contact numbers for the PI and study physicians and describe circumstances under which the subject should contact the investigators immediately and/or seek emergency hospital treatment.

e. ***Physician judgement:** Should the requirement for abstinence from contraindicated medications and supplements not be adhered to by the subject prior to biopsy, the study physician may use clinical judgement to determine the potential for an impact on subject safety and the acceptability of undergoing muscle biopsy. These decisions will be made on a case by case basis considering the product consumed, time-frame of consumption, and other individual risk factors for bleeding possessed by the subject.

13. Blood Draw: Blood draws will be performed by a qualified study staff member or GRECC nurse.

a. **Procedure:** Blood will be drawn nine times over the course of the study from each subject. The blood will most likely be drawn from the antecubital vein (≤ 60ml or 2 ounces); however other sites may be used at the discretion of the person drawing blood and assent of the subject. Fasting is not a requirement.

b. **Benefit:** The purpose of the blood draws are for screening for subject eligibility and safety, and for determining the effects of the nutritional supplement on blood-based measures of immune function. If requested, subjects will be provided the results of their clinical blood tests which may provide an educational benefit.

c. **Risk:** Blood draw in the proposed amount is a minimal risk procedure. There is a risk of mild discomfort or pain or feelings of lightheadedness. Fainting is possible. The blood draw will likely cause a small bruise. There is also a small risk of infection.

d. **Minimization of Risk:** The risk of the blood draw will be minimized by the skill of the person drawing the blood. The person drawing blood will utilize standard aseptic technique and follow VA policy for hand hygiene and protective coverings. Sharps will be handled appropriately and disposed of properly. Snacks (e.g. peanut butter crackers and juice) are offered to subjects to minimize the feelings of light-headedness after blood draw and biopsy.

14. Other Human Subject Data Collected

a. During participation subjects will be asked to maintain their “normal” diet and to not make any conscious efforts to lose or gain weight. Weight will be measured at study entry and upon completion.

b. Use of Certain Medications: it is unknown how certain medications (specifically those used to control inflammation) and other age-associated medical issues will influence the variables of interest to the study. Due to this possibility, medications and medical problems will be recorded and used as potential confounding variables in the statistical or exploratory analyses.

15. Computed Tomography Scan of the Thigh

a. **Procedure:** Muscle Cross-Sectional Area and composition will be determined by the hospital imaging service using computed tomography (CT) scan of the mid-thigh at pre-training, post-training, and at the end of the study. The service performed CT scans for our previous study which showed that size increased (P=0.002, 7.4 ± 2.0%) in older adults after 12-weeks of

training [3]. Subjects are carefully positioned in the scanner and boney land marks are used to ensure precise mid-thigh location for each scan. Analysis of scans will be completed using a program such as Slice-O-matic (Montreal, Canada) to discern between bone, fat, and muscle.

CT scan data is transferred from the Imaging service to the study on a CD which is not encrypted per se. However, the risk of an unintended recipient accessing the data is particularly low because specialized software is required to view the data. Furthermore, the data is a cross-sectional image of the thigh which looks like a slice of ham (i.e. not sensitive). The information will be labeled with the subject study ID, not name or SSN. Transport of the disk from the LR to NLR VA will occur in a locked bag and the disks will be maintained according to CAVHS regulations.

b. **Benefit:** The CT scan of the thigh is of no direct benefit to the subject though the measure provides important scientific knowledge of the effects of exercise training, detraining, and nutritional supplementation in older adults.

c. **Risk:** The procedure will expose subjects to radiation. The total amount of radiation the subjects will be exposed to per scan (6 mSv) is approximately the same as the average exposure an individual has from natural sources per year. Having three scans in one year creates a very small increase (from 44.9% to 44.95%) in the risk of cancer for the average 70 year old male (www.xrayrisk.com).

d. **Minimization of Risk:** There are no exclusion criteria specific to this procedure. Risk is low in the study population as compared to younger populations and individuals who may become pregnant.

16. Data Safety and Monitoring Plan

a. The following plan has been adapted from "Guidelines for Data and Safety Monitoring for Clinical Trials Not Requiring Traditional Data Monitoring Committees" [55].

b. The study has eligibility criteria that have been precisely defined, as described above, to ensure enrollment of subjects who possess an appropriate risk/benefit ratio. The conservative inclusion and exclusion criteria were developed with input from multiple study physicians. The criteria select of individuals with relatively low risk of adverse events from the study procedures and relatively high probability of benefit from participation. These criteria are assessed and documented in the Medical History and Physical Form prior to active participation. Physician clearance to participate is documented on this form and CPRS.

c. Safety monitoring will be performed on a frequent and regular basis from enrollment until at least 30 days post-participation for each subject. Monitoring occurs by CPRS query and subject self-report or exam at each research visit as well as during telephone contacts. Thus, during participation monitoring per subject will occur at an average rate of more than once per week.

d. Monitoring will be proportional to the risk of the phase of the study for the individual. Thus, monitoring may occur four times per week per subject if biopsy and exercise are involved but less than once per week during post-training follow-up when only nutritional supplementation is involved.

e. Expected adverse events are well-defined as described above and in the informed consent document. All serious adverse events and unexpected adverse events possibly related to participation are also monitored for. The results of monitoring are documented on the submitted monitoring checklist as well as in a CPRS note. This form has both specific expected adverse events and open ended questions for unexpected symptoms as well as space for a detailed note to chart.

f. Appropriate action relative to the severity of the event is taken by the study team immediately when an event is identified. This may range from documentation by the coordinator (e.g. mild muscle soreness) to safety examination by the study physician (e.g. elevated blood pressure after exercise). This safety evaluation will include the measures necessary for the study physician to judge that the subject can safely continue participation, needs treatment, or should be withdrawn from the study.

g. The study procedures (exercise, supplementation, etc) can and will be halted at any time a concern for the subject is identified. Halted procedures will not resume until the concern has been alleviated. If concerns cannot be alleviated then the study physicians may judge to withdraw the subject, modify the protocol, suspend enrollment, or halt the study. This is a relatively small single site study which allows these decisions to be made in real-time as they occur rather after aggregate data collection after interim analysis.

h. All adverse events are brought to the attention of the principal investigator and, as needed to the study physicians. Adverse events will be reported to the IRB according to CAVHS policy.

i. The study has also stipulated that a member of the study team will not be blinded to subject randomization to supplement or placebo. This person will not be involved with assessment of any outcome measures but will be responsible for monitoring of subject product consumption compliance as it relates to the individual, the study, and the pharmacy.

j. The sample size is justified by power calculations presented in this protocol.

k. The study team members possess no conflicts of interest with regard to the study protocol and the procedures and products involved.

17. Data Handling and Recording Plan

a. The study team has undergone peer-review by the VA Rehabilitation R&D Service to determine that the team is composed of appropriately qualified individuals to supervise the overall conduct of the trial, to handle the data, to verify the data, to conduct the statistical analyses, and to prepare any study reports.

b. Data collected directly from the subject or their CPRS record is recorded in real-time directly onto the submitted data collection forms (e.g. medical history form, exercise training log, procedure note) by authorized members of the study team. Each of these forms has been submitted for IRB approval.

c. These forms are adapted from those used in previous studies and thus are validated for the ability to reliably capture the required data.

d. Data is transferred (recorded) from the data collection forms into an electronic database (Excel spreadsheet). Data entry is performed by a so delegated member of the study team. The process is performed in duplicate (i.e. double data entry) so that the data can be readily checked for content validity, completeness, accuracy, and consistency. Changes to the electronic database can be made if needed by the principal investigator or the study team member who entered the data.

e. The database variables will be determined by the principal investigator based on the study outcomes collected from or about the subjects per protocol. Changes to the database variable fields will only be made with approval from the principal investigator.

f. Data collection forms are maintained according to VA research data policy (i.e. indefinitely at this time). Thus, data will not be disposed of or deleted and can be referenced should a question arise from the electronic database.

g. The paper and electronic records containing HIPAA elements are only accessible to authorized members of the study team who are within the VA. This information is maintained behind double locks in the GRECC and on the limited access GRECC server. This server receives frequent backup to ensure safety of content.

h. Information, paper or electronic, containing HIPAA identifiers will not be shared with the UAMS members of the study team or other individuals outside the VA with the exception of potential communication between VA and non-VA physicians to ensure subject safety.

i. The electronic database is coded and does not contain HIPAA elements. The data is intentionally transferred without dates to meet this criterion. The cross-walk file will not leave the VA and will not be shared within the VA with any unauthorized individuals.

j. The study has assigned a member(s) of the study team to be not blinded. This person will monitor and record subject compliance and is the study point of contact initial questions concerning the supplement/placebo. The responsibility this study monitor is to safeguard the blinding from the other investigators, promote subject compliance, and maintain the integrity of the data.

k. Certain data elements are transformed during transfer from paper to electronic (e.g. birthdate to age, subject start and end date to time of participation). However, it is will always be possible if necessary to compare the original data and observations with the processed data.

l. The subject code used in the electronic database is an unambiguous random number that allows identification of all the data reported for each subject. The code is not related to any HIPAA element.

m. It is understood that CAVHS and the VA have ownership of all data.

n. The study will purchase the investigational products from a VA-authorized vendor and have them shipped directly to the VA pharmacy. The pharmacy will maintain accountability for receipt, storage, and dispense of these products as described in the clinical trial product information sheet. The pharmacy and study monitor will also maintain accountability for return of unused investigational products.

LABORATORY ASPECTS OF THE STUDY

Study Outcomes: The study will examine the effects of the nutritional supplement on measures of immune function and muscle health. The immune response to three types of stimulation will be measured: antibody response to vaccination, immune response in muscle and blood to a bout of exercise, and an in vitro response for immune cell proliferation. Muscle adaptation to training will be measured at the whole body (i.e. muscle cross-sectional area, strength, and function) and cellular levels. The blood and muscle tissue collected from the subjects at the described time points will be used for laboratory analyses of variables related to immune function and muscle adaptation to exercise training. The basis for the expectations that each measure will be affected by the ingredients of the nutritional supplement (β-hydroxy- β-methylbutyrate, arginine, and glutamine) and/or resistance exercise is provided.

The specific proposed method of measurement or measure of immune function (and associated hypothesis) in muscle or blood may change for practical and scientific reasons and not be considered a deviation so long as the measure: 1) addresses a molecular or cellular trait potentially impacted by aging and muscle health or the improvements of muscle health by supplementation or exercise; 2) is not genetic testing; 3) and the results will not be communicated back to the subjects or used to influence their medical care in any way.

BLOOD MEASURES

A. **Response to Vaccination:** β-hydroxy-β-methylbutyrate has been used in animal science to improve the response to vaccination [32]. The study will determine if 2-weeks of supplementation improves the response of older adults to the TDAP vaccine. The response to *pertussis* is of primary interest though the responses to the tetanus and diphtheria antigens will also be measured. Serum antibody titers will be compared between pre-vaccination and two time points post-vaccination. Enzyme-linked immunosorbent assays are available for measuring antibodies to each antigen (IBL International, Toronto, Ontario).

B. **T-Cell Subpopulations:** Glutamine and arginine may be conditionally essential amino acids in the contexts of aging and exercise that are needed for optimal T-Cell function [34;56]. Aging decreases the ratio of naïve helper (CD4+) to cytotoxic (CD8+) T-Cells [57]; and though the levels of both cell populations increase shortly after moderate exercise for older adults, their levels do not reach the pre-exercise levels of young adults [58]. The proposed study will determine if 2-weeks of supplementation increases the CD4+ to CD8+ ratio or increases the response to acute exercise. A regulatory t-cell subset (CD4+, CD25+, FoxP3+) will also be examined since these cells function as regulators of immune function that prevent an uncontrolled immune response or autoimmunity [59]. Assessment of T-cell subsets will be carried out by flow cytometry using fluorochrome-linked antibodies specific to each marker (BD Biosciences, San Jose, CA). Additionally, if an alteration in inflammatory status is indicated by the cytokine analysis, then the Th17 subset (CD4+, CCR6+, CD161+, RORα+) will be evaluated. This pro-inflammatory subset has not been studied following supplementation or exercise and the results could provide for the first time information on whether alteration in Th17 t-cells accompanies improvement in immune status.

C. **Cytokine and Chemokines:** Immune responses are either predominated by pro-inflammatory Th1 cytokines (e.g. IFN-γ, TNF-α, IL-2, and IL-17) or anti-inflammatory Th2 cytokines (e.g. IL-10 and IL-4) and chemokines depending on the nature of the stimulus. However, arginine and glutamine may be able to limit the production of these factors [36]. Since aging is thought to increase basal cytokine levels and impair the cytokine response to exercise [60], the study will determine if 2-weeks of supplementation decreases baseline cytokine levels and increases the response to acute exercise. Cytokines and chemokines will be measured using the multiplex Luminex array system (Bio-Rad, Hercules, CA) and confirmed using high sensitivity enzyme linked immunosorbent assays (R&D Systems, Minneapolis, MN).

D. **Immune Cell Proliferation:** In vitro models indicate that glutamine and arginine are important to immune cell proliferation [34;61]. Similarly, human trials of β-hydroxy- β-methylbutyrate, arginine, and glutamine have shown that monocytes, lymphocytes, and T-cells increase after treatment [27;46]. Proliferative capacity of lymphocytes also increases shortly after moderate exercise but the change is significant only in young and not older adults [58]. This study will determine if 2-weeks of supplementation increases the in vitro proliferative capacity of peripheral blood mononuclear cells at either baseline or after acute resistance exercise. After the in vivo effect of the supplement is determined, the results will be confirmed using the supplement or its individual ingredients to treat cells in vitro. Proliferation will be measured by flow cytometry based on uptake of a fluorescent dye (carboxyfluorescein succinimidyl ester) after treatment with phorbol ester and calcium ionophore as was used in Finding 5.

MUSCLE MEASURES

E. **Macrophages:** Evidence from animal models indicates that arginine and glutamine metabolism is involved with macrophage activation that mediates inflammation or anti-inflammation and growth [35;37]. Our preliminary results indicate that the majority of macrophages residing in muscle perform the latter of these two functions, i.e. anti-inflammation and growth. However, muscle of

older adults contains fewer macrophages and the numbers do not increase in response to acute exercise. The study will determine if 2-weeks of supplementation increases the number of macrophages present in muscle at baseline or in response to acute resistance exercise. Macrophages will be counted in muscle cross section by immunohistochemistry using antibodies to detect the CD163 marker (Cell Sciences, Canton, MA). Additional markers (CD68 and CD206) will be used to confirm the specificity of the CD163 marker to macrophages.

F. **Cytokines and Growth Factors:** Our preliminary results show that older muscle contains elevated basal levels of cytokines and diminished levels of growth factors. The effect of arginine and glutamine on cytokine expression is expected to be the same in tissue as it is systemically. The study will determine if 2-weeks of supplementation decreases baseline cytokine levels and increases the response to exercise. Since chronic inflammation is thought to dampen growth factor production [62], the study will determine if supplementation also increases growth factor levels at baseline and in response to exercise. IL1β, IL10, and IGF1 are of primary interest though the other cytokines, and growth and remodeling factors *including MuRF and MAFbx* which will also be measured. Gene expression will be measured at the mRNA level using total RNA extracted from whole muscle by quantitative real-time PCR. Assays will utilize SYBR green chemistry and standard curves as was used in Findings 1-4 (Figures 2-5).

G. **Nuclear Factor-kB (NFkB) and Phosphatidylinositol 3-Kinase Signaling (PI3K):** Direct assessment of protein and protein activity in human muscle has proven difficult for cytokines and growth factors including IGF1, one of the most commonly studied regulators of muscle mass. For this reason, the study will evaluate muscle for cytokine (NFkB) and growth factor (PI3K) activity by evaluating signal transduction pathways involved in regulation of muscle hypertrophy and atrophy [63]. The study will determine if 2-weeks of supplementation decreases NFkB signaling and increases PI3K signaling at baseline and in response to exercise. NFkB activity will be assessed by electrophoresis mobility shift assays to detect muscle nuclear protein binding to the NFkB DNA site as used in Figure 9A or as follows for PI3K. PI3K activity will be assessed by Western Blot based phosphorylation assays (Millipore, Billerica, MA).

H. **Additional Signaling Pathway Members:** The regulation of inflammation and growth in macrophages and muscle cells is regulated by a complex network of signaling molecules. Thus, in addition to NFkB and PI3K, other factors will also be measured for effects of supplementation and exercise [24;25;64]. Of main interest are FOXO1, STAT3, AKT, S6K, and ERK1 and 2. The activity of these factors is regulated, in part, by their phosphorylation state. Matched antibody pairs are available to measure the ratio of these phosphorylated to un-phosphorylated proteins by Western Blot (Cell Signaling Technology, Boston, MA). Pathway scanning kits are also available which include other related molecules in the cascade. Muscle remains from the nutritional supplement pilot study presented in the introduction. This muscle will enable an initial broad assessment of the signaling pathways affected by nutritional supplementation. The results will guide any changes in the specific factors chosen for assay in muscle collected for the proposed study.

I. **Satellite Cells and Myonuclei:** Muscle adapts to resistance training through increases in satellite cell numbers and addition of nuclei to the myofiber [21]. However, these adaptations are diminished in older adults [22]. The study will determine if 15-weeks of supplementation increases the number of satellite cells and myonuclei per myofiber in muscle. Satellite cells (Pax7+, CD56+, DAPI+) and myonuclei (Dystrophin plus DAPI staining) will be counted using established staining methods [21].

J. **Fiber Size:** Muscle also adapts to resistance training by muscle fiber hypertrophy. However, this growth is hindered in individuals, particularly older adults, who have a diminished satellite cell and myonuclei response to training [22]. The study will determine if 15-weeks of supplementation increases the effects of resistance training on myofiber size. Changes in fiber size will be

measured microscopically as the average percent increase in fiber cross-sectional area from baseline to post-training.

STATISTICAL PLAN: The sample size (N=50) provides greater than 80% power for Aims 1-3 to detect mean differences between groups of 0.8 to 0.85 standard deviation (SD) units at a 5% level of significance. Power calculations were based on using a one-sided t-test to compare independent groups using data from our work and others [2;23;65] though non-parametric tests or transformations will be used as necessary for data containing outliers. There is no formal plan to adjust for multiple comparisons. However, multiple correlation analysis will be used to determine the correlation between endpoints and any possible inflation in the overall Type I error rate. This analysis will allow testing of the overall hypothesis that measures of immune function (Aim 1) will be positively correlated with adaptations to exercise (Aim 2) and retention of muscle strength, mass, and function after exercise training is halted (Aim 3). The hypotheses are stated for each aim and the time points for comparison are listed in Table 6.

Table 6. Time points are lettered for clear reference by the stated hypotheses.

BASELINE MEASURES (WEEKS 1-5)
A. Blood draw at study enrollment for measuring pre-vaccination antibody titers
B. Assessment of muscle strength, mass, and function at baseline
C. Blood drawn prior to acute exercise at baseline
D. Muscle biopsy prior to acute exercise at baseline
E. Blood drawn 1 hr after acute exercise at baseline
F. Muscle biopsy 72 hrs after acute exercise at baseline
POST-SUPPLEMENT RUN-IN MEASURES (WEEK 8)
G. Blood drawn prior to acute exercise after 2-weeks of supplementation
H. Muscle biopsy prior to acute exercise after 2-weeks of supplementation
I. Blood drawn 1 hr after acute exercise after 2-weeks of supplementation
J. Muscle biopsy 72 hrs after acute exercise after 2-weeks of supplementation
MEASURES DURING TRAINING PERIOD (WEEKS 9-20)
K. Blood draws at weeks 1 and 2 post-vaccination for measuring antibody response
POST-TRAINING MEASURES (WEEK 21)
L. Assessment of muscle strength, mass, and function after completion of training
M. Muscle biopsy 72 hrs after final exercise bout of training
MEASURES DURING POST-TRAINING FOLLOW-UP (WEEKS 22-4)
N. Assessments of strength, mass, and function after 16 and 26 weeks of detraining

Aim 1 Power: Aim 1 will determine if the supplement consumption for 2-weeks improves baseline measures of immune function or the immune responses to vaccination or acute exercise. The representative measures chosen to calculate power are change in muscle macrophage content after exercise (Finding 2) and change in CRP levels post-supplementation (Pilot Study Finding 1). Przybyla et al detected a 29% increase in CD163+ macrophages in young subjects after exercise but no change in older subjects [2]. Power for this aim is calculated to detect at least a 29% greater increase in macrophages in the supplement than the placebo group. Przybyla et al used a nonparametric test to lessen the effects of outliers. To be more conservative here, the calculations here dropped the most extreme high and low data values before estimating effect sizes. With that approach, the 29% increase corresponds to an effect size of 0.8 SD units (mean/SD = 0.128/0.159). Similar results were found using CRP data from Pilot Finding 1. The effect size for change in CRP is 0.85 SD units (mean/SD = 0.665/0.780 for log-transformed data). Having at least 20 subjects per group will provide 80% or greater power to detect a mean shift of 0.8-0.85 SD units at a significance level of 5% for the following Aim 1 hypotheses:

a. The response to vaccination will be greater in the supplement than the placebo group. The change between times K and A (Table 6) will be compared between groups.

b. The resting ratio of CD4+ to CD8+ t-cells will increase in the supplement but not in the placebo group. The change between times G and C will be compared between groups.

c. The change in t-cell subsets after acute exercise will be greater in the supplement than the placebo group. The change between times I and G will be compared to the change between times E and C for both groups.

d. Resting cytokine levels will decrease in the supplement but not in the placebo group. Change will be compared between groups for blood (times G and C) and muscle (times H and D). *Similar results are predicted for the MuRF and MAFbx atrophy-related transcripts.*

e. The increase in cytokine levels after acute exercise will be greater in the placebo group than the supplement group. For both groups, change between times I and G will be compared to the change

between E and C for blood, and for muscle the change between times J and H will be compared to the change between times F and D.

f. The proliferative capacity of peripheral blood mononuclear cells will be greater in the supplement than the placebo group. The change between times G and C will be compared between groups. The change between times I and E will also be compared between groups.

g. The resting number of muscle CD163+ macrophages will increase in the supplement but not in the placebo group. The change between times H and D will be compared between groups.

h. The number of muscle CD163+ macrophages will increase after acute exercise in the supplement but not in the placebo group. The change between times J and H will be compared to change between times F and D for both groups.

i. Resting NFkB activity will decrease and PI3K activity will increase in the supplement group but not in the placebo group. The change between time points H and D will be compared between groups.

j. In response to acute exercise, NFkB activity will be lower and PI3K activity will be greater in the supplement group than in the placebo group. The change between times J and H will be compared to change between times F and D for both groups. *Responses similar to NFkB are predicted for FOXO1, and responses similar to PI3K are expected for STAT3, AKT, P38 MAPK, and ERK 1 and 2.*

Aim 2 Power: Aim 2 will determine whether supplement consumption increases muscle adaptation to 12-weeks of resistance training. The representative response measure for this objective is post-training percent increase in muscle strength. In a similar study, Trappe et al sought to improve strength gain by resistance training plus drug treatment. The drug treatment group gained significantly more strength by 25.1% than the group receiving placebo (26.4% vs. 21.1%, $P<0.05$) [23]. The previous findings presented here (Finding 4) indicate that older subjects completing a 12-week training program but not taking supplements will experience an average strength gain of 25 ± 8%. If treatment with the proposed supplement improves strength similarly to the Trappe drug treatment, subjects in the supplement group will improve strength by an average of 31% (i.e., 25.1% greater than those of the placebo group). This translates to an approximate effect size of 0.8 SD units (0.25*0.251/0.08) if variances are pooled for the groups compared. With 20 subjects per group, there will be 80% power to detect a mean shift of 0.8 SD units using a significance level of 5% for the Aim 2 hypotheses:

a. The amount of strength gained from 12-weeks of training will be greater in the supplement than the placebo group. The change between times L and B will be compared between groups.

b. The amount of muscle size gained from 12-weeks of training will be greater in the supplement than the placebo group. The change between times L and B will be compared between groups.

c. The increase in muscle function after 12-weeks of training will be greater in the supplement than the placebo group. The change between times L and B will be compared between groups.

d. The increase in muscle satellite cells after 12-weeks of training will be greater in the supplement than the placebo group. The change between times M and D will be compared between groups.

e. The increase in muscle myonuclei after 12-weeks of training will be greater in the supplement than the placebo group. The change between times M and D will be compared between groups.

f. The increase in muscle fiber size after 12-weeks of training will be greater in the supplement group than the placebo group. The change between times M and D will be compared between groups.

Aim 3 Power: Aim 3 will determine if supplement consumption increases the retention of exercise-derived benefits post-training during 26 weeks of long-term follow-up. The representative response measure for this objective is the strength gain retained during long-term follow-up after exercise training is completed. Lemmer et al observed an average gain in muscle strength of 27% ($P<0.01$) in older men after strength training which is similar the 25% gain presented here in Finding 4 [65]. However, at

31-weeks of post-training follow-up, strength had decreased to be about 9% above pre-training levels. Assuming the same level of variation among our subjects in retention of strength gain as the observed 8% SD for strength gain in Finding 4, then a 0.8 SD effect size would be about 6.4% (0.8*0.08), translating to our supplement group retaining strength of 12.4% above pre-training levels. With 20 subjects per group, there will be 80% power to detect a mean shift of 0.8 SD units using a significance level of 5%. If the SD for retention of strength is smaller than 8%, then we will have higher power to detect the same difference or equal power to detect smaller differences for the following Aim 3 hypotheses:

a. The change in strength during the detraining period will be smaller in the supplement than the placebo group. The change between times N and L will be compared between groups.
b. The change in muscle size during the detraining period will be smaller in the supplement than the placebo group. The change between times N and L will be compared between groups.
c. The change in muscle function will be smaller in the supplement than the placebo group. The change between times N and L will be compared between groups.

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