CIRCRES/2022/321094D/R2

The Amino Acid Homoarginine Inhibits Atherogenesis by Modulating T-Cell Function

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Short title: Homoarginine Reduces Atherosclerosis

Subject Terms: Atherosclerosis, Basic Science Research, Inflammation

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Subject codes: Atherosclerosis, inflammation, animal models of human disease, basic science research

Abstract

Background: Amino acid metabolism is crucial for inflammatory processes during atherogenesis. The endogenous amino acid homoarginine (HA) is a robust biomarker for cardiovascular outcome and mortality with high levels being protective. However, the underlying mechanisms remain elusive. We investigated the effect of HA-supplementation on atherosclerotic plaque development with a particular focus on inflammation.

Methods: Female *apolipoprotein (Apo) E*-deficient mice were supplemented with HA (14 mg/L) in drinking water starting two weeks before and continuing throughout a six week period of Western-type diet feeding. Control mice received normal drinking water. Immunohistochemistry and flow cytometry were used for plaque- and immunological phenotyping. T cells were characterized using mass spectrometry-based proteomics, by functional *in vitro* approaches, e.g., proliferation and migration/chemotaxis assays as well as by super-resolution microscopy.

Results: HA-supplementation led to a 2-fold increase in circulating HA concentrations. HA-treated mice exhibited reduced atherosclerosis in the aortic root and brachiocephalic trunk. A substantial decrease in $CD3^+$ T cells in the atherosclerotic lesions suggested a T cell-related effect of HA-supplementation, which was mainly attributed to $CD4^+$ T cells. Macrophages, dendritic cells, and B cells were not affected. $CD4^+$ T-cell proteomics and subsequent pathway analysis together with *in vitro* studies demonstrated that HA profoundly modulated the spatial organization of the T-cell actin cytoskeleton and increased filopodia formation via inhibition of Myh9. Further mechanistic studies revealed an inhibition of T-cell proliferation as well as a striking impairment of the migratory capacities of T cells in response to relevant chemokines by HA, all of which likely contribute to its atheroprotective effects.

Conclusion: Our study unravels a novel mechanism by which the amino acid HA reduces atherosclerosis, establishing that HA modulates the T-cell cytoskeleton and thereby mitigates T-cell functions important during atherogenesis. These findings provide a molecular explanation for the beneficial effects of HA in atherosclerotic cardiovascular disease.

Non-standard Abbreviations and Acronyms:

Arg – L-arginine
CCL19 – C-C motif chemokine ligand 19
CCL21 – C-C motif chemokine ligand 21
CCR7 – C-C motif chemokine receptor 7
CXCL10 – C-X-C motif chemokine ligand 19
CXCR3 – C-X-C motif chemokine receptor 3
EM – Effector memory T cells
eNOS - Endothelial nitric oxide synthase
FCA – Fibrous cap atheroma
HA – Homoarginine
LDLr – Low density lipoprotein receptor
MLC2 – Myosin light chain 2
MS – Mass spectrometry
Myh9 – Myosin heavy chain 9
PIT – Pathological intimal thickening
RLC – Regulatory light chain
STED – Stimulated emission depletion

Introduction

Atherosclerosis is a dyslipidemia-driven, chronic inflammatory disease that involves the interplay of various immune cells and immunological processes.[1] In humans, the *Canakinumab Anti-Inflammatory Thrombosis Outcome Study* (CANTOS) trial[2], the *COLchicine Cardiovascular Outcomes Trial* (COLCOT)[3] and *Low Dose Colchicine for secondary prevention of cardiovascular disease* (LoDoCo2)[4] demonstrated that regulation of immunological processes on top of lipid-lowering therapy, e.g., statins or PCSK (proprotein convertase subtilisin/kexin type)-9 inhibitors, reduces cardiovascular (CV) events emphasizing the immune system's critical role in atherosclerosis. Moreover, the negative results of the *Cardiovascular Inflammation Reduction Trial* (CIRT) established that atherosclerosis-specific inflammatory pathways must be targeted considering low-dose methotrexate therapy had no effect on CV outcomes.[5] Although the CANTOS, COLCOT and LoDoCo2 trials underline the clinical importance of targeting inflammation in atherosclerosis, treatment with the IL-1ß antibody canakinumab or colchicine did not improve overall mortality. Moreover, canakinumab-treatment led to increased numbers of infections.[2] Thus, novel therapeutic options to specifically target the inflammatory response during atherogenesis are urgently needed. Recent experimental data showed that, besides the innate immune system, adaptive immunity also plays a crucial role in the progression of atherosclerosis.[6,7] Corroborating this data, single-cell immune landscaping of human atherosclerotic plaques revealed that plaques from symptomatic patients had a distinct subset of $CD4^+$ T cells and T cells that were differently activated and differentiated as compared to asymptomatic patients.[8] Adaptive immune cells, specifically T cells, may therefore have a high potential to be used as immunotherapeutic target for atherosclerosis.

The endogenous amino acid homoarginine (HA) has been identified as a strong biomarker with low circulating levels associated with increased risk of CVD.[9-11] Data from the population-based Dallas Heart Study provided further insights by revealing an inverse association of plasma HA with subclinical vascular disease such as aortic wall thickness and plaque burden.[12] Recently, we identified oral HA-supplementation to be curative in murine models of acute ischemic stroke[13] and chronic heart failure[14,15] suggesting a direct beneficial effect of HA on CV outcomes. A *first in man* study has already proven that HA-supplementation is safe and well tolerated in humans.[16] Accordingly, a randomized clinical trial was recently initiated to study HA-supplementation in patients suffering from acute ischemic stroke (NCT03692234). Although supplementation of this amino acid seems to promote CV health, the underlying mechanisms remain elusive. Considering that

atherosclerosis is the common cause of CVD, including its clinical manifestations such as acute ischemic stroke or post-myocardial infarction heart failure, we hypothesized that HA-supplementation protects against atherosclerosis.

In the current study, we sought to investigate the effect of HA-supplementation on atherosclerotic plaque development and to identify key mechanisms by which HA exerts its effects. Here, we show that HA-supplementation reduces atherosclerotic plaque development via modulation of $CD4^+$ T cells. HA binds to Myh9, inhibits regulatory light chain phosphorylation, and profoundly modulates $CD4^+$ T-cell actin polarization, which in turn impairs actin-dependent functions such as T-cell activation, mobility, migration and proliferation, explaining reduced plaque burden. These findings provide a mechanistic explanation for the beneficial effects of the amino acid HA on atherosclerotic CVD.

Methods

Data Availability.

All data are presented in the text and the supplement. Data are available from the corresponding author upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[38] partner repository with the dataset identifier PXD023960.

Please see detailed Methods and Material and the Major Resources Table in the Supplemental Materials.

Results

Homoarginine-supplementation reduces atherosclerosis

To dissect the mechanisms underlying the beneficial effect of HA on CVD, we set out to directly assess HA-supplementation in a pre-clinical atherosclerosis model. Applying the established HA dosage regime[13,14], *apolipoprotein (Apo) E*-deficient mice were supplemented with HA (14 mg/L) in drinking water starting two weeks before and continuing throughout a six week-course of Western-type diet (HA-treated) whereas control mice received normal drinking water. Liquid chromatography-tandem mass spectrometry measurements confirmed that the dosage applied led to a 2-fold increase of HA levels in plasma and spleen of HA-treated mice (Figure 1A and Supplemental Figure 1A) without altering other related amino acids, such as L-arginine (Arg, Supplemental Figure 1A-B). No significant differences were observed in body weight, basic hematologic parameters or lipid levels and composition

(Supplemental Table S1 and Supplemental Figure 1C) between HA-treated and control animals. Strikingly, HA-supplementation resulted in a 24% reduction of atherosclerotic lesion volume in the aortic root (Figure 1B). Lesion size in the brachiocephalic trunk was also significantly reduced (Supplemental Figure 2A). The content of $Mac3^+$ macrophages (Figure 1D) and α-SMA^+ smooth muscle cells (Figure 1E) were not significantly altered in plaques of HA-treated mice. Detailed plaque phenotyping using the Virmani classification[17] revealed that HA-supplementation delayed the progression of atherosclerosis as HA-treated mice displayed a shift towards earlier stages, i.e. pathological intimal thickening (PIT), and a decrease in more advanced fibrous cap atheroma (FCA) as compared to control mice (Figure 1C). Further analysis of cellular composition of atherosclerotic plaques revealed a significant decrease of lesional $CD3^+$ T cells (Figure 1F), which could be mainly attributed to a decrease in $CD4^+$ T cells (Supplemental Figure 2B), suggesting a T cell-related effect of HA-supplementation.

Homoarginine modulates T-cell biology

In-depth analysis demonstrated that HA-supplementation indeed profoundly modulates T-cell biology. Corroborating our observation in the atherosclerotic lesion, we observed a decrease in splenic $CD3^+$ T cells (Figure 2A) which was attributed to a decrease in $CD4^+$, and not $CD8^+$ T cells (Figure 2B and Supplemental Figure 2B-E). This decrease in $CD4^+$ T cells could be accounted for by a decrease in effector memory (EM) T cells (Figure 2C).

Mechanistically, the reduced abundance of EM $CD4^+$ T cells in HA-treated mice was likely due to impaired proliferation, as reflected by their decreased Ki67 and 7-AAD levels in the spleen (Figure 2D-E). These data were confirmed *in vitro*, where splenic $CD4^+$ T cells isolated from HA-treated mice, that were stimulated with CD3/CD28 antibodies showed reduced proliferation after 72 hours (Figure 2F). Apoptosis assessment by flow cytometry and immunohistochemistry unraveled a moderate decrease in apoptotic (Annexin V^+) $CD4^+$ T cells in the spleen (Supplemental Figure 3A) and no statistical difference in the number of apoptotic cells in the lesions (Supplemental Figure 3B), supporting our hypothesis that the observed reduction in $CD4^+$ T cells is mediated by impaired proliferation rather than by increased cell death. Since T cells develop and mature in the thymus, we also investigated a potential effect of HA on thymic development. Flow cytometry-based analysis of the CD4/CD8 double-negative (DN) fractions revealed no statistical differences in DN1, DN2, DN3 and DN4 subsets in atherosclerotic HA-treated mice as compared to controls (Supplemental Figure 3C).

In accordance with the impairment in T-cell proliferation upon HA-treatment, we observed a significant reduction in CD25 expression on $CD4^+$ T cells, which is considered a marker of T-cell activation (Supplemental Figure 4A). Moreover, transcripts of activation markers, including C-X-C motif chemokine receptor 3 (CXCR3), its ligand C-X-C motif chemokine ligand 10 (CXCL10) as well as expression of the cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) showed reduced expression in the spleen of HA-treated atherosclerotic mice as compared to controls (Figure 3A). In line, we detected a decrease of the pro-inflammatory cytokines IFN-γ and interleukin (IL)-12p70 in plasma of HA-treated mice (Figure 3B) as well as in the supernatant of *in vitro*-cultured and CD3/CD28-activated splenic $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice (Figure 3C). As lower activation may lead to changes in T-cell polarization, we investigated whether HA-supplementation affects T-cell polarization and observed no statistical changes in T-cell subpopulations (T helper (h) 1, Th2, Th17 and regulatory T cells (Tregs)) isolated from spleens from atherosclerotic HA-treated mice and controls (Supplemental Figure 4B). Since CD25 is also a phenotypic marker for Tregs, we specifically studied whether the observed reduction in CD25 expression on $CD4^+$ T cells (Supplemental Figure 4A) may reflect a potential effect of HA-treatment on Treg functionality. Although CD25 expression on splenic Tregs was slightly lower in the HA-treated animals, the fractions of $CD25^+$ vs. $CD25^-$ Tregs were similar (Supplemental Figure 4C). Also, the expression of the Treg transcription factor Helios, which is proposed to be a marker of thymic derived Tregs and thus allow to decipher specific effects on thymic ($Helios^+$) and peripheral-induced ($Helios^-$) Tregs, was not significantly affected upon HA-treatment (Supplemental Figure 4D). We further performed a Treg suppression assay using Tregs and CFSE-labelled conventional responder $CD4^+$ T cells both isolated from spleens of HA-treated mice and controls. Suppressive capacities of HA-treated and control Tregs were similar (Supplemental Figure 4E). Notably, in accordance with our previous findings (Figure 2D-F), conventional $CD4^+$ T cells from HA-treated mice showed diminished proliferative capacity as compared to controls, suggesting that the beneficial effect of HA on atherogenesis is mediated by impaired proliferation and not by Treg-induced suppression of T-cell activation.

Homoarginine does not affect macrophage-, dendritic cell-, or B-cell biology

Since atherogenesis is orchestrated by various immune cells, we further investigated potential effects of HA on antigen presenting cells (APCs), specifically macrophages, dendritic cells (DC), and B cells. As described previously, lesional macrophages showed no statistical

difference related to HA-treatment. Likewise, flow cytometric analysis of F4/80$^+$ macrophages in the spleen did not show significant differences in number and activation between HA-treated and control mice (Supplemental Figure 5A). Further phenotyping of the various macrophage subsets (M1-type for CD38 expression, M2-type for CD206 expression and foam cells for CD36 (class-B Scavenger receptor) expression) also revealed no statistical changes on macrophage polarization by HA (Supplemental Figure 5B). Since we observed a moderate decrease in apoptotic CD4$^+$ T cells (Supplemental Figure 3A-B), we also elucidated a potential effect of HA-treatment on efferocytosis, but the percentage of engulfed apoptotic cells was similar between bone marrow-derived macrophages isolated from HA-treated and control mice (Supplemental Figure 5C).

A detailed look into DCs showed no statistical difference in the total number of splenic conventional DCs (CD11c$^+$, MHC-II^{high} cells) between HA-treated and control mice (Supplemental Figure 5D). Likewise, no statistical difference on the maturation of bone marrow-derived DCs (BMDC) was detected (Supplemental Figure 5E). Finally, we sought to investigate whether the effect on T cells *in vivo* is direct or indirect through interactions with APCs. Therefore, we performed a DC/OTII T cell co-culture experiment using HA-treated OTII-transgenic mice. DCs were loaded with the ovalbumin (OVA) peptide and co-cultured with naïve CFSE-labelled HA-OTII CD4$^+$ T cells. In contrast to HA's effect on antigen-independent T-cell proliferation (Figure 2F), antigen-dependent T-cell proliferation was not significantly affected by HA-treatment (Supplemental Figure 5F).

Using a flow cytometry-based approach we further determined the proportion of absolute counts of B cells in spleen as well as germinal center-positive B cells, but no statistical differences were detected between HA-treated and control mice (Supplemental Figure 5G-H). To investigate a potential effect of HA-treatment on B-cell responses, we performed an immunoglobulin ELISA. Corroboratively, circulating levels of IgG, IgM, and IgE were not affected by HA-treatment (Supplemental Figure 5I). Accordingly, *Rag1*-deficient *ApoE*$^{-/-}$ mice, lacking T and B cells, did not show any improvement of atherosclerotic disease after HA-treatment (Supplemental Figure 6 and Supplemental Table 3). Altogether, these data suggest a direct, antigen-independent effect of the applied HA dosage regime on CD4$^+$ T cells.

Homoarginine interferes with the actin cytoskeleton in T cells

To further elucidate the molecular function of HA in T cells, we tested whether HA is incorporated into CD4$^+$ T cells. Using stable isotope labelled [$^{13}C_7{}^{15}N_4$]-HA, we observed a

concentration-dependent accumulation of [$^{13}C_7{}^{15}N_4$]-HA in $CD4^+$ T cells (Supplemental Figure 7A), most likely via the SLC7A amino acid transporters[18], suggesting a potential intracellular mode of action of HA. Hence, we performed mass spectrometry (MS)–based proteomics on $CD4^+$ T cells directly isolated from spleens, lymph nodes and blood of atherosclerotic HA-treated and control mice. Gene ontology analysis identified various pathways that were linked to the modulation of T-cell signaling through the actin cytoskeleton (Figure 3D, Supplemental Figure 7B and Supplemental Table 4). Following this proteomic outcome, we stained splenic $CD4^+$ T cells from atherosclerotic HA-treated and control mice that were cultured for 72 hours including CD3/CD28 activation with fluorescent phalloidin to label the actin cytoskeleton. Subsequent 3D stimulated emission-depletion (STED) optical nanoscopy unveiled a striking effect of HA-treatment on the spatial actin organization in $CD4^+$ T cells (Figure 4). Specifically, we observed an enrichment of actin surface/volume ratio (Figure 4A) indicating a general expansion of the actin cytoskeleton after HA-treatment. $CD4^+$ T cells from HA-treated mice showed profound alterations in cytoskeleton morphology, namely irregular shapes as compared to native "spherical" appearance of cells from control mice including a profound increase in filopodia formation (Figure 4B). Further computational analysis showed that $CD4^+$ T cells from HA-treated mice had an overall different spatial orientation (Figure 4C).

Taken together, these data suggest that HA modulates $CD4^+$ T-cell biology leading to a loss of specific actin polarization inside the cells, which in turn might impede effective actin functions in T-cell activation, movement and proliferation.

Homoarginine regulates T-cell migration

To relate this remodeling in actin cytoskeleton organization to functional activity, we studied whether HA affects T-cell migration. We investigated the effect of 5 days *in vitro* HA-treatment on the migratory behavior of Jurkat T cells towards the chemokine C-X-C motif chemokine ligand CXCL10 using a transwell migration assay and found a significant reduction in migratory capacity of HA-treated cells as compared to control cells (Figure 5A). To relate this finding to a primary cell culture setting, we also used CD3/CD28-activated murine $CD4^+$ T cells isolated from spleens of *ApoE*$^{-/-}$ mice and again observed that 5 days *in vitro* HA-treatment profoundly inhibited T-cell migration towards the chemokines CXCL10 and C-C motif chemokine ligand CCL19 (Figure 5B). Finally, we combined an Ibidi chamber-based migration assay with confocal microscopy to further characterize and track the migration of CD3/CD28-activated and fluorescent labelled $CD4^+$ T cells isolated from

atherosclerotic HA-treated and control mice induced by the chemokine CCL19. Track-plots of cells showed substantial differences in 2D tracks between the two groups (Figure 5C). Quantification of track lengths revealed that cells from HA-treated mice exhibited much shorter tracks (Figure 5D), indicating an impairment in mobility and consequently in migratory capabilities. Corroborating this data, we found a downregulation of the homeostatic chemokines CCL19 and CCL21 and their common receptor CCR7 in spleens of HA-treated mice (Figure 5E), which was attributed to T cells, since the counterparts of T cells, such as DCs were not affected by HA-treatment (Supplemental Figure 5). The effect of HA on T-cell mobility could also affect subsequent T-cell functions and may be ascribed to a direct modification of the actin cytoskeleton.

Homoarginine inhibits Myh9

To further investigate how HA-treatment impacts the actin cytoskeleton in $CD4^+$ T cells and subsequent T-cell function, we evaluated promising targets identified by the previous proteomics experiments (Figure 3D, Supplemental Figure 7B and Supplemental Table 4). Specifically, we examined the actin-binding protein Myh9 (Myosin heavy chain 9, non-muscle myosin heavy chain IIa), which is known to play a role in cytoskeleton organization and lamellopodia formation.[19,20]

First, we performed *in silico* molecular docking and dynamics simulations of Myh9-ligand complexes. Docking of HA with mouse Myh9 showed that HA, Arg and the well-characterized Myh9 inhibitor blebbistatin[21] bind at the same pocket of Myh9. Since HA and Arg are quite flexible, two different binding modes were selected for further analysis (Figure 6A). Binding free energy (BFE) of HA was even lower than that of blebbistatin, indicating that HA – in contrast to Arg which showed higher binding free energy – interferes with Myh9 to explain the profound effects of HA on the actin cytoskeleton and in turn T-cell function. Likewise, HA's inhibitory effect on $CD4^+$ T-cell migration was more pronounced than blebbistatin's, and no additive effect of HA and blebbistatin was observed (Figure 6B), indicating that HA acts in a mode resembling that of blebbistatin.

Non-muscle myosin IIa (Myh9) comprises of two heavy chains, two essential light chains and two regulatory light chains (RLC or MLC). MLC phosphorylation is well-established as regulatory mechanism and correlates with myosin activity.[22] We studied the effect of HA-treatment on MLC2 phosphorylation in our *in vivo* model using $CD4^+$ T cells isolated from

atherosclerotic HA-treated and control mice and observed a reduction in phosphorylation events on Serine (Ser)-19 as revealed by western blot (Figure 6C) and on Threonine (Thr)-18/Ser-19 identified by immunofluorescence microscopy (Figure 6D). Altogether, our data suggest that HA binds to Myh9, reduces MLC phosphorylation and thus likely Myh9 activity.

Discussion

Our study unravels an unexpected mechanism by which the amino acid HA reduces atherosclerosis, i.e., via the regulation of $CD4^+$ T cells. Specifically, we show that HA-supplementation modulates the spatial organization of the actin cytoskeleton in $CD4^+$ T-cells, impeding T-cell mobility, migration, activation and proliferation via inhibition of Myh9. This was associated with a reduction of effector $CD4^+$ T cells leading to the attenuation of atherosclerosis.

During the past decade a large body of epidemiological and clinical evidence accumulated that circulating HA is a strong biomarker for CV outcome and mortality with high levels being protective.[9-11,23] Previous experimental mouse studies have shown that the supplementation of 14 mg/L HA via the drinking water reduces stroke size in a model of middle cerebral artery occlusion[13] and preserves cardiac function in a model of post-ischemic myocardial infarction (MI).[14] In humans, HA is related to atherosclerotic CVD, as there is an inverse association of plasma HA with subclinical measures of atherosclerosis such as aortic wall thickness and plaque burden.[12]

Although various hypotheses had been postulated and pursued, the underlying mechanism has remained elusive. Due to its similarity to the amino acid Arg, HA was suggested to serve as an alternative substrate for endothelial nitric oxide synthase (eNOS) leading to an increase in protective NO.[24-26] However, using stable isotope labelled [$^{15}N_4{}^{13}C_7$]-HA and [$^{15}N_2$]-Arg for oral HA- and Arg-supplementation, we did not observe an accumulation of urinary [^{15}N]-nitrate (Supplemental Figure 7D), suggesting that our dosage regime of 14 mg/L HA via drinking water has no effect on *in vivo* NO production by NOS and that other mechanisms are in place.

In the present paper, we found that HA-supplementation inhibited $CD4^+$ T-cell proliferation and restrained T-cell activation. Although single cell sequencing and/or CyTOF technologies might have uncovered further potential effects of HA-treatment on T-cell subpopulations, we can safely conclude that HA-supplementation affects the antigen-independent proliferation

capacity of $CD4^+$ T cells, independent of T-cell life-span. We identified a profound effect of HA on the migratory behavior of $CD4^+$ T cells. In both *in vitro* and *ex vivo* experimental settings, HA inhibited responses to chemotactic gradients, such as CXCL10 and CCL19 for murine $CD4^+$ T cells. Accordingly, HA-treated mice also showed a downregulation of the T-cell homing machinery, as reflected by reduced expression of the homeostatic chemokines CCL19 and CCL21 and their common receptor CCR7. A previous study reported that *LDLr*-deficient mice with systemic CCR7-deficency exhibited less atherosclerosis. This study further observed a reduced migration of $Ccr7^{-/-}$ T cells into the inflamed aorta compared with $Ccr7^{+/+}$ T cells, indicating that entry of T cells into the vascular wall during atherogenesis require CCR7.[27] These data further corroborate a favorable effect of CCL19/CCL21/CCR7 downregulation on plaque burden observed in our HA-treated mice.

On the other hand, another study also described the regulation of the CCL19/CCL21/CCR7 machinery in atherosclerosis and reported that CCL19 and CCL21 expression is upregulated within atherosclerotic lesions of $ApoE^{-/-}$ mice as well as in human atherosclerotic carotid plaques. While CCL19 and CCL21 were upregulated, CCR7 expression was found to be reduced in the periphery, suggesting – in contrast to our findings – that CCR7 and its ligands CCL19 and CCL21 are differentially regulated.[28] In line with this, another study found that genetic deletion of *Ccr7* in $ApoE^{-/-}$ mice exacerbates atherosclerosis by increasing T-cell migration and accumulation in atherosclerotic lesions,[29] which – at first sight – seems to contradict our findings. In this context, however, it is noteworthy that HA-treatment of $ApoE^{-/-}$ mice also led to a decrease in *Cxcr3* expression and CXCL10-induced migration, both of which are primarily hallmarks of activated $CD4^+$ T cells. This indicates that HA-treatment can affect both CCR7-dependent migration (e.g. of naïve T cells) as well as CCR7-independent migration (e.g. of activated T cells), thus reconciling our findings with these studies.

In line with the impairment in migration, we discovered that HA expanded the actin cytoskeleton within T cells, leading to extensive filopodia formation. Regulation of actin dynamics is important for $CD4^+$ T-cell development and homeostasis, activation, mobility, migration, and effector function[30], and in our study, HA-induced dysregulation of actin dynamics resulted in a decrease in $CD4^+$ effector cells, impaired T-cell proliferation and migration, resulting in beneficial effects on atherosclerosis.

The current findings clearly warrant further investigation into the molecular mechanism, by which HA modulates the actin cytoskeleton, especially as a direct effect of HA on actin (de-) polymerization could be excluded (Supplemental Figure 7C). In T cells, regulation of the actin-binding protein Myh9 (Non-muscular myosin IIa), one of the top hits identified by our proteomic studies, has been shown to be crucial for T-cell motility.[31] Following this path, the profound effects of HA on T-cell motility resemble previous T-cell studies using *Myh9* siRNAs and/or blebbistatin.[21,31,32] Along with the molecular docking and dynamics simulations, we provide strong evidence that HA exerts its effects via inhibition of Myh9 by reducing phosphorylation of its regulatory light chain MLC2. Interestingly, although structurally similar, *in silico*, Arg did not reveal significant interaction with Myh9, which may explain the selective benefits of HA. Of particular note, smiliar results of docking simulations were obtained for human Myh9-ligand complexes (data not shown), indicating that our mechanistic observations in mice can potentially be translated to humans and may thus explain the beneficial effects of HA on CV outcome.

This attenuation of atherosclerosis is in apparent contradiction to recent findings by Rodionov et al., where HA-supplementation did not affect lesion size in low-density lipoprotein receptor (LDLr) whc ('wicked high cholesterol') mice,[33] a mouse model primarily used to study familial hypercholesterolemia.[34] However, the initial purpose of the Rodionov study was to investigate the effect of HA-supplementation on coronary artery calcification mediated by tissue-nonspecific alkaline phosphatase. In contrast, our study specifically aimed at interrogating the effect of HA on atherogenesis using *ApoE*$^{-/-}$ mice, a model primarily designed to study the inflammatory mechanisms underlying the initiation and progression of atherosclerosis.[35] Furthermore, the differences in dosage and diet regimes, i.e. waiving 2 weeks of pre-treatment with HA and a shorter period of Western-type diet (4 weeks vs. 6 weeks) may explain the different outcomes. Here, it is worthwhile to mention that our study only includes female animals. Since we and others never observed sex-dependent differences in the predictive value of HA in humans or sex-dependent differences of HA-treatment in experimental models, we believed that in accordance with proper animal welfare this is valid reason to use only female mice in the present *in vivo* study.

In the body, HA is mainly synthesized from lysine and Arg catalyzed by the enzyme L-arginine:glycine amidinotransferase (AGAT).[13,36] In line with previous mouse experimental HA-supplementation studies[13], the applied dosage of 14 mg/L HA via the drinking water did

not affect lysine and Arg concentrations (Supplemental Figure 1A-B) and had no counter-regulatory effect on local *Arg1* or AGAT (encoded gene *Gatm*) expression in the spleen of our mice (Supplementary Figure 7E). In the future, it will be intriguing to study the effect of AGAT- and subsequent HA-deficiency on atherosclerosis with a specific focus on $CD4^+$ T-cell function and its actin cytoskeleton. In addition to this predominant AGAT-catalyzed synthesis, endogenous HA may be also synthesized by a "lysine-urea cycle", with argininosuccinate lyase (ASL) catalyzing the conversion from homoargininosuccinate to homoarginine.[37] In the present study, HA-supplementation had no impact on local *Asl* expression (Supplemental Figure 7E).

In synopsis, we found a novel mechanism by which the amino acid HA impedes atherogenesis specifically by modulating the actin cytoskeleton in $CD4^+$ T cells leading to reduced $CD4^+$ T-cell activation, migration and proliferation, via inhibition of the actin-binding protein Myh9. These data provide experimental support and for the first time a mechanistic explanation for the beneficial effects of HA on CVD outcomes observed in a large variety of clinical and epidemiological studies. Our data suggest that oral HA-supplementation could be considered as putative immunotherapeutic approach for patients at high risk for atherosclerotic CVD. Further clinical studies are required to validate whether HA-supplementation could be harnessed to target T-cell function during atherogenesis.

Sources of Funding This study was supported by the Deutsche Forschungsgemeinschaft [Grant numbers DFG Einzelförderung 451372580 to D.A.; CRC 1123 to D.A., E.L. (A5), A.I., R.Z. (Z2), J.D. (A10), R.A.T.M. (Z1), D.S. (B5), C.W. (A1, A10, Z3); INST409/150-1FUGG to C.W., R.M.] and the German Center for Cardiovascular Research (DZHK) [Promotion of Women Scientist grant 81X1600210 to D.A.]. This study was also supported by the British Heart Foundation [Programme Grant (RG/18/12/34040) to C.A.L.], the EU [Horizon 2020, REPROGRAM to E.L.] and the European Research Council [ERC consolidator grant to E.L, ERC advanced grant to C.W.]. C.W. is a Van de Laar professor of atherosclerosis.

Acknowledgement We thank Sigrid Unterlugauer, Debra McAndrew, Yonara Freire Soares Marques and Yvonne Jansen for their excellent technical assistance, Maria Aslani for the Circos Plot as well as Winnie Vos and Bram van Os for their valuable input on T-cell phenotyping. The graphical abstract was created with Biorender.com

Authors' contribution D.A., and E.L. conceived the study. D.A., E.L. and C.W. contributed to the design and implementation of the research. K.N., M.L., C.A.B., Y.W., I.A.K., C.B., C.A.L., R.M., J.W.M.H., A.J., and D.A. performed HA experiments. KW performed MD stimulations. Y.L. and S.M. performed IHC experiments. E.S. measured amino acid concentrations. I.F. and A.I. performed mass spec proteomics and C.A. and R.Z performed MS data and subsequent pathway analyses. M.B. and R.T.A.M. performed imaging experiments. S.H. and J.D. provided input for the discussion. D.S. perfomed statistical analysis. M.L., C.W., E.L. and D.A. wrote the manuscript.

Disclosures The authors have no relevant financial or non-financial interests to disclose.

Ethics approval All mouse studies were approved by the local ethics committees (Regierung von Oberbayern ROB-55.2-2532.Vet_02-15-156 and ROB-55.2-2532.Vet_02-17-180) and conducted in accordance with the institutional and national guidelines for the care and use of laboratory animals.

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Figure Legends

Figure 1: Homoarginine-supplementation reduces atherosclerosis. (A) Homoarginine (HA) plasma concentrations of HA-treated (HA, 14 mg/L via drinking water) and control (normal drinking water) *apolipoprotein E*-deficient (*ApoE*$^{-/-}$) mice after 6 weeks of Western diet (n=5 vs. 5). (B-F) Phenotypic characterization of plaque burden in the aortic root. (B) Quantification of atherosclerotic lesion areas in the aortic root (n=9 vs. 8) together with representative microphotographs from oil-red O-stained cross-sections; scale bar 500 µm. (C) Characterization of atherosclerotic plaque morphology using Virmani classification: fibrous cap atheroma (FCA), pathological intima thickening (PIT) and intimal xanthoma (IX) (n=27 vs. 24 plaques from 9 vs. 8 mice). (D-F) Immunofluorescent staining assessing the cellular composition of cross-sections of the aortic root for (D) macrophages (Mac-3^{+} area, n=9 vs. 8) and (E) vascular smooth muscle cells (α-smooth muscle actin, α-SMA^{+} area, n=8 vs. 8; 1 ROUT outlier excluded). (F) Quantification of T cells (CD3ε^{+}, red) in cross-sections of the aortic root (n=9 vs. 8) together with representative microphotograph; scale bar: 50 µm; DAPI: 4´,6-Diamidin-2-phenylindol as DNA stain, AF: Autofluorescence. Data are represented as mean ± standard deviation; comparisons were assessed by Mann-Whitney *U* (A, B and D), Chi-square (C), and Student's *t* test (E and F).

Figure 2: Homoarginine inhibits T-cell activation and proliferation. (A-E) Flow cytometric (FC) analysis of splenic T cells isolated from atherosclerotic HA-treated and control mice. Proportion of (A) CD3ε^{+} and (B) CD4^{+} T cells in the spleen (n=9 vs 8). Proportion of (C) CD4^{+} naïve and effector memory (EM) cells in the spleen (n=9 vs. 8) together with representative FC gating strategies for naïve and EM CD4^{+} T cells using CD62L and CD44. (D-F) Effect of HA on T-cell proliferation: (D) FC analysis of Ki-67 in CD4^{+} EM T cells (n=12 vs. 11 from 2 independent experiments) together with representative FC histograms for Ki-67. (E) FC analysis of 7-Aminoactinomycin (7-AAD) in CD4^{+} EM T cells (n=12 vs. 11 from 2 independent experiments) together with representative FC histograms for 7-AAD. (F) *In vitro* proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labelled splenic CD4^{+} T cells isolated from atherosclerotic HA-treated and control mice followed by FC analysis of CFSE-dilution (n=5 vs. 5). A.U. arbitrary unit. Data are represented as mean ± standard deviation; comparisons were assessed by Student's-*t* test (A-D), Mann-Whitney *U* test (E) with correction for multiple comparisons by FDR (F).

Figure 3: Homoarginine modulates T-cell biology. (A) Relative gene expression of the activation marker *Cxcr3*, *Cxcl10*, *Ifn-γ* and *Tnf-α* in the spleen of atherosclerotic HA-treated and control mice (qRT-PCR normalized to *18S* rRNA; n=8 vs. 7). Cytokine concentrations of INF-γ and IL-12p70 in **(B)** plasma of atherosclerotic HA-treated and control mice (n=8 vs. 11 for INF-γ; 2 ROUT outlier excluded and n=9 vs. 11 for IL-12p70; 1 ROUT outlier excluded) as well as **(C)** supernatant of splenic $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice after 72 h *in vitro* culture (n=9 vs. 9 for INF-γ; 1 ROUT outlier excluded and n=9 vs. 10 for IL-12p70). **(D)** Circos plot showing differently regulated proteins in $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice as determined by mass spectrometry. From outside to inside: blood (red), lymph node (blue) and spleen (green); gene name of significantly regulated proteins compared to controls; proteins that are regulated in ≥ two tissues are highlighted in bold; heatmap represents Log_2 fold change (FC) of proteins compared to controls from -0.5 (dark blue) to +0.5 (dark red); ribbons represent the seven most abundant gene ontology terms in molecular function with actin binding and actin filament binding (blue), GTPase binding (red), ubiquitin-like protein ligase binding (orange), phospholipid binding (green), phosphatase binding (violet), transcription coactivator activity (yellow) and unfolded protein response (grey; for spleen n=4 technical x 4 biological vs. 4 technical x 5 biological replicates, for lymph node n=4 technical x 4 biological vs. 4 technical x 5 biological replicates, for blood n=1 technical x 3 biological vs. 1 technical x 2 biological replicates). Data are represented as mean ± standard deviation; comparisons were assessed by Student's-*t* tests with correction for multiple comparisons by FDR (A), Student's-*t* test (B left), and Mann-Whitney *U* test (B right and C).

Figure 4: Homoarginine remodels actin cytoskeleton in T cells. (A-C) $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice were cultured for 72 hours, stimulated via CD3/CD28 activation, stained with fluorescent phalloidin to label the actin cytoskeleton and subsequently imaged with 3D stimulated emission-depletion (STED) microscopy. Analysis of **(A)** F-actin surface to volume ratio after 3D segmentation (n=25 cells/group) together with representative images of the cell volume after 3D reconstruction. Colour code indicates fluorescent intensity for Phalloidin and represents the density of the actin cytoskeleton. **(B)** Analysis of filopodia formation per cell (n=9 vs. 12 cells/group). **(C)** Probability (%) of angular distribution of phalloidin-labelled actin fibres together with representative images after computational skeletonization. Colour code indicates preponderant angular orientation of actin fibres (n=17 vs. 18 cells/group). Data are

represented as mean ± standard deviation; comparisons were assessed by Mann-Whitney *U* test (A), Student's *t* test (B), and F-test of sum-of-squares to compare least-square regression models based on Gaussian equations (C).

Figure 5: Homoarginine inhibits T-cell migration. (A) T-cell migration of Jurkat cells cultured for five days with or without 14 mg/L HA (n=6 vs. 6). (B) T-cell migration of murine $CD4^+$ T cells cultured for five days with or without 14 mg/L HA followed by activation via CD3/CD28 stimulation (n=5 vs. 5). Migration assays were performed in transwell chambers in the presence and absence of the chemoattractants C-X-C motif chemokine ligand CXCL10 or C-C motif chemokine ligand CCL19 at indicated concentrations. The number of migrated cells was determined by flow cytometry and the chemotactic index for control and HA-treated T cells was normalized to the corresponding cell count at baseline condition (w/o chemokine). (C-D) Microscopic ibidi chamber-based migration assay of splenic $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice, cultured and activated via CD3/CD28 stimulation (n=231 vs. 164 cells). (C) Representative 2D track plots of cells during a 30 minute-stimulation with chemokine CCL19. (D) Quantification of track lengths after 30 minutes recording. (E) Relative gene expression of homeostatic chemokines *Ccl19*, *Ccl21* and its common receptor *Ccr7* in the spleen of atherosclerotic HA-treated and control mice (qRT-PCR normalized to *18S* rRNA; n=8 vs. 7). Data are represented as mean ± standard deviation or median ± interquartile range (5D); comparisons were assessed by Mann-Whitney *U* test (A, B and D) and Student's-*t* test (E) with correction for multiple comparisons by FDR.

Figure 6: Homoarginine inhibits Myh9. (A) *In silico* molecular docking and dynamics simulations of mouse Myh9-ligand complexes. Binding of blebbistatin, HA and Arg to mouse Myh9 is expressed as binding free energy (BFE). (B) T-cell migration of murine $CD4^+$ T cells cultured for five days with or without 14 mg/L HA followed by activation via CD3/CD28 stimulation and with or without 1 hour 50 µM blebbistatin (B)-treatment (n=5). Migration assays were performed in transwell chambers in the presence and absence of CCL19 at indicated concentrations. The number of migrated cells was determined by flow cytometry and the chemotactic index was normalized to the corresponding cell count at baseline condition (w/o chemokine). (C-D) Homoarginine reduces Myh9 regulatory light chain (MLC2) phosphorylation. Myosin light chain (MLC)2 phosphorylation of splenic $CD4^+$ T cells isolated from atherosclerotic HA-treated and control mice detected via (C) western blot (Serine (Ser)-19; n=4 vs. 4) as well as via (D) immunofluorescence microscopy (Threonine

(Thr)-18/Ser-19, n= 153 vs. 216 cells) including representative images (top image: control; bottom image: 14 mg/L HA; scale bar: 10 Jim; DAPI: 4´,6-Diamidin-2-phenylindol as DNA stain). Data are represented as mean ± standard deviation; comparisons were assessed by Kruskal-Wallis H with Dunn's *post hoc* test (B), Student's-*t* test (C), and Mann-Whitney *U* test (D).

Novelty and Significance

What is known?

- Atherosclerosis is a chronic inflammatory disease that involves the interplay of various immune cells and immunological processes.
- The endogenous amino acid homoarginine is a strong biomarker for cardiovascular outcome with high levels being protective.
- Oral homoarginine supplementation is curative in murine models of acute ischemic stroke and chronic heart failure.

What new information does this article contribute?

Although homoarginine has been established as a strong biomarker and its supplementation promotes cardiovascular health in mice, the underlying mechanisms remain elusive. Since atherosclerosis is a common cause of cardiovascular disease, we investigated whether homoarginine-supplementation protects against atherosclerosis. Our study shows that homoarginine-supplementation reduces atherosclerotic plaque development via modulation of the adaptive immune system, specifically $CD4^+$ T cells. Mechanistically, homoarginine binds to myosin IIa and profoundly modulates $CD4^+$ T-cell actin polarization, which in turn impairs actin-dependent functions such as T-cell activation, mobility, migration and proliferation, explaining the reduced plaque burden. These findings provide for the first a mechanistic explanation for the beneficial effects of the amino acid homoarginine on atherosclerotic cardiovascular disease. A *first in man* study has already proven that homoarginine-supplementation is safe and well tolerated in humans. Our data suggest that oral homoarginine-supplementation could be considered as putative immunotherapeutic approach for patients at high risk for atherosclerotic cardiovascular disease.

Figure 1

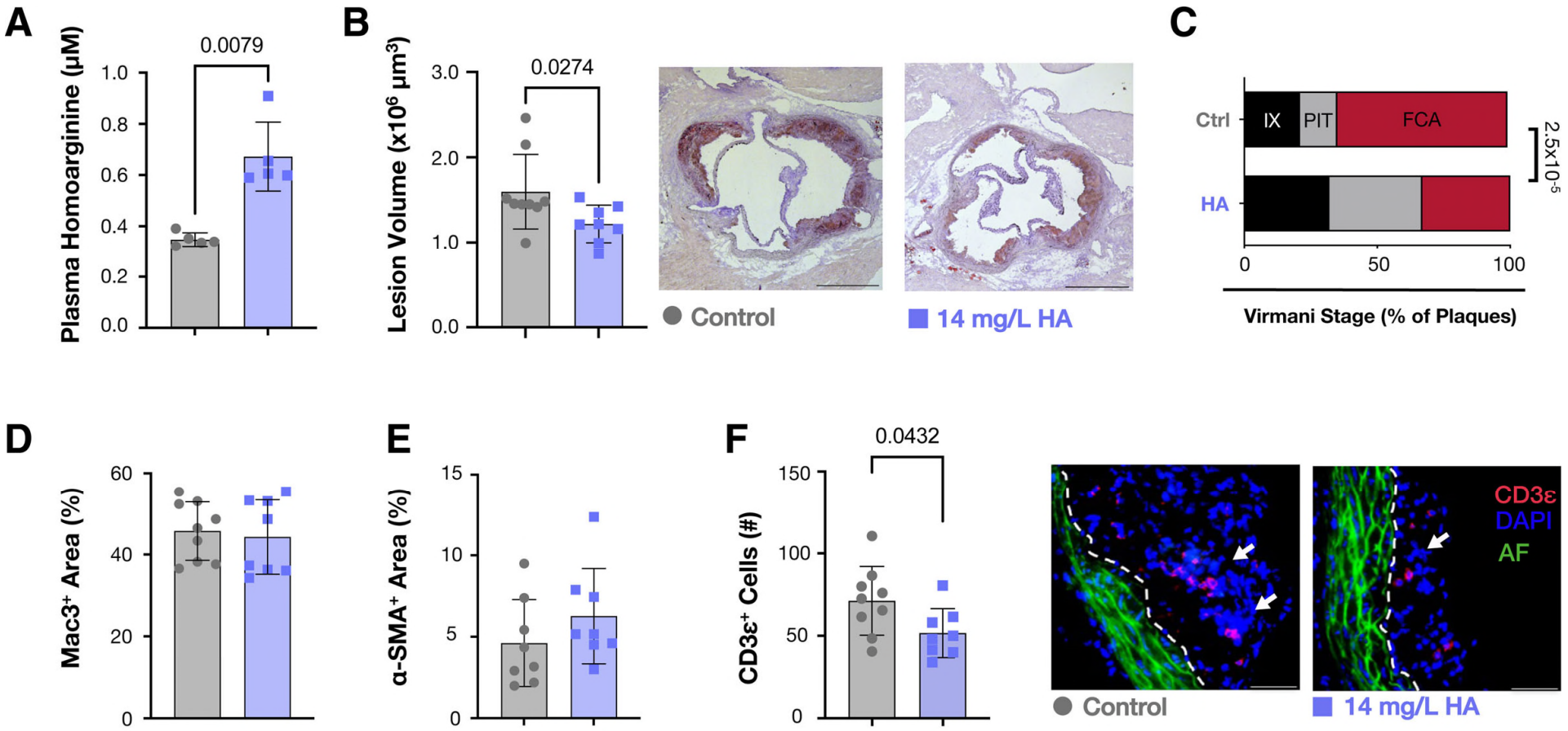

A
Plasma Homoarginine (μM)
0.0079
1.0
0.8
0.6
0.4
0.2
0.0
B
Lesion Volume (x10⁶ μm³)
0.0274
3.0
2.0
1.0
0.0
Control
14 mg/L HA
C
Ctrl
IX
PIT
FCA
HA
2.5x10⁻⁵
0
50
100
Virmani Stage (% of Plaques)
D
Mac3⁺ Area (%)
60
40
20
0
E
α-SMA⁺ Area (%)
15
10
5
0
F
CD3ε⁺ Cells (#)
0.0432
150
100
50
0
CD3ε
DAPI
AF
Control
14 mg/L HA

Figure 2

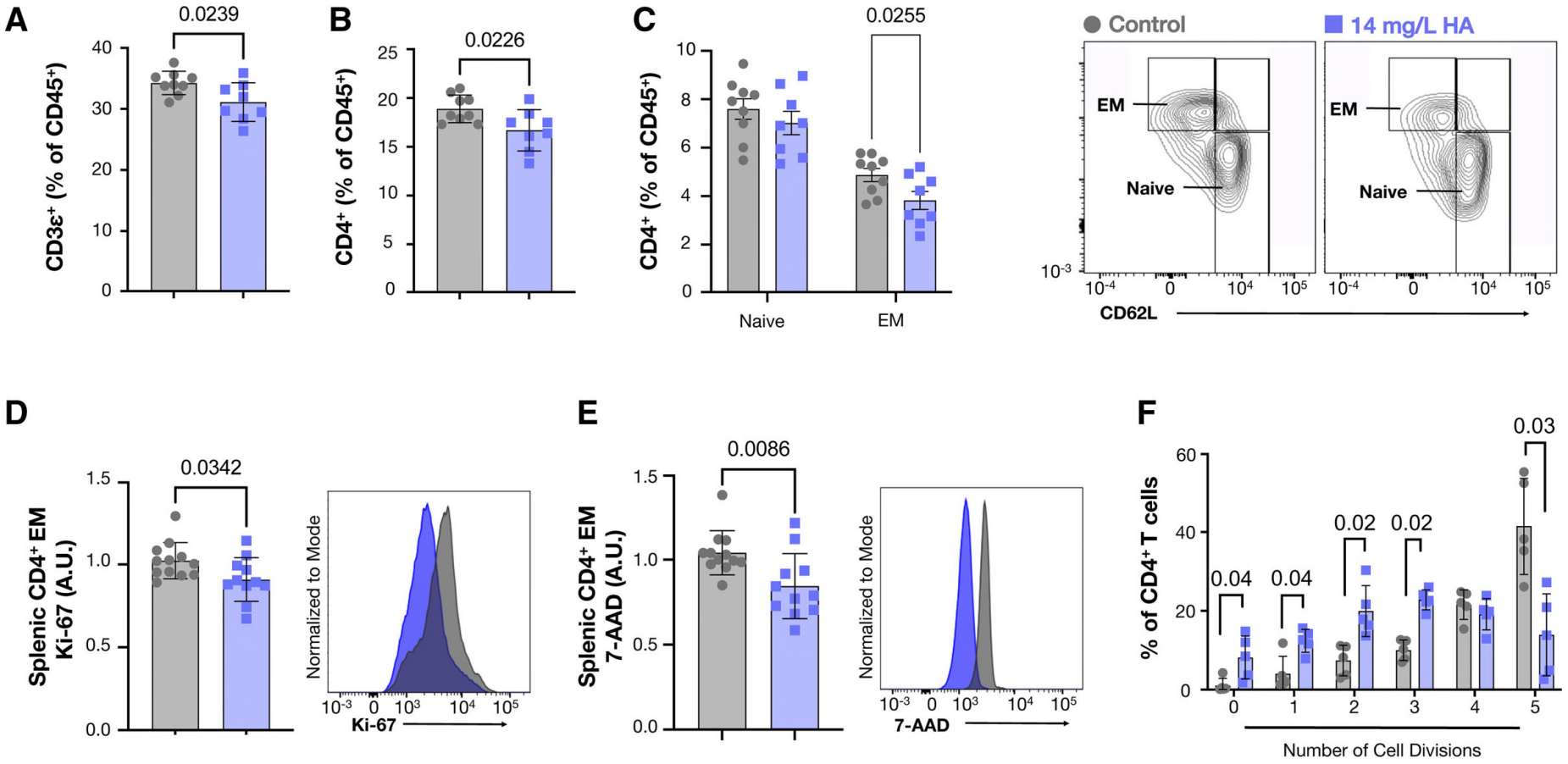

A
0.0239
CD3ε⁺ (% of CD45⁺)
B
0.0226
CD4⁺ (% of CD45⁺)
C
0.0255
CD4⁺ (% of CD45⁺)
Naive
EM
Control
14 mg/L HA
EM
Naive
EM
Naive
CD62L
D
0.0342
Splenic CD4⁺ EM Ki-67 (A.U.)
Normalized to Mode
Ki-67
E
0.0086
Splenic CD4⁺ EM 7-AAD (A.U.)
Normalized to Mode
7-AAD
F
0.03
0.02
0.02
0.04
0.04
% of CD4⁺ T cells
Number of Cell Divisions

Figure 3

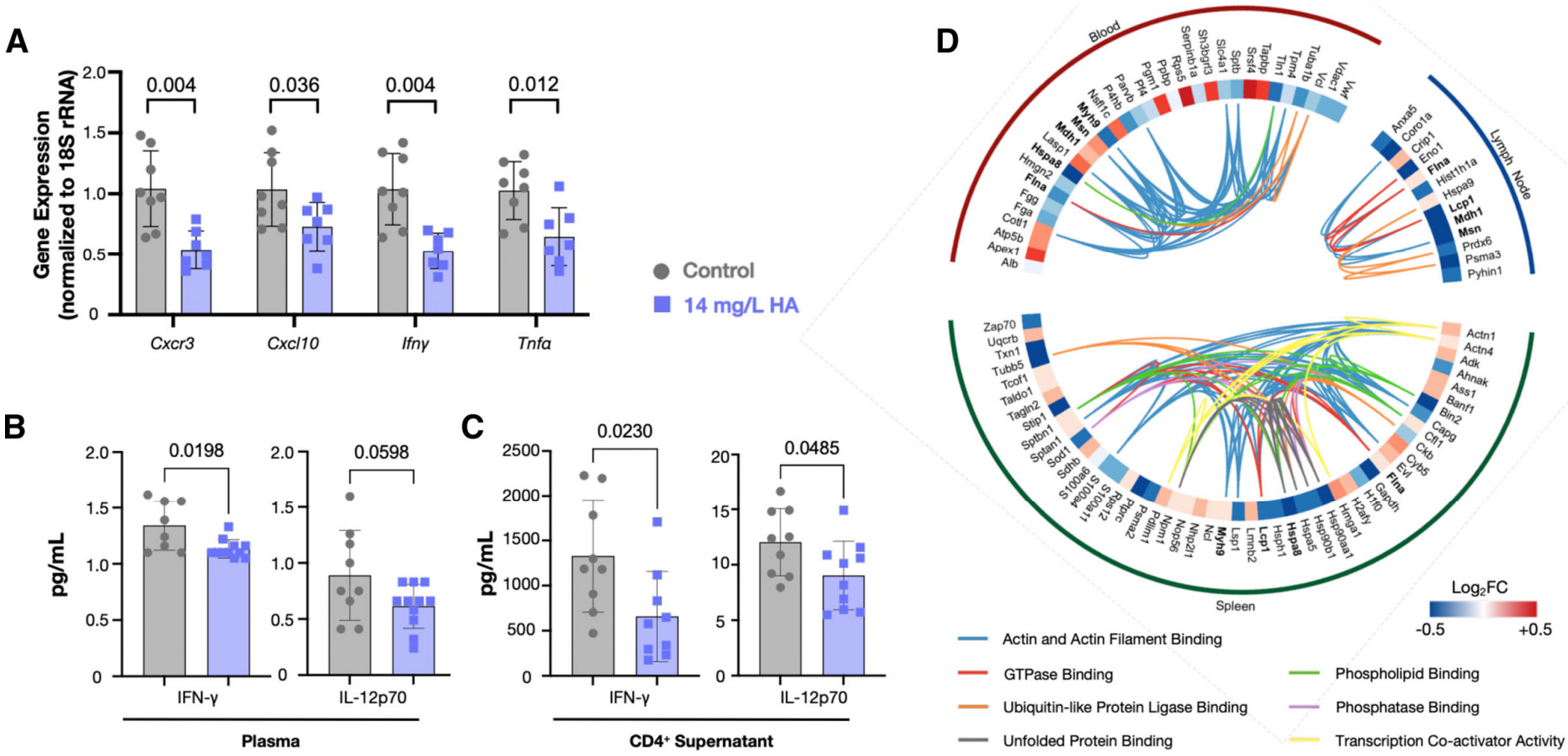

A
Gene Expression (normalized to 18S rRNA)
0.004
0.036
0.004
0.012
Cxcr3
Cxcl10
Ifnγ
Tnfa
Control
14 mg/L HA
B
pg/mL
0.0198
0.0598
IFN-γ
IL-12p70
Plasma
C
pg/mL
0.0230
0.0485
IFN-γ
IL-12p70
CD4+ Supernatant
D
Blood
Lymph Node
Spleen
Log2FC
-0.5
+0.5
Actin and Actin Filament Binding
GTPase Binding
Ubiquitin-like Protein Ligase Binding
Unfolded Protein Binding
Phospholipid Binding
Phosphatase Binding
Transcription Co-activator Activity

Figure 4

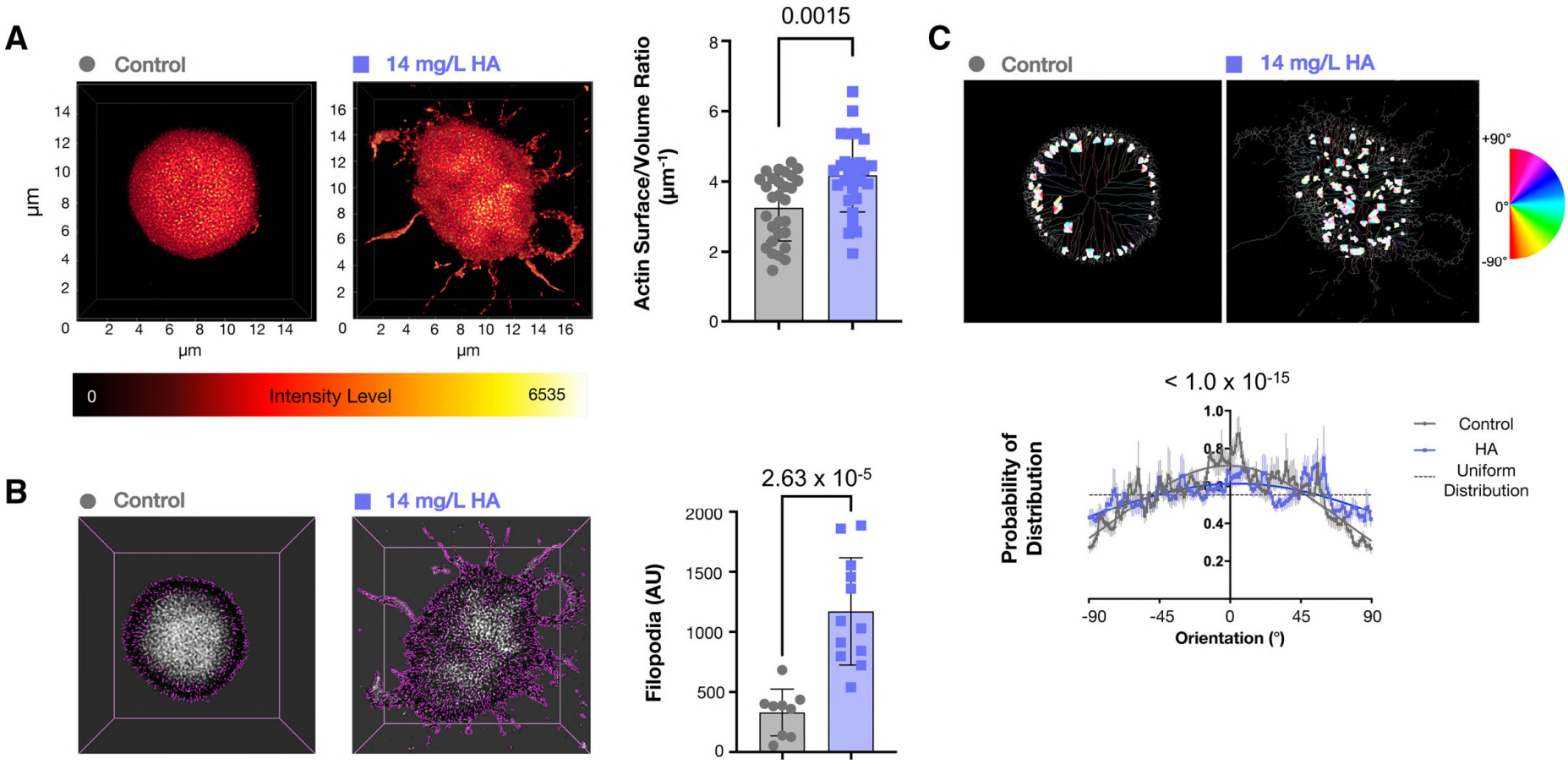

A
Control
14 mg/L HA
µm
0
Intensity Level
6535
0.0015
Actin Surface/Volume Ratio (µm-1)
B
Control
14 mg/L HA
2.63 x 10-5
Filopodia (AU)
C
Control
14 mg/L HA
+90°
0°
-90°
< 1.0 x 10-15
Probability of Distribution
Control
HA
Uniform Distribution
Orientation (°)

Figure 5

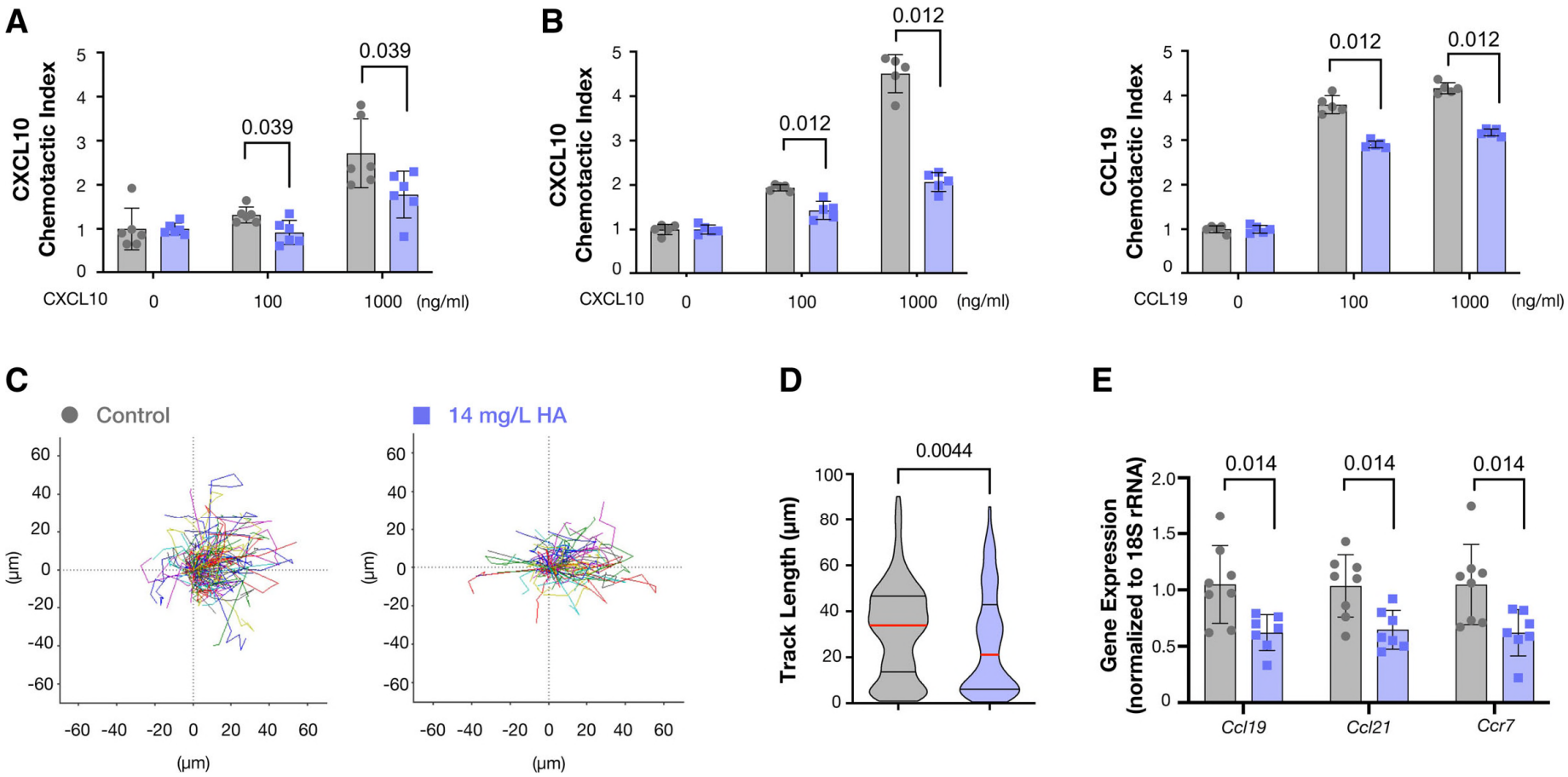
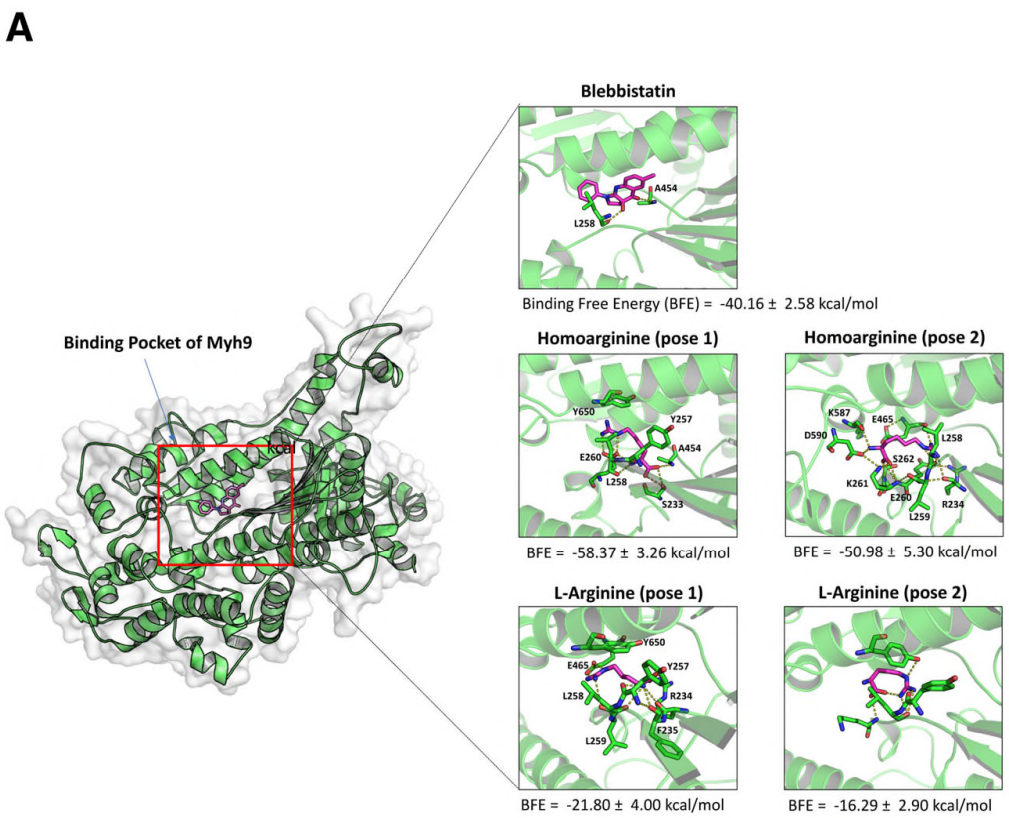
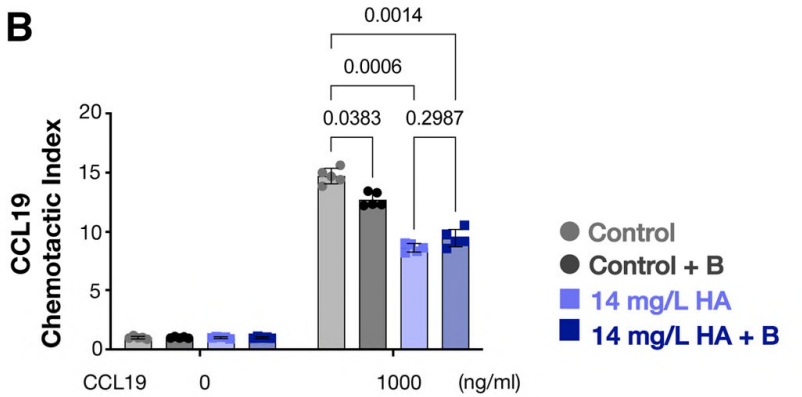
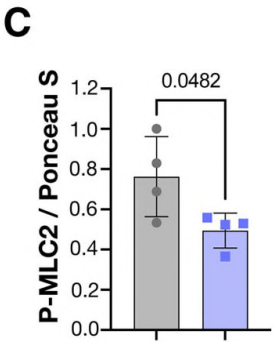
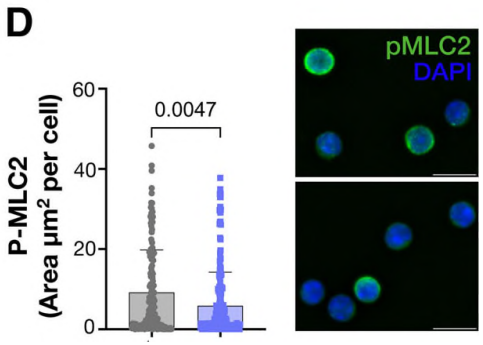

A
CXCL10 Chemotactic Index
0.039
0.039
CXCL10
0
100
1000
(ng/ml)
B
CXCL10 Chemotactic Index
0.012
0.012
CXCL10
0
100
1000
(ng/ml)
CCL19 Chemotactic Index
0.012
0.012
CCL19
0
100
1000
(ng/ml)
C
Control
14 mg/L HA
(μm)
D
Track Length (μm)
0.0044
E
Gene Expression (normalized to 18S rRNA)
0.014
0.014
0.014
Ccl19
Ccl21
Ccr7

Figure 6


A
Binding Pocket of Myh9
Blebbistatin
A454
L258
Binding Free Energy (BFE) = -40.16 ± 2.58 kcal/mol
Homoarginine (pose 1)
Y650
Y257
E260
A454
L258
S233
BFE = -58.37 ± 3.26 kcal/mol
Homoarginine (pose 2)
K587
E465
D590
L258
S262
K261
E260
R234
L259
BFE = -50.98 ± 5.30 kcal/mol
L-Arginine (pose 1)
Y650
E465
Y257
L258
R234
F235
L259
BFE = -21.80 ± 4.00 kcal/mol
L-Arginine (pose 2)
BFE = -16.29 ± 2.90 kcal/mol


B
0.0014
0.0006
0.0383
0.2987
CCL19 Chemotactic Index
20
15
10
5
0
CCL19
0
1000
(ng/ml)
Control
Control + B
14 mg/L HA
14 mg/L HA + B


C
0.0482
P-MLC2 / Ponceau S
1.2
1.0
0.8
0.6
0.4
0.2
0.0


D
0.0047
P-MLC2
(Area µm² per cell)
60
40
20
0
pMLC2
DAPI