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# Identification of a leucine-mediated threshold effect governing macrophage mTOR signaling and cardiovascular risk

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AUTHOR CONTRIBUTIONS

XZ, DK, BM and BR designed the study. XZ, DK, SJJ, AF, JS, VS, IS, EY, AR, YSY, AP, AY, OR, SE, JDS, MS, AD, JC, NOS, AJ, IJL, BM and BR contributed to data acquisition, data analysis, and data interpretation. All authors contributed to the writing of the paper. BR is the guarantor of this work, had full access to all the data in the study, and assumes full responsibility for the integrity of the data and the accuracy of the data analysis.

DECLARATION OF INTEREST STATEMENT

All authors declare they have no conflict of interest.

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## Abstract

High protein intake is common in Western societies and is often promoted as part of a healthy lifestyle. However, amino acid-mediated mammalian target of rapamycin (mTOR) signaling in macrophages has been implicated in the pathogenesis of ischemic cardiovascular disease. In a series of clinical studies on male and female participants (NCT03946774 and NCT03994367) that involved graded amounts of protein ingestion together with detailed plasma amino acid analysis and human monocyte/macrophage experiments, we identify leucine as the key activator of mTOR signaling in macrophages. We describe a threshold effect of high protein intake and circulating leucine on monocyte/macrophage wherein only protein in excess of ~25 grams/meal induces mTOR activation and functional effects. By designing specific diets modified in protein and leucine content representative of intake in the general population, we confirm this threshold effect in mouse models and find ingestion of protein in excess of ~22% of dietary energy requirements drives atherosclerosis in male mice. These data demonstrate a mechanistic basis for the adverse impact of excessive dietary protein on cardiovascular risk.

### Keywords

mTOR; autophagy; macrophage; dietary protein; leucine; atherosclerosis; cardiovascular risk

## INTRODUCTION

In animal models, high-protein diets have been linked to the development of atherosclerosis. More than a century ago it was discovered that rabbits that were fed a protein-rich diet developed intimal lesions within the aorta[1]. Since that time, a substantial body of research across a variety of animal models, including rabbits, rodents (mice, rats, hamsters) and non-human primates (monkeys), has established a clear connection between high dietary protein intake and atherosclerotic plaque formation and progression[2]. We have recently conducted a unique mechanistic study in the atherosclerosis-prone ApoE$^{-/-}$ mouse model and found that high-protein feeding promotes atherogenesis via amino acid-mediated mTORC1 signaling and subsequently impaired autophagy and mitophagy in macrophages[3]. These findings establish a direct link between dietary protein intake, macrophages, and atherogenesis. However, the precise mechanisms underlying amino acid-mediated mTORC1 activation in macrophages are still unclear. An increasing number of regulatory proteins have been implicated as sensors of distinct intracellular amino acid concentrations which trigger mTORC1 signaling[4–6]. We therefore hypothesized that the stimulatory (and deleterious) effect of high protein intake on mTORC1 in macrophages might be due to select “pathogenic” amino acids. Moreover, we hypothesized the presence of a dose-response relationship between protein intake (and/or specific amino acids) and the magnitude of mTORC1 activity. Addressing these unresolved questions is critical as the atherogenic potential of high protein intake observed in animal models has important clinical implications.

High protein intake is common in people living in Western societies. In addition, high protein intake is generally considered healthy and has therefore become popular. The recommended daily intake of protein to maintain nitrogen balance is 0.8 g/kg/d, which corresponds to about 11% of total energy requirements (Figure 1A)[7]. Higher protein intake is recommended for older adults to overcome the age-related impairment of muscle and bone protein synthesis[8,9]. On average, people in Western societies consume about a third more protein than the recommended daily protein intake and about one quarter of the population consumes even more than twice the recommended amount (i.e. more than 1.6 g/kg/d or 22% of daily energy as protein) (Figure 1A)[10–14]. About two-thirds of protein in Western-type diets is derived from animal sources[11,15–20]. The results from several observational studies suggest high total protein intake and high animal protein intake are both associated with increased cardiovascular disease mortality, although high protein intake from plant sources may not cause adverse health outcomes[16,21]. However, no mechanistic studies linking high protein intake to cardiovascular pathologies in people exist. Thus, our understanding of the relationship between dietary protein intake and atherosclerosis remains incomplete.

The purpose of the present study was to evaluate whether high protein intake activates the amino acid-mTORC1-autophagy signaling pathway in people, similar to our observation in ApoE$^{-/-}$ mice[3]. In addition, we evaluated the dose-response relationship between protein intake and this mTOR signaling pathway, including downstream effects, and the amino acid specificity of mTORC1 activation in both mice and people. We found that the amino acid-mTORC1-autophagy mechanism is active in human monocytes-macrophages and that there is a critical dose above which protein intake activates this deleterious signaling pathway and atherogenesis. Importantly, we identified leucine as the critical amino acid modulator of this response. These results highlight a mechanism, present in both mice and people, whereby high dietary protein intake, via increases in plasma leucine, induces dose-dependent activation of a signaling pathway in monocytes/macrophages that is involved in the pathogenesis of atherosclerosis. This discovery provides a key mechanistic link between high dietary protein intake and atherosclerotic cardiovascular disease risk that can help redefine diet recommendations and can perhaps be leveraged as a therapeutic strategy.

## RESULTS

In order to determine the effects of high protein intake on mTORC1-autophagy signaling in human circulating monocytes, we conducted two distinct clinical studies (Extended Data Figure 1A). In the first study, we evaluated extremes of protein intake by comparing the effects of liquid meals that contained either 10% or 50% of energy as protein on monocyte mTORC1 activation in a cohort of participants. In the second study, we utilized a more “real-world” scenario, by evaluating these outcomes in participants who consumed either a standard protein mixed meal or a mixed meal with modestly increased protein content (15% kcal vs 22% kcal).

Our signaling analysis was focused on CD14$^{+}$CD16$^{-}$ monocytes as they comprise the largest proportion of circulating monocytes and are the predominant subtype that differentiate into atherosclerotic plaque macrophages[22–25]. We used both flow cytometry analysis of

peripheral blood mononuclear cells (PBMCs) and magnetic beads for efficient platelet-depleted $CD14^{+}CD16^{-}$ monocyte isolation (Extended Data Figure 1B). In our prior mouse studies[3], we established that monocyte/macrophage mTORC1 was activated after high protein gavage and the most robust activation was observed between 1 h to 3 h after gavage. Thus, similar time-points were used in our study in people to assess the effects of ingesting meals with varying protein content on plasma amino acid concentrations as well as monocyte mTORC1 signaling and downstream effects by fluorescence-activated cell sorting (FACS) analysis, Western blotting, and immunofluorescence microscopy (summarized in Extended Data Figure 1A with further details and results for each study described below).

## Impact of very high protein intake on mTORC1-autophagy signaling in human circulating monocytes

Fourteen participants with average body mass index (BMI) in the overweight range (Table 1) were studied on two occasions after a 12 h overnight fast. On one occasion participants consumed a low protein liquid meal (500 kcal, 10% of energy as protein, 17% as fat, and 73% as carbohydrate); on the other occasion they consumed a very high protein liquid meal (500 kcal, 50% of energy as protein, 17% as fat, and 33% as carbohydrate) (Figure 1B). Blood samples were collected before and at 1 h and 3 h after ingesting the meal to determine plasma amino acid concentrations and to isolate monocytes (Figure 1B). Total amino acid concentration in plasma increased after consuming the very high protein but not the low protein liquid meal (Figure 1C). We also measured plasma triglyceride (TG) concentration, because TG is an established atherosclerotic disease risk factor, and found plasma TG concentration was not different after the very high- and low protein meals (Extended Data Figure 1C).

The effects of the very high-protein versus the low-protein liquid meal on mTORC1 activation in isolated monocytes were determined by both Western blot and FACS analysis, using phosphorylation of ribosomal protein S6 (downstream target of mTORC1) as the readout. Ingesting the very high-protein, but not the low protein meal, progressively increased mTORC1 signaling during the 3 h postprandial period (Figure 1D–E and Extended Data Figure 1D). In addition, we assessed the co-localization of mTOR and LAMP2—a classical marker of mTORC1 activation—in monocytes from a subset of the participants (5 of 14) and found mTOR and LAMP2 were co-localized after the very high-protein meal but not the low-protein meal Extended Data Figure 1E). We then used immunofluorescence microscopy to measure the intensity of monocyte LC3 (microtubule-associated protein 1A/1B-light chain 3), a key marker of autophagy[26]. The very high-protein, but not the low protein meal led to progressive loss of LC3 signal intensity, indicative of mTORC1-mediated autophagy inhibition (Figure 1F).

The culmination of results from these initial experiments lent insight into the kinetics of protein-induced mTORC1 activation in circulating monocytes and provided a preliminary gauge of effects at proportional extremes of dietary protein intake on human monocyte/macrophage mTORC1-autophagy signaling. These initial results suggest that acute very high protein intake induces monocyte mTORC1 activation and reciprocal suppression of autophagy within 1 h to 3 h of protein ingestion.

## Impact of high protein mixed meal intake on mTORC1-autophagy signaling in human circulating monocytes

To assess the effect of high protein intake in a “real-world” scenario, we studied nine participants with overweight (Table 1) on two separate occasions (Figure 2A). On one occasion, the participants consumed a 450 kcal standard meal, which contained 15% of energy as protein (16 grams of protein), which represents the average meal protein content in the general population[10–14]; 35% of meal energy content was fat, and 50% was carbohydrates. On the other occasion, the participants consumed a 450 kcal high protein meal, which contained 22% of energy as protein (25 grams), which represents the upper quartile of protein intake (Figure 2A)[10–14]; 30% of meal energy content was fat, and 48% was carbohydrates. Blood samples to determine plasma amino acid concentrations and for isolating monocytes for signaling and other assays were collected before ingesting the meal and at 1 h and 2 h afterwards (Figure 2A). Plasma TG concentration was not different after the two meals (Extended Data Figure 1F). Total plasma amino acid concentration increased after consuming the 22% protein but not the 15% protein meal (Figure 2B), and postprandial S6 phosphorylation was greater after the 22% protein than the 15% protein meal (Figure 2C). Similarly, LC3 immunofluorescence signal intensity decreased from baseline at 2 h after consuming the 22% protein, but not the 15% protein meal (Figure 2D). These results suggest a fully functional and activated mTORC1-autophagy signaling axis in circulating human monocytes and demonstrate that high protein intake-induced monocyte mTORC1 activation with subsequent inhibition of autophagy occurs even within the normal range of meal protein intake.

## Plasma amino acid profiles after low, standard, high and very high protein intake

In the two clinical studies we conducted, both very high protein (50% of energy) and moderately high protein (22% of energy) meals, compared with the respective control meals that contained <16% of energy as protein, increased total circulating amino acid concentration and activated monocyte mTORC1 signaling and caused reciprocal inhibition of autophagy (Figures 1 and 2). In order to assess potential differential contributions of select amino acids to mTORC1 activation, we evaluated differences and similarities in the postprandial plasma amino acid concentration profiles in the two studies (Extended Data Figures 2 and 3). In the first cohort of participants, plasma concentrations of almost all amino acids rose significantly in the postprandial period after consuming the very high protein (50% of energy), but not the low protein (10% of energy) meal (Figures 3A, C, and E and Extended Data Figure 2). In the second cohort, only some plasma amino acid concentrations were higher after consuming the high protein (22% energy) compared with the standard protein (15% energy) meal (Figures 3B, D, and F, Extended Data Figure 3). The concentrations of seven amino acids (leucine, isoleucine, valine, methionine, threonine, serine, and arginine) increased after both the very high protein (50% of energy) liquid meal and the high protein (22% of energy) mixed meal (Figures 3A, B). Thus, we focused on these seven amino acids in our subsequent analysis looking for dose-dependent effects in human monocytes and monocyte-derived macrophages.

## Leucine is the most consequential amino acid with regard to mTORC1 activation in human monocyte-derived macrophages

To determine which of the seven amino acids that were higher after both very high and high protein meal compared with respective lower protein meal intake (Figure 4A) contributes to mTORC1 signaling in monocytes/macrophages, we utilized cultured human monocyte-derived macrophages (HMDMs) as a tool to study the macrophage-specific mTORC1 response to these individual amino acids and assess dose-effects. HMDMs are an ideal cell type in this evaluation given they are readily derived from circulating human monocytes and differentiate into mature macrophages over 12 days developing the classic pro-inflammatory markers CD11b/CD18 and CD68 (Extended Data Figures 4A–C)[27–29]. Furthermore, HMDMs manifest robust mTORC1 activation and downstream autophagy inhibition in response to amino acid stimulation (Extended Data Figures 4D–F).

We first compared the effects of each of the seven amino acids on mTORC1 signaling in HMDMs by applying them in equimolar (2 mM) concentration; we chose 2 mM, because it is within the range commonly used to activate mTORC1 in cultured cells[30,31]. Western blot for the mTORC1 target S6 showed leucine was by far the most robust mTORC1 inducer, activating mTORC1 nearly 4-fold above the other amino acids (Figure 4B). Other surrogate markers of mTORC1 activation, including co-localization of mTOR with the lysosomal marker LAMP2 (Figure 4C) and suppression of autophagosome formation as gauged by LC3 puncta via microscopy (Figure 4D and Extended Data Figure 4F), also confirmed leucine as the overwhelming mTORC1 inducer in HMDMs. In addition, the phosphorylation of ULK1 in HMDMs followed the pattern of S6 phosphorylation after incubation with the seven amino acids (Extended Data Figure 4G), while p-AMPK was unaffected by amino acid treatment (Extended Data Figure 4G), implying the amino acid-induced inhibitory effects on autophagy are mediated via mTORC1 signaling.

In order to compare the effects of the seven candidate amino acids with regard to mTORC1 activation in a physiologically relevant context, we repeated the experiments on HMDMs by using the respective peak plasma concentrations of each of these amino acids we observed after the very high protein (50% of energy) meal (Extended Data Figure 2). Western blots for the mTORC1 target S6, co-localization of mTORC1 with the lysosomal marker LAMP2, and LC3 puncta formation by microscopy, all revealed leucine as the most robust and predominant inducer of mTORC1-autophagy signaling (Figures 4E–G and Extended Data Figure 4H). It is notable that leucine was the predominant activator of mTORC1 signaling, even though it was applied at a 25% lower concentration (~450 μM vs ~550 μM) than the second closest inducer, isoleucine. Moreover, the branched-chain amino acids (BCAAs) are often considered in unison when discussing their effects on physiology and cell signaling, but our data demonstrate leucine's ability to stimulate mTORC1 signaling in macrophages is unique in contrast to the modest effects of the other BCAAs (isoleucine and valine).

## Threshold effects govern leucine-mediated mTORC1 activation and downstream sequela in human monocyte-derived macrophages

Given that we identified leucine as the most potent amino acid activator of mTORC1 and plasma leucine concentration rises after high protein intake in a dose-dependent manner

(Figure 3C–D and Extended Data Figures 2 and 3), we posited that a discernable leucine threshold effect could be detected by examining its mTORC1-inducing activity across a range of leucine concentrations (0, 100, 300, and 600 μM) seen after meal intake in our feeding studies (Figures 1 and 2 and Extended Data Figures 2 and 3). Western blot analysis revealed a clear dose-dependent effect of leucine-mediated mTORC1 activation in HMDMs, as measured by phosphorylation of ribosomal protein S6 (Figure 5A) and ribosomal protein S6 kinase (pS6K) (Extended Data Figure 5A), with a noticeable step-up in activation between 100 μM and 300 μM leucine (Figure 5A). An obvious dose-dependent threshold effect was also observed for mTORC1-LAMP2 co-localization (Figure 5B) and inhibition of autophagy (diminished LC3 puncta formation) and mitophagy (reduced co-localization of the mitochondrial marker COXIV with the autophagosome marker LC3) (Figures 5C, D). Similarly, such threshold effects were seen with all downstream functional sequela of mTORC1 activation in macrophages (Figures 5E–G). These included leucine's dose-threshold-related and synergistic effect on 1) rotenone-induced mitochondrial dysfunction, as determined by quantitation of MitoTracker Red versus Green by FACS (Figure 5E and Extended Data Figure 5B), 2) reactive oxygen species (ROS) production, using dihydroethdium (DHE), a fluorescent probe specific for the detection of ROS, by microscopy (Figure 5F), and 3) enhanced apoptosis, as gauged by Caspase-3/7 staining of macrophages treated with FCCP, the potent mitochondrial uncoupler and inducer of mitochondrial apoptosis (Figure 5G).

The culmination of these findings demonstrates that leucine has a dose-dependent threshold effect on HMDM mTORC1 activation and detrimental downstream effects on autophagy/mitophagy, accumulation of dysfunctional mitochondria, ROS generation, and resultant apoptosis. Importantly, the threshold concentration upon which leucine promotes such detrimental mTORC1 signaling occurs consistently between 100 μM and 300 μM concentrations, which is within the range of postprandial leucine concentrations we observed already after the high protein (22% of energy as protein or 25 grams of protein) compared with the standard protein (15% of energy) mixed meals (Extended Data Figure 3) and well below the postprandial plasma leucine concentration after the very-high protein (50% of energy) meal (Extended Data Figure 2).

## Leucine is the most consequential amino acid with regard to mTORC1 activation in murine monocytes/macrophages

Based upon our findings in human monocytes and HMDMs, we sought to establish if a similar leucine-dependent mTORC1 activation occurs in mice in vivo as well as in cultured murine macrophages. We had previously shown that gavage of mice with 3.2 g of protein/kg body weight results in a significant rise in serum amino acids (including a rise in leucine to a peak of ~1 mM) with ensuing mTORC1 activation in circulating monocytes and tissue macrophages[3]. In order to mimic circulating amino acid concentrations more on par with those found in our human studies, we reduced the protein gavage by 50% to 1.6 g of protein/kg and conducted a time-course evaluation of serum amino acid concentrations and monocyte mTORC1 activation by FACS (Figure 6A). Only four amino acids (Leu, Ile, Val, and Thr) were significantly increased compared with the vehicle control gavage (Figures 6B and 6C and Extended Data Figure 6A). Notably, the amino acids that were increased

after protein gavage in the mice included the same ones that were increased in plasma from people after high protein meal intake (Figure 3). Furthermore, a significant increase in mTORC1 activation in blood monocytes was observed in both male and female mice after gavage (Figure 6D and Extended Data Figure 6B), mimicking our findings in people.

In order to determine which amino acid was the predominant contributor to mTORC1 activation in murine monocytes/macrophages, we selected these four amino acids plus methionine and lysine, which we had also used in the HMDM experiments (Figure 4), and incubated murine bone marrow-derived macrophages (BMDMs) with each individual amino acid at the peak serum concentration observed after protein gavage (Extended Data Figure 6). Similar to the effects we observed with HMDMs, Western blot for phosphorylation of ribosomal protein S6 revealed that leucine is by far the most dominant mTORC1 activator in relation to the other amino acids, some of which were applied at equal or even greater concentrations and did not activate mTORC1 (Figure 6E). Having demonstrated the importance of leucine in mTORC1 activation of cultured macrophages, we evaluated whether leucine is indeed the driver of monocyte/macrophage mTORC1 signaling after protein gavage in vivo. To this end, we gavaged mice with 0.8 g of protein/kg, 1.6 g of protein/kg, and also 0.8 g of protein/kg supplemented with free leucine to make up the difference in leucine content between the 0.8 g protein/kg and 1.6 g protein/kg gavage. As expected, 1.6 g protein/kg gavage led to greater mTORC1 activation than 0.8 g protein/kg gavage, but more importantly, 0.8 g protein/kg supplemented with leucine led to mTORC1 activation on par with 1.6 g protein/kg (Figure 6F). These results indicate that the difference in leucine content accounted for the entire difference in mTORC1 activation between the two protein gavage doses. Overall, these data demonstrate that metabolism of dietary protein and the postprandial rise in serum amino acids and activation of monocytes/ macrophage mTORC1 in mice and cultured murine monocytes/macrophages follows very similar trajectories to those observed in people and human circulating monocytes/ HMDMs, which establishes the critical role for leucine in this physiology.

## Impact of varying dietary protein intake on atherosclerosis in a mouse model

Having established a dose-dependent threshold effect of dietary protein and circulating leucine on mTORC1 and deleterious downstream signaling in both human and murine monocytes/macrophages, we next set out to determine the consequence of escalating protein intake on atherogenesis in $ApoE^{-/-}$ mice in vivo. We used three Western diets which have lower or higher protein contents than the standard Western diet commonly used in murine atherosclerosis studies[32]. The standard Western diet contains 15% of energy (kcal%) as protein, 43 kcal% as carbohydrate, and 42 kcal% as fat) and can be assumed to be the equivalent of the recommended daily protein intake in people, because mice fed this diet consume the same amount of protein (in g/kg) as mice fed standard chow. We used: 1) a low-protein Western diet (7 kcal% protein, 51 kcal% carbohydrate, 42 kcal% fat), 2) a moderate-protein Western diet (21 kcal% protein, 36 kcal% carbohydrate, 42 kcal% fat), and 3) a high-protein Western diet (46 kcal% protein, 11 kcal% carbohydrate, 43 kcal% fat). Because total food intake differs when mice are fed these different diets, we measured daily food and protein intakes and found the mice consumed about 0.4, 1.0 g, and 1.5 g of protein/d on the low, moderate, and high protein Western diets, respectively (Figure 7A).

Our observation of daily protein intake in mice is consistent with what we have shown before[3]. Specifically, compared with total daily protein intake in mice fed the standard Western diet, mice fed the moderate-protein Western diet ate about 20% more protein whereas those fed the high-protein Western diet ate about twice as much and those fed the low-protein Western diet ate about half (Figure 7A, left panel). Therefore, the moderate-protein and high-protein Western diets in mice were equivalent to the average and high daily protein intake in Western societies and the 15% protein and 22% protein mixed meals in our human studies (compare Figure 8A, right panel with Figure 3A, both showing relative protein intake levels of our mouse and human studies superimposed on the average daily intake in the general population)[9, 11–14]. In addition, we included a low-protein Western diet, which provided the essential amount of protein required for mice, because of the purported health benefits of dietary protein restriction[33–37]. These diets were therefore used to study their atherogenic potential in the ApoE$^{-/-}$ mouse model over an 8 week period (Figure 7B), as we have previously done with the standard Western and high-protein Western diet[3]. Total body weight gain was inversely proportional to protein intake, with the lowest body weight at 8 weeks coinciding with the highest protein Western diet (Figure 7C). The differences in body weight among the groups were due to differences in fat mass whereas lean body mass was not different among groups (Extended Data Figure 7A). Serum cholesterol concentration increased markedly as a result of Western diet feeding and was not different between the low-, moderate-, and high-protein Western diet groups (Extended Data Figure 7B). On the other hand, serum triglyceride concentration was not affected significantly by low-, moderate-, and high-protein Western diet feeding (Extended Data Figure 7C).

At 8 weeks of Western diet feeding, atherosclerotic lesion size was quantified by using oil red O (ORO)-staining of aortic root sections. Compared with the low-protein diet, the high-protein, but not the moderate-protein Western diet increased plaque burden (Figure 7D). Similarly, characterization of plaque content showed higher macrophage content in lesions from the high-protein Western diet group as gauged by MOMA-2 staining (Figure 7E), along with more apoptotic cells via TUNEL staining (Figure 7F) and greater area of acellular debris indicative of larger necrotic cores (Figure 7G). Immunofluorescence staining for pS6 in plaque macrophages indicated a linear trend in mTOR activation from the low- to the moderate- to the high-protein Western diet (Figure 7H). Taken together, these results further support a dose-dependent threshold effect of dietary protein intake on macrophage mTORC1 activation and atherogenesis.

Finally, we confirmed that increased dietary leucine is both necessary and sufficient to confer the pro-atherogenic effect of high protein intake in vivo. For a period of 8 weeks, ApoE KO mice were randomized to feeding by 6 different diets, including: 1) moderate-protein Western Diet [called MP-WD], 2) high-protein Western Diet [called HP-WD], 3) MP-WD to which leucine, but no other amino acids, was added to match the leucine content of the HP-WD [called MP-WD+Leu], 4) MP-WD to which amino acids were added to match the total AA content of the HP-WD [called MP-WD+AA], 5) MP-WD to which additional amino acids, except for leucine, were added to match the contents of all amino acids, except leucine, in the HP-WD [called MP-WD+AA (Normal Leu)], and 6) nitrogen-adjusted version of MP-WD+AA (normal Leu), which consisted of the MP-WD with additional amino acids, except leucine, to make it isonitrogenous with the

HP-WD [called MP-WD+AA (Normal Leu (IsoN))] (Figure 8A and Supplemental Table). Serum cholesterol and triglyceride concentrations were not different among the different diet groups (Extended Data Figures 7D,E). Lipid-rich atherogenic lesions, expressed as proportion of total tissue alongside the pinned aorta and average lesion size in aortic root sections were significantly greater in the HP WD, MP WD+AA and MP WD+Leu groups compared with the MP WD whereas the MP WD + AA (Normal Leu) and the MP WD + AA (Normal Leu (IsoN)) did not affect atherogenic lesion size compared with the MP-WD (Figure 8B,C). Similar effects were also observed in other plaque phenotyping measures including macrophage content, apoptotic cell content, and necrotic core size (Extended Data Figure 7F–H).

## DISCUSSION

Atherosclerosis is the leading cause of myocardial infarction and stroke and accounts for more than 25% of all-cause mortality[38–40]. Results from studies in animal models suggest high protein intake is atherogenic[2,32], and the adverse effect of high protein intake on vascular health is driven by amino acid-mediated activation of mTORC1 in macrophages[3]. Here, we evaluate the dose response relationship between dietary protein intake and the amino acid-specificity of this amino acid-mTOR-autophagy mechanism in human monocytes/macrophages. We identify leucine as the key amino acid responsible for activating mTOR in macrophages and discover a threshold effect of high protein (and leucine) intake on this deleterious signaling pathway wherein dietary protein intake greater than about 25 grams per meal (or about ≥22% of energy requirement) activates mTOR signaling in monocytes/macrophages. Finally, by designing specific mouse diets with graded protein contents that represent the mouse equivalent of average and high dietary protein intakes in the general US population, we demonstrate the presence of a dietary protein threshold effect in driving atherosclerosis in mouse models that equates to about ≥25 g per meal, or about ≥22% of total energy intake. The culmination of our work highlights a mechanism whereby dietary protein intake, and specifically leucine intake, induces dose-dependent mTORC1-mediated inhibition of the autophagy-lysosomal degradation in monocytes/macrophages to promote atherogenesis (summarized in Figure 8D). This discovery provides a key mechanistic link between dietary protein and atherosclerotic cardiovascular disease risk that can help redefine dietary protein intake recommendations and can perhaps be leveraged as a therapeutic strategy.

Several aspects of our findings are unique and worthy of discussion. First, we identified leucine as the predominant activator of mTOR in monocytes/macrophages and the critical amino acid underlying atherosclerotic lesion development. Second, we established that leucine is both necessary and sufficient to cause the mTOR-mediated adverse effect of high protein intake on atherogenesis in vivo. Prior studies have implicated BCAAs in the progression of metabolic and cardiovascular disease risk[41–43]. However, the focus has thus far largely remained on BCAAs as a whole with little distinction between the functional effects of leucine, isoleucine, or valine. Recent data on the effects of individual BCAA on metabolic function[44] and mortality[45] corroborate the concept that BCAAs have independent cardiometabolic effects and should not be considered in unison. For example, restriction of dietary isoleucine and valine leads to metabolic benefits in mice while

restriction of leucine is inconsequential[44]. The deleterious role of isoleucine and valine was mechanistically linked to hepatic FGF21-UCP1 signaling with no relation to the mTORC1 pathway[44]. Furthermore, the hepatic catabolism of isoleucine and valine (and not leucine) was implicated in this process[44], suggesting that differences in the metabolic fates of these amino acids needs to be considered in follow up mechanistic studies.

Our data supports the presence of a threshold effect of leucine concentration on mTORC1 activation and downstream sequela in monocytes/macrophages of both mice and humans. Prior studies evaluating the physiological effects of leucine-mTORC1 signaling have largely focused on skeletal muscle and its impact on muscle protein synthesis[46]. In muscle, an increase in plasma leucine concentration of about 2-times basal values drives muscle mTORC1 activation, whereas lower plasma leucine levels have no or only marginal effects[47–49]. Although these results demonstrate that the degree of circulating leucine levels govern its impact on mTORC1 signaling under physiological conditions, our data now suggest that a leucine threshold for mTORC1 signaling in macrophages can have pathophysiological consequences, driving an aherogenic environment that consists of autophagy dysfunction, generation of ROS, and activation of pro-apoptotic pathways. It is noteworthy to point out that the threshold of dietary leucine-activated mTORC1 signaling in macrophages we identified appears to coincide with the maximal stimulatory effect of dietary protein on muscle protein synthesis at about 25-30 g per meal[50,51], suggesting a complex integrated metabolic network of multi-organ physiological functions that ensure optimal health. Nevertheless, it is often recommended to consume more protein to prevent the age-associated declines in skeletal-muscular mass and function[8,9,52]. A holistic, multi-organ and tissue/cell-specific assessment will be needed to define the exact threshold of the delicate balance between dietary proteins beneficial and adverse effects on health to help define optimal dietary protein intake.

The observations we made have important clinical implications. The Institute of Medicine, the World Health Organization, and the Food and Agriculture Organization of the United Nations have established a recommended amount of protein intake (0.8 g/kg/d or ~11% of daily energy requirements) to maintain nitrogen balance[7,50]. In addition, much higher protein intake (up to about 35% of daily energy requirements) are considered acceptable[7,10,50]. Furthermore, it has been proposed that middle-aged and older adults consume at least 25 g to 30 g of protein, particularly leucine-rich proteins, with each meal and a total of at least 1.0–1.2 g/kg/d to prevent age-associated declines in muscle mass[8,51], even though the effectiveness of this approach has not been firmly established[50]. High protein intake is also recommended to help people lose weight[53,54]. Many Americans and adults in other Western cultures already consume such high amounts (≥1.2 g/kg/d) of protein[10–14]. Moreover, the majority of protein in the diet of people living in Western Societies is derived from leucine-rich animal proteins[11,15–19]. Here, we find such high protein intake, and the corresponding leucine intake, have potential detrimental effects on vascular health (Figure 9). The adverse effects of high protein intake on atherogenesis in our mouse studies were present even though mice who consumed the high protein diet gained less body fat and total body weight. The results from the present study help explain the increased cardiovascular disease mortality associated with high total and high animal

protein, but not high plant protein intake in several population studies[16,21,55] and provide a direct potential mechanistic link for this association.

Our study has some limitations. First, we used Apo-E$^{-/-}$ mice, because they are prone to atherosclerosis. However, it is possible that these mice are not representative of other mice, because genotype affect metabolic, physiologic, and molecular response to protein restriction[56]. Nevertheless, we also studied HMDM and collected monocytes from people after they consumed meals with different amounts of protein and found the same effects of dietary protein/amino acids on mTORC1 signaling and downstream effects. Therefore, we are confident that our mouse model has translational value. Secondly, most of the mice in our study were male and it is possible that the responses of female mice to high protein intake differ from those of male mice. However, we consider this unlikely because we found high protein intake activates monocyte/macrophage mTORC1 in both male and female mice. Third, Study 1, our initial very high protein proof-of-concept study, involved liquid meals rather than mixed meals. Although the metabolic response to a liquid meal may differ from that of a mixed meal, it does not diminish the clinical implications of the results from this study, because liquid meals are commonly used as meal supplements or even meal substitutes. Lastly, our studies of protein ingestion and amino acid effects on human monocytes/macrophages were based on middle-aged participants without overt cardiovascular disease. It is possible that protein and leucine intake thresholds, the magnitude of leucine-mediated mTORC1 signaling towards autophagy and downstream sequelae are different in older adults or those with overt cardiovascular disease. Comparisons of monocyte/macrophages in older adults or participants with and without cardiovascular disease would be informative follow up studies and particularly important because these individuals may be recommended to consume dietary protein supplementation. Furthermore, we studied circulating monocytes and HMDMs, but not plaque macrophages in people. Although amino acid-dependent mTOR signaling could be different in plaque macrophages, our prior studies in mice have shown macrophages from various tissue beds, including atherosclerotic plaques and spleen, have similar robust elevations in leucine uptake and mTORC1 signaling upon dietary protein intake[3].

We should also note some limitations in our proposed mTOR mechanism. We did not evaluate the intracellular sensors which link leucine to mTORC1 activation in macrophages. Amino acid-mediated induction of mTOR occurs through formation of the lysosome-bound Ragulator complex and subsequent recruitment of the mTOR complex (mTORC1) to the lysosome surface[57,58]. Further regulation takes place by distinct GTPase Activating Proteins Toward Rags (GATOR) complexes which can either inhibit or sustain amino acid-mediated mTORC1 activation and downstream signaling[58]. The mechanism by which distinct amino acids are sensed and regulate mTORC1 is complicated and not completely defined but there have been insights gained in the past few years on the network of intracellular amino acid sensors and transporters[59]. Given the significant abundance of leucine in dietary protein and its role as an essential amino acid, it is thus not surprising that leucine appears to have multiple mechanisms to regulate mTORC1. For example, Sestrins (SESN) act as cytoplasmic sensors of leucine[4], leucyl-tRNA synthetase 1 (LRS) is important for lysosomal mTORC1 activation[60], solute carrier 38A9 (SLC38A9) transports leucine from the lysosomal lumen to the cytoplasm to activate mTORC1 through Sestrins[61], and the leucine

metabolite acetyl-CoA activates mTOR through acetylation of Rag GTPase[62]. Furthermore, macropinocytosis could be involved in amino acid transport into lysosomes and downstream mTORC1 activation[63,64]. The relative importance of each of these processes in determining intracellular leucine concentrations and facilitating leucine-mediated mTORC1 activation is cell-type specific[62,65]. Although we have identified leucine as the key determinant of mTORC1 activation in monocytes/macrophages and demonstrated the presence of distinct thresholds above which signaling takes place, the operative intracellular mechanism(s) activating mTORC1 are unknown and would be a critical follow-up to our current studies.

In our study, a low protein Western diet did not prevent or cause less atherosclerosis Apo-E$^{-/-}$ mice than a moderate protein Western diet, suggesting protein intake within a wide range of physiological and potentially even insufficient protein intakes does not have any cardiovascular consequences. This observation contrasts with the improved cardiometabolic function biomarkers in mice that are protein-restricted[33,44,66,67]. It is possible that there are physiological pathway-specific differences in the effect of dietary protein. It is also possible that there are moue strain-specific difference in the effect of dietary protein on physiological functions. In addition, the duration of feeding the diets could affect the outcomes.

In conclusion, our work has uncovered a mechanism by which high-protein intake, through an increase in plasma leucine, causes mTORC1-mediated inhibition of monocyte/macrophage autophagy and subsequent atherogenesis. This has important clinical and public health implications because protein intake at any level above the minimum recommended daily intake of 0.8 g/kg/d is considered acceptable and safe and has become popular[7,50]. However, the results from our studies suggest high protein intake should be considered with caution. Additional mechanistic and prospective clinical trials that evaluate the effects of different amounts and types of dietary proteins on the atherogenic signaling pathways we have identified are needed to ensure current dietary guidelines concerning protein intake are appropriate or revised accordingly.

## MATERIALS AND METHODS

The content of this section describes our previously published protocols with some noted modifications.[3]

### Human Studies

**Study participants—**A total of twenty-three men and women completed the studies, which were approved by the Institutional Research Board of the Human Research Protection Office of Washington University School of Medicine in St. Louis, Missouri and were registered on ClinicalTrials.gov (NCT03946774 and NCT03994367). All participants provided written informed consent before enrolling in the study and completed a screening evaluation that included a medical history and physical examination, and standard blood tests after they fasted for 12 hours overnight. For the human subjects studies, we initially chose the sample size based on the robustness of mTOR activation following exposure to amino acids and high- and low-protein oral gavage in our mouse models. A post-hoc power analysis we conducted with the data we obtained from our study determined that the sample size was sufficient to detect a statistically significant difference in mTOR activation

(assessed as phospho-S6 content) after ingesting the very high protein and standard protein liquid meals. The postprandial phospho-S6 content in monocytes 3 h after ingesting the very high protein liquid meal containing 50% of total energy as protein was 2.3 ± 2.2 (mean ± SD) times greater than after ingesting the corresponding control meal containing 10% of total energy as protein. Using these data and assuming <0.05 probability of Type I error, 90% power, and two-sided testing with a paired study design, we estimate that 13 participants are needed to detect a 2.3 fold difference. Ten participants would be sufficient to detect the same effect size assuming 80% power. Participants were eligible to participate in the study if they met the following inclusion criteria: i) age ≥21 years and ≤70 years and ii) body mass index ≥25.0 and ≤35.0 kg/$m^2$. Potential participants were excluded if they: i) were vegetarian or vegan or had intolerance to any ingredients in the study meals, ii) had a history of intestinal resection or rerouting; iii) had a disease or used medications that could affect the study outcome measures; iv) had significant (± 3%) body weight changes during three months before the study, v) consumed more than one alcoholic drink per day; or vi) engaged in structured exercise for more than 90 minutes per week. Participant flow is depicted in Extended Data Figure 8.

**Study 1 – Very-high protein liquid meal study—**Fourteen participants (Table 1) completed this study protocol (NCT03946774). Participants were enrolled between October 1, 2019 and December 17, 2020. Each participant completed two (standard and very high protein) 500 kcal liquid meal tests after an overnight fast in random order approximately one to two weeks apart in the Clinical and Translational Research Unit (CTRU) at Washington University School of Medicine in St. Louis, MO. The liquid meals were prepared in the CTRU Metabolic Kitchen by using varying amounts of Boost Plus® (a commercial nutrition supplement beverage, Nestlé), Unjury® (a commercial protein isolate product, Unjury Inc), nonfat dry milk powder, Sol Carb (a commercial nutritional carbohydrate polymer, Medica Nutrition), canola oil, and water. The standard meal contained 10% of total energy as protein, 17% as fat, and 73% as carbohydrates; the high protein meal contained 50% of energy as protein, 17% as fat, and 33% as carbohydrates. Participants were instructed to consume the meal within 5 min after baseline blood samples to determine plasma amino acid and TG concentrations and to isolate monocytes. Additional blood samples to determine plasma amino acid and TG concentrations and to isolate monocytes were collected at 1 h and 3 h after consuming the meal. In two participants, blood samples were also collected in CTRU at 30 min, 2 h, and 4 h after consuming the meal to initially establish the time-course of mTORC1 activation in monocytes.

**Study 2 – High protein mixed meal study—**Nine participants (Table 1) completed this study protocol, which represented an ancillary sub-study of a larger clinical trial (NCT03994367). Participants were enrolled between November 15, 2019 and December 2, 2020. Each of them completed two (standard and high protein) 450 kcal mixed meal tests after an overnight fast in random order approximately one to two weeks apart in the CTRU at Washington University School of Medicine in St. Louis, MO.The meals, which contained “real” food (potatoes, beans, onions, carrots, corn, bacon, fats, broth, spices) and were liquified by homogenization for consistency, were prepared in the CTRU Metabolic Kitchen. The standard mixed meal contained 15% of total energy as protein and the high

protein mixed meal contained 22% of energy as protein. The additional protein in the high protein meal was a blend of animal protein isolates (egg, chicken, beef, whey) and replaced both carbohydrates and fat in proportion to their contribution to non-protein energy content in the standard meal. The standard meal therefore contained a total of 450 kcal of which 15% were protein (17 grams), 50% were carbohydrates, and 35% were fat, and the high protein meal contained a total of 450 kcal of which 22% were protein (25 grams), 48% were carbohydrates, and 30% were fat. Participants were instructed to consume the meal within 30 min after baseline blood samples were collected to determine plasma amino acid and TG concentrations and to isolate monocytes. Additional blood samples to determine plasma amino acid and TG concentrations and to isolate monocytes were collected at 1 h and 2 h after starting the meal.

**Outcomes—**The primary outcome was the evaluation of mTORC1 signaling in circulating monocytes. The secondary outcome was the quantification of serum amino acids from participants. We chose mTORC1 signaling in circulating monocytes as the primary outcome because we found in animal models that mTORC1 activation drives atherosclerosis. Serum amino acid concentrations were the secondary outcome to determine the relationship between specific amino acids and monocyte mTORC1 activation. For evaluation of mTORC1 signaling in circulating monocytes, whole blood was collected in chilled tubes containing EDTA. For analysis with Western Blotting, whole blood was collected in chilled tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs) were isolated via centrifugation with Ficoll-Paque PLUS. CD14+ monocytes were isolated using the EasySep Human Monocyte Enrichment Kit. The analysis followed a standard Western Blotting protocol while primary antibodies for pS6 and S6 were utilized. For FACS-based analysis, PBMCs were isolated via centrifugation with Ficoll-Paque PLUS and stained with fluorochrome-conjugated macrophage markers CD45, CD14, and CD16. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% saponin, and incubated with p-S6 primary antibody followed by Alexa Fluor® 488 secondary antibody. All samples were analyzed using the BD Biosciences Canto II or LSR II flow cytometer and quantified using FlowJo software. The evaluation of mTORC1 signaling was based on the ratio of pS6/S6 intensity in analysis with Western Blotting and MFI of pS6 in FACS analysis. To determine amino acid concentrations, plasma was isolated from each sample by centrifugation and was then stored at −80 C until analysis. Gas chromatography-mass spectrometry (GC-MS) was used to quantify amino acid (except arginine) concentrations in human plasma. Arginine concentration in plasma was quantified by using liquid chromatography/tandem mass spectrometry.

**Blood sample processing and isolation of human circulating monocytes—**Whole blood was collected in chilled tubes containing EDTA (BD Vacutainer) and plasma from each sample was isolated by centrifugation within 30 min and was then stored at −80 °C until analysis. Peripheral blood mononuclear cells (PBMCs) were isolated via centrifugation with Ficoll-Paque PLUS (GE Healthcare). CD14+ monocytes were isolated using the EasySep Human Monocyte Enrichment Kit (19059, StemCell Technologies). FACS analysis was utilized to evaluate the isolation efficiency.

**Human Monocytes Derived Macrophages (HMDMs) Culture and Treatment—** PBMCs were isolated via centrifugation with Ficoll-Paque PLUS (GE Healthcare) through blood buffy coat from Blood Bank in Washington University in St. Louis. Fresh monocytes were isolated by CD14+ beads selection of PBMCs, then plated in RPMI and 20% fetal bovine serum with 100ng/ml M-CSF (Peprotech). On Day 1, Day 4, Day 6, and Day 9, fresh medium was added. On Day 12, differentiation efficiency was evaluated by using FACS and immunofluorescence analysis, and the following treatments were performed on fully differentiated macrophages: FCCP (20 μM; Sigma, C2920) and rotenone (40 μM; Sigma, R8875).

**Amino Acid Starvation and Stimulation of Cells—**Amino acid-rich RPMI 1640 (Sigma, R8758) and amino acid-free RPMI 1640 (US Biological Life Science, R8999-04A) media containing 10% dialyzed FBS (Sigma, F0392) were used for macrophage cell culture experiments. When testing the specific effect of individual amino acids on mTORC1 signaling, autophagy and macrophage functional assays, amino acid-free RPMI 1640 was supplemented with an amino acid concentration indicated.

**Mouse Studies—**Animal protocols were approved by the Washington University Animal Studies Committee (Protocol #22112260). All mice used in this study were on C57BL/6J background and ordered from Jackson Lab. ApoE genotyping was performed using standard PCR techniques. Mice housed in a specific pathogen–free barrier facility at 22-24 °C and 30% humidity with a standard light-dark cycle (12:12) and routine checks of each animal's health status. Mice were weaned at 3 weeks of age to a standard mouse chow diet (Labdiet, 5053). For *in vivo* atherosclerosis-related-experiments, male mice of the stated genotype were started at ~8 weeks of age with low-protein (TD140106: 0.15% cholesterol, 7% energy protein, 42% energy fat, 51% energy carbohydrate), moderate-protein (TD140105: 0.15% cholesterol, 21% energy protein, 42% energy fat, 36% energy carbohydrate) or high protein (TD04524: 0.15% cholesterol, 46% energy protein, 43% energy fat, 11% energy carbohydrate) Western-type diets (all from Harlan). For the evaluation of both the sufficiency and the requirement of a high amount of leucine in the pathogenesis of atherosclerosis associated with a high protein Western Diet (HP WD), mice were randomized to 6 different diets, including ApoE KO mice were randomized to 6 different diets, including: 1) moderate-protein Western Diet [called MP-WD], 2) high-protein Western Diet [called HP-WD], 3) MP-WD to which leucine, but no other amino acids, was added to match the leucine content of the HP-WD [called MP-WD+Leu], 4) MP-WD to which amino acids were added to match the total AA content of the HP-WD [called MP-WD+AA], 5) MP-WD to which additional amino acids, except for leucine, were added to match the contents of all amino acids, except leucine, in the HP-WD [called MP-WD+AA (Normal Leu)], and 6) nitrogen-adjusted version of MP-WD+AA (normal Leu), which consisted of the MP-WD with additional amino acids, except leucine, to make it isonitrogenous with the HP-WD [called MP-WD+AA (Normal Leu (IsoN))]. The detailed content of each diet was shown in Supplement Table 1. For the evaluation of plasma amino acid and TG concentrations and mTORC1 signaling in blood monocytes after gavage with different diets, both male and female C57BL/6J mice at ~8 weeks of age were used. Mice were fasted for 4 hours and then given by gavage a solution that contained either 0.8 g protein per

kg or 1.6 g protein per kg or a solution that contained 0.8 g protein per kg plus leucine to match the total leucine content of the solution with 1.6 g protein per kg. Blood was collected for isolation of PBMCs and to determine plasma amino acid, cholesterol, and TG concentrations at time points indicated. The dosage of protein for gavage was chosen based on results we obtained previously[3] with the goal to mimic postprandial circulating amino acid concentrations observed in our human studies. Atherosclerosis experiments on ApoE-null mice produce lesions with standard deviation of ~20%. Using $\alpha$=0.05 and power=20%, detecting differences of 25-30% in lesion area between 2 cohorts of ApoE-null mice requires n=7-10 mice. All atherosclerosis studies in this manuscript exceeded the minimum number of mice required by these sample size calculations and are denoted in the figures/figure legends. For *in vitro* experiments, macrophages derived from male and female mice aged between 2 to 6 months were used and within each experiment only mice with the same sex and similar age were compared.

**Mouse Macrophage Isolation, Culture and Treatment—**Standard techniques were used to isolate thioglycollate-elicited peritoneal macrophages and bone marrow-derived macrophages. Briefly, male or female mice at ~8 weeks of age were injected with 4% sterile thioglycollate media (Sigma, T9032) intraperitoneally, and 4 days later, lavaged peritoneal macrophages were counted and plated (DMEM with 10% fetal bovine serum). Where indicated, the following treatments were performed on macrophages: FCCP (20 μM; Sigma, C2920) and rotenone (40 μM; Sigma, R8875). Treated cells were harvested at various times for protein isolation using standard techniques, stained with antibodies for FACS analysis, or fixed with 4% paraformaldehyde for immunofluorescence microscopy.

**Fluorescence Activated Cell Sorting (FACS)—**FACS analysis for mitochondria dysfunction and ROS levels was performed on cultured HMDMs. Cultured macrophages were plated (Greiner 665102), treated with the indicated reagents, and incubated with MitoTracker Red CMXRos (250 nM; Life Technologies, M7512), MitoTracker Green FM (200 nM; Life Technologies, M7514) at 37°C for 30min. Cells were collected and resuspended in FACS buffer (2% FBS in PBS) for subsequent flow cytometry.

FACS analysis for phosphorylated S6 ribosomal protein (phospho-S6) was performed on human blood monocytes and mouse blood monocytes. For human blood monocytes, PBMCs were isolated via centrifugation with Ficoll-Paque PLUS and stained with fluorochrome-conjugated macrophage markers CD45, CD14, and CD16 (Biolegend, 1:200). Mouse blood leukocytes were isolated and stained with fluorochrome-conjugated monocytes markers CD45, CD11b, and Ly-6C (Biolegend, 1:200). Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% saponin, and incubated with p-S6 (Cell Signaling Technology 4856S, 1:200) followed by Alexa Fluor® 488 secondary antibody (Invitrogen A11008; 1:500). All samples were analyzed using the BD Biosciences Canto II or LSR II flow cytometer with BD FACSDiva software v 6.0 and quantified using FlowJo software v 10.8. The Gating strategies were shown in Extended Data Fig. 9.

**Immunofluorescence Microscopy—**Immunofluorescence (IF) imaging of macrophages and frozen-tissue sections was performed as follows. Cells or tissues were fixed with 4% paraformaldehyde, blocked and permeabilized (1% BSA, 0.2 %

milk powder, 0.3% Triton X-100 in TBS; pH 7.4), and incubated with the antibodies sequentially. Specificity of staining was tested in control experiments either by omitting primary antibodies or using samples from knock-out mice where available. The following primary antibodies were used in 1:250 dilutions: MOMA2 (AbDSerotec, MCA519C, 1:250), LC3 (MBL, PM036, 1:250), p-S6 (Cell Signaling Technology 4856, 1:100), mTOR (Cell Signaling Technology, 2983, 1:250), Lamp2 (Abcam, ab13524, 1:250), COX IV (Abcam, ab14744, 1:200) and CD68 (Biorad, MCA1957, 1:250). Species-specific fluorescent secondary antibodies (Alexa Fluor® 488 Goat anti-Rabbit IgG (H+L), A11008; Alexa Fluor® 488 Goat anti-Rat IgG (H+L), A11006; Alexa Fluor® 594 Goat anti-Rat IgG (H+L), A11007; Alexa Fluor® 594 Goat anti-Mouse IgG (H+L), A11005) were obtained from Invitrogen/Life Technologies (1:250). CellEvent Caspase-3/7 Green Detection Reagent (Life Technologies, C10423), DeadEndFluorometric TUNEL System (Promega, G3250), Dihydroethidium (DHE) (Life Technologies, D11347) were used according to the manufacturer's protocol. A Zeiss LSM-700 confocal microscope was used for image acquisition (ZEN Black Edition software (2011) and images quantified using were quantified using ZEN Blue Edition software (2011) (Carl Zeiss AG). .

**Western Blotting—**Cells were lysed in a standard RIPA lysis buffer (150 mM NaCl; 10 mM Tris-HCl, pH 7.2; 0.1% Triton X-100; 1% sodium deoxycholate; 5 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich, 4693132001) and phosphatase inhibitors on ice. Lysed samples were centrifuged at 10,000 *g* for 10 min. Standard techniques was used for protein quantification, separation, transfer, and blotting. The following primary antibodies were used: p-S6K (9234, 1:1000), S6K (2708, 1:2000), p-S6 (2215, 1:1000), S6 (2217, 1:2000), p-ULK1 (14202, 1:2000), p-AMPKα (2535, 1:1000), AMPKα (2532, 1:2000), and HRP-linked anti-rabbit IgG (7074, 1:2000) were all from Cell Signaling Technology. ULK1 (A7481, 1:2000) was ordered from Sigma-Aldrich. were all from Cell Signaling Technology. Western blot images were obtained with Bio-Rad ChemiDocMP Imaging system and the densitometry was quantified with ImageJ bundled with 64-bit Java 1.8.0.

**Quantification of Serum/Plasma Amino Acid and Cholesterol Concentrations —**Gas chromatography-mass spectrometry (GC-MS) was used to quantify amino acid (except arginine) concentrations in human and mouse plasma, and macrophages isolated from mouse spleen and aorta as we have previously described[68,69]. Arginine concentration in plasma was quantified by using liquid chromatography/tandem mass spectrometry[70]. Serum cholesterol were assayed from blood obtained from mice after 6-hour fast per manufacturer's protocols (Thermo Scientific TR13421). Serum TG concentration was determined by using a commercial kit (Thermo Scientific TR22421).

**Atherosclerotic Lesion Assessment—**Quantification of atherosclerosis at the aortic root was as follows. PBS-perfused hearts were placed in a cryostat mold containing tissue freezing medium. 10 μm thick sections were taken from the samples beginning just caudal to the aortic sinus and extending into the proximal aorta. Slides were fixed with 4% paraformaldehyde and stained with Oil Red O. Images were taken by EVOS XL Core Cell

Imaging system and Oil Red O positive regions were quantified using ZEN Blue Edition software (2011) (Carl Zeiss AG).

**Randomization—**Atherosclerosis experiments in mice were conducted on littermate mice housed in the same facility born within 2-3 weeks of each other. Allocation of mice to each treatment group was random. For in vitro experiments, cells were randomly allocated into different groups. A staff member of the Razani lab who was not part of the study team created and maintained the random allocation of study participants by using a random number generator. In the human subjects studies, the order of the different test meals was randomized. A staff member of the Center for Human Nutrition who was not part of the study team created and maintained the random allocation of study participants by using a random number generator.

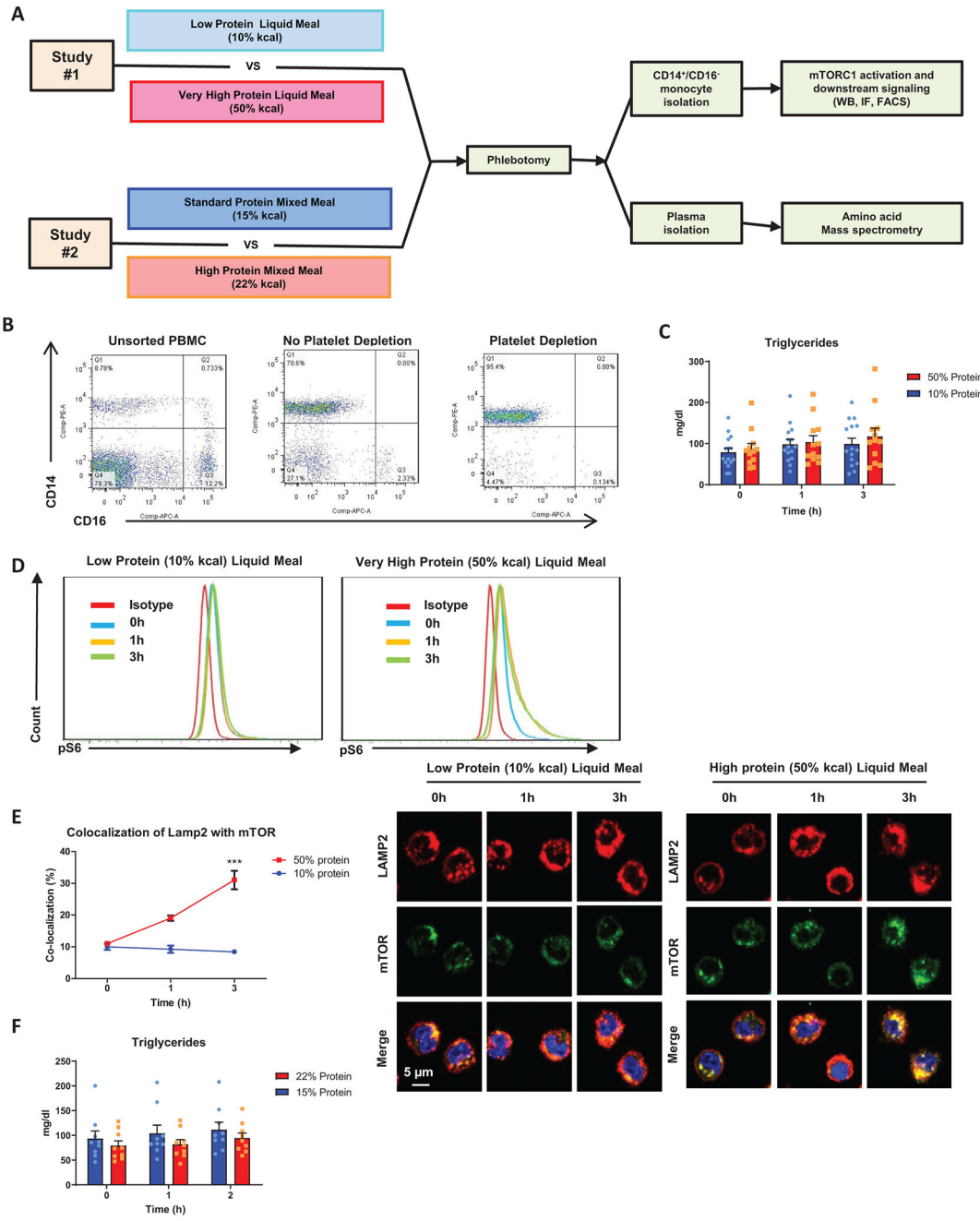
**Blinding—**Operators for data collection were blinded. Investigators were blinded to group allocation during data analyses.

**Data exclusions—**No data were excluded from the analyses.

**Reporting on sex—**We studied both men and women, but sex and/or gender was not considered in the data analysis because we had no reason to test sex- or gender-based hypotheses. Participants' sex was determined by self-report and participants' sex is clearly noted in Table 1 and relevant figure legends. For the evaluation of plasma amino acid and triglyceride concentrations and mTORC1 signaling in blood monocytes after gavage with different diets, both male and female C57BL/6J mice were used. For in vitro experiments, macrophages derived from male and female mice aged between 2 to 6 months were used. Within each experiment only mice with the same sex and similar age were compared.

**Statistical Analyses—**Statistical significance of group and/or treatment effects and group by treatment interactions were evaluated by using the Student t-test or ANOVA (for multiple groups/conditions) followed by either Dunnett's test (when multiple groups are compared with a single control) or Tukey's multiple comparison test. Data distribution was assumed to be normal but this was not formally tested. Data are presented as mean ± SEM. Statistical significance is represented as follows: *P ≤ 0.05, **P < 0.01, ***P < 0.001, NS=not significant.

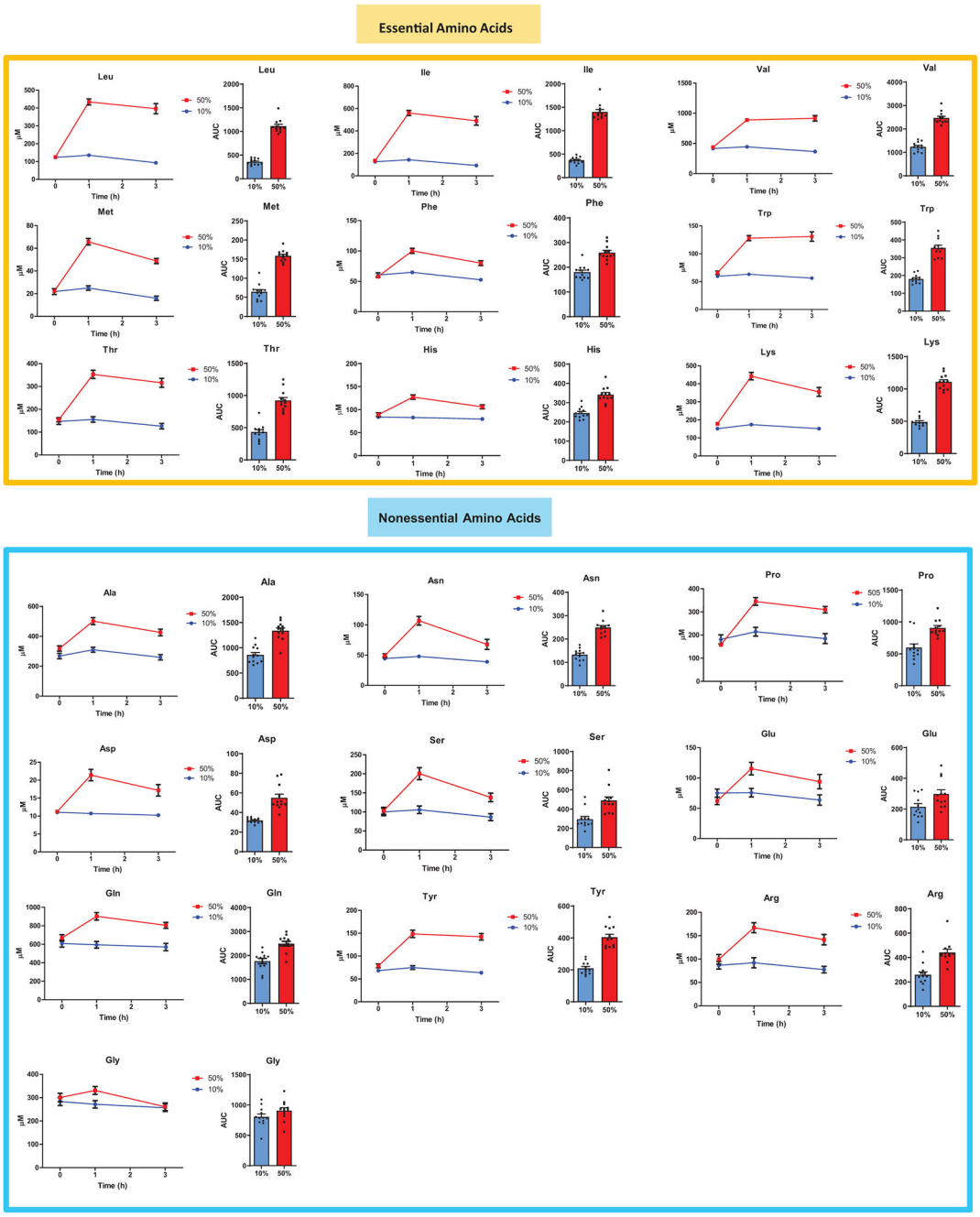
## Extended Data




**Extended Data Figure 1.**

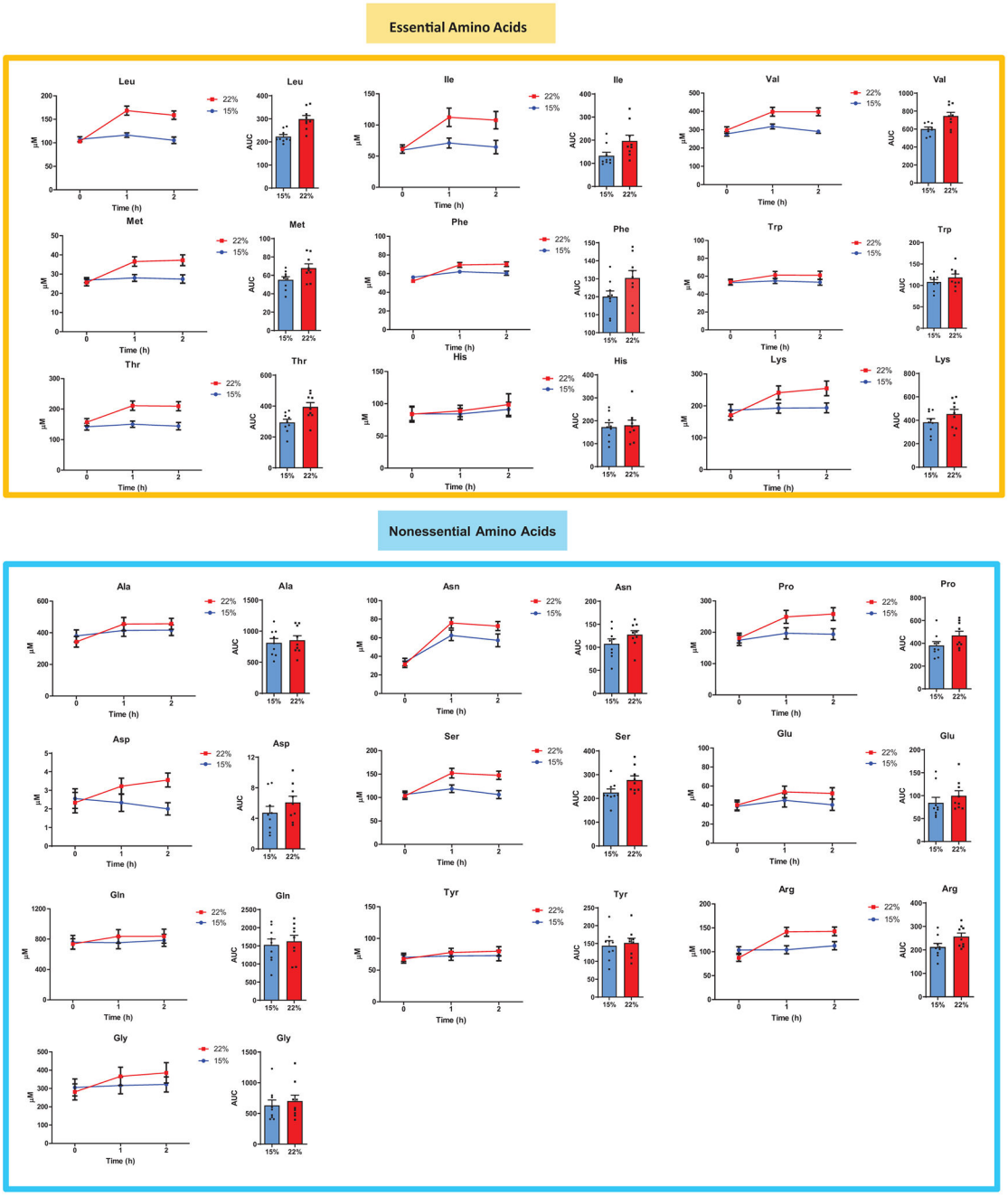
**(A)** Study #1 evaluated the effect of consuming extremes of protein intake as liquid meals (10% kcal protein vs 50% kcal protein) on monocyte mTORC1 activation and downstream functional events. Study #2 focused on a “real-world” setting and evaluated the effects of a standard mixed meal and a high-protein mixed meal (15% kcal vs 22% kcal as protein) on the same outcomes. Blood samples from participants were obtained at baseline

and at defined time intervals after meal consumption, plasma was isolated for evaluating amino acid mass concentration, and CD14+/CD16− monocytes were isolated for mTORC1 signaling evaluation using Western blotting (WB), immunofluorescence microscopy (IF), and fluorescence-activated cell sorting (FACS). **(B)** Representative FACS pictures showing high efficiency of circulating monocytes isolation from participants' blood. **(C)** Plasma triglyceride (TG) concentration before and after participants (n=12) consumed a very high protein (50% kcal) or a low protein (10% kcal) liquid meal (time as indicated). **(D)** Representative FACS pictures for Fig 2D. **(E)** Quantification of mTORC1-LAMP2 colocalization in isolated circulating monocytes participants (n=5) consumed a very high protein (50% kcal) or a low protein (10% kcal) liquid meals (time as indicated). P <10e-8 and determined by Two-way ANOVA with Sidak's multiple comparisons. Representative pictures on the right. **(F)** Plasma TG concentration before and after participants (n=9) consumed a standard mixed meal (15% kcal protein) or a high protein (22% kcal protein) mixed meal (time as indicated). For all graphs, data are presented as mean ± SEM. ***P < 0.001.



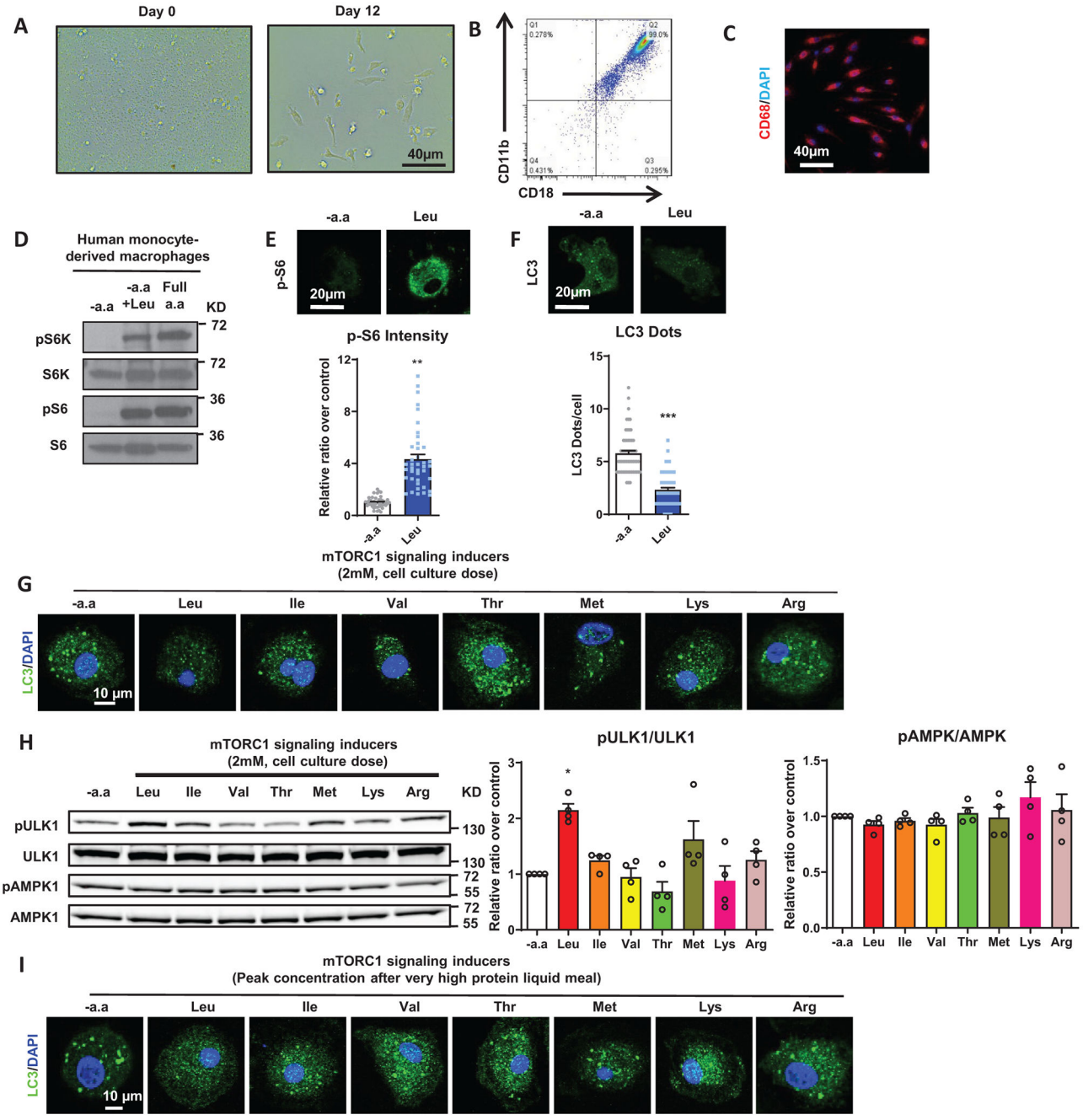

**Extended Data Figure 2.**

Plasma concentrations of 20 amino acids before and after participants (n=12) consumed a very high protein (50% kcal) or a low protein (10% kcal) liquid meal (time as indicated). For all graphs, data are presented as mean ± SEM.




**Extended Data Figure 3.**

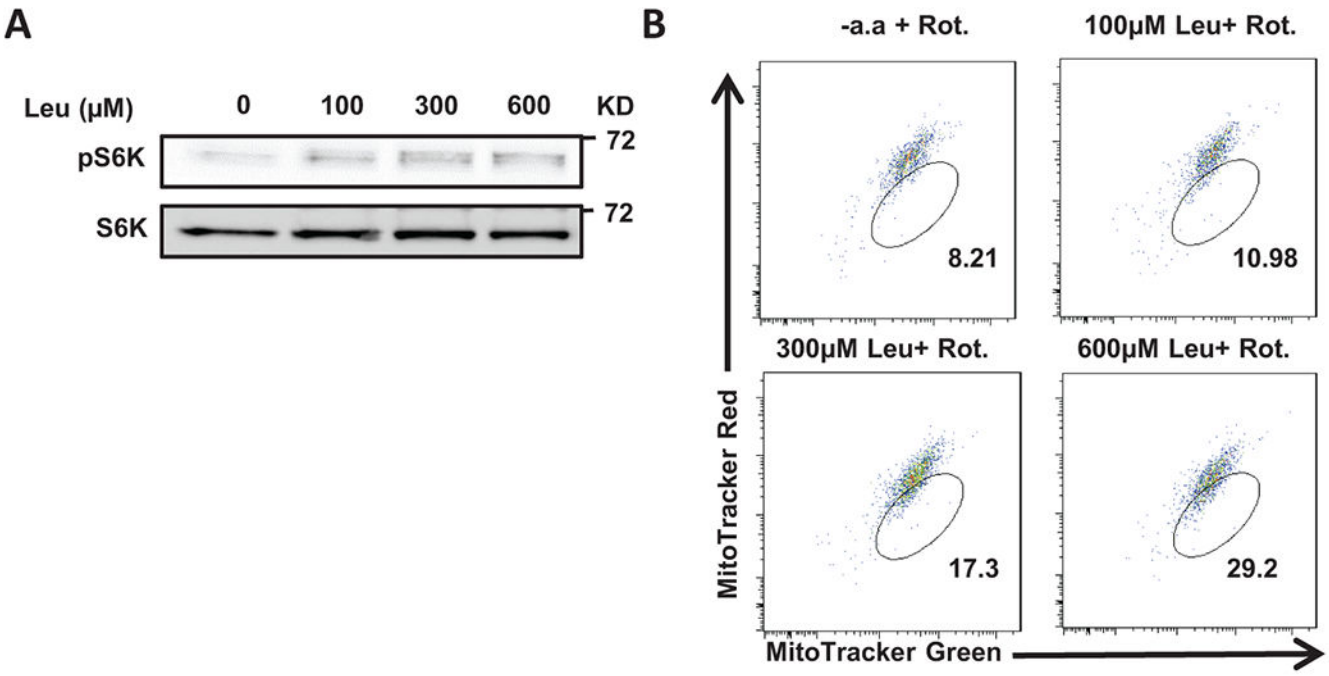
Plasma concentrations of 20 amino acids before and after participants (n=9) consumed a standard mixed meal (15% kcal protein) or a high protein (22% kcal protein) mixed meal (time as indicated). For all graphs, data are presented as mean ± SEM.




**Extended Data Figure 4.**

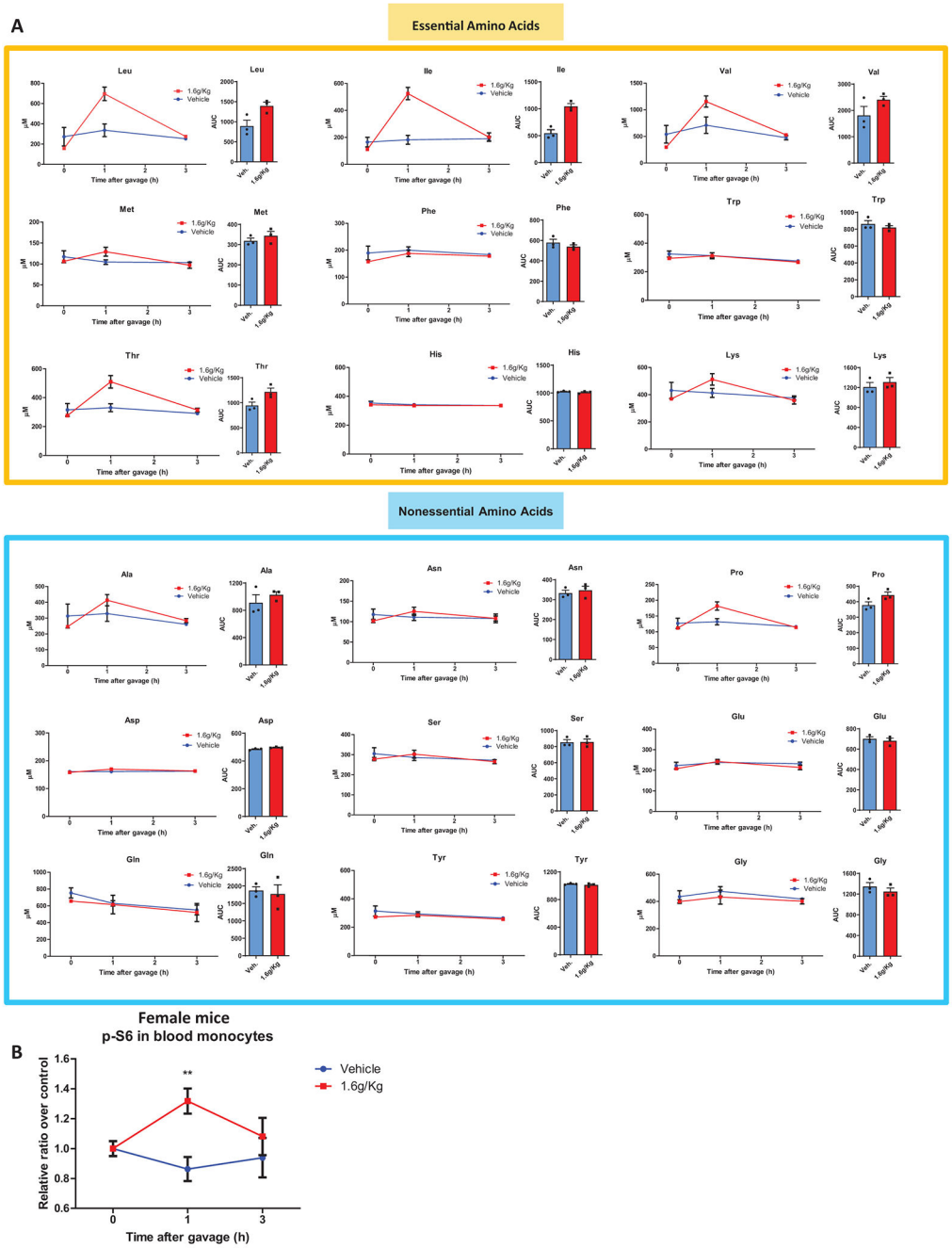
**(A)** Representative images of cultured human monocytes-derived macrophages (HMDMs) from isolated human circulating monocytes before and after 12 days of differentiation. The picture represents for three independent experiments. **(B-C)** FACS (B) and immunofluorescence (C) analysis of differentiated HMDMs by macrophage markers CD11b, CD18, and CD68. The picture represents for three independent experiments. **(D)** Western blot analysis of mTOR activation in HMDM (using phospho-S6 and -S6K) after treatment with leucine (2mM) or full amino acids. The picture represents

for three independent experiments. **(E-F)** Immunofluorescence microscopy analysis and quantification of mTORC1 activation (using phospho-S6) (E) and autophagy inhibition (using LC3 puncta formation) (F) in HMDMs treated with 2 mM leucine. The IF data (E and F) were obtained from three independent experiments with analysis of n ≥ 15 cells per experiment (n=45 in Group -aa and n=40 in Group Leu for E; n=58 in Group –aa and n=58 in Group Leu for F). P<10e-8 for E and F. **(G)** Representative images of LC3 in HMDMs for Figure 5D. **(H)** Immunoblot analysis of the phosphorylation of AMPK and ULK1 in HMDMs by the selected amino acids (applied at 2mM). n=4 independent experiments per group. P=0.00132 and determined by One-way ANOVA with Dunnett`s multiple comparisons. **(I)** Representative images of LC3 in HMDMs for Figure 5G. For all graphs, data are presented as mean ± SEM, **P < 0.01 and ***P < 0.001.



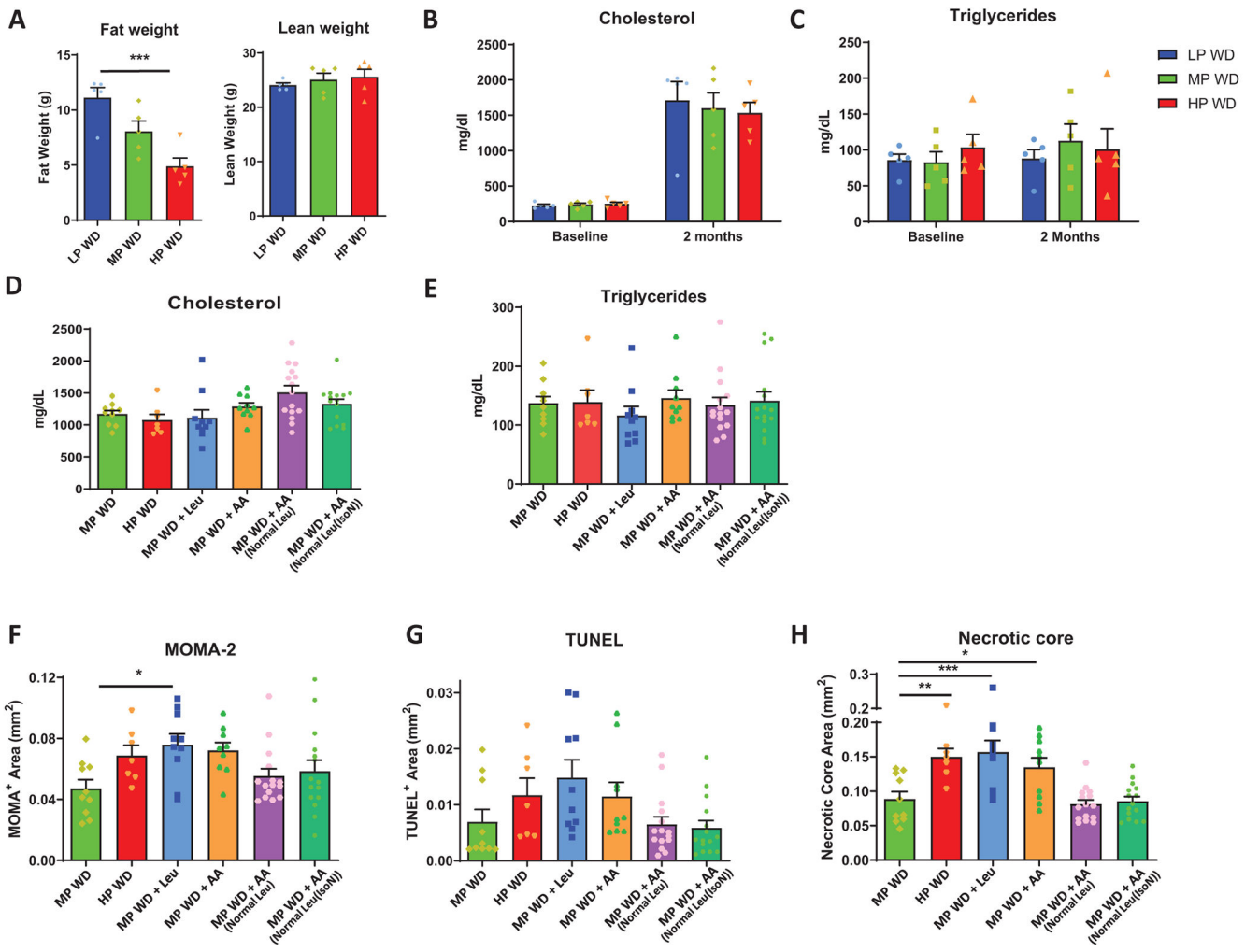

**Extended Data Figure 5.**

(**A)** Western blot analysis of the dose-dependent effect of leucine on mTORC1 activation (using phospho-S6K) in human monocyte-derived macrophages (HMDMs). The picture represents for three independent experiments. **(B)** Representative FACS plots for Fig 6E.




**Extended Data Figure 6.**

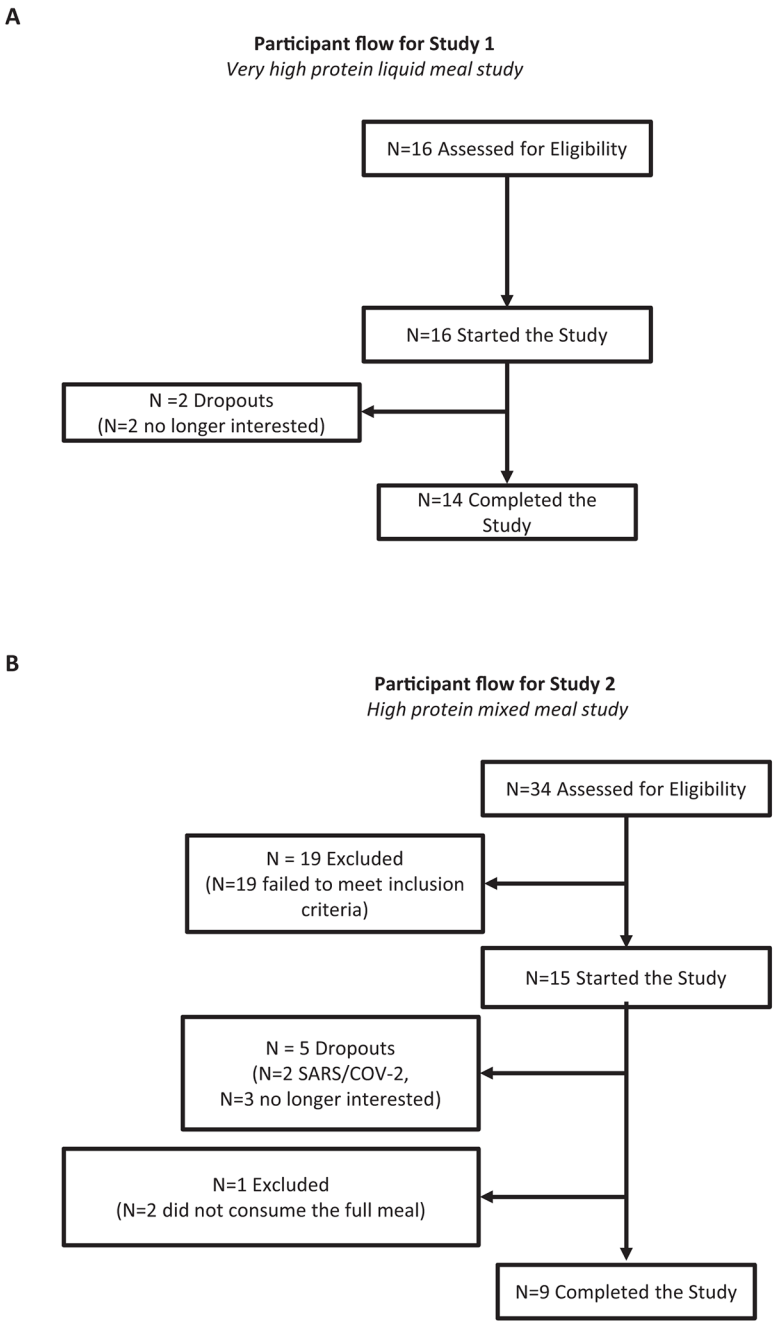
**(A)** Plasma concentrations of 20 amino acids before and after male mice were gavaged with a solution that contained 1.6 g/kg protein or vehicle (times as indicated). n=3 mice for each group. **(B)** FACS analysis of mTORC1 activation in blood monocytes (using phosphorylated S6) from female mice gavaged with the same protein solution compared with vehicle (times as indicated). n=4 mice for each group. P=0.00829 and determined by Two-way ANOVA with Sidak's multiple comparisons. For all graphs, data are presented as mean ± SEM. **P < 0.01.




**Extended Data Figure 7.**

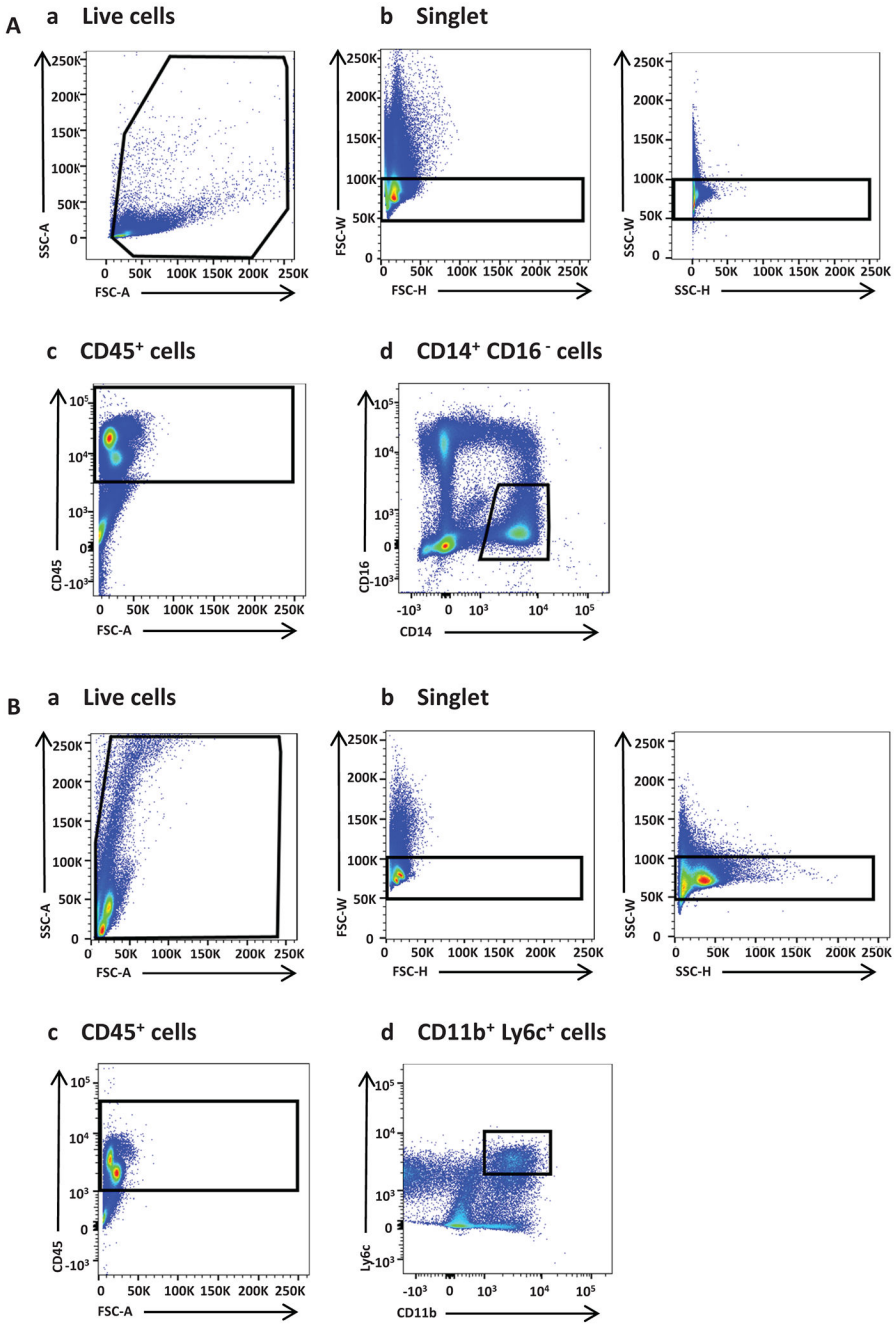
**(A)** Body composition (fat and lean weights) of ApoE$^{-/-}$ mice fed Western diets with low protein (LP WD), moderate protein (MP WD), or high protein (HP WD) contents for 8 weeks. n=5 mice for each group. P=0.00057. **(B-C)** Serum cholesterol (B) and triglyceride (C) concentrations in ApoE$^{-/-}$ mice before and after placement on low, moderate, or high protein Western diets for 8 weeks. n=5 mice for each group. **(D-E)** Serum cholesterol (D) and triglyceride (E) concentrations after ApoE$^{-/-}$ mice were fed six different diets with varying protein and leucine contents, including: 1) a moderate-protein Western Diet (MP WD, n=10), 2) a high-protein Western Diet (HP WD, n=7), 3) a MP WD to which an amount of leucine, but no other amino acids, was added to match the leucine content of the HP WD (MP WD+Leu, n=10), 4) a MP WD to which amino acids were added to match the total amino acid content of the HP WD (MP WD+AA, n=10), 5) a MP WD to which amino acids, except for leucine, were added to match the content of all amino acids, except for leucine, of the HP WD (MP WD+AA (Normal Leu), n=15), and 6) an isonitrogenous MP-WD+AA-Leu diet (MP WD+AA (Normal Leu (IsoN), n=15) which consisted of the MP-WD+AA-Leu diet with additional amino acids to match the total amino acid nitrogen content of the HPWD. **(F-H)** Plaque composition quantified by immunofluorescence

(IF) microscopy of aortic root sections for (F) macrophages (MOMA-2), (G) apoptosis (TUNEL+), and (H) necrotic core (MP WD, n=10; HP WD, n=7; MP WD+Leu, n=10; MP WD+AA, n=10; MP-WD+AA(Normal Leu), n=15; isoMP-WD+AA, n=15 biologically independent animals). P=0.0159 for F, and P= 0.000329, 0.02219, and 0.00417 for G. For all graphs, data are presented as mean ± SEM, and determined by One-way ANOVA with Dunnett's multiple comparisons. *P ≤ 0.05, **P < 0.01 and ***P < 0.001.




**Extended Data Figure 8.**

**(A,B)** CONSORT diagram of participant enrollment in Trial NCT03946774 (A) and Trial NCT03994367 (B).




**Extended Data Figure 9.**

**(A,B)** Gating strategy used to assess human (A) and mouse (B) circulating monocytes. **(A)** Dead cells and cell aggregates were removed in (a) and (b) to enrich single live cells, (c) human leukocytes were selected using CD45, and (d) human circulating monocytes were enriched by CD11b and F4/80 gating for further analysis. **(B)** Dead cells and cell aggregates

were removed in (a) and (b) to enrich single live cells, (c) mouse leukocytes were selected using CD45, and (d) mouse circulating monocytes were enriched by CD11b and Ly6c gating for further analysis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Role of the funders/sponsors.**

The funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

## Data Availability

All source data used to generate the figures are available as part of the manuscript files and from the corresponding authors upon reasonable request.

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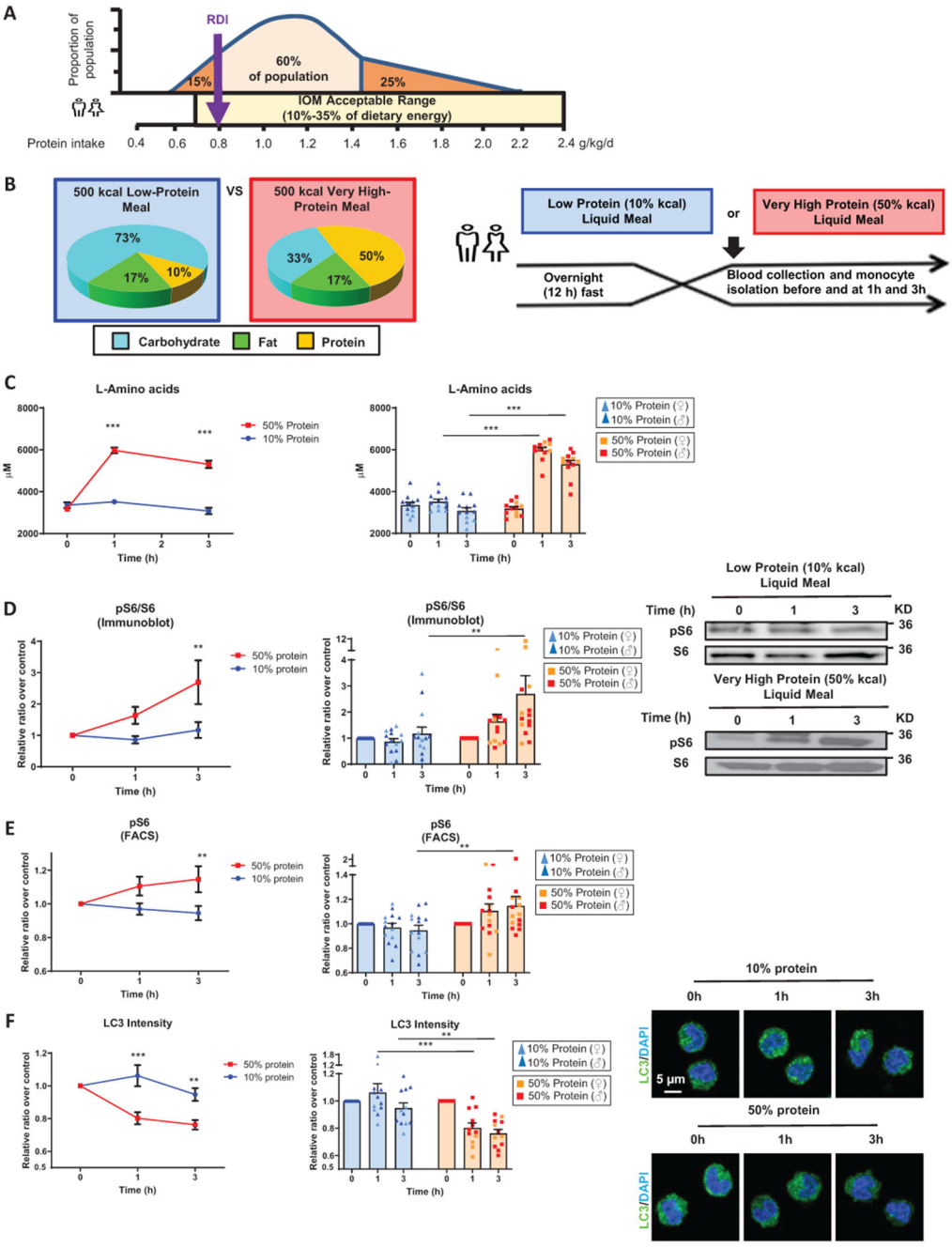



**Figure 1. Very high-protein intake induces mTORC1 activation and inhibition of autophagy in isolated human monocytes.**

**(A)** Protein intake distribution in the U.S. population. The Recommended Daily Intake (RDI) for protein is 0.8 g/kg/d. About 15% of the population consumes less and 85% of the population consumes more than the RDI. Average protein intake is about 15% of total energy and nearly 25% of the population consumes at least twice the RDI. (B) Schematic of the cross-over feeding study design. Fourteen participants (8 men, 6 women) consumed both very high protein and low protein liquid meals. The low protein meal provided 500 kcal of energy, 10% kcal as protein, 17% kcal as fat, and 73% kcal as carbohydrate. The very high

protein meal provided 500 kcal of energy, 50% kcal as protein, 17% kcal as fat, and 33% kcal as carbohydrate. **(C)** Total plasma amino acid concentration before and at 1 h and 3 h after consuming the low and very high protein meals (n=12, $P<10^{-6}$). **(D-E)** Before and after consuming the low and very high protein meals, mTORC1 activation in isolated circulating monocytes was evaluated by (D) Western blot (n=14) and (E) FACS analysis (n=14) of phosphorylated ribosomal protein S6 **(F)** IF microscopy analysis of LC3 autophagy marker in isolated circulating monocytes before and after consuming the low and very high protein meals (n=12, P=0.00435 for D, P=0.00619 for E, P=0.00001 and 0.0018 for F). n ≥ 100 cells were analyzed in Figures E. For all graphs, data are presented as mean ± SEM, and determined by Two-way ANOVA with Sidak's multiple comparisons. **P < 0.01 and ***P < 0.001.

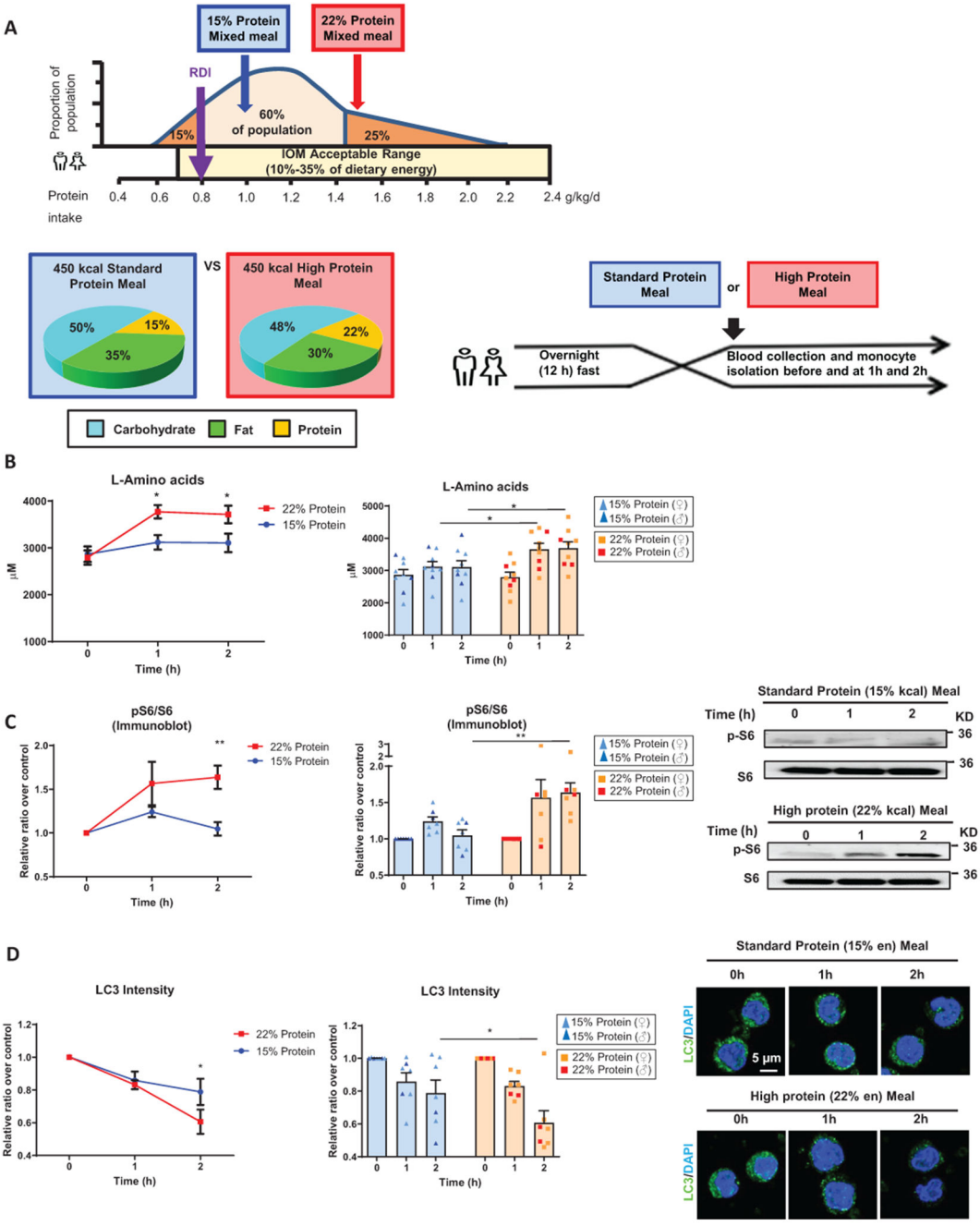
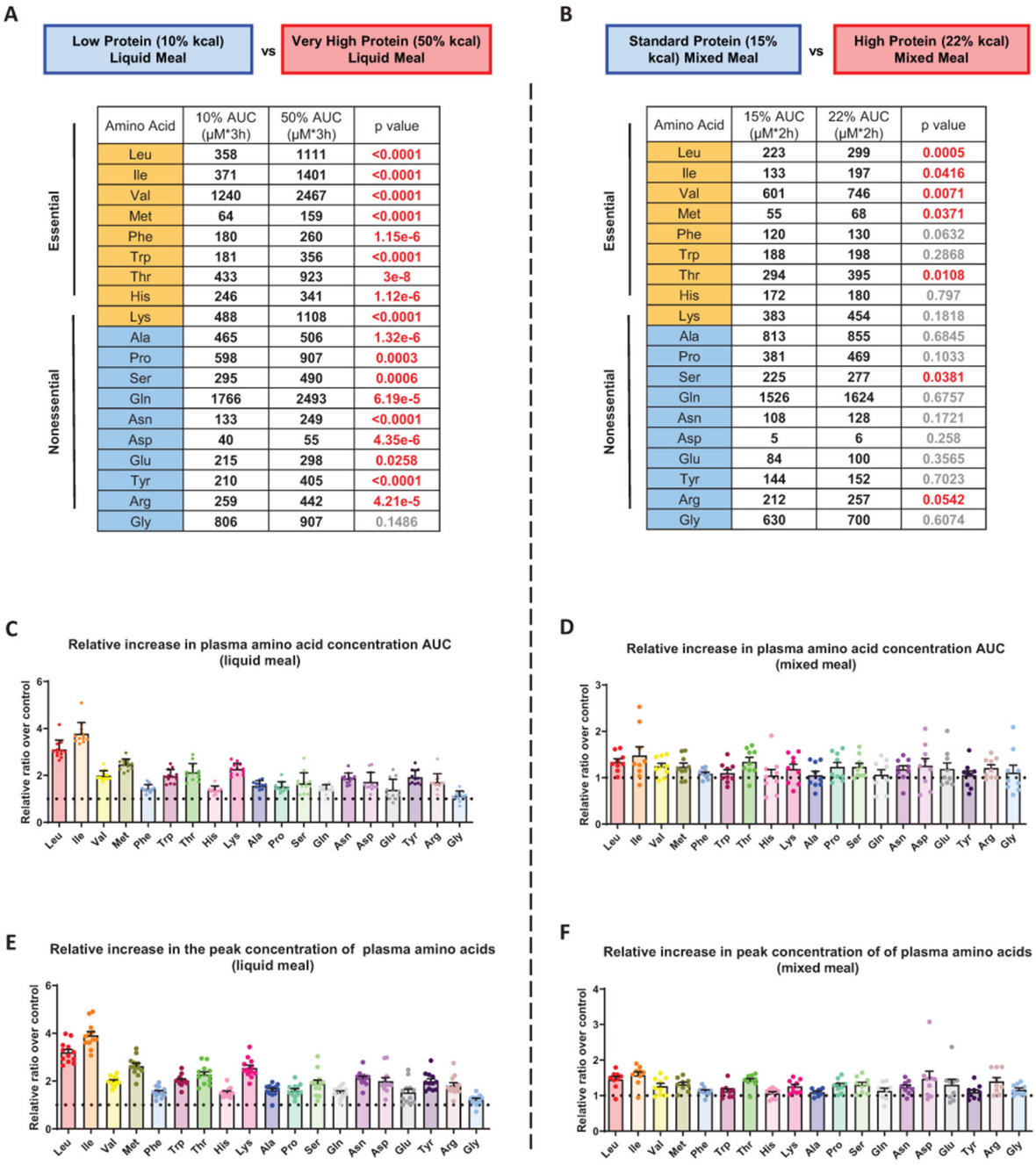



**Figure 2. High-protein intake induces mTORC1 activation and inhibition of autophagy in isolated human monocytes.**

**(A)** Schematic of the cross-over feeding study design. Nine participants (3 men, 6 women) consumed both high-protein and standard protein mixed meals. The standard meal provided 450 kcal of energy, 15% kcal as protein, 35% kcal as fat, and 50% kcal as carbohydrate. The high protein meal provided 450 kcal of energy, 22% kcal as protein, 30% kcal as fat, and 48% kcal as carbohydrate. (B) Total plasma amino acid concentration before and at 1 h and 2 h after consuming the standard and high protein meals (n=9, P=0.026795 and 0.04162).

(**C)** Western blot analysis of mTORC1 activation (using phosphorylated S6) in isolated circulating monocytes before and after consuming the standard and high protein meals (n=7, P=0.00484). (**D)** Immunofluorescence microscopy analysis of the autophagy marker LC3 (using LC3 puncta formation) in isolated circulating monocytes before and after consuming the standard and high protein meals (n=7, P=0.0472). For all graphs, data are presented as mean ± SEM, and determined by Two-way ANOVA with Sidak's multiple comparisons.. *P ≤ 0.05, **P < 0.01.




| | Amino Acid | 10% AUC (μM*3h) | 50% AUC (μM*3h) | p value |
|---|---|---|---|---|
| Essential | Leu | 358 | 1111 | <0.0001 |
| | Ile | 371 | 1401 | <0.0001 |
| | Val | 1240 | 2467 | <0.0001 |
| | Met | 64 | 159 | <0.0001 |
| | Phe | 180 | 260 | 1.15e-6 |
| | Trp | 181 | 356 | <0.0001 |
| | Thr | 433 | 923 | 3e-8 |
| | His | 246 | 341 | 1.12e-6 |
| Nonessential | Lys | 488 | 1108 | <0.0001 |
| | Ala | 465 | 506 | 1.32e-6 |
| | Pro | 598 | 907 | 0.0003 |
| | Ser | 295 | 490 | 0.0006 |
| | Gln | 1766 | 2493 | 6.19e-5 |
| | Asn | 133 | 249 | <0.0001 |
| | Asp | 40 | 55 | 4.35e-6 |
| | Glu | 215 | 298 | 0.0258 |
| | Tyr | 210 | 405 | <0.0001 |
| | Arg | 259 | 442 | 4.21e-5 |
| | Gly | 806 | 907 | 0.1486 |

| | Amino Acid | 15% AUC (μM*2h) | 22% AUC (μM*2h) | p value |
|---|---|---|---|---|
| Essential | Leu | 223 | 299 | 0.0005 |
| | Ile | 133 | 197 | 0.0416 |
| | Val | 601 | 746 | 0.0071 |
| | Met | 55 | 68 | 0.0371 |
| | Phe | 120 | 130 | 0.0632 |
| | Trp | 188 | 198 | 0.2868 |
| | Thr | 294 | 395 | 0.0108 |
| | His | 172 | 180 | 0.797 |
| Nonessential | Lys | 383 | 454 | 0.1818 |
| | Ala | 813 | 855 | 0.6845 |
| | Pro | 381 | 469 | 0.1033 |
| | Ser | 225 | 277 | 0.0381 |
| | Gln | 1526 | 1624 | 0.6757 |
| | Asn | 108 | 128 | 0.1721 |
| | Asp | 5 | 6 | 0.258 |
| | Glu | 84 | 100 | 0.3565 |
| | Tyr | 144 | 152 | 0.7023 |
| | Arg | 212 | 257 | 0.0542 |
| | Gly | 630 | 700 | 0.6074 |

**Figure 3. Plasma amino acid concentration profiles after low, standard, high and very high protein intake**

**(A)** Plasma amino acid concentration area under the curve (AUC, over 3 hours – denoted as μM*3h) after participants consumed the low and very high protein meals (10% kcal and 50% kcal as protein, respectively) described in Figure 2. (**B)** Plasma amino acid concentration area under the curve (AUC, over 2 hours – denoted as μM*2h) after participants consumed the standard and high protein (15% kcal and 22% kcal as protein, respectively) mixed meals described in Figure 3. (**C)** Relative increase in plasma amino acid

AUC after the very high protein (50% kcal) compared with the low protein (10% kcal) meal (n=12). (**D)** Relative increase in plasma amino acid concentration AUC after the high protein (22% kcal) compared with the standard protein (15% kcal) meal (n=9). (**E)** Relative increase in the peak plasma amino acid concentrations (1h) after consuming the very high protein (50% kcal) compared with the low protein (10% kcal) meal (n=12). (**F)** Relative increase in peak of plasma amino acid concentrations (1h) after consuming the high protein (22% kcal) compared with the standard protein (15% kcal) meal (n=9). For all graphs, data are presented as mean ± SEM, and determined by unpaired two-sided Student's t-test for A and B.

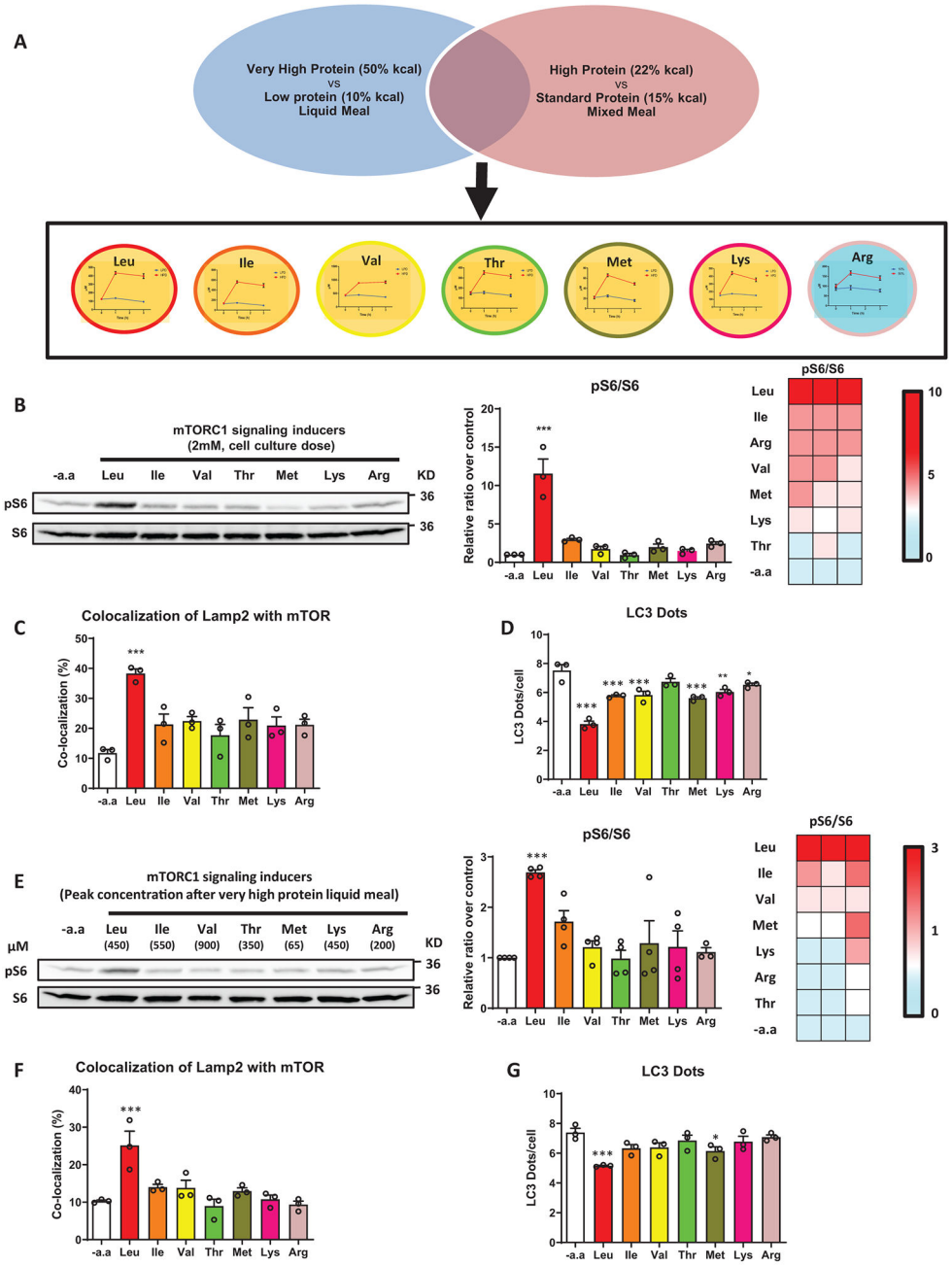



**Figure 4. Identification of leucine as the most consequential amino acid for mTORC1 activation in human monocyte derived macrophages (HMDM)**

**(A)** List of 7 plasma amino acids that were increased after ingestion of both the very high protein and high protein meals compared with the low protein and standard protein meals, respectively. **(B-C)** Quantification of mTORC1 activation in HMDMs by the selected amino acids (2 mM) using (B) Western blot analysis (assessed by phosphorylated S6) and (C) immunofluorescence (IF) microscopy (assessed by co-localization of mTOR and Lamp2). n=3 independent experiments. P<0.0001 for B and C. **(D)** Quantification of the autophagy

marker LC3 in HMDMs using IF microscopy upon stimulation by the selected amino acids (2 mM). n= 3 independent experiments per group. P<0.0001, 0.00023, 0.00031, <0.0001, 0.00114, 0.02942. (**E-F)** Quantification of mTORC1 activation in HMDMs by the selected amino acids (applied at concentrations that correspond to their peak plasma concentration after consuming the very high protein meal described in Figure 1 and Extended Data Figure 2) using (E) Western blot analysis (assessed by phosphorylated of S6) (n=4 independent experiments, P=0.00017) and (F) IF microscopy (assessed by co-localization of mTOR and Lamp2) (n=3 independent experiments, P=0.00016). (**G)** Quantification of the autophagy marker LC3 in HMDMs using IF microscopy upon stimulation by the selected amino acids (applied at concentrations that correspond to their peak plasma concentration after consuming the very high protein meal). n=3 independent experiments per group. P=0.0002, 0.0297. The IF data (C, D, F, and G) were obtained from three independent experiments with analysis of n ≥ 100 cells per experiment. For all graphs, data are presented as mean ± SEM, determined by One-way ANOVA with Dunnett‘s multiple comparisons. *P ≤ 0.05, **P < 0.01 and ***P < 0.001.

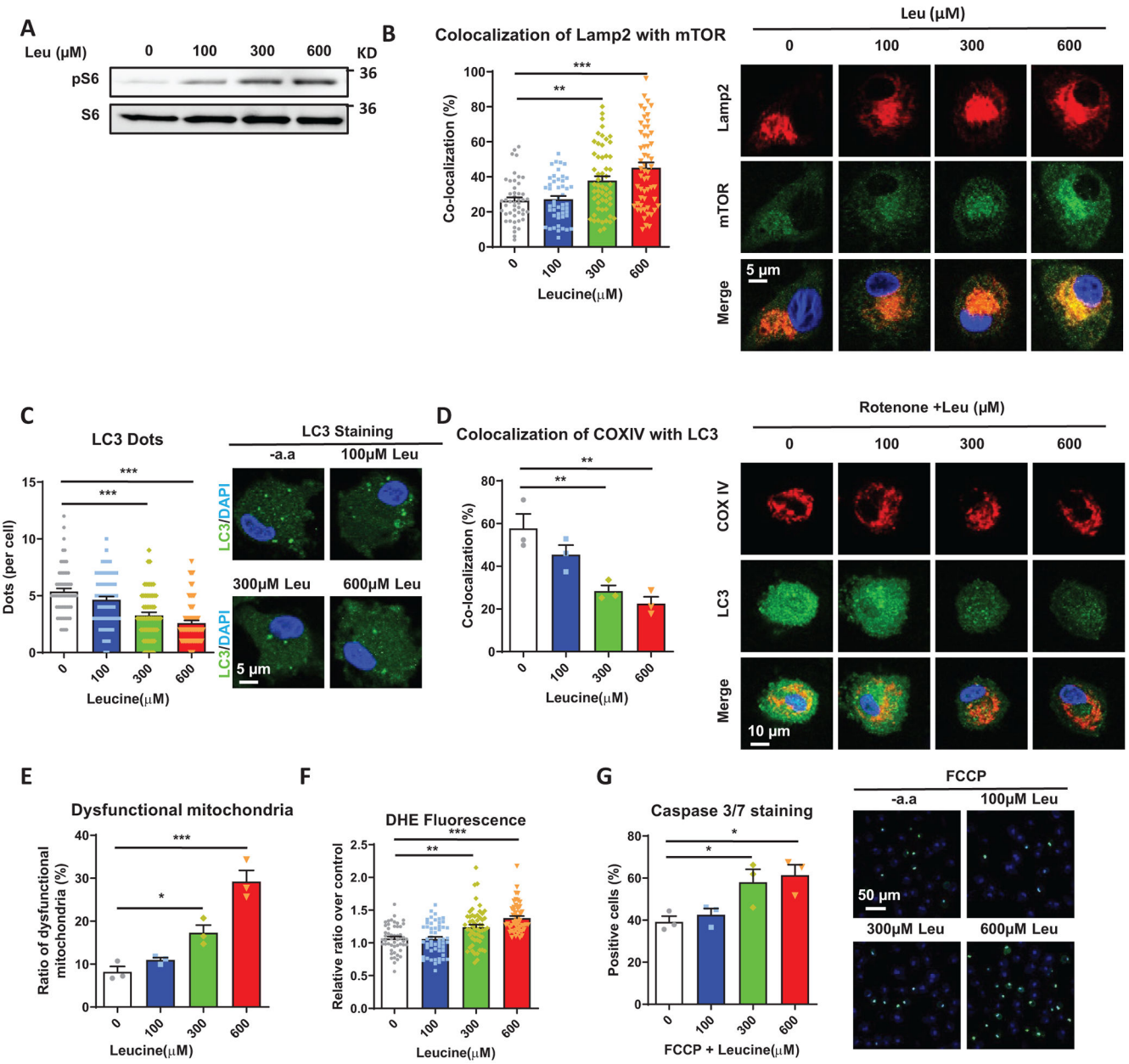



**Figure 5. Leucine-mediated mTORC1 activation and downstream sequela in Human monocyte-derived macrophages (HMDMs)**

**(A)** Western blot analysis of the dose-dependent effect of leucine on mTORC1 activation (assessed by phosphorylation of S6). **(B)** Immunofluorescence (IF) microscopy analysis of the dose-dependent effect of leucine on mTORC1 activation (assessed by co-localization of mTOR and Lamp2). P=0.0035, P<0.0001. **(C,D)** IF microscopy analysis of the dose-dependent effect of leucine on (C) autophagy inhibition (assessed by quantitation of LC3 puncta formation) and (D) mitophagy inhibition (assessed by co-localization of mitochondrial marker COXIV with LC3). P<0.0001 for C, and P=0.00464 and 0.0015 for D. **(E,F)** Dose-dependent effect of leucine on rotenone-induced (E) mitochondrial dysfunction

by FACS analysis and (F) intracellular ROS (assessed by quantitation of DHE-staining via fluorescence microscopy). P=0.01465 and P< 0.0001 for E, and P=0.00103 and P< 0.0001 for F. **(G)** IF microscopy analysis of the dose-dependent effect of leucine in FCCP (carbonylcyanide p-trifluoromethoxyphenyl hydrazone)-induced apoptosis (quantified as percentage of caspase-3/7 positive cells). P=0.04123 and 0.01883. The IF data (B, C, D, F, and G) were obtained from three independent experiments with analysis of n ≥ 40 cells per experiment (B: n=47,48,55, 56 for each group; C: n=60, 61,62, 64 for each group; F: n=51,54,55,53 for each group). The FACS data (E) was obtained from three independent experiments. For all graphs, data are mean ± SEM, and determined by One-way ANOVA with Dunnett's multiple comparisons.. *P ≤0.05, **P < 0.01 and ***P < 0.001.

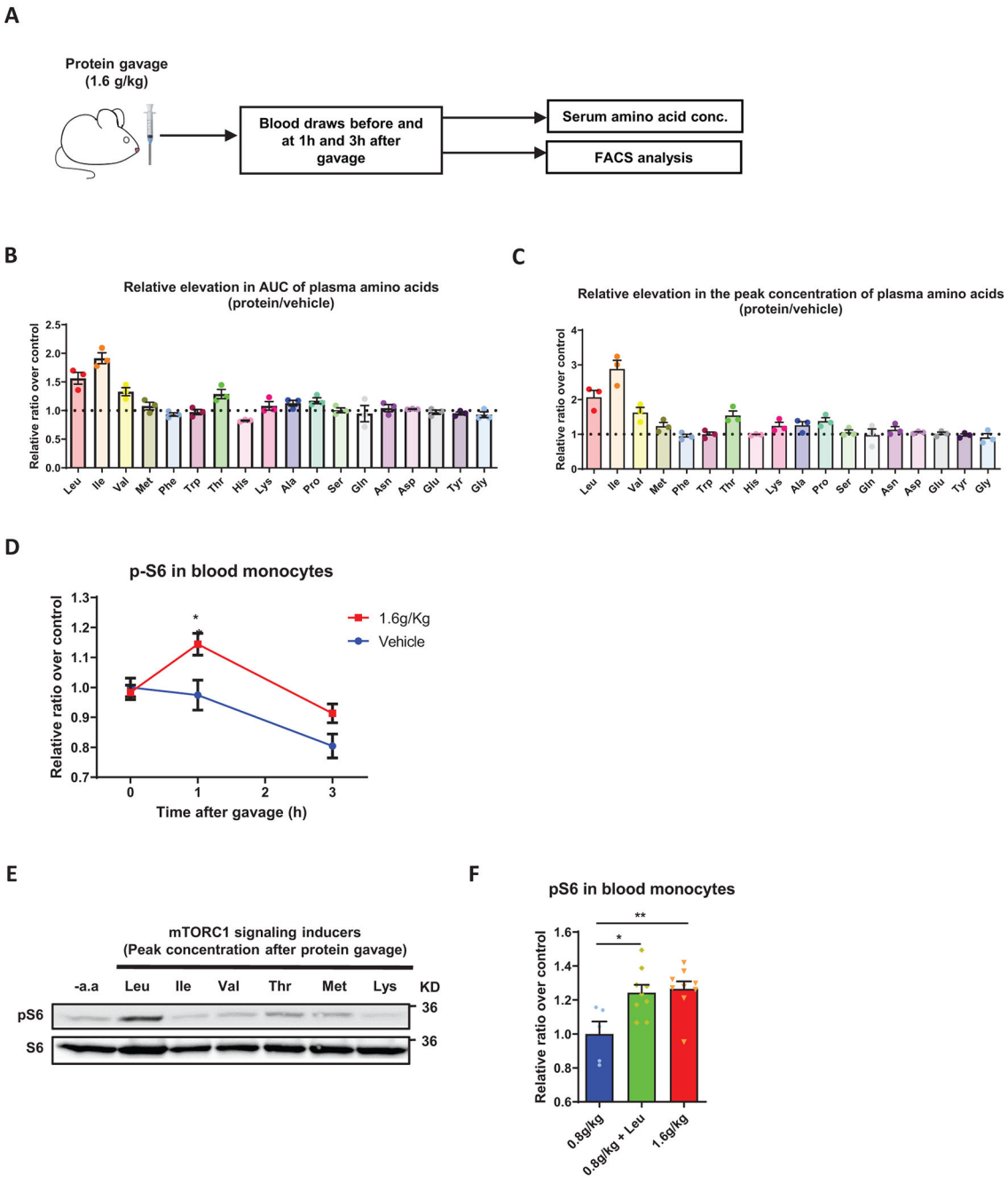



**Figure 6. Leucine is the most consequential amino acid for mTORC1 activation in murine monocytes/macrophages in vitro and in vivo.**

(**A**) Summary of the experimental protocol for gavaging mice with liquid protein (1.6 g/kg) and assessment of serum amino acids and blood monocyte mTORC1 activation. (**B**) Relative increase in serum amino acid concentration areas under the curve (AUC, over 3 hours) in mice (n=3) gavaged with protein compared with vehicle. (C) Relative increase in the peak plasma amino acid concentrations in mice (n=3) gavaged with protein compared with vehicle for 1 hour. (**D**) FACS analysis of mTORC1 activation in blood

monocytes (using phosphorylated S6) from male mice (n=6) gavaged with protein compared with vehicle. P=0.00773. (**E)** Western blot analysis of mTORC1 activation (assessed by phosphorylated S6) in bone marrow-derived macrophages stimulated by selected amino acids with highest excursion in (B) (applied at concentrations that correspond to their peak serum concentration). (**F)** FACS analysis of mTORC1 activation in blood monocytes (using phosphorylated S6) from mice gavaged with 0.8 g/kg protein (n=5), 1.6 g/kg protein (n=9), and 0.8 g/kg protein plus enough leucine to make up the difference in the leucine content of the 0.8g/kg and 1.6g/kg protein (n=9, P=0.01094 and 0.0058.). For all graphs, data are presented as mean ± SEM, determined by Two-way ANOVA with Sidak's multiple comparisons for D, or by One-way ANOVA with Dunnett's multiple comparisons for F. *P ≤ 0.05, **P < 0.01.

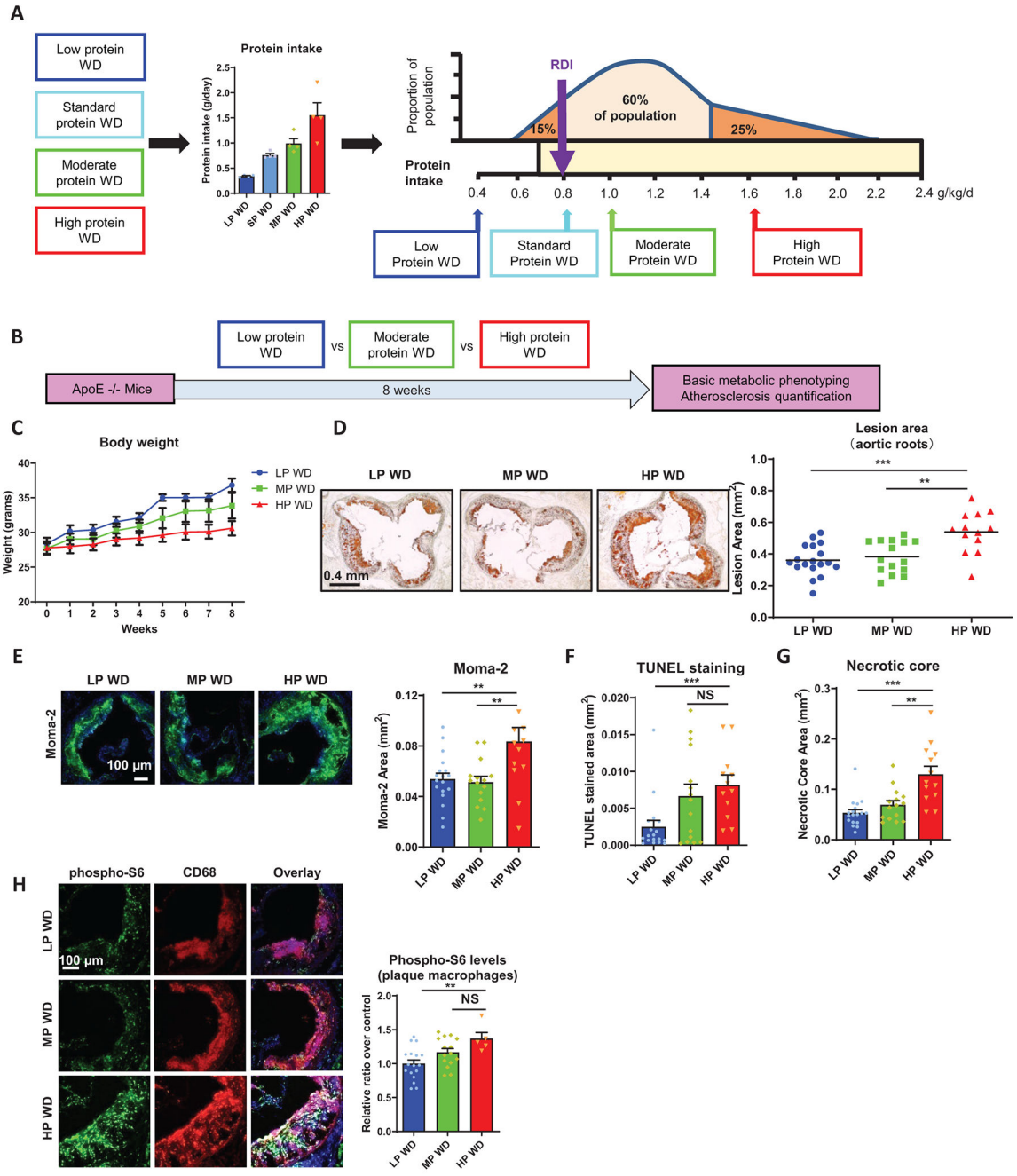



**Figure 7. Impact of varying dietary protein intake on atherosclerosis in a mouse model**

**(A)** Comparison of daily protein intake from mice fed 4 Western diets (WD) (n=4 for each group) containing different amounts of protein (left) and superimposing these diets on the protein intake curve of people with the murine standard WD corresponding to the human recommended daily intake (RDI) (right). **(B)** Summary of the experimental protocol for in vivo assessment of atherosclerosis in ApoE$^{-/-}$ mice using diets with protein content below that of standard WD (low protein WD) and above standard WD (moderate protein WD and high protein WD). **(C)** Total body weight of ApoE$^{-/-}$ mice fed Western diets

with low protein (LP WD), moderate protein (MP WD), or high protein (HP WD) for 8 weeks. n=5 for each group. **(D)** Quantification of atherosclerotic plaque burden in Oil Red O–stained aortic root sections from ApoE$^{-/-}$ mice fed the low, moderate, or high protein Western diets for 8 weeks (LP WD=18, MP WD=15, HP WD=13); representative roots are shown on the left, lesion areas are shown on the right. P=0.0002 and 0.0013. **(E-G)** Plaque composition quantified by immunofluorescence (IF) microscopy of aortic root sections for (E) macrophages (MOMA-2) (LP WD=18, MP WD=15, HP WD=13 biologically independent animals, P=0.0095, 0.0071, and 0.0015.), (F) apoptosis (TUNEL+) (LP WD=18, MP WD=15, HP WD=12 biologically independent animals, P<0.0001 and P=0.2562), and (G) necrotic core (LP WD=18, MP WD=15, HP WD=13, P<0.0001). **(H)** IF microscopy analysis of pS6 levels in macrophages located inside atherosclerotic plaques from mice fed the low, moderate, or high protein Western diets for 8 weeks (LP WD=18, MP WD=15, HP WD=5 biologically independent animals, P=0.0067 and 0.0770). For all graphs, data are presented as mean ± SEM, and determined by two-sided Mann-Whitney U-test. *P ≤ 0.05, **P < 0.01 and ***P < 0.001.

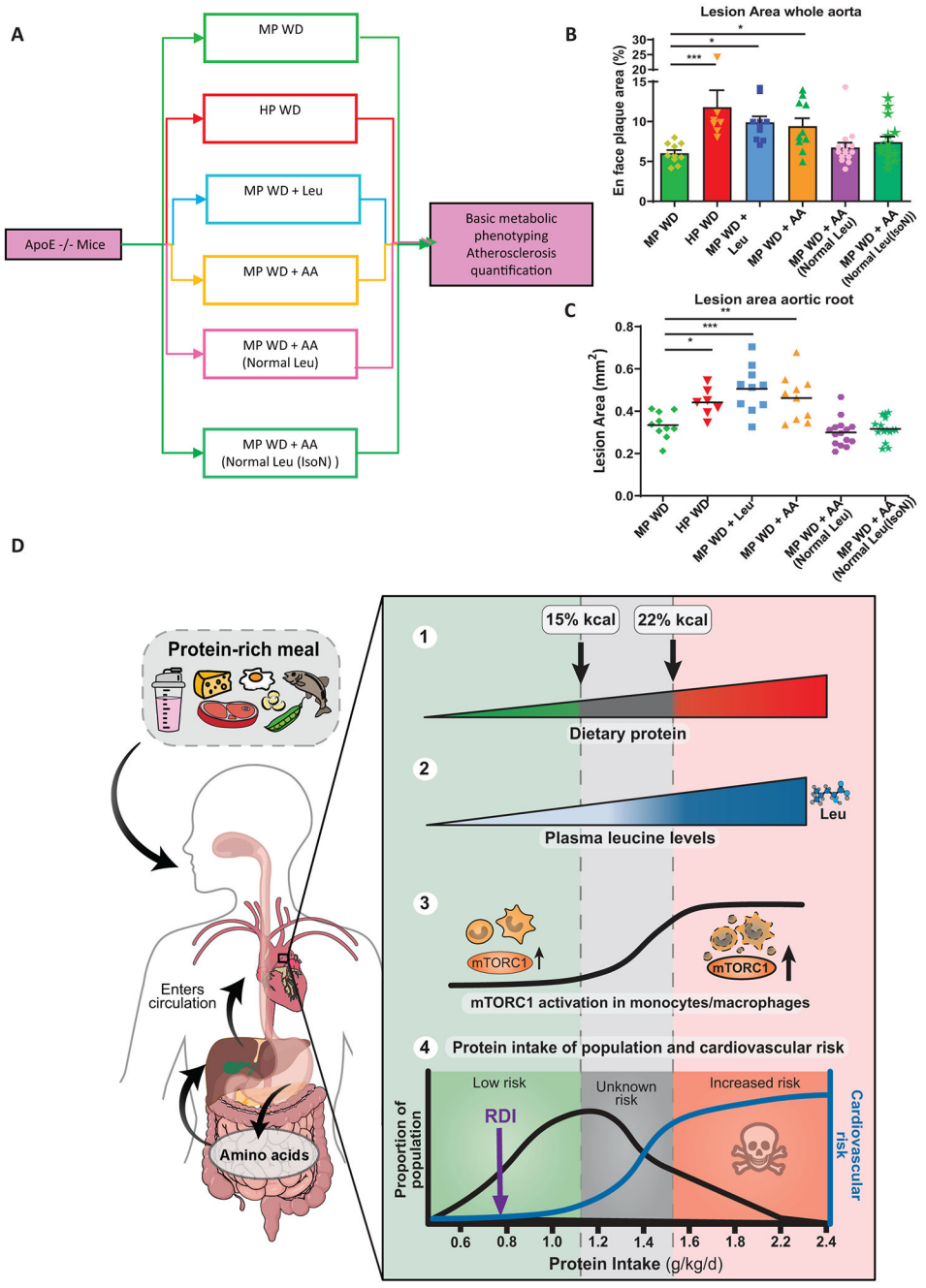



**Figure 8. Effects of dietary protein and leucine intake on macrophage mTORC1 signaling and atherosclerotic cardiovascular risk**

(A) Summary of the experimental protocol for in vivo assessment of atherosclerosis in ApoE−/− mice using six different diets with varying protein, amino acid, and leucine contents, including: 1) moderate-protein Western Diet [called MP-WD], 2) high-protein Western Diet [called HP-WD], 3) MP-WD to which leucine, but no other amino acids, was added to match the leucine content of the HP-WD [called MP-WD+Leu], 4) MP-WD to which amino acids were added to match the total AA content of the HP-WD [called MP-WD+AA], 5) MP-WD to which additional amino acids, except for leucine, were

added to match the contents of all amino acids, except leucine, in the HP-WD [called MP-WD+AA (Normal Leu)], and 6) nitrogen-adjusted version of MP-WD+AA (normal Leu), which consisted of the MP-WD with additional amino acids, except leucine, to make it isonitrogenous with the HP-WD [called MP-WD+AA (Normal Leu (IsoN))]. (B-C) Quantification of atherosclerotic plaque burden in Oil Red O–stained en face aorta (B) and aortic root sections (C) from ApoE−/− mice fed the diets indicated (MP-WD, n=10; HP-WD, n=7; MP-WD+Leu, n=10, MP-WD+AA, n=10, MP-WD+AA (Normal Leu), n=15; MP-WD+AA (Normal Leu (IsoN)), n=15). P=0.00082 0.01902, and 0.04849 for B, and P=0.03232, P<0.0001, P=0.00273 for C. (D) Ingestion of a protein-rich meal (containing ~25 g or about ≥22% kcal protein) leads to a rise in circulating amino acids to levels which trigger mTORC1 activation and downstream signaling in monocytes/macrophages. This phenomenon is unique to leucine (and not other amino acids) and mediates the deleterious functional impact of this signaling on elevated atherosclerosis and cardiovascular risk. For all graphs, data are presented as mean ± SEM, and determined by One-way ANOVA with Dunnett's multiple comparisons. *P ≤ 0.05, **P < 0.01 and ***P < 0.001.

**Table 1.**

Participant characteristics

| | **Study 1** | **Study 2** |
|---|---|---|
| | **Very high protein liquid meal study** | **High protein mixed meal study** |
| N (M/F) | 14 (8/6) | 9 (3/6) |
| Age (years) | 41 ± 3 | 41 ± 5 |
| Body mass index (kg/m$^2$) | 27.9 ± 1.3 | 28.4 ± 1.2 |

Data are mean ± SEM.

Abbreviations: BMI, body mass index; F, female; M, male.