Mechanical stimuli-defined TNFα endocytosis governs mesenchymal stem cell homeostasis

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Acknowledgments

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Abstract

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine responsible for immune regulation and is considered to execute its function mainly through its receptor-mediated canonical signal pathway. Mesenchymal stem cells (MSCs) are the heterogeneous primitive cells initially discovered residing in the adult bone marrow stroma, possessing self-renewal and multiple differentiation potential and critically maintaining multiple tissue/organ homeostasis. The interplay between MSCs and immune cytokines via the receptors on the MSC surface has increasingly been recognized; increasing evidence has shown that MSCs produce a certain amount of cytokines by themselves with little understanding of the role of MSC-derived cytokines.

In this study, we, for the first time, reveal a non-inflammatory, non-canonical role of MSC-derived TNFα by showing that TNFα-deficient MSCs exhibit impaired self-renewal and differentiation due to upregulated mTOR phosphorylation. Mature TNFα is internalized into the cytoplasm via endocytosis after being cleaved by the TNFα-converting enzyme and shedded into the extracellular microenvironment. We further find that cytoplasmic TNFα binds to Rictor, a component of mTOR complex 2, to restrain mTOR activation.

A complex regulatory network and signaling pathways are involved in governing MSC fate commitment. Mechanical stimuli, including physical cues from the matrix and applied forces, account for one critical extrinsic factor controlling MSC fate determinations. Microgravity conditions, such as astronauts in spaceflight missions and bedridden
patients, are reported to result in progressive bone loss, but the therapeutics have yet to be established. In our study, we use hindlimb unloading (HU) mice to mimic the microgravity condition and find that HU mice resulted in reduced TNFα endocytosis and impaired cell function in MSCs as well as osteopenia phenotype. Rapamycin therapy rescues MSCs impairment and osteopenia in HU mice by blocking mTOR activation.

Collectively, our findings identify a previously unrecognized role of TNFα in maintaining MSC homeostasis via receptor-independent endocytosis to finetune mTOR signaling homeostasis. A mechanical stimuli-dependent and receptor-independent endocytosis of TNFα is required to maintain mTOR equilibrium and therefore safeguard MSC homeostasis. Rapamycin may be a promising therapy for hypodynamia-induced osteoporosis in astronauts and bedridden patients.
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- ALP: Alkaline Phosphatase
- ANC: All Nuclear Cells
- ANOVA: one-way analysis of variance
- BFR: bone formation rate
- BrdU: Bromodeoxyuridine
- BSA: albumin from bovine serum
- CFU-F: Colony Forming Unit - Fibroblasts
- CME: clarin-mediated endocytosis
- Co-IP: Co-Immunoprecipitation
- DMEM: Dulbecco’s modified Eagle’s medium
- EDTA: ethylenediaminetetraacetic acid
- ELISA: enzyme-linked immunosorbent assay
- ERK: extracellular signal-regulated kinase
- FBS: fetal bovine serum
- FDA: Food and drug administration
- FITC: fluorescein isothiocyanate
- GFP: green fluorescent protein
- H&E: hematoxylin and eosin
- HA/TCP: hydroxyapatite/tricalcium phosphate
- HEPES: hydroxyethyl piperazine ethanesulfonic acid
- **HRP**: horseradish peroxidase
- **HU**: hindlimb unloading
- **IFNγ**: interferon-gamma
- **IL**: interleukin
- **IL-6**: interleukin 6
- **JNK**: c-Jun N-terminal kinase
- **LPL**: lipoprotein lipase
- **MAR**: mineral apposition rate
- **MSCs**: Mesenchymal Stem Cells
- **mTOR**: mechanistic/ mammalian target of rapamycin
- **mTORC**: mTOR complex
- **NCE**: non-clarin endocytosis
- **OCN**: osteocalcin
- **OPG**: osteoprotegerin
- **p-cJun**: phosphorylated cJun
- **p-ERK1/2**: phosphorylated ERK1/2
- **p-JNK**: phosphorylated JNK
- **p-p38**: phosphorylated p38
- **p-p50**: phosphorylated p50
- **p-p53**: phosphorylated p53
- **p-p65**: phosphorylated p65
- **p-P70S6K**: phosphorylated P70S6K
- **p-Raptor**: phosphorylated Raptor
• p-Rictor: phosphorylated Rictor
• p-Sin1: phosphorylated Sin1
• p-Smad3: phosphorylated SMAD3
• MAPK: mitogen-activated protein kinase
• PBS: phosphate buffer saline
• PFA: paraformaldehyde
• PI3K: phosphoinositide 3-kinase
• PM: plasma membrane
• PPARγ: peroxisome proliferator-activated receptor gamma 2
• PTEN: phosphatase and tensin homologue
• qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction
• RANK: nuclear factor kappaB
• RANKL: nuclear factor kappaB ligand
• RME: receptor-mediated-endocytosis
• RUNX2: runt-related transcription factor 2
• SD: standard deviation
• siRNA: small interfering RNA
• TACE: the TNFα-converting enzyme TNFR: TNF receptor
• TEM: Transmission electron microscopy
• TGF-β: transforming growth factor-beta
• TNFα: Tumor necrosis factor-alpha
• mTNFα: recombinant mouse TNFα
• pro-TNFα: precursor TNFα
- **hTNFα**: recombinant human mature TNFα
- **pro-hTNFα**: recombinant human precursor TNFα
- **WT**: wild-type
- **α-MEM**: alpha-minimum essential medium
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Introduction

Tumor necrosis factor alpha (TNFα)

TNFα, initially discovered as a factor associated with the killing of tumor cells, is a pro-inflammatory cytokine responsible for immune regulation (1-5). Transmembrane TNFα, also referred to as precursor TNFα (pro-TNFα), is a 26 kDa protein expressed on the cell surface of monocytes/macrophages, NK cells, activated T cells, and a diverse array of non-immune cells such as endothelial cells, muscle cells, and fibroblasts. Pro-TNFα is cleaved by the TNFα-converting enzyme (TACE) on the cell membrane, and then soluble mature TNFα is shed into the extracellular environment (6-8). Production, secretion, and functional execution of inflammatory cytokines involve a series of fine-tuning molecular mechanisms (9). The mechanism is particularly elaborate for TNFα, the prototypic member of the TNF superfamily ligands, which plays a crucial function in regulating a variety of biological processes, not only limited to the pro-inflammation (9, 10).

The biologically active TNFα transduces the canonical signals in an autocrine/paracrine mode through two distinct transmembrane receptors, TNF receptor 1 (TNFR1) and TNFR2 (11-13) (Figure 1). The expression of various receptors of TNF may vary between cell types and tissues (14). There are at least five different types of signaling pathways mediated by TNFα/TNFR, including NF-κB, apoptosis, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK), and c-Jun N-terminal kinase (JNK) (9). Although considerable advances have been made in the
understanding of biology and the clinical role of the TNFα for its canonical pathway, it is largely unknown about its biological role in non-canonical pathways.

Figure 1. Schematic picture of TNFα, TNF-receptors, and canonical TNFR downstream pathways. Transmembrane TNFα, also referred to as precursor TNFα (pro-TNFα), is expressed on the cell surface. Pro-TNFα is cleaved by the TNFα-converting enzyme (TACE) on the cell membrane, and then soluble mature TNFα is shed into the extracellular environment. Cell signaling of TNFα is mediated through interaction with two distinct transmembrane receptors, TNF receptor 1 (TNFR1) and TNFR2. Both pro-TNFα and mature TNFα are able to activate TNFR1 and TNFR2 and the downstream signal pathways.
Bone Marrow Mesenchymal Stem Cells (MSCs)

Bone marrow mesenchymal stem cells (MSCs) are the heterogeneous primitive cells initially discovered in the adult bone marrow stroma, which possess self-renewal and multi-differentiation potential and critically maintain multiple tissue/organ homeostasis (15). Understanding the regulatory mechanisms of MSC behaviors in physiology and pathology represents a fundamental scientific issue with implications for deciphering disease pathogenesis and establishing an appropriate stem cell-based therapeutic (15). Bone marrow MSCs play an important role in maintaining bone marrow homeostasis; the interplay between cytokines and MSCs determines MSCs’ differentiation traits. Particularly over the years, the notion that MSCs closely interact with the immune system has increasingly been recognized, during which the function of MSCs is tightly governed by multiple inflammatory cytokines (16-18). Within an optimal concentration range, cytokines elicit MSC immunomodulation and prime MSCs to show improved efficacy in immune therapies. Cytokines secreted by activated immune cells can regulate MSC function via their receptors on the membrane. We have previously reported that tumor necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) synergistically impair MSC stemness in osteopenia and diminish tissue regeneration (17, 19), whereas others have documented that immunomodulation of MSCs is elicited by IFNγ combined with TNFα and interleukins (ILs) (20, 21). Interestingly, other than producing various immunosuppressive factors, MSCs are also known to express and secrete a broad spectrum of inflammatory cytokines and chemokines, which are claimed in responses to extrinsic stresses, such as hypoxia and immune mediators (22-25), contributing to their immunomodulatory capability. However, it is largely unknown whether MSCs can release...
inflammatory cytokines in physiology, and the functional purposes of MSC-derived inflammatory cytokines remain unclear in this field.

**Figure 2. MSCs interplay with the immune system.** MSCs re-educate the immune cells to induce the generation of regulatory immune cells with tolerogenic properties. These regulatory immune cells, such as Tregs, Bregs, regulatory APC, and NK cells, will gather to create a tolerogenic environment suitable to modulate the immune response. These cells could then use multiple regulatory pathways with a central role for IL-10 to finally establish immunomodulation (Figure adapted and modified from Najar. M. et al. 2016, *Cytotherapy* (18)).
**Mechanical stimuli and Microgravity**

All cells, whether individually or in a tissue, experience and respond to intracellular and extracellular mechanical stimuli (26). Cell-associated forces include osmotic pressure and the forces generated by the cytoskeleton as it pushes and pulls against the plasma membrane and the intracellular organelles. External forces can be static, incremental, or cyclical, including hydrostatic pressure, shear stress, twisting, compression, and high-frequency vibrations. The cell responds to these stimuli by modifying its division rate, death, differentiation, movement, signal transduction, gene expression, secretion, and endocytosis (26).

Bone marrow MSCs are exposed to a variety of mechanical stimuli in vivo, such as fluid shear and tissue deformation, which induce mechanical stress in the cell membrane and cytoskeleton. Mechanical stimuli, including physical cues from the matrix and applied forces, account for another critical extrinsic factor controlling MSC fate determinations, particularly in the bone where mechanical loading is constantly adapted and coordinated (27). In this regard, bone marrow MSCs are especially mechanosensitive and mechanoresponsive for their functional regulation and bone maintenance (28). Those mechanical stimulations affect bone marrow MSC proliferation, self-renewal, and differentiation through mechanosensing and mechanotransduction (27).

Gravity has been a constant factor throughout the evolution of life on the earth; it would not be surprising if altered gravitational force led to deviations in biological systems (29).
Mechanical unloading (microgravity) of cells affects cell morphology, cytoskeletal arrangement, gene expression (i.e., downregulating IL-6 and TNFα), receptor-mediated-endocytosis (RME), and various signaling pathways (29-32). Bone cell morphology is significantly modified following exposure to microgravity to adapt to the new mechanical environment (32). Under microgravity, the bone cells have reduced transcription and translation of cytoskeletal and cytoskeletal-associated proteins (33, 34), and decreased focal adhesion formation, together resulting in the increased formation of osteoclast resorption pits (35). Furthermore, the actin cytoskeleton of osteoblasts subjected to microgravity exposure completely collapsed (36), significantly impacting multiple downstream signaling pathways, most notably, the inhibition of bone morphogenic protein (BMP) signaling axis (33, 37). Severe disruption of cell function and tissue atrophy have been reported in both cultured cells and animals when exposed to microgravity during space missions (32, 38).

Importantly, astronauts in spaceflight and bedridden patients suffered a progressive loss of bone mass due to a lack of mechanical stimuli, but the therapeutics have yet to be established (39). Accordingly, the hindlimb unloading (HU) mouse model has been widely used as a ground-based model for understanding the biological response of the musculoskeletal and immune systems to spaceflight and microgravity environments (40, 41). HU mice represent a well-established hypodynamia model mimicking the condition of weightlessness; they have been demonstrated to experience muscle and bone mass atrophy, as well as increased bone marrow adipose tissue, recapitulating the osteopenia phenotype seen in human unloading. In human unloading conditions, the bone marrow MSCs demonstrate osteogenic inhibition due to coordinated responses of multiple
Notably, mechanical unloading also induces general humoral alterations in the organism, in which the onset of systemic inflammation is observed with increased susceptibility to autoimmune disorders (45). Further dissecting the mechanisms of mechanical regulation of MSCs with potential inflammatory cytokine reactions would help unravel novel molecular targets for counteracting microgravity-induced osteopenia.

Figure 3. The morphology and physiology of adherently growing cells altered after microgravity exposure. Cytoskeleton components of actin, microtubules, and intermediate filaments are displayed in inset circles. In adherent cells, microtubules form radiation arrangements near the nucleus. Actin fibers anchor to cell membranes. Intermediate filaments form a loose network around the nucleus. Among cells under
microgravity influence, the microtubules are shortened and curved. Fewer actin fibers but more condensed intermediate filaments are observed. This illustration was inspired by long-term thyroid cell culture in a simulated microgravity environment (Figure adapted from Bradbury P. et al. 2020, *Frontiers in Cell and Development Biology* (32)).

**Figure 4. Effect of microgravity on bone marrow tissue and cells.** Diagram showing the osteoblastic and osteoclastic activity regulation under normal and microgravity conditions. For example, osteoprotegerin (OPG) is reduced by osteoblast cells and increased secretion of nuclear factor-κB ligand (RANKL) due to the effect of microgravity. Microgravity affects MSCs to inhibit the differentiation of osteoblast and bone formation.
When more RANKL is available to attach to the RANK receptor on the pre-osteoclast, there is more osteoclast activation and bone resorption. (Figure adapted from Singh K. et al. 2018, Reach (46)).

**Endocytosis**

Endocytosis is a cellular physiological process by which cells absorb molecules that cannot pass through the plasma membrane and constitutes the major cell communication machinery with the extracellular environment (47, 48). Although endocytosis was initially discovered and studied as a relatively simple process of transporting molecules across the plasma membrane, it was subsequently found to be inextricably linked with almost all aspects of the cellular signaling (48). Endocytic pathways integrate diverse signals, thereby contributing to a higher level of cellular and organismal organization. In this way, endocytosis and cell signaling are intertwined in many biological processes, such as motility and cell fate determination (47).

The complexity of the endocytosis system kicks off at the plasma membrane (PM), where multiple entry portals have been described. A rough classification is based on the size of the initial membrane invagination. Large particles (>500 nm) are taken up by phagocytosis, as generally is the case for bacteria or apoptotic cells, whereas fluid uptake occurs by macropinocytosis. Both processes involve large rearrangements of the PM guided by actin cytoskeleton remodeling and coordinated by the stepwise involvement of RHO-GTPases. Micropinocytic events are instead characterized by smaller invagination
(<200nm) and include clathrin-mediated endocytosis (CME) and non-clathrin endocytosis (NCE) (48, 49).

Receptors at the plasma membrane are the first-line sensors of extracellular signals. Not surprisingly, the regulation of their surface levels immediately impacts how a cell responds to environmental stimuli. There are several mechanisms through which endocytosis controls receptor signaling, such as 1) by regulation of receptor availability at the cell surface, 2) by regulation of ligand accessibility to the receptor, and 3) by regulation of the assembly of plasma membrane-specific platforms. In addition, the biological output of a specific signal can be controlled not only by the internalization of receptors and/or ligands into endosomal organelles but also by the routes through which receptors reach the different compartments (reviewed in Ref (48)).

All cells experience and respond to mechanical stimuli, such as changes in plasma membrane tension, shear stress, hydrostatic pressure, and compression (26). Mechanical force-induced membrane tension alternation has been reported to affect the endocytosis process by bending the membrane inward to form the endocytic vesicles, especially clathrin-mediated endocytosis, which has been reported to be enhanced by a transient mechanical stimulus. Clathrin-mediated endocytosis can be blocked by Pitstop® 2, a specific clathrin-mediated endocytosis inhibitor (26, 50, 51).
Figure 5. Multiple portals of entry into the mammalian cell. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors, and lipids), and the mechanism of vesicle formation (Figure adapted from Conner S. 2003, *Nature* (49)).
Figure 6. Endocytosis controls signaling. Examples are provided of how endocytosis and recycling control signaling at different levels and cellular locations. A: endocytosis regulates signaling at the plasma membrane. Endocytosis extinguishes signals by routing plasma membrane receptors to degradation. B: different endocytic routes modulate signal duration. Several receptors can be internalized through CME and NCE, and the relative partitioning of receptors between the two entry routes determines the final biological output. C: endosomes act as signaling platforms. The signaling endosome hypothesis was originally proposed in neurons where endosomes were postulated to serve as platforms for the assembly and transport of protein complexes for long-range signal transmission (Figure adapted from Sigismund S. et al. 2012, Physiol Rev (48)).

mTOR signaling

Rapamycin was first isolated in 1972 from the bacterium Streptomyces hygroscopicus and was initially identified as an antifungal agent and an immunosuppressant, with later discoveries to possess anti-tumor properties (52). Subsequently, the mechanistic/mammalian target of rapamycin (mTOR) was uncovered as the molecular target of rapamycin, with multiple studies revealing mTOR as a master regulator of cells, which senses nutrients and growth factors and integrates downstream cascades to coordinate cell growth, metabolism, and autophagy (53). It has further been increasingly noticed that mTOR signaling is involved in the development and maintenance of various stem and progenitor cell populations (53). mTOR signaling functions through two distinct complexes, mTORC1 and mTORC2, which contain shared and unique partners. Each
The complex contains mTOR, mLST8/GβL, and deutor (54-56). mTORC1 contains exclusively Raptor (Kog1 in budding yeast) and PRAS40. Raptor functions as a scaffolding protein that links the mTOR kinase with mTORC1 substrates to promote mTORC1 signaling. PRAS40 functions in an incompletely defined and controversial regulatory capacity as a mTORC1 inhibitor, competitive substrate, or both (56). mTORC1 is considered a master cell growth and metabolism regulator, controlling cellular protein synthesis, autophagy, lipid synthesis, mitochondrial metabolism, and biogenesis (56).

The roles of mTORC2 function are still largely unknown (56, 57). mTORC2 exclusively contains Rictor, mSin1, mLST8, and Protor-1. Although the signaling pathways for mTORC2 activation are not well characterized, it is known to participate in cell survival, metabolism, and proliferation through activation of Akt (58-60). Particularly, mTOR regulation of MSCs has yielded conflicting results with distinct roles of mTORC1 and mTORC2 in MSC lineage commitments (61). (Figure 7).

It is evident that mTOR has an important role in modulating innate and adaptive immune responses. mTOR regulates cytokine production by antigen-presenting cells in response to inflammatory stimuli through PI3K/Akt/mTOR cascade, suggesting a pivotal role for this molecule in determining the nature of T cell responses. (62) However, there is no direct interplay between mTOR complexes and cytokines reported in the literature.
Figure 7. mTORC1 versus mTORC2. Distinct rapamycin sensitivities, partner proteins, substrates, and cellular functions distinguish the two known mTOR signaling complexes, mTORC1 and mTORC2 (Figure adapted from Foster K. and Fingar D. 2010, *Journal of Biological Chemistry* (56)).
Hypothesis

Our preliminary data show that MSC-derived TNFα is essential to maintain MSC function via TNFR-independent endocytosis to keep mTOR signaling equilibrium. Endogenous pro-TNFα undergoes proteolytic cleavage by TACE at the cell membrane to release soluble mature TNFα. Under mechanical force stimuli, mature TNFα can be endocytosed into the cytoplasm through Clathrin-mediated endocytosis and to maintain MSC homeostasis via mTOR signaling restraint (Fig. 8). Loss of physiologic mechanical stimuli in hindlimb unloading (HU) mice results in hyperactivation of mTOR in MSCs and osteopenia. TNFα deficiency or lack of mechanical stimuli results in an aberrant mTOR activation in bone marrow MSC and alters their lineage differentiation to an adipogenesis-preferred impaired status. Therefore, we hypothesize that optimal TNFα maintains MSC homeostasis through mechanotransduction-mediated endocytosis to directly bind with mTOR complexes within the cytoplasm. Our study will unveil an unknown underlying mechanism of mechanotransduction-mediated cytokine endocytosis to interact with cellular molecules in regulating stem cell function.
Figure 8. Diagram showing TNFα regulation of MSC homeostasis via a receptor-independent manner through endocytosis. TACE: TNFα-converting enzyme
Specific Aims

Our preliminary data show that MSC-derived TNFα is essential to maintain MSC function via TNFR-independent endocytosis to keep mTOR signaling equilibrium. Loss of physiologic mechanical stimuli in hindlimb unloading (HU) mice results in mTOR activation in MSCs and osteopenia. Therefore, we hypothesize that optimal TNFα maintains MSC homeostasis through mechanotransduction-mediated endocytosis to bind directly with mTORC2.

Specific Aim 1. To investigate how TNFα maintains MSC homeostasis and dissect the underlying mechanism. Our preliminary data showed that MSCs derived from TNFα−/−, but not TNFR−/−, mice show MSCs dysfunction in vitro with a significantly hyperactivated mTOR signaling. Recombinant TNFα, at a physiological concentration, can be endocytosed into the cytoplasm and rescue the dysfunction of TNFα-deficient MSCs and plays an important role in maintaining MSC homeostasis through a TNFR-independent pathway but by mTOR restraint.

Aim1a: To evaluate the bone phenotype of TNFα−/− mice in vivo. We will further assess the bone phenotype in TNFα−/− and TNFR−/− mice to investigate whether in vivo lineage alteration of bone marrow MSCs happened in TNFα deficient mice.

Collect femurs from WT, TNFα−/−, and TNFR−/− mice:

- microCT analysis to investigate the trabecular bone volume;
- H&E staining to evaluate the morphology of the distal femur area;
• Oil Red O staining to evaluate the fatty tissue inside the distal femur bone marrow area;
• Alkaline phosphatase (ALP) staining to evaluate the ALP-positive osteoblasts in the distal femur;
• Double calcein labeling assay to assess the bone turnover rate.

Aim1b: To investigate whether endocytosis is critical for mTNFα rescue. Based on our preliminary data, we detected TNFα protein in TNFα−/− bone marrow MSC lysates after mTNFα treatment; we hypothesized that mTNFα could be internalized via endocytosis. We will investigate how endocytosis is involved in the rescue process.

Isolate and culture bone marrow MSCs from WT, TNFα−/−, and TNFR−/− mice:
• Use ProtOn™ Fluorescein Labeling Kit to label recombinant mTNFα in vitro by fluorescent isothiocyanate (FITC), then MSCs were exposed to FITC-TNFα for 24 hours and subsequently examined by cell imaging analysis and semi-quantification to confirm the internalization of mTNFα;
• Use Pitstop® 2 as an inhibitor of endocytosis to see if it would block the internalization of mTNFα;
• Use Pitstop® 2 as an endocytosis inhibitor to see if it would diminish the rescue of mTNFα treatment in TNFα−/− MSCs in terms of cell proliferation and differentiation functions.

Aim1c: To dissect the underlying mechanism of how TNFα inhibits mTOR activity. Our preliminary data verified that TNFα−/− MSCs exhibited significantly
upregulated phosphorylation levels of both mTORC2 and mTORC1, including phosphorylated Rictor (p-Rictor), phosphorylated Sin1 (p-Sin1), and phosphorylated Raptor (p-Raptor), as well as TORC1 downstream effector phosphorylated P70S6K (p-P70S6K), which were able to be suppressed by mTNFα treatment. We will further dissect the underlying mechanism of how TNFα inhibits mTOR activity.

Collect cell lysates from WT, TNFα−/−, and mTNFα-treated TNFα−/− bone marrow MSCs:

• Use immunoprecipitation to investigate if cytoplasmic TNFα is directly bound to the mTOR complex.
• Immunoprecipitation with an anti-mTOR antibody after whole cell lysates were collected to harvest purified and enriched mTOR complexes. To measure the mTOR kinase activity by an ELISA-based activity assay based on the levels of P70S6K phosphorylation and mTORC1 substrate.
• To measure the mTOR kinase activity in the presence of different concentrations of recombinant TNFα to confirm the mTOR inhibition effect of TNFα.

**Specific Aim 2. To investigate how mechanical force stimuli regulate MSCs homeostasis and identify a practical approach to treat/prevent bone loss in disuse osteoporosis.** All mesenchymal tissues are subjected to mechanical forces and respond to mechanical stimulation. Exercise training has been shown to alter pro- and anti-inflammatory cytokines levels and maintain bone homeostasis. Since our preliminary data showed that loss of physiologic mechanical stimuli in HU mice resulted in TNFα deficiency
and aberrant mTOR activation in bone marrow MSCs. In vitro cell culture also showed similar MSC dysfunction from HU mice and $TNF\alpha^{-/-}$ mice. In this aim, we will address how mechanical force stimuli regulate MSCs homeostasis and identify a practical approach to treat/prevent bone loss in hypodynamia patients, such as astronauts and bedridden patients.

**Aim 2a: To evaluate the bone phenotype in HU mice.** The bone marrow niche and homeostasis of bone architecture will be examined after the hindlimb unloading procedure to give us more clues on the osteopenia phenotype and impaired MSC homeostasis.

Collect femurs from control and HU mice:

- microCT analysis to investigate the **trabecular bone volume**;
- H&E staining to evaluate the **morphology** of the distal femur area;
- Oil Red O staining to assess the **fatty tissue** inside the distal femur bone marrow area;
- Alkaline phosphatase (ALP) staining to evaluate the ALP-positive **osteoblasts** in the distal femur;
- Double calcein labeling assay to assess the **bone turnover rate**.

**Aim 2b: To investigate the effect of mechanical stretch on MSCs function in vitro.** As mechanical stimuli have been reported to enhance CME, we used a Flexcell stretching system to examine the effect of mechanical stimuli on MSC’s endocytosis. Our data showed that mechanical stretching promoted TNF\alpha endocytosis and restrained
mTOR signal activity. How mechanical stretch and upgraded endocytosis rated would affect MSC function will be investigated.

Apply 2 hr 15% stretching on WT bone marrow MSCs:

- MSCs were exposed to 488-BSA or FITC-TNFα before stretch loading and subsequently examined by cell imaging analysis and flow cytometry analysis to assess the general and specific mTNFα endocytosis rates;
- BrdU staining to evaluate the cell proliferation;
- Alizarin Red staining and western blotting for osteogenic regulators (e.g., RUNX2, ALP, and OCN) to evaluate the MSCs osteogenesis capacity;
- Oil Red O staining and western blotting for adipogenic regulators (e.g., PPARγ and LPL) to evaluate the MSCs adipogenesis capacity.

**Aim 2c: To investigate whether the rescue of impaired MSCs function and bone phenotype in HU mice could be achieved by targeting mTOR restraint.** As an FDA-approved drug, rapamycin, a specific inhibitor of mTOR complexes, will be used as a novel therapeutic drug candidate to treat HU mice. We will investigate whether rapamycin treatment could ameliorate the bone marrow niche and homeostasis of bone architecture in HU mice by downregulating mTOR signaling.

Collect femurs from control, HU, and rapamycin-treated HU mice:

- microCT analysis to investigate the trabecular bone volume;
- H&E staining to evaluate the morphology of the distal femur area;
• Oil Red O staining to evaluate the **fatty tissue** inside the distal femur bone marrow area;

• Alkaline phosphatase (ALP) staining to evaluate the ALP-positive **osteoblasts** in the distal femur;

• Double calcein labeling assay to assess **the bone turnover rate**.
Materials and Methods

**Animals**

Female C57BL/6J (wildtype (WT), No. 000664), B6.129S-\textit{Tnfrsf1a\textasciitilde{tm}1Gkl}\textasciitilde{J} (\textit{TNFa\textasciitilde--/}, No. 003008), B6.129S-\textit{Tnfrsf1b\textasciitilde{tm}1Gkl}\textasciitilde{J} (\textit{TNFR\textasciitilde--/}, No. 003243), B6.129S7-\textit{Ilng\textasciitilde{tm}1Tsj}\textasciitilde{J} (\textit{IFNy\textasciitilde--/}, No. 002287), B6.129S2-\textit{Il6\textasciitilde{tm}1Kopf}\textasciitilde{J} (\textit{IL-6\textasciitilde--/}, No. 002650), NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wij</sup>/SzJ, (NSG, No. 005557), B6.Cg-Tg(Prrx1-cre)1Cjt/J (\textit{Prx1-Cre}, No. 005584) and B6.Cg-\textit{Gt(ROSA)26Sor\textasciitilde{tm}9(CAG-tdTomato)Hze}\textasciitilde{J} (\textit{tdTomato}, No. 007909) mice were purchased from Jackson Lab and maintained in a C57BL/6J background for least ten backcrosses. \textit{Prx1-Cre} and \textit{tdTomato} transgenics were interbred to get \textit{Prx1-Cre;tdTomato} mice for tracing mesenchymal cells \textit{in vivo}. Genotyping was performed by PCR using tail samples from mice and primer sequences provided by the Jackson Laboratory. Age-matched female littermates were used in all experiments. Female immunocompromised nude mice (Beige \textit{nu/nu} XIDIII) were purchased from Harlan. Mice were housed in a pathogen-free condition, maintained on the standard 12-h light-dark cycle, and received food and water \textit{ad libitum}. All animal experiments were performed under institutionally approved protocols for the use of animal research (University of Pennsylvania, Protocol No. 805478).

**Hindlimb unloading**

WT and \textit{Prx1-Cre;tdTomato} mice at 8-week old were subjected to continuous tail suspension for 2 weeks, which was performed according to previous statements (44).
Briefly, mice were individually caged and suspended by the tail using a strip of adhesive surgical tape attached to a chain hanging from a pulley. Mice were suspended at a 30° angle to the floor with only the forelimbs touching the floor, allowing mice to freely move and access food and water (Figure 9). At sacrifice, hindlimb bones were sampled for indicated analyses.

Figure 9. Schematic hindlimb unloading mice model.

**Isolation and culture of mouse bone marrow MSCs**

Isolation and culture of MSCs from mouse bone marrow were performed according to our previous protocol (63, 64). Briefly, whole bone marrow cells from the femora and tibia were seeded, incubated overnight, and rinsed with phosphate buffer saline (PBS) to remove the non-adherent cells. The adherent cells were cultured with alpha-minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, USA) at 37°C in a humidified atmosphere of 5% CO₂. MSCs were digested with 0.25% trypsin (Invitrogen, USA) and passaged for functional experiments after seeding at appropriate densities.

**Isolation and culture of mouse T cells and macrophages**

For the culture of mouse T cells, splenocytes were collected and treated with ACK lysis buffer (Lonza, Switzerland) to remove red blood cells. Naïve T cells were isolated by
5 μg/ml plate-bound anti-mouse CD3 antibody (eBioscience, USA). Activated T cells were obtained by stimulating naïve T cells for 48 h with 2 μg/ml soluble anti-mouse CD28 antibody (eBioscience, USA) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, 10 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, USA). Th1 cells were obtained by treating activated T cells with 20 ng/ml IL-2 (PeproTech, USA), 20 ng/ml IL-12 (PeproTech, USA), and 10 μg/ml anti-IL-4 blocking antibody (BioLegend, USA).

Mouse macrophages were isolated by seeding 2×10⁶ femoral ANCs in 6-well plates with 20 ng/ml macrophage-colony stimulating factor (M-CSF; PeproTech, USA) in DMEM supplemented with 15% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, USA). After 48 h, non-adherent cells were removed, and the adherent macrophages were cultured for another 48 h with 20 ng/ml M-CSF in the presence or absence of 1 ng/ml LPS (Millipore, USA).

**Chemical treatments**

Chemical reagents and treatments were as below: Recombinant mouse TNFα (PeproTech, USA), mouse RANKL (PeproTech, USA), human TNFα (R&D Systems, USA), and human pro-TNFα (R&D Systems, USA) were added at indicated concentrations, with a physiological concentration at 1 ng/ml used in most experiments. TAPI-2 (Millipore, USA) was used at 120 nM. Pitstop® 2 (Abcam, UK) was added at 12
μM. Rapamycin (Abcam, UK) was used at 50 nM in vitro and 1.5 mg/kg/d in vivo intraperitoneally for two weeks (65).

**TNFα overexpression and knockdown in vitro**

For knockdown of TNFα in vitro, serum-starved MSCs were treated with TNFα siRNA or vehicle siRNA control (Santa Cruz Biotechnology, USA) transfected by the Lipofectamine RNAiMAX reagent (Invitrogen, USA), according to the manufacturer’s instructions. For overexpression of TNFα, green fluorescent protein (GFP)-TNFα fusion protein expression plasmids were kindly provided by Dr. Jennifer Stow (Addgene plasmid No. 28089). Empty plasmids with the same backbone were used as the control. Plasmids were transduced using Lipofectamine LTX with Plus reagent (Life Technologies, USA) according to the manufacturer’s instructions.

**In vitro cyclic stretching**

MSC monolayers grown on collagen-coated Flexcell plates were subjected to cyclic stretches (15% elongation at 0.5 Hz) using a Flexcell Fx-4000T tension unit (Flexcell International, USA) for 2-hour periods. This vacuum-driven device applied biaxial strain to cells regulated by a computer-controlled program. The inhibitor Pitstop® 2 was added half hour before stretching with a serum-free medium and was present during the cyclic stretching in inhibition groups. Control wells were plugged at the bottom by rubber capping without the application of any stretches.

**Colony forming unit - fibroblast (CFU-F) assay**
Forming of fibroblastic colonies (CFU-F) by MSCs was evaluated accordingly (64). Briefly, a total of 1.5 × 10^6 all nuclear cells (ANCs) from bone marrow were seeded in 60 mm culture dishes and cultured for 16 days. The colonies were washed with PBS, fixed with 2% paraformaldehyde (PFA; Sigma-Aldrich, USA), and stained with 0.5% toluidine blue solution (Sigma-Aldrich, USA). The number of cell colonies was counted under microscopy, and those with more than 50 cells were considered as colonies.

**BrdU cell proliferation assay**

Analysis of MSC proliferation was performed using BrdU labeling (63). MSCs were seeded onto 8-well chamber slides (Thermo Fisher Scientific, USA) at 2 × 10^4 cells per well. After adherence, BrdU labeling reagent (Invitrogen, USA) was added to the media at 1:100 for 48 h. Cells were then fixed with 70% Ethanol, denatured with 2 N HCl, and stained with a BrdU Staining Kit (Invitrogen, USA) according to the manufacturer’s instruction. Fluoroshield Mounting Medium with DAPI (Abcam, UK) was used for counterstaining and mounting. The BrdU-positive cells from five fields per sample were quantified using the ImageJ software (National Institute of Health, USA) and were presented relative to the total number of cells.

**Osteogenic differentiation**

For analysis of osteogenic differentiation capability (64), MSCs were cultured under osteogenic inductive media including 2 mM β-glycerophosphate (Sigma-Aldrich, USA), 100 μM L-ascorbic acid phosphate (Wako, Japan), and 10 nM dexamethasone (Sigma-Aldrich, USA). After four weeks of induction, *in vitro* mineralization was detected by 1%
Alizarin Red S (Sigma-Aldrich, USA) staining. The positively stained areas were quantified using the ImageJ software (National Institute of Health, USA) shown as percentages of the total areas. Protein expression levels of osteogenic marker genes were also examined by the Western blot assay, as stated below.

**Adipogenic differentiation**

For the adipogenic differentiation (63), MSCs were cultured under adipogenic inductive media containing 500 nM isobutylmethylxanthine (Sigma-Aldrich, USA), 60 μM indomethacin (Sigma-Aldrich, USA), 500 nM hydrocortisone (Sigma-Aldrich, USA), 10 μg/ml insulin (Sigma-Aldrich, USA) and 100 nM L-ascorbic acid phosphate (Wako, Japan). After seven days of induction, lipid droplets were stained with Oil Red O (Sigma-Aldrich, USA). The positively stained cells were quantified using the ImageJ software (National Institute of Health, USA) and shown as percentages of the total cells. Protein expression levels of adipogenic marker genes were also examined by the Western blot assay, as stated below.

**Ectopic tissue formation**

Ectopic tissue formation was performed to analyze MSC function in vivo, as previously stated (63). $4 \times 10^6$ MSCs were mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc., USA) as a carrier and were subcutaneously implanted into 8-week-old immunocompromised mice (NOD.Cg-Prkdc<sup>scid</sup> Ii2rg<sup>tm1Wjl</sup>/SzJ, Jackson lab). At eight weeks after implantation, the implants were harvested, fixed in 4% PFA, decalcified with 10% ethylenediaminetetraacetic acid (EDTA, pH 7.4), embedded in
paraffin, and sectioned into 6 μm slices. Sections were then stained with hematoxylin and eosin (H&E) staining, and de novo bone areas formed were analyzed using the ImageJ software (National Institute of Health, USA) and shown as percentages of the total areas.

**Endocytosis analysis**

Recombinant mouse TNFα (PeproTech, USA), human TNFα (R&D Systems, USA), and human pro-TNFα (R&D Systems, USA) were labeled by FITC using the ProtOn™ Fluorescein Labeling Kit (Vector Laboratories, Italy) according to the manufacturer's instructions. FITC-labeled TNFα and pro-TNFα were then used to treat MSCs on coverslips at 1 ng/ml for 24 hours. Cells were then fixed in 4% PFA, and the coverslips were mounted using Fluoroshield Mounting Medium with DAPI (Abcam, UK). The FITC-positive cells from five fields per group were quantified and were shown as percentages of the total cell numbers. FITC intake rates were analyzed using flow cytometric analysis, as stated below.

**Flow cytometric analysis**

For analysis of in situ MSCs, ANCs from hindlimbs of *Prx1-Cre;tdTomato* mice were collected and stained with FITC anti-mouse TNFα antibody (BioLegend, USA) at 1:100 or PerCP anti-mouse p-mTOR antibody (Thermo Fisher Scientific, USA) at 1:100 for 60 min on ice using the Intracellular Staining Permeabilization Wash Buffer (BioLegend, USA). For analysis of MSC endocytosis, MSCs after FITC-labeled TNFα and pro-TNFα treatments were collected and fixed in 2% PFA. For analysis of MSC surface markers, cultured MSCs were collected and stained with PE-conjugated antibodies to CD73.
(Thermo Fisher Scientific, USA), CD90 (BioLegend, USA), CD105 (Thermo Fisher Scientific, USA), CD146 (Thermo Fisher Scientific, USA), CD166 (Thermo Fisher Scientific, USA), Stem cell antigen 1 (Sca1; BD Biosciences, USA), CD45 (Thermo Fisher Scientific, USA) and a PerCP-conjugated antibody to CD34 (BioLegend, USA) at 1:100 for 60 min on ice. All samples were analyzed using FACS^Calibur with the CellQuest software (BD Bioscience, USA).

**qRT-PCR**

Total RNA was isolated from cultured cells using the miRNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III (Life Technologies, USA). Real-time PCR was performed using the SYBR Green Supermix (Bio-Rad, USA) on a CFX96™ Real-Time PCR System (Bio-Rad, USA).

The primers for mouse *TNFα* were:

Forward, 5'-CCTGTAGCCCACGTCGTAG-3';

Reverse, 5'-GGGAGTAGACAAGGTACAACCC-3'.

The primers for mouse *GAPDH* were:

Forward, 5'-TGTGTCCGTCGTGGATCTGA-3';

Reverse, 5'-TTGCTGTGTGAAGTGCAGGAG-3'.

**Western blot**
Western blot assay was performed accordingly (63, 64). Cultured MSCs were lysed in the RIPA Lysis Buffer System with protease and phosphatase inhibitors (Santa Cruz Biotechnology, USA). Protein levels were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). A total of 20 μg protein was separated by SDS-PAGE (Invitrogen, USA) and was transferred to 0.2 μm nitrocellulose membranes (Millipore, USA). The membranes were then blocked with 5% non-fat dry milk and 0.1% Tween-20 for 1 hour, followed by incubation overnight at 4°C with the following primary antibodies:

Antibodies to cJun, p-p53, p53, p-NFκB (p50), NFκB (p50), osteocalcin (OCN), poly(ADP-ribose) polymerase (PARP) and peroxisome proliferator-activated receptor gamma (PPARγ) were purchased from Santa Cruz Biotechnology, USA and were used at concentrations of 1:200; Antibodies to TNFR2, runt-related transcription factor 2 (RUNX2), PI3K, PTEN, p-mTOR, mTOR, p-Akt, Akt, Caspase 3, p-cJun, cJun N-terminal kinase (JNK), p-JNK, p-NFκB (p65), NFκB (p65), p-p38, p38, active-β-catenin, β-catenin, p-ERK1/2, ERK1/2, p-P70S6K, P70S6K, p-Rictor, p-Raptor, Raptor and p-Sin1 were obtained from Cell Signaling Technology, USA and were used at concentrations of 1:1000; The antibody to lipoprotein lipase (LPL) was purchased from Thermo Fisher Scientific, USA and was used at a concentration of 1:1000; The antibody to TNFR1, alkaline phosphatase (ALP), p-Smad3, Smad3 and GFP were purchased from Abcam, UK and were used at concentrations of 1:1000; Antibodies to Rictor and Sin1 were purchased from Bethyl Laboratories, USA and were used at concentrations of 1:1000; The antibody to β-Actin was purchased from Sigma-Aldrich, USA and was used at a concentration of 1:1000. The membranes were then washed and incubated for 1 h at
room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, USA). Immunoreactive proteins were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate, SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) and Autoradiography Film (Labscientific Inc., USA). All Western blotting data were verified by more than three independent experiments.

ELISA

Supernatants of cell culture media and bone marrow were collected after centrifuging at 12,000 rpm for 15 min. Concentrations of TNFα were analyzed using a Mouse ELISA MAX™ Deluxe kit (BioLegend, USA), according to the manufacturer’s instructions.

Co-IP assay

Cultured MSCs were lysed in the RIPA Lysis Buffer System with protease and phosphatase inhibitors (Santa Cruz Biotechnology, USA). Protein levels were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). 1 μg of the control IgG and 20 μl of re-suspended protein A/G PLUS-agarose were added to protein lysates and incubated at 4℃ for 30 min. Pellet beads were centrifuged at 2,500 rpm for 5 min at 4℃, and the supernatants with total cellular protein (450 μg) were collected. Primary antibodies were added at 1:100 and incubated overnight at 4℃ followed by incubation with 20 μl of re-suspended protein A/G PLUS-agarose for 2 hours. Immunoprecipitates were then collected after centrifuging at 2,500 rpm for 5 min at 4℃ and were re-suspended in 30 μl electrophoresis sample buffer. 10 μl aliquots were
subjected to Western blot analysis. All Co-IP data were verified by more than three independent experiments.

**mTORC1 activity analysis**

Whole-cell lysates were collected from MSCs, followed by co-IP with an anti-mTOR antibody. Purified and enriched mTOR complexes were used to measure kinase activity by the K-LISA™ mTOR Activity Kit (Millipore, USA), according to the manufacturer's instructions.

**Calcein labeling assay**

Calcein labeling histomorphometric analysis was performed, as stated before (64). Mice were intraperitoneally injected with Calcein (Sigma-Aldrich, USA) at 15 mg/kg prepared in 2% sodium bicarbonate solution at ten days and three days before sacrifice. Bone formation analyses using mineral apposition rate (MAR) and bone formation rate (BFR) were performed according to the standardized nomenclature for bone histomorphometry under fluorescence microscopy (IX71; Olympus, Japan).

**Bone marrow adiposity analysis**

Femora were fixed in 4% PFA and decalcified with 10% EDTA (pH 7.4), followed by cryosection and staining with Oil Red O solution. Bone marrow adipocytes surrounding the trabecular areas were analyzed. Positively stained areas were quantified using the ImageJ software (National Institute of Health, USA) and shown as percentages of the total areas.
**Micro-CT analysis**

At sacrifice, femora were fixed in 4% PFA and scanned using a high-resolution Scanco μCT35 scanner (Scanco Medical AG, Switzerland) with a voxel size of 20 μm at 70 kV and 200 μA. Images were then reconstructed, and data were analyzed by measuring BMD and BV/TV ratio.

**Transmission electron microscopy**

MSCs monolayer was fixed in 0.5% glutaraldehyde for 2 hours, followed by dehydration and polymerization. The ultrathin sections were placed on copper grids and stained with uranyl acetate and lead citrate. The grids were studied and photographed under a transmission electron microscope.

**Statistics, scientific rigor, and reproducibility**

To determine the number of specimens for the proposed experiments, a power analysis was conducted based on the “Resource Equation” method because the effect size was unknown. Experiments were performed using female animals only to exclude phenotypic differences between sexes. Animals were randomly allocated to groups, and no animals were excluded from the data analysis. All experiments had appropriate control groups to ensure robust and unbiased results. All phenotypes described in *in vivo* experiments were representative of a minimum of 3 littermate pairs (n=3) per group for each experiment. Comparisons between two groups were analyzed by independent unpaired two-tailed Student’s t-test, and the comparisons between more than two groups were analyzed by one-way ANOVA with the Bonferroni adjustment. Analyses were
conducted in a blinded and randomized fashion by at least two examiners to ensure unbiased results. The p-values less than 0.05 were considered statistically significant. All data will be stored electronically in secured servers. All data are represented as the means ± standard deviation (SD).
Results

TNFα is required to maintain bone marrow MSC homeostasis through TNF receptor-independent pathway.

To investigate whether and to what extent MSC-produced cytokines can regulate stem cell properties in bone marrow MSCs at the physiological status, we used TNFα global gene deletion (TNFα−/−), interferon-gamma global gene deletion (IFNγ−/−), and interleukin-6 global gene deletion (IL-6−/−) mice as cytokine-deficient models, which represents three of the most widely studied inflammatory cytokines with extensive biological function, to examine MSC function (9). We found that bone marrow MSCs from TNFα−/−, but not IFNγ−/− nor IL-6−/− mice, showed a significantly reduced proliferation rate as assessed by the number of colony forming unit-fibroblasts (CFU-F; Figure. 10A and B) and bromodeoxyuridine (BrdU) labeling assay (Figure 10 C and D).

Figure 10. Proliferation capacities of WT, IFNγ−/− and IL-6−/−, TNFα−/−, and TNFR−/− bone marrow MSCs. (A) Toluidine blue staining showed the number of CFU-F in WT, IFNγ−/−, and IL-6−/− mice bone marrow all nuclear cells (ANCs; 1.5×10⁶ cells, n=3). (B) Toluidine blue staining showed the number of CFU-F from WT, TNFα−/−, and TNFR−/− mice bone
marrow ANCs (1.5×10^6 cells, n=3). (C) The proliferation rate of bone marrow MSCs was assessed by BrdU incorporation assay. There was no significant decrease in colony formation nor BrdU-positive cells among WT, IFNγ^{-/-}, and IL-6^{-/-} bone marrow MSCs. Scale bar = 100 μm. (D) The proliferation rate among WT, TNFα^{-/-}, and TNFR^{-/-} bone marrow MSCs was assessed by BrdU incorporation assay. The percentage of positive cells was significantly decreased in TNFα^{-/-} compared to WT and TNFR^{-/-} bone marrow MSCs (n=3). Scale bar = 100 μm. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01. CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine.

When cultured under osteoinductive conditions, bone marrow MSCs from TNFα^{-/-}, but not IFNγ^{-/-} nor IL-6^{-/-}, showed decreased osteogenic differentiation capacity, as indicated by reduced mineralized nodule formation and expression of osteogenic markers runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP), and osteocalcin (OCN; Figure 11 A, B, D, and E). Using an established in vivo bone marrow MSC implantation assay, in which 4×10^6 bone marrow MSCs with carrier hydroxyapatite tricalcium phosphate (HA/TCP) particles were subcutaneously implanted into immunocompromised mice, we revealed that bone marrow MSCs from TNFα^{-/-}, but not IFNγ^{-/-} or IL-6^{-/-} mice regenerated significant less new bone than the wild-type (WT) group at 8 weeks post-implantation (Figure 11 C and F).
Figure 11. Osteogenic differentiation capacity of WT, IFNγ−/−, IL-6−/−, TNFα−/−, and TNFR−/− bone marrow MSCs. (A) Alizarin red staining showed mineralized nodule formation in WT, IFNγ−/− and IL-6−/− bone marrow MSCs when cultured under osteoinductive conditions (n=3). (B) Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in WT, IFNγ−/− and IL-6−/− bone marrow MSCs. β-actin was used as a protein loading control (n=3). A two-tailed Student t-test was used to compare treatment and WT groups to quantify Western blotting. (C) Subcutaneous implantation of bone marrow MSCs in immunocompromised mice (n=3) showed that bone (B), bone marrow (BM), and connective tissue (CT) were generated around HA/TCP (HA) at eight weeks after implantation. Scale bar =100 μm. A semi-quantitative analysis showed the amount of bone formation in WT, IFNγ−/− and IL-6−/− bone marrow MSCs implants. (D) Alizarin red staining showed mineralized nodule formation of WT, TNFα−/−, and TNFR−/− bone marrow MSCs cultured under osteoinductive conditions (n=3). (E) Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in WT, TNFα−/−, and TNFR−/− bone marrow MSCs. β-actin was used as a protein
loading control (n=3). (F) Subcutaneous implantation of WT, *TNFα*−/−, and *TNFR*−/− bone marrow MSCs in immunocompromised mice (n=3) showed that bone (B), bone marrow (BM), and connective tissue (CT) were generated around HA/TCP (HA) at 8 weeks after implantation. Scale bar = 100 μm. A semi-quantitative analysis showed the amount of bone formation in bone marrow MSCs implants. Error bars represent the s.d. from the mean values. N.S. not significant, *p*<0.05, **p**<0.01. RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; HA/TCP: hydroxyapatite/tricalcium phosphate.

In contrast, when induced under adipogenic conditions, *TNFα*−/− bone marrow MSCs exhibited a significantly elevated adipogenic differentiation capacity, as indicated by the number of Oil red O-positive cells and up-regulated expression of adipogenic regulators peroxisome proliferator-activated receptor gamma 2 (PPARγ) and lipoprotein lipase (LPL; Figure 12 A-D). *IFNγ*−/− and *IL-6*−/− bone marrow MSCs showed similar adipogenic capacities to what was observed in the WT group (Figure 12 A-D).
Figure 12. Adipogenic differentiation capacity of WT, \textit{IFN}\gamma^{-/-}, \textit{IL}-6^{-/-}, \textit{TNF}\alpha^{-/-}, and \textit{TNFR}\kappa^{-/-} bone marrow MSCs. (A) Oil red O staining showed the capacity of WT, \textit{IFN}\gamma^{-/-}, and \textit{IL}-6^{-/-} bone marrow MSCs to form lipid droplets when cultured under adipogenic conditions (n=3). Scale bar = 100 \mu m. (B) Western blot analysis showed the expression levels of adipogenic genes \textit{PPAR}\gamma and \textit{LPL} in WT, \textit{IFN}\gamma^{-/-}, and \textit{IL}-6^{-/-} in WT, \textit{IFN}\gamma^{-/-} and \textit{IL}-6^{-/-} bone marrow MSCs. \beta-actin was used as a protein loading control (n=3). To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. (C) Oil red O staining showed the capacity of WT, \textit{TNF}\alpha^{-/-}, and \textit{TNFR}\kappa^{-/-} bone marrow MSCs to form lipid droplets when cultured under adipogenic conditions (n=3). Scale bar = 100 \mu m. (G) Western blot analysis showed the expression levels of adipogenic genes \textit{PPAR}\gamma and \textit{LPL} in WT, \textit{TNF}\alpha^{-/-}, and \textit{TNFR}\kappa^{-/-} bone marrow MSCs (n=3).
For quantification of Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

Based on the results above, we concluded that the deficiency of TNFα, but not IFNγ nor IL-6, impaired bone marrow MSC proliferation and differentiation capacities in vitro. Since TNFα is generally recognized as exerting many biological effects by binding to TNF receptors (TNFR) 1 and 2, TNFR1/2 knockout (TNFR−/−) mice were used as a TNFR-deficient model to examine stem cell properties of bone marrow MSCs. Bone marrow MSCs from WT, TNFα−/− and TNFR−/− mice were isolated and confirmed knockout efficacy by Western blot analysis (Figure 13).

Figure 13. Gene knockout efficacy of TNFα−/− and TNFR−/− mice. Western blot analysis showed the expression levels of TNFα, pro-TNFα, TNFR1, and TNFR2 in WT, TNFα−/−, and TNFR−/− bone marrow MSCs. All experimental data were verified in at least three
independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, * p<0.05.

To examine the bone marrow cellularity in WT, TNFα−/− and TNFR−/− mice, bone marrow ANCs were isolated from the femurs, and cells negative for CD34 and CD45 were gated for MSC surface epitope profiling. Flow cytometric analysis showed no significant alteration in the percentages of non-hematopoietic cells in WT, TNFα−/−, and TNFR−/− bone marrow (Figure 14 A and B).

Figure 14. Bone marrow cellularity of WT, TNFα−/− and TNFR−/− mice. (A) Flow cytometric analysis demonstrated the cellularity of bone marrow in WT, TNFα−/− and TNFR−/− mice. CD34 and CD45 double-negative cells were gated out from the ANCs as
the non-hematopoietic population for MSCs surface marker epitope profiling. (B) Flow cytometric analysis showed the surface epitope profiles of WT, $TNF\alpha^{-/}$ and $TNFR^{-/}$ bone marrow MSCs. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant. ANCs: all nuclear cells.

Interestingly, we found that $TNF\alpha^{-/}$ and $TNFR^{-/}$ bone marrow MSCs exhibited different phenotypes when cultured in vitro. $TNFR^{-/}$ MSCs exhibited a similar CFU-F number (Figure 10 B) and proliferation rate (Figure 10 D) to those observed in WT controls. $TNFR^{-/}$ MSCs only showed slightly reduced mineralized nodule formation (Figure 11 D) and similar expression levels of osteogenic markers RUNX2 and ALP (Figure 11 E) when cultured under osteogenic inductive conditions, as well as similar capacity for in vivo new bone formation (Figure 11F). TNFR deficiency failed to affect adipogenic differentiation, as shown by the numbers of Oil Red O positive cells and the expression levels of adipogenic regulators, PPARγ, and LPL (Figure 12 C and D).

To confirm in vivo lineage alteration of bone marrow MSCs, we examined the osteogenic condition of the trabecular bone tissue in WT, $TNF\alpha^{-/}$, and $TNFR^{-/}$ mice. We found a significant reduction in the number of osteoblasts (Figure 15 A) and the bone turnover rate (Figure 15 B ) in $TNF\alpha^{-/}$ mice compared to WT controls, which were not observed in $TNFR^{-/}$ mice. On the other hand, Oil Red O staining showed a greatly enlarged fatty marrow area in the bone marrow of $TNF\alpha^{-/}$, but not $TNFR^{-/}$ mice (Figure 15 C). These data indicate that TNFα is required for maintaining bone marrow MSC and
bone marrow architecture homeostasis, which appears to be independent of TNFR signaling.

Figure 15. Bone marrow niche in WT, TNFα−/−, and TNFR−/− mice. (A) ALP staining showed the number of ALP-positive osteoblasts in the distal femur of WT, TNFα−/−, and TNFR−/− mice (n=3). Scale bar = 200 μm. (B) Calcein double labeling of the distal femur showed the bone turnover rate in WT, TNFα−/−, and TNFR−/− mice (n=3). Scale bar = 100 μm. Mineral apposition rate (MAR) is the distance between the midpoints of the two labels divided by the time between the midpoints of the interval. Bone formation rate per bone surface (BFR/BS) is the volume of mineralized bone formed per unit of time and per unit of the bone surface. It’s calculated as the product of MAR and MS/BS (BFR=MAR *
(MS/BS)). Mineralizing surface per bone surface (MS/BS) represents the percentage of bone surface exhibiting mineralizing activity. (C) Oil red O staining showed the number of adipocytes in the distal femur of WT, \( \text{TNF} \alpha^{-/-} \), and \( \text{TNFR}^{-/-} \) mice (n=3). Scale bar = 500μm. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, **p<0.01, ***p<0.005.

**MSCs produce and release TNF\( \alpha \) *in vitro* and *in vivo*.**

TNF\( \alpha \) is mainly produced by the immune system, such as T helper type 1 cells (Th1), neutrophils, NK cells, and macrophages, and a diverse array of non-immune cells, such as endothelial cells, muscle cells, and fibroblasts. To investigate whether and to what extent MSCs can produce inflammatory cytokines at the physiological status, we cultured mouse bone marrow MSCs and analyzed their production of TNF\( \alpha \), compared to the expression levels from naïve T cells, activated T cells, Th1, macrophages, and LPS-treated macrophages. A combination of quantitative real-time polymerase chain reaction (qRT-PCR), western blot analysis, and enzyme-linked immunosorbent assay (ELISA) was applied respectively for the detection of mRNA expression, protein expression, and secretion levels of TNF\( \alpha \) (Figure 16 A-C). We found that bone marrow MSCs expressed less amount of TNF\( \alpha \) than Th1 and macrophages, but a similar level to that observed in activated T cells and a higher level than naïve T cells.
**Figure 16.** MSCs produce and release TNFα. 1x10^6 bone marrow MSCs were cultured in a 6-well plate for two days before sample collection. (A) qPCR analysis showed TNFα mRNA levels in WT bone marrow MSCs compared with naïve T cells, activated T cells, Th1 cells, macrophages, and LPS-stimulated macrophages. (B) Western blot analysis showed cytoplasmic TNFα in WT bone marrow MSCs compared with naïve T cells, activated T cells, Th1 cells, macrophages, and LPS-stimulated macrophages. For quantification of Western blotting, a two-tailed Student t-test was used for the comparison between immune cells and MSCs, respectively. (C) ELISA analysis showed the TNFα concentration in the culture supernatant of WT bone marrow MSCs compared with naïve T cells, activated T cells, Th1 cells, macrophages, and LPS-stimulated macrophages. Examination of mRNA expression levels, protein expression levels, and secreted concentrations of TNFα in different cell types. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

To confirm that TNFα-positive MSCs are distributed in the bone marrow, we used Prx1-Cre;tdTomato reporter mice to show that Prx1 and TNFα double-positive cells were detected in the bone marrow and culture-expanded bone marrow-derived MSCs (Figure 17), suggesting that in vivo bone marrow MSCs indeed produce TNFα.
Figure 17. TNFα co-localized with Prx1+ cells in the femur and cultured bone marrow MSCs. Immunofluorescent staining in Prx1-Cre;tdTomato reporter mice showed co-localization of TNFα and Prx1+ cells. Scale bar = 20 μm. All experimental data were verified in at least three independent experiments.

**MSCs produce TNFα for functional maintenance.**

Next, we investigated how TNFα maintains MSC homeostasis in an *in vitro* cell culture system. We examined the dosage response of TNFα/− MSCs to different concentrations of recombinant mouse TNFα (mTNFα) and measured the levels of TNFα in the supernatants and cell lysates 24 hours after treatment. The results showed that mTNFα, at a concentration of 1ng/ml, was able to reestablish the bone marrow MSC physiological concentration (Figure 18 A) without activating canonical TNFR downstream signaling.
(e.g., p53 and NFκB pathways; Figure 18 B). Exogenous 1 ng/ml recombinant mTNFα could mimic the physiologic function of endogenous TNFα in maintaining MSC function.

Figure 18. 1 ng/ml mTNFα is considered a physiological concentration for bone marrow MSCs. 0.5x10^6 bone marrow MSCs were cultured in a 6-well plate and then exposed to a concentration gradient of recombinant mouse TNFα (mTNFα) for 24 hours. (A) ELISA showed the concentration of TNFα in WT and TNFα^-/- bone marrow MSC supernatants. (B) Western blot analysis showed phosphorylated p53 (p-p53), total p53, phosphorylated p65 (p-p65), total p65, and cytoplasmic TNFα in WT and TNFα^-/- bone marrow MSCs exposed to indicated concentration of mTNFα. β-actin was used as a protein loading control. For quantification of Western blotting, a two-tailed Student t-test was used for the comparison between immune cells and MSCs, respectively. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.
Then we applied 1ng/ml mTNFα as the physiological concentration to assess whether exogenous TNFα treatment could rescue the TNFα−/− bone marrow MSC phenotype. We confirmed that 1ng/ml mTNFα treatment failed to activate TNFR downstream signalings, such as p53, caspases apoptosis, p38MAPK, NFκB, or c-Jun/JNK pathways (Figure 19).

**Figure 19. Physiological concentration TNFα fails to activate TNFR downstream signals.** (A-B) Western blot analysis showed the expression levels of p-p53, p53, caspase3, PARP, phosphorylated p38 (p-p38), p38, p-p65, p65, phosphorylated p50 (p-p50), p50, phosphorylated cJun (p-cJun), cJun, phosphorylated JNK (p-JNK), and JNK in WT, TNFα−/−, and 1ng/ml mTNFα-treated TNFα−/− MSCs. β-actin was used as a protein loading control. For quantification of Western blotting, a two-tailed Student t-test was used.
for the comparison between immune cells and MSCs, respectively. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant. JNK: c-Jun N-terminal kinase; NFκB: nuclear factor kappaB.

Therefore, we applied mTNFα at 1 ng/ml and explored whether this “physiological” level of TNFα would benefit MSCs. We have further used the receptor activator of nuclear factor kappaB ligand (RANKL), another member of TNF superfamily cytokines important for bone homeostasis (66), at the same dose of 1 ng/ml to treat MSCs as a control for TNFα to confirm that other TNF superfamily members could not substitute the effect of TNFα in maintaining MSC homeostasis. We discovered that 1 ng/ml TNFα, but not RANKL, was effective in rescuing the dysfunction of MSCs derived from TNFα−/− mice, as proved by complete recovery of colony formation, proliferation, and adipogenesis with partially recovered osteogenic differentiation (Figure 20 A-D).
Figure 20. Physiological concentration of mTNFα rescued MSCs dysfunction from TNFα deficiency. (A) Toluidine blue staining showed the number of CFU-F from WT, TNFα−/−, mTNFα-treated TNFα−/−, and RANKL-treated TNFα−/− bone marrow ANCs (1.5×10⁶ cells, n=3). (B) Proliferation rates of WT, TNFα−/−, mTNFα-treated TNFα−/−, and RANKL-treated TNFα−/− MSCs (n=3) were assessed by BrdU incorporation assay. Scale bar = 100 μm. (C) Alizarin red staining showed the mineralized nodule formation in WT, TNFα−/−, mTNFα-treated TNFα−/−, and RANKL-treated TNFα−/− MSCs (n=3) after cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in WT, TNFα−/−, mTNFα-
treated $TNF\alpha^{-/-}$, and RANKL-treated $TNF\alpha^{-/-}$ MSCs (n=3). β-actin was used as a protein loading control. (D) Oil red O staining showed the lipid droplets formation in WT, $TNF\alpha^{-/-}$, mTNFα-treated $TNF\alpha^{-/-}$, and RANKL-treated $TNF\alpha^{-/-}$ MSCs (n=3) when cultured under adipogenic conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes $PPAR\gamma$ and $LPL$ in WT, $TNF\alpha^{-/-}$, mTNFα-treated $TNF\alpha^{-/-}$, and RANKL-treated $TNF\alpha^{-/-}$ bone marrow MSCs (n=3). β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student $t$-test was used to compare treatment and WT groups. N.S. not significant, *$p<0.05$. RANKL: nuclear factor kappaB ligand; CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

In addition, we used GFP-TNFα plasmid to overexpress TNFα in $TNF\alpha^{-/-}$ MSCs to rescue impaired stem cell functions (Figure 21 A). Overexpression of TNFα in $TNF\alpha^{-/-}$ bone marrow MSCs significantly increased their proliferation rate (Figure 21 B), promoted their osteogenic differentiation (Figure 21 C), and reduced their adipogenic differentiation (Figure 21 D).
Figure 21. Overexpression of TNFα in $TNF\alpha^{-/-}$ bone marrow MSC rescued impaired MSC functions. (A) Western blotting showed GFP and TNFα expression in $TNF\alpha^{-/-}$ MSC, indicating successful plasmid overexpression. (B) The proliferation rate of MSCs was assessed by BrdU incorporation assay. There was a significant increase in the number of BrdU-positive cells in the TNFα overexpression group. Scale bar = 100 μm. (C) Alizarin red staining showed mineralized nodule formation in MSCs cultured under osteoinductive conditions. Western blot analysis showed the expression levels of osteogenic genes
RUNX2, ALP, and OCN in MSCs. (D) Oil red O staining showed the capacity of MSCs to form lipid droplets when cultured under adipoinductive conditions. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare control and overexpression groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase; OE: overexpress.

**TNFα exerts MSC homeostasis in a TNFR-independent manner.**

To exclude the potential effect of TNFR on TNFα-mediated MSC homeostasis maintenance, a small interfering RNA (siRNA) was used to knock down TNFα expression in TNFR−/− MSCs and then used mTNFα to rescue their cell function. After confirming that TNFα siRNA effectively reduced TNFα expression by Western blot analysis (Figure 22 A), 1ng/ml mTNFα was applied for rescue. The results showed that TNFα knockdown dampens TNFR−/− MSC proliferation and differentiation. Proliferation and adipogenesis could be fully rescued with partial rescue in osteogenesis by mTNFα treatment in TNFα-deficient MSCs (Figure 22 B-D). Knockdown of TNFα in TNFR−/− MSC followed by extrinsic TNFα rescue also revealed that TNFα is indispensable, but independent from TNFR signaling, for MSC homeostasis.
Figure 22. Knockdown of TNFα in TNFR⁻/⁻ bone marrow MSC dampened MSC functions, which could be reversed by mTNFα treatment. (A) Western blotting showed pro-TNFα and TNFα expression in MSC, indicating successful siRNA knockdown. (B) The proliferation rate of siControl- and siTNFα-treated bone marrow MSCs were assessed by BrdU incorporation assay. siTNFα (n=3) showed a significantly
decreased proliferation rate compared to the siControl group. Scale bar = 100 μm. (C) Alizarin red staining showed mineralized nodule formation in siControl- and siTNFα-treated MSCs when cultured under osteoinductive conditions. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (D) Oil red O staining showed the capacity of siControl- and siTNFα-treated MSCs to form lipid droplets when cultured under adipoinductive conditions. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and siControl groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

**MSC uptake TNFα via endocytosis**

In our previous data, we noticed that when treating TNFα−/− MSCs with mTNFα, the levels of TNFα detected in culture media were always much lower than what we initially added (Figure 18 A) and that intracellular TNFα could be found in cell lysates (Figure 18 B). These data indicated that TNFα might be uptaken by deficient MSCs despite not stimulating the receptors. To address this hypothesis, we labeled TNFα with fluorescein isothiocyanate (FITC) for MSC treatments and discovered that FITC-labeled mTNFα
(FITC-TNFα) was indeed uptaken by TNFα−/− MSCs (Figure 23 A). Additionally, we detected FITC-labeled TNFα in TNFR−/− MSCs, suggesting that TNFα endocytosis is independent of TNF receptors (Figure 22 A). Next, to examine whether the endocytotic processes mediated TNFα uptake, we used Pitstop® 2, an inhibitor of clathrin-mediated endocytosis (CME) (67), to block endocytosis. We identified that Pitstop® 2 effectively inhibited, but not blocked, TNFα uptake by MSCs via immunofluorescence (IF) microscopy and Western blot analysis (Figure 22 A and B), suggesting that TNFα uptake was at least partially through CME.

**Figure 23.** MSCs uptake TNFα through CME. (A) TNFα−/−, Pitstop® 2-treated TNFα−/−, and TNFR−/− bone marrow MSCs were exposed to FITC-TNFα for 24 hours and
subsequently examined by IF imaging analysis and semi-quantification (n=3). Scale bar = 20 μm. (B) Western blotting showed the TNFα level in *TNFα*<sup>−/−</sup> MSC lysate after being cultured with mTNFα for 24h. The presence of Pitstop® 2 reduced the levels of TNFα in *TNFα*<sup>−/−</sup> MSCs (n=3). β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p*<0.05.

Next, we examined whether TNFα endocytosis regulates MSC function. As expected, Pitstop® 2 treatment further significantly diminished the rescuing effects of TNFα on *TNFα*<sup>−/−</sup> MSCs, leading to remaining proliferative and differentiation dysregulation (Figure 23 A-C). The above results collectively suggested that TNFα safeguarded MSC homeostasis via a receptor-independent manner through endocytosis (Fig. 2L).
Figure 24. TNFα endocytosis regulates MSC function. (A) Proliferation rates of WT, TNFα−/−, and mTNFα-treated TNFα−/− as well as mTNFα and Pitstop® 2 double-treated TNFα−/− MSCs (n=3) were assessed by BrdU incorporation assay. (B) Alizarin red staining showed mineralized nodule formation in WT, TNFα−/−, and mTNFα treated TNFα−/− as well as mTNFα and Pitstop® 2 double-treated TNFα−/− MSCs (n=3) after cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN (n=3). (C) Oil red O staining showed the lipid droplets formation in WT, TNFα−/−, and mTNFα treated TNFα−/− as well as mTNFα and Pitstop® 2 double-treated TNFα−/− MSCs (n=3) when cultured under adipoinductive
conditions for seven days. Scale bar = 100 μm. Western blotting showed the expression levels of adipogenic genes PPARγ and LPL (n=3). β-actin was used as a protein loading control. For quantification of Western blotting, a two-tailed Student t-test was used for the comparison between treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01, ***p<0.005. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

Figure 25. Diagram showing TNFα regulation of MSC homeostasis via a receptor-independent manner through endocytosis.

**Endocytosed TNFα restrains mTOR signaling.**

Next, we dissected the underlying mechanism by which TNFα regulates MSC homeostasis. In screening the important signaling pathways modulating MSC behaviors,
we discovered that the mammalian target of rapamycin (mTOR) signaling changed in correspondence to TNFα deficiency and replenishment (Figure 26 A). In contrast, the canonical Wnt pathway, the transforming growth factor-beta (TGF-β) pathway, and the extracellular signal-regulated kinase (ERK) pathway were not influenced by TNFα as shown by the expression levels of active β-catenin, phosphorylated Smad3 (p-Smad3), and phosphorylated ERK1/2 (p-ERK1/2; Figure 26 B).

Figure 26. Aberrant mTOR signaling was detected in TNFα−/− MSCs. (A) Western blot analysis showed the expression levels of p-mTOR, mTOR, and TNFα in WT, TNFR−/−, TNFα−/−, and mTNFα-treated TNFα−/− MSCs. (B) Western blot analysis showed the expression levels of active β-catenin, β-catenin, p-Smad3, Smad3, p-ERK1/2, and ERK1/2 in MSCs. β-actin was used as a protein loading control. For quantification of
Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

Aberrant activation of mTOR signaling was detected in TNFα−/− MSCs, as revealed by the elevated p-mTOR level (Figure 26 A). We then checked the signal molecules upstream of the mTOR pathway and unexpectedly found no aberrant activation of phosphatase and tensin homologue (PTEN) or phosphoinositide 3-kinase (PI3K)-p110 (Figure 27 A); however, the phosphorylation level of Akt (p-Akt) was elevated in TNFα−/− MSCs (Figure 27 B). It is known that mTOR functions in two distinct kinase complexes, termed mTOR complex1 (mTORC1) and mTOR complex 2 (mTORC2); Akt is a downstream target of mTORC2 that regulates mTORC1 activity (60). Western blot analysis verified that TNFα−/− MSCs exhibited significantly upregulated phosphorylation levels of mTORC2 and mTORC1, including phosphorylated Rictor (p-Rictor), phosphorylated Sin1 (p-Sin1), and phosphorylated Raptor (p-Raptor), as well as mTORC1 downstream effector, phosphorylated P70S6K (p-P70S6K), which were inhibited by mTNFα treatment (Figure 27 B and C).
Figure 27. Upstream and downstream of mTOR signaling. (A) Western blot showed the expression levels of PI3K and PTEN, representing the upstream of mTOR signaling in WT, TNFR<sup>−/−</sup>, TNFα<sup>−/−</sup>, and mTNFα-treated TNFα<sup>−/−</sup> MSCs. (B) Western blot analysis showed the expression levels of p-Akt, Akt, and mTORC2 components p-Raptor, Raptor, and p-P70S6K in MSCs. (C) Western blot analysis showed the expression levels of mTORC2 components p-Rictor, Rictor, p-Sin1, and Sin1 MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.
Moreover, TNFα siRNA-treated WT bone marrow MSCs showed aberrant mTOR activation, which was also fully suppressed by mTNFα treatment (Figure 28).

**Figure 28.** mTOR signaling in siTNFα bone marrow MSCs. (A) Western blot analysis showed the levels of mTORC2 components p-Rictor, Rictor in MSC. (B) Western blot analysis showed the expression levels of p-Akt, Akt and mTORC1 components p-Raptor, Raptor, and p-P70S6K in MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.
Endocytosed TNFα binds to mTORC2.

To decipher how TNFα regulates mTOR signaling in MSCs, we hypothesized that TNFα interacted with mTOR complexes directly, given the unaffected upstream factors, PI3K and PTEN (Figure 27 A). To address this hypothesis, we applied a protein co-immunoprecipitation (co-IP) assay to examine the potential binding of TNFα with mTOR complexes. We found that TNFα bound with mTORC2 proteins, including mTOR, Rictor, and Sin1 (Figure 29 A and B), whereas TNFα failed to bind with Raptor, a mTORC1 protein, in WT and mTNFα-treated TNFα−/− MSCs, (Figure 29 C).
Figure 29. co-IP showed TNFα binds to mTORC2 rather than mTORC1. (A) TNFα was immunoprecipitated from WT, \( \text{TNF} \alpha^{-/-} \), and mTNFα-treated \( \text{TNF} \alpha^{-/-} \) MSC lysates. Subsequently, a Western blot analysis was performed using anti-mTOR, -Rictor, and -Sin1 antibodies. Immunoprecipitation assay showed that cytoplasmic TNFα bound to mTORC 2. (B) mTOR, Rictor, and Sin1 were immunoprecipitated from WT MSC lysates, and Western blot analysis was subsequently performed to confirm the interaction between TNFα and mTORC 2. (C) TNFα was immunoprecipitated from WT, \( \text{TNF} \alpha^{-/-} \), and mTNFα-treated \( \text{TNF} \alpha^{-/-} \) MSC lysates. Subsequently, a Western blot was used to show a lack of cytoplasmic TNFα binding to the mTORC1 component, Raptor. To quantify Western blotting, relative protein levels were normalized to the input; a two-tailed Student t-test was used to compare the immunoprecipitated group and the input. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

To examine whether TNFα regulates mTOR kinase activity, we next performed an ELISA-based activity assay followed by immunoprecipitation of mTOR antibody to measure the kinase activity of mTORC1 in WT, \( \text{TNF} \alpha^{-/-} \), \( \text{TNF} \alpha^{-/-} \), and mTNFα-treated \( \text{TNF} \alpha^{-/-} \) MSCs. The results showed that \( \text{TNF} \alpha^{-/-} \) MSCs had more significant mTORC1 kinase activity than WT and \( \text{TNF} \alpha^{-/-} \) controls. The elevated mTORC1 kinase activity was down-regulated by mTNFα treatment (Figure 30 A). Furthermore, we showed that mTNFα inhibited mTORC1 kinase activity in a dose-dependent manner (Figure 30 B). These converging lines of evidence prompted us to hypothesize that TNFα may directly bind to mTORC2 to regulate mTORC2 signaling and subsequently be capable of modulating mTORC1 activity.
Figure 30. mTOR kinase activity measured by an ELISA-based activity assay. (A) Whole-cell lysates were collected from WT, TNFR<sup>−/−</sup>, TNFα<sup>−/−</sup>, and mTNFα-treated TNFα<sup>−/−</sup> MSCs (n=3), followed by immunoprecipitation with an anti-mTOR antibody. Purified and enriched mTOR complexes were used to measure mTOR activity based on the levels of P70S6K phosphorylation and mTORC1 substrate. (B) mTOR kinase activity was determined in a different concentration of mTNFα (n=3). Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, ***p<0.005.

**Mature TNFα, but not pro-TNFα, restrains aberrant mTOR activation.**

There are two forms of TNFα: 26kDa transmembrane pro-TNFα and 17kDa mature soluble TNFα. pro-TNFα is cleaved by the TNFα-converting enzyme (TACE) on the cell membrane and becomes mature TNFα, which is then released into the extracellular environment. It is unknown why MSCs need to endocytose TNFα from the extracellular environment instead of utilizing cytoplasmic pro-TNFα to restrain mTOR activation. In order to dig into this question, the TACE inhibitor, TAPI-2, was used to block the cleavage of pro-TNFα. We found that TNFα concentration in the cell culture supernatant was
reduced, the level of pro-TNFα was increased, and mature TNFα was decreased in the cytoplasm simultaneously (Figure 31 A and B).

![Image](image_url)

**Figure 31.** TAPI-2 treatment suppressed the conversion of pro-TNFα to mature TNFα. (A) ELISA assay showed the TNFα concentration in the cell culture supernatant of WT and TAPI-2 treated WT MSCs. (B) Western blot analysis showed the expression levels of pro-TNFα and TNFα in WT and TAPI-2-treated WT MSCs. β-actin was used as a protein loading control. A two-tailed Student t-test was used to compare treatment and WT groups to quantify Western blotting. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01, ***p<0.005.

We have also confirmed that the release and uptake cycle of TNFα contributed to mTOR suppression in MSCs, as inhibiting TACE by TAPI-2 and inhibiting CME by Pitstop® 2 resulted in mTOR signaling activation, mimicking the effects of TNFα deficiency (Figure 32).
Figure 32. The release and uptake cycle of TNFα contributed to mTOR suppression.

Western blotting showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, and P70S6K in WT, TAPI-2 treated WT, and TNFα−/− MSCs (n=3). β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01, ***p<0.005.

The data above suggested that only mature TNFα, but not pro-TNFα, was capable of restraining activation of the mTORC2/Akt/mTORC1 cascade in MSC. We next applied recombinant human mature TNFα and human precursor TNFα (pro-TNFα) to rescue impaired TNFα−/− MSCs. We first used FITC-labeled cytokines to confirm that both TNFα and pro-TNFα could be internalized by MSCs (Figure 33 A and B), and then we found that only TNFα, but not pro-TNFα, downregulated the mTORC2/Akt/mTORC1 cascade (Figure 33 B).
Figure 33. MSCs internalize hTNFα and pro-hTNFα. (A) *TNFα*−/− bone marrow MSCs were exposed to FITC-TNFα and FITC-pro-TNFα for 24 hours and subsequently examined by cell imaging analysis and quantification. Scale bar = 20 μm. (B) Western blotting showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, P70S6K, pro-TNFα, and TNFα in WT, *TNFα*−/−, TNFα-treated *TNFα*−/−, and pro-TNFα-treated *TNFα*−/− MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

Meanwhile, cell function assays revealed that pro-TNFα treatment failed to rescue CFU-F number, proliferation rate, and osteogenic and adipogenic differentiation in *TNFα*−/−. 
MSCs (Figure 34 A-D). These data indicated that endocytosed TNFα bound to mTORC2 and restrained activation of mTOR signaling in MSCs, which might be responsible for functional regulation.

Figure 34. pro-hTNFα treatment failed to rescue TNFα deficiency cell phenotype. (A) Toluidine blue staining showed the number of CFU-F from WT, TNFα−/−, TNFα-treated TNFα−/−, and pro-TNFα-treated TNFα−/− bone marrow ANCs (1.5×10⁶ cells). (B) The proliferation rate of bone marrow MSCs was assessed by BrdU incorporation assay. Scale bar = 100 μm. (C) Alizarin red staining showed mineralized nodule formation in WT, TNFα−/−, TNFα-treated TNFα−/−, and pro-TNFα-treated TNFα−/− MSCs when cultured under
osteoinductive conditions. Western blot analysis showed the expression levels of osteogenic genes \textit{RUNX2}, \textit{ALP}, and \textit{OCN} in MSCs. (D) Oil red O staining showed the capacity of WT, \textit{TNF}\textsubscript{α\textsuperscript{-/-}}, TNF\textsubscript{α}-treated \textit{TNF}\textsubscript{α\textsuperscript{-/-}}, and pro-TNF\textsubscript{α}-treated \textit{TNF}\textsubscript{α\textsuperscript{-/-}} MSCs to form lipid droplets when cultured under adipoinductive conditions. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes \textit{PPARγ} and \textit{LPL} in MSCs. β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01. CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

Furthermore, we showed that exogenous TNF\textsubscript{α}, but not pro-TNF\textsubscript{α}, bound with mTORC2 proteins in MSCs (Figure 35 A), and mTORC1 kinase activity was downregulated by \textit{in vitro} treatment with TNF\textsubscript{α}, but not pro-TNF\textsubscript{α} (Figure 35 B).
Figure 35. mature TNFα binds to mTORC2 rather than pro-TNFα. (A) TNFα was immunoprecipitated from WT, TNFα−/−, hTNFα-treated TNFα−/−, and pro-hTNFα-treated TNFα−/− MSC lysates (n=3), and subsequent Western blot analysis was used to detect mTOR, Rictor, pro-TNFα, and TNFα. To quantify Western blotting, relative protein levels were normalized to input, a two-tailed Student t-test was used to compare treatment and WT groups. Immunoprecipitation assay revealed that pro-hTNFα failed to bind to mTORC2. (B) mTOR kinase activity was determined in the presence of hTNFα or pro-hTNFα by an ELISA-based activity assay (n=3). All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

Figure 36. Diagram showing mature TNFα finetunes mTOR signaling by direct binding to mTORC2 after being internalized into the cytoplasm.
**Mechanical stimuli boost TNFα endocytosis.**

Next, we aimed to examine how the release and uptake of TNFα are coupled for MSC regulation. MSCs are exposed to various mechanical stimuli *in vivo* (28), which have been reported to influence the cellular endocytic processes (50, 51). As mechanical stimuli have been reported to enhance CME (50, 51), we hypothesized that mechanical force loading induces TNFα endocytosis to maintain MSC homeostasis. We first examined whether mechanical stretch loading affected MSC's intake of FITC-labeled mTNFα (FITC-TNFα) using a Flexcell Fx-4000T tension unit, followed by flow cytometry and IF microscopy. The results showed that the stimulus of a 2-hour *in vitro* mechanical stretch significantly boosted TNFα endocytosis in both WT (Figure 37 A and C) and *TNFα*−/− MSCs (Figure 37 E and G). Then we used Alexa Fluor™ 488-conjugated albumin from bovine serum (488-BSA) to confirm that the general endocytosis rate was also boosted by mechanical stretching in WT (Figure 37 B and D) and *TNFα*−/− MSCs (Figure 37 F and H). Pitstop® 2, a CME inhibitor, attenuated the stretch-stimulated endocytosis process (Figure 37 A-H).
Figure 37. Mechanical force upregulates TNFα and overall endocytosis rate. (A and B) Flow cytometric analysis indicated increased rates of TNFα endocytosis (A) and albumin endocytosis (B) after 2-hour of mechanical stretching of WT MSCs. These changes were abolished by Pitstop® 2 treatment. (C and D) Fluorescence microscopy analysis and semi-quantification showed that 2-hour mechanical stretching increased TNFα (C) and albumin (D) endocytosis rates in WT MSCs, and these changes were abolished by Pitstop® 2 treatment. Scale bar = 50 μm. (E and F) Flow cytometric analysis showed increased rates of TNFα endocytosis (A) and albumin endocytosis (B) after 2-hour of mechanical stretching of TNFα−/− MSCs; this increased TNFα endocytosis was blocked by Pitstop® 2 treatment. (G and H) Fluorescence microscopy analysis and semi-quantification showed that 2-hour mechanical stretching increased TNFα (G) and albumin (H) endocytosis rates in TNFα−/− bone marrow MSCs. These changes were abolished by Pitstop® 2 treatment. Scale bar = 50 μm. All experimental data were verified in at least
three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

To investigate whether mechanical force regulates the TNFα shuttle across the MSC membrane, we applied 2-hour cyclic mechanical stretching for two consecutive days to MSCs and examined TNFα levels during force loading at indicated time points. Compared to the control group, the mechanical stretch-loaded group experienced a sharp drop of TNFα in the cell culture supernatant during mechanical stretching, as assessed by ELISA analysis, along with an increase to the normal levels during the resting periods (Figure 38 A). Correspondingly, Western blot analysis showed an elevated cytoplasmic TNFα protein level immediately after mechanical stretching and diminished upon resting (Figure 38 B). These data suggested constitutive production and release of TNFα by MSCs with inducible internalization triggered by mechanical force.

Figure 38. Extracellular and intracellular TNFα level fluctuates during mechanical stretching. (A) ELISA assays showed the concentration of TNFα in the cell culture media of control and mechanical force-loaded MSCs at indicated time points in a cyclic mechanical force-loading system (n=3). Concentration values were normalized to the...
respective original values. (B) Western blotting showed cytoplasmic TNFα levels at indicated time points in a cyclic mechanical force loading system (n=3). β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

In dissecting whether force-driven dynamics of TNFα contribute to MSC regulation, we examined and revealed changes in mTOR signaling activation following stretch-provoked TNFα fluctuations (Figure 39).

**Figure 39. mTOR signaling fluctuates during mechanical stretching.** Western blotting showed the expression levels of mTORC2/Akt/mTORC1 at indicated time points in a cyclic mechanical force loading system (n=3). β-actin was used as a protein loading control. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.
Next, we aimed to examine whether stretching-induced TNFα endocytosis promoted MSC functions. Real-time PCR analysis showed that cyclic stretching failed to increase TNFα mRNA level, suggesting that mechanical stretching may directly elevate cytoplasmic TNFα without upregulating its gene expression (Figure 40 A). Further cellular functional analyses demonstrated that intermittent stretch at 2-hour per time, twice a week, significantly promoted proliferation and osteogenic differentiation of MSCs with inhibition of adipogenesis (Figure 40 B-D).

**Figure 40. Mechanical stretching promoted MSC functions.** (A) qPCR analysis showed the expression level of TNFα mRNA in control and mechanical stretch-loaded MSCs. (B) The proliferation rate of control and mechanical stretch-loaded MSCs was assessed by BrdU incorporation assay. Scale bar = 100 μm. (C) Alizarin red staining
showed mineralized nodule formation of control and mechanical stretch-loaded bone marrow MSCs after being cultured under osteoinductive conditions for four weeks. Western blot analysis showed osteogenic genes RUNX2, ALP, and OCN expression levels in mechanical stretch-loaded and control MSCs. (D) Oil red O staining showed lipid droplet formation in control and mechanical stretch-loaded MSCs cultured under adipogenic conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. All experimental data were verified in at least three independent experiments. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

To further confirm that mechanical stretching restrained mTOR signaling via TNFα endocytosis, we applied mechanical stretching to TNFα−/− MSCs and then examined the mTOR signaling. The results showed no significant alteration between the stretch-loading and control groups (Figure 41 A). mTNFα treatment followed by mechanical stretching restrained p-mTOR, p-Rictor, p-Akt, p-Raptor, and p-P70S6K expression, whereas this restraint of mTOR signaling was diminished with the presence of Pitstop® 2 (Figure 41 B). These data suggest that endocytic TNFα is intensified during mechanical stretching and exerts its role in tuning mTOR signaling activity. Collectively, these results indicated that mechanical force drove TNFα endocytosis and promoted MSC function (Figure 42).
Figure 41. Mechanical stretching restrained mTOR signaling via TNFα endocytosis. (A) Western blotting showed that control and mechanical stretch-loaded *TNFα*<sup>−/−</sup> MSCs expressed a similar level of mTORC2/Akt/mTORC1. (B) Western blotting showed the expression levels of TNFα and mTORC2/Akt/mTORC1 in MSCs. β-actin was used as a protein loading control. All experimental data were verified in at least three independent experiments. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p*<0.05.
Figure 42. Diagram showing mechanical force governs TNFα endocytosis and therefore regulates MSC homeostasis via a receptor-independent manner through mTOR signaling.

**Rapamycin protects MSC function against TNFα deficiency.**

The results above prompted us to investigate whether inhibition of mTOR signaling contributes to the functional regulation of MSCs by TNFα and whether pharmaceutical mTOR suppression serves as an effective approach to rescue the functional decline of MSCs under TNFα deficiency. In this regard, we tested rapamycin, a potent and specific chemical inhibitor of mTOR complexes and an FDA-approved drug in clinics (65), on TNFα−/− MSCs.
To evaluate whether inhibition of mTOR signaling rescues the functional decline of MSCs in TNFα−/− mice in vivo, rapamycin was intraperitoneally administered at a dosage of 1.5 mg/kg per day to TNFα−/− mice at eight weeks of age for fourteen consecutive days and the samples were harvested at ten weeks of age for further evaluation (Figure 43 A). After two weeks of rapamycin administration, bone marrow MSCs demonstrated remarkable recovery despite TNFα deficiency, forming more fibroblastic colonies ex vivo (Figure 43 B). Furthermore, MSCs derived from rapamycin-treated TNFα−/− mice showed a fully recovered proliferative capacity (Figure 43 C). For differentiation, MSCs derived from rapamycin-treated TNFα−/− mice preferred osteogenesis over adipogenesis, with a full recovery in adipogenesis and partial recovery in osteogenesis (Figure 43 D and E).
Figure 43. Rapamycin infusion rescues TNFα-deficient MSCs. (A) The experimental outline describing mTOR inhibitor rapamycin was i.p. injected into TNFα−/− mice at a dose of 1.5 mg/kg/d for 14 days. Then the femurs and bone marrow MSCs were harvested for further analysis. (B) Toluidine blue staining showed the number of CFU-F from WT, TNFα−/−, and rapamycin-treated TNFα−/− mice bone marrow ANCs (1.5×10⁶ cells). (C) The proliferation rate of bone marrow MSCs was assessed by BrdU incorporation assay. Scale bar = 100 μm. (D) Alizarin red staining showed the mineralized nodule formation of bone marrow MSCs when cultured under osteoinductive conditions for four weeks.
Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (E) Oil red O staining showed lipid droplet formation in bone marrow MSCs cultured under adipogenic conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. All experimental data were verified in at least three independent experiments. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01, ***p<0.005. CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

Molecular examinations in ex vivo MSCs confirmed that rapamycin treatment repressed mTOR signaling under TNFα deficiency by downregulating the phosphorylation level of P70S6K in MSCs from TNFα−/− mice (Figure 44).
**Figure 44. Rapamycin infusion inhibits mTORC1 activity.** Western blot analysis showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, and P70S6K in bone marrow MSCs from WT, TNFα−/−, and rapamycin-treated TNFα−/− mice (n=3). β-actin was used as a protein loading control. To quantify western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

To further confirm that rapamycin treatment ameliorates the lineage alteration of bone marrow MSCs *in vivo*, we examined the osteogenic condition of the trabecular bone in WT, TNFα−/− and rapamycin-treated TNFα−/− mice. We found that the reduced number of osteoblasts (Figure 45 A) and bone turnover rate (Figure 45 B) in TNFα−/− mice could be partially rescued by rapamycin treatment. On the other hand, Oil Red O staining showed a significantly reduced fatty marrow area in the bone marrow tissue in rapamycin-treated TNFα−/− mice compared to the untreated group (Figure 45 C).
Figure 45. Rapamycin infusion rescues TNFα-deficient bone marrow phenotype.

(A) ALP staining showed the number of ALP-positive osteoblasts in the distal femur of WT, TNFα−/−, and rapamycin-treated TNFα−/− mice (n=3). Scale bar = 100 μm. (B) Calcein double labeling of the distal femur showed the bone turnover rate in WT, TNFα−/−, and rapamycin-treated TNFα−/− mice (n=3). Scale bar = 100 μm. (C) Oil red O staining showed the number of adipocytes in the distal femur of WT, TNFα−/−, and rapamycin-treated TNFα−/− mice (n=3). Scale bar = 500 μm. All experimental data were verified in at least three independent experiments. Error Scale bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01, ***p<0.005. Rapa: rapamycin; MAR: mineral apposition
rate; BFR/BS: Bone formation rate per bone surface (BFR=MAR x (MS/BS)); MS/BS: mineralizing surface per bone surface.

In order to exclude potential off-target effects of rapamycin treatment, we used rapamycin to treat TNFα−/− MSCs. We found that rapamycin can rescue impaired MSC function (Figure 46) and restrain mTOR signaling (Figure 47) in vitro as well.
Figure 46. Rapamycin rescues TNFα-deficient MSCs in vitro. (A) Toluidine blue staining showed the number of CFU-F from WT, TNFα−/−, and in vitro rapamycin-treated TNFα−/− bone marrow ANCs (1.5×10^6 cells). (B) The proliferation rate of bone marrow MSCs was assessed by BrdU incorporation assay. Scale bar = 100 μm. (C) Alizarin red staining showed the mineralized nodule formation of bone marrow MSCs when cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (D) Oil red O staining showed lipid droplet formation in bone marrow MSCs cultured under adipoinductive conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. All experimental data were verified in at least three independent experiments. To quantify Western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01. CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.
Figure 47. Rapamycin treatment inhibits mTORC1 activity in cultured MSC. Western blot analysis showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, and P70S6K in MSCs from WT, TNFα−/−, and rapamycin-treated TNFα−/− mice (n=3). β-actin was used as a protein loading control. To quantify western blotting, a two-tailed Student t-test was used to compare treatment and WT groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

Mechanical unloading induces functional decline of MSCs and bone loss counteracted by rapamycin.

Inspired by the above data, we intended to investigate the pathophysiological implication of mechanical force-induced TNFα endocytosis. It is well known that a lack of
mechanical stimuli, for example, in astronauts and bedridden patients, may cause progressive loss of bone mass (68). Hindlimb unloading (HU) of mice has been widely used as a ground-based model to simulate the effects of spaceflight and microgravity environments, in which osteopenia and MSC dysfunction have been reported (41, 44).

To examine whether the bone loss in HU mice is mainly attributed to TNFα-associated MSC deficiency, we generated Prx1-Cre;tdTomato reporter mice, in which Prx1+ bone marrow MSCs were tdTomato positive (Figure 48 A). We isolated all nuclear cells (ANCs) from the reporter mouse bone marrow and performed flow cytometry analysis. There was significantly downregulated TNFα and upregulated P-mTOR expression of p-mTOR in Prx1+ ANCs after 14 days of hindlimb unloading compared to the control group (Figure 48 B). This finding was confirmed in an ANC smear experiment showing a significantly decreased TNFα level (Figure 48 C) and elevated mTOR activity (Figure 48 D) in tdTomato+ cells in the HU group. In addition, an increased level of TNFα in HU mouse bone marrow plasma was found compared to the control group (Figure 48 E). These findings indicated diminished TNFα dynamics with mTOR signaling activation in bone marrow MSCs under mechanical unloading.
Figure 48. Hindlimb unloading mice exhibit altered TNFα expression and mTOR activity in the bone marrow. (A) The experimental outline describes the generation of Prx1-Cre;tdTomato reporter mice and the hindlimb unloading (HU) model. (B) Flow cytometric analysis showed the expression levels of TNFα p-mTOR in Prx1-tdTomato-positive ANCs isolated from the reporter mouse (n=3) bone marrow after 14 days HU. (C and D) Immunofluorescent staining and semi-quantification showed downregulated expression of TNFα (C) and upregulated expression of p-mTOR (D) in Prx1+ cells in bone marrow ANCs of HU mice. Scale bar = 25 μm. (E) ELISA analysis showed the TNFα concentration in the bone marrow plasma from control and HU mice. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05, **p<0.01. HU: hindlimb unloading.
Considering the above findings, we continued to evaluate whether inhibiting mTOR signaling by rapamycin infusion could counteract the putative functional decline of MSCs and osteopenia phenotype upon hindlimb suspension by downregulating mTOR signaling. Rapamycin was intraperitoneally administered to HU mice for fourteen consecutive days, after which the samples were harvested for further evaluation. As expected, the HU group showed hyperactivated mTOR signaling of MSC when compared to the control group, and rapamycin administration prevented activation of mTOR signaling upon hindlimb suspension (Fig. 49). Furthermore, rapamycin treatment in vivo protected MSCs against HU-induced functional impairments, demonstrating improved colony formation, proliferation and osteogenic differentiation in MSCs derived from rapamycin-treated mice with suppressed adipogenesis as a partial rescue in cell function (Figure 50).

**Figure 49.** Rapamycin prevented activation of mTOR signaling of MSCs upon hindlimb suspension. Western blot analysis showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, and P70S6K in bone
marrow MSCs from Control, HU, and rapamycin-treated HU mice (n=3). β-actin was used as a protein loading control. A two-tailed Student t-test was used to compare treatment and control groups to quantify western blotting. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. HU: hindlimb unloading; Rapa: rapamycin.

Figure 50. Rapamycin infusion protected MSCs against HU-induced functional impairments. (A) The experimental outline describes the generation of Prx1-
Cre;tdTomato reporter mice. mTOR inhibitor rapamycin was i.p. injected into hindlimb unloading mice at a dose of 1.5 mg/kg/d for 14 days. Subsequently, the femurs and bone marrow MSCs were harvested for further analysis. (B) Toluidine blue staining showed the number of CFU-F from control, HU, and rapamycin-treated HU mice bone marrow ANCs (1.5×10^6 cells). (C) Proliferation rates of bone marrow MSCs were assessed by BrdU incorporation assay. Scale bar = 100 μm. (D) Alizarin red staining showed the mineralized nodule formation in bone marrow MSCs when cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (E) Oil red O staining showed lipid droplet formation in bone marrow MSCs cultured under adipoinductive conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. For quantification of western blotting, a two-tailed Student t-test was used for the comparison between treatment and control groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. ANCs: all nuclear cells; CFU-F: Colony Forming Unit - Fibroblasts; BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase; HU: hindlimb unloading; Rapa: rapamycin.

Furthermore, in vivo osteogenic and adipogenic capacities of WT, HU, and rapamycin-treated HU mice were examined. We found that rapamycin infusion substantially prevented the decline of bone formation in HU mice. The reduced number of osteoblasts (Figure 51 A) and bone turnover rate (Figure 51 B) were fully rescued by rapamycin treatment in the HU mice. MicroCT analysis showed that rapamycin treatment
significantly increased the BMD and BV/TV ratio in HU mice (Figure 51 C). Histological analysis showed that the decreased trabecular bone volume in HU mice was completely rescued by rapamycin treatment (Figure 51 C). Rapamycin also completely rescued bone marrow adiposity revealed by adipocytes staining in the bone marrow (Figure 51 D).

**Figure 51. Rapamycin infusion substantially prevented the decline of bone formation in HU mice bone marrow.** (A) ALP staining showed the number of ALP-positive osteoblasts in the distal femur of control, HU, and rapamycin-treated HU mice. Scale bar = 100 μm. (B) Calcein double labeling of the distal femur showed the bone turnover rate in mice. Scale bar = 100 μm. (C) MicroCT 3-D construction image and H&E staining showed the BMD, BV/TV ratio, and trabecular bone volume in the distal femur of mice (n =5). (D) Oil red O staining showed the number of adipocytes in the distal femur of mice. Scale bar = 500μm. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not
significant, *p<0.05, **p<0.01, ***p<0.005. HU: hindlimb unloading; Rapa: rapamycin; MAR: Mineral apposition rate; BFR/BS: Bone formation rate per bone surface (BFR=MAR x (MS/BS)). MS/BS: Mineralizing surface per bone surface.

Finally, we identified rapamycin as an effective therapy for preserving bone mass despite mechanical unloading. Collectively, these data indicated that mechanical unloading induced functional decline of MSCs and bone loss based on TNFα and mTOR signaling dysregulation, which was counteracted by rapamycin (Figure 52). Inhibition of mTOR activity, such as rapamycin treatment, may prevent progressive bone loss experienced by astronauts in spaceflight and bedridden patients.

Figure 52. Diagram illustrating that rapamycin might be a therapeutic target to maintain MSC and bone marrow homeostasis by restraining mTOR signaling in mechanical unloading conditions.
**TNFα endocytosis is related to the aging process.**

It is well known that mTOR signaling contributes to the aging process (69-77). We, therefore, tested a hypothesis that TNFα endocytosis-mediated mTOR suppression may partially rescue aged bone marrow MSCs. As expected, activated mTOR signaling was detected in bone marrow MSCs from 20-month-old (20M) mice when compared to 3-month-old (3M) control mice, as assessed by Western blotting (Figure 53). This activated mTOR expression could be suppressed by *in vitro* mTNFα treatment (Figure 53).

**Figure 53. Exogenous TNFα rejuvenated aging MSC by mTOR suppression.** Western blot analysis showed the expression levels of p-mTOR, mTOR, p-Rictor, Rictor, p-Akt, Akt, p-Raptor, Raptor, p-P70S6K, P70S6K, pro-TNFα, and TNFα in bone marrow MSCs from 3-month-old and 20-month-old mice (n=3). β-actin was used as a protein loading control. To quantify western blotting, a two-tailed Student t-test was used to compare treatment and control groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.
Since prominent changes in cell size are one of the major attributes that distinguish senescent cells from their normal counterparts (78), we used transmission electron microscopy (TEM) analysis to show enlarged cell and nuclei size in 20M MSCs compared to 3M MSCs and mTNFα-treated 20M MSCs (Figure 54).

Figure 54. TEM image and nuclei analysis of aged MSC. Two-dimensional cell and cell nuclear size analysis by transmission electron microscopy (TEM) of 3M, 20M, and mTNFα-treated 20M MSCs. Scale bar = 4 μm. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. ***p<0.005.

Furthermore, we found that in vitro exogenous TNFα treatment was able to increase the proliferation rate (Figure 55 A), upregulate the osteogenic differentiation capacity (Figure 55 B), and downregulate the adipogenic differentiation capacity of aged bone marrow MSCs (Figure 55 C).
Figure 55. exogenous TNFα improved aged MSC functions. (A) Proliferation rates of 3M, 20M, and mTNFα-treated 20M MSCs were assessed by BrdU incorporation assay. Scale bar = 100 μm. (B) Alizarin red staining showed the mineralized nodule formation in bone marrow MSCs when cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (C) Oil red O staining showed lipid droplet formation in bone marrow MSCs cultured under adipoinductive conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. To quantify western blotting, a two-tailed Student t-test was used to compare treatment and 3M groups. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.
Age-related downregulation of endocytosis was reported in rodent thyroid, liver, and neural systems (79-81). Thus, we next questioned whether the aberrant activation of mTOR in aged MSCs was related to the reduction of endocytic TNFα. Using IF microscopy and flow cytometry, we found that aged MSCs showed a reduced endocytic capacity compared to the control group, and mechanical stretching was able to intensify the TNFα and BSA endocytosis in aged MSCs (Figure 56).

Figure 56. Mechanical stimuli enhance aged MSC endocytosis. (A and C) Fluorescence microscopy analysis and semi-quantification showed decreased TNFα (A) and albumin (B) endocytosis in aged MSCs, which could be intensified by 2-hour
mechanical stretching. Scale bar = 50 μm. (B and D) Flow cytometric analysis showed decreased rates of TNFα endocytosis (B) and albumin endocytosis (D) of aged MSCs, which could be rescued by 2-hour mechanical stretching. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05.

Interestingly, without adding additional TNFα, cyclic stretch failed to alleviate activated mTOR signaling (Figure 57 A) and improve proliferation and differentiation in aged MSCs (Figure 57 B-D). These findings indicate that endocytosis of TNFα, rather than overall endocytosis, is the key to rescuing aged MSCs.
Figure 57. Mechanical stretch failed to improve aged MSC function. (A) Western blot analysis showed the expression levels of p-mTOR, mTOR, p-P70S6K, P70S6K, pro-TNFα, and TNFα in aged bone marrow MSCs. (B) Proliferation rates of MSCs were assessed by BrdU incorporation assay. Scale bar = 100 μm. (C) Alizarin red staining showed the mineralized nodule formation in bone marrow MSCs when cultured under osteoinductive conditions for four weeks. Western blot analysis showed the expression levels of osteogenic genes RUNX2, ALP, and OCN in MSCs. (D) Oil red O staining showed lipid droplet formation in bone marrow MSCs cultured under adipogenic
conditions for seven days. Scale bar = 100 μm. Western blot analysis showed the expression levels of adipogenic genes PPARγ and LPL in MSCs. β-actin was used as a protein loading control. A two-tailed Student t-test was used to compare stretch and control groups to quantify western blotting. All experimental data were verified in at least three independent experiments. Error bars represent the s.d. from the mean values. N.S. not significant, *p<0.05. BrdU: bromodeoxyuridine; RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin; PPARγ: peroxisome proliferator-activated receptor gamma 2; LPL: lipoprotein lipase.

Collectively, we found that endogenous pro-TNFα undergoes proteolytic cleavage by TACE at the cell membrane to release soluble mature TNFα. Under mechanical force stimuli, mature TNFα is endocytosed into the cytoplasm through CME and binds to mTORC2 to maintain MSC homeostasis via fine-tuning mTOR signaling. TNFα deficiency or lack of mechanical force stimulation results in an aberrant mTOR activation in bone marrow MSC and alters their lineage differentiation to an adipogenesis-preferred impaired status (Figure 58).
Figure 58. Endogenous pro-TNFα undergoes proteolytic cleavage by TNFα-converting enzyme (TACE) at the cell membrane and then is released as soluble mature TNFα. Mature TNFα is internalized into the cytoplasm through clathrin-mediated endocytosis, modulated by mechanical force stimuli. Cytoplasmic TNFα binds to mTORC2 and fine-tunes mTOR signaling to maintain MSC homeostasis. TNFα deficiency or lack of mechanical force leads to aberrant mTOR signaling activation in MSCs and alters their lineage differentiation to an adipogenesis-preferred status. Rapamycin treatment may serve as an effective therapy for TNFα deficiency- and hypodynamia-induced MSC impairment.
Discussion

MSC and cytokines

The interplay between cytokines and MSCs may determine MSC’s differentiation traits. MSCs interact with the immune system for functional modulation and tissue/organ homeostatic maintenance (15, 20). The regulatory framework of MSC function involves dynamic interactions of MSCs with the surrounding niche. MSCs secrete a broad spectrum of cytokines to modulate niches and respond to various environmental cues for behavioral adaptations (15, 82). On the one hand, the niche factors, immune components, and exceptionally high concentrations of inflammatory cytokines profoundly govern MSC behaviors in resident and transplanted tissues. They diminish MSC function in pathophysiological processes to induce tissue deterioration, such as osteoporosis (17, 19). Bone marrow MSCs are known to produce a trivial amount of TNFα and to express TNFR1 and TNFR2 (19, 24). Previous studies showed that a high concentration of TNFα, owing to an inflammatory environment caused by the immune response, induces MSC apoptosis and impairs MSC-based regeneration via activating TNFR pathways (17, 19).

On the other hand, inflammatory cytokines also prime MSCs for function when MSCs are infused for the immunomodulation (20, 21). Interestingly, In this regard, despite evidence suggesting that MSCs secret inflammatory cytokines as a pro-inflammatory functional state (22), it remains elusive whether MSC-derived inflammatory cytokines influence their behaviors in physiology. Inflammatory cytokines play crucial roles in regulating various basic biological processes in the organism, whereas the effects and
mechanisms of inflammatory cytokines safeguarding the stem cell pool remain largely unexplored (17, 19, 20).

**TNFα maintains MSC homeostasis at a physiological concentration.**

In this study, we, for the first time, reveal that MSCs produce and release low levels of TNFα, which is indispensable for the functional maintenance of MSCs. The autocrine/paracrine mode of TNFα regulation on MSCs indicates that MSCs create a beneficial physiological niche for their homeostasis. Studies have also reported that other inflammatory cytokines, such as IFNγ and IL-6, serve as autocrine regulators of MSCs. Still, the observations were performed in the differentiation process or under inflammatory conditions (25, 83). Here, we identify that genetic depletion of only TNFα, but not IFNγ or IL-6, results in MSC impairments, suggesting that TNFα possesses the functional peculiarity among inflammatory cytokines to safeguard stemness of MSCs.

We further uncover a new mechanism mediating the beneficial effects of low levels of TNFα on MSCs, which is proved to be a receptor-independent manner. The receptor-independent mechanism(s) mediating the effects of inflammatory cytokines have not been revealed. We found that TNFα−/−, but not TNFR−/−, MSCs showed impaired differentiation and proliferation. It is generally believed that TNFα binds with TNFRs to induce signaling cascades; however, our data suggest that TNFα, at a physiological concentration, plays a vital role in MSC homeostasis using a TNFR-independent mechanism. Based on these findings, we defined a physiologic range of TNFα, which
provides appropriate intracellular and extracellular levels of TNFα without activating canonical TNFR-mediated signaling.

Furthermore, we used TNFα siRNA to knock down TNFα expression in WT MSCs. We found similar phenotypes to those observed in TNFα−/− MSCs, confirming that a physiological concentration of TNFα is required to maintain MSC homeostasis. In support of this conclusion, we found that adding exogenous mTNFα can rescue impaired TNFα−/− MSCs. Although it is believed that immune cells, including monocytes/macrophages and T helper cells, are the major source of TNFα, we found that static macrophages and naïve T cells, in the absence of stimuli, expressed and secreted a minimal amount of TNFα, suggesting that MSCs may be a major source of the physiological TNFα in the extracellular microenvironment that ensures mature TNFα internalization. This physiological concentration of TNFα is in demand for functional homeostasis of MSCs and shows particular significance in vivo for bone maintenance.

**Cytokine endocytosis**

Here, inspired by simple observations of the differential influences of TNFα and TNFR deficiencies on MSC homeostasis, as well as the decreased TNFα compared to the initially added concentrations in the MSC culture media, we discover that physiological TNFα is endocytosed into MSCs to function with failure to stimulate the TNFR downstream signaling. Therefore, the novel endocytic mechanism mediating TNFα effects would be considered irrelative to the canonical TNFα role as an inflammatory
cytokine. Accordingly, in low and high levels, TNFα function through differential ways to modulate different molecular targets, respectively contributing to stem cell regulations in physiology and pathology, as also indicated in previous studies (19, 84).

As recognized, endocytosis integrates nutrient internalization, diverse signaling transduction, and composition of the plasma membrane, thus contributing to cellular and organismal functioning as a fundamental life process (85). For stem cells, it has been reported that clathrin-mediated endocytosis (CME) is critical for balancing TGF-β and ERK signaling outputs to regulate embryonic stem cell pluripotency and fate choices. Endocytosis is also reported to be linked to autophagy to restrain intestinal stem cell proliferation (86, 87). We have further documented that MSCs use the endocytosis mechanism to uptake apoptotic extracellular vesicles for functional maintenance (63). Together with the present study’s findings, these works establish endocytosis as a dynamic yet constitutive way indispensable for orchestrating stem cell behaviors.

Prior to achieving TNFR-independent regulation, pro-TNFα has to be processed by TACE to form mature TNFα (7), which is secreted into the extracellular microenvironment and then internalized into the cytoplasm by mechanical force-induced endocytosis. Application of TACE inhibitor can significantly block soluble TNFα shedding and therefore achieve therapeutic effects for animal models with inflammatory diseases such as rheumatoid arthritis (88, 89). Un-cleaved pro-TNFα can be internalized but fails to affect MSC function, suggesting that TNFα endocytosis is a dynamic process that regulates the microenvironment to maintain stem cell homeostasis. Whether there is a non-canonical
mechanism underlying TNFα endocytosis that may be associated with the inward bending of the membrane to form the endocytic vesicle remain to be elucidated in future studies.

**Mechanical stimuli and endocytosis**

The mechanical properties of the stem cell niche critically influence cell shape and fate decisions, which are mediated by the cytoskeleton and the related mechanosensing pathway (90). Changes in the cytoskeleton also contribute to the endocytic process, which requires dynamic adaptations of the plasma membrane and transportation of endosomal vesicles (85). Not surprisingly, endocytosis has been reported to be influenced by the applied forces (50, 51), confirmed in our study for the uptake of TNFα.

Decreased membrane tension has been described to activate endocytosis since it reduces resistance to inward bending of the membrane, which is necessary to form endocytic vesicles (26, 50). In this study, we used a cyclic stretching system, which involves a relatively short-duration stretching force that is repeatedly applied and released, to evaluate the results of mechanical loading on endocytosis. We found that mechanical stimuli intensify MSC’s endocytic activity, controlling TNFα levels in the cytoplasm and extracellular microenvironment and then fine-tuning mTOR signaling, which upregulates the osteogenesis and downregulates adipogenesis in bone marrow MSCs. Our results propose an underlying mechanism to explain the previous finding that mechanical force stimulation promotes osteogenesis and decreases adipogenesis in MSCs (91-93).
However, further work is needed to elucidate how mechanical force promotes the endocytosis process and to specify the underlying machinery molecules responding to force stimuli.

**TNFα and mTOR signaling**

After endocytosis, mature TNFα in the cytoplasm directly binds to mTORC2 to exert a suppressive effect on the mTORC2/Akt/mTORC1 pathway, ensuring mTOR signaling is under control. This represents a previously unknown function of this cytokine: TNFα governs stem cell homeostasis by binding with cytoplasmic protein instead of membrane cytokine receptors. mTOR functions through two distinct complexes, mTORC1 and mTORC2 (54). mTORC1 is considered a master cell growth and metabolism regulator, controlling cellular protein synthesis, autophagy, lipid synthesis, mitochondrial metabolism, and biogenesis. The roles of mTORC2 function are still largely unknown (57). mTORC2 comprises six different proteins: mTOR, Rictor, mSin1, mLST8, Protor-1, and Deptor. Although the signaling pathways for mTORC2 activation are not well characterized, it is known to participate in cell survival, metabolism, and proliferation through activation of Akt (58-60). Our findings suggest that a lack of TNFα in MSCs results in aberrant activation of mTORC2, thereby leading to mTORC2/Akt/mTORC1 cascade activation. It has been demonstrated that the downstream signal of mTORC1, ribosomal S6 kinase 1 (S6K1), positively regulates the expression of adipogenic gene PPARγ (94). However, the role of mTOR signaling in osteogenesis is still controversial (61, 95) since it has been shown to have both inhibitory (65, 96-98) and stimulatory effects (99-104).
Our results support the notion that TNFα deficiency-induced aberrant mTOR activation in MSCs leads to impaired lineage differentiation, downregulated osteogenesis, and upregulated adipogenesis. Our findings indicate for the first time a non-inflammatory role of TNFα in governing stem cell fates, paving an avenue for future studies on physiological TNFα-mediated tissue/organ development and homeostatic control.

**Microgravity**

Since gravity has been a constant factor throughout the evolution of life on the earth, it would not be surprising if altered gravitational force led to deviations in biological systems. We hypothesized that MSCs rely on constant mechanical stimuli to regulate TNFα endocytosis, establishing and maintaining the balance between the extracellular environment and cytoplasm without activating TNFR signaling. Accordingly, it is speculated that loss of mechanical stimuli causes endocytosis failure and MSC dysfunction, indicating force-driven membrane dynamics as one essential characteristic of living cells.

Mechanical unloading (microgravity) of cells affects cell morphology, gene expression (i.e., downregulating IL-6 and TNFα), receptor-mediated-endocytosis (RME), and various signaling pathways (29-31, 105). At the organismal level, severe symptoms of skeletal muscle atrophy and bone loss have been observed when exposed to microgravity during space missions (38, 39). However, approaches to counteracting mechanical unloading-induced tissue alterations are currently limited. HU mice represent a well-established
hypodynamia model mimicking the condition of weightlessness; they have been demonstrated to experience muscle and bone mass atrophy and increased bone marrow adipose tissue (42-44). Our data show that HU mice exhibit MSC lineage alteration and osteopenia phenotype due to aberrant mTOR signal activation caused by TNFα deficiency in the MSC cytoplasm, which is related to hypodynamia-induced endocytosis reduction.

**Therapeutic approach identification for hypodynamic osteopenia**

Furthermore, we identify rapamycin, a specific mTOR inhibitor, as a therapeutic agent to treat hypodynamic osteopenia. Rapamycin was first isolated in 1972 from the bacterium *Streptomyces hygroscopicus* and was initially identified as an antifungal agent and an immunosuppressant, with later discoveries to possess anti-tumor properties (53). Due to its anticancer and immunosuppressive activities, rapamycin is used in the treatment of cancers such as renal cell carcinoma, breast cancer, non-small cell lung tumors, and glioblastoma (106-109), as well as autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis, and scleroderma (110-113). Notably, these diseases are usually associated with osteoporosis (114). In rapamycin-treated Fbn1+/− systemic sclerosis mice, mTOR inhibition helped to upregulate osteogenesis, downregulate adipogenesis of bone marrow MSCs, and restore functional bone homeostasis (65, 115).

It is noteworthy that previous studies showed that prolonged rapamycin treatment could result in both mTORC1 and mTORC2 inhibition (116, 117). However, in this study,
we observed only inhibition of mTORC1, but not mTORC2, in MSCs after chronic rapamycin treatment. Aberrant mTORC2 activation resulting from TNFα deficiency led to upregulated p-P70S6K activity through a mTORC2/Akt/mTORC1 cascade. Using rapamycin to control aberrant mTOR activation in MSCs may be a practical approach. Future work must be done to propel rapamycin therapy in hypodynamia osteopenia.

**TNFα and aging**

The elevated level of TNFα may contribute to age-related diseases, mainly *via* the NFκB pathway downstream of the TNFR signaling (118-121). Inhibition of mTORC1 can preserve, and perhaps even rejuvenate, stem-cell function in various tissues, suggesting that mTORC1 may play an important role in the aging process (71). In this study, we used a low concentration of mTNFα, without activating TNFR downstream signal, to rescue aged bone marrow MSCs *via* mTOR suppression, suggesting that the physiological level of TNFα may be able to alleviate aging phenotypes.

**In summary**, we have uncovered a previously unrecognized physiological role of TNFα in bone marrow MSC homeostasis. MSCs endow the cytoplasm with mature TNFα and use a receptor-independent pathway to modulate mTOR activity to ensure cell homeostasis, dependent on constant mechanical stimuli to activate TNFα endocytosis. Lack of physiologic mechanical stimuli, such as in HU mice, causes hypodynamia-induced TNFα deficiency in MSCs, leading to aberrant mTOR activation and MSC lineage
differentiation alteration. In addition, we identified that rapamycin might serve as an effective therapy for TNFα-deficiency and hypodynamia-induced loss of bone mass in bedridden patients and astronauts in spaceflight, *vice versa*, TNFα may serve a therapeutic effect in mTOR-hyperactivated senescent MSCs. Taken together, these findings suggest that dynamic TNFα release and endocytosis safeguard MSC homeostasis and identify an osteoanabolic approach for hypodynamia osteopenia.
Future Plan

As an orthodontist, my research interest lies in the translational research area of mechanical force-related MSC biology and growth modification to solve clinical problems. **Distraction osteogenesis (DO)** is a widely used technique in plastic and orthopedic surgery to treat craniofacial defective syndrome patients. Mechanical force is applied to a fractured bone during this process to enhance the regenerative processes and induce new bone formation. Similarly, **rapid maxillary expansion (RPE)**, another form of osteogenic distraction between two halves of the maxilla, is widely used by orthodontists in treating transverse deficient patients. In the clinic, we observed that patients responded differently to maxillary expansion, especially in adults. Although the literature is abundant on the clinical process of DO and maxillary expansion, there is a distinct lack of study on the underlying biological mechanism governing this osteogenic distraction process. It is proposed in the literature that differences in calcification patterns of the mid-palatal suture, craniofacial architecture, and age are contributing factors affecting the outcome of maxillary expansion; however, the biological basis underlying is still not well understood.

From what we’ve found in this study, I would next step to investigate **the roles of MSC resided in the mid-palatal suture and their interplay with surrounding immune cells during expansion stimuli**. Using Gli1-Cre\textsuperscript{ERT2};tdTomato reporter mouse model to label MSCs within mid-palatal sutures and investigate how suture-derived MSCs respond to expansion/stretching stimuli in vivo and in vitro. Compare the expansion and control groups using flow cytometry analysis, single-cell RNA sequencing, and MSC culture to
identify if there will be any MSC lineage trajectories and cell population shifts in response to RPE. Transcriptomics analysis may provide a new paradigm to precisely depict a mechanical stimuli-related MSC modulatory machinery from the surrounding microenvironment during maxillary expansion. If a target is found, we will pursue a practical therapy strategy to increase the expansion range and enhance the stability after expansion in transverse deficient patients.
Conclusions

- Bone marrow MSCs produce and secrete TNFα \textit{in vivo} and \textit{in vitro} to safeguard bone homeostasis. Lack of TNFα leads to impaired proliferation and altered lineage differentiation capacity in MS. Physiologic concentration of TNFα is in demand for functional homeostasis of MSCs and shows particular significance \textit{in vivo} for bone maintenance.
- TNFα executes its safeguard role in a receptor-independent manner. TNFα is internalized into the cytoplasm through endocytosis.
- Mechanical stimuli enhance endocytosis activity. Under microgravity conditions, the uptake of TNFα is reduced; MSC experiences a similar phenotype as TNFα-deficiency cells.
- Cytoplasmic mature TNFα, rather than pro-TNFα, binds to mTORC2 in the cytoplasm to restrain mTOR activity, therefore maintaining MSC functional homeostasis.
- Rapamycin infusion rescues TNFα-deficiency and hindlimb suspension mice phenotype via mTOR signaling suppression, providing us with a promising therapeutic strategy to treat hypodynamia osteopenia.
- Senescent MSCs show elevated mTOR activity. Exogenous TNFα, but not mechanical stimuli, rescues impaired senescent MSC function \textit{in vitro}, indicating the cytoplasmic TNFα level, rather than endocytosis activity, is associated with senescent cell functional phenotype.
References


24. T. Kinnaird et al., Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* **94**, 678-685 (2004).


54. R. Loewith *et al.*, Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell* **10**, 457-468 (2002).


58. D. A. Guertin *et al.*, Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* **11**, 859-871 (2006).


67. S. Nandadasa et al., Secreted metalloproteases ADAMTS9 and ADAMTS20 have a non-canonical role in ciliary vesicle growth during ciliogenesis. Nature communications 10, 953 (2019).


77. J. J. Wu et al., Increased Mammalian Lifespan and a Segmental and Tissue-Specific Slowing of Aging after Genetic Reduction of mTOR Expression. Cell Reports 4, 913-920 (2013).


80. H. Gerber, H. J. Peter, H. Studer, Age-related failure of endocytosis may be the pathogenetic mechanism responsible for "cold" follicle formation in the aging mouse thyroid. Endocrinology 120, 1758-1764 (1987).


