Application for the startup prize of the Austrian society of Rheumatology

Project-Title

**Arginine and the metabolic control of osteoclast generation**

Applicant

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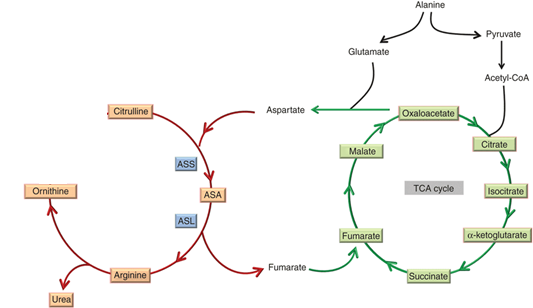
# 1. Background

* 1. **. Osteoclasts and Osteoclastogenesis**

Osteoclasts are giant, multi-nucleated cells, which derive from the monocyte-macrophage lineage via a multi-stage process that is influenced by various hormones and cytokines (1). The main function of osteoclasts is to regulate bone turnover, a function that they carry out through formation of a specialized, bone-facing membrane consisting of the “sealing membrane” and the “ruffled border” (2). Attachment of the osteoclast to the bone by the sealing membrane is facilitated via actin-ring formation by podosomes, enabling the osteoclast to resorb bone material (3). The ruffled border in turn ensures vesicular fusion to deliver lysosomal enzymes and the uptake of degradation products (4). Even though osteoclasts are immensely large cells formed via fusion of precursors, bone degradation is impossible via phagocytosis and has to be performed extracellularly (5). This is achieved via extracellular acidification by the resorption compartment that lies between the bone surface and the ruffled border. The extracellular acidification causes the demineralization of the bone matrix and exposure of the underlying organic components of the bone, which mostly consist of type I collagen (6). Stimulation with macrophage-colony stimulating factor (M-CSF) induces RANK (Receptor Activator of NF-κB transcription factor) expression in osteoclast precursors of the myeloid linage. Binding of the ligand (RANKL) to RANK leads to the activation of various signaling cascades such as nuclear factor kB (NFκB) and the nuclear factor of activated T-cells cytoplasmic calcineurin-dependent 1 (NFATc1) causing the expression of osteoclast-related genes such as cathepsin K, tartrate-resistant acid phosphatase (TRAP) and the calcitonin receptor, leading to osteoclast differentiation and maturation (7-9). However, many aspects regulating these processes are still poorly understood.

* 1. **Arginase and Arginine in macrophage biology**

The metabolism of innate immune cells in relationship to their polarization and differentiation status has been of great interest, especially with a focus on L-Arginine (L-Arg) turnover in macrophages and has been extensively reviewed (10). The semi-essential amino acid can be metabolized via either Nitric Oxide Synthase (NOS) or Arginase I (ArgI) (11). The different fates of L-Arg are important for macrophage plasticity, as described in the M1/M2 polarization paradigm, which broadly divides macrophages into either proinflammatory (“classically activated”, M1, expressing iNOS) or anti-inflammatory (“alternatively activated”, M2, expressing Arginase) cell subsets. These subsets arise from specific stimulation of the cells and are characterized by varying expression levels of cytokines, thereby influencing the adaptive immune compartment (11). Generally, M1 macrophages are involved in host defense, while M2 macrophages are involved in wound healing and tissue repair. While breakdown of L-Arg via ArgI yields ornithine and urea, NOS catalyzes the production of citrulline and nitric oxide (NO). L-Arg therefore acts as a precursor for several immunological and homeostatic relevant metabolites like the polyamines and proline and therefore plays an important role in host defense and macrophage biology (Figure 1) (12).



Arginase I

Sdss

NO

iNOS

CAT2

Arginine

Polyamines

**Ornithine Decarboxylase**

Figure 1: Overview over the different fates of intracellular Arginine. Arginine can be either preferentially metabolized via NOS or Arginase I. In addition, there is a connection to the tricarboxylic acid cycle (TCA cycle), also non as citric acid cycle, a central mechanism for energy generation in a cell. ASL: Argininosuccinate lyase; ASS: Argininosuccinate synthase; NOS: nitric oxide synthase; OAT: Ornithine Aminotransferase; ODC: Ornithine decarboxylase; CAT2: cationic amino acid transporter 2 (Adapted after Erez et al, 2011).

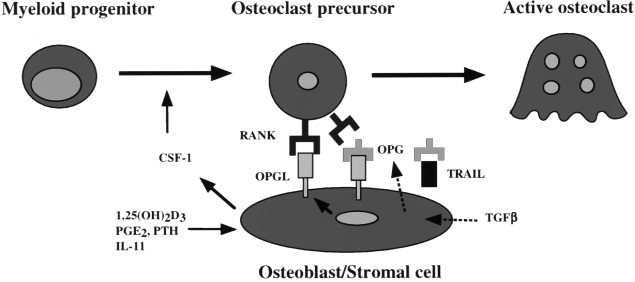
* 1. **Arginase and Arginine in osteoclast biology**

Although NO, a metabolic product of Arginine, has been implicated to regulate osteoclastogenesis, the role of Arginine itself, as well as Arginase I are not understood.

# Preliminary Data:

Osteoclasts can be in vitro differentiated by adding M-CSF and RANKL to previously isolated bone marrow cells. Interestingly, after screening for targets regulated by M-CSF and RANKL signaling, we discovered a significant decrease in Arginase mRNA levels in osteoclasts compared to the M-CSF only treated osteoclast precursors (Figure 2).





M-CSF

M-CSF+RANKL

Day 3: osteoclast

Precursor

Day 0: Myeloid progenitor

Day 7:

Osteoclast

Figure 2: Expression levels of ArgI after induction of osteoclastogenesis. Messenger RNA levels of Arginase I decrease after addition of RANKL on day 3 of osteoclast differentiation. Data are normalized to HPRT and Day 3 of differentiation, presented as mean ± SEM and n=5 mice per gp, and are two combined independent experiments. \* represent p < 0.05

### Hypothesis:

We hypothesize that Arginase I is an essential regulator of osteoclast development by regulating the availability of Arginine. We speculate that Arginine is an essential amino acid that is required for the generation of osteoclasts.

### Possible significance of the findings:

Understanding the regulation of osteoclast development in the context of arthritis and osteoporosis is of great medical importance. In our experiments, we aim to describe the role of a single amino acid, L-Arginine, which might serve as essential factor that regulates the differentiation of osteoclasts by controlling the metabolic activity of osteoclasts and their precursors. With the identification of such metabolic signals, completely novel therapeutic strategies could potentially be developed.

## Specific Aims:

The main aim is to investigate how Arginase I inhibits osteoclast generation. We therefore plan the following experiments:

1. Does recArgI induce cell death?

For this purpose, we will perform Annexin V/7-AAD stainings, which allow detection of live cells (Annexin V-, 7-AAD-), early apoptotic cells (Annexin V+, 7-AAD-), late apoptotic cells (Annexin V+, 7-AAD+) and necrotic cells (Annexin V-, 7-AAD+).

1. As Arginase I metabolizes Arginine to ornithine and urea (see Figure 1), addition of recArgI on one hand depletes L-Arginine, on the other hand generates ornithine and urea. We therefore want to test, whether enzymatic products of Arginase I or depletion of L-Arginine is responsible for inhibiting osteoclastogenesis.
   * We will test, whether addition of ornithine and/or urea affects osteoclast generation, i.e. whether addition of ornithine or urea mimics the effect of recArgI on osteoclast generation.
   * We will use L-Arginine deficient medium to differentiate osteoclasts. If the mechanism by which recArgI inhibits osteoclastogenesis is depletion of L-Arginine, osteoclastogenesis should be inhibited also when using culture media devoid of L-Arginine.
2. We will investigate how treatment with recArgI affects critical signaling events during osteoclastogenesis (especially RANKL-induced signaling), such as MAP-kinase activation and mTOR activation using Western blot.
3. We will analyze the effect of L-Arg restriction on transcription in osteoclast precursors by qPCR, analyzing genes such as NFATc1, calcitonin receptor, c-fos and others.
4. We will investigate, whether re-supplementation with L-Arginine can rescue the inhibitory effect of recArgI I on osteoclast generation and the metabolic activity of osteoclasts.

## Selected Methods

Osteoclast generation:

Bone marrow cells of both genotypes will be isolated and cultured for 3 days in 100 ng/ml M-CSF to enrich for monocytes/macrophages and will be then cultured in 10% FCS/DMEM supplemented with 30 ng/mlM-CSF and 50 ng/ml RANKL (both from R&D Systems McKinley Place NE, Minneapolis) for another 3 to 4 days. Osteoclasts will be detected by the presence of TRAP+ multinucleated cells (≥ 3 nuclei). Urea and ornithine will be purchased by Sigma-Aldrich.

qPCR:Total RNA will be isolated from cultivated dendritic cells or osteoclasts using theRNeasy Mini kit (QIAGEN). 1 µg total RNA will be used forfirst strand cDNA synthesis (Amersham Biosciences) and 1 µlcDNA will then be used for PCR with specific primers.

Western blotting:

OCs will be stimulated with RANKL 50 ng/ml or medium alone ranging from 5, up to 120 minutes for the activation of MAP-kinases, for induction of transcription factors stimulation will be longer (up to 48 hours). After stimulation, OCs will be lysed in Laemmli buffer, and proteinswill be separated by electrophoresis on 10% SDS-polyacrylamidegels. Proteins will be blotted onto polyvinylidene difluoride membraneand, after blocking with 5% dry milk/0.1% Tween 20, incubatedwith primary Abs in the same solution. Bound Abs will be detectedby anti-IgG conjugated with peroxidase and subsequent chemiluminescent detection. Activation of MAP-kinases such as ERK1/2, p38 and JNK will be monitored using phosphorylation specific antibodies. Induction and activation of NF-κB, c-FOS, c-JUN and induction of NFATc1 will also be tested.

Seahorse:

For real-time analysis of oxygen consumption rate (OCR), macrophages will be analyzed with an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). In this assay, basal oxygen consumption can be established by measurement of OCR. Cells will be differentiated for 2 days with 100 ng/ml M-CSF, harvested and then plated in Seahorse 24 well dishes. After another 24 h incubation with 30 ng/ml M-CSF, cells will be stimulated with 50 ng/ml RANKL and/or 1000 ng/ml recArgI. Respiration will be measured 24 h after RANKL addition.

1. **Work plan and outlook:**

**6.1. General information on Laboratories and Investigators:**

These experiments will be performed at the Dpt. of Rheumatology, Medical University of Vienna, headed by Prof. Josef Smolen. The laboratory is fully equipped to carry out the proposed studies. The experiments will be conducted by a Julia Brunner, a PhD student, who has gathered all the necessary skills to perform the proposed experiments, both in osteoclast biology as well as in working with recombinant Arginase I, which is reflected in her publication record. She will be supervised by Dr. Stephan Blüml.

**Work plan:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **weeks** | **1-6** | **7-12** | **13-18** | **19-24** |
| **Aim 1** |  |  |  |  |
| **Aim 2** |  |  |  |  |
| **Aim 3** |  |  |  |  |
| **Aim 4** |  |  |  |  |
| **Aim 5** |  |  |  |  |

**6.2. Outlook:**

We hypothesize that Arginine metabolism essentially regulates osteoclastogenesis. With the preliminary data and the results obtained from this proposal, we will write a grant application for the FWF including the following large aims:

* In order to analyze the global transcriptional response of osteoclast precursor cells in response to RANKL, will perform RNA-sequencing from osteoclasts treated with recArgI, L-Arg deficient medium and medium resupplied with L-Arg, to screen for changes in gene expression induced by extracellular Arginine levels.
* Analyze osteoclast development in mice deficient for known cellular transporters of L-Arginine (CAT2, SLC38A9)
* Test the efficacy of recArgI in inhibiting osteoclastogenesis *in vivo* (in models of arthritis and osteoporosis)
* Analyze Arginase I levels in patients with arthritis and osteoporosis

1. **Requested budget and justification:**

**Total animal care**: € 4.000

**Costs for disposables and reagents:**

**Cell culture** (general cell culture reagents, plastic ware, media, pipets, serum)

€ 2.830

**Requested personnel:**

Funding for one PhD student for 6 months is requested.

The PhD student will be in charge of experimental procedures and communicate with the P.I.s on a daily basis.

PhD student: € 18.170

**Total: € 25.000**

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