Research Title: Establishing the Immunoporotic role of "Breg-Treg-Th17" cell axis in Probiotics mediated modulation of Bone Health in post-menopausal osteoporosis.

Introduction: Osteoporosis is a systemic-skeletal disorder that is mainly characterized by enhanced fragility of bones leading to increased rates of fractures in the hip, spine etc. Notably, the prevalence of osteoporosis related fragility fractures is higher in the aged population as compared to other diseases. Among the global population, greater prevalence of osteoporosis has been observed in postmenopausal women (Hadji et al., 2013). ***It has been estimated that 30% of men and 50% of women, over the age of 50 are highly susceptible to enhanced bone loss and fractures***. ***According to International Osteoporosis Foundation (IOF), one third of the female and one fifth of the male will suffer from osteoporosis related factures in their lifetime. It has also been evaluated that the economic burden of osteoporosis by the end of 2025 will reach 25.3 billion in USA alone. According to Indian survey reports, total population of India is approximately 1.3 billion, the second most populated country in the world. Around 10% (approximately 13 million) of the Indian population is over the age of 50 with either having osteoporosis (T score value < -2.5) or with low bone mass (T score value between -1.0 and -2.5), with huge un-estimated economic burden.*** Thus, new and safer-therapies are need of the hour for better management and treatment of osteoporosis related bone loss in the long run. This can only be possible when we advance our research in the field of "Osteoimmunology", which had already established the importance of immune system in inflammatory responses including bone loss in osteoporosis***. In lieu of the growing involvement of immune system in osteoporosis, our group had recently coined the term "Immunoporosis" i.e. Immunology of Osteoporosis (Srivastava et al., 2018) to highlight the specific role of immune system in pathology of osteoporosis.*** Various factors produced during immune responses are capable of profoundly affecting regulation of bone homeostasis. Accumulating evidence lends support to the theory that bone destruction associated with osteoporosis and rheumatoid arthritis is caused by the enhanced activity of osteoclasts, resulting from the imbalance/impairment of Tregs and Th17 cells. A slight disturbance in the critical balance between Treg-Th17 cells gives rise to various inflammatory conditions including osteoporosis. A study reported in 2013, proven that Bregs ($CD19^{+}CD24^{+}CD38^{hi}$) plays a vital role in regulating proliferation, differentiation and immunological functions of Th17 and Tregs cells via secreting IL-10 and IL-35 cytokines. It has been demonstrated that in active cases of autoimmune diseases viz. RA and in patients with inflammatory diseases showed deformities in the functions of Bregs. Our recent lab findings (Dar et al., 2018a, Dar et al., 2018b, Dar et al., 2018c; Sapra et al., 2022a, Sapra et al., 2022b) clearly indicated that the delicate balance between Tregs-Th17 immune cells gets disturbed in post-menopausal osteoporotic experimental mice model. But whether this resulted imbalance is due to the alteration in Bregs subset has not been investigated till date. ***Thus, the present study for the first time will elucidate the role of Breg population in regulating this delicate Tregs-Th17 cell balance (i.e. the "Breg-Treg-Th17 cell axis") in both control and patients with post-menopausal osteoporosis in preclinical and clinical model of osteoporosis.*** The Gut microbiota (GM) plays a primary role in the induction, education, and function of the host immune system. Recent studies have shown that GM-associated metabolites (GAMs) such as SCFAs (acetate, propionate, and butyrate) have the potential to modulate regulatory B cells (Bregs) (Rosser et al., 2020). Dysbiosis-induced "Leaky Gut" is one of the major contributing factors in postmenopausal osteoporosis (PMO) (XU et. al., 2020). Probiotics, defined as live commensal microorganisms such as bacteria which when administered in adequate amounts can confer health benefits on the host by altering the composition of the GM. Manipulation of the GM with probiotics is gaining public attention and faecal transplants along with various other selective therapeutic interventions targeting the GM are likely to become increasingly common shortly. Since the intestinal microenvironment favors the development and differentiation of Bregs but whether the observed dysbiosis in the case of osteoporosis promotes bone deterioration via modulating Bregs frequencies has not been investigated till date. ***The fact that the intestinal microenvironment favors Bregs generation lead us to hypothesize that probiotics via their effect on Bregs could have the potential to modulate bone health in PMO.*** Surprisingly, no study till date has ever analyzed this connection (**Figure 1)**.

Objectives:

1. To elucidate the role of Bregs in modulating osteoclastogenesis (*in vitro).*
2. Establishing the Role of immunoporotic "Breg-Treg-Th17" cells axis in preclinical model of PMO.
3. To delineate the therapeutic potential of probiotics in modulating "Breg-Treg-Th17" cell axis in preclinical model of PMO.
4. To investigate the cellular, immunological and molecular mechanism of probiotics induced Bregs in modulating "Treg-Th17" cell-axis.
5. To investigate the status of immunoporotic "Bregs-Tregs-Th17" cell axis and bone loss in osteoporotic patients

Material and Methods:

1. **Objective 1:** Bone marrow cultures (osteoclast), Positive selection of B cells, Bregs differentiation, co-culturing of B cells and bone marrow cells, Flow cytometry.
2. **Objective 2:** Post-menopausal osteoporotic mice model development, flow cytometry, magnetic separation of B and T cells, Micro CT, SEM, FTIR, AFM etc.
3. **Objective 3:** Post-menopausal osteoporotic mice model development, Probiotics administration, Real time, Gut permeability, flow cytometry, magnetic separation of B and T cells.
4. **Objective 4:** HPLC or LC-MS of probiotics conditioned media and its administration in post-menopausal osteoporotic

mice model.

5. **Objective 5:** PBMCs isolation from whole blood, ELISA, Flow cytometry etc.

Results: In the present study, we examined the anti-osteoclastogenic potential of Bregs under *in vitro* conditions and observed that Bregs suppress the RANKL induced osteoclastogenesis in a dose dependent manner. Moving ahead in our study, we investigated the role of Bregs in modulating RANKL induced osteoclastogenesis under *in vitro* conditions. Excitingly, we observed that Bregs inhibited the differentiation of osteoclasts from BMCs in a dose dependent manner. Also, our *in vitro* data suggested that Bregs exhibit the potential of suppressing functional activity (bone resorption) of matured osteoclasts. We were further keen to know whether the observed suppression of osteoclastogenesis by Bregs is cytokine dependent (soluble factors) or require cell to cell contact. Thus, we employed trans-well system for our cultures and observed that the anti-osteoclastogenic potential of Bregs is primarily mediated by soluble factors. These data clearly establish that Bregs inhibit osteoclast differentiation via soluble mediators. Our group recently reported that Bregs population significantly reduce in osteoporotic mice model along with its tendency to produce IL-10 cytokine. In addition, our present study confirmed the anti-osteoporotic potential of Bregs as the percentage of Bregs is inversely correlated with the bone mineral density (BMD) of osteoporotic patients thus indicating towards its bone health enhancing potency. Moving ahead, Tregs and Th17 cell differentiation is regulated by Bregs, and we observed that deficiency of Bregs further reduced the percentage of Tregs and augments the Th17 cell population in post-menopausal osteoporotic mice model and patients. Moreover, serum cytokine data further supported the imbalance of Treg/Bregs and Th17 cells in case of osteoporosis. In addition, our co-culture experimental data indicated that Bregs from osteoporotic mice model lost the potential to regulate proliferation of T cells as supported by the reduced proliferation in the control group where healthy Bregs were co-cultured with the autologous T cells but in osteoporotic condition we observed more proliferation of T cells. Taken together, our present study for the first time establishes that Bregs exhibits anti-osteoclastogenic potential *in vitro*. Moreover, reduction in Bregs number observed in osteoporotic patients may also be one of the prime contributing factors towards inflammatory bone loss observed in the postmenopausal osteoporotic mice model. In addition, this study for the first time highlights the immunoporotic role of probotics in skeletal homeostasis and emphasizes the probiotics as a novel osteoprotective agent for the treatment and management of osteoporosis. Probiotics supplementation significantly enhances the BMD, bone strength, and micro-architecture of bones via modulating both the osteoclastogenesis and differentiation of Tregs, Bregs, and Th17 cells, thereby suggesting toward the pivotal role of the "Breg–Treg–Th17" cell axis in postmenopausal osteoporotic mouse models.

Statistical Analysis: Statistical differences between the distinct groups were evaluated by employing analysis of variance (ANOVA) with subsequent analysis via Student's t-test paired or unpaired as appropriate. All the values in the data are expressed as mean ± SEM (n = 6). Statistical significance was determined as $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***p, 0.001) with respect to the indicated groups.

Discussion: Supplementation of probiotics in ovx (preclinical model of osteoporosis) mice significantly enhanced bone health via modulating the nexus between Bregs, Tregs, and Th17 immune cells. Of note, it has been observed that the deficiency in estrogen hormones significantly enhanced the level of inflammatory cytokines such as IL-17 while substantially reducing the level of anti-osteoclastogenic cytokines such as IL-10, thereby augmenting bone loss in ovx mice. Importantly, our serum cytokine data further attest to the immunomodulatory potential of probiotics where administration of probiotics significantly reduced the levels of IL-17 cytokine (signature cytokine of Th17 cells) along with simultaneous induction of IL-10 cytokine (signature cytokine of Treg and Breg cells). Collectively, our present study for the first time highlights the immunoporotic role of probiotics in skeletal homeostasis and emphasizes the probiotic as a novel osteoprotective agent for the treatment and management of osteoporosis. Probiotics supplementation significantly enhances the BMD, bone strength, and micro-architecture of bones via modulating both the osteoclastogenesis and differentiation of Tregs, Bregs, and Th17 cells, thereby suggesting toward the pivotal role of the "Breg–Treg–Th17" cell axis in postmenopausal osteoporotic mouse models. However, the secretory metabolites responsible for the immunoporotic potential of probiotics are still warranted, thereby opening new avenues for further research.

Impact of Research: The current study has the potential to provide ***novel insights for the development of effective future therapeutics for treating and managing post-menopausal osteoporosis***. To date, due to the heterogeneous nature of immune cell responses, researchers are unable to ascertain how to harness the potential functions of these cells for therapeutic interventions. ***The proposed study will thus be instrumental in gaining further insights into this interesting and novel field of bone biology (termed as "Osteo-microbiology": Osteoimmunology and Microbiology) and will have an immense clinical implication in near future.*** The administration of various probiotic strains can thus open up new avenues in the treatment of various inflammatory bone conditions such as osteoporosis and rheumatoid arthritis by modulating the delicate balance between the gut microbiota and immune system (viz. Bregs).

Rationale

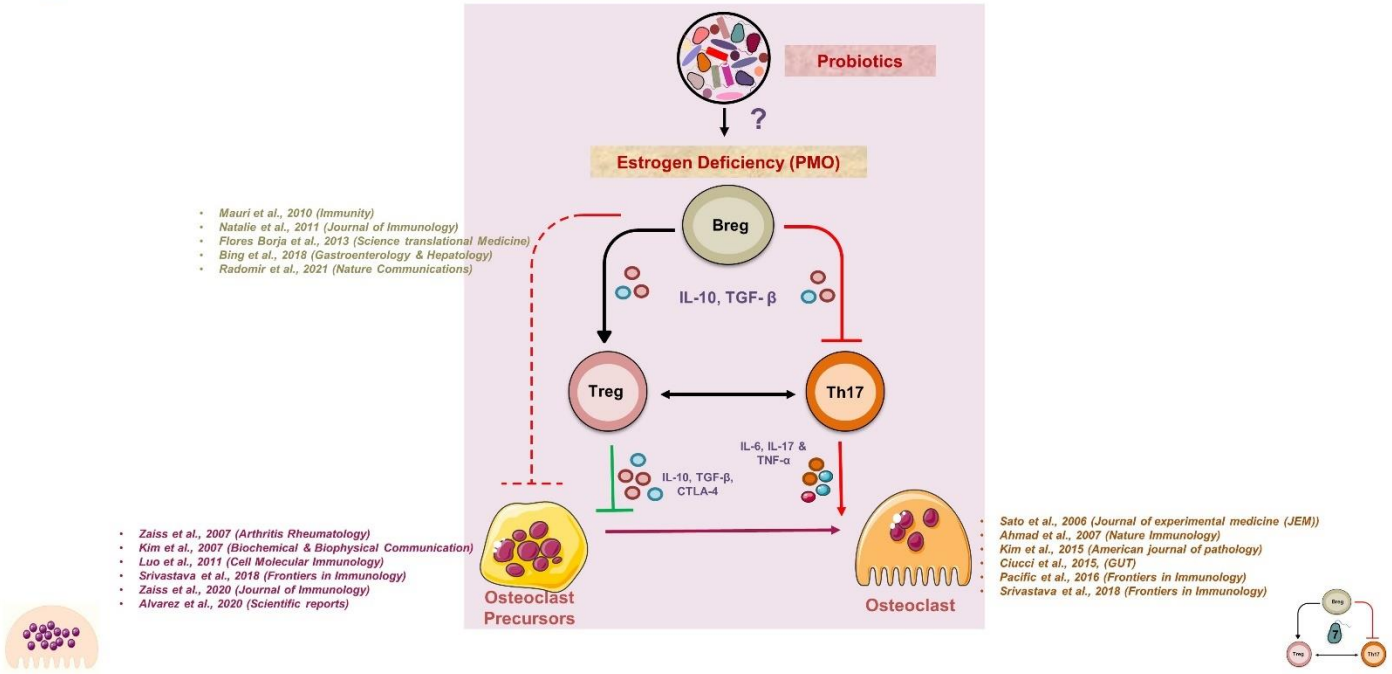

Figure 1: Rationale of the study: First we want to look for the bone health modulatory potential of Bregs and how it further regulates the Tregs and Th17 cells under estrogen deficient osteoporotic conditions. Whether probiotics administration can alters the Bregs-Treg-Th17 cell axis and modulate the bone health under osteoporotic conditions.

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