Beneficial Effects of Sodium Butyrate on Bone Remodeling in a Mouse Model of Osteogenesis Imperfecta

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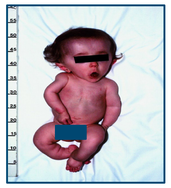
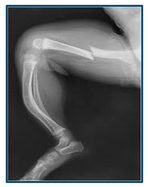
Osteogenesis Imperfecta (OI)

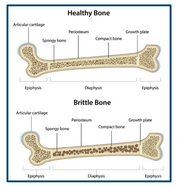
- Imperfect birth of bones
- Characterized by reoccurring bone fractures, low bone mass and bone fragility.
- Incidence is 1 in 20,000 live births.
- The severity of OI ranges from perinatal lethal and severely deforming types to very mild forms without deformity.
- Now referred to as a “Collagen-related disorder” as OI can be caused by mutations in Type I collagen (Classical OI; autosomal dominant) as well as mutations in non-collagenous genes whose protein products interact with collagen (Non-classical; Autosomal recessive).
- No cure for OI; only symptomatic treatment is given.



Clinical presentation



Radiologic presentation



Bone Structure

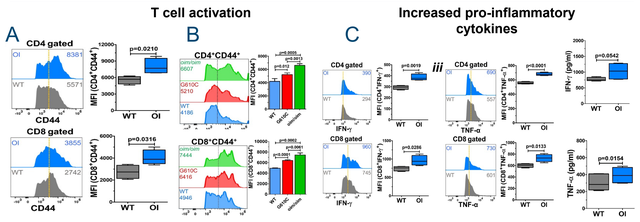
T Lymphocyte subsets and Bone

- T lymphocytes could be essential in regulating the homeostasis, survival and function of not only osteoclasts but osteoblasts as well *(Takayanagi 2015, Lei et al, 2015)*.
- Using mice lacking functional lymphocytes, it has been demonstrated that T cells play a role in bone regeneration, with fracture calluses of deficient mice exhibiting lower levels of bone markers, leading to poor bone quality *(Toben et al, 2011; Nam et al, 2012)*.
- Delayed fracture healing significantly correlated with enhanced levels of terminally differentiated $CD8^+$ effector memory T cells in peripheral blood *(Reinke et al, 2013)*.
- Massive infiltration of T cells in the fracture callus especially near newly forming bone has been shown *(Konnecke et al, 2014)*.
- T lymphocyte–derived RANK-L/OPG is a mediator of the osteoclast formation and bone loss *(Kong et al, 1999; Teng et al 2000)*.
- Tregs are important immunosuppressive cells that can also regulate the functioning of osteoblasts and osteoclasts.
- Markedly improved calvarial defect repair was observed on delivery of Tregs along with bone marrow mesenchymal stem cells *(Liu et al, 2011)*.
- FOXP3-overexpressing mice, characterized by increased numbers of Tregs, have high bone volume and low osteoclast number *(Zaiss et al, 2010)*.
- Bisphosphonates, which have been widely used as a treatment for OI, have been shown to cause an elevation in the cytokine cascade of Tregs (IL-10 and TGF-β) in the peripheral blood of osteoporotic patients *(Talaat et al 2015)*.

Immune Profile in OI

- This has never been examined before.
- Clues from literature
  - Pediatric OI patients present a hyperinflammed condition with increased platelet counts *(Salter et al, 2018)*.
  - Potential activation of inflammatory pathways seen in OI mice *(Rauch et al, 2018)*.
  - Serum levels of TNF-α is increased in mice, suggesting chronic inflammation *(Matthews et al, 2017)*.

Activated Phenotype of T Cells and Enhanced Effector Cytokine Secretion



Activated phenotype of T cells and increased effector cytokines levels in OI mice. (A) Spleen-derived naïve T cells from WT and OI mice were stained using fluorochr… CD44 antibody, acquired by FACS, and analyzed by FlowJo software. Data from one of three experiments with a similar result is shown in *left panel*. The dotted vertical line indicates the mean position of the peak in WT T cells to compare relative expression between samples visually. Mean MFI values (from all experiments) for CD44 in OI T cells are significantly greater than those seen in WT T cells *(right panel)*. This can be seen in both CD4 *(upper panel)* and CD8 *(lower panel)* cells. Data are presented as Mean ± Standard Deviation. (B) Spleen-derived naïve T cells from WT, G610C, *oim/oim*, stained with CD44 antibody, acquired by FACS, and analyzed by FlowJo software. MFI values for CD44 in G610C were significantly greater than WT but significantly lower than *oim/oim* both in CD4 and CD8 populations. Dotted vertical line indicates the mean position of the peak in WT T cells. (C) Splenic T cells from WT and OI mice were activated for three days using anti-CD3 and anti-CD28 antibodies before being re-stimulated with similar stimuli in the presence of Golgi-Plug. Intracellular staining was done with fluorochrome-conjugated antibodies, and data were acquired using FACS. Data from one of three experiments with a similar result is shown in *the left panels*. The numerical values within each display are MFI, and the dotted vertical line indicates the mean position of the peak in WT T cells. Mean MFI values (from all experiments) show that there is a significantly greater expression of both IFN-γ and TNF-α in OI T cells as compared to WT T cells *(right panels)* in both CD4 *(upper panel)* and CD8 *(lower panel)* cells. Data are presented as Mean ± Standard Deviation. Also, a trend for significant increase is seen in IFN-γ levels *(upper panel)* while a significant increase is seen in TNF-α levels *(lower panel)* in OI mice serum compared to WT mice serum. Data are presented as Mean ± Standard Deviation.

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Acknowledgements

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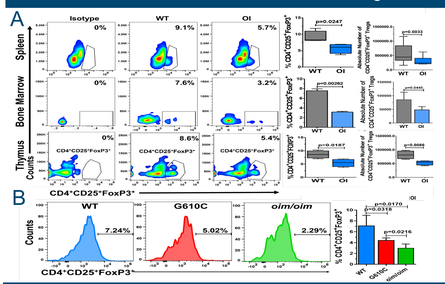
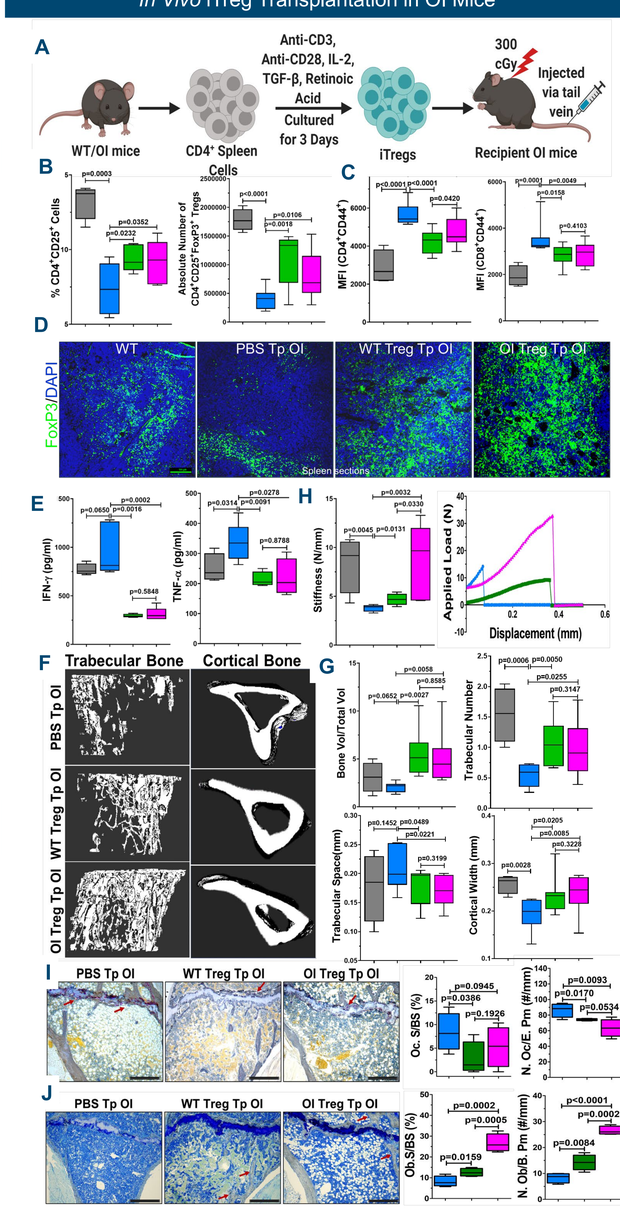
Reduced Numbers of Tregs in OI Mice



(A) Reduced Treg numbers in OI mice. Cells from the spleen, bone marrow and thymus were stained using fluorochrome-conjugated CD4, CD25, and FoxP3 antibodies to compare the percent of Tregs between WT and OI mice. Flow cytometric plots of the representative data, and the graph of the cumulative data of percentages of Tregs as well as the absolute number of Tregs, demonstrates that there is a significant decrease in the number of Tregs both in the spleen and the thymus of OI mice compared to WT mice. Data are presented as Mean ± Standard Deviation.

(B) Reduced number of Tregs in G610C mice. Number of nTregs (analyzed by flow as $CD4^+CD25^+FoxP3^+$) in G610C mice were lower as compared to WT but were higher than that seen in *oim/oim*.

In Vivo iTreg Transplantation in OI Mice



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Treg Transplantation in OI mice restore T cell tolerance and improves bone parameters. (A) WT and OI iTreg's transplanted via tail vein into irradiated OI mice (n=7-9). (B) Percentage and absolute number of Tregs after WT/OI Treg transplantation. (C) Graphs for splenic T cells stained for CD4, CD8 and CD44 after WT/OI Treg transplantation. (D) FoxP3 staining in spleen sections from WT, OI and OI mice transplanted with WT and OI Treg's. (E) INF-γ and TNF-α in serum after WT/OI Treg transplantation. (F) *Ex-vivo* micro-CT for trabecular and cortical bone after WT/OI Treg transplantation. (G) Graphs for bone morphometric parameters. (H) 3-point bending of femur showing stiffness and deflection curve. (I) Histomorphometry showing osteoclast numbers after transplantation in images-(arrows) and graphs (Oc.S/BS, N.Oc/E.Pm). (J) Bone histomorphometry shows increased trabeculae (images: arrows) and osteoblast numbers, Ob.S/BS, N.Ob/B.Pm after transplantation.

Question?

Can we increase Treg numbers in OI mice without transplantation?

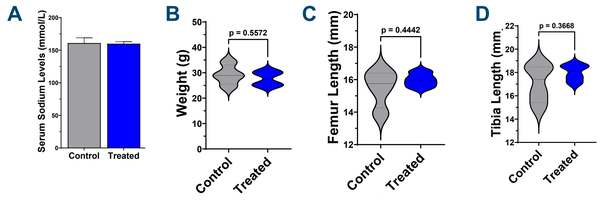
Use of Sodium Butyrate to Increase Treg Numbers

- Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation (Arpaia et al, Nature, 2013).
- Butyrate produced in the gut following Lactobacillus rhamnosus GG ingestion, or butyrate fed directly to germ-free mice, induced the expansion of Tregs (Tyagi et al, Immunity 2018).
- Parathyroid hormone-dependent bone formation requires butyrate production by intestinal microbiota, which increases Tregs (Li et al, J Clin Invest, 2020).

Sodium Butyrate Treatment in *oim/oim* Mice

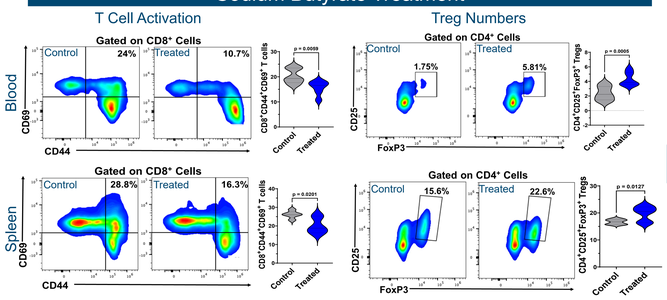
- 10mM sodium butyrate in drinking water.
- n=4 in control and treated group
- Treatment continued for 6 months.
- Water intake was the same between the control and treated group.

Serum Sodium Levels, Weight and Bone length after Sodium Butyrate Treatment



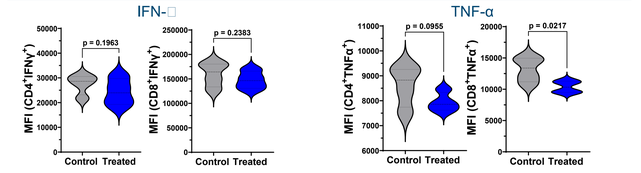
Serum sodium levels, weight and bone length in oim/oim mice after sodium butyrate treatment for 6 months. There was no change in the serum levels of sodium in the treated mice versus the control mice. Similarly, the weight, femur length and tibia length was not different between treated and control mice. Data are presented as Mean ± Standard Deviation.

T Cell Activation and Treg Numbers in Blood and Spleen after Sodium Butyrate Treatment



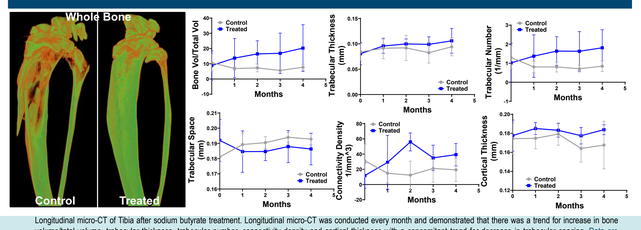
T cell activation and Treg numbers in blood and spleen after sodium butyrate treatment for 6 months. There was a decrease in activation of T cells as shown by a significantly decreased percentage of cells expressing the activation markers CD44 and CD69, in both blood and spleen. The percentage of Tregs shows a significant increase in both blood and spleen after sodium butyrate treatment for 6 months. Data are presented as Mean ± Standard Deviation.

Effector Cytokine Secretion by T Cells in Spleen after Sodium Butyrate Treatment



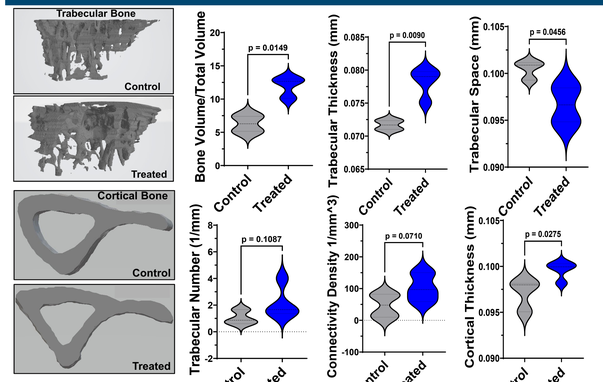
Effector cytokine secretion by T cells in spleen after sodium butyrate treatment for 6 months. There was a decrease (though not significant) in IFN-γ secretion by T cells. A significant decrease in TNF-α secretion by T cells is seen 6 months after starting treatment with sodium butyrate. Data are presented as Mean ± Standard Deviation.

Longitudinal Micro-CT of Tibia after Sodium Butyrate Treatment



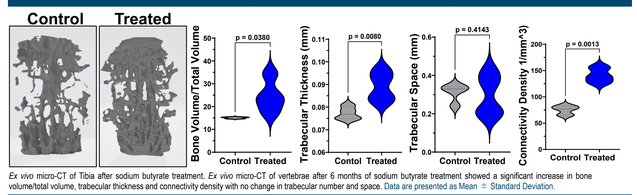
Longitudinal micro-CT of Tibia after sodium butyrate treatment. Longitudinal micro-CT was conducted every month and demonstrated that there was a trend for increase in bone volume/total volume, trabecular thickness, trabecular number, connectivity density and cortical thickness with a concomitant trend for decrease in trabecular spacing. Data are presented as Mean ± Standard Deviation.

Ex-Vivo Micro-CT of Tibia after Sodium Butyrate Treatment



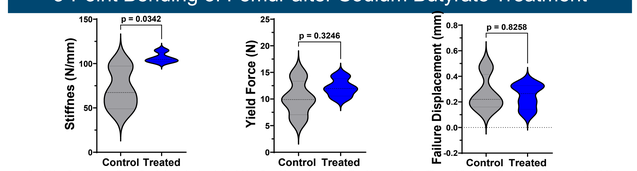
Ex vivo micro-CT of Tibia after sodium butyrate treatment. *Ex vivo* micro-CT after 6 months of sodium butyrate treatment showed a significant increase in bone volume/total volume, trabecular thickness and cortical thickness with a concomitant significant decrease in trabecular spacing. There was a trend for increase in trabecular number and connectivity density. Data are presented as Mean ± Standard Deviation.

Ex-Vivo Micro-CT of Spine after Sodium Butyrate Treatment



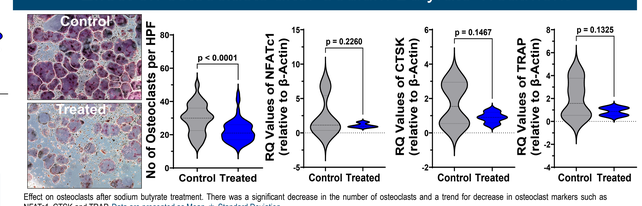
Ex vivo micro-CT of Tibia after sodium butyrate treatment. *Ex vivo* micro-CT of vertebrae after 6 months of sodium butyrate treatment showed a significant increase in bone volume/total volume, trabecular thickness and connectivity density with no change in trabecular number and space. Data are presented as Mean ± Standard Deviation.

3-Point Bending of Femur after Sodium Butyrate Treatment



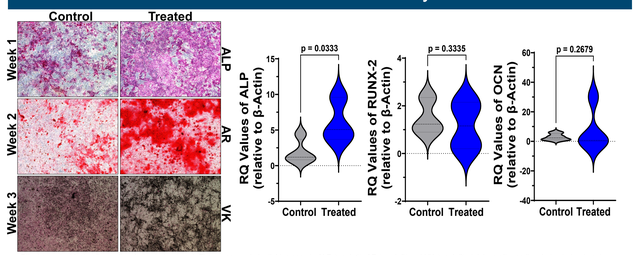
3-point bending of femur after sodium butyrate treatment. 3-point bending of femur shows a significant increase in the stiffness of the bone with a trend for increase in the yield force and the yield displacement. Data are presented as Mean ± Standard Deviation.

Effect on osteoclasts after Sodium Butyrate Treatment



Effect on osteoclasts after sodium butyrate treatment. There was a significant decrease in the number of osteoclasts and a trend for decrease in osteoclast markers such as NFATc1, CTSK and TRAP. Data are presented as Mean ± Standard Deviation.

Effect on osteoblasts after Sodium Butyrate Treatment



Effect on osteoblasts after sodium butyrate treatment. There was a substantial increase in ALP (week 1), AR (week 2) and VK (week 3) stainings after sodium butyrate treatment. The expression of osteoblastic markers such as ALP was significantly increased while Runx-2 and OCN did not change. Data are presented as Mean ± Standard Deviation.

Conclusions

- New evidence indicates that there is an immunological/inflammatory component in OI.
- There was a quantitative decrease in Tregs in OI mice.
- Restoring Treg numbers by systemic transplantation from WT or OI mice into OI mice or by non-invasive measures such as giving sodium butyrate orally, reduced T cell activation and effector cytokine secretion, increased Treg numbers, correlated with significant improvements in tibial trabecular and cortical bone parameters, along with increased stiffness of femur. Furthermore, an increase in osteoblast differentiation and a decrease in osteoclast number were observed in Treg transplanted OI mice.

Understanding contribution of Tregs to osteogenesis in OI could help to develop immunotherapy-based treatment modalities for OI