INTRODUCTION:
The molecular and cellular regulation of fracture healing is not completely understood, yet such knowledge is critical to developing treatments to optimize bone repair and remodeling. One area of continued interest is osteoimmunology: studying the interactions between pathways of the immune system and their interplay with bone healing. Specifically, immunological regulation of the osteoblast (OB) has been a particular poorly understood topic to date. While it is well known that there exists an interaction between OB and osteoclast (OC) cells through RANK-RANKL signaling, the role of lymphocytes and cytokines in OB biology during fracture healing remains unclear.

To this end, mice lacking the recombining activating genes 1 and 2 (Rag-) were chosen as a model for studying this interaction as they are unable to form T-cell (TC) or B-cell (BC) receptors and hence, completely lack mature TC and BC lymphocytes. In turn, they are devoid of any lymphocytic sources of interleukins, providing a model of fracture healing in the complete absence of these factors. Previous literature is suggestive of a lymphocytic role in fracture healing, noting that mice lacking the subset of γδTCs develop better fracture calluses. The objective of this study is to use the Rag-/- mouse to elucidate the physiological role of TCs in fracture healing, specifically on OB differentiation. It is hypothesized that TCs and/or their subtypes positively influence OBs leading to improved fracture healing.

MATERIALS AND METHODS:
In vitro studies: Since OBs differentiate from mesenchymal progenitors during the early phases of fracture healing, we examined if the presence or absence of TCs would alter OB differentiation using primary mesenchymal stromal cells harvested from the bone marrow of both WT and Rag-/- mice. OB primary cultures were grown in a 37°C incubator, stained with Alizarin red and also underwent RNA extraction and analysis. Cells at 5.0X10^5/mL were seeded per plate with α-MEM and treated with IL-17α (20ng/ml, R&D systems) and TGFβ (10ng/ml) for 4 days prior to RNA extraction and analysis of osteoblastic markers. Mice: 6.2597-Rag1tm1Mom/J (Rag-) and C7BL/6J (WT) male 12-week old mice were used (Jackson Laboratory). All studies followed Canadian Council of Animal Care (CCAC) guidelines. Standard pre-stabilized midshaft Tibias were harvested and at 10 and 20 days. For the OB cell line MC3T3-E1, 2.5X10^5 cells were seeded in 6-well plates with α-MEM, and treated with IL-17 (20ng/ml, R&D systems) and TGFβ (10ng/ml) for 4 days prior to RNA extraction and analysis of osteoblastic markers. Fracture analysis: Tibias at 3, 7, 14, 21, 28 and 35 days post-fracture. Tibias harvested at 3 and 7 days were fixed in 4% paraformaldehyde, decalified in 10% EDTA (pH 7.4), and embedded in paraffin for immunohistochemistry (IHC) using CD3 staining. Tibias harvested at 3 and 7 days also underwent total RNA isolation from their callus. The liquid nitrogen frozen callus was smashed by BioPulverizer (MidSci) and total RNA was extracted using TRIZOL (Invitrogen). Quantitative RT-PCR was performed with StepOnePlus system (Applied Biosystems) using SYBR Green (Bio-Rad). Tibias harvested at 14 and 28 days were administered calcein green and distances of bone formation between mineralization fronts were measured. Tibias harvested at 28 and 35 days were used for standard torsional biomechanical testing and μCT-based stereologic analysis.

RESULTS:
T-lymphocytes are normally present in the early phase of fracture repair: IIHC using an antibody against CD3, a marker ubiquitously expressed in TCs, confirmed the presence of TCs at high levels three days following a fracture in WT mice, and returning to baseline by 7 days (data not shown). Rag-/- mice however, had a marked absence of TC during this time frame. There are fewer mineralized osteoblasts in Rag-/- mice compared to WT: Based on the primary OB culture, OB CFUs derived from the bone marrow stromal cells of Rag-/- mice had fewer CFUs compared to WT. RT-PCR data confirmed a decline in expression of OB differentiation markers. Callus formation is prolonged in Rag-/- mice: The presence of a larger callus in Rag-/- mice at 28 days as reflected by increased TV (total callus volume, p=0.002) measurements compared to WT was confirmed on μCT. Tissue mineral density (TMD) at 28 days was significantly lower in the Rag-/- mice (p=0.004). These differences were no longer significant at 35 days. Calcein studies showed increased distance of bone formation by WT compared to Rag-/- mice. The expression of pro-inflammatory genes is attenuated during early fracture repair: Expression of pro-inflammatory cytokines IL-6, G-CSF, and IL-17F were decreased while expression of anti-inflammatory cytokines IL-10 and TGFβ was found to be increased compared to WT serum levels (IL-6, G-CSF and IL-10 data shown).

DISCUSSION:
As few studies exist on the interaction between the immune system and fracture healing, recent studies on Rag-/- and γδTC-/- have only begun characterizing the role of TC on fracture healing. We have focused in this study, specifically on OB cell differentiation and maturation. We found that a lack of TCs results in fewer OBs and a prolonged proliferative phase resulting in lower levels of markers of OB differentiation. This indicates a crucial role for TCs in the regulation of OB maturation. The cytokine expression data furthermore provided support for the idea that an imbalance of pro- and anti-inflammatory cytokines is the underlying mechanism. For this reason, we directly treated OB cells in culture with these cytokines to observe their effects on maturation. Indeed, IL-17 alone, promoted increased expression of markers of maturation highlighting for the first time, a key role for the pro-inflammatory cytokine IL-17F in fracture healing. This suggests a novel model in fracture healing processes whereby IL-17F, known to be secreted by the T-helper cell 17 subset of TCs, stimulates OB maturation. Future studies will be aimed at testing this model of early fracture healing.

SIGNIFICANCE:
This study provides a novel mechanism of interaction between the immune system and bone healing, specifically on how TCs enable OB maturation via IL-17F. This may provide future molecular targets that may have clinical applications to improve bone healing in those with nonunion or risks of impaired fracture healing.