The American Journal of Pathology, Vol. 182, No. 5, May 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

SHORT COMMUNICATION

Absence of B Cells Does Not Compromise Intramembranous Bone Formation during Healing in a Tibial Injury Model

Liza J. Raggatt,* Kylie A. Alexander,*† Simranpreet Kaur,* Andy C. Wu,* Kelli P.A. MacDonald,† and Allison R. Pettit*

From the University of Queensland Centre for Clinical Research, The University of Queensland, Herston; and the Queensland Institute for Medical Research,† Brisbane, Australia*

Accepted for publication January 28, 2013.

Address correspondence to Allison R. Pettit, Ph.D., Mater Medical Research Institute, Level 4, Translational Research Institute, 37 Kent St., Woolloongabba, QLD 4102 Australia. E-mail: apettit@mmri.mater.org.au.

Previous studies have generated conflicting results regarding the contribution of B cells to bone formation during physiology and repair. Here, we have investigated the role of B cells in osteoblast-mediated intramembranous anabolic bone modeling. Immunohistochemistry for CD45 receptor expression indicated that B cells had no propensity or aversion for endosteal regions or sites of bone modeling and/or remodeling in wild-type mice. In the endocortical diaphyseal region, quantitative immunohistology demonstrated that young wild-type and B-cell deficient mice had similar amounts of osteocalcin$^+$ osteoblast bone modeling surface. The degree of osteoblast-associated osteomac canopy was also comparable in these mice inferring that bone modeling cellular units were preserved in the absence of B cells. In a tibial injury model, only rare CD45 receptor positive B cells were located within areas of high anabolic activity, including minimal association with osterix$^+$ osteoblast-lineage committed mesenchymal cells in wild-type mice. Quantitative immunohistology demonstrated that collagen type I matrix deposition and macrophage and osteoclast distribution within the injury site were not compromised by the absence of B cells. Overall, osteoblast distribution during normal growth and bone healing via intramembranous ossification proceeded normally in the absence of B cells. These observations support that *in vivo*, these lymphoid cells have minimal influence, or at most, make redundant contributions to osteoblast function during anabolic bone modeling via intramembranous mechanisms. *(Am J Pathol 2013, 182: 1501–1508; http://dx.doi.org/10.1016/j.ajpath.2013.01.046)*

Fractures, resulting from injury or underlying disease-related bone fragility, are common and costly medical afflictions.[1] Cells of the immune and osseous systems have a complex dynamic interplay and have multilevel influence on the fate and functionality of each other. Identification of the key immune cell participants in bone healing is important in appropriately focusing research toward exploiting this promising strategy for manipulating bone repair.

It has been previously reported that fracture healing in mice was accelerated in the absence of both B and T cells (recombination activating gene (*RAG*)$-1^{-/-}$ mice[2]), suggesting that the involvement of both of these cells dampened the bone repair process.[3] However, the reported data on fracture healing differences were subtle and had no consistent trends across the time course or between related outcome measurements. Based on qualitative assessments, it was concluded that the B-lymphocyte population was likely responsible for the reported alterations in fracture healing, even though the model lacked both T and B cells.[3] This conclusion was supported by an earlier study showing enhanced osteogenesis using two different models of *de novo* bone formation (subcutaneous endochondral bone induction in response to recombinant bone morphogenetic protein-2 and bone marrow ablation) in B-cell deficient μMT mice.[4] Absence of B cells enhanced bone growth outcomes in both models, potentially through augmenting the early recruitment and proliferation of mesenchymal progenitors.[4] Again, improvement in bone formation

This work was supported by National Health and Medical Research Council (NHMRC) project grants 455941 and 631484 (A.R.P. and L.J.R.), an NHMRC Dora Lush Postgraduate Scholarship 409914 (K.A.A.), and an NHMRC Career Development Award 519744 (A.R.P.).

http://dx.doi.org/10.1016/j.ajpath.2013.01.046

outcomes were relatively subtle in this study, suggesting that B cells may have only a secondary or accessory role in restricting anabolic bone formation.

A more recent publication by Nam et al[5] that also assessed fracture healing in *RAG-1*$^{-/-}$ mice came to the alternate conclusion that the larger, low density callus generated in these mice when compared to wild-type mice, was more immature and therefore represented delayed fracture healing. T cells were noted within the fracture hematoma at early stages of healing.[5] B-cell distribution was not assessed in this study and somewhat surprisingly was not considered as potentially contributing to the study outcomes.[5] Similarly, in human fracture tissues collected at multiple healing stages, high numbers of fracture associated T cells were observed particularly in early stage fracture tissues in association with the hematoma. Minimal presence of B cells in the fracture samples was observed.[6] However, it was specifically noted that T cells were "excluded from areas of bone and cartilage formation," according to Andrew et al.[6,p465] Overall, the accumulated data are highly conflicting with respect to lymphocyte contributions to bone fracture repair.

The literature regarding the outcome of B- and/or T-cell deficiency on physiological bone growth is also inconsistent. Mice lacking either B cells (μMT mice[7,8]) or T cells (nude mice[8]) have been reported to be osteoporotic. The osteoporotic mechanism was ascribed to T-cell support of B-cell osteoprotegerin (a specific inhibitor of bone resorption) production, which constitutes as much as 50% of the basal osteoprotegerin levels within bone marrow.[8] However, others have reported no overt bone phenotype in μMT mice[9] or *RAG-1*$^{-/-}$ mice.[9,10] Other relevant data adding to the confusion with respect to the contribution of B cells to bone anabolism include reports indicating the following: B cells are a major source of receptor activator of NF-κB ligand within the bone marrow[11]; the klotho mutant mouse model of aging that exhibits low turnover osteopenia is associated with a reduced number of bone marrow B cells[11]; CD45 receptor (CD45R)$^{+}$ B cells stimulated osteoclast formation[11]; CD19^{+} B cells inhibit osteoclastogenesis and osteoclast survival via transforming growth factor-β expression[12]; and anabolic actions of estrogen administration were blunted in B-cell deficient μMT mice, suggesting minor pro-anabolic contributions of B cells in this model.[7] It is clear that further studies are required to assist in dissecting the contributions of B cells to bone physiology, healing, and pathology.

We have previously used a tibial injury model to assess osteoimmunology contributions to intramembranous ossification during bone healing.[13] This model, while not a true fracture, has the advantage of being highly reproducible when compared to traditional fracture models and is not complicated by marrow ablation/injury and subsequent anabolic outcomes due to intramedullary pin insertion.[5,14] It heals similarly to fully stabilized fractures through direct bridging of the defect via intramembranous ossification.[13] We used the tibial injury model in B-cell deficient μMT mice to specifically address whether B cells contribute to direct anabolic modeling and subsequent bone healing.

Materials and Methods

Animals

B-cell deficient μMT mice (homozygous for the immunoglobulin heavy constant mu (*Igh-6*)^{tm1Cgn} targeted mutation, C57Bl/6 background) and C57Bl/6 control animals were obtained from institutional or commercial (Animal Resources Centre, Canning Vale, Western Australia) breeding colonies. Animals were maintained on standard chow with diurnal light-dark cycle. The University of Queensland Ethics Committee approved all animal protocols.

Tibial Bone Injury Model

As previously described,[13,15] the 11- to 12-week-old mice were anesthetized and an incision was made in the left hind limb over the medial aspect of the proximal tibia. A 0.8-mm diameter hole was created in the distal tibial crest that penetrated through both cortices using a 21-gauge needle.[13,15]

Tissue Collection, Histology, and Immunohistochemistry

Left hind limbs were dissected and skin and extraneous muscle were removed. Limbs were fixed for 24 hours in 4% paraformaldehyde (Sigma, St Louis, MO) at 4°C and decalcified in 14% ethylenediaminetetraacetic acid (Sigma) pH 7.2 at 4°C for a minimum of 2 weeks. Specimens were processed and paraffin embedded using a Shandon Pathcenter Processor and embedding station (Thermo Electron Corporation, Waltham, MA). Five micrometer standard microtome sectioning was performed in the sagittal plane. Routinely more than 400 μm (50%) of the injury site was sampled for subsequent analysis, which provides accurate representation of the entire response.[13] Immunohistochemistry was performed on deparaffinized and rehydrated sections as previously described[16] with specific primary antibodies: anti-F4/80 (rat IgG2b clone CI:A3-1; AbD Serotec, San Diego, CA), anti-type 1 collagen (CT1) (rabbit polyclonal; US Biological, Swampscott, MA), anti-osteocalcin (rabbit polyclonal; Enzo Life Sciences, Exeter, UK), anti-osterix (rabbit polyclonal; Abcam, Cambridge, MA), and CD45R (common name B220, rat IgG2a; BD Pharmingen, Franklin Lakes, NJ), or relevant isotype control antibodies (normal ratIgG2b; AbD Serotec), ratIgG2a (BD Pharmingen), and normal rabbit IgG (Santa Cruz Biotechnology Inc.). All sections were counterstained using Mayer's hematoxylin (Sigma) and mounted using permanent mounting media (Thermo Fisher Scientific, Waltham, MA). Staining was assessed using a Nikon eclipse 80i microscope with a Nikon D5-Ri1 camera and NIS-elements imaging software version 3.1 (Tokyo, Japan). Histological staining for tartrate resistant acid phosphatase (TRAP) activity, a marker of osteoclasts and their precursors, was performed as previously described.[17]