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Research Paper

Doxorubicin induces bone loss and modifies multiple cell populations *in vivo* – Implications for modelling of bone metastasis

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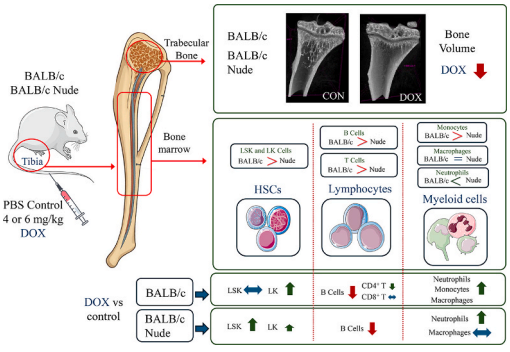
HIGHLIGHTS

- Doxorubicin (DOX) is used to limit tumour growth *in vivo* but the agent also has negative impact on bone structure.
- We demonstrate differential effects of DOX on the bone microenvironment in immunocompromised and immunocompetent mice.
- DOX caused significant trabecular bone loss in both groups, the effect was most prominent in immunocompetent mice.
- Multiple bone marrow cell populations were affected by DOX, including immune cells, with differences between the groups.
- Our findings highlight the importance of model selection, especially for therapeutic studies focused on bone.

GRAPHICAL ABSTRACT

Differential effects of doxorubicin (DOX) on bone and bone marrow cell populations in immunocompetent and immunocompromised mice



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ABSTRACT

Doxorubicin (DOX), commonly used to treat breast cancer, is associated with cardiotoxicity and has negative effects on other organ systems, including the skeleton. DOX-induced bone damage has been demonstrated in murine models; however, results are conflicting due to the use of different doses, schedules, and rat/mouse strains. As DOX is used to limit tumour progression in models of skeletal metastasis, it is paramount to determine how the agent affects the bone microenvironment in the relevant mouse strains, to enable correct interpretation of DOX effects in tumour studies. We have therefore investigated the effects of DOX on bone structure and a range of bone and bone marrow cell populations, comparing immunocompetent and immunocompromised mice.

Groups of 7-week-old female BALB/c and BALB/c Nude mice were treated with either saline (control), 4 or 6 mg/kg DOX weekly for four weeks. Effects on bone volume and structure was determined using *ex vivo* μCT, a panel of bone marrow cell populations were quantified by flow cytometry and osteoblast/osteoclast numbers were assessed using bone histomorphometry.

DOX caused trabecular bone loss, with immunocompetent BALB/c mice being more sensitive to DOX than the immunocompromised BALB/c nude counterparts. The 6 mg/kg dose of DOX altered the ratio of bone marrow immune and haematopoietic cell populations in both groups, increasing the numbers of hematopoietic cells and progenitors, decreasing B cells and increasing the number of neutrophils. Bone marrow macrophage and monocyte numbers were increased following DOX treatment in BALB/c nude mice only. Our data demonstrate

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that DOX impacts a number of cell types in the bone microenvironment, highlighting the importance of considering treatment-induced bone effects when using DOX in models of bone metastasis.

1. Introduction

It is well established that many anti-cancer agents may have detrimental effects on bone, reviewed by D'Oronzo *et al* [1]. Doxorubicin (DOX) is an anthracycline chemotherapy agent used to treat a range of cancer types, including breast, lung, gastric and ovarian tumours [2]. The primary mechanism of action of DOX is targeting topoisomerase II, ultimately resulting in DNA double-strand breakage and cell death [3]. In addition, DOX induces oxidative stress by directly generating reactive oxygen species (ROS), that in turn can damage DNA as well as other cellular components. Studies using a range of cancer cells and dosing regimens have demonstrated that DOX can induce senescence, autophagy, pyroptosis, ferroptosis, or necrosis of cancer cells [4,5].

Despite clinical success as an anti-cancer agent, DOX treatment is associated with various side effects, including cardiac dysfunction, liver and reproductive toxicity. In addition, chemotherapy regimens that included anthracyclines have been shown to cause bone loss in patients with early breast cancer [6–8]. However, as DOX was given as part of combination chemotherapy, e.g. together with cyclophosphamide, the effects specifically due to DOX on bone parameters could not be established. Similarly, a number of *in vivo* studies reporting negative effects on bone of anthracycline-containing chemotherapy have used combinations of agents, hence the contribution of DOX to these could not be ascertained [9,10]. DOX is not tumour cell-specific and has the ability to affect a range of cellular processes; both *in vitro* and *in vivo* studies have demonstrated negative effects on bone and bone cells [11]. However, results are conflicting and the use of different doses, schedules, rat/mouse strains, as well as the presence/absence of tumours, hampering assessment of the effects of DOX on the bone microenvironment. Rana *et al* demonstrated that weekly treatment of 5-week-old female BALB/c mice with 5 mg/kg DOX for 3 weeks caused a significant reduction in trabecular bone volume compared to control [12]. In addition, DOX (5 mg/kg weekly for 4 weeks) increased bone lesion size in an orthotopic 4 T1 bone metastatic breast cancer model and *in vitro* studies showed that DOX increased osteoclast formation from spleen and bone marrow samples. The same dose and schedule were used by Park et al to show that in 10-week old C57BL/6J mice, DOX-induced bone loss could be prevented by the autophagy inhibitor 3MA, providing evidence that DOX causes bone loss in part through increased reactive oxygen species (ROS), resulting in stimulating osteoclast autophagy and activity [5]. Induction of autophagy in the heart has also been reported in rat studies of DOX-induced cardiotoxicity [13]. Fonseca *et al* treated 8-week-old male Wistar rats with 2 mg/kg DOX weekly for seven weeks and found that DOX had negative effects on bone structure of the femur, reducing both cortical and trabecular bone volume [14]. DOX has been shown to cause more severe bone loss than ovariectomy (OVX). After sham or OVX, Yao *et al* treated 6-week-old FVB/NJ mice with 5 mg/kg DOX weekly for 4 weeks and analysed trabecular bone volume and cortical thickness at endpoint. The DOX treated group displayed significantly lower trabecular bone density than the OVX group, demonstrating that DOX has a negative impact on bone beyond just causing a reduction in oestrogen levels through a lowering of the uterine weight. They also found that DOX induced bone cell senescence, and that clearance of senescent cells with AP20187 could partially prevent DOX-induced bone loss, suggesting that targeting the p38MAPK-MK2 axis may be a way to reduce the negative effects of DOX on bone [15].

There is evidence that DOX may cause bone loss by increasing inflammation. Wang *et al* investigated how a single dose of 5 mg/kg DOX i.p. caused increased osteoclast number, decreased bone formation and a substantial bone loss in 10-week WT mice, with particular focus on the role of the innate immune response [16]. Assessing the acute response to DOX, they found that the number of circulating lymphocytes and monocytes were decreased 2 h post treatment, whereas neutrophil levels and neutrophil extracellular trap (NET) components were elevated. In addition, serum levels of the pro-inflammatory cytokines IL-1β, IL-18, IL-6, and TNF-α were elevated 3 days after DOX administration. They concluded that inflammasomes are key players in DOX-induced bone loss.

Several other studies have investigated whether DOX affects immune cell populations to contribute to off-target effects, however these mainly focussed on analysis of numbers of circulating immune cells. A comparison of blood samples obtained from breast cancer patients before and after DOX treatment showed that upregulation of neutrophil-specific genes was associated with early stages of DOX-induced cardiotoxicity, suggesting this could be utilised as a potential biomarker of this off-target effect [17]. In mice, elevated levels of neutrophil infiltration in the heart have been reported after DOX treatment, and that depletion of neutrophils reduced DOX-induced cardiotoxicity, highlighting that immune cells are significantly impacted by this agent [18]. Less is known about the effects of DOX on cells of the adaptive immune system, however a significant reduction in the numbers of B and T cells were reported in breast cancer patients 2 weeks after administration of anthracycline-containing chemotherapy. B cells appeared to be particularly sensitive, falling to 5.4 % of pre-chemotherapy levels [19].

Many *in vivo* studies investigating the wider effects of DOX have focussed either on monitoring cardiotoxicity or analyses of immune cells in the circulation. However, to assess the specific impact of DOX on bone metastasis, it is essential to map the effects on the cellular components of the bone microenvironment that may contribute to therapeutic response and/or mediate potential side effects. A particular strength of our study is that we have analysed the effects of DOX in both bone and bone marrow samples obtained from BALB/c and BALB/c Nude mice following 4 weeks of weekly treatment with a therapeutic dose. We have established that with some difference between the two groups, DOX affects a range cell types in the bone microenvironment, including immune cells, in addition to causing significant trabecular bone loss. Our data suggest that the impact of anti-cancer agents on bone and bone marrow populations should be considered when investigating the effects of therapy in models of bone metastasis.

2. Materials and methods

2.1. In vivo studies

Seven-week-old female BALB/c and BALB/c nude mice (Charles River, UK) were used to assess the effects of DOX on bone and the bone marrow microenvironment. Mice were housed in a controlled environment with a 12 h light/dark cycle at 22 °C and provided with ad libitum access to water and food. All experiments included in this manuscript were approved by the Research Ethics Committee for animal experimentation at the University of Sheffield, UK and were carried out to local guidelines and with UK Home Office approval under the authority PPL 70/8964 and P99922A2E held by Professor Penelope D Ottewell, University of Sheffield, UK. To evaluate the effects of DOX on bone and bone marrow, female (n = 8/group) BALB/c (immunocompetent) and BALB/c Nude (immunocompromised) mice were treated either with saline (control), 4 or 6 mg/kg DOX once weekly via intra-venous injection (i.v.) (Fig. 1). Animals were culled 48 h after administration of the last treatment, hind limb bones collected and either fixed with 4 % paraformaldehyde for μCT analyses or bone marrow was extracted for quantification of cell populations by flow cytometry.

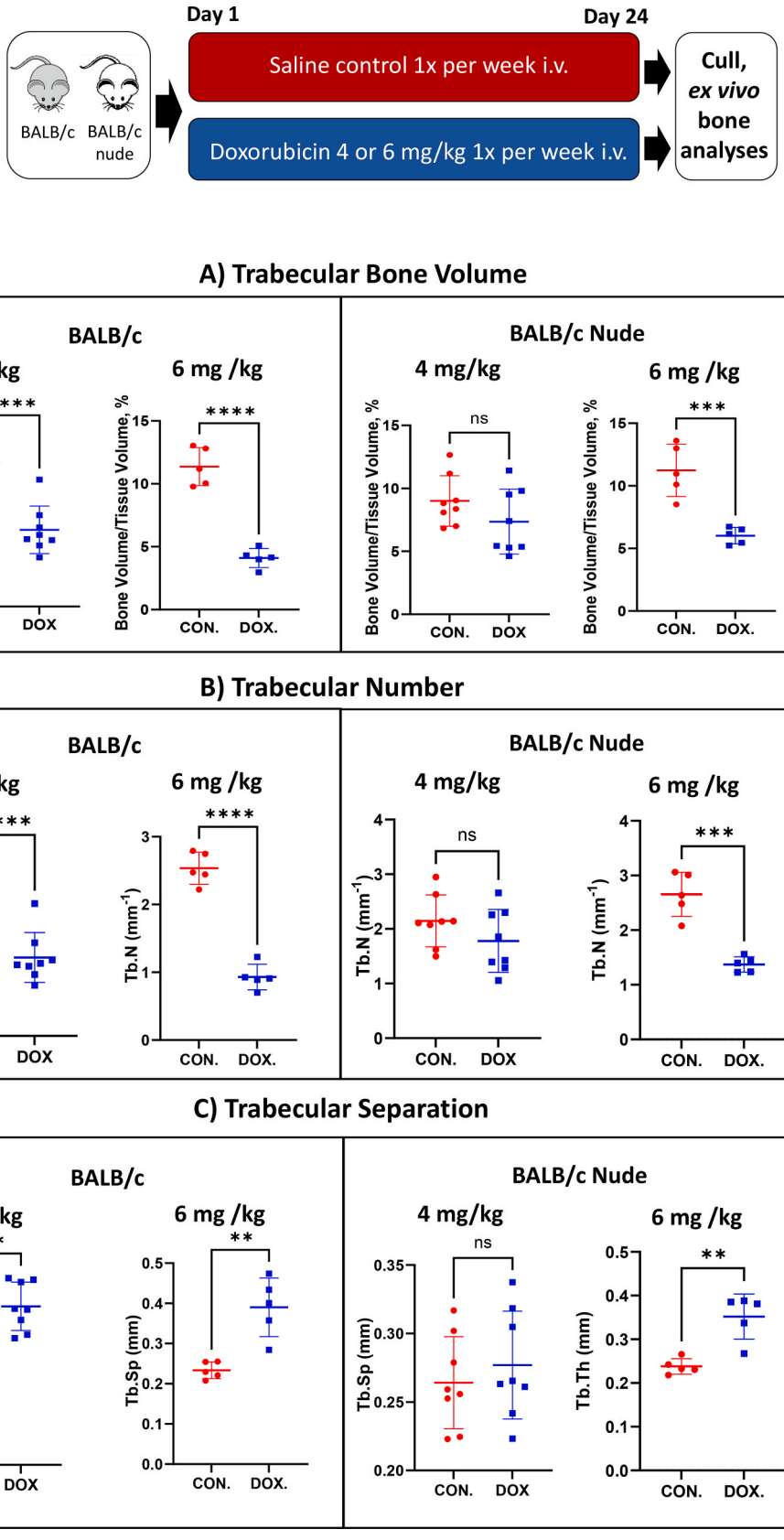

Fig. 1. Effects of doxorubicin on bone structure 7-week-old female BALB/c (left panels) and BALB/c Nude (right panels) mice received either saline (CON) or doxorubicin (DOX, 4 or 6 mg/kg) weekly up to day 24. Effects were compared to saline for the following: **A)** trabecular bone volume (BV/TV%), **B)** trabecular number (Tn/mm^{-1}), **C)** trabecular bone separation (mm), **D)** trabecular bone thickness (mm), **E)** Cortical bone volume (mm). **F)** Shows examples of 3D reconstructed uCT images Data are shown as Mean ± SD, n = for 8 for 4 mg/kg DOX and n = 5 for 6 mg/kg DOX. ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$, ns is non-significant.

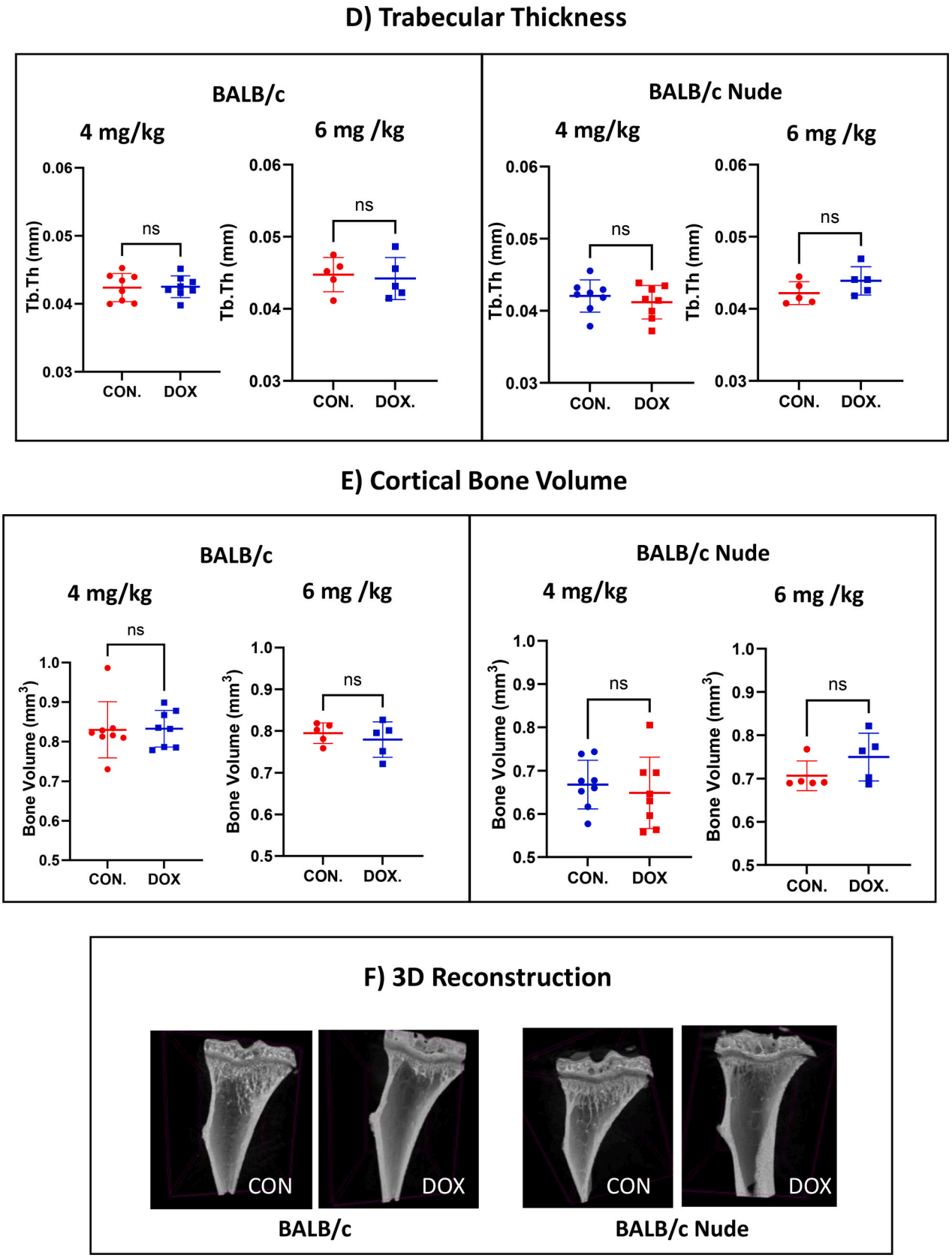

Fig. 1. (*continued*).

2.2. Microcomputed tomography (μCT) analysis

To establish whether DOX treatment had affected the bone volume or structure, microcomputed tomography (μCT) was performed on the proximal tibia using a Skyscan 1172 X-ray computed microtomography scanner (Bruker, Aartselaar, Belgium). A 2016x1344 camera resolution

with a 0.5 Al filter and 4.3 μm pixel size settings were used, and all bones were scanned 180° with a default 0.7 rotation step. SkyScan software was used to obtain 2D images that were reconstruction into a 3D image using NRecon software.

Quantification of the bone structural values (Trabecular Bone Density, Number, Thickness, Bone Separation and Cortical Bone Volume) with CTan software, the reference point was selected where the spongy bridge on the trabecular bone had broken. 0.2 mm from the reference point is the offset for trabecular bone (1 mm for cortical bone). After the offset value, 1 mm of the bone was set as the height to analyse. After selecting the correct bone region, we selected the region of interest (ROI), either the trabecular or cortical area was selected. The same ROIs were defined for all samples and then analysed with the batch manager. In the analyses, all samples were run through "Thresholding", "Despeckle", and "3D Analysis" in sequence. The thresholding value was set to a minimum of 80–85 and a maximum of 255. Despeckle was set to remove white speckles of less than 10 voxels. Default settings were used for 3D analysis of trabecular bone thickness, number, and separation measurements.

2.3. Bone histomorphometry

Osteoclasts and osteoblasts were quantified on histological sections following TRAP staining of decalcified tibiae. TRAP staining of osteoclasts on histological sections (3 μm) and identification of osteoblasts using morphological criteria were done as previously described [29]. Osteoclast and osteoblast number/mm trabecular bone surface was then scored on two non-serial sections using a Leica RMRB upright microscope, a 10 × objective and OsteoMeasure software (Osteometrics). In order to determine bone cell number per mm/trabecular bone all trabecular surfaces 125 μm away from the growth plate were scored.

2.4. Extraction of bone marrow

Bone marrow extraction was done by placing isolated bones in ice-cold PBS followed by five washes in 100 % and then 70 % alcohol. The top end of the bone was carefully removed with scissors and placed into sterilised 0.2 ml PCR tubes where the bottom had been pierced. Tubes containing bones were then placed into 1,5 ml Eppendorf tubes that contained 200 μl sterile PBS with 1 % penicillin and streptomycin. Tubes were centrifuged at 5000g for 5 min to flush the bone marrow into the PBS and analysed by flow cytometry.

2.5. Flow cytometry

To quantify the bone marrow populations, fresh bone marrow samples were analysed by flow cytometry and the markers listed in table 1a, using a BD LSR II flow cytometer and FlowJo software v10.8.0 (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and compensation control was performed using Invitrogen UltraComp eBeads.

Bone marrow was washed in ice-cold PBS supplemented with 1 % v/v FBS. Samples were aliquoted and incubated with fluorochrome-conjugated antibodies (supplementary table 1) and live/dead dyes (diluted 1:100/1 million cells) for 45 min. Gates were established using samples that were unstained or labelled with each single stain and fluorescence minus one (FMO: staining the sample with all except one stain). Samples were stained with live/dead dye (Zombie UV) at ambient temperature for 30 min and incubated with antibodies on ice for 45 min. After each staining step, samples were washed with ice-cold FACS buffer and centrifuged at 5000g for 5 min to remove the supernatant prior to flow cytometry analyses.

2.6. Gating strategies for flow cytometry analysis

The gating strategies for haematopoietic populations, lymphocytes and myeloid populations using side scatter area (SSC-A), forward scatter area (FSC-A) and height (FSC-H) and the markers are shown in supplementary figure 1. All positive or negative gating was determined using fluorescence minus one (FMO) and a single stain for the markers listed in table 1. A total number of 100,000 cells were gated from BALB/c mice and 700,000 from BALB/c nude mice, of which 80 % and 64 % were CD45+, respectively.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0. The results were compared using *t*-test analysis to determine their significance (*P $\leq$ 0.05, **P $\leq$ 0.01, ***P $\leq$ 0.001 and **** P $\leq$ 0.0001).

3. Results

3.1. Effects of DOX on bone volume and structure

Previous studies using murine models have reported negative effects of DOX on bone, inducing bone loss and reduced bone formation, however there is a lack of direct comparisons between mouse models most commonly used in breast cancer bone metastasis studies. We therefore initially determined the effects of two doses of DOX in tumour-free, age-matched, immunocompetent and immunocompromised mice. Groups of 7-week-old female BALB/c and BALB/c Nude mice were treated either with saline, 4 or 6 mg/kg DOX once weekly for four weeks (see experimental outline, Fig. 1). These dosing schedules were chosen as they have been shown to cause significant reduction of subcutaneous breast tumour growth *in vivo* [20]. No toxicity was noted; there was no significant difference in weight between animals in the saline group compared to the DOX treated groups throughout the experimental period (supplementary figure 2). Animals were culled on day 24 and hind limbs collected for μCT analysis to determine structural differences. As shown in Fig. 1, DOX induced significant trabecular bone loss in both groups of mice, but there was a noticeable difference in drug sensitivity between them. In immunocompetent mice, 4 mg/kg DOX was sufficient to induce a significant reduction in both trabecular bone volume (CON: 11.35 ± 1.42 vs DOX: 6.09 ± 1.89, p $\leq$ 0.0001) and trabecular number (CON: 2.68 ± 0.34 vs DOX:1.43 ± 0.46, p $\leq$ 0.0001), reflected in a corresponding increase in trabecular separation (CON: 0.18 ± 0.02 vs DOX:0.23 ± 0.03, p: 0.0048) (Fig. 1, left hand panels). We carried out bone histomorphometry on a limited subset of samples and found that the bone loss was associated with an increased number of osteoclasts compared to control (supplementary figure 3). It is important to note that changes in bone volume and structure depends on both bone cell number and their activity, hence the end-point uCT analyses shown in Fig. 1 are a more accurate measure of the impact of DOX on bone than the bone histomorphometry. In contrast, a reduction in bone volume was only seen in immunocompromised animals following treatment with the higher 6 mg/kg dose of DOX (Fig. 1, right hand panels). In addition, 6 mg/kg DOX had a stronger negative impact on both trabecular bone volume and number in immunocompetent- (BV/TV: CON: 11.67 ± 1.51 vs DOX:4.10 ± 0.76p $\leq$ 0.0001 and TB.N: CON: 2.54 ± 0.24 vs DOX:0.93 ± 0:19, p $\leq$ 0.0001) compared to immunocompromised mice (BV/TV: CON:11.24 ± 2.09 vs DOX:6.02 ± 0.66, p:0.0007 and TB.N: CON:2.65 ± 0.4 vs DOX:1.37 ± 0.14, p:0.0002), demonstrating that sensitivity to the drug differed between the groups. Trabecular thickness and cortical bone volume was unaffected by treatment with either dose of DOX in both groups of mice, (Fig. 1, D and E).

3.2. Bone marrow cell populations differ between immunocompromised and competent mice

Multiple bone marrow cell populations are implicated in cancer treatment response, with cells of the haematopoietic and immune niches of particular interest, as they are proposed to form integral parts of the

metastatic niche [21,22]. As cell populations within these compartments are continuously replenished, they are highly likely to be affected by agents like DOX that block DNA replication. In order to establish which bone marrow populations that were modified by the DOX treatment schedules used in our studies, it was important to first determine how the main cell populations differed between untreated BALB/c and BALB/c Nude mice. LSK cells (Lineage (−), Sca-1(+), C-Kit(+)) (defined as HSCs and progenitors) and LK cells (Lineage (−), Sca-1(−), C-Kit(+)), common myeloid populations, granulocyte macrophage progenitors and megakaryocytes-erythrocyte progenitors, are the main components of the HSCs niche. Due to lack of the Foxn1 gene, nude mouse strains have no functional T Lymphocytes, yet they do have immature T lymphocytes and functional myeloid populations. Thus, cell numbers of these selected population were compared, to establish the differences or similarities between BALB/c and BALB/c nude mice. As shown in Fig 2., flow cytometric analysis of the different populations showed that the number of both LSK and LK cells (as percentage of live CD45$^+$ve cells) were significantly lower in the BALB/c Nude compared to the BALB/c mice (LSK: BALB/c: 0.54 ± 0.13 vs BALB/c Nude: 0.19 ± 0.05, p:<0.0001 and LK: BALB/c: 2.97 ± 0.62 vs BALB/c Nude: 1.86 ± 0.35, p: 0.0006). The percentage of B cells of live CD45$^+$ bone marrow cells in BALB/c mice were significantly higher than in BALB/c Nude mice (BALB/c: 40.99 ± 7.16, BALB/c Nude: 17.42 ± 6.58, p:<0.0001). In BALB/c mice, only a very small percentage of the live CD45$^+$ cells were found to be either CD8$^+$ T (0.11 ± 0.04) or CD4$^+$ T (cells 0.014 ± 0.005), whereas these populations were undetectable in the BALB/c nude mice. The percentage of macrophages was also not significantly different (BALB/c: 1.45 ± 0.14, BALB/c Nude: 1.66 ± 1.7, p: ns:0.073), although the monocyte percentage was significantly lower in the BALB/c Nude mice compared to BALB/c (BALB/c: 0.91 ± 0.18, BALB/c Nude: 0.01 ± 0.02, p:<0.0001). Neutrophils were significantly higher in the BALB/c Nude compared to BALB/c mice (BALB/c: 23.21 ± 3.1, BALB/c Nude: 42.8 ± 5.92, p:<0.0001).

These studies demonstrated that there were significantly lower numbers of haematopoietic stem cells and their progenitors (LSK and LK cells) and B Lymphocytes in the immunocompromised mice. These differences should be taken into consideration when comparing the effects of therapeutic agents in murine models.

3.3. *DOX affects haematopoietic stem and progenitor cell numbers*

In addition to bone volume and structure, haematopoietic stem cells and their progenitors (LSK and LK cells, respectively) in the bone marrow may be affected by DOX treatment, causing unwanted side effects on haematopoiesis. Therefore, we explored the effects of DOX treatment on these populations, using the 6 mg/kg dose that had caused bone loss in both BALB/c and BALB/c Nude mice. As shown in Fig. 3.A, flow cytometric analysis demonstrated that 6 mg/kg DOX did not affect LSK cell numbers in BALB/c mice (CON: 0.54 ± 0.13, DOX: 0.54 ± 0.09p: ns). However, LSK cell numbers (as percentage of live cells) were significantly increased in DOX treated BALB/c Nude mice compared to control (CON: 0.19 ± 0.05, DOX:0.34 ± 0.09p: 0.0009). The number of LK cells were increased by DOX in both BALB/c and BALB/c Nude mice (BALB/c; CON:2.97 ± 0.62, DOX:7.345 ± 1.48p:<0.0001, BALB/c Nude; CON:1.86 ± 0.35, DOX:2.27 ± 0.35p: 0.035) (Fig. 3B). Taken together, these data demonstrate that in both groups of mice, DOX may alter the ratio of haematopoietic cells, with a shift towards increased numbers of progenitor cells detected.

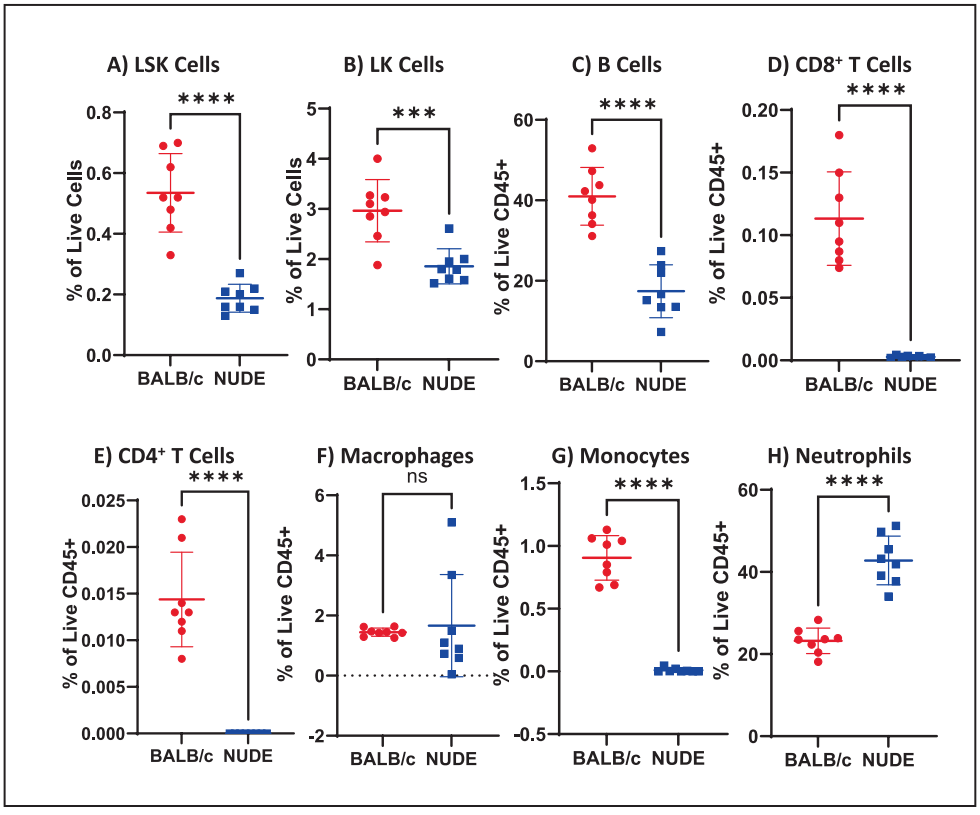

Fig. 2. Comparison of bone marrow cell populations between immunocompetent and immunocompromised mice. Comparison of **A)** LSK Cell and **B)** LK Cell data as % of live cells, isolated from BALB/c (red) and BALB/c Nude (blue) bone marrow, **C)** B Lymphocytes, **D)** CD8$^+$ T Lymphocytes, **E)** CD4$^+$ T Lymphocytes, **F)** Macrophages, **G)** Monocytes and **H)** Neutrophils as % of live CD45$^+$ cells. T-Test was used for statistical analysis, ns is non-significant, *** is $p < 0.001$, **** is $p < 0.0001$ and data show Mean ± SD, n = 8 for BALB/c and BALB/c Nude mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

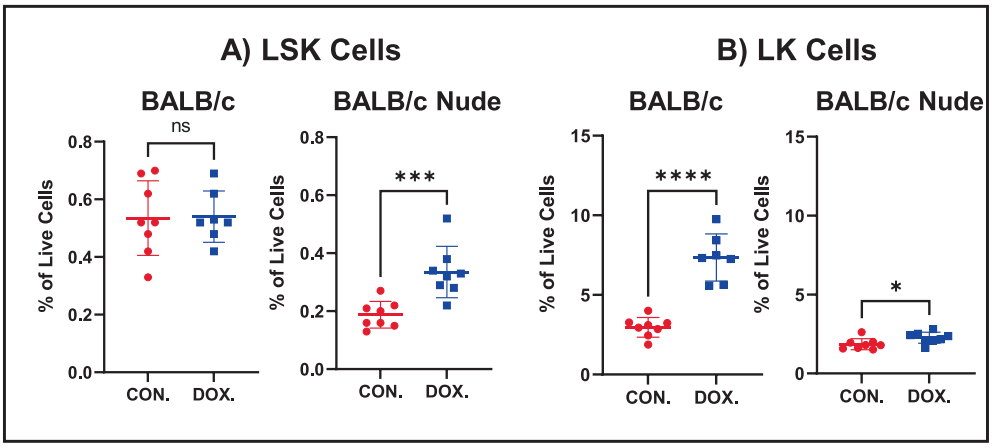

Fig. 3. Effects of doxorubicin on the hematopoietic stem cells and their progenitors in BALB/c and BALB/c Nude mice. Flow cytometry analysis of bone marrow cell populations of 7-week-old female BALB/c and BALB/c Nude mice treated with either saline (CON.) or 6 mg/ kg doxorubicin (DOX.) Effects on the **A)** LSK cells and **B)** LK cells (% of live cells) were compared using T-Test. ns is non-significant, * ≤ 0,05, *** ≤0.001 and **** ≤0.0001. Data shown as Mean ± SD n = for 8 for all groups.

3.4. Immune cell populations in the bone marrow are affected by DOX

Having established that haematopoietic cells were affected by 6 mg/ kg DOX in both groups of mice, we next explored the effect on a panel of immune cells (both innate and adaptive) present in the bone marrow. Immune responses involve highly proliferative processes that are affected by agents like DOX, potentially modifying cancer progression and treatment effects. It is therefore important to establish how DOX affects immune populations in murine model systems prior to carrying out *in vivo* studies to assess effects of anti-cancer agents.

As shown in Fig. 4A, B Lymphocyte numbers (as percentage of Live $CD45^+$ cells) were significantly reduced following DOX treatment in both groups (BALB/c; CON:40.99 ± 7.16 vs DOX:12.36 ± 3.51p:<0.0001, BALB/c Nude; CON:17.42 ± 6.58 vs DOX:5.86 ± 3.98p: 0.0008). As expected, $CD8^+$ (cytotoxic) and $CD4^+$ (helper) T cell numbers were only slightly altered in BALB/c mice; $CD8^+$ T cells were reduced by DOX (CON:0.1 ± 0.06, DOX:0.05 ± 0.01, p: 0.02, Fig. 4B), whereas $CD4^+$ T cells were increased (CON:0.13 ± 0.07, DOX:0.2 ± 0.04p: 0.01, Fig. 4C). Analysis of the innate immune cell populations (macrophages, monocytes, and neutrophils) showed that DOX treatment affected these cells differently in BALB/c vs BALB/c Nude mice (Fig. 4 D-F). Macrophages, monocytes and neutrophils were significantly increased in the DOX treated BALB/c group compared to control (Macrophages: CON: 1.45 ± 0.14, DOX:2.53 ± 0.41, p:<0.0001; Monocytes CON: 0.91 ± 0.18, DOX:7.15 ± 0.98, p: <0.0001; Neutrophils: CON: 23.21 ± 3.1, DOX:43.53 ± 3.71, p: <0.0001), whereas the only significant difference found in BALB/c Nude mice following DOX treatment was an increase in the neutrophil population (CON: 42.8 ± 5.92 vs DOX: 54.98 ± 9.58, p: 0.0085, Fig. 4F). Collectively, these data demonstrate that DOX treatment resulted in alterations in both adaptive and innate immune cell populations in the bone marrow, but the effect differed significantly between immune-competent and −compromised mice.

4. Discussion

DOX is widely used to treat breast cancer, alone or combined with bone-targeted agents. It is also commonly used to slow tumour progression in bone metastasis *in vivo* models, limiting tumour burden and extending the period available for studies of novel therapeutic agents or combinations [23,24]. It is well-established that cardiotoxicity and myelosuppression may limit the use of DOX in patients, and numerous additional off-target effects have been reported from both clinical and pre-clinical studies, including bone loss in breast cancer patients [6,25,26]. The latter is of relevance for investigations using bone metastasis models, where DOX-induced bone loss may have significant impact on the interpretation of results. For example, it may be impossible to distinguish between tumour- and DOX-induced bone loss, hampering the analysis of anti-cancer effects. Our study focussed on characterising the effects of DOX on the bone microenvironment in tumour-free animals, to provide important baseline information and guidance for researchers aiming to investigate the effects of anti-cancer therapies in murine models of bone metastasis.

A number of studies have reported DOX effects on bone cells *in vitro*, including increased osteoclastogenesis [11,27] and decreased osteoblastogenesis [12], indicating that DOX treatment would disrupt the fine-tuned balance between bone formation and resorption, resulting in net bone loss. This is further supported by *in vivo* studies reporting negative effects on bone volume/structure following DOX treatment, however a wide range of doses and schedules have been used, as well as different strains, ages and sex of animals. In addition, DOX is frequently used in combination with other agents (e.g. cyclophosphamide), with results showing that DOX-containing chemotherapy regimens have detrimental impact on bone, including in patients [6,9,25,26]. Hence there is a significant body of evidence supporting that DOX negatively impacts bone, however, it is unclear what cell types are affected, how the effects relate to the dosing schedule used, or the age and immune cell populations of the animals. Here we have addressed some of these issues by establishing the effects of DOX on bone volume and structure in tumour-free immunocompetent and immunocompromised mice, as well as on a panel of cells isolated from the bone marrow following four weeks of DOX treatment *in vivo*.

Our initial experiments were designed to establish whether DOX had differential effects in immunocompetent vs immunocompromised mice, and to identify a dose of DOX that caused significant bone loss in both groups. We used a 4-week treatment schedule where i.v. DOX was administered weekly, previously shown to be well tolerated and to reduce the growth of subcutaneously implanted breast tumour xenografts [20]. We were particularly interested in assessing DOX effects on trabecular bone in the proximal tibia, an area of bone colonised by breast tumour cells following intracardiac injection, the most commonly used model of bone metastasis [28,29]. In agreement with previously published studies, we found significant DOX-induced bone loss in both groups, with immunocompetent mice being more sensitive to this agent. Limited bone histomorphometry suggested that DOX increased osteoclast number in immunocompetent animals, but the effects on osteoclast and osteoblast activity during the 4-week treatment period was not measured as it would require longitudinal blood sample collection for

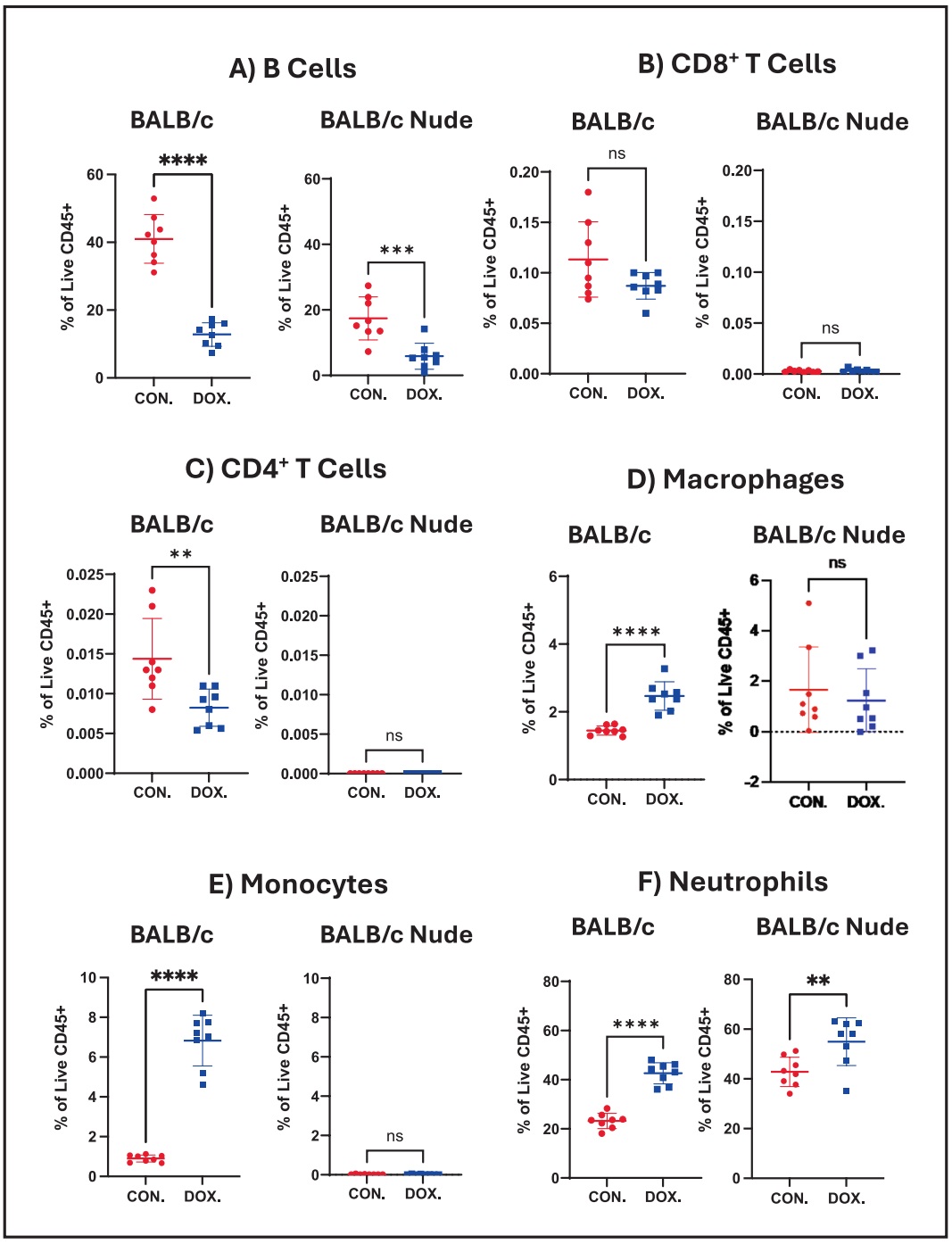

Fig. 4. Effects of doxorubicin on bone marrow immune cell populations in BALB/c and BALB/c Nude mice. Flow cytometry analysis of bone marrow cell populations of 7-week-old female BALB/c and BALB/c Nude mice treaded with either saline (CON.) or 6 mg/ kg doxorubicin (DOX.) Effects (% of live CD45$^+$ cells) on **A)** B cells, **B)** CD8$^+$ T cells, **C)** CD4$^+$ T cells, **D)** macrophages, **E)** monocytes and **F)** neutrophils were compared using T-Test. ns is non-significant, * ≤0.05, ** ≤0.01, *** ≤0.001 and **** ≤0.0001. Data shown as Mean ± SD n = for 8 for all groups.

bone turnover markers. It is therefore important to note that the design of our study only allowed us to measure the impact of treatment on bone at the endpoint.

We assessed bone loss after several cycles of DOX treatment, although there have been reports of bone effects caused by a single dose of 5 mg/kg DOX [16]. Lower doses than this may not be sufficient to cause bone loss; we have previously demonstrated that a single administration of 2 mg/kg DOX to BALB/c nude mice has no significant effect on bone volume or structure, either in the presence or absence of breast tumours [24]. We chose to use a 'clinical' dosing regimen of repeated weekly DOX treatment for four weeks, to mimic the conditions used in to limit tumour growth in bone. We did not seek to capture the acute effects of DOX, hence analyses were carried out on samples collected 48 h after the last drug administration. Our findings highlight the dilemma that researchers face of either using a chemotherapy agent like DOX to reduce tumour progression and mimic the clinical setting, thereby introducing treatment-induced bone loss into their model systems, or allowing tumour growth to progress unimpeded which in turn limits the study duration. An additional option is to add an anti-resorptive agent (e.g. zoledronic acid); this agent is generally well

tolerated and shown to cause significant reduction in both tumour- and treatment-induced bone loss *in vivo* [23]. In the clinical setting, anti-resorptive agents are used to prevent treatment-induced bone loss (e.g. in patients receiving chemotherapy or endocrine therapy for early breast cancer), as well as to reduce cancer-induced bone loss in metastatic breast and prostate cancer [30]. However, zoledronic acid reduces tumour growth in bone [23,31] and modifies a range of cell types in the bone microenvironment in model systems [32–34]. Addition of zoledronic acid will therefore interfere with the interpretation of effects of anti-cancer agents on tumour growth in bone.

We also wanted to establish the effects of DOX on different bone marrow cell populations following *in vivo* treatment, as this is likely to cause differential effects depending on the immune competency of the animal. There is very little information available in this area, most studies have focussed on analyses of DOX-mediated impact on osteoblasts/osteoclasts [12,16,24], on circulating immune cells [19] or serum levels of bone turnover markers [26]. To establish the baseline level, we first quantified a selection of bone marrow cells in untreated mice of both groups. As expected, we found marked differences in several populations, most notably lower numbers of monocytes, B, LK and LSK cells and higher numbers of neutrophils in BALB/c nude mice compared to BALB/c. We detected very low levels of T cells only in BALB/c mice, in agreement with those reported by Hensel *et al* in their comprehensive characterisation of immune populations in different sites (including bone) across multiple mouse strains [35].

Next, we collected bone marrow samples obtained from the long bones of mice that had undergone 4 weeks of DOX treatment. Analysing the same cell populations as was done for untreated animals, we determined that DOX caused significant increases in LSK cells (immunocompromised mice) and LK cells (both groups). These results suggest that stem and progenitor populations are less sensitive to DOX, maybe because of their non-proliferative nature, compared to other bone marrow cell populations [36]. Further studies would be needed to establish if these DOX-induced changes to progenitor and stem cell population results in functional differences in the mature progeny and whether the increases we observed represent a compensatory mechanism. Our data show that DOX modifies bone marrow cell populations but does not reveal the spatial and temporal effects, the availability of novel methodology and technology may be used to identify these in more detail. For example, mapping of the transcriptional landscape of the bone marrow microenvironment at single cell resolution has yielded some insight into stress responses (including to therapy), with the authors suggesting that future studies should focus on clarifying the functional consequences of niche heterogeneity on aberrant stem cell functions [37].

We next analysed a number of immune cell populations and found a significant reduction of the proportion of B cells following DOX treatment in both groups of mice. Our results are in agreement with those reported from clinical studies that have showed lymphocyte depletion in breast cancer patients after anthracycline therapy [19], with one study reporting B cell numbers dropping to as low as 5.4 % of pre-chemotherapy levels [38]. The slight decrease we observed in $CD4^+$ T cells induced by DOX in BALB/c mice are in line with a report that DOX inhibits *ex vivo* proliferation of T cells isolated from healthy human donors [39]. However, it seems likely that the effect of DOX on T cells in tumour-free bone marrow is minor and the effects of DOX on T cells need to be explored further in immunocompetent models of tumour growth in bone.

The impact of DOX on cells of the innate immune system were more pronounced, causing a significant increase in monocytes and macrophages, but only in immunocompetent animals. The increase in monocyte numbers was particularly prominent, suggesting that an inflammatory response to DOX was initiated. We are unaware of other studies of DOX effects on monocytes and macrophages in bone marrow, however Zhang *et al* have reported macrophage infiltration into the heart in a murine model following DOX treatment and proposed that this contributed to cardiomyopathy [40]. A recent review that described the proposed roles of macrophages in development and progression of bone metastases highlighted significant gaps in our knowledge [41]; based on analysis of a large number (70 +) of published datasets containing single cell omics of tumour-associated macrophages, the authors identified seven different subpopulations with distinct molecular signatures and note that their potential role in bone metastasis remain to be established. Our findings that DOX treatment increases both monocyte and macrophage numbers in bone in immunocompetent animals suggest that treatment-induced alterations to innate immune cell populations should also be carefully monitored, including mapping effects on different macrophage subsets. Future studies should investigate whether DOX contributes to the inflammatory response in bone metastasis, e.g. whether monocyte and/or macrophage infiltration exacerbates the release of pro-inflammatory cytokines and chemokines known to contribute to cancer-induced bone disease in breast and prostate cancer (reviewed by Göbel, [42]).

DOX also caused a significant increase in the number of bone marrow neutrophils in both groups of mice. As reported for macrophages, neutrophil infiltration in the heart following DOX treatment is proposed to be involved in mediating cardiotoxicity [43], but there are no reports of this happening in bone. Neutrophils have been shown to have both pro- and anti-tumour effects (reviewed by McFarlane, [44]) and their action is highly dependent on the microenvironment, however their specific involvement in bone metastasis remains to be established. A recent murine study has identified a novel mechanism of DOX-induced bone loss through activation of inflammasomes, innate immune system sensors that regulate the activation of caspase-1 and induce inflammation [16,45]. Demonstrating that a single dose of DOX (5 mg/kg) caused a spike in neutrophil numbers 2 h post treatment and induced NET formation, the authors propose that the AIM2 and NLRP3 inflammasomes contribute to the detrimental effects of DOX on bone. As inflammation is increased with the presence of tumours in bone, contributing to cancer-induced bone loss, these data suggest that DOX may exacerbate this process.

5. Summary and conclusion

This study confirms the negative effects of DOX on bone structure and integrity *in vivo*, mediated through loss of trabecular volume and number, accompanied by changes in bone marrow cell populations, and identifies significant differences in DOX response between immunocompetent and immunocompromised mice. The data imply that DOX-induced bone destruction is dose-dependent and impacted by the immunological status of the host, and that multiple cell types in bone are affected by this agent.

Our study has some limitations; further work is required to fully characterise the impact of DOX on the bone microenvironment in the cancer setting, including whether the observed changes in low-abundance populations are biologically meaningful. Other cell types involved in development and progression of bone metastases may be affected by DOX besides those studied here; the precise molecular mechanisms involved are likely to be complex and were not the focus of our study. For example, we did not explore how DOX modifies the level of cytokines known to regulate bone turnover (e.g. IL1B, IL6), nor did we explore the impact of DOX in male mice, allowing us to identify to what extent the bone loss was secondary to a DOX-induced reduction in uterine weight and thereby lower oestrogen levels in the female mice. We did not aim to identify the specific molecular mechanisms involved, but to provide evidence that chemotherapy agents have a multitude of effects in the bone microenvironment that need to be considered in any studies of treatment that impacts on bone metastases in murine models. Future investigations should focus on clarifying the molecular mechanisms behind DOX-induced bone destruction, notably the roles of reactive oxygen species (ROS) and TGFβ, as well as induction of inflammation. Understanding these pathways could lead to the

development of targeted therapeutics to minimise the harmful effects of DOX on bone.

Overall, this research contributes to a greater understanding of the effects of chemotherapy on bone health, underlining the need for comprehensive measures to safeguard bone strength and function in cancer patients undergoing DOX treatment.

CRediT authorship contribution statement

Veli Kaan Aydin: Writing – review & editing, Visualization, Investigation, Funding acquisition, Formal analysis. **Lubaid Saleh:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Penelope Dawn Ottewell:** Writing – review & editing, Supervision, Formal analysis. **Ingunn Holen:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement.

All experiments included in this manuscript were approved by the Research Ethics Committee for animal experimentation at the University of Sheffield, UK and were carried out to local guidelines and with UK Home Office approval under the authority PPL 70/8964 and P99922A2E held by Professor Penelope D Ottewell, University of Sheffield, UK.

All authors have reviewed and approved the final version of the manuscript and confirm the accuracy of this statement.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2025.100736.

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