Defective Bone Repair in C57Bl6 Mice With Acute Systemic Inflammation

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Abstract

Background Bone repair is initiated with a local inflammatory response to injury. The presence of systemic inflammation impairs bone healing and often leads to malunion, although the underlying mechanisms remain poorly defined. Our research objective was to use a mouse model of cortical bone repair to determine the effect of

systemic inflammation on cells in the bone healing microenvironment.

Question/Purposes (1) Does systemic inflammation, induced by lipopolysaccharide (LPS) administration affect the quantity and quality of regenerating bone in primary bone healing? (2) Does systemic inflammation alter vascularization and the number or activity of inflammatory cells, osteoblasts, and osteoclasts in the bone healing microenvironment?

Methods Cortical defects were drilled in the femoral diaphysis of female and male C57BL/6 mice aged 5 to 9 months that were treated with daily systemic injections of LPS or physiologic saline as control for 7 days. Mice were euthanized at 1 week (Control, n = 7; LPS, n = 8), 2 weeks (Control, n = 7; LPS, n = 8), and 6 weeks (Control, n = 9; LPS, n = 8) after surgery. The quantity (bone volume per tissue volume [BV/TV]) and microarchitecture (trabecular separation and thickness, porosity) of bone in the defect were quantified with time using microCT. The presence or activity of vascular endothelial cells (CD34), macrophages (F4/80), osteoblasts (alkaline phosphatase [ALP]), and osteoclasts (tartrate-resistant acid phosphatase [TRAP]) were evaluated using histochemical analyses.

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Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

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Results Only one of eight defects was bridged completely 6 weeks after surgery in LPS-injected mouse bones compared with seven of nine defects in the control mouse bones (odds ratio [OR], 0.04; 95% CI, 0.003–0.560; $p = 0.007$). The decrease in cortical bone in LPS-treated mice was reflected in reduced BV/TV ($21\% \pm 4\%$ vs $39\% \pm 10\%$; $p < 0.01$), increased trabecular separation ($240 \pm 36 \mu\text{m}$ vs $171 \pm 29 \mu\text{m}$; $p < 0.01$), decreased trabecular thickness ($81 \pm 18 \mu\text{m}$ vs $110 \pm 22 \mu\text{m}$; $p = 0.02$), and porosity ($79\% \pm 4\%$ vs $60\% \pm 10\%$; $p < 0.01$) at 6 weeks postoperative. Defective healing was accompanied by decreased CD34 (1.1 ± 0.6 vs 3.4 ± 0.9 ; $p < 0.01$), ALP (1.9 ± 0.9 vs 6.1 ± 3.2 ; $p = 0.03$), and TRAP (3.3 ± 4.7 vs 7.2 ± 4.0 ; $p = 0.01$) activity, and increased F4/80 (13 ± 2.6 vs 6.8 ± 1.7 ; $p < 0.01$) activity at 2 weeks postoperative.

Conclusion The results indicate that LPS-induced systemic inflammation reduced the amount and impaired the quality of bone regenerated in mouse femurs. The effects were associated with impaired revascularization, decreased bone turnover by osteoblasts and osteoclasts, and by increased catabolic activity by macrophages.

Clinical relevance Results from this preclinical study support clinical observations of impaired primary bone healing in patients with systemic inflammation. Based on our data, local administration of VEGF in the callus to stimulate revascularization, or transplantation of stem cells to enhance bone turnover represent potentially feasible approaches to improve outcomes in clinical practice.

Introduction

Bone regenerates by primary or secondary fracture healing depending on the mechanical stability of the fracture. Primary fracture healing requires absolute stability. It is associated with minimal callus formation and direct bridging of fracture ends [15]. Secondary fracture healing occurs in the case of relative stability and shows a combination of endochondral and intramembranous bone formation [14]. A healing fracture and the immune system responsible for inflammatory processes share numerous signaling and regulatory mechanisms [13, 45]. As the initial phase of the bone-healing cascade, inflammation plays a pivotal role in the process of fracture repair during which an early scaffold of the callus is formed [15, 29]. It triggers synthesis of the extracellular matrix, stimulates revascularization, and recruits mesenchymal stem cells (MSCs) and other progenitor cells [15, 25]. The immune system also exhibits important crosstalk with bone cells during the later phases of secondary bone repair to orchestrate chondrogenesis and endochondral ossification [13, 15]. Furthermore, the macrophages and several inflammatory

cytokines are necessary for differentiation of bone marrow MSCs into osteogenic cells that will regenerate and mineralize bone [10, 18].

Systemic inflammatory conditions, such as those associated with orthopaedic polytrauma, disrupt the localized and highly regulated inflammatory phase of bone healing and are proposed to underlie clinical complications such as malunion and nonunion [10]. In a rat model it was shown that repair of a stabilized femoral osteotomy in the presence of acute systemic inflammation induced by chest trauma was associated with an increase in granulocytes and a decrease in macrophages during the early soft callus phase of healing [38]. In another study conducted in neutrophil-deficient mice it was concluded that this type of granulocyte was not involved in the disruption of bone repair leading to malunion in the presence of systemic inflammation [27]. Others have investigated bone repair in a mouse model of chronic inflammation and reported reduced vascularization, increased numbers of macrophages, and impaired remodeling of the callus [1]. These studies begin to address the mechanisms underlying impaired bone healing in the presence of systemic inflammation. In the current study we used a mouse model of bone repair to determine: (1) whether systemic inflammation, induced by lipopolysaccharide (LPS) administration affects the quantity and quality of regenerating bone in primary bone healing, and (2) whether systemic inflammation alters vascularization and the number or activity of inflammatory cells, osteoblasts, and osteoclasts in the bone-healing microenvironment?

Materials and Methods

Overview

To answer our first question regarding the effect of LPS-induced systemic inflammation on the repair of a cortical bone defect, we generated a mechanically stable, cortical window defect in skeletally mature mouse femurs. MicroCT was used to quantify the amount and quality of bone bridging the defect at 1, 2, and 6 weeks postoperative. To answer our second question regarding the effect of systemic inflammation on revascularization and the presence of immune and bone cells in the fracture callus, we quantified staining for vascular endothelial cells, VEGF, macrophages, osteoclasts, and osteoblasts during the same times.

Animals and Surgery

All animal procedures were performed in strict accordance with a protocol approved by the McGill Facility Animal Care Committee, in keeping with the guidelines of the

Canada Council on Animal Care. Skeletally mature female and male C57BL6 mice aged 5 to 8 months were purchased from Charles River Laboratories (Montreal, Quebec, Canada). Surgery was performed essentially as described previously [6, 16] to generate reproducible, mechanically stable defects. The femur was exposed by blunt dissection and the periosteum carefully removed from the anterolateral aspect of the proximal femur using the third trochanter as the proximal landmark. Bilateral unicortical defects measuring 2 mm long $\times$ 1 mm wide were drilled on the outer aspect of the femur with a 1-mm burr on a Stryker drill (Stryker Canada Inc, Hamilton, Ontario, Canada). One group of mice received intraperitoneal injections of 25 μ g LPS diluted in 0.1 mL phosphate buffered saline (PBS) for 7 days starting immediately postoperative. This dose is one-tenth of the lethal dose in mice and was chosen to prevent damage of other organs and allow the mice to survive indefinitely [22]. A second group of mice received an intraperitoneal injection of 0.1 mL PBS as Controls. Cohorts of Control and LPS-injected mice were euthanized at 1 week (Control, $n = 7$; LPS, $n = 8$), 2 weeks (Control, $n = 7$; LPS, $n = 8$), and 6 weeks (Control, $n = 9$; LPS, $n = 8$) postoperative. These times were selected to analyze bone healing during the inflammatory, regenerative, and remodeling phases of mouse bone healing, using established criteria [14]. Femurs were dissected, fixed overnight at 4° C in fresh 4% paraformaldehyde, and stored in PBS at 4° C until microCT analyses.

Measurement of Serum Tumor Necrosis Factor (TNF)- α

Serum TNF- α was used as an index of systemic inflammation in mice euthanized at 1 week postoperative [6]. Mice were exsanguinated 2 hours after the final LPS injection, when the highest serum concentration reportedly is found [50], and the serum was assayed using an enzyme-linked immunosorbent assay (ELISA kit; R&D Systems, Minneapolis, MN, USA). One hundred microliters of serum or standard (31.25 to 2000 pg/mL) was added to a plate coated with mouse TNF- α capture antibody and incubated before adding mouse TNF- α detection antibody. Diluted streptavidin-horseradish peroxidase then was added followed by the addition of tetramethylbenzidine chromogen. After adding 50 μ L of 2 N H₂SO₄, the absorbance of each well was measured at 450 nm and 570 nm.

MicroCT Scans and Analyses

MicroCT scans and quantitative analyses were used to assess bone bridging across the defect and bone architecture according to protocols established in the laboratory

[6, 16]. Scans were captured using a Skyscan 1172 MicroCT scanner (Bruker, Kontich, Belgium) with a 0.5-mm aluminum filter at a voltage of 50 kV, current of 200 μ A, and 5 μ m spatial resolution. Bridging of the bone defect was defined as continuity of bone tissue between the proximal and distal ends of the defect in the mid-sagittal plane, and new bone covering at least 95% of the defect in the coronal plane. Two different regions of interest (ROI) were selected for analysis of bone volume per tissue volume (BV/TV). ROI A covered a manually delineated cylinder of medullary tissue adjacent to the window defect and ROI B covered the rectangle of new bone occupying the window defect (Fig. 1). The architecture of bone in ROI B was defined using the following parameters: trabecular number, trabecular thickness, trabecular separation, connectivity density (three-dimensional connectivity of bone structures), percentage total porosity, and the number of closed pores.

Histology, Histochemistry, and Immunohistochemistry

Histologic analyses were performed to assess the morphologic features of new bone with time, neovascularization of the repair tissue, and the presence or activity of mast cells, macrophages, osteoclasts, and osteoblast in the repair tissue. Femurs were decalcified in 10% EDTA and embedded in paraffin for histochemical and immunochemical analyses essentially as described previously [46]. Serial 5- μ m thick sections were cut and stained with hematoxylin and eosin to observe morphologic features and with acidified toluidine blue stain (pH 2.3) to observe mast cells [6] in the repair tissue adjacent to the window defect. Alkaline phosphatase (ALP)-positive osteoblasts were observed by staining with Naphthol AS-TR phosphate, N,N-dimethyl formamide, and nitroblue tetrazolium /bromochloroindolyl phosphate in tris-malate buffer (pH 9.3). Osteoclasts and other mononuclear tartrate-resistant acid phosphatase (TRAP)-positive cells were observed by staining with Naphthol AS-TR phosphate, sodium nitrite, sodium tartrate, and pararosaniline hydrochloride in acetate buffer (pH 5.0). Images were captured on a Zeiss Axioskop 40 microscope (Carl Zeiss Canada Ltd, Toronto, Ontario, Canada), and staining concentration quantified using Image J software (NIH, Bethesda, MD, USA), as described previously [6]. Adjacent sections of decalcified bone were used for immunohistochemical analyses using the following antisera and methyl green as counterstain: CD34 to mark vascular endothelial cells (R&D Systems), VEGF to mark angiogenesis (Abcam, Cambridge, MA, USA), Cathepsin K to mark osteoclasts (Abcam), F4/80 to mark M1

(proinflammatory) and M2 (antiinflammatory, proangiogenic) macrophages (Abcam), and Arginase-1 to mark only M2 macrophages (Santa Cruz Biotechnology Inc, Dallas, TX, USA). Quantification of staining in the ROI (color range: saturation = 0–255; hue = 0–255; brightness = 0–252) was expressed as a percentage of the total area.

Statistical Analyses

The average value for left and right femurs was determined for all parameters, and SPSS Version 21 (IBM Corporation, Armonk, NY, USA) was used for the chi-square test (distal bone formation and bridging) and the Mann-Whitney U test (all other parameters) was used for unpaired samples. Differences were considered significant at a p less than 0.05.

Fig. 1A–L Bilateral femoral window defects were generated in skeletally mature mice, and daily intraperitoneal injections of phosphate buffered saline (PBS) or lipopolysaccharide (LPS) were administered for the first 7 days postoperative. Cohorts of mice were euthanized at 1, 2, or 6 weeks postoperative. Femurs were isolated and scanned at a resolution of 5 μ m on a Skyscan 1172 microCT instrument. Representative images of the mid-sagittal region with the defects delineated by arrowheads are shown at 1, 2, and 6 weeks postoperative. The upper arrowhead indicates the proximal end of the defect while the lower arrowhead indicates the distal end of the defect. In mice injected with PBS (Control) new bone was seen at the proximal end of the defect at (A) 1 week postoperative, (B) at proximal and distal ends at 2 weeks, and (C) bridging the gap at 6 weeks postoperative. In mice injected with LPS there was little new bone at the (D) proximal end at 1 week or at the (E) distal end at 2 weeks, and (F) most defects failed to bridge by 6 weeks postoperative. (G) Distal bone formation and (H) cortical bridging were decreased in LPS-treated mice (grey bars) compared with Control mice (white bars) at all times. Quantitative analyses of new bone were performed in the (I) medullary canal and in the (K) cortical gap and the results are presented as bar graphs. Bone volume per tissue volume (BV/TV) in the (J) medulla and (L) cortex of LPS-treated mice also was reduced at 1 and 2 weeks postoperative. By 6 weeks postoperative medullary bone was similar but cortical bone remained reduced in the LPS-treated mice compared with Control mice. Results are expressed as median with 25th to 75th quartiles and ranges. Significantly different from LPS-injected mice; * $p < 0.01$; ** $p < 0.001$; ROI = region of interest.

Results

Relationship of LPS-induced Systemic Inflammation to Bone Healing

The dose of LPS used to induce systemic inflammation during the first week of bone repair resulted in elevated circulating TNF- α , a reduction of bone in repair tissue, and impaired bridging of the defect by 6 weeks postoperative. Although mice in the LPS-treated group exhibited decreased activity and lethargy compared with the Control group for 1 to 2 days after surgery, none of the mice experienced serious complications and mortality was comparable (four per group). At 1 week postoperative, 2 hours after the last injection, serum TNF- α was 44 ± 23 pg/mL in Control mice injected with PBS and 254 ± 152 pg/mL in LPS-injected mice (95% CI, 93–326 pg/mL; $p < 0.001$). MicroCT scans of the mid-sagittal region of defects showed better healing of the defect in Control mice (Fig. 1A–C) compared with LPS-treated mice at the same times (Fig. 1D–F). Bone repair was initiated at the proximal pole by 1 week postoperative in Control mice but was not visible until 2 weeks postoperative in LPS-injected mice. By 6 weeks postoperative the gap was bridged (Fig. 1C) in the majority of Control mice whereas a gap remained in the majority of LPS-treated mice (Fig. 1F). Distal bone formation (Fig. 1G) was evident in one of

Table 1. Quantitative microCT analyses

Parameter	1 Week postoperative			2 Weeks postoperative			6 Weeks postoperative		
	Control (n = 7)	LPS (n = 8)	p Value (95% CI)	Control (n = 7)	LPS (n = 8)	p Value (95% CI)	Control (n = 9)	LPS (n = 8)	p Value (95% CI)
Trabecular number (mm)	1.2 ± 0.2	0.3 ± 0.2	< 0.001 (0.7–1.1)	4.0 ± 1.1	0.8 ± 0.5	< 0.001 (2.3–4.1)	3.6 ± 0.9	2.7 ± 0.7	< 0.165 (0.01–1.8)
Trabecular thickness (µm)	30 ± 3	27 ± 5	< 0.189 (–1.7 to 7.7)	36 ± 4	34 ± 5	< 0.779 (–3.1 to 7.1)	110 ± 22	81 ± 18	< 0.017 (6.4–51.6)
Trabecular separation (µm)	298 ± 23	352 ± 38	0.029 (18–90)	209 ± 49	332 ± 53	< 0.001 (272–386)	171 ± 29	240 ± 36	< 0.002 (32–106)
Connectivity density (µm ⁻³)	0.9 ± 0.3	0.2 ± 0.2	0.001 (0.4–1.0)	2.0 ± 0.4	0.4 ± 0.3	< 0.001 (1.2–2.0)	0.2 ± 0.2	0.4 ± 0.2	< 0.165 (–0.4 to 0.02)
Total porosity (%)	96 ± 1	99 ± 1	< 0.001 (1.9–4.1)	86 ± 4	97 ± 2	< 0.001 (7.6–14.5)	60 ± 10	79 ± 4	< 0.002 (11–27)
Number of closed pores	15 ± 9	3 ± 5	< 0.009 (4.0–20.0)	47 ± 12	17 ± 13	< 0.001 (16–44)	305 ± 237	140 ± 49	0.128 (–20 to 349)

LPS = lipopolysaccharide.

seven (14%) Control mice at 1 week postoperative, five of seven (71%) at 2 weeks, and eight of nine (89%) at 6 weeks postoperative, compared with zero of seven at 1 week, one of seven (13%) at 2 weeks (odds ratio [OR], 0.133; 95% CI, 0.014–1.318; $p = 0.020$), and three of eight (38%) at 6 weeks (OR, 0.075; 95% CI, 0.006–0.936; $p = 0.027$) in LPS-treated mice. Quantitative data for bridging of the gap (Fig. 1H) showed three of seven (43%) bones bridged at 2 weeks in the Control group and zero of eight in the LPS-treated group. By 6 weeks postoperative bridging had occurred in seven of nine (78%) mice in the Control group and one of eight (13%) in the LPS-treated group (OR, 0.04; 95% CI, 0.003–0.560; $p = 0.007$). There was more bone (BV/TV) in the femoral medulla (Fig. 1I, J) of Control mice compared with LPS-treated mice at 1 week ($6.1\% \pm 1.4\%$ vs $0.9\% \pm 0.3\%$; 95% CI, 4.1%–6.3%; $p < 0.001$) and 2 weeks ($17.2\% \pm 4.5\%$ vs $6.1\% \pm 2.7\%$; 95% CI, 6.8%–15.0%; $p < 0.001$) postoperative, but there was no difference at 6 weeks ($14.6\% \pm 6.8\%$ vs $15.2\% \pm 8.6\%$; 95% CI, –9.3% to 8.3%; $p = 1.000$) postoperative. In a similar manner BV/TV in the cortex (Fig 1K, L) was higher in Control than in LPS-injected mice at 1 week ($3.6\% \pm 0.6\%$ vs $0.8\% \pm 0.7\%$; 95% CI, 2.0%–3.5%; $p < 0.001$) and 2 weeks ($14.0\% \pm 3.5\%$ vs $2.9\% \pm 2.1\%$; 95% CI, 7.9%–14.3%; $p < 0.001$), which was maintained at 6 weeks ($39.0\% \pm 10.0\%$ vs $21.5\% \pm 4.2\%$; 95% CI, 9.7%–26.3%; $p = 0.002$) postoperative. Additional data describing the microarchitecture of bone in the window defect show that Control mice had more trabeculae (trabecular number) that were better connected to one another (connectivity density) with lower porosity and more osteocyte lacunae (closed pores) in initial woven bone occupying the window defect compared with mice treated with LPS at 1 and 2 weeks postoperative (Table 1). At 6 weeks postoperative trabeculae were thicker with fewer spaces in Control compared with LPS-treated bone (Table 1).

Vascularization, Cellularity, Anabolism, and Catabolism

LPS-induced systemic inflammation was associated with a reduction in the number of CD34-positive vascular endothelial cells and mast cells in repair tissue at 1 week postoperative, reduced ALP and TRAP activity, and an increase F4/80 staining of all macrophages relative to Arg-1 stained M2 macrophages. Sections of decalcified bone harvested at the mid-sagittal region of the femurs and stained with hematoxylin and eosin were used to evaluate the morphologic features of repair tissue (Fig. 2). At 1 week postoperative condensed connective tissue occupied the marrow cavity adjacent to the bone defect in Control (Fig. 2G) and LPS-treated (Fig. 2H) mice. At 2 weeks

Fig. 2A–R Representative images of 5- μ m sections from the mid-sagittal region of decalcified, paraffin embedded femurs were stained with hematoxylin and eosin and adjacent sections stained with acidified toluidine blue to identify purple granular mast cells. Low-magnification images of hematoxylin and eosin-stained sections at 1 week for (A) Control and (B) lipopolysaccharide (LPS)-treated mice, 2 weeks for (C) Control and (D) LPS-treated mice, and 6 weeks after surgery for (E) Control and (F) LPS-treated mice show the margins of the defect (arrowheads) and the region of tissue (box) where high-magnification images were taken. At 1 week postoperative (G) Control mice had newly formed bone (arrow) on the endosteal side, and dense, fibrous tissue (asterisk) on the periosteal side of the defect, while (H) LPS-treated mice had only condensed mesenchyme. At 2 weeks postoperative (I) Control femurs showed a clear pattern of

bone trabeculae (arrows) surrounded by marrow, and in the defect of (J) LPS-treated mice, disorganized islands of bone (arrows) in mesenchyme are seen. At 6 weeks postoperative the trabecular bone matures into woven bone with a normal cortical shape in the (K) Control group whereas in the (L) LPS-treated group there are large lacunae in the cortical bone and fibrous tissue remaining in the gap (asterisk in Illustration F). Mast cells (arrowheads) appear more numerous in (M) Control than in (N) LPS-treated bones at 1 week postoperative, whereas equivalent numbers were seen at 2 weeks after surgery in the (O) Control and (P) LPS-treated groups. By 6 weeks postoperative mast cell numbers and location were still similar in (Q) Control and (R) LPS-treated mice. Scale bars = 500 μ m (A–F), 50 μ m (G–L) and 20 μ m (M–R).

postoperative trabeculae of woven bone were seen in Control (Fig. 2I) femurs whereas islands of bone in condensed connective tissue were seen in LPS-treated bones (Fig. 2J). By 6 weeks postoperative the bone bridging the defect resembled that of the intact cortex in Control mice (Fig. 2K), whereas LPS-treated mice exhibited residual trabeculae in the medullary cavity and gaps filled with fibrous tissue (Fig. 2L). Mast cells, whose granules stain purple with acidified toluidine blue, were seen primarily in loose connective tissue at 1 week (Fig. 2M, N), around sinusoids and bordering new bone at 2 weeks (Fig. 2O, P),

and in marrow spaces at 6 weeks postoperative (Fig. 2Q, R) in both groups. Quantification of the purple granular cells revealed more mast cells in Control than in LPS-treated mice at 1 week postoperative, but with no differences between the groups thereafter (Table 2).

Quantitative data for histochemical (TRAP, ALP) and immunochemical (CD34, F4/80, Arg-1) staining at 1, 2, and 6 weeks postoperative showed differences between the Control and LPS-treated mice in all parameters at 1 week postoperative but in none of the parameters at 6 weeks postoperative (Table 2). More staining for TRAP (Fig. 3A,

Table 2. Quantitative analyses of cellular composition

Stain and target	1 Week postoperative			2 Weeks postoperative			6 Weeks postoperative		
	Control	LPS	p Value (95% CI)	Control	LPS	p Value (95% CI)	Control	LPS	p Value (95% CI)
	(n = 7)	(n = 8)		(n = 7)	(n = 8)		(n = 9)	(n = 8)	
Acid TB: mast cells/ mm ²	2.1 ± 1.0	0.5 ± 0.6	0.009 (0.6–2.5)	8.4 ± 5.2	9.8 ± 5.8	0.613 (7.6–4.8)	9.4 ± 9.2	10.0 ± 6.2	1.000 (10.9–17.8)
VEGF: angiogenesis	0.5 ± 0.3	0.5 ± 0.3	1.000 (–3.4 to 3.4)	1.5 ± 1.5	0.9 ± 0.8	0.818 (0.7–1.9)	1.7 ± 0.3	1.8 ± 0.8	1.000 (0.8–0.6)
CD34: vascular endothelium	5.0 ± 1.1	2.3 ± 0.9	0.001 (1.6–3.8)	3.4 ± 0.9	1.1 ± 0.6	< 0.001 (1.5–3.1)	0.5 ± 0.1	0.4 ± 0.2	0.286 (0.1–0.3)
F4/80: M1/M2 macrophage	7.4 ± 3.4	16.5 ± 4.6	< 0.001 (4.5–13.7)	6.8 ± 1.7	13.0 ± 2.6	< 0.001 (3.7–8.7)	2.5 ± 0.5	2.6 ± 1.1	0.413 (1.1–0.9)
Arg-1: M2 macrophage	1.2 ± 0.3	1.6 ± 0.5	0.072 (0.9–0.1)	0.6 ± 0.4	4.5 ± 3.2	< 0.001 (1.3–6.5)	0.7 ± 0.2	0.8 ± 0.3	0.413 (0.4–0.2)
Cathepsin K: collagen 1	0.07 ± 0.02	0.06 ± 0.02	0.121 (0.01–0.03)	0.9 ± 0.3	0.3 ± 0.2	< 0.001 (0.4–0.9)	0.1 ± 0.1	0.2 ± 0.1	0.730 (0.2–0.1)
TRAP: OC and fibrous marrow	1.6 ± 0.8	0.7 ± 0.2	0.004 (0.3–1.5)	7.2 ± 4.0	3.3 ± 4.7	0.014 (2.7–9.0)	0.6 ± 0.3	0.7 ± 0.7	0.413 (0.2–0.1)
TRAP: against new bone only	0.05 ± 0.03	1.3 ± 2.6	0.002 (0.9–3.4)	0.4 ± 0.3	0.8 ± 0.7	1.000 (1.0–0.2)	0.01 ± 0.01	0.04 ± 0.03	0.286 (0.1–0.5)
ALP: OB enzyme	24.7 ± 11.0	3.9 ± 3.4	< 0.001 (12.0–29.6)	6.1 ± 3.2	1.9 ± 0.9	0.025 (1.7–6.7)	0.9 ± 0.3	1.3 ± 0.5	0.190 (0.9–0.1)

Acid TB = acidified toluidine blue; VEGF = vascular endothelial growth factor; TRAP = tartrate resistant acid phosphatase; ALP = alkaline phosphatase; OC = osteoclast; OB = osteoblast.

B), ALP (Fig. 3C, D), and CD34 (Fig. 3E, F) was seen in Control compared with LPS-treated mice at 2 weeks postoperative, whereas F4/80 (Fig. 3G, H) and Arg-1 (Fig. 3I, J) staining were lower in Control mice. The absolute activity of TRAP positive catabolic cells was higher in the Control mice compared with the LPS-treated mice at 1 week ($1.6\% \pm 0.8\%$; 95% CI, 0.3%–1.5%; $p = 0.004$) and 2 weeks ($7.2\% \pm 4.0\%$ vs $3.3\% \pm 4.7\%$; 95% CI, 2.7%–9.0%; $p = 0.014$) postoperative. When normalized to new bone surfaces there was relatively higher activity in the LPS-treated mice at 1 week postoperative (1.3 ± 2.6 vs 0.05 ± 0.03 ; 95% CI, 0.9–3.4; $p = 0.002$) but with no differences thereafter (Table 2).

Discussion

Current approaches to the treatment of orthopaedic, maxillofacial, and dental injuries often are complicated by the limited regenerative capacity of bone, which results in delayed healing and malunions. Recent studies suggest that modulation of the immune system offers a noninvasive option to enhance bone repair [42, 44, 48]. Immune cells and inflammatory cytokines have been found to have a major influence on the success of cell transplantation-based

therapies like tissue-engineered scaffolds seeded with MSCs [44]. Despite progress in the field of osteoimmunology in general, the interactions between cells of the immune system and those involved in bone repair remain underexplored. Our study builds on our previous work that investigated the effect of mast-cell deficiency in KitW-sh mice on the inflammatory phase of bone repair using a mouse model of a mechanically stable femoral defect [6]. The current study used the same preclinical model to investigate the effect of systemic inflammation, evidenced by elevated circulating TNF α , on bone repair. The data indicate that LPS-induced systemic inflammation reduced the amount (BV/TV) and impaired the quality (trabecular separation and porosity) of bone regenerated at 6 weeks postoperative in skeletally mature mice. At 2 weeks postoperative the effects were associated with reduced expression of CD34 (marker for vascular endothelial cells), reduced ALP (osteoblasts) and TRAP (osteoclasts), and increased expression of F4/80 and arginase-1, which was indicative of a relative increase in catabolic macrophages.

We acknowledge several limitations to our study. First, the results are not directly applicable to humans as a murine model was used. However, the immune response to LPS-induced systemic inflammation in mice has been well characterized in rodents [1, 12, 23, 27, 49], and shown to be

Fig. 3A–J Mid-sagittal sections were harvested from decalcified, paraffin-embedded femurs of Control and lipopolysaccharide (LPS)-treated mice euthanized at 2 weeks postoperative and adjacent sections stained for quantitative analyses of cell composition. Tartrate resistant acid phosphatase (TRAP)-positive osteoclasts (red) adjacent to bone spicules (green) were more numerous in bones harvested from (A) Control compared with (B) LPS-treated mouse femurs. Alkaline phosphatase (ALP)-positive osteoblasts also were more numerous in (C) Control compared with (D) LPS-treated mouse femurs. (E) Newly

formed blood vessels (asterisk) lined with CD34-positive vascular endothelial cells were more numerous in Control femurs than in those from (F) LPS-treated mice. The total number of F4/80-positive macrophages was lower in (G) Control bone compared with that from (H) LPS-treated mice. A similar pattern was seen with arginase-1 positive M2 macrophages, which were fewer in (I) Control femurs than in femurs from (J) LPS-treated mice. Scale bar represents 50 μ m for all images.

comparable to that seen in humans [8]. LPS introduced into the intraperitoneal cavity enters the systemic circulation via the portal vein and activates the immune system [5], which was confirmed by monitoring serum TNF α in our study. This mimics the clinical situation in cases of severe trauma when the gut barrier is impaired and gram negative bacteria containing LPS enter the blood stream [11, 41]. Second, we focused mainly on the cells that have been shown to play an important role in the inflammatory, regenerative, and remodeling phases (1–6 weeks postoperative) of the repair process. Bone repair in femoral defects proceeds from proximal (3–7 days postinjury) to distal (7–14 days postinjury). The delay in initiation of repair at the proximal and distal ends of the defect in LPS-treated mice was most likely a consequence of impaired revascularization, particularly at the distal pole which is less well vascularized in the first place [7]. Additional experiments analyzing the cells and their interactions during the first few days of healing are warranted but were beyond the scope of this work. Third, although histologic and immunohistochemical analyses provide relevant information regarding the quantity and location of cells and enzymes, additional methods such as fluorescence-activated cell sorting will be required to confirm the results. Fourth, coculture experiments with the cells involved in a controlled ex vivo environment will be required to uncover the molecular pathways responsible for the abnormalities.

In a similar study performed in rats, femoral fractures were fixed with an intrafemoral nail, which provides limited stability when used in clinical practice, and resulted in secondary bone healing [40]. Administration of LPS for 7 days after surgery during the inflammatory stage resulted in impaired healing of the fractures at 6 weeks postoperative, as indicated by reductions in bone mineral content and density and decreased biomechanical strength at the fracture site. Although the animal model (rat vs mouse), mechanism of bone repair (secondary vs primary), and outcome measures (bone mineral density and biomechanical strength vs quantitative microCT and histopathology) in that study differed from our study, the conclusion was the same. LPS-induced systemic inflammation resulted in decreased quantity and impaired quality of regenerated bone. These observations emphasize not only the long-term deleterious effect of systemic inflammation during the early phase of bone repair but also the importance of defining the cellular microenvironment of healing bone.

To further explore the cellular mechanisms underlying impaired bone healing in the LPS-treated mice, we performed histochemical and immunochemical analyses to identify cells involved in the processes of revascularization (CD34), removal of debris (F4/80), repair (ALP), and remodeling (TRAP). CD34, ALP, and TRAP activity were reduced and F4/80 activity increased in the LPS-treated mice at 2 weeks postoperative, but no difference was seen

in these parameters between the Control and LPS-treated mice at 6 weeks postoperative. This could reflect the limited duration of the LPS treatment to the first week of healing. Importantly, it indicates that early disruption of cellular activity in the bone-healing microenvironment will have a lasting, deleterious effect on the repair process. Osteoclasts are a major constituent of the bone-repair process that share a common circulating precursor with monocytes and macrophages [26]. In chronic, systemic inflammatory conditions like rheumatoid arthritis, elevated levels of proinflammatory mediators increase osteoclast activity and promote bone loss [39]. Although the absolute activity of osteoclasts was lower in the LPS-treated mice compared with the Control mice, their relative activity when compared with the surface of new bone was increased. The relative TRAP activity is the more important value in this case [30], as osteoclasts are found only adjacent to new bone and there was less new bone in the LPS-treated mice. ALP is a recognized marker for osteoblasts and other cell types in the bone microenvironment including hypertrophic chondrocytes, MSCs, and vascular pericytes [17, 20]. The reduction in ALP at 1 and 2 weeks postoperative in LPS-treated mice was most likely responsible for the reduction in bone volume observed by microCT analysis. A plausible explanation would be the presence of LPS-induced systemic inflammation and elevated circulating TNF- α , which has been reported to inhibit the differentiation of MSCs in bone-forming osteoblasts by suppressing *Cbfa1* [19]. Moreover, resolution of the inflammatory phase of bone healing is proposed to be necessary for effective osteoblast function [33]. The concurrent expression of peak levels of ALP-positive osteogenic and CD34 vascular endothelial cells at 1 week postoperative in the condensing mesenchyme at the site of repair supports the hypothesis that there is crosstalk between the lineages destined to become bone and vascular tissue [4].

In fractures, revascularization of the injured tissue is controlled mainly by VEGF [21, 24, 31]. Using a murine model of fracture repair and secondary bone healing it was shown that systemic administration of the antiinflammatory indomethacin enhanced revascularization of the fracture callus at 7 days postoperative, but bone healing was impaired at 28 days postoperative [31]. Others used an ovine femoral osteotomy model in which mechanical instability led to an increase in inflammatory cells in and adjacent to the defect, accompanied by a reduction in angiogenic factors, including VEGF [43]. In the current study using a mechanically stable defect in the mouse femur, a reduction in vascularization (CD34 immunoreactivity) was not accompanied by any change in VEGF immunoreactivity in LPS-treated mice at 7 and 14 days postoperative. The different species and/or models of bone

repair in previous work might explain the differences in VEGF expression. Alternatively, angiopoietin, a protein expressed during the early phase of bone healing, could have been altered in the LPS-induced mice to result in a reduction in revascularization [28].

Effective revascularization and bone repair also depend on a physiologic balance between M1 and M2 macrophages [2, 47]. The F4/80 antibody recognizes inflammatory M1 and proangiogenic, tissue repair-inducing M2 subtypes whereas the Arg-1 antibody recognizes only M2 macrophages. F4/80 immunoreactivity was increased in LPS-treated mice at 7 and 14 days postoperative while Arg-1 immunoreactivity did not become elevated until 14 days postoperative. LPS induces M1 macrophage differentiation and activity but has little effect on M2 macrophages [32], which indicated increased F4/80 immunoreactivity at 7 days postoperative was indicative of a relative increase in M1 over M2 macrophages in LPS-treated mice. This relative increase in M1 over M2 macrophages during the first week of healing could have had a negative effect on bone repair at 6 weeks postoperative. The increase in Arg-1 immunoreactivity in LPS-treated mice at 14 days postoperative localized almost exclusively to the fibrous tissue in the window defect, which was much more abundant in these animals compared with the control animals and most likely reflected the profibrotic effect of the M2a macrophage subset [3, 34–36]. Similar macrophage-induced increases in fibrous tissue formation leading to defective healing of skin wounds have been associated with LPS-induced systemic inflammation [37]. In addition to macrophages, mast cells have been implicated in the immune response to trauma in a mouse model of acute systemic inflammation [9]. The systemic inflammatory response and end organ (liver, lung) damage 6 hours after being subjected to hemorrhagic shock and femoral fracture were less severe in mast cell-deficient *KitW-sh* mice compared with wild type mice. Cai et al. [9] concluded from their data that mast cells play a key role in the systemic inflammatory response. In previous work we characterized healing of the femoral bone defect used in the current study in mast cell-deficient *KitW-sh* mice [6]. In both studies we showed impaired revascularization using CD34 immunoreactivity at 7 and 14 days postoperative. In contrast, F4/80 immunoreactivity was decreased in the previous study and increased in the current study. The results imply that mast cells and macrophages interact with one another during the inflammatory phase of bone repair.

The current *in vivo* mouse study shows that transient LPS-induced systemic inflammation compromised revascularization and osseous repair resulting in defective healing of a cortical window defect. Short-term alterations in the activity of vascular endothelial cells, macrophages, osteoclasts, and osteoblasts during the first 2 weeks of

healing had long-term deleterious effects on bone repair evaluated at 6 weeks postoperative. Based on our study, future clinical investigations might evaluate local administration of VEGF into the fracture callus to stimulate revascularization, or transplantation of stem cells to enhance bone turnover [44]. Our results also emphasize the potential for immunotherapy aimed at shifting the balance to favor M2 over M1 macrophages during the early inflammatory phase as a potential therapeutic intervention to improve bone healing. Definition of the molecular pathways whereby cells in the bone healing microenvironment interact with one another during the repair process awaits future studies focused on bone marrow-derived cells in a controlled ex vivo environment.

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