Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration

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Tissue injury and the healing response lead to the release of endogenous danger signals including Toll-like receptor (TLR) and interleukin-1 receptor, type 1 (IL-1R1) ligands, which modulate the immune microenvironment. Because TLRs and IL-1R1 have been shown to influence the repair process of various tissues, we explored their role during bone regeneration, seeking to design regenerative strategies integrating a control of their signalling. Here we show that IL-1R1/MyD88 signalling negatively regulates bone regeneration, in the mouse. Furthermore, IL-1β which is released at the bone injury site, inhibits the regenerative capacities of mesenchymal stem cells (MSCs). Mechanistically, IL-1R1/MyD88 signalling impairs MSC proliferation, migration and differentiation by inhibiting the Akt/GSK-3β/β-catenin pathway. Lastly, as a proof of concept, we engineer a MSC delivery system integrating inhibitors of IL-1R1/MyD88 signalling. Using this strategy, we considerably improve MSC-based bone regeneration in the mouse, demonstrating that this approach may be useful in regenerative medicine applications.
Although, the advancement of regenerative medicine will play a vital role in meeting the future healthcare challenges, the promises of regenerative therapies remain largely unrealized. For designing effective regenerative medicine strategies, we should better understand the interactions between the multiple actors that shape a regenerative environment. In particular, tissue injury is generally associated with an immune response, which is most likely a key regulator of the healing process. Hence, in-depth understanding of the role of the immune system during tissue repair and regeneration could provide clues to therapeutic avenues for restoring damaged tissues, and controlling the immune regulations of tissue healing may become an attractive option in regenerative medicine.

Unlike most tissues, bone possesses an innate capacity to regenerate following injury. The majority of bony injuries, when properly treated by re-apposition, heal without a permanent lesion. However, many clinical indications remain that require therapeutic intervention to augment bone regeneration such as large craniomaxillofacial defects, bone degeneration in patients with osteonecrosis, distal tibial fractures and periodontal disease. Autologous bone grafting is currently the gold standard, but this approach is associated with numerous drawbacks, including donor-site morbidity, the availability of limited grafting material and compromised bone quality in patients with osteoporosis. Therefore, extensive efforts have been made to develop bone regenerative strategies using various combinations of cells, growth factors and biomaterials. However, only few of these strategies have translated into clinical practice and none of them have become a standard in regenerative medicine. Efficacy, safety, practical, cost-effectiveness and regulatory issues often prevent the widespread therapeutic use of bone regenerative therapies. In addition, one of the major challenges lies in the limited understanding of the cellular and molecular mechanisms that should be targeted to promote bone regeneration. Especially, understanding and subsequently controlling the immune regulations of bone regeneration could be crucial to improve the effectiveness of bone regenerative therapies.

Commonly, tissue injury and the healing response lead to the release of various endogenous danger signals including Toll-like receptor (TLR) and interleukin-1 receptor, type 1 (IL-1R1) ligands, which modulate the immune microenvironment. These danger signals are involved in the recruitment and the activation of immune cells engaged in host defence. In addition, TLRs and IL-1R1 have been shown to influence the repair process of several tissues. For example, the injury promoting effects of TLR4 are apparent in many organs, as seen by the protection of TLR4-mutant or -deficient mice after hepatic, renal, cardiac and cerebral ischemia reperfusion. Similarly, IL-1R1 signalling critically regulates infarct healing and disruption of IL-1 signalling can improve the quality of wound healing.

In this study, we explore the role of TLRs and IL-1R1 during bone regeneration, seeking to design regenerative strategies integrating a control of their signalling. We show that IL-1R1 signalling via the adaptor protein MyD88 negatively regulates bone regeneration, in the mouse. IL-1β is released at the bone injury site and inhibits the regenerative capacities of mesenchymal stem cells (MSCs). Mechanistically, IL-1R1/MyD88 signalling impairs MSC migration, proliferation and differentiation into osteoblasts, by inhibiting the Akt/GSK-3β/catenin pathway. Furthermore, we propose a MSC delivery system integrating inhibitors of IL-1R1/MyD88 signalling. Using this approach, we significantly improve MSC-based bone regeneration in a mouse critical size calvarial defect model, demonstrating that this approach may be useful in regenerative medicine applications.

**Results**

**IL-1R1/MyD88 signalling negatively regulates bone regeneration.** To evaluate the role of TLRs and IL-1R1 during bone regeneration, we first analysed regeneration in mice deficient for MyD88 and TRIF, which are key adaptor proteins involved in TLR and IL-1R1 signalling transduction. Among the available orthotopic models used for evaluating bone regeneration, we chose the critical size calvarial defect, because this model has gained a widespread reputation and use in the published literature, reporting valuable data from basic and applied research. Moreover, the critical size calvarial defect model is very reliable and displays low variability, making it ideal to compare bone regeneration in multiple knockout mice. Critical size calvarial bone defects (5 mm diameter) were created and filled with a standard fibrin matrix. The fibrin matrix was used to ensure clot formation in the defect and to standardize the model, while the procedure did not significantly influence bone regeneration (Supplementary Fig. 1). Remarkably, 4 weeks post surgery, Myd88−/− mice displayed a faster regeneration characterized by a better coverage of the defect with mineralized bone compared to wt mice, suggesting that TLR1, TLR2, TLR4-9, and/or IL-1R1 influence bone regeneration, since their signalling depends on MyD88 (ref. 12). When bone regeneration was assessed in mice lacking MyD88 signaling (Supplementary Fig. 2). While very few Myd88−/− bone marrow MSCs could have been transferred in wt mice (0.001–0.01% of injected cells), Myd88−/− MSCs most likely did not populate the bone marrow of irradiated wt mice, since the vast majority of MSCs does not go beyond lung and liver following intravenous injection. Interestingly, Myd88−/− chimeric mice did not display better healing (Fig. 1e,f), suggesting that IL-1R1/MyD88 signalling negatively regulates bone regeneration by acting on tissue-resident cells. Since osteoclasts derive from bone marrow monocytes, they were lacking MyD88 in Myd88−/− chimeric mice. Therefore, we hypothesized that IL-1R1/MyD88 signalling negatively regulates bone regeneration by acting on osteoblasts and/or skeletal stem/progenitor cells including MSCs.

To test whether IL-1R1/MyD88 signalling influences MSC-driven bone regeneration, we delivered syngeneic MSCs derived from bone of wt, Myd88−/− or Il1r1−/− mice into calvarial defects of wt mice, and we analysed regeneration after 4 weeks. MSCs from five isolates were pulled together and expanded for 12). When bone regeneration was assessed in mice lacking MyD88 signaling (Supplementary Fig. 2). While very few Myd88−/− bone marrow MSCs could have been transferred in wt mice (0.001–0.01% of injected cells), Myd88−/− MSCs most likely did not populate the bone marrow of irradiated wt mice, since the vast majority of MSCs does not go beyond lung and liver following intravenous injection. Interestingly, Myd88−/− chimeric mice did not display better healing (Fig. 1e,f), suggesting that IL-1R1/MyD88 signalling negatively regulates bone regeneration by acting on tissue-resident cells. Since osteoclasts derive from bone marrow monocytes, they were lacking MyD88 in Myd88−/− chimeric mice. Therefore, we hypothesized that IL-1R1/MyD88 signalling negatively regulates bone regeneration by acting on osteoblasts and/or skeletal stem/progenitor cells including MSCs.

To test whether IL-1R1/MyD88 signalling influences MSC-driven bone regeneration, we delivered syngeneic MSCs derived from bone of wt, Myd88−/− or Il1r1−/− mice into calvarial defects of wt mice, and we analysed regeneration after 4 weeks. MSCs from five isolates were pulled together and expanded for 3 passages (Supplementary Fig. 3), before being delivered in bone defects through a fibrin matrix. Delivering wt MSCs resulted into a very slight improvement of regeneration compared with treatment without cells. In contrast, delivering Myd88−/− or Il1r1−/− MSCs induced significantly better regeneration (Fig. 1g,h). To check if the regeneration stimulated by Myd88−/− and Il1r1−/− MSCs relied on a better intrinsic capacity of these cells to differentiate towards osteoblasts or to proliferate, we compared them with wt MSCs in vitro. Without IL-1R1 ligands added to the cell culture medium, Myd88−/−, Il1r1−/− and wt MSCs displayed better healing (Fig. 1c,d), indicating that IL-1R1/MyD88 signalling negatively regulates bone regeneration.
MSCs showed equal ability to differentiate and to proliferate (Supplementary Fig. 4). Therefore, we hypothesized that the better regeneration induced by Myd88<sup>−/−</sup> and Il1r1<sup>−/−</sup> mice was due to their inability to sense IL-1R1 ligands present at the injury site.

**IL-1β signalling via IL-1R1/MyD88 inhibits MSC functions.** Using a cytokine array, we profiled the cytokines present in the bone defect microenvironment during the first 2 weeks following injury (Supplementary Fig. 5). Importantly, among IL-1R1 ligands, IL-1β as well as IL-1 receptor antagonist (IL-1Ra) were present at relatively high concentration within the first 10 days (Fig. 2a,b). After 10 days, IL-1β concentration starts to decline, while IL-1Ra concentration drastically increases afterwards, suggesting that the IL-1R1/MyD88 signalling occurs for at least 1 week post injury. Moreover, to gain insights about the cell types...
releasing IL-1β, we quantified the concentration of the cytokine in calvarial defects of mice where monocytes/macrophages were depleted by clodronate liposomes34, since these cells are known to release a large amount of IL-1β (ref. 26). In mice depleted of monocytes/macrophages (Supplementary Fig. 6), the concentration of IL-1β was about twice lower during the first 6 days following injury (Fig. 2c), demonstrating that monocytes/macrophages significantly contribute to the presence of IL-1β.

Next, we explored the impact of IL-1R1/MyD88 signalling on the regenerative capacities of MSCs. We focused on colony formation, proliferation, migration, differentiation and trophic function (for example, secretion of growth factors), considering that these functions are critical for the regenerative potential of MSCs. When primary (uncultured) bone-derived MSCs were seeded in the presence of IL-1β (1 ng ml⁻¹), fibroblast colony-forming units (c.f.u.-F) and the average size of colonies were significantly lower (Fig. 3a,b; Supplementary Fig. 7a–c). In addition, IL-1β (1 ng ml⁻¹) drastically inhibited the differentiation of the colonies towards osteoblasts (Fig. 3c; Supplementary Fig. 7d). Then, we analysed the effect of IL-1β on proliferation, migration, differentiation and trophic function, of bone-derived MSCs that have been expanded for three passages. Secretion of trophic factors from MSCs was not affected by IL-1β (Supplementary Fig. 8). Yet, IL-1β considerably inhibited MSC proliferation, when cells were co-stimulated with serum or with platelet-derived growth factor-BB (PDGF-BB), which is potent proliferation and migration factor for MSCs (Fig. 3d; Supplementary Fig. 9a). Similarly, transwell migration of MSCs towards serum or PDGF-BB was inhibited by IL-1β (Fig. 3e; Supplementary Fig. 9b). Regarding MSC differentiation, a single stimulation with a low concentration of IL-1β (1 ng ml⁻¹) severely inhibited their differentiation into osteoblasts (Fig. 3f,g; Supplementary Fig. 9c) and into chondrocytes (Supplementary Fig. 10). In addition, proliferation, migration and osteoblastic differentiation of MSCs lacking IL-1R1 and/or MyD88 were not affected by IL-1β (Supplementary Fig. 11), confirming that the IL-1R1/MyD88 signalling pathway is responsible for the inhibitory effects of the cytokine. Notably, IL-1β inhibited colony formation, proliferation, migration and osteoblastic differentiation of bone marrow-derived MSCs (Supplementary Fig. 12).

**Figure 3 | IL-1β inhibits c.f.u.-F proliferation migration and osteoblastic differentiation of MSCs.** (a,b) Primary MSCs were seeded with or without IL-1β (1 ng ml⁻¹) during 6 days. Graphs show c.f.u.-F and average size of colonies. Data are means ± s.e.m. (n = 6 independent isolates). Representative wells (9 cm²) are shown in b. (c) Primary MSCs were seeded in osteogenesis induction medium (OIM) for 28 days with or without IL-1β (1 ng ml⁻¹) during 6 days). After 28 days, matrix mineralization was revealed with alizarin red staining. Representative wells are shown (2 cm²). (d) MSC proliferation was stimulated with 10% serum or PDGF-BB (5 ng ml⁻¹), in the presence of IL-1β at increasing concentration. After 72 h, cell number increase was measured. (e) Migration of MSCs through a transwell was induced by 10% serum or by PDGF-BB (5 ng ml⁻¹), in the presence of IL-1β (1 ng ml⁻¹) during 6 h. After 6 h, the number of cells per square millimetre that passed through the transwell was counted. (f) MSCs in OIM were treated once with IL-1β (1 ng ml⁻¹) during 4 days. After 7 and 14 days, expression of osteoblast-specific genes was determined by quantitative PCR. Fold changes in gene expression relative to MSCs cultured in normal medium are shown. Alp, alkaline phosphatase; Runx2, runt-related transcription factor 2; Ibcp, integrin-binding sialoprotein. For d–f, data are means ± s.e.m. (n ≥3). **P < 0.01, ***P < 0.001; Student’s t-test. (g) MSCs in OIM were treated once with IL-1β (1 ng ml⁻¹) during 4 days. After 28 days, matrix mineralization was revealed with alizarin red staining. Representative wells (2 cm²) are shown.
(OIM) were treated once with IL-1β, in the presence of 5 ng ml⁻¹ of IL-1β. After 72 h, the cell number increase was measured. Data are means ± s.e.m. (n = 3).

(b) Osteoblast migration through a transwell was induced by 10% serum or PDGF-BB (5 ng ml⁻¹), in the presence of IL-1β (1 ng ml⁻¹) or wt MSCs. After 6 h, the number of cells per square millimetre that passed through the transwell was measured. Data are means ± s.e.m. (n = 3). (c) Osteoblasts in osteogenesis induction medium (OIM) were treated once with IL-1β (1 ng ml⁻¹ during 4 days).

Figure 4 | IL-1β slightly inhibits proliferation migration and differentiation of osteoblasts. (a) Osteoblast proliferation was stimulated with 10% serum or PDGF-BB (10 ng ml⁻¹), in the presence of 5 ng ml⁻¹ of IL-1β. After 72 h, the cell number increase was measured. Data are means ± s.e.m. (n = 3).

(b) Osteoblast migration through a transwell was induced by 10% serum or PDGF-BB (5 ng ml⁻¹), in the presence of IL-1β (1 ng ml⁻¹). After 6 h, the number of cells per square millimetre that passed through the transwell was measured. Data are means ± s.e.m. (n = 3). (c) Osteoblasts in osteogenesis induction medium (OIM) were treated once with IL-1β (1 ng ml⁻¹ during 4 days). (d) After 28 days, matrix mineralization was revealed with alizarin red staining. Representative wells are shown. For a-c, data are means ± s.e.m. (n≥3). *P<0.05, **P<0.01; Student’s t-test.

IL-1β impairs the Akt/GSK-3β/β-catenin pathway in MSCs. To gain insights on the molecular mechanism by which IL-1R1/MyD88 signalling inhibits MSC proliferation and migration, we used an antibody array to analyse the phosphorylation state of key intercellular signalling molecules following co-stimulation with IL-1β and PDGF-BB (Supplementary Fig. 15). Among the proteins tested, the strongest phosphorylation signal induced by PDGF-BB was for the serine/threonine kinase Akt. In contrast, Akt phosphorylation at Thr308 and Ser473 considerably decreased over time, when cells were co-stimulated with IL-1β (Fig. 6a), indicating that IL-1R1/MyD88 signalling inhibits the Akt pathway. Then, since Wnt/β-catenin signalling is critical for the differentiation of MSCs into osteoblasts, we investigated if IL-1R1/MyD88 signalling disturbs this pathway, by analysing the phosphorylation state of β-catenin following stimulation with IL-1β. IL-1β induced β-catenin phosphorylation, indicating that β-catenin was undergoing degradation. Confirming β-catenin degradation, the total concentration of β-catenin decreased gradually over time (Fig. 6b). In addition, we examined whether β-catenin degradation induced by IL-1R1/MyD88 signalling is linked to a decrease of Akt activity. We focused on GSK-3β, which is a kinase within the β-catenin destruction complex, because Akt inactivates GSK-3β by phosphorylation of Ser9 (ref. 38). In MSCs that were stimulated with IL-1β, the concentration of phosphorylated GSK-3β gradually decreased over time (Fig. 6c), indicating that IL-1R1/MyD88 signalling inhibits the Akt/GSK-3β/β-catenin singling pathway.

To verify that Akt activity and β-catenin directly contribute to MSC proliferation, migration, differentiation and MSC-driven bone regeneration, we used specific inhibitors for Akt and β-catenin, respectively, MK-2206 and XAV-939. MK-2206 directly inhibits Akt phosphorylation at Ser473 and Thr308, while XAV-939 promotes β-catenin degradation through stabilization of axin39. Firstly, both inhibitors were confirmed to be effective in MSCs (Supplementary Fig. 16). Then, we found that Akt
inhibition by MK-2206 significantly inhibits proliferation, differentiation and migration of MSCs (Fig. 6d–f). Moreover, similarly to the effect IL-1β, MK-2206 induced the dephosphorylation of GSK-3β (Fig. 6c). Promotion of β-catenin degradation by XAV-939 had no significant effect on MSC proliferation (Fig. 6d) and migration (Fig. 6e), but significantly reduced MSC differentiation into osteoblasts (Fig. 6f). Lastly, we verified that Akt and β-catenin pathways are important for bone regeneration stimulated by MSC delivery. As a model, we treated calvarial defects with Il1r1/Mdyd88−/− MSCs co-delivered with MK-2206 or XAV-939 and we analysed regeneration after 4 weeks. Since Il1r1/Mdyd88−/− MSCs are not responsive to IL-1, their Akt activity and β-catenin level were affected by the inhibitors, but not by IL-1 present in the defect microenvironment. The regeneration induced by Il1r1/Mdyd88−/− MSCs co-delivered with inhibitors was significantly lower compared with regeneration-induced Il1r1/Mdyd88−/− MSCs delivered alone (Fig. 6g,h), showing that both Akt and β-catenin pathways are critical for MSC-driven bone regeneration.

Inhibition of IL-1R1/MyD88 signalling promotes regeneration.

Since we found that IL-1R1/MyD88 signalling represses the regenerative capacities of MSCs, we thought to inhibit this pathway to create a pro-regenerative niche supporting endogenous and transplanted stem cells. As a proof of concept, we engineered a cell delivery system based on fibrin matrix functionalization with IL-1R1/MyD88 signalling inhibitors. We chose two different strategies: (i) delivering the natural inhibitor of IL-1R1, IL-1Ra; (ii) delivering a MyD88 inhibitory peptide, RDVLPGTCVNS (MyD88-I)40, which inhibits MyD88 homodimerization. To deliver MyD88-I accurately, MyD88-I was engineered to be covalently crosslinked into fibrin matrix and to translocate into cells. A fibrin-binding sequence derived from antennapedia/N terminus of the peptide, followed by a plasmin/matrix metalloproteinase-sensitive sequence 42 (VPMSMRGG) and a fibrin-binding sequence derived from plasmin inhibitor41 (2-PI1-8, NQEQVSPL) was added at the N terminus of the peptide, followed by a plasmin/matrix metalloproteinase-sensitive sequence 42 (VPMSMRGG) and a membrane translocation sequence derived from antennapedia/homeobox protein43 (RIQIKWFQNRMKWKK). Therefore, the peptide is covalently crosslinked into fibrin, during the natural polymerization process of the matrix41. Then, following matrix remodelling, the peptide is released by proteases, translocates into cells and ultimately inhibits IL-1R1 signalling (Fig. 7a). First, we confirmed that the engineered peptide could inhibit IL-1R1/MyD88 signalling, as shown by its ability to significantly impair the release of cytokines from MSCs following stimulation with IL-1β (Supplementary Fig. 17). Next, we used the critical calvarial defect model again for testing the cell delivery system, since this versatile model allows for evaluation of biomaterials and bone tissue engineering approaches within a reproducible orthotopic site54,55. Bone defect were treated with or without MSCs co-delivered with IL-1Ra (1 µg) or z2P1,a,MyD88-I (4 µg). Analysis of bone regeneration after 2 month revealed that delivering IL-1Ra or z2P1,a,MyD88-I alone improves bone regeneration compared with treatment with fibrin only (Fig. 7b,c). Strikingly, treatment with MSCs co-delivered with IL-1Ra or z2P1,a,MyD88-I led to a marked increase of bone tissue deposition as compared with treatment with MSCs only, with no signs of bone overgrowth and yielding coverage at 78% and 93%, respectively (Fig. 7b,c).

Discussion

Tissue injury and the following healing process are almost always accompanied with an immune response. Therefore,
understanding the immune regulations of tissue repair and regeneration could be fundamental for designing effective regenerative therapies. Particularly, the regenerative capacities of endogenous and transplanted stem cells could rely on the inflammatory/immune microenvironment at the injury site. For example, it has been shown that recipient T cells negatively regulate bone formation by autologous MSCs, in the mouse. Moreover, MSC-mediated bone regeneration in...
the rat has been enhanced, by inhibiting of NF-κB, which is a major transcription factor regulating both the innate and adaptive immune response. As another example, we recently found that self-RNA released after radiation-induced injury promote crypt stem cells death through TLR3 signalling. Since tissue injury and the healing response usually lead to the release of endogenous danger signals such as TLR and IL-1R1 ligands, and because TLRs and IL-1R1 have been shown to influence the innate immune response. As another example, we recently found that self-RNA released after radiation-induced injury promote crypt stem cells death through TLR3 signalling.

While some TLRs have been shown to have a role in the repair of certain organs including brain, heart, liver, kidney, and skin, all TLR knockout mice that we tested displayed normal bone regeneration in specific pathogen-free conditions. In contrast, MyD88- and IL-1R1-deficient mice exhibited better bone regeneration, indicating that IL-1R1/MyD88 signalling negatively regulates the regenerative capacity of MSCs. Since IL-1R1/MyD88 signalling is well known to modulate the activity of immune cells, we first suspected that this signalling was inhibiting bone regeneration by acting on the immune cells mobilized after injury. However, using the chimeric mouse model where bone marrow-derived immune cells and osteoclasts are deficient for MyD88, we found that IL-1R1/MyD88 signalling inhibits bone regeneration by acting on tissue-resident cells except osteoclasts. An ideal way to determine if IL-1R1/MyD88 signalling negatively regulated the regenerative capacity of MSCs in vivo would have been to analyse bone regeneration in a mouse having only these cells deficient for IL-1R1 or MyD88. Unfortunately, due to the lack of very specific markers for MSCs, there are currently no accurate methods to create such conditional knockout mouse. Therefore, as a model to explore whether IL-1R1/MyD88 signalling inhibits MSC-driven bone regeneration, we compared bone healing stimulated by the local delivery of bone-derived MSCs from wt, Il1r1−/− or Myd88−/−. We found that a much better regeneration is induced by Il1r1−/− or Myd88−/− MSCs, indicating that IL-1R1/MyD88 signalling inhibits bone regeneration induced by MSCs.
contribute to the presence of IL-1β in the defect microenvironment. In addition, in the case of transplanted MSCs, the stem cells could also release IL-1β. Importantly, we found that IL-1β significantly impairs colony formation of freshly isolated (primary) MSCs, as well as their differentiation towards osteoblasts. Although MSCs phenotype and properties can change during in vitro expansion, a strong inhibitory effect of IL-1β was also observed in cultured (expanded) MSCs, indicating that IL-1β affects both primary and in vitro-expanded MSCs. Notably, while primary bone-derived MSCs and primary bone marrow-derived MSCs were reported to differ in stem cells antigen-1 and nestin expression, c-Jun-N-terminal kinase and osteoblastic differentiation potential38,49, the inhibitory effects of IL-1R1/MyD88 signalling on bone-derived MSC and bone marrow-derived MSC were the same. IL-1β also inhibited proliferation, migration and differentiation of osteoblasts, but the inhibitory effects of IL-1β on osteoblasts were less strong, compared with MSCs, suggesting that IL-1R1/MyD88 signalling most likely inhibits bone regeneration by acting principally on stem/progenitor cells. Tracing the fate of transplanted wt, Myd88−/− and Il1r1−/−MSCs, we could demonstrate that both proliferation and osteoblastic differentiation of MSCs delivered in bone defect are impaired by IL-1R1/MyD88 signalling. Moreover, when we examined the mobilization of stem/progenitor cells following bone injury, Myd88−/− and Il1r1−/− mice—having no cells responsive to IL-1β—mobilized a higher percentage of cells having typical MSC markers. The cell population detected within the defects most likely contained MSCs, while other cell types that also express MSC markers such as pericytes50 could have been detected. Still, IL-1R1/MyD88 signalling probably inhibits the mobilization of MSCs in the defect, since IL-1β strongly inhibits MSC migration in vitro, and because Il1r1−/− and Myd88−/− mice recruit more MSC-like cells after bone injury.

Exploring the signalling mechanism by which IL-1β inhibits MSC regenerative functions, we first found that IL-1β promotes the dephosphorylation of Akt. Therefore, since Akt is a central node in cell signalling downstream many growth factor receptors such as PDGF and fibroblast growth factor receptors38,39, node in cell signalling downstream many growth factor receptors the dephosphorylation of Akt. Therefore, since Akt is a central node in cell signalling downstream many growth factor receptors, we first found that IL-1R1/MyD88 signalling inhibits Akt by interfering with Akt phosphorylation and dephosphorylation. Interestingly, Akt can also be activated by growth factors, thus leading to Akt activation

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Methods

Animals: All animals were kept under specific pathogen-free conditions. WT C57BL/6 mice were purchased from SLAC Japan. Myd88b−/−, Il1r1−/− (ref. 59), Traf3−/− (ref. 60), Tlr5−/− (ref. 62), Tlr7−/− (ref. 63), Tlr2/4/9−/− (refs 64–66) and Il1r1−/− (ref. 59) mice were backcrossed onto a C57BL/6 background for more than eight generations. All animal experiments were performed with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University).

Calvarial defect model. Mice used for surgery were 10–12 weeks old. Mice were anaesthetized with isoflurane. The top of their head was shaved and a longitudinal incision was performed to reveal the skull. Bone tissue was exposed by retracting the soft tissues. Using a drill, two craniotomy defects (5 mm diameter) were created in the parietal bones of the skull on each side of the sagittal suture line. The defects were washed with saline and covered with a fibrin matrix polymerized atop the dura (40 μl per defect, 10 mg/ml fibrinogen (Embryonic Research Laboratories), 2 U/ml thrombin (Sigma–Aldrich), 5 mM CaCl2, 25 μg/ml aprotinin (Roche), 2 x 105 MSCs). For Akt and β-catenin inhibition experiments, MK-2206 or XAV-939 were added to the MSC mix to have a final concentration of 1 mM per fibrin matrix. Then, the soft tissue was closed with stitches. As a painkiller, mice received a subcutaneous injection of Tramadol (100 mg kg−1).

Microcomputed tomography. Skulls were scanned with a Scan-Xmate RB808S5110 system (Comscan Techno Co., Ltd) for screening and with a microCT 40 (Scanco Medical AG) operated at energy of 70 kVp and intensity of 145 mAs for detailed measurements. Scans were performed at high-resolution mode resulting in a nominal isotropic resolution of 30 μm. After reconstruction, a 3D Gaussian filter (sigma 1.2, support 1) was applied to all images. Bone was segmented from background using a global threshold of 22.4% of maximum grey value. Afterwards, cylindrical masks were placed manually at the defects. Bone volume within these masks was calculated using a standardized procedure developed for quantitative bone morphometry46. Coverage was calculated on a dorso-ventral projection of the cervical area46.

Chimeric mice. Bone marrow cells (1 x 108) from 5–week-old Myd88b−/− or wt mice were intravenously injected into lethally irradiated 4-weeks-old recipient wt mice. Mice were placed on 10 ml−1 neomycin sulfate for 2 weeks post radiation. After 6 weeks, most of bone marrow-derived immune cells and osteoclasts in bone tissue should lack Myd88 (ref. 69). Mice were used for cranial injury from femurs was harvested to confirm the absence of Myd88 in chimeric mice.

Release of cytokines and inflammatory-associated molecules following bone injury. Calvarial defect (4 mm diameter) were treated with a fibrin matrix as described for the calvarial defect model. After 1, 3, 6, 10 and 15 days, the partially remodelled matrix and the bone tissue surrounding the defect (1 mm farther) was collected. A complete 5 mm diameter calvarial bone tissue was collected (Day 0). Fibroin matrices and tissue samples were incubated in 1 ml of tissue protein extraction reagent (T-PER, Thermo Scientific) supplemented with protease inhibitor cocktail (one tablet of protease inhibitor cocktail (Roche) for 10 ml). The lysed cells were centrifuged at 3,000g for 5 min and the supernatant was analysed using ELISA for Myd88 (MyBioSource).

Isolation of bone-derived MSCs. Long bones of arms and legs of wt, Myd88b−/− and Il1r1−/− mice (6–8 weeks old) were detached from the body trunk. All muscles and cartilages were removed. The bones were rinsed with PBS and cut into three pieces, to have one diaphysis part and two metaphyses/epiphyses parts. The bone marrow within the medullary cavity of the diaphyses was flushed out using a 27-Gauge needle attached to a 10 ml syringe filled with α-MEM containing 1% fetal bovine serum (FBS). Bones pieces, including diaphyses and metaphyses/epiphyses were washed with PBS and cut into 1–3 mm3 pieces. Bone chips were digested in a 25 cm2 cell culture-treated flask with 3 ml of α-MEM medium containing 2 mM l-glutamine, 100 mg ml−1 penicillin/streptomycin, 10% FBS, and 1 mg ml−1 collagenase type II (Life Technologies). Bone chips were digested for 1 h at 37°C to release most of hematopoietic cells on the inner interface of the bones. Then, the bone chips were washed with MSC culture medium (α-MEM, 2 mM l-glutamine, 100 mg ml−1 penicillin/streptomycin, 10% heat inactivated MSC-certified FBS ( Gibco)) and incubated at 37°C with 5% CO2 in 6 ml of MSC culture medium. Bone chips were kept in culture for 6 days with one medium change at day 3. Then, the MSCs that have migrated out of the bone chips and formed colonies were detached using trypsin/EDTA. Cells were split at a ratio of 1:3 (passage 0) and the bone chips were reseeded together for allowing MSCs to continue migrating out for 3 more days. Five independent isolates were pulled together and MSCs were further expanded for three passages before storage. The expression of MSC-specific surface marker was verified using flow cytometry. MSCs were stained with anti-human CD14, CD29, CD44, CD75 and CD90.1. Cells were washed twice with flow cytometry buffer and further analysed by flow cytometry (BD FACSCanto II). For cells used in colonies formation assay, bone pieces were gently digested in a mortar and pestle (in washing buffer containing 1 x 106 MSCs and 1 mM EDTA). Disrupted bone was rinsed with PBS containing 2% FBS and 1 mM EDTA until bone fragments turned white. Then, bone fragments were transferred to a 100 mm dish containing a collagenase solution (2 ml of 0.25% collagenase Type I (Stemcell Technologies) in PBS containing 20% FBS). Using a scalpel, bone pieces were cut into small pieces (1–2 mm) and transferred into a 50 ml polypolyethylene tube with 10 ml collagenase solution. Bone fragments were digested at 37°C under agitation for 45 min. Then, 30 ml of washing buffer was added and supernatant was filtered through a 70 μm cell strainer. Cells were centrifuged at 300 x g for 10 min and resuspended at 500 μl of medium. Lastly, CD45+ cells were depleted (MagCellect Mouse Mesenchymal Stem Cell Isolation Kit, R&D Systems) before colonies formation assays.

MSC isolation from bone marrow. Long bones of arms and legs of wt (6–8 weeks old) were detached from the body trunk. All muscles and cartilages were removed and the bones were rinsed with PBS. The bone marrow was flushed out using a 27-Gauge needle attached to a 10 ml syringe filled with α-MEM containing 1% FBS. Bone marrow cells were washed through a 70 μm filter mesh and transferred in tissue culture-treated plate at a density of 2.5 x 107 cells per ml. Then, cells were incubated at 37°C with 5% CO2 in a humidified chamber for 4 h. Non-adherent cells were removed by changing the medium to culture medium (α-MEM containing 2 mM l-glutamine, 100 mg ml−1 penicillin/streptomycin, and 10% FBS). Thereafter, medium was replaced every 8 h. After 72 h, cells were washed with PBS and new medium was added. Then, medium changed every 2–3 days. After 2–3 weeks, MSC colonies formed were detached using trypsin/EDTA (passage 0). MSCs were expanded until 3 passages. For cells used in colonies formation assay, CD45+ cells were depleted (MagCellect Mouse Mesenchymal Stem Cell Isolation Kit, R&D Systems) before colonies formation assays.

Colony formation assay. Primary cells were seeded in six-well plates (50 bone-derived cells) or 12-well plates (200 bone marrow-derived cells) and cultured in medium (α-MEM, 100 ml−1 penicillin/streptomycin, 20% FBS) with or without 1 ng ml−1 of IL-1β for 12 days. Medium was changed once after 6 days. Then, cells were washed with PBS and stained with an ice-cold crystal violet solution (0.5% in methanol). The number of colony (> 50 cells) was counted and their size was measured using ImageJ software. For osteoblastic differentiation of colonies, primary cells (50 bone-derived cells) were seeded in 24-well plate with osteogenesis induction medium (α-MEM with 2 mM of l-glutamine, 10% FBS, 100 mg ml−1 penicillin/streptomycin, 50 μM ascorbate-phosphate, 10 mM β-glycerolphosphate and 100 ng ml−1 of human BMP-2 (R&D Systems)) or with without 1 ng ml−1 of IL-1β. After 28 days, cells were fixed with ice-cold methanol and calcified nodules were stained using a calcified nodule staining kit (AK-21; GE Healthcare Life Sciences). A measured volume over 1 x 106 was considered as a positive signal.

Monocytes/macrophages depletion. One day before surgery, 200 μl of clodronate liposomes or empty liposomes (Anionic liposomes, FormuMax Scientific Inc.) was intravenously injected in wt mice. Additional 50 μl of clodronate liposomes or empty liposomes was intravenously injected right before surgery and every 2 days until day 6. The concentration of IL-1β into the defect was measured by enzyme-linked immunosorbent assay (ELISA) as described above. The efficiency of clodronate liposomes depletion was measured by measuring the percentage of CD11b+ cells in the spleen by flow cytometry (anti-mouse CD11b, Biogene).

Osteoblast isolation. Cells from 3-day-old mice were isolated and further digested in α-MEM containing enzymes (0.1% collagenase type II (Life Technologies), 0.2% dispase (Sigma–Aldrich)) at 37°C for 20 min in a shaking water bath to release calvarial cells. The supernatant containing released cells was transferred in a new tube, centrifuged at 300 x g, and the pellet was resuspended in medium (α-MEM, 100 ml−1 penicillin/streptomycin, 10% FBS). The calvariae were digested three more times for a total of four digestions. Digestions 3 and 4 containing osteoblasts were pulled together and transferred in a tissue
culture-treated plate at a density of 3 × 10^5 cells ml⁻¹. Calvarial cells were then maintained in culture for 2 weeks in osteogenesis inducing medium (α-MEM with 2 mM l-glutamine, 10% FBS, 100 μg ml⁻¹ penicillin/streptomycin, 50 μM ascorbate-phosphate, 10 mM β-glycerophosphate, 100 mM L-ascorbate and 2 mM ofL-glutamine). For Akt and β-catenin inhibition experiments, MK-2206 and XAV-939 were added instead of IL-1β at a concentration ranging from 0 to 10 μM. Total Akt and phosphorylated Akt were quantified using ELISA (InviteOne ELISA, affimmetrix eBioscience) according to the manufacturer’s instructions. Total GSK-3β and phosphorylated GSK-3β were quantified using ELISA (InviteOne ELISA, affimmetrix eBioscience) according to the manufacturer’s instructions.

Migration assays. Migration assays were performed as described previously. Briefly, solutions containing murine PDGF-BB (5 ng ml⁻¹) or without 1 ng ml⁻¹ of murine IL-1β were added to the bottom side of a collagen type I (C4243, Sigma-Aldrich) coated transwell (8 μm pore size, Millipore). For Akt and β-catenin inhibition experiments, MK-2206 or XAV-939 were added instead of IL-1β at a concentration of 10 μM. Directly after, murine IL-1β (Peprotech) was added at concentrations ranging from 1 to 1,000 ng ml⁻¹. For Akt and β-catenin inhibition experiments, MK-2206 (AdooQ Bioscience) or XAV-939 (AdooQ Bioscience) were added instead of IL-1β at a concentration of 10 μM. After 24 h, the number of cells that migrated to the bottom side of the membrane were counted. Membranes of each insert were removed and mounted on microscopy glass slides using Vectashield (Vector Laboratories) mounting medium containing DAPI. The number of cells that migrated to the bottom side of the membrane was counted.

Osteoblastic differentiation assays. Cells were seeded on 24-well plate at 70–80% confluency using 10% FBS with 5 ng ml⁻¹ of murine IL-1β (1 ng ml⁻¹). For Akt and β-catenin inhibition experiments, MK-2206 or XAV-939 were added instead of IL-1β at a concentration of 10 μM. After 4 days, osteogenesis inducing medium was replaced (without IL-1β). Medium was changed every 3–4 days. Seven or 14 days following stimulation with IL-1β, total RNAs were isolated, using RNAeasy Plus Mini Kit (Qiagen) and reverse transcription was performed using ReverTra Ace (Toyobo Co., Ltd.). Quantitative PCR was performed with an ABI PRISM 7500 using TaqMan Assay with the following primers: Alpl Mouse, Mm00475834_m1; Runx2 Mouse, Mm00501580_m1; Oster1 Mouse, Mm00442594_m1; Col1a1 Mouse, Mm00434612_m1; Gapdh Mouse, Mm00180665_m1. For extracellular matrix mineralization analysis, MSCs were cultured for 28 days in osteogenesis inducing medium. Then, cells were fixed with methanol and calcified nodules were stained using a calcified nodule staining kit.

Chondrodermat differentiation of MSCs. MSCs were transferred in a 15 ml conical tubes (250,000 cells per tube), centrifuged at 200g for 5 min and resupended in D-MEM/F12. MSCs were centrifuged again and resupended in 0.5 ml of D-MEM/F12 containing 100 μg ml⁻¹ of penicillin/streptomycin, 1% ITS supplement, 1 × chondrogenic supplement (R&D Systems), with or without murine IL-1β (1 ng ml⁻¹). Cells were centrifuged on more time at 200g for 5 min to form a pellet. The cap of the tubes were loosen to allow gas exchange and the tubes were incubated upright at 37°C with 5% CO₂. The medium was replaced every 3 days (without IL-1β). After 28 days, the spheroids were washed with PBS and fixed with 10% formalin for 60 min. Spheroids were washed twice with water and stained with Alcian Blue 8 GX (Sigma-Aldrich, 0.1 mg ml⁻¹ in ethanol/acetic acid solution (3:2)) overnight at room temperature in the dark. Spheroids were destained three times with an ethanol/acetic acid solution (3:2) and resupended in PBS, before imaging.

Trophic factors secreted by MSCs. MSCs were seeded in six-well plates and cultured until 70–80% confluency. Cells were starved for 24 h with medium containing 1% FBS and further stimulated with PBS or IL-1β (1 ng ml⁻¹). After 24 h supernatants were collected and stored at −80°C. Trophic factors were detected using an antibody array focusing on angiogenic factors (Mouse Angiogenesis Antibody Array, R&D Systems) according to the manufacturer’s instructions, using 400 μl of supernatant. The chemiluminescent signals were detected using ImageQuant LAS 4000 and quantified with ImageQuant TL software (GE Healthcare Life Sciences). A measured volume over 1 × 10⁶ was considered as a positive signal.

Intracellular signalling assays. MSCs were seeded in six-well plate and cultured until 60–70% confluency. Cells were starved for 24 h with medium containing 1% FBS. Then, cells were stimulated with murine PDGF-BB (5 ng ml⁻¹), murine IL-1β (1 ng ml⁻¹), or with both for 10 min to 24 h. Phosphorylation and cleavage of intracellular signalling molecules were detected using an antibody array (PathScan Intracellular Signalling Array, Cell Signalling) according to the manufacturer’s instructions. The chemiluminescent signals were detected using ImageQuant LAS 4000 and quantified with ImageQuant TL software (GE Healthcare Life Sciences).

Akt signalling assay. MSCs were seeded in six-well plate and cultured until 60–70% confluency. Then, cells were stimulated with murine IL-1β (1 ng ml⁻¹) or PBS for 0 to 72 h. For Akt and β-catenin inhibition experiments, MK-2206 or XAV-939 were added instead of IL-1β at concentrations ranging from 0 to 10 μM. Total Akt and phosphorylated Akt were quantified using ELISA (InviteOne ELISA, affimmetrix eBioscience) according to the manufacturer’s instructions. Total Wnt/β-catenin and GSK-3β signalling assays. MSCs were seeded in six-well plate and cultured until 60–70% confluency. Then, cells were stimulated with murine IL-1β (1 ng ml⁻¹) or PBS for 4 to 72 h. For Akt and β-catenin inhibition experiments, MK-2206 or XAV-939 were added instead of IL-1β at a concentration ranging from 0 to 10 μM. Total β-catenin and phosphorylated β-catenin were quantified using ELISA (InviteOne ELISA, affimmetrix eBioscience) according to the manufacturer’s instructions.

Osteoblastic differentiation of transplanted MSCs. As a non-differentiated control, MSCs were seeded in cell culture plate with α-MEM containing 10% FBS and 5 ng ml⁻¹ of PDGF-BB. Medium was renewed after 4 days. Seven days after transplantation, the partially remodelled fibrous matrices containing MSCs were removed from the defect and incubated in 1 ml of an enzyme solution (trypsin (10 mg ml⁻¹) and collagenase II (1 mg ml⁻¹)) for 1 h at 37°C. Then, the digested matrix was resuspended in 10 ml of α-MEM containing 10% serum, passed through a cell strainer and centrifuged. The cells were resuspended in 1 ml of red blood cell lysis buffer (Sigma-Aldrich), incubated at room temperature for 5 min, and resuspended in 10 ml of flow cytometry buffer. For non-proliferating cells and wt proliferating cells controls, cells were detached from cell culture plate using trypsin/EDTA and resuspended in flow cytometry buffer. Cells were washed twice with flow cytometry buffer and further analysed by flow cytometry (BD FACSanto II).

Peptide inhibitor of IL-1R1/MyD88 signalling (zP1z,BMyD88-I). The peptide zP1z,BMyD88-I (NQEQVSPVLSMGRGGQKJIFQFNRMKWRKDDYLVPG TCVNS) was synthesized by GeneScript and verified as 99.5% by HPLC. The peptide was further dialyzed against HEPES buffer (20 mM HEPES, 0.15 M NaCl, pH 7.5). The capacity of the peptide to inhibit IL-1R1/MyD88 signalling was assessed by monitoring the release of cytokines from MSCs following stimulation with murine IL-1β. MSCs seeded in 24-well at 70–80% confluency were stimulated with 10 ng ml⁻¹ of IL-1β together with zP1z,BMyD88-I at increasing concentrations. The peptide concentration of the IL-6, CCL2, CXCL1 and CXCL2 released were measured using ELISA (Mouse IL-6, CCL2/IFN-MCP-1, CXCL1/KC and CXCL2/MP-2; DuoSets, R&D Systems).
MSC delivery system containing inhibitors of IL-1R1/MyD88 signalling. Fibrin matrices with or without wt MDCs were delivered into cranial defects as described for the calvarial defect model (2 × 10^6 cells per defect). Fibrin matrices (40 μl per defect, 10 mg ml⁻¹ fibrinogen, 2 μl ml⁻¹ of thrombin, 5 mM CaCl₂, 25 μg ml⁻¹ aprotinin) were functionalized with 4 μg of α2PI, α2-M, MyD88-I or 1 μg of murine IL-1Ra (R&D Systems). After 8 weeks, bone regeneration was analysed by microCT as described above.

References

11. Schindeler, A., McDonald, M. M., Bokko, P. & Little, D. G. Bone remodeling (aprotinin) were functionalized with 4 μg of α2PI, α2-M, MyD88-I or 1 μg of murine IL-1Ra (R&D Systems). After 8 weeks, bone regeneration was analysed by microCT as described above.
12. Schindeler, A., McDonald, M. M., Bokko, P. & Little, D. G. Bone remodeling (aprotinin) were functionalized with 4 μg of α2PI, α2-M, MyD88-I or 1 μg of murine IL-1Ra (R&D Systems). After 8 weeks, bone regeneration was analysed by microCT as described above.

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Author contributions

M.M.M. designed the research, conducted the majority of the experiments, analysed the data, and wrote the manuscript. K.M. participated to microCT measurements, chimeric mouse generation, osteoblast isolation, and experimental design. G.K. and R.M. participated to microCT measurements and analyses. T.S. and O.T. helped with knockout mouse colonies and experimental design. S.A. supervised the research.

Additional information

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