Immune responses are crucial not only for host defence against pathogens but also for tissue maintenance and repair after injury. Lymphocytes are involved in the healing process after tissue injury, including bone fracture and muscle damage. However, the specific immune cell subsets and mediators of healing are not entirely clear. Here we show that $\gamma\delta$ T cells produce IL-17A, which promotes bone formation and facilitates bone fracture healing. Repair is impaired in IL-17A-deficient mice due to a defect in osteoblastic bone formation. IL-17A accelerates bone formation by stimulating the proliferation and osteoblastic differentiation of mesenchymal progenitor cells. This study identifies a novel role for IL-17-producing $\gamma\delta$ T cells in skeletal tissue regeneration.
The immune system protects the body by eradicating pathogenic microorganisms but has alternative physiological and pathological roles in a variety of biological systems including the musculoskeletal system. T cells are important in the pathogenesis of erosive arthritis and spondyloarthropathy accompanied with enhanced bone formation. In addition, inflammatory reactions are involved in ectopic bone formation in fibro dysplasia ossificans progressiva and one report indicates that regulatory T cells are crucial for muscle repair. Bone repair after injury is also associated with immune reactions.

Fractured bone regenerates through a cascade of events: haematoma formation, inflammation, callus formation and bone remodelling. On bone fracture, blood vessels near the injury site rupture, resulting in the formation of a haematoma. The haematoma is infiltrated by immune cells, including neutrophils, macrophages and lymphocytes, which induce acute inflammation. After the resolution of inflammation, mesenchymal progenitor cells accumulate around the fractured site to form granulation tissue. Neovascularization in the injury site is also observed. The mesenchymal progenitor cells differentiate into chondrocytes and osteoblasts, to undergo endochondral and intramembranous ossification, forming callus that bridges bone fragments. The callus is replaced by mature bone tissue by bone remodelling in the later stage of repair so that the fractured bone restores its original shape and function.

As T cells are present in the haematoma and mice deficient in lymphocytes reportedly exhibit delayed or accelerated bone fracture healing, it is suggested that T cells contain multiple subsets with different functions in bone repair. Effector memory CD8+ T cells have been reported to delay fracture healing. However, the specific T-cell subsets that promote healing and the mediating factors involved remain to be elucidated.

γδ T cells are innate-like lymphocytes that are distributed preferentially to peripheral tissues and can exert tissue-regenerative functions. Here we show that interleukin (IL)-17A is highly induced immediately after bone injury and promotes bone regeneration by accelerating osteogenesis via its effects on injury-associated mesenchymal cells. Furthermore, we reveal that γδ T cells (T cell receptor (TCR) nomenclature of Helig and Tonegawa) proliferate in the injury site and function as the crucial producer of IL-17A in fracture healing.

Results

IL-17A is induced in the repair tissue after bone injury. To determine which type of T cells are involved in the bone regeneration that occurs after injury, we analysed the messenger RNA (mRNA) expression of the T-cell-related cytokines in the bone regeneration process following the introduction of a femoral cortical bone defect by drill-hole injury (Supplementary Fig. 1a). This model essentially recapitulates the intramembranous bone formation process, enabling a simplified quantification of repaired bone and the preservation of the bone marrow as the result of not using a fixation system. After bone injury, massive proliferation of fibroblastic cells in the drill hole, along with an infiltration of inflammatory cells and vascularization occurred (Supplementary Fig. 1b–d). Regenerating skeletal muscle cell layer (Supplementary Fig. 1c) and a thickening periosteum (Supplementary Fig. 1d) were observed extending into the proliferating fibroblastic cell layer. As the regenerative tissue in the drill hole was continuous with the one around the bone, we harvested the cells from the tissues in the drill hole, periosteum and injured skeletal muscle, and defined them as the cells of the repair tissue.

There was no significant increase in the expression of Ifng, Il4, Il17f or Il22 in the bone marrow or the repair tissue; however, the expression of Il17a in the repair tissue, not in the bone marrow, was significantly increased 2 days after injury (Fig. 1 and Supplementary Fig. 1e). These results suggest that IL-17A plays a role in the process of bone regeneration after injury.

IL-17A promotes bone regeneration after injury. To determine the role of IL-17A in the regenerative process after injury, we assessed bone regeneration using Il17a−/− mice by measuring the level of closure of the drill hole with micro-computed tomography (CT). We found that 14 and 21 days after injury, the volume of the newly formed bone tissue in the drill hole was smaller in the Il17a−/− mice than wild-type mice (Fig. 2a,b).

The bone mineral content within the drill hole was lower in the Il17a−/− mice than in the wild-type mice (Fig. 2c,d). Although it has been suggested that IL-17F is involved in fracture repair, there was no significant difference in bone regeneration in the wild-type and Il17f−/− mice (Supplementary Fig. 1f–i).

We then evaluated bone formation by osteoblasts and bone resorption by osteoclasts at the injury site using histomorphometry. New bone was formed as trabecula-like structures in the drill hole in both the wild-type and Il17a−/− mice (Fig. 2e). To investigate the number of bone-forming osteoblasts on the bone surface, we measured the ratio of the bone surface covered by cuboidal osteoblasts, which are known to have a high capacity for synthesizing bone matrix. The ratio of the cuboidal osteoblast surface to the bone surface was lower in the Il17a−/− mice than in the wild-type mice (Fig. 2f,g). There was no significant difference in the ratio of the eroded surface and the number of osteoclasts between the wild-type and Il17a−/− mice during the process of bone regeneration (Supplementary Fig. 2a–c). These results suggest that IL-17A promotes bone regeneration by activating osteoblastic bone formation without affecting osteoclastic bone resorption.

Injury tissue contains IL-17A-responsive P2S cells. The differentiation of mesenchymal cells is regulated by various cytokines. Among these cytokines, IL-6 and tumour necrosis factor, which are induced by IL-17A, are known to have the capacity to promote bone fracture healing. However, there was no significant difference in the expression of Il6 and Tnfα in the repair tissues of wild-type and Il17a−/− mice (Supplementary Fig. 3a), indicating that IL-17A promotes bone formation independently of these cytokines.

We analysed the expression of the IL-17A receptor IL-17RA on the cells of the repair tissue after the haematopoietic cell...
**Figure 2** | IL-17A promotes bone regeneration after injury by enhancing osteoblast function. (a) Micro-CT images of the drill holes in the wild-type and Il17a<sup>−/−</sup> mice after injury. Scale bar, 500 µm. (b) Quantification of bone formation in the drill holes (n = 4 – 8 per time point per genotype). (c) Visualization of the bone mineral density of the newly formed bone in the drill holes. Images were constructed by colouring the micro-CT images according to the CT values. The lines denote the original shapes of the drill holes. Scale bar, 500 µm. (d) Bone mineral content in the drill holes (n = 4 – 8 per time point per genotype). (e) Histological images of the femur of the wild-type and Il17a<sup>−/−</sup> mice stained with HE staining. The lines indicate the edge of the drill hole. Scale bar, 500 µm. (f) Bone surface lined with cuboidal osteoblasts (dotted lines) on the newly formed bone. Scale bar, 50 µm. (g) Cuboidal osteoblast surface per bone surface. The bone surface covered with bone-forming osteoblasts was quantified by bone histomorphometric analysis (n = 3 per time point per genotype, 6 representative sections per each femur). Statistical analysis was carried out using Student’s t test for each time point in Fig. 2. Error bars denote the mean±s.e.m. *P<0.05; NS, not significant.
population and endothelial cell population positive for CD45, Ter119, CD11b, CD31 or CD34 had been gated away. IL-17RA was expressed on the mesenchymal cells in the repair tissue and the ratio of IL-17RA-expressing mesenchymal cells was significantly increased after the injury (Fig. 3a). Most of the IL-17RA+ mesenchymal cells in the injury tissue were positively stained for both platelet-derived growth factor receptor (PDGFR)-α and Sca-1 (Fig. 3b). PDGFRα± Sca-1− cells (P2S cells) are thought to be an enriched population of mesenchymal stem cells (MSCs)14. Mesenchymal cells in the repair tissue were highly positive for other MSC markers: CD44, Integrin β1 and CD31 (Fig. 3b). We further confirmed that IL-17RA+ mesenchymal cells and P2S cells were actually present in the drill hole even when we removed the outer layer of the repair tissue (Supplementary Fig. 3b).

**IL-17A enhances osteoblastogenesis in vitro.** The above results led us to examine the effects of IL-17A on injury-associated mesenchymal cells. The activity of alkaline phosphatase (ALP), an osteoblast differentiation marker, and mineralization were upregulated by IL-17A in both the presence and absence of bone morphogenetic protein (BMP)-2 (Fig. 4a,b). IL-17A decreased mineralization and bone nodule formation in murine calvarial cells (Supplementary Fig. 4a–c) consistent with a previous report15, suggesting that IL-17A exerts distinct effects on bone formation depending on the cellular origin.

How does IL-17A regulate osteoblastic bone formation? 5-Bromodeoxyuridine (BrdU) incorporation assay showed that IL-17A significantly increased the proliferation of the injury-associated mesenchymal cells (Fig. 4c). Consistent with this, immunohistological analysis showed that the number of BrdU+ cells within the drill hole in Il17aΔ/Δmice was significantly lower than that in the wild-type mice (Fig. 4d). We analysed the effect of IL-17A on the mRNA expression of osteoblast genes. The expression of Runx2 and Sp7, the essential transcription factors for osteoblastogenesis, was unchanged, whereas the mRNA of certain osteoblast genes including Alpl and Colla1 was upregulated (Fig. 4e). Thus, IL-17A promotes bone formation in injury-associated mesenchymal cells through the stimulation of both osteoblast proliferation and differentiation. To examine the contribution of soluble factors released from injury-associated mesenchymal cells, we performed a comprehensive mRNA expression analysis on P2S cells stimulated with IL-17A and/or BMP-2. Among the soluble factors and their receptors related to osteoblast differentiation, Fgf2, Pdgfa, Pdgfc and Tgfb1 were found to be upregulated by these cytokines, suggesting that IL-17A may indirectly promote osteoblastogenesis via these factors at least in part (Supplementary Fig. 4d).

**Vγ6+ γδ T cells produce IL-17A on bone injury.** The cellular source of IL-17A following bone injury was next explored. Immunohistological analysis of the repair tissue in IL-17A–GFP reporter mice (Il17aΔ/Δmice) showed that IL-17A+ cells were observed around the drill hole and the injured muscle, but they were hardly detected in the bone marrow (Fig. 5a,b and Supplementary Fig. 5a). The number of IL-17A+ cells significantly increased 2 and 7 days after injury and the density of these cells was higher in the drill hole than in the injured muscle at day 2 (Fig. 5c).

To characterize the IL-17A-producing cells after injury, we analysed the cells harvested from the repair tissue in Il17aΔ/Δ mice. By flow cytometric analysis, we found that most of the IL-17A+ cells were CD3ε+ TCRγδ+ γδ T cells (Fig. 5d and Supplementary Fig. 5b,c). The number of γδ T cells and the ratio of IL-17A+ cells increased in the repair tissue after injury (Fig. 5e and Supplementary Fig. 5d). The proliferation of γδ T cells in the repair tissue was remarkably increased 2 days after the injury (Fig. 5f). Although regulatory T cells were suggested to be involved in bone fracture healing16, Foxp3+ cells did not

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**Figure 3 | Mesenchymal cells in the injury site contain IL-17A-responsive P2S cells.** (a) The ratio of IL-17RA+ cells in CD45− Ter119− CD11b− CD31− CD34+ mesenchymal cells in the repair tissues of non-treated and injured mice (n = 6 per time point). (b) The expression of MSC markers on IL-17RA+ mesenchymal cells of the repair tissue (n = 3–6). Most of the IL-17RA+ mesenchymal cells were P2S cells positive for both platelet-derived growth factor receptor-α (PDGFRα) and Sca-1. Statistical analysis was carried out using Student's t-test. Error bars denote the mean ± s.e.m. *P < 0.05.
Statistical analysis was carried out using Student’s t-test. All the data were analyzed by immunofluorescence and the ratio of BrdU incorporation assay. Approximately 70% of the γδ T cells in the repair tissue after the injury expressed Vγ6 (Fig. 6a). Furthermore, over 80% of IL-17A+γδ T cells were Vγ6+ cells (Fig. 6a). Vγ6+γδ T cells exhibited a remarkable increase in number 2 days after the injury (Fig. 6b). These results indicate that Vγ6+γδ T cells play a key role in IL-17A production in musculoskeletal tissue, to promote bone regeneration after injury.

**Discussion**

Although it has long been recognized that the immune system is closely related to bone fracture healing, the mechanisms linking them have been poorly understood. In this study, we found that IL-17A is highly induced after bone injury. Bone regeneration was impaired in Il17a−/− mice, due to the decrease in osteoblastic bone formation. IL-17A promoted osteoblastogenesis of mesenchymal cells harvested from the repair tissue, in which P2S cells were enriched. We also found that Vγ6+γδ T cells were the major source of IL-17A in bone regeneration process. Thus, our data demonstrate that Vγ6+γδ T cells produce IL-17A to promote osteoblastic bone formation in bone regeneration.

To specifically examine the role of γδ T cells in bone regeneration, we analyzed the bone regeneration in Tcrd−/− mice, which are deficient in γδ T cells. Bone regeneration was significantly impaired in Tcrd−/− mice (Supplementary Fig. 6a–d), indicating that γδ T cells play a crucial role in bone regeneration. In the injury tissue of Tcrd−/− mice, Il17a expression was significantly lower than that of wild-type mice but not completely abrogated (Supplementary Fig. 6e). Analysis of surface markers of IL-17A-producing CD3ε+ cells by flow cytometry revealed that there was a Thy-1+ population in both wild-type and Tcrd−/− mice, suggesting that type 3 innate lymphoid cells may compensate for the loss of IL-17A production by γδ T cells (Supplementary Fig. 6f).

Among the cytokines related to IL-17A, the expression of Il1b and Il23p19, which were reported to activate γδ T cells to produce IL-17A18,19, was robustly upregulated in the cells of the repair tissue in the early stage of bone regeneration, suggesting their contribution to IL-17A production (Supplementary Fig. 7).

γδ T cells are divided into subsets expressing distinct TCR-Vγ chains, with each subset having its own characteristic tissue distribution and cytokine production pattern.20,21 Approximately 70% of the γδ T cells in the repair tissue after the injury expressed Vγ6 (Fig. 6a). Furthermore, over 80% of IL-17A+γδ T cells were Vγ6+ cells (Fig. 6a). Vγ6+γδ T cells exhibited a remarkable increase in number 2 days after the injury (Fig. 6b). These results indicate that Vγ6+γδ T cells play a key role in IL-17A production in musculoskeletal tissue, to promote bone regeneration after injury.
arthritis: IL-17A derived from T \textsubscript{H17} cells in inflamed synovium stimulates receptor activator of nuclear factor-κB ligand (RANKL) expression on synovial fibroblasts, as well as local inflammation, resulting in exaggerated osteoclastic bone resorption\textsuperscript{2,24,25}. However, in the present study, osteoclastic bone resorption in \textit{Il17a}\textsuperscript{-/-} mice was not affected (Supplementary Fig. 2a–c). This is possibly because the number of IL-17A-producing cells in the repair tissue was very small in the late phase when osteoclastogenesis occurred (Fig. 5c).

IL-17A-blocking antibody has been shown to be effective for chronic inflammatory diseases associated with excessive bone formation including spondyloarthropathy and psoriatic arthritis,
T cells in the injury site are enriched in the VγγTRAF2/4/5/6, which activates nuclear factor-
recruitment of Act1 and TNF receptor-associated factor to the short term after bone injury, it is likely to be that IL-17A
Considering the results that high induction of IL-17A is limited
osteoblast proliferation and/or differentiation. It is reported that
exert negative effects on the osteoblastogenesis in the culture of
target cell types. In certain previous studies, IL-17A was shown to
involve endochondral bone formation, in which cartilage is
formed within the bone defect and replaced by the bone.
Thus, it is demonstrated that there is a crucial role for
IL-17A-producing γδ T cells to contribute to the maintenance of not only the epithelial
barrier but also the supportive tissue underneath. γδ T-cell-based
immunotherapy has long been studied in the treatment of cancer.
Although it is necessary to develop the method to stimulate
tissue-specific γδ T cells, this study suggests IL-17-producing γδ T cells to be an effective therapeutic target for bone fracture healing.

Methods
Animals and in vivo experiments. Mice were kept under specific pathogen-free
conditions and all the experiments were performed with the approval of the
Institutional Review Board at the University of Tokyo. C57BL/6 mice were
purchased from Clea Japan, Inc. Il17a−/− mice and Il17f−/− mice were
previously generated on a C57BL/6 background34. Il17a−/−; Foxp3−/− mice were previously generated on a
C57BL/6 background35. Ter119−/− mice were obtained from the Jackson
Laboratory. For analyses on bone regeneration, female mice of 6–8 weeks old were
used. For the analyses on inflammation, female and male mice of 6–8 weeks old
were used.
For drill-hole injury, as described previously13, mice were anaesthetized with an
intraperitoneal injection of pentobarbital sodium. Hair on the thigh was removed
using an electric shaver and the skin underneath was disinfected with ethanol. An
approximately 10-mm longitudinal skin incision was made immediately above and
parallel to the femur. The bone surface was exposed by splitting the muscle
mesiodistally and removing the periosteum. A drill hole with a diameter of
0.8–1.0 mm was made on the anterior portion of the diaphysis of the femur.
For the proliferation assay, mice were injected intraperitoneally with 150
μl of BrdU (Sigma) solution at the concentration of 10 mg ml−1 in saline (Otsuka
Pharmaceutical Factory, Inc.), 1 day before killing.
For isolation of cells from mice. Tissues were obtained from the femur as depicted in
Supplementary Fig. 1a. Bone marrow cells were harvested from the femur by
introducing PBS into the bone marrow cavity. Erythrocytes were depleted by
haemolysis using ammonium chloride (Sigma). To collect the repair tissue cells, the
skin, skeletal muscles and periosteum were removed from the femur and the tissues in
the drill hole, periosteum and skeletal muscle were digested in a collagenase
(WAKO) solution at the concentration of 1 mg ml−1 dissolved in RPMI (Gibco).
Debris was removed by filtration using 70 μm mesh (BD) and by density
centrifugation using 40% Percoll (GE Healthcare) solution in RPMI, followed by
haemolysis. Neonatal murine calvarial cells were isolated as described previously35; neonatal mice were killed by anaesthesia and disinfected by immersion in ethanol.
Calvaria were dissected and digested in a digestion solution, in which 1 mg ml−1 of

Figure 6 | IL-17A-producing γδ T cells in the injury site are Vγ6+ cell subset. (a) The usage of Vγ chain of γδ T cells in the repair tissue after injury (n = 3). 17D1 antibody recognizes Vγ5+ and Vγ6+ cells, but there were virtually no Vγ5+ γδ T cells observed, indicating IL-17A-producing γδ T cells in the injury site are enriched in the Vγ6+ cell subset. (b) The number of 17D1+ γδ T cells (upper) and IL-17A-producing 17D1+ γδ T cells (lower) in the repair tissue before and after injury (n = 3 per time point). Statistical analysis was carried out using Student’s t test. Error bars denote the mean ± s.e.m.
*P < 0.05.
collagenase and 2 mg ml⁻¹ of dispase were dissolved in α-MEM (Gibco). Debris was removed by filtration using 70 μm mesh and osteoblastic cells were collected.

Quantitative reverse transcriptase–PCR. Total RNAs of the cells prepared as described above were extracted using TRIzol (Life Technologies) according to the manufacturer’s instructions. First-strand complementary DNAs were synthesized using Superscript III reverse transcriptase (Invitrogen). Quantitative real-time reverse transcriptase–PCR was performed with a LightCycler (Roche) using SYBR Green (TOYOBO) according to the manufacturer’s protocol. The expression level of miRNA was normalized with that of GAPDH. The primers for analysis of gene expression by real-time reverse transcriptase–PCR were as follows: for GAPDH, 5'-GCCGTATTAGCACAAGCTT-3' and 5'-TGAGCTGTCAAGTCTT-3'; for TGF-β3, 5'-GCCCTACAGACAGTGCTACTT-3' and 5'-TTAGTGGTCTTGCTACCC-3'; for CAA, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'; for Il6, 5'-CAATTGCCATTGCACAAC-3' and 5'-CAGAATTGCCATTGCACAAC-3'; for Il1b, 5'-CAACGAGTGCTACTTGGTTAGGA-3' and 5'-GGACTCATGGTGGCTCAGAA-3'; for Sp7, 5'-GGTAGGGAGCTGGGTTAAGG-3' and 5'-GG TAGGGCTGATGTAC-3'; for Il17a, 5'-CCTCACAGCAACGAAGAACA-3' and 5'-CCACGAGTGCTACTTGGTTAGGA-3'; for Tnfa, 5'-TGAGCTCATTGAATGCT-3' and 5'-GGATCCAGAGCTTGAAGAGCT-3'; for Il4, 5'-CAGAATTGCCATTGCACAAC-3' and 5'-CAGAATTGCCATTGCACAAC-3'; for Il23p19, 5'-TCCCTCTGTGATCTGGGAAG-3' and 5'-GGACTCATGGTGGCTCAGAA-3'; for Tgfb1, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'; for Il17f, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'; for Il17d, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'; for Tgfb3, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'; for Il22, 5'-ACACGATGCTCTTGGTAAAG-3' and 5'-GAGCTCCGTCTGAAAGAGCT-3'.

Histological and histomorphometric analyses. Non-decalcified frozen sections of 5 μm thickness were prepared. The sections were incubated with paraformaldehyde were freeze embedded with carboxymethyl cellulose in a cooled plate. The sections were incubated with 4% paraformaldehyde underwent were washed away from the section before staining. The sections were incubated with monoclonal antibody 17D1 specific for TCR-Vγ1 T cells (Biolegend) and Alexa Fluor® 488 can bind with 647-conjugated goat anti-mouse antibody respectively. Monoclonal antibody 17D1 was used at a dilution of 1/200. Primary antibody solution containing a rabbit anti-Fcγ polyclonal antibody (Abcam) at 4°C for 1 h, followed by incubation with a secondary antibody solution containing goat anti-rabbit IgG antibody conjugated with fluorescein dye (Life Technologies) for 1 h. Nuclei were stained with Hoechst33342. Micro-CT analysis. CT scanning was performed using a Micro-CT (Skyscan 1174, Bruker). Data were acquired on an Ion Proton (Thermo Fisher) and analysed using CLC Genomics Workbench (CLC).

RNA sequencing. Total RNA of cultured Ps cells was extracted using Maxwell 16 LEV simplyRNA Cells and Tissue Kit (Promega). cDNA was synthesized and amplified using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech Laboratories). Data were acquired on an Ion Proton (Thermo Fisher) and analysed using CLC Genomics Workbench (CLC).

Statistical analysis. P-values were calculated using Student’s t-test or analysis of variance (ANOVA) with Dunnett’s (for one-way ANOVA) or Tukey’s (for two-way ANOVA) multiple-comparison test. Differences with a P-value of < 0.05 were considered significant (*P < 0.05; **P < 0.01; NS, not significant, throughout the paper). All the data are expressed as mean ± s.e.m.

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Author contributions
T.O. performed most of the experiments, interpreted the results and prepared the manuscript. K.O. provided advice on project planning and data interpretation, and contributed to the manuscript preparation. T. Na. and T. Ni. contributed to project planning, and data interpretation. Y.I. and S.H. provided genetically modified mice. H.T. directed the project and wrote the manuscript.

Additional information
Accession codes: The RNA-Seq data have been deposited in GEO under the accession code GSE77172.

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