The microbiota maintain homeostasis of liver-resident γδT-17 cells in a lipid antigen/CD1d-dependent manner

Fenglei Li1, Xiaolei Hao2, Yongyan Chen1, Li Bai1, Xiang Gao3, Zhexiong Lian1, Haiming Wei1,2, Rui Sun1,2,4 & Zhigang Tian1,2,4

The microbiota control regional immunity using mechanisms such as inducing IL-17A-producing γδ T (γδT-17) cells in various tissues. However, little is known regarding hepatic γδT cells that are constantly stimulated by gut commensal microbes. Here we show hepatic γδT cells are liver-resident cells and predominant producers of IL-17A. The microbiota sustain hepatic γδT-17 cell homeostasis, including activation, survival and proliferation. The global commensal quantity affects the number of liver-resident γδT-17 cells; indeed, E. coli alone can generate γδT-17 cells in a dose-dependent manner. Liver-resident γδT-17 cell homeostasis depends on hepatocyte-expressed CD1d, that present lipid antigen, but not Toll-like receptors or IL-1/IL-23 receptor signalling. Supplementing mice in vivo or loading hepatocytes in vitro with exogenous commensal lipid antigens augments the hepatic γδT-17 cell number. Moreover, the microbiota accelerate nonalcoholic fatty liver disease through hepatic γδT-17 cells. Thus, our work describes a unique liver-resident γδT-17 cell subset maintained by gut commensal microbes through CD1d/lipid antigens.

1 Institute of Immunology and the Key Laboratory of Innate Immunity and Chronic Disease (Chinese Academy of Science), School of Life Science and Medical Center, University of Science and Technology of China, Hefei 230027, China. 2 Hefei National Laboratory for Physical Sciences at Microscale, Hefei, Anhui 230027, China. 3 Model Animal Research Center, Nanjing University, Nanjing, Jiangsu 210061, China. 4 Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, China. Correspondence and requests for materials should be addressed to Z.T. (email: tzg@ustc.edu.cn).
he liver is situated in a unique systemic circulation system that receives blood from both the hepatic artery and the portal vein, making this organ a prime location for both metabolic and immune function. However, the precise mechanism that connects the microbiota and the hepatic immune response is seldom reported. Bacterial translocation and pattern-associated molecular pattern (PAMP) transport are the two main events that have been observed in the liver-gut axis. However, the proposed mechanisms will remain elusive until the soluble factors from the microbiota and their cellular targets in liver-gut axis are determined.

The liver is enriched in innate immune cells, including γδT cells at a frequency of 3–5% (5 to 10-fold greater than in other tissues or organs) within total liver lymphocytes. γδT cells function as a bridge between innate and adaptive immunity because they express a rearranged T-cell receptor (TCR) that recognizes certain antigens and can also rapidly secrete pro-inflammatory cytokines including interleukin (IL)-17A upon stimulation. By producing IL-17A to recruit neutrophils and enhance adaptive immunity, IL-17A-producing γδT (γδT-17) cells have an important role in host defense against bacterial, fungal and viral infections, as well as stress, tumour surveillance and autoimmune diseases. However, although hepatic γδT cells are involved in several liver immune diseases, their physiological characteristics, and why the liver contains such high levels of γδT cells, are unknown.

CD1d, a typical lipid presentation molecule for natural killer T (NKT) cells, can also present lipid antigens to the γδTCR and activate γδT cells. A γδT cell subset in human blood can respond to CD1d-presented sulfamate, a lipid antigen present in both hosts and bacteria. Another γδT cell subset in the mouse duodenum can respond to exogenous lipid antigens including phosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) presented by CD1d. The liver constantly encounters microbial lipid components, and crosstalk occurs between CD1d and liver NKT cells; however, little is known regarding the role of γδT cells in this process.

Here we compare γδT cells originating from several organs and identify a liver-resident γδT-cell population that predominantly produces IL-17A. The microbiota maintain hepatic γδT-17 cell homeostasis, the underlying mechanism of which involves microbiota lipid antigens presented by hepatocyte-expressed CD1d, but not PAMPs or cytokine signals. Moreover, liver-resident γδT cells responding to the microbiota contribute to nonalcoholic fatty liver disease (NAFLD).

Results
Hepatic γδT cells produce IL-17A. Compared with other immune organs and tissues, hepatic γδT cells predominantly produced high levels of IL-17A, similar to γδT cells from the peritoneal cavity (PC) and lung and significantly higher than those from inguinal lymph nodes (iLNs), the spleen, the thymus, small intestine intraepithelial lymphocytes (IEL), colon IEL and mesenteric LN (mLN) (Fig. 1a,c). In terms of phenotype, hepatic γδT cells exhibited mixed Vγ chain usage, which was also distinct from γδT cells of other organs (Fig. 1b). They were in a more active and mature state, as indicated by higher percentages of CD44highCD62L− cells and lower CD24 expression (Fig. 1c). Corresponding with their high IL-17A expression levels, hepatic γδT cells expressed low levels of CD27 (Fig. 1c), which is a fate determinant of γδT cells to express IFN-γ (γδT-1) but not IL-17A (γδT-17). However, unlike γδT cells of the PC and lung, hepatic γδT cells rarely expressed cytokine receptors including CD121, CD25 and CD127 (Fig. 1c). Interestingly, neonatal mice had low levels of γδT-17 but high levels of γδT-1 cells in the liver (Fig. 1d). As the mice aged, the hepatic γδT-17 cell frequency increased, while that of γδT-1 cells decreased, suggesting that hepatic γδT-17 cells might be induced after birth (Fig. 1d). Overall, hepatic γδT cells exhibited a unique composition and phenotype, indicating that they represent a distinct γδT-cell subtype.

We further characterized the trafficking and homing tendencies of hepatic γδT cells. GFP+ splenic and CD45.1+ hepatic lymphocytes were transferred separately or together into C57BL/6γδTCR−/− mice (Supplementary Fig. 1a). One day after transfer, splenic γδ T cells travelled randomly into both the spleens and the livers of recipient mice. In contrast, hepatic γδT cells selectively homed only to the liver, and co-transfer with splenic lymphocytes did not change this homing tendency. We confirmed this result by transferring purified hepatic γδT cells into Rag1−/− mice and found that hepatic γδ T cells, particularly γδT-17 cells, homed back to the liver but not to other organs (Supplementary Fig. 1b). To further explore whether hepatic γδT cells reside in the liver, CD45-congenic mice were surgically joined by parabiosis and evaluated for chimerism in various cell populations 2 weeks later. While conventional T cells exhibited substantial chimerism (Fig. 1e), the livers of CD45.1 parabiont mice contained almost all CD45.1+ γδT cells with very few, if any, CD45.2+ γδT cells; vice versa was observed in CD45.2 parabiont mice (Fig. 1e). Interestingly, PC and thymic γδT cells were also locally retained, but lung, spleen and iLN γδT cells were mutually exchanged (Supplementary Fig. 1c).

Compared with circulating γδT cells, most of the chemokine receptors and integrin molecules were expressed at lower levels on hepatic γδT cells, but C-X-C chemokine receptor 6 (CXCR6) was expressed at extremely high levels on hepatic γδT cells (Supplementary Fig. 2a). However, it was not involved in the liver homing of hepatic γδT cells because the numbers of hepatic γδT cells and γδT-17 cells were not changed in Cxcr6−/− mice (Supplementary Fig. 2b), although CXCR6 was important for the liver homing of natural killer (NK) and NKT cells. Moreover, the liver residency of hepatic γδT cells was absent in Il17a−/− mice. WT and Il17a−/− mice were surgically joined by parabiosis; 2 weeks later, the WT mouse liver contained fewer Il17a−/− mouse-derived γδT cells, but the Il17a−/− mouse liver contained nearly 50% γδT cells derived from the WT mouse (Supplementary Fig. 2c). These results suggest that γδT cell liver homing might be related to their IL-17 expression ability in unknown mechanism.

In summary, these data collectively indicate that liver-resident γδT cells are indeed a unique γδT-cell subset with low expression levels of cytokine receptors, and that predominantly produce high levels of IL-17A and are retained in the liver.

The microbiota maintain hepatic γδT-17 cell homeostasis. To explore if the microbiota might influence liver-resident γδT-17 cells, antibiotics were used to clear mouse gut bacteria as previously reported. The antibiotic (Abx)-treated mice had a sharp decrease in commensal microbes, as indicated by the 99% reduction in cultivable bacteria, 95% reduction in bacterial DNA and elongated caecum (Supplementary Fig. 3a–c). Moreover, Abx-treated mice displayed a normal peripheral immune response (Supplementary Fig. 3d). Although the total number and frequency of hepatic B, T, NK, NKT and regulatory T (Treg) cells were normal in Abx-treated mice (Supplementary Fig. 3e,f), the accumulation of liver-resident γδT-17 cells during ontogeny was blocked in mice treated with antibiotics beginning in utero through the pregnant mother (Fig. 2a). The reduction in CD27 expression on hepatic γδT cells was also arrested, but the decline of hepatic γδT-1 cells was not affected (Fig. 2a).
To directly observe the interaction between hepatic γδT-17 cells and the microbiota, germ-free (GF) mice were used, which had even more significantly decreased numbers of hepatic γδT-17 cells than Abx-treated SPF mice (Fig. 2b). In addition, treating GF mice with antibiotics did not further reduce their hepatic γδT-17 cell numbers, suggesting that antibiotics were not directly toxic to γδT-17 cells (Fig. 2b). Reconstituting Abx-treated mice with gut commensal microbes was sufficient to restore their reduced hepatic γδT-17 cell numbers to a normal level (Fig. 2b). The kinetics of hepatic γδT-17 cell recovery in commensal microbioreconstructed GF mice were also studied. Commensal microbioreconstitution recovered the hepatic γδT-17 cell number gradually, reaching a similar level to that in SPF mice after 4 weeks of reconstruction (Fig. 2c). These results indicate that the accumulation of hepatic γδT cells relies on microbiota.

The reduced hepatic γδT-17 cells in Abx-treated mice and GF mice were not restricted by Vγ chain usage (Fig. 2d). Consistent with the reduced IL-17A expression by γδT cells from Abx-treated mice and GF mice, they were less differentiated and less activated, as indicated by the lower proportions of CD27+CCR6+ and CD44hiCD62Llow cells (Fig. 2e). Moreover, enhanced apoptosis and reduced proliferation of hepatic γδT cells were also observed after Abx treatment (Fig. 2e). To better understand the specific influence of the microbiota on hepatic lymphocytes, an in vivo BrdU incorporation method was used to assay the cell proliferation. Only γδT-17 cells, but not NK, αβT or even IL-17A-negative γδT cells, displayed reduced BrdU incorporation and Ki67 expression in Abx-treated mice, indicating that the microbiota specifically promoted the proliferation of hepatic γδT-17 cells, but not all lymphocytes, in the liver (Fig. 2f). Together, these data indicated that the microbiota sustain liver-resident γδT-17 cell homeostasis by maintaining their normal activation, survival and proliferation.

Global commensal load affects hepatic γδT-17 cell number. To screen for bacteria that influence liver-resident γδT-17 cell numbers, different combinations of antibiotics (A, ampicillin; V,
Figure 2 | The microbiota maintain the pool of hepatic γδ-T-17 cells in steady state. (a) FACS analysis of IL-17A, CD27 and IFN-γ expression by hepatic γδ-T cells at the indicated B6 mouse ages over time. The mice began treatment with water alone (Water) or containing antibiotics (Abx) in utero through the pregnant mother (n = 7/time point). (b) Five-week-old adult SPF B6 mice and GF mice were fed water alone (Water) or containing antibiotics (Abx) for 4 weeks. Abx-pretreated SPF mice were co-housed with normal mice for an additional 4 weeks to reconstitute their gut bacteria (Restored), and the hepatic γδ-T-17 cell numbers were evaluated by FACS (n = 4 for SPF mice, n = 5 for GF mice). (c) Ten-week-old GF mice were co-housed with SPF mice to restore their commensal microbes, and the hepatic γδ-T cell number was detected at the indicated time points post co-housing (n = 9, 4, 5, 10; from left to right). The frequency of IL-17A expression by each hepatic γδ-T-cell subtype (n = 10, 7, 5; from top to bottom) (d) and the frequency of hepatic γδ-T cells with the phenotype CD44hiCD62L−, CD27−CCR6+, Annexin-V+7AAD− and Ki67+ (n = 5 per group) (e) from Water-treated mice, Abx-treated mice and GF mice were analysed by FACS. (f) Control and Abx-treated mice were i.p. injected with 1 mg BrdU three times at 2-day intervals, and BrdU incorporation and Ki67 expression by hepatic γδ-T-17 (CD3γδTCRγ−IL-17+), IL-17A negative γδ-T (CD3γδTCRγ−IL-17−), NK (CD3NK1.1+) and 5βT (CD3γ TCRβ+) cells were evaluated by FACS. Differences between γδ-T-17 cells and other cells from control mice (indicated as #) and differences between Water- and Abx-treated mice of each subtype of cells (indicated as **) are shown (n = 6, 4; Water, Abx). The data are representative of more than three independent experiments. The mean ± s.e.m. is shown (*P < 0.05; **P < 0.01; ***P < 0.001 one-way ANOVA with post hoc test.).

vancomycin; N, neomycin; M, metronidazole) were used to treat mice. Different antibiotic mixtures had different targets (Supplementary Fig. 4a,b); thus, we produced a series of mice with commensal microbes at various clusters (Supplementary Fig. 4c) and diversities (Supplementary Fig. 4d). Although different compositions of microbes were induced in the mice (Fig. 3a), there was almost no correlation between the γδ-T-17 cell number and the antibiotic type (Fig. 3b) or the bacteria species diversity (Fig. 3c).

Interestingly, the mice with low levels of microbes always had small numbers of hepatic γδ-T-17 cells, and the mice with high levels of microbes always had large numbers of hepatic γδ-T-17 cells (Fig. 3b). Indeed, both the numbers and the proliferation percentages of hepatic γδ-T-17 cells positively correlated with the global microbe DNA loads (Fig. 3d). These results suggest that the global bacterial load is a key factor in the homeostasis of liver-resident γδ-T-17 cells.

E. coli, a typical type of commensal microbe, was chosen to further explore this hypothesis. E. coli was completely deleted in eight groups of mice, including the A, V, AM, VN, AVM, AVN, VNM and AVNM groups (Fig. 4a), but some mice without E. coli (V, AVM and AVN groups) still had a comparable level of hepatic γδ-T-17 cells as normal mice (Fig. 3b), suggesting that

CD1d presents commensal lipid antigen to hepatic γδ-T-17 cell. To investigate the mechanism by which the microbiota/E. coli promote liver-resident γδ-T-17 cell accumulation, microbiota-derived PAMPs were evaluated. Though TLR agonists, such as poly(I:C) (a TLR3 ligand), could increase the hepatic γδ-T-17 cell number in WT mice (Supplementary Fig. 5a), administration of Pam3Csk4 (a TLR1/2 ligand), LPS (a TLR4 ligand), CpG (a TLR9 ligand), curdulan (a Dectin-1 ligand) or poly(I:C) could not restore hepatic γδ-T-17 cells in Abx-treated mice (Supplementary Fig. 5b,c). Moreover, TLR2/TLR4/TLR9 deficiency did not influence hepatic γδ-T-17 cells (Supplementary Fig. 5d). The second microbiota-associated candidate for regulating hepatic γδ-T-17 cells was cytokine signalling. However, the levels of liver cytokines (Supplementary Fig. 5e) and the cytokine receptor...
expressions on hepatic γδT cells (Supplementary Fig. 5f) were unchanged in Abx-treated mice, though CD1d1 expression on PC γδT cells was reduced (Supplementary Fig. 5f), as previously reported21. Neutralizing antibodies against IL-1β and IL-23 in vivo reduced the number of PC γδT-17 cells, but not hepatic γδT-17 cells (Supplementary Fig. 5g). Thus, PAMPs and cytokine signals do not support the homeostasis of liver-resident γδT-17 cells.

The liver is rich in lipid antigens and CD1d; thus, we speculated that CD1d/lipid antigen complexes interact with liver-resident γδT-17 cells in the liver. WT and Cdl1d−/− mice were co-housed to share the same microbiota, but Cdl1d−/− mice had a decreased number of hepatic γδT-17 cells with lower proportions of CD27−CCR6+ cells compared with WT mice, similar to the result observed in Abx-treated WT mice (Fig. 5a). Co-housed NKT-deficient Jα18−/− mice had similar hepatic γδT-17 cell numbers to WT mice, demonstrating that CD1d sustained hepatic γδT-17 cells directly and independently of NKT cells (Fig. 5b). Moreover, Abx-treatment did not further downregulate the number and frequency of hepatic γδT-17 cells in Cdl1d−/− mice (Fig. 5a). Collectively, these data indicate that the microbiota maintain the homeostasis of liver-resident γδT-17 cells mainly through CD1d signalling.

To explore whether CD1d-associated lipid antigens indeed have a role in this process, Abx-treated WT mice were injected with previously reported CD1d-presenting bacterial lipid antigens, including E. coli cardiolipin (CL), PG, PE and total polar lipid extract from E. coli. After receiving exogenous E. coli-derived lipids, the liver-resident γδT-17 cell number, proliferation and CD27−CCR6+ expression levels partially but markedly recovered in Abx-treated WT mice but not in Cdl1d−/− mice (Fig. 5c), indicating that lipid antigens act in a CD1d-dependent manner. Moreover, lipid antigens still induced the recovery of the hepatic γδT-17 cell numbers in Abx-treated IL-1/IL-23-neutralized mice (Supplementary Fig. 5h) and TLR2/TLR4/TLR9-deficient mice (Supplementary Fig. 5i), which further suggested that lipid antigens maintain the pool of liver-resident γδT-17 cells in a TLR/cytokine signal-independent manner.

Figure 3 | Commensal microbe load is positively correlated with hepatic γδT-17 cell numbers. Mice were fed for 4 weeks with water alone (Water) or containing the following antibiotics for 4 weeks (A = ampicillin; V = vancomycin; N = neomycin; M = metronidazole); all four (AVNM), each one alone or in various combinations (AV, AN, AM, VN, VM, NM, AVN, AVM, VNM and ANM). (a) Genomic bacterial DNA was isolated from faecal samples, and the V5–V6 hypervariable region of 16S rDNA was amplified and sequenced. The average percentages of reads for the three experimental groups are shown.

(b) The left axis indicates the number of hepatic γδT-17 cells determined by FACS, and the right axis indicates the DNA load of bacteria in the faeces. (c) The Shannon index of diversity relative to species was calculated, and Pearson correlation curves with the γδT-17 cell number and KI67 expression are shown. (d) The Pearson correlation curves between the γδT-17 cell number and KI67 expression with the bacterial DNA load. The data are representative of more than three independent experiments (n = 7 mice per group). Either a representative plot or the mean ± s.e.m. is shown.
Some microbiota metabolic molecules may reach the liver via the portal vein. To explore if microbiota lipid antigens could reach the liver, 14C-glucose was used to label E. coli that was then intragastrically delivered into mice. After 6 h, there was high radioactivity in the lipids extracted from the livers of mice transferred with 14C-glucose labelled E. coli but not in the mice transferred with unlabelled E. coli (Fig. 5d). This suggests that microbial lipids can enter the liver at steady state, which allows them to encounter hepatic γδT-17 cells in the liver. γδT-17 cells from both the liver and the spleen were stained with CD1d tetramers loaded with different lipid antigens (PE, PG, CL and GD) tetramer-positive T-17 cells in the liver decreased markedly after microbiota depletion (Fig. 5f). These results indicate that hepatocytes can recognize microbiota-derived lipid antigens through CD1d.

Next, we asked which cells expressed CD1d stimulate hepatic γδT-17 cells. Using fetal liver chimeric mice, we found that the number of hepatic γδT-17 cells decreased only when CD1d-restricted lipid antigens presented by CD1d were reduced number of endogenous lipid antigens presented by CD1d on Abx-treated hepatocytes. After supplying exogenous lipid antigens, WT or Abx-treated, but not Cdl1d−/−, hepatocytes induced an approximately twofold increase in the γδT-17 cell number compared with those without exogenous lipid antigens added (Fig. 6e). These results indicate that hepatocytes can directly present commensal/E. coli lipid antigens through CD1d to promote liver-resident γδT-17 cell expansion.

**Hepatic γδT-17 cell accelerate HFD-induced NAFLD progression.** Using an Ilt7ra−/− mouse, Harley et al. showed that IL-17A signalling could accelerate NAFLD through recruiting neutrophils and inducing nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS, which ultimately induced hepatocellular damage. We wondered if the predominant liver-resident γδT-17 cell subtype was the source of IL-17A during NAFLD, and, importantly, whether the microbiota promoted NAFLD through these cells.

High-fat diet (HFD)-fed mice displayed elevated numbers of γδT-17 cells in several organs, including the liver (Fig. 7a, Supplementary Fig. 6a), suggesting that hepatic γδT-17 cells might be one of the main sources of IL-17A in the liver during NAFLD. Though Tcrd−/− mice a body weight increase comparable to that of WT mice (Supplementary Fig. 6b), they were protected from NAFLD by displaying reduced steatohepatitis, reduced liver damage and a catabatic glucose dysmetabolism (Fig. 7b–d). The reduced NAFLD symptoms in Tcrd−/− mice were also displayed in another mouse NAFLD model induced by high-fat/high-carbohydrate diet (HFFC) (Fig. 7e), and transfer
of HFHCD-fed mice with WT hepatic γδT cells, but not with \( \text{Il}17a^{-/-} \) hepatic γδT cells, accelerated NAFLD in \( \text{Tcrd}^{-/-} \) mice (Fig. 7e). These results demonstrate that hepatic γδT-17 cells can accelerate NAFLD.

Next, we asked whether the microbiota were involved in hepatic γδT-cell expansion during NAFLD. Both HFD and HFHCD triggered the increase and proliferation of hepatic γδT-17 cells (Fig. 8a,b). However, after depleting commensal microbes, Abx-treated mice that started with lower hepatic γδT-cell numbers had only slight increases compared with the baseline of control mice, and the numbers was far lower than the levels observed in HFD/HFHCD-induced NAFLD mice (Fig. 8a,b). Another candidate for microbiota associated-IL-17A production was Th-17 cells; unlike Th-17 cells in the intestine26 and skin27, which can be induced to proliferation by their local microbiota, the numbers of hepatic Th-17 cells did not decrease in Abx-treated mice, and HFD and HFHCD did not trigger increased hepatic Th-17 cells, indicating the irrelevant role of hepatic Th-17 cells in this process (Fig. 8c). The downstream effectors of IL-17A during NAFLD25, the numbers of neutrophils (Fig. 8d), and the mRNA levels of NADPH oxidase enzymes (Fig. 8e) also decreased in the livers of Abx-treated mice.

More importantly, Abx-treated mice exhibited a similar alleviation of NAFLD as \( \text{Tcrd}^{-/-} \) mice fed with HFD/HFHCD,
as indicated by the reduced steatohepatitis (Fig. 9a,b), the reduced ALT level (Fig. 9c), the elevated body weight (Fig. 9d) and the increased hepatic triglyceride level, elevated body weight as indicated by the reduced steatohepatitis (Fig. 9a,b), the reduced ALT level, elevated hepatic triglyceride level, elevated body weight and the elevated ALT level (Fig. 9c), the elevated body weight (Fig. 9d) and the increased hepatic triglyceride level, elevated body weight. This together with the mechanism of liver γδT-cell homeostasis found by us, further indicate that the host–microbiota interaction at distinct barrier sites occurs in a tissue-specific manner.

IL-17A production by γδT cells is often elicited by cytokines or TLRs without prior antigen exposure (termed ‘natural γδT’ cells)33, and we showed that TLR and IL-1/IL-23 signalling was not responsible for liver γδT-17 cell homeostasis in a physiological state (Supplementary Fig. 5). These two seemingly contradictory facts together exposed the mystery between γδT-cell antigen recognition and TLR/cytokine recognition. Increasing numbers of studies have indicated that encountering antigen is a prerequisite for γδT cells to respond to inflammatory cytokines34,35. Until now, the antigen specificity or antigen requirement of the ‘natural γδT-17’ cells has remained unknown or controversial30,28. However, although γδT cells in the mucosa and epithelium are well-suited to recognize microbial components, their recognized antigens remain scarce30. In our study, we demonstrate for the first time that commensal lipid antigens can reach the liver (Fig. 5d) and be presented by CD1d to the γδTCR of liver-resident γδT cells (also a type of ‘natural γδT-17’ cells), which supports the development and homeostasis of this cell subset.

The lipid antigens that specifically link CD1d and the γδT-17 cell receptor are bacterial CL36, synthetic α-GalCer37, sulfatide11,38, synthetic phosphatidylcholine, PE, PG39, and synthetic natural PE39. Among these, CL, PG and PE can be derived from both bacteria and stressed host cells40,41. Indeed, E. coli CL,
were assessed from the 4th week to the 10th week during HFHCD treatment, and the serum ALT level and GTT curve were evaluated. Furthermore, CD1d expression on hepatocytes is hepatocytes, which are also critical lipid metabolic factories. CD1d-expressing cells include hepatocytes, Kupffer cells, and DCs, but the strongest expression is found on CD1d-positive cells in Hepatitis B patients. However, this expression is not a direct result of the cross-reactivity of CD1d-lipid antigens with other antigens recognized by the TCR of NKT cells. Several types of hepatic cells, including endothelial cells, Kupffer cells and DCs, can express CD1d, but the strongest expression is found on CD1d-positive cells in Hepatitis B patients. However, this expression is not a direct result of the cross-reactivity of CD1d-lipid antigens with other antigens recognized by the TCR of NKT cells.

PG and PE could partly recover the decreased hepatic γδT-17 cells in Abx-treated mice (Fig. 5c); nevertheless, none of these antigens could completely recover the decline, suggesting that other lipid antigens may also have roles in this process. We showed that liver γδT-17 cells could also be stained by CD1d-tetramer (an improved form of α-GalCer) (Fig. 5e), which may be a result of the cross-reactivity of the CD1d-lipid antigens recognized by the γδTCR. The cross-reactivity of CD1d-phospholipids and CD1d-α-GalCer, as well as that of CD1d-CL and CD1d-phospholipids, were also observed in other γδTCR studies.

Several types of hepatic cells, including endothelial cells, Kupffer cells and DCs, can express CD1d, but the strongest expression is found on CD1d-positive cells in Hepatitis B patients. However, this expression is not a direct result of the cross-reactivity of CD1d-lipid antigens with other antigens recognized by the TCR of NKT cells. Several types of hepatic cells, including endothelial cells, Kupffer cells and DCs, can express CD1d, but the strongest expression is found on CD1d-positive cells in Hepatitis B patients. However, this expression is not a direct result of the cross-reactivity of CD1d-lipid antigens with other antigens recognized by the TCR of NKT cells.

is an interesting topic. For example, although CD1d can present α-GalCer to both γδT cells and NKT cells, the mode of γδTCR-CD1d-α-GalCer recognition appears to be markedly different from that of NKT recognition. Thus, the difference between NKT and γδT cells in CD1d recognition of microbiota-derived lipid antigens requires further investigation.

NAFLD is induced by chronic inflammation in obesity, and the progression of NAFLD is the comprehensive result of hepatic immune cells, including Kupffer cells, NK cells, and NKT cells, and liver metabolic cells, including hepatocytes and hepatic stellate cells. However, although the metabolic relationship between the microbiota and fatty liver has been shown, how the microbiota influences the hepatic immune response during NASH remains unclear. In this study, we showed that microbiota-maintained liver-resident γδT-17 cells were the main source of IL-17A and could significantly accelerate NAFLD (Fig. 7). An interesting finding was the uncoupling of decreased liver inflammation (Fig. 9a–c) and increased body weight (Fig. 9d) in Abx-treated mice, which was also found by other groups in Il17a<sup>−/−</sup> mice and Abx-treated mice. However, this uncoupling was not found in Tcrd<sup>−/−</sup> mice, who
displayed reduced liver inflammation (Fig. 7b–e) but comparable body weights to those of WT mice, as shown by us (Supplementary Fig. 6b) and others. 51 This suggests that different mechanisms are used by IL-17A to promote hepatic inflammation and lipid metabolism and that the lipid metabolism pathway might be compensated for by other signals in Tcrd−/− mice.

After depleting commensal microbes, hepatic γδT-17 cells did not increase (Fig. 8a), but they did increase in the presence of microbiota during NAFLD. Nevertheless, same as γδT-17 cells in the liver, γδT-17 cells in the gut and other tissues also increase during NAFLD (Supplementary Fig. 6a), mainly because NAFLD is a systemic disease with inflammation in multiple organs. 52 Thus, though there is no mutual exchange or circulation between hepatic γδT cells (Fig. 1e) and gut γδT cells, 53 at steady state, we still cannot fully exclude the possibility that a portion of gut γδT-17 cell would traffic to the liver in the disease state. However, instead of the cellular traffic model, we depict a molecular traffic model by displaying that E. coli lipid can reach the liver from gut (Fig. 5d); then the lipid antigen can be presented by hepatocyte-expressed CD1d to directly stimulate hepatic γδ T cells (Fig. 6c–e) and ultimately alleviate hepatic γδT-17 cell clones that are specific to CD1d-lipid antigen tetramer (Fig. 5e). Nevertheless, four possible mechanisms exist for the accumulation of hepatic γδT-17 cell

---

**Figure 8 | The microbiota promote the expansion of liver-resident γδT-17 cells during HFD/HFHCD-induced NAFLD.** Normal water-fed mice and Abx-treated B6 mice were placed on an HFD (LFD as control) and an HFHCD (LFLCD as control) for 24 weeks. (a) The hepatic γδT-17 cell number, (b) Ki67 expression of hepatic γδT cells, (c) hepatic Th-17 cell number and (d) neutrophil number in the liver were detected by FACS. (e) Water- and Abx-treated B6 mice were placed on an HFD or an HFHCD for 12 weeks. The hepatic mRNA expression levels of Nox2, p47phox, p67phox and p22phox were evaluated. The data are representative of three independent experiments with five mice per group and shown by the mean ± s.e.m. (*P < 0.05; **P < 0.01; ***P < 0.001 one-way ANOVA with post hoc test.).
during NAFLD from our analysis. First, the excessive accumulation of fat-related lipid antigens during NAFLD may further affect hepatic γδT-17 cells; because it is difficult to distinguish the sources of lipids in vivo, there is still more work to do. Second, because NAFLD is always accompanied by the over growth of commensal microbes47–49, an elevated global microbe load or several specific bacteria may induce a higher number of hepatic γδT-17 cells as described in our study. Third, HFD-induced inflammation may stimulate the microbiota-maintained hepatic γδT-17 cells as a bystander effect. Forth, there may be a portion of γδT-17 cells activated in other tissues (for example, gut) traffic into the liver.

Similar to that observed in the mouse liver, healthy human livers also contain a distinct Vδ3+ γδT-cell subset51; interestingly, they recognize CD1d and release IL-17A after activation55. However, their detailed characteristics and precise mechanisms are unclear. Using a mouse model, our work demonstrates for the first time that hepatic γδT cells are a unique liver-resident subset. Hepatocyte CD1d could present gut commensal lipid antigens to hepatic γδT cells, which drove them to predominantly produce IL-17A and maintained their haemostasis. Our results reveal a novel crosstalk between metabolism and immunity in the liver–gut axis and also suggest a tissue-specific interaction between the microbiota and immune cells in the liver (Supplementary Fig. 7).

Methods
Mice. C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center (SLAC, Chinese Academy of Sciences); Rag1−/− mice were obtained from the Model Animal Research Center (Nanjing University); Cxcr6−/− and CD45.1+

Figure 9 | The microbiota accelerate HFD/HFHCD-induced NAFLD through hepatic γδT-17 cells. (a–e) Mice were treated as described in Fig. 8. (a) Representative liver histology (H&E staining, scale bar, 200 μm); arrowheads indicate the steatohepatitis foci of inflammation with clusters of inflammatory cells, and the red rectangle indicates a 4× zoom of the gated region. (b) Numbers of steatohepatitis foci counted in a (n = 5 per group). (c) Serum ALT level, (d) body weight curve and (e) glucose tolerance test (GTT) and insulin tolerance test (ITT) curves are shown (n = 5 per group). (f–i) HFD-fed, Abx-treated mice were either injected with IL-17A (500 ng, once per week) or transferred with hepatic γδT cells (2 × 10⁴, once per 2 weeks) from the 4th week to the 24th week of HFD treatment. (f) The serum ALT level, (g) hepatic triglyceride level, (h) body weight curve and (i) GTT curve are shown (n = 5 per group). The data are representative of three independent experiments and shown by the mean ± s.e.m. (*P < 0.05; **P < 0.01; ***P < 0.001 one-way ANOVA with post hoc test (b,c,f,g), two-way ANOVA test (d,e,h,i).
mice were purchased from the Jackson Laboratory. Tetr−/− mice were a gift from Dr Zhilan Yin (Nankai University); TgR2−/−, TgR9−/− and TgRβ−/− mice were a gift from Dr Zhixiong Liu (University of Science and Technology of China (USTC)); and Jazb1−/− and CD1d−/− mice were a gift from Dr Shaobo Su (Sun Yat-sen University). Animal Feed High-Tech Co. Ltd. (Huizhou, China) were approved by Local Ethics Committee for Animal Care and Use at University of Science and Technology of China. Germ-free mice were purchased from SLAC and housed in the germ-free facility at SLAC according to their animal care regulations. The sample size was determined by the ‘resource equation’ method, taking into account the possible reduction of diet/drink treatment.

Mouse treatment. Six- to ten-week-old male mice were used in most of the experiments with exceptions, such as the use of neonatal mice in the ontogeny experiment and the use of 30-week-old mice in the HFD/HFHCID-induced NAFLD experiment. Some viral mice were depleted using antibodies as previously reported50, the detailed method are followed. Four kinds of antibodies including ampicillin (1 g l−1), vancomycin (0.5 g l−1), neomycin sulfate (1 g l−1) and metronidazole (1 g l−1) were dissolved in sterile water and stored in 4°C no more than a week before using. This antibiotic-contained water was supplied as drinking water to adult mice and pregnant mice for more than 4 weeks and was changed every 3 days. The volume of drinking water and the body weights of the mice were monitored twice a week. Mice exhibiting more than a 30% decline in body weight were removed. After antibiotic treatment stopped, Abx-treated mice were co-housed with mice for 4 weeks to reconstitute commensals.

For the PAMP restoration experiment, WT mice and Abx-treated mice were intraperitoneally (i.p.) injected with 50 µg curdlan, 50 µg Pam3csk4, 100 µg LPS, 100 µg poly(I:C) or 50 µg CpG (all from Sigma) and harvested 1 day later. For cytokine neutralization, the mice were intravenously (i.v.) injected with 50 µg control IgG, anti-IL-23 or anti-IL-23 antibody twice at 3-day intervals and harvested 3 days later. For the lipid antigen restoration experiment, the mice were i.p. injected with 20 µg E. coli Cl, PG or PE or 50 g E. coli poliar liquid extract (all from Avanti Polar Lipids, Alabama) six times at 2-day intervals and harvested 2 days after the last injection. For macrophage and DC depletion, the mice were i.v. injected with 200 µl CLEMMP-Lip (Vrije Universiteit) twice at 3-day intervals and harvested 3 days later. All of the mice receiving the above treatment were randomly assigned to different groups, but the investigators were not blinded to the treatments or genotypes.

Bacterial diversity analysis. Fresh stool samples were collected and weighed, and bacterial DNA was extracted from the stool using a QiAamp Fast DNA Stool Mini Kit (Qiagen). The 16S rRNA gene was analysed to determine the bacterial composition and diversity using an Illumina MiSeq (Novogene Bioinformatics Technology Co., Ltd.).

For bacterial titre analysis, fresh stools were collected and homogenized in sterile PBS. The serially diluted homogenates were plated onto blood agar plates (for total cultivable bacterial determination) or eosin methylene blue (EMB; for E. coli determination) plates at 37°C for 24 h. The colonies were distinguished by their biochemical reactions and counted.

Faeces/E. coli transfer. Mice were treated with antibiotics for 4 weeks, and their antibiotic-containing water was replaced with antibiotic-free water. Then, the mice were intragastrically administered 0.2 ml fresh faeces (50 mg ml−1) or 1010 intact14C-labelled c.f.u. of E. coli that was isolated from an EMB plate and expanded in LB. The mice were analysed 3 weeks later.

14C labelling of E. coli. E. coli was 14C labelled as previously reported50 with a slight modification. Briefly, E. coli was isolated from fresh mouse faeces using an EMB plate, expanded in M9 minimal medium to OD600 = 0.4 and spiked with 25 µCi/ml 14C-glucose (NECO42V290UC, PerkinElmer) for 16 h. A total of 1010 c.f.u. of unlabelled or 14C-labelled E. coli was intragastrically delivered to the mice. The livers were collected 6 h later, the lipids in the liver were extracted using a methanol/chloroform (2:1) mixture and radioactivity was assessed.

Parabiosis. CD45.1−/− and CD45.2−/− mice were joined by parabiosis for 2 weeks as previously described57. Age matched and co-housed mice were anaesthetic and pre-injected and at various times post injection of glucose or insulin. The glucose levels in the blood were assayed by an automated glucose meter (LifeScan). Serum ALT and AST were determined using an automated Chemray 240 clinical analyzer (Rayto, Shenzhen, China). Liver samples that were fixed in 4% paraformaldehyde and paraffin-embedded were sliced into 5-µm-thick sections and stained with H&E. Lipids in the frozen liver tissue were extracted using a methanol/chloroform (2:1) mixture and dissolved in isopropyl alcohol. The triglyceride level was detected using a kit (Hulii, China).

Cell preparation. Mice were killed, and the iNs, spleens, livers, thymi, intestines, colons and mesenteric LNs were collected. Mononuclear cells (MNCs) from each organ were then separated as previously described with slight changes58. Briefly, LNs and thymi were passed through a 200-µm steel mesh and washed with PBS. Spleens were first passed a 200-µm steel mesh then RBC lysed and washed with PBS. Livers were passed a 200-µm steel mesh and the cell pellet were collected in the flow through, the MNCs in the pellets were isolated by gradient centrifugation with 40 and 70% Percoll. Lungs were first excised and minced to small pieces, then digested with 0.1% collagenase I for 1 h at 37°C, the large pieces of lungs were removed by filtering and the MNCs in the flow through were obtained by gradient centrifugation with 40 and 70% Percoll. Intestines and colons were surgically excised the peyer’s patch and then excised to small pieces, digested with 1 mM DTT for 15 min at 37°C and passed a 200-µm mesh, IEL in the flow through were isolated by gradient centrifugation with 40 and 70% Percoll. The unfiltered tissue was further digested with collagenase IV for 1 h at 37°C and filtered through a 100-µm mesh, the flow through was isolated by gradient centrifugation with 40 and 70% Percoll. PC MNCs were obtained by peritoneal lavage using cold PBS. Hepatic γδT cells were purified using a mouse TCRγδ+ T-Cell Isolation Kit (Miltenyi Biotec).

Fetal liver MNC chimera. To construct a hematopoietic chimera, 2 × 106 fetal liver MNCs from an E18.5 mouse were transfused to lethally irradiated 10-week-old mice (11 Gy, 1 day before transfer). Hepatic γδT cells were analysed 20 weeks later.

In vitro co-culture. Hepatocytes were separated using a previously described two-step perfusion method59. Briefly, mice were anaesthetized and the portal vein was cannulated. The liver was first digested with 0.5 mM EGTA and then digested with 0.05% collagenase I, washed with PBS, Livers were passed a 200-µm steel mesh and the cell pellet were collected in the flow through, the MNCs in the pellets were isolated by gradient centrifugation with 40 and 70% Percoll. The unfiltered tissue was further digested with collagenase IV for 1 h at 37°C and filtered through a 100-µm mesh, the flow through was isolated by gradient centrifugation with 40 and 70% Percoll. PC MNCs were obtained by peritoneal lavage using cold PBS. Hepatic γδT cells were purified using a mouse TCRγδ+ T-Cell Isolation Kit (Miltenyi Biotec).

Flow cytometry analysis. freshly isolated MNCs were blocked and incubated with the indicated fluorescent mAbs for 30 min at 4°C. The cells were stained with TROPHIC Animal Feed High-Tech Co. #TP26301 + carbohydrates (18.9 g l−1 sucrose + 23.1 g l−1 fructose) in drinking water) and LFLCD (fat 12.5% kcal, carbohydrate 68.1% kcal, protein 19.4% kcal; TROPHIC Animal Feed High-Tech Co. #TP26323 + carboxylate-free drinking water). Fresh food was supplied twice a week, and the food and drink consumption were quantified throughout the experiment. The body weights of the mice were monitored weekly.

For the IL-17A and hepatic γδT-cell transfer experiment, Abx-treated mice were i.v. injected with IL-17A protein (PeproTech, 500 ng, once per week) or purified hepatic γδT cells (2 × 106, once per 2 weeks) from the 4th week on an HFD until harvesting at the 24th week. Tetr−/− mice were i.v. injected with purified hepatic γδT cells (2 × 106, once per week) from the 4th week on a HFFCD until harvesting at the 10th week.

For the GTT test, mice were fasted for 12 h and i.p. injected with 1 g kg−1 glucose; for the ITT test, mice were fasted for 3 h and i.p. injected with 0.5 mg kg−1 human fast-acting insulin ( Lilly France). Tail vein blood was collected both pre-injection and at various times post injection of glucose or insulin. The glucose levels in the blood were assayed by an automated glucose meter (LifeScan). Serum ALT and AST were determined using an automated Chemray 240 clinical analyzer (Rayto, Shenzhen, China). Liver samples that were fixed in 4% paraformaldehyde and paraffin-embedded were sliced into 5-µm-thick sections and stained with H&E. Lipids in the frozen liver tissue were extracted using a methanol/chloroform (2:1) mixture and dissolved in isopropyl alcohol. The triglyceride level was detected using a kit (Hulii, China).

Fetal liver MNC chimera. To construct a hematopoietic chimera, 2 × 106 fetal liver MNCs from an E18.5 mouse were transfused to lethally irradiated 10-week-old mice (11 Gy, 1 day before transfer). Hepatic γδT cells were analysed 20 weeks later.
analysed using FlowJo software with a gating strategy showed in Supplementary Fig. 8.

**BrDU incorporation.** Mice were i.p. injected with 1 mg BrDU three times at 2-day intervals. The BrDU+ cell frequency was evaluated according to the FITC BrDU Flow Kit instructions (BD Pharmingen).

**Quantitative RT-PCR.** Total RNA from liver tissue was extracted using TRIzol reagent (Invitrogen). Gene expression was analysed according to the instructions of the SYBR Premix Ex Taq kit (Takara) and quantified using the ΔΔCT method. All primers (Supplementary Table 2) were synthesized by Sangon (Shanghai, China).

**Statistics.** Student’s t-test for two groups and a one-way ANOVA for more than two groups were used to determine statistically significant differences. A two-way ANOVA test was used to determine differences in the GTT and IPT tests. Differences achieving values of P<0.05 were considered statistically significant.

**Data availability.** The data that support the findings of this study are available within the article and/or Supplementary Information files or from the corresponding authors on request.

**References**
1. Racanelli, V. & Rehermann, B. The liver as an immunological organ. Hepatology 43, 554–562 (2006).
2. Gao, B., Jeong, W. I. & Tian, Z. Liver: An organ with predominant innate immunity. Hepatology 57, 729–736 (2008).
3. Jenne, C. N. & Kubes, P. Immune surveillance by the liver. Nat. Immunol. 14, 996–1006 (2013).
4. Kojima, M., Honda, K., Noguchi, M. & Ta J. Y. Innate immune surveillance by the liver. Nature 505, 55–62 (2014).
5. Chassaing, B., Etienne-Mesmin, L. & Gewirtz, A. T. Microbiota-liver axis in metabolic disease. Nature 529, 62–67 (2016).
6. Ivanov, I. I. et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349 (2008).
7. Naik, S. et al. Characterized control of skin immunity by resident commensals. Science 337, 1115–1119 (2012).
8. Chien, Y. H., Zeng, X. & Prinz, I. The natural and the inducible: interleukin (IL)-17-producing gammadelta T cell subsets. Trends Immunol. 34, 151–154 (2013).
9. Zeng, X. et al. gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. Immunity 37, 524–533 (2012).
10. Zeng, X. et al. Gamma delta T cells recognize haptons and mount a hapten-specific response. Elife 3, e03609 (2014).
11. Dieude, M. et al. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. J. Immunol. 186, 4771–4781 (2011).
12. Uldrich, A. P. et al. CD1d-lipid antigen recognition by the gammadelta TCR. Nat. Immunol. 14, 1137–1145 (2013).
13. Luoma, A. M. et al. Crystal structure of Vdelta1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human gammadelta T cells. J. Immunol. 187, 1051–1063 (2011).
14. Joyce, S. A. et al. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proc. Natl Acad. Sci. USA 111, 7421–7426 (2014).
15. Mehta, P. et al. NALFD: a multisystem disease. J. Hepatol. 62, S47–S56 (2015).
53. Young, A. J., Marston, W. L. & Dudler, L. Subset-specific regulation of the lymphatic exit of recirculating lymphocytes in vivo. J. Immunol. 165, 3168–3174 (2000).

54. Kenna, T. et al. Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver. Clin. Immunol. 113, 56–63 (2004).

55. Mangan, B. A. et al. Cutting edge: CD1d restriction and Th1/Th2/Th17 cytokine secretion by human Vdelta3 T cells. J. Immunol. 191, 30–34 (2013).

56. Gomez de Agüero, M. et al. The maternal microbiota drives early postnatal innate immune development. Science 351, 1296–1302 (2016).

57. Liu, K. et al. Origin of dendritic cells in peripheral lymphoid organs of mice. Nat. Immunol. 8, 578–583 (2007).

58. Wang, J. et al. Lung natural killer cells in mice: phenotype and response to respiratory infection. Immunology 137, 37–47 (2012).

59. Chen, Y. et al. Increased susceptibility to liver injury in hepatitis B virus transgenic mice involves NKG2D-ligand interaction and natural killer cells. Hepatology 46, 706–715 (2007).

Acknowledgements
This work was supported by the Ministry of Science & Technology of China (973 Basic Science Project 2013CB944902, 2013CB530506), the Natural Science Foundation of China ((#81401366, #91429303, #31230025), a General Financial Grant from the China Postdoctoral Science Foundation (2014M550351) and a Special Financial Grant from the China Postdoctoral Science Foundation (2015T80661). We thank the National Institutes of Health Tetramer Core Facility for providing the CD1d tetramers.

Author contributions
F.L. designed and performed most of the experiments and wrote the manuscript; X.H. and Y.C. designed and performed some of the experiments; L.B., X.G. and Z.L. designed the experiments and supplied important mouse model; H.W. and R.S. designed and supervised the experiment, and revised the manuscript; and Z.T. provided the study strategy, designed and supervised the experiments, wrote the manuscript and financially supported the study.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Li, F. et al. The microbiota maintain homeostasis of liver-resident γδT-17 cells in a lipid antigen/CD1d-dependent manner. Nat. Commun. 8, 13839 doi: 10.1038/ncomms13839 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017
Erratum: The microbiota maintain homeostasis of liver-resident γδT-17 cells in a lipid antigen/CD1d-dependent manner

Fenglei Li, Xiaolei Hao, Yongyan Chen, Li Bai, Xiang Gao, Zhexiong Lian, Haiming Wei, Rui Sun & Zhigang Tian

Nature Communications 8:13839 doi: 10.1038/ncomms13839 (2017); Published 9 Jan 2017; Updated 6 Apr 2017

The HTML version of this Article previously published had an incorrect volume number of 7; it should have been 8. This has now been corrected in the HTML; the PDF version of the paper was correct from the time of publication.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017