of feeding. These observations led us to set the time point for estimating liver steatosis and injury and inflammation or liver fibrosis during the course of feeding at week 5 or week 15, respectively. At the end of the indicated periods, the mice were weighed and anesthetized with pentobarbital sodium, and then their abdomens were opened. Following blood sampling via the inferior caval vein, the portal vein and inferior caval vein were cut to enable blood outflow and then the liver was removed, weighed, and processed for further analyses. All animal experimental protocols were approved by the Institute of Experimental Animal Science, Osaka University. To evaluate the levels of liver injury, serum alanine aminotransferase (ALT) activities were measured as previously described [23]. To determine the levels of steatosis, total lipids were extracted from the liver and then triglyceride content was measured as previously described [24].

Flow cytometric analysis

Liver mononuclear cell populations were prepared as previously described [11, 23]. Cell surface staining of the prepared cells was performed as described [11, 23], using the following antibodies or tetramers: fluorescein isothiocyanate-conjugated anti-CD49b (DX5), phycoerythrin-conjugated anti-CD4 (H129.19), peridinin chlorophyll protein-conjugated anti-CD8α (53-6.7), and allophycocyanin-conjugated anti-TCRβ (H57-597) monoclonal antibody, or fluorescein isothiocyanate-conjugated anti-TCRβ, phycoerythrin-conjugated anti-CD4, peridinin chlorophyll protein-conjugated anti-CD45R/B220 (RA3-6B2) monoclonal antibody, and allophycocyanin-conjugated mouse-CD1d tetramers loaded with α-galactosylceramide. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). Mouse CD1d tetramer was obtained from Proimmune (Oxford, UK) and the loading with α-galactosylceramide was performed following the manufacturer's protocol. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the CELLQuest program (Becton Dickinson). iNKT cells were detected on electronically gated CD45R/B220- TCRβ+ CD1d-tetramer-reactive cells.

RNA isolation and analysis

Total RNA was isolated from frozen liver tissues by using an RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Complementary DNA was synthesized from isolated RNA using SuperScript III and random hexamer (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) normalized to beta-actin.

Histological evaluation

The removed liver was partly fixed in 10% formalin for staining with hematoxylin–eosin (H&E), Sirius-Red, or Oil-red-O, or it was immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen for immunohistochemical staining. Sirius-Red staining was performed to assess liver fibrosis, which was quantified by the extent of the area, using image-analysis software, WinROOF (Mitani, Fukui, Japan). Intracellular lipid was stained with Oil-red-O. To evaluate the infiltration of CD4+ cells or CD8+ cells into the liver, acetone-fixed fresh-frozen tissue sections were immunostained with anti-mouse CD4 (H129.19) or anti-mouse CD8α (53-6.7) monoclonal antibody, respectively, using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. The sections were developed with diaminobenzidine (DAB) substrate (Vector Laboratories) and then counterstained with hematoxylin. Antibody against CD4 or CD8 was purchased from BD Biosciences.

Statistical analysis

The statistical significance of differences between two groups was determined by applying the Mann–Whitney *U*-test. Statistical significance was defined as $P < 0.05$. All data are shown as mean ± standard error of the mean (SEM).

Results

Lipid accumulation in the liver induced by the HFD was independent of the presence or absence of iNKT cells

To investigate the role of iNKT cells in the development of diet-induced steatosis/steatohepatitis, we fed the ND or HFD to WT and KO mice for 5 weeks. The HFD increased the body weight by around 30% at week 5 in both WT and KO mice, while the ND increased it by around 14% (HFD-fed WT mice 31.6 ± 2.4%, HFD-fed KO mice 29.7 ± 5.6%, ND-fed WT mice 15.5 ± 0.6%, ND-fed KO mice 13.5 ± 1.1%; $n = 5$). The weight gains with the HFD or ND were not significantly different between WT and KO mice. Evaluation of the liver weight at week 5 showed that the HFD-fed WT or KO mice possessed significantly heavier livers than the ND-fed WT or KO mice, respectively, without any significant differences between the WT and KO mice (HFD-fed WT mice 1.95 ± 0.06 g, HFD-fed

KO mice 1.89 ± 0.07 g, ND-fed WT mice 1.52 ± 0.04 g, ND-fed KO mice 1.50 ± 0.06 g; $n = 5$).

We next performed Oil-red-O staining of liver sections from the mice to examine whether the absence of iNKT cells would affect the HFD-induced lipid accumulation in the liver. The staining showed that the HFD, compared with the ND, induced marked lipid retention in hepatocytes in both WT and KO mice (Fig. 1a). Evaluation of the liver triglyceride level demonstrated that the HFD, compared with the ND, clearly induced triglyceride accumulation in the livers of both WT and KO mice, without a significant difference between these groups of mice (Fig. 1b).

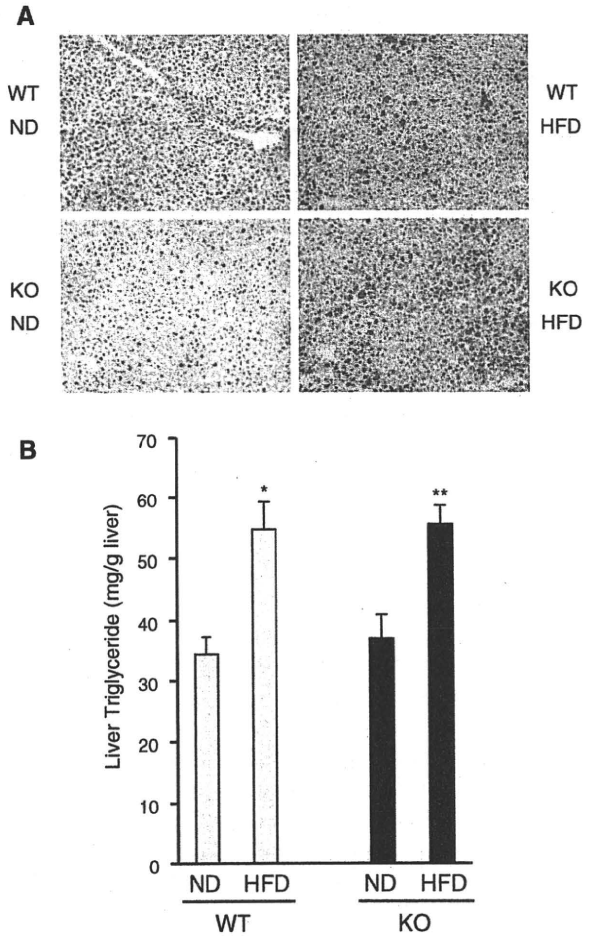

Fig. 1 Lipid accumulation in the liver induced by high-fat diet (*HFD*). Livers were obtained from BALB/c wild-type (*WT*) and BALB/c Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or an HFD for 5 weeks. **a** Lipid accumulation in liver sections was visualized by Oil-red-O staining. Representative images are shown (×200). **b** Hepatic triglyceride levels were quantified. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. $*P < 0.05$ versus WT fed ND. $**P < 0.05$ versus KO fed ND

Collectively, these results suggested that the absence of iNKT cells did not affect the level of HFD-induced steatosis.

HFD augmented liver injury and inflammation in the absence of iNKT cells

To examine the levels of liver injury, we measured ALT activity in serum from WT and KO mice fed the ND or HFD at week 5 after the start of being fed the diets. The serum ALT level in the HFD-fed WT mice (35.8 ± 1.98 IU/l) was significantly higher than that in the ND-fed WT mice (25.2 ± 0.66 IU/l) (Fig. 2a). The serum ALT level in the HFD-fed KO mice (174.8 ± 61.2 IU/l) was also significantly higher than that in the ND-fed KO mice (36.4 ± 7.48 IU/l). It was also higher than that in the HFD-fed KO mice at week 2 (83.3 ± 16.5 IU/l). Of note is the finding that the magnitude of the increase in ALT level at week 5 was clearly much higher in KO (4.9-fold) than in

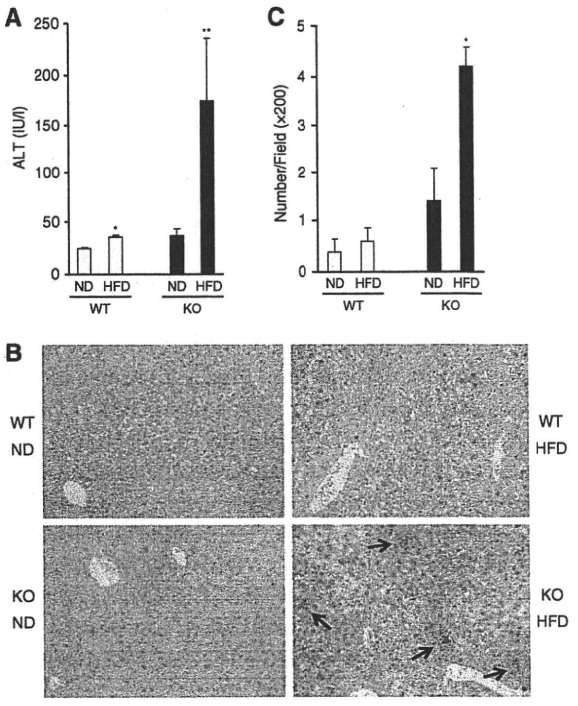

Fig. 2 Liver injury and inflammation exacerbated by HFD in the absence of invariant natural killer T (*iNKT*) cells. Serum and livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Serum alanine aminotransferase (*ALT*) levels were measured. $*P < 0.05$ versus WT fed ND. $**P < 0.05$ versus KO fed ND. **b** Liver tissues were stained with hematoxylin–eosin. Representative images are shown (×200). *Arrows* indicate the inflammatory foci. **c** The numbers of the foci were counted in five different fields per section. $*P < 0.05$ versus KO fed ND. All data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments

WT mice (1.5-fold), even though the serum ALT level in the ND-fed KO mice was modestly higher than that in the ND-fed WT mice.

We next conducted histological analyses of liver sections from the mice. H&E staining revealed that the livers from KO mice fed the HFD possessed not only steatotic areas but also scattered inflammatory foci composed of gathering nonparenchymal cells (Fig. 2b). Although inflammatory foci were also observed in the livers from KO mice fed the ND, a larger number of foci could clearly be seen in KO mice fed the HFD than in KO mice fed the ND (Fig. 2c). In contrast, WT mice fed the HFD, as well as those given the ND, showed few inflammatory foci. Taken together, these results indicated that the HFD augmented liver inflammation in KO mice but not in WT mice.

The HFD enhanced hepatic inflammation-related gene expression in the absence of iNKT cells

To understand the underlying mechanisms of the hepatic inflammation induced by the HFD in the absence of iNKT cells, we first examined the levels of several cytokines and chemokines in the livers from mice at week 5 after they had been started on the diets. Real-time RT-PCR analyses revealed that the messenger RNA expression of tumor necrosis factor (TNF)-α, IFN-γ, IL-10, chemokine (C–C motif) ligand (CCL) 2 and 4, and chemokine (C–X–C motif) ligand (CXCL) 9 and 10 were remarkably upregulated by the HFD, compared with the ND, in KO but not in WT mice (Fig. 3), although these values in KO mice fed the ND were modestly higher than those in WT mice fed the ND. In contrast, the messenger RNA expression of IL-4, -5, and -13 did not show any detectable levels in the livers from both WT and KO mice fed either the ND or HFD.

The HFD altered the proportions of subpopulations in liver mononuclear cells from KO mice, but not in those from WT mice

We next examined the phenotype of mononuclear cells in livers from mice at week 5 of feeding. Flow cytometric analyses demonstrated that the proportion of CD4+ TCRβ+ CD4 T cells was lower in KO mice fed the ND than in WT mice fed the ND (Fig. 4a), which might have resulted from a lack of iNKT cells partly composed of CD4+ cells [5]. The proportion of CD49b+ TCRβ− NK cells or CD8+ TCRβ+ CD8 T cells was higher in KO mice fed the ND than in WT mice fed the ND. The HFD did not lead to any significant changes in the proportion of hepatic CD4 T, CD8 T, NK, or iNKT cells in WT mice. In contrast, the HFD induced significant increases in the proportion of hepatic CD4 T cells and CD8 T cells, but not of NK cells, in KO mice. We then examined the distribution of these cells

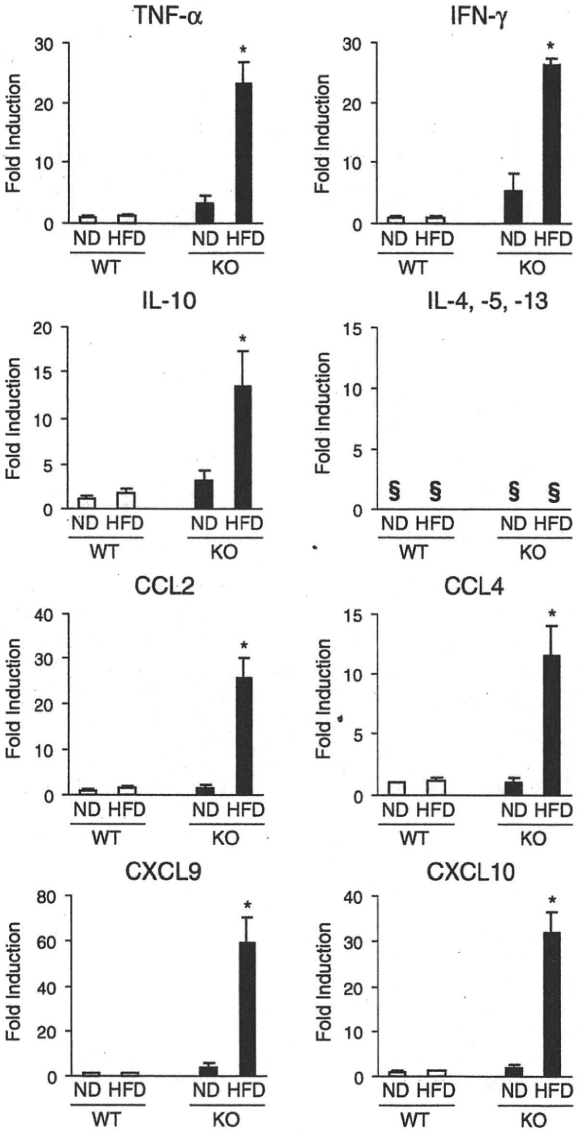

Fig. 3 Inflammatory cytokine and chemokine gene expression in the liver. Liver tissues were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. Liver RNA levels of the indicated genes and beta-actin as a control were analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR). Data are shown as the fold increase of HFD-fed WT, ND-fed KO, or HFD-fed KO compared with ND-fed WT mice, with means ± SEM from five mice in each group. Data are representative of more than four independent experiments. *TNF-α* Tumor necrosis factor alpha, *IFN-γ* interferon gamma, *IL* interleukin, *CCL* chemokine (C–C motif) ligand, *CXCL* chemokine (C–X–C motif) ligand. §, not detected. $*P < 0.05$ versus KO fed ND

in the liver. Immunohistochemical examination revealed that CD4+ cells and CD8+ cells formed foci surrounding hepatocytes in the livers of KO mice (Fig. 4b), which partly corresponded to the inflammatory foci observed in the

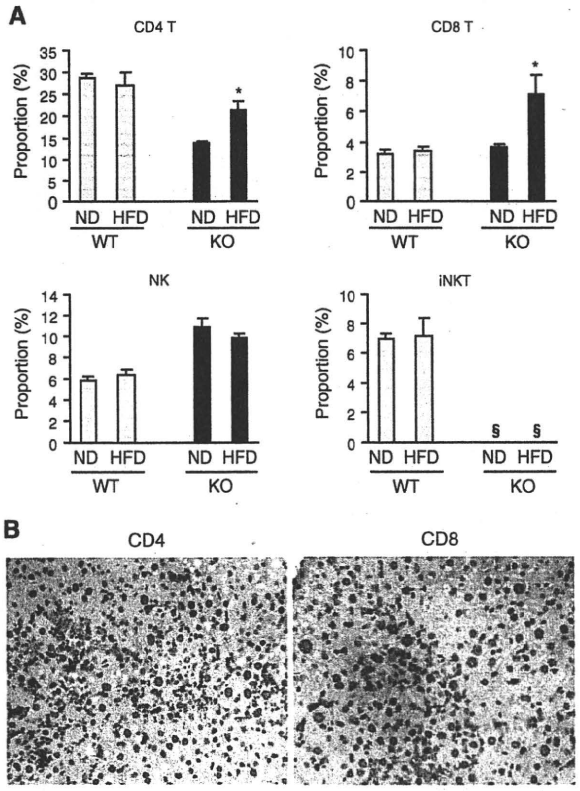

Fig. 4 Involvement of CD4 T and/or CD8 T cells in liver inflammation. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Prepared mononuclear cells from the livers were stained with cell markers indicated in "Materials and methods". Proportions of the indicated cell population were analyzed by flow cytometry. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. §, not detected. $^*P < 0.05$ versus KO fed ND. **b** Liver sections were analyzed by immunohistochemical staining for CD4- or CD8-positive cells. Representative images are shown (×200)

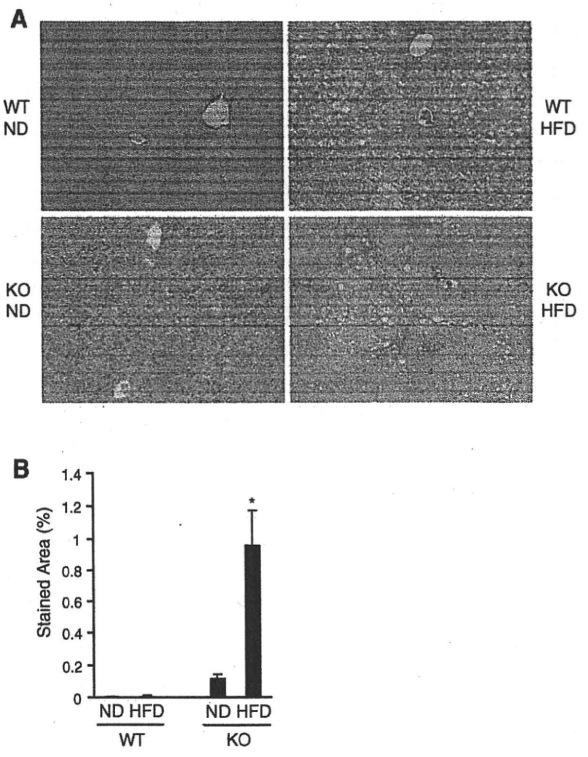

Fig. 5 Liver fibrosis following inflammation in the absence of iNKT cells. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 15 weeks. **a** Liver tissues were stained with Sirius-Red to assess liver fibrosis. Representative images are shown (×200). **b** The stained areas were evaluated in five different fields per section. Data shown are means ± SEM from five mice in each group. Data are representative of more than two independent experiments. $^*P < 0.05$ versus KO fed ND

H&E-stained liver sections of KO mice. WT mice fed either the ND or HFD did not display such foci consisting of stained cells in the livers. Collectively, these results suggested that CD4 T and/or CD8 T cells played a role in the HFD-enhanced liver inflammation in KO mice.

The HFD led to the development of liver fibrosis in the absence of iNKT cells

Persistent hepatic inflammation causes fibrotic changes in the liver [25]. To investigate whether inflammation with steatosis due to the HFD in KO mice would induce fibrosis in the liver, we fed the ND or HFD to WT and KO mice for a longer period of 15 weeks. H&E staining and Oil-red-O staining showed that KO mice fed the HFD possessed the inflammatory foci, together with lipid retention in the liver, at week 15, as well as showing these findings at week 5, and that WT mice fed the HFD showed lipid retention, but few inflammatory foci, in the liver at week 15 (data not shown). Sirius-Red staining revealed clear fibrosis in the livers from KO mice fed the HFD and also in the livers from KO mice fed the ND, but to a much lesser extent (Fig. 5a). In contrast, the staining showed no obvious fibrosis in the livers from WT mice fed the HFD or in those given the ND. Quantitative analyses to evaluate the stained areas also showed that the HFD-fed KO mice possessed significantly greater areas of hepatic fibrosis than the ND-fed KO mice, while WT mice fed either the ND or HFD had few fibrotic areas (Fig. 5b). Taken together, these results indicated that the HFD led to the development of liver fibrosis accompanied by steatohepatitis in KO mice.

Discussion

An increasing amount of evidence suggests that iNKT cells play a role in immune responses in the liver [12], although

the exact implication of that role is controversial. iNKT cells, for instance, have been reported to play a critical role in animal models of liver injury induced by concanavalin A, α-galactosylceramide, or salmonella infection [26–28], suggesting a proinflammatory role of these cells. On the other hand, iNKT cells have been very recently implicated in the suppression of liver damage in a mouse model of cholestasis [29], suggesting an anti-inflammatory role of these cells. The present study, using iNKT cell-deficient mice fed an HFD, demonstrated that the HFD led to the development of steatohepatitis with fibrosis in the absence of iNKT cells, while the HFD led to steatosis but not steatohepatitis in the presence of these cells. This suggests that iNKT cells play a critical role in suppressing the development of inflammation and fibrosis in the steatotic liver.

Our real-time RT-PCR analyses demonstrated that CCL2, CCL4, CXCL9, and CXCL10 were remarkably upregulated by the HFD in KO mice but not in WT mice (Fig. 3). CCL2 or CCL4 has the ability to attract predominantly Th1 cells via chemokine (C–C motif) receptor 2 or 5, respectively. CXCL9 and CXCL10 also attract predominantly Th1 cells via chemokine (C–X–C motif) receptor 3 [30, 31]. Indeed, Th1 cytokines such as TNF-α and IFN-γ were remarkably upregulated by the HFD in KO mice but not in WT mice. Although IL-10, which is one of the anti-inflammatory cytokines, was also upregulated by the HFD in KO mice but not in WT mice, the upregulation of IL-10 may have counteracted the upregulation of the proinflammatory Th1 cytokines TNF-α and IFN-γ. Our flow cytometric analyses and immunohistochemical analyses showed that the proportions of CD4 T and CD8 T cells were increased (Fig. 4a) and that these cells also accumulated to form foci (Fig. 4b) in the livers of KO mice fed the HFD. Bigorgne et al. [32] reported that HFD-induced obesity in leptin-deficient ob/ob mice rendered hepatic mononuclear cells, particularly CD4 T and CD8 T cells, sensitive to chemokines such as CXCL12 and CXCL13, which attract T cells, suggesting an important role of chemokines in liver inflammation with steatosis. Although the sources of the chemokines upregulated in our model were not clear, these chemokines presumably play an important role in the infiltration of proinflammatory cells in the liver of the KO mice fed the HFD. iNKT cells suppress the production of these chemokines directly or indirectly; thus, they may prevent steatohepatitis induced by an HFD.

The liver can be anatomically exposed to gut-derived contents, such as food antigens and bacterial products, via the portal vein [33, 34]. Once these entities flow into the liver, they can activate a variety of cells in the liver, which may be associated with certain types of liver disease [33, 34]. Gut-derived food-antigens can activate T cells [33] and gut-derived bacterial products can stimulate all resident cells in the liver, such as hepatocytes, Kupffer cells, stellate cells, and dendritic cells, via toll-like receptors [33–36]. Moreover, fat itself, particularly saturated fatty acids, stimulates an immune response in the liver [37, 38]. On the other hand, the liver is an immune-tolerogenic organ, in which immune-suppressive cells may play a critical role to keep this organ immunologically silent [33]. The present study demonstrated that liver inflammation was greatly exacerbated—where CD4 T and/or CD8 T cells infiltrated to form foci surrounding damaged hepatocytes—by an HFD in the absence of iNKT cells. This suggests a suppressive role of iNKT cells in the development of liver inflammation with steatosis. Thus, iNKT cells may play an important role in keeping the liver immunologically silent, and the absence of iNKT cells together with steatosis may elicit a break of hepatic immune tolerance, resulting in the activation of CD4 T and/or CD8 T cells to provoke liver inflammation. Consistent with this speculation is the observation that the absence of iNKT cells, even without steatosis, caused modest liver inflammation.

In conclusion, iNKT cells suppress liver inflammation progressing to fibrosis that is exacerbated by HFD-induced steatosis, thus contributing to the maintenance of immune homeostasis in the liver. This study has shed some light on iNKT cells as immunoregulatory cells and their key role in the pathogenesis of NAFLD.

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Research Article

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Altered interferon-α-signaling in natural killer cells from patients with chronic hepatitis C virus infection☆

Takuya Miyagi[1,2], Tetsuo Takehara[1], Kumiko Nishio[1], Satoshi Shimizu[1], Keisuke Kohga[1], Wei Li[1], Tomohide Tatsumi[1], Naoki Hiramatsu[1], Tatsuya Kanto[1], Norio Hayashi[1,2,*]

[1]*Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan;* [2]*Global Centers of Excellence Program, Frontier Biomedical Science Underlying Organelle Network Biology, Osaka University, Suita, Osaka, Japan*

Background & Aims: Natural killer (NK) cells play an important role in the immune response against virus infection. Interferon (IFN)-α, an essential component in therapy against hepatitis C virus (HCV) infection, regulates NK cell function. However, it remains obscure how chronic HCV infection (CHC) modifies intracellular IFN-α signaling in NK cells. We investigated IFN-α signaling in NK cells in patients with CHC.

Methods: Peripheral blood mononuclear cells were obtained from patients with CHC and healthy subjects (HS) as controls.

Results: The expression level of signal transducer and activator of transcription (STAT) 1, a key molecule of IFN-α signaling, was clearly higher in NK cells from the CHC patients than in those from HS. The phosphorylation level of STAT1 with IFN-α stimulation was significantly greater in NK cells from the CHC patients than in those from the HS, while that of STAT4 was significantly less. These phosphorylation levels of STAT1 and STAT4 positively and negatively correlated with the STAT1 level in NK cells, respectively. The IFN-α induced messenger RNA level of the suppressor of cytokine signaling 1, which is a downstream gene of phosphorylated-STAT1, was clearly greater in NK cells from the CHC patients than in those from the HS, while that of IFN-γ, which is a downstream gene of phosphorylated-STAT4, was clearly lower.

Conclusions: These results indicate altered IFN-α signaling in NK cells in CHC patients, suggesting that this alteration is associated with the persistence of HCV infection and resistance to IFN-α therapy.

Keywords: Natural killer cells; Interferon; Hepatitis C virus; Signal transducer and activator of transcription.

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* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.

E-mail address: hayashin@gh.med.osaka-u.ac.jp (N. Hayashi).

Abbreviations: NK, natural killer; IFN, interferon; STAT, signal transducer and activator of transcription; HCV, hepatitis C virus; CHC, chronic hepatitis C virus infection; HS, healthy subject; SOCS, suppressor of cytokine signaling; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; IL, interleukin; pSTAT, phosphorylated-signal transducer and activator of transcription; RT-PCR, reverse transcription polymerase chain reaction; NKT, natural killer T; ISG, interferon stimulated gene.

Introduction

Natural killer (NK) cells play an important role in innate immune responses against a variety of viral infections by directly killing infected cells with cytotoxic molecules such as perforin and granzyme [1]. The cells also have a great ability to secrete a key cytokine, interferon (IFN)-γ, which activates subsequent adaptive immune responses as well as inhibits viral replication [1,2]. Another major component in innate immune responses during viral infections is IFN-α, which is the most abundant cytokine released during viral infections [3]. In addition to its anti-viral effects, IFN-α activates NK cells to induce IFN-γ production via activation of the signal transducer and activator of transcription (STAT) 4, as well as its cytotoxic ability via activation of STAT1 [4–7].

Hepatitis C virus (HCV) causes persistent infection in more than 70% of infected patients. Whereas some of the patients show a carrier-like state, most develop chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, which is why HCV infection is a worldwide health problem [8]. The administration of IFN-α is a well-established anti-viral therapy for HCV infection. More than 90% of patients with acute HCV infection respond to IFN-α based therapy, while only around 50% of patients with chronic HCV infection (CHC) do [9–12], suggesting a mechanism by which persistent HCV infection leads to resistance to IFN-α based therapy. NK cell number has been demonstrated to decrease in patients with CHC, while it is controversial whether NK cell functions are impaired in patients with CHC [13–15]. It thus remains unclear whether perturbation of NK cells is involved in the persistence of CHC as well as resistance to the therapy [13–15].

In the present study, we investigated how chronic HCV infection modifies intracellular IFN-α signaling in NK cells. The expression level of total STAT1, a key molecule of IFN-α signaling, was clearly higher in NK cells from patients with CHC than in those from healthy subjects (HS). Phosphorylation of STAT1, resulting in induction of the suppressor of cytokine signaling (SOCS) 1 messenger RNA (mRNA) expression in response to IFN-α was clearly greater in NK cells from the CHC patients than

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in those from the HS. On the other hand, the phosphorylation of STAT4, resulting in induction of IFN-γ mRNA expression in response to IFN-α, was clearly less in NK cells from the CHC patients than in those from the HS. NK cell degranulation was significantly enhanced in response to IFN-α in the HS, but not in the CHC patients. These findings suggest altered IFN-α signaling in NK cells in patients with CHC. The alteration of IFN-α signaling might be associated with the persistence of chronic HCV infection and resistance to IFN-α therapy.

Materials and methods

Subjects

Twenty-six patients with CHC (HCV RNA genotype 1) and 26 healthy volunteers were enrolled in this study. The profile of these subjects is shown in the Supplementary data and Supplementary Table 1. The study was approved by the ethical committee of Osaka University Hospital.

Isolation of peripheral blood mononuclear cell (PBMC) populations

PBMCs were isolated from fresh heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation as described [16].

In vitro stimulation of cells

Prepared cells were unstimulated or stimulated with either natural human IFN-α, recombinant human IFN-γ or recombinant human interleukin (IL)-12. The details are provided in the Supplementary data.

Cell lysates and Western blot analysis

Prepared cells were lysed as described [17]. A 25 μg sample of protein was separated on 10% SDS polyacrylamide gels and transferred onto PVDF membrane. Monoclonal anti-STAT1 antibody (1/Stat1) was purchased from BD Biosciences (San Jose, CA, USA). Polyclonal anti-β-actin antibody from Abcam (Cambridge, MA, USA) was used as the loading control. Detection of immunolabeled proteins was performed as described [17].

Flow cytometric analysis

The staining of prepared cells was performed as described [16,18]. Briefly, cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (UCHT1) and biotin-conjugated anti-CD56 antibody (B159), fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and cold pure methanol, and then stained with phycoerythrin-conjugated anti-phosphorylated-STAT (pSTAT) 4 (pY693) (38/p-Stat4) and Alexa Fluor® 647-conjugated anti-pSTAT1 (pY701) antibody (4a), or phycoerythrin-conjugated anti-STAT1 (1/Stat1) antibody alone, or the corresponding isotype control, followed by staining with peridinin chlorophyll protein-conjugated streptavidin (BD Biosciences). All antibodies were purchased from BD Biosciences. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the FlowJo program (Tree Star Inc., Ashland, OR, USA).

NK cell enrichment from PBMCs

To obtain pure populations of $CD56^+$ $CD3^-$ NK cells from PBMCs, NK cells were negatively isolated by magnetic cell sorting with a human NK cell isolation kit (Miltenyi Biotec, Gladbach, Germany). The purity of the isolated population was confirmed using FACS analysis and was more than 90%.

RNA isolation and analysis

Total RNA isolation and the real-time reverse transcription polymerase chain reaction (RT-PCR) analysis are presented in detail in the Supplementary data, Supplementary Fig. 1, and Supplementary Table 2.

NK cell degranulation assay

NK cell degranulation was assessed as described [19], with minor modifications. Details and representative data are provided in the Supplementary data and Supplementary Fig. 2.

Statistical analysis

The statistical significance of differences between the patient and control groups or that of changes due to IFN-α stimulation in the NK cell degranulation assay was determined by applying unpaired or paired Student's *t*-test, respectively. Correlations were assessed using the Pearson product–moment correlation coefficient. The statistical significance was defined as $p < 0.05$.

Results

NK cells from CHC patients showed a higher level of STAT1 expression than those from HS

Murine NK cells have been reported to exhibit a lower level of STAT1 expression than non-NK cells [17,18]. Also, the increase of STAT1 expression level in murine NK cells has been observed in viral infection [18]. We examined whether similar findings could be observed for human NK cells. Western blot analyses revealed that human NK cells from representative HS have a clearly lower level of STAT1 expression than non-NK cells (Fig. 1A). We then examined whether chronic HCV infection affected the STAT1 expression level in NK cells. It was clearly higher in NK cells from the CHC patients than in those from the HS (Fig. 1B).

We next examined STAT1 expression level within individual cells by flow cytometry. Consistent with the results of Western blot analyses, flow cytometric analyses demonstrated that NK cells from a representative healthy subject had a lower level of STAT1 expression than non-NK cells such as T cells or natural killer T (NKT) cells (Fig. 2A). NK cells from a representative CHC patient displayed a clearly higher level of STAT1 expression than

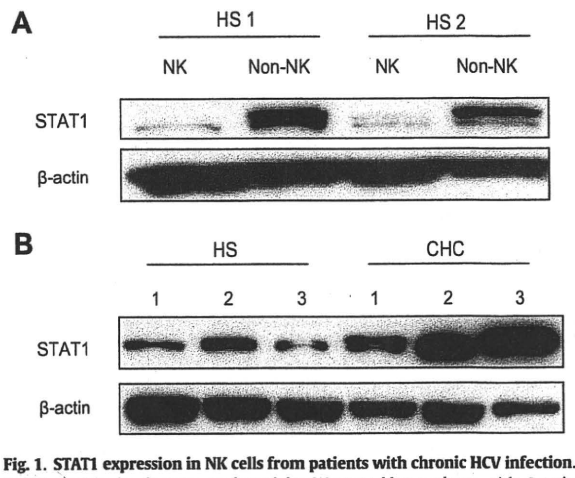

Fig. 1. STAT1 expression in NK cells from patients with chronic HCV infection. STAT1 protein levels were evaluated by Western blot analyses with β-actin measurement as a loading control. PBMCs were obtained from patients with chronic HCV infection (CHC) and healthy subjects (HS). NK cells and non-NK cells were purified from those cells. (A) STAT1 protein levels in NK cells and non-NK cells from two representative HS are shown. (B) STAT1 protein levels in NK cells from three other representative HS and three representative patients are shown.

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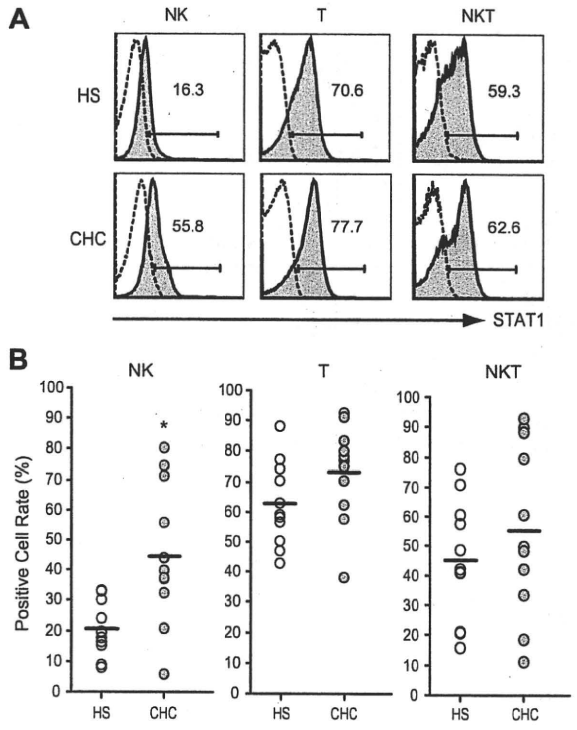

Fig. 2. STAT1 expression level in NK, T or NKT cells from patients with chronic HCV infection. STAT1 protein level was evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells, $CD56^-$ $CD3^+$ T cells, and $CD56^+$ $CD3^+$ NKT cells. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Representative histograms from a patient and an HS are shown. Dotted lines show staining with the isotype control. Solid lines with shaded areas show staining with the antibody. Numbers are percentages of positive cells (positive cell rate) determined based on the isotype control staining. (B) Comparison of STAT1 expression level between the patients with CHC ($n = 11$) and HS ($n = 11$) are shown as positive cell rates. Each circle represents data for an individual. Horizontal bars represent means. *$p < 0.005$ vs. HS.

those from the healthy subject. Fig. 2B summarizes the profile of the intracellular STAT1 expression level of NK, T, and NKT cells. The intracellular STAT1 expression level of NK cells from the CHC patients was significantly higher than that from the HS, while that of T cells or NKT cells did not show any significant difference.

Altered activation of STAT1/4 occurred in response to IFN-α in NK cells from CHC patients

Activation of STAT1/4 in response to IFN-α in murine NK cells has been reported to shift from pSTAT4 dominant to pSTAT1 dominant as the intracellular STAT1 expression level increases [18]. This led us to examine the activation of STAT1/4 in response to IFN-α in human NK cells using samples from CHC patients and HS. Although IFN-α can phosphorylate both STAT1 and STAT4, IFN-γ can phosphorylate STAT1 and IL-12 can phosphorylate STAT4 in NK cells [1,7]. We examined the phosphorylation level of STAT1/4 in response to IFN-α, compared with IFN-γ or IL-12, in NK cells from the subjects by flow cytometry. It was found that IFN-α phosphorylated STAT1 more strongly in NK cells from the representative CHC patient than in those from the representative HS, while IFN-γ did not (Fig. 3A). On the other hand, IFN-α phosphorylated STAT4 more weakly in NK cells from the CHC patient than in those from the HS, while IL-12 did not. Fig. 3B summarizes the profile of STAT1/4 phosphorylation level in NK cells in response to IFN-α, IFN-γ or IL-12. IFN-α, but not IFN-γ, phosphorylated STAT1 significantly more strongly in NK cells from the CHC patients than in those from the HS. IFN-α, but not IL-12, phosphorylated STAT4 significantly more weakly in NK cells from the CHC patients than in those from the HS. These results suggested altered signaling of IFN-α, but not of IFN-γ or of IL-12, in NK cells of patients with CHC.

We then examined the relationship between STAT1 expression level and STAT1/4 phosphorylation level in response to IFN-α in NK cells from the CHC patients. The phosphorylation level of STAT1 in response to IFN-α correlated significantly and positively with the STAT1 expression level in NK cells ($R^2 = 0.67$, $p < 0.003$), while that of STAT4 correlated significantly and negatively ($R^2 = 0.49$, $p < 0.02$) (Fig. 4). These results suggested that, as in murine NK cells, the activation of STAT1/4 in response to IFN-α in human NK cells shifts from a preference for pSTAT4 to one for pSTAT1 as intracellular STAT1 expression level increases.

Altered induction of interferon stimulated gene (ISG) expression occurred in response to IFN-α in NK cells

We examined how the altered activation of STAT1/4 in response to IFN-α in NK cells affected the induction of downstream gene expression. Real-time RT-PCR analyses revealed that the induction level of SOCS1 mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT1, was clearly greater in NK cells isolated from the CHC patients than in those from the HS, and that the induction level of IFN-γ mRNA expression with IFN-α stimulation, which is a downstream gene of pSTAT4, was clearly lower (Fig. 5). On the other hand, the mRNA induction level of perforin or granzyme B, which is a cytotoxic molecule induced by IFN-α via pSTAT1, was not greater but modestly lower, suggesting negative regulation by the large induction of SOCS1, which is a negative regulator of the pSTAT1 pathway [20].

Altered activation of NK cells occurred in response to IFN-α

To examine how the altered IFN-α signaling in NK cells affected the activation of NK cells in response to IFN-α, we evaluated the NK cell degranulation ability in response to IFN-α. NK cell degranulation assay showed that CD107a expression, as a marker of degranulation, in the presence of K562 cells was significantly up-regulated in response to IFN-α in NK cells from HS, but not in those from CHC patients (Fig. 6), suggesting altered NK cell activation in response to IFN-α in CHC patients.

Discussion

Both IFN-α and IFN-γ have been observed in sera from patients with CHC [21,22]. Their production may be induced by a host response to HCV within the liver, which would make these IFNs detectable in the systemic circulation of patients with CHC. The present study has shown that NK cells from patients with CHC display higher levels of STAT1 expression compared to those from HS (Fig. 1B and Fig. 2). STAT1 itself is one of the ISGs, whose

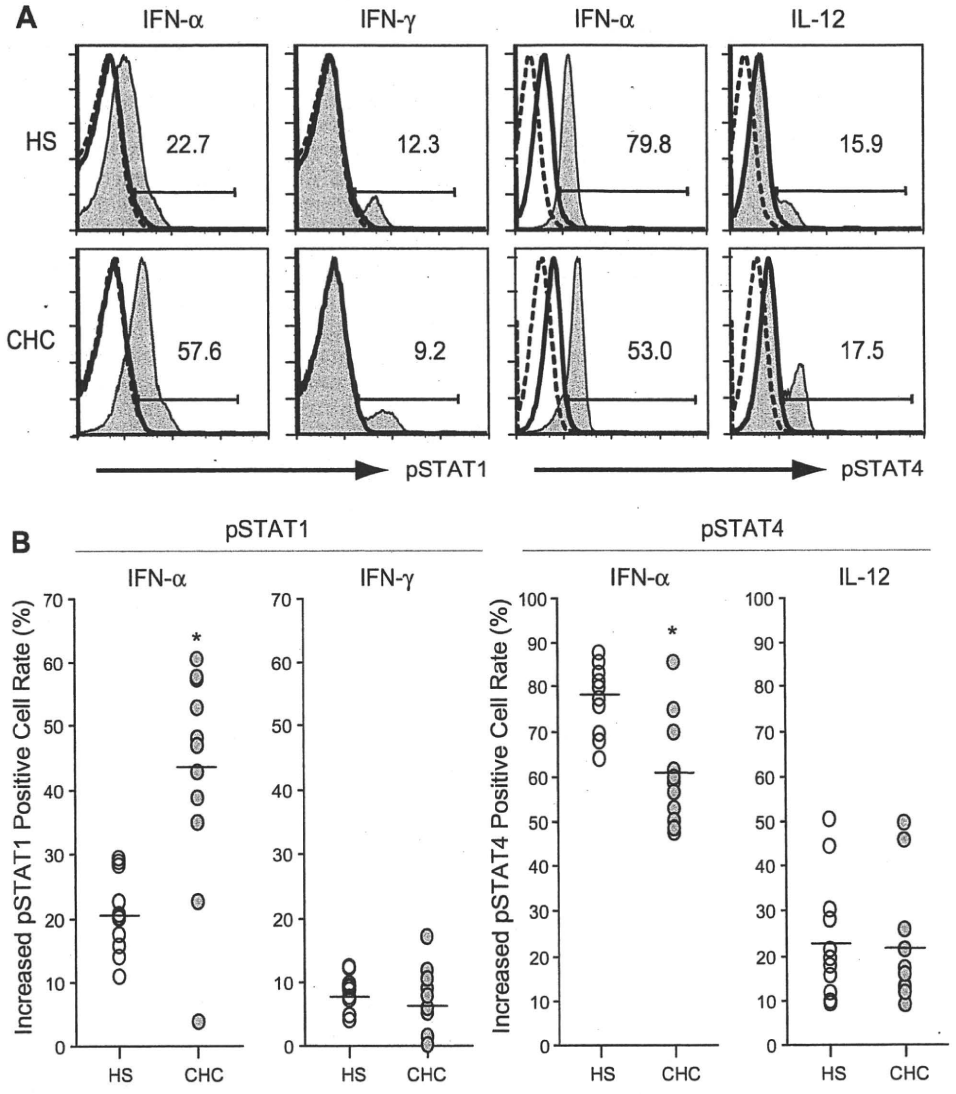

Fig. 3. Altered activation of STAT1/4 in response to IFN-α, but not to IFN-γ or IL-12 in NK cells from patients with chronic HCV infection. pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry with isotype control staining. PBMCs were derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). (A) Prepared PBMCs were unstimulated or stimulated with natural IFN-α, IFN-γ or IL-12 for 90 min *in vitro*, and then collected. pSTAT1 and pSTAT4 protein levels were evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells. Representative histograms of a patient and an HS are shown. Dotted lines show staining of stimulated cells with isotype control. Thick lines show staining of unstimulated cells with the antibody. Thin lines with shaded areas show staining of stimulated cells with the antibody. Positive cell rates were determined based on the staining with isotype controls. Numbers are increased positive cell rates which were determined by subtracting the positive cell rate of unstimulated cells from those of stimulated cells. (B) Comparison of pSTAT1/4 level in response to IFN-α, IFN-γ or IL-12 between the patients with CHC ($n = 11$) and the HS ($n = 11$) are shown as increased pSTAT1/4 positive cell rate. Each circle represents individual data. Horizontal bars represent means. *$p < 0.001$ vs. HS.

expression is up-regulated by IFN-α or IFN-γ [23,24]. It is thus possible that the higher level of STAT1 in NK cells from the CHC patients was up-regulated by IFN-α and/or IFN-γ induced by a chronic host response to HCV.

Our real-time RT-PCR analyses showed that the induction level of SOCS1 mRNA in response to IFN-α was significantly greater in NK cells from the CHC patients than in those from the HS (Fig. 5). This finding is consistent with the observation that the phosphorylation level of STAT1 in response to IFN-α was significantly stronger in NK cells from the CHC patients than in those from the HS (Fig. 3), because SOCS1 is a downstream gene of pSTAT1 [20]. On the other hand, SOCS1 is an inducible negative regulator which inhibits further activation of the pSTAT1 pathway [20]. It is therefore possible that this greater up-regulation of SOCS1 owing to the greater level of STAT1 phosphorylation in response to IFN-α finally results in a weaker response to IFN-α, that is, a weaker induction of ISGs via the pSTAT1 pathway. Indeed, an increase of the STAT1 level,

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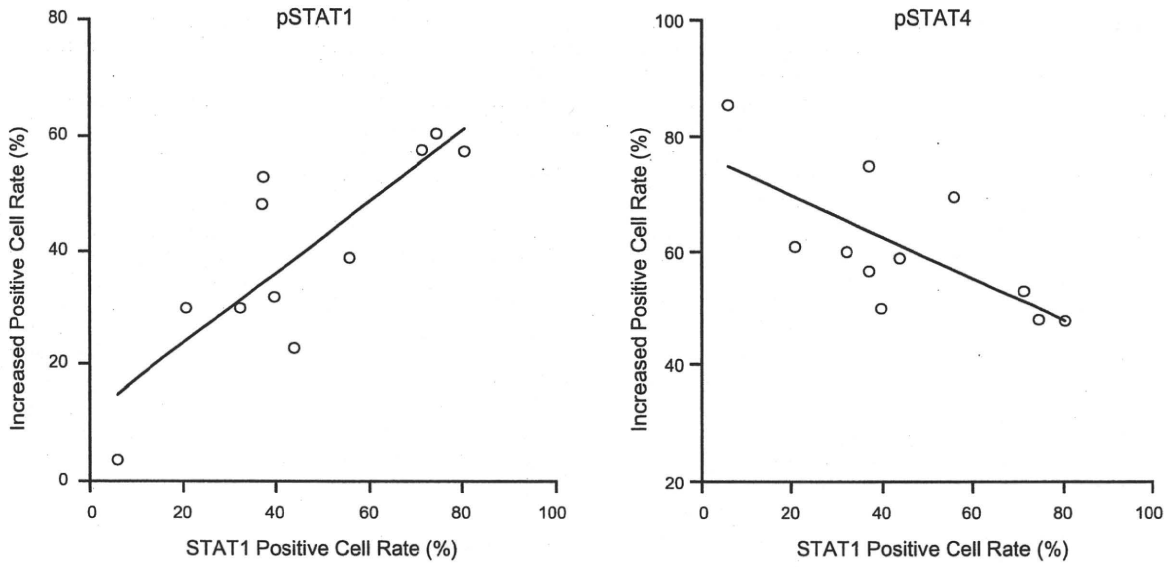

Fig. 4. Correlation between STAT1 level and pSTAT1/4 level in response to IFN-α in NK cells from patients with chronic HCV infection. The relationship was statistically analyzed between intracellular STAT1 level in NK cells and increased pSTAT1/4 level in response to IFN-α in NK cells from patients with chronic HCV infection. Each circle represents individual data. The lines represent regression lines.

which is itself a downstream gene of pSTAT1, by IFN-α based therapy, correlated negatively in vivo with the basal STAT1 expression level before therapy (Miyagi et al. unpublished data). The higher basal STAT1 expression causes a greater level of STAT1 phosphorylation in response to IFN-α, which is followed by a greater level of SOCS1 induction and then might result in a lower increase of STAT1 level. Since perforin and granzyme B are also ISGs from the pSTAT1 pathway [6,25], it is reasonable that the mRNA induction of perforin and granzyme B in response to IFN-α in NK cells from the CHC patients was not significantly greater but modestly lower than those from the HS (Fig. 5). Indeed, the enhancement of degranulation by IFN-α, that is, the increase of CD107a expression, in the presence of K562 cells in response to IFN-α, was significant in NK cells from the HS, but not in those from the CHC patients (Fig. 6).

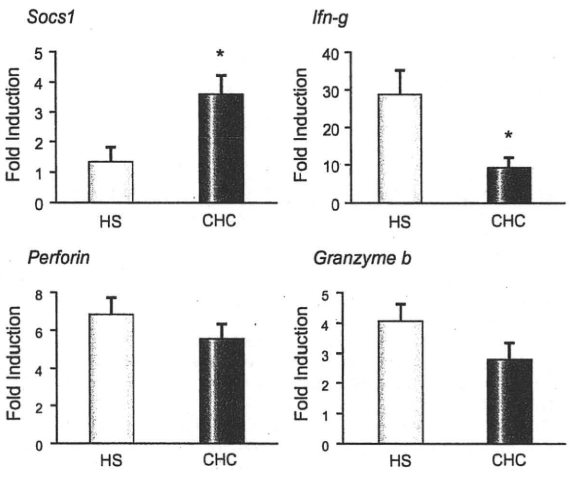

Fig. 5. Induction of ISGs in NK cells in response to IFN-α *in vitro*. NK cells were purified from PBMCs derived from patients with chronic HCV infection (CHC) and healthy subjects (HS). Isolated NK cells were untreated or treated with natural IFN-α *in vitro* for 3 h, and then collected. The collected cell RNA level of the indicated genes and β-actin as a control were analyzed by real-time RT-PCR. Data are shown as the fold increase of treated cells compared with untreated cells, with means ± standard error of the mean from five subjects in each group. *p <0.05 vs. HS.

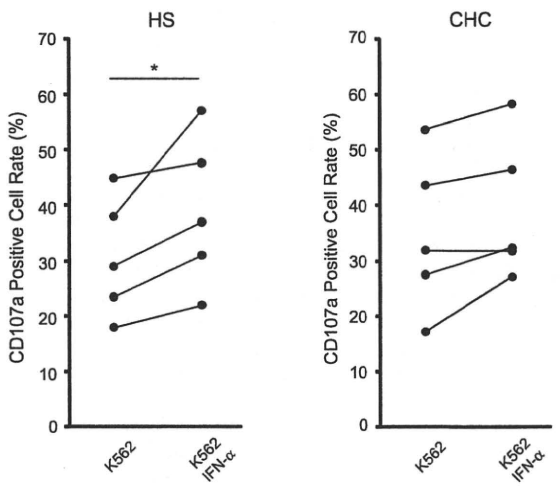

Fig. 6. Increase of NK cell degranulation by IFN-α stimulation. PBMCs derived from patients with chronic HCV infection (CHC) and healthy subjects (HS) were treated with or without IFN-α in the presence of K562 cells. The CD107a expression of NK cells were evaluated by flow cytometry, electronically gating on $CD56^+$ $CD3^-$ NK cells. Data are shown as the frequency of CD107a-positive NK cells treated with or without IFN-α. Each circle represents individual data. *p <0.05.

Patients who were clear of HCV due to IFN-α based therapy exhibited a significant increase in NK cell numbers and activity in the peripheral blood as well as in the liver compared to those who were not able to become clear of the virus [26–28]. IFN-γ produced from NK cells have been reported to suppress HCV replication in vitro [29]. IFN-γ from NK cells is also considered to activate the subsequent adaptive immune response during virus infection as well as inhibit viral replication [1,2,7]. These findings suggest the involvement of NK cells with IFN-γ in the response to IFN-α based therapy. The present study showed that the level of STAT4 phosphorylation in response to IFN-α in NK cells correlated negatively with the intracellular STAT1 level in NK cells (Fig. 4), and that IFN-γ induction, which is one of the downstream genes of pSTAT4, was clearly weaker in NK cells from the patients with CHC than in those from the HS (Fig. 5). The lower activation in CHC patients of the pSTAT4-to-IFN-γ pathway in response to IFN-α in NK cells compared to those from the HS might be one of the mechanisms which make CHC patients resistant to IFN-α based therapy.

Ahlenstiel et al. have recently reported that chronic exposure to HCV-induced IFN-α caused NK cells to become functionally polarized towards a cytotoxic phenotype, but that lacked an increase in IFN-γ production [30]. Moreover, Oliviero et al. showed that NK cells from CHC patients were of a predominantly activating phenotype, and that these phenotypic changes were associated with enhanced cytotoxic activity and defective IFN-γ production [31]. These reports would be associated with our finding that NK cells from the CHC patients displayed a high level of STAT1 expression. Cytotoxic molecules such as perforin and granzyme, as well as STAT1, belong to the ISGs [6,25]. A high level of STAT1 in NK cells in the CHC patients might correspond to a high level of cytotoxic molecules in NK cells resulting in enhanced cytotoxic activity at the basal level. Indeed, the CD107a expression at basal level in NK cells from the CHC patients seemed to be modestly higher than that in NK cells from the HS (Fig. 6), although NK cells from the CHC patients did not significantly increase the CD107a expression in response to IFN-α. On the other hand, our present study showed that STAT1 expression level in NK cells correlated negatively with the activation of STAT4 to produce IFN-γ in response to IFN-α in NK cells. A high level of STAT1 in NK cells would also cause defective IFN-γ production in the NK cells of patients with CHC.

Recent studies have revealed that the higher level of ISGs in hepatocytes as well as in PBMCs before IFN-α based therapy is associated with resistance to this therapy [32–33]. The present study demonstrated that NK cells from CHC patients displayed higher levels of STAT1, which is one of the ISGs, compared with those from HS (Fig. 2). Those who had a higher STAT1 level in NK cells showed less STAT4 phosphorylation and more STAT1 phosphorylation in response to IFN-α, resulting in less IFN-γ induction and more SOCS1 induction. Thus, it is possible that those who have a higher STAT1 level in NK cells would display less response to IFN-α based therapy. Our preliminary data with a small number of patients treated with IFN-α based therapy revealed a tendency for those who had a higher STAT1 level in NK cells to not respond well to the therapy in the context of HCV clearance by week 8 after initiation of therapy (Supplementary Fig. 3).

In the present study, we found that the expression level of total STAT1, a key molecule of IFN-α signaling, was clearly higher in NK cells from the patients with CHC than in those from the HS. The phosphorylation levels of STAT1 and STAT4 with IFN-α stimulation were altered in NK cells from the CHC patients, compared with those from the HS. The induction of IFN-γ mRNA expression with IFN-α stimulation, which is one of the downstream genes of pSTAT4, was clearly weaker in isolated NK cells from the CHC patients than in those from the HS. The induction of SOCS1 mRNA expression, which is one of the downstream genes of pSTAT1, was clearly stronger. The enhancement of NK cell degranulation, the increase of CD107a expression, in response to IFN-α was significant in the HS, but not in the CHC patients. These results indicate that IFN-α signaling in NK cells is altered in CHC patients, suggesting that this alteration of IFN-α signaling is associated with the persistence of chronic HCV infection and resistance to IFN-α therapy. The basal total STAT1 level in NK cells might enable the prediction of the outcome of IFN-α therapy against HCV infection, and thus could serve as a molecular target for more effective IFN-α based therapy.

Conflict of interests

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.03.018.

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IκBζ is essential for natural killer cell activation in response to IL-12 and IL-18

Tohru Miyake[a,b,c], Takashi Satoh[a,b], Hiroki Kato[a,b], Kazufumi Matsushita[a,b], Yutaro Kumagai[a,b], Alexis Vandenbon[d], Tohru Tani[c], Tatsushi Muta[e], Shizuo Akira[a,b,1], and Osamu Takeuchi[a,b]

[a]Laboratory of Host Defense, [d]Laboratory of Systems Immunology, WPI Immunology Frontier Research Center, and [b]Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; [c]Department of Surgery, Shiga University of Medical Science, Seta-tsukinowacho, Shiga 520-2192, Japan; and [e]Laboratory of Cell Recognition and Response, Graduate School of Life Sciences, Tohoku University, Sendai 980-8578, Japan

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IκBζ, encoded by *Nfibiz*, is a nuclear IκB-like protein harboring ankyrin repeats. IκBζ has been shown to regulate IL-6 production in macrophages and Th17 development in T cells. However, the role of IκBζ in natural killer (NK) cells has not be understood. In the present study, we found that the expression of IκBζ was rapidly induced in response to IL-18 in NK cells, but not in T cells. Analysis of *Nfkbiz*$^{-/-}$ mice revealed that IκBζ was essential for the production of IFN-γ production and cytotoxic activity in NK cells in response to IL-12 and/or IL-18 stimulation. IL-12/IL-18–mediated gene induction was profoundly impaired in *Nfkbiz*$^{-/-}$ NK cells. Whereas the phosphorylation of STAT4 was normally induced by IL-12 stimulation, STAT4 was not recruited to the *Ifng* gene regions in *Nfkbiz*$^{-/-}$ NK cells. Acetylation of histone 3 K9 on *Ifng* regions was also abrogated in *Nfkbiz*$^{-/-}$ NK cells. IκBζ was recruited on the proximal promoter region of the *Ifng* gene, and overexpression of IκBζ together with IL-12 activated the *Ifng* promoter. Furthermore, *Nfkbiz*$^{-/-}$ mice were highly susceptible to mouse MCMV infection. Taken together, these results demonstrate that IκBζ is essential for the activation of NK cells and antiviral host defense responses.

IFN-γ | transcription | virus infection | STAT4

Antiviral host defense is mediated by coordinated action of multiple cell types, such as innate immune cells, T cells, and natural killer (NK) cells (1, 2). In response to virus infection, NK cells induce apoptosis of infected cells by expressing perforins and granzymes and produce high amounts of IFN-γ (3–5). Various cytokines are important for activating NK cells to produce IFN-γ and increasing their cytotoxic activity. Type I IFNs produced from innate immune cells as well as cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21 are critical for NK-cell activation. Furthermore, NK-cell receptors harboring immunoreceptor tyrosine-based activation motifs (ITAMs) or those interacting with ITAM-containing adaptor proteins induce cytolytic activity and IFN-γ production (4). For instance, antibody-coated target cells are recognized by FcgRIII, which mediates antibody-dependent cellular cytotoxicity and IFN-γ production.

The expression of the *Ifng* gene is one of the hallmarks of NK-cell activation. Because *Ifng* is also highly expressed in T helper 1 (Th1) cells, the mechanisms of the *Ifng* promoter activation has been extensively studied in Th1 cells and NK cells (6, 7). Stimulation of NK cells with IL-12 and IL-18 leads to production of IFN-γ as well as elevation of cytotoxic activity (8, 9). It has been shown that sequence elements conserved among species (also known as conserved noncoding sequences, CNSs) present up to 100 kb from the transcription start site (TSS) of *Ifng* are involved in controlling the expression of IFN-γ (7, 10–12). Various transcription factors are recruited to the CNS of *Ifng*. IL-12 induces phosphorylation and nuclear translocation of STAT4, which is recruited to the *cis*-regulatory region of *Ifng* (7, 13, 14). IFN-γ–inducible T-box transcription factor (T-bet) is critical for inducing *Ifng* by controlling histone acetylation in Th1 cells (11, 15, 16). T-bet is also required for the development of NK cells (17). In T cells, T-bet–dependent chromatin remodeling of the *Ifng* locus induces recruitment of the NF-κB p65 subunit to *Ifng cis*-elements (12).

IL-18 is structurally related to IL-1β and known to work together with IL-12 to activate Th1 cells to produce IFN-γ (18). However, IL-18 does not induce Th1 development by itself, unlike IL-12. Recent reports show that IL-18 is involved in the differentiation of Th2 cells and Th17 cells in murine experimental models, in addition to Th1 (18, 19). Although IL-18 is reported to activate AP-1 via MAP kinases to express *Ifng* (20), the mechanism of how IL-18 potentiates IFN-γ production in NK cells is yet to be clarified.

IκBζ, also known as INAP or MAIL, is a nuclear factor belonging to the Bcl-3 family, which contains a nuclear localization domain in the N terminus and C-terminal ankylin repeats (21). IκBζ is encoded by the *Nfkbiz* gene, and the expression of *Nfkbiz* is rapidly induced in response to various Toll-like receptor (TLR)/IL-1 receptor (IL-1R) stimuli in macrophages (22). The expressed IκBζ interacts with NF-κB p50 subunit and positively regulates expression of a set of genes including *Il6*, *Il12b*, *Csf2*, *Defb2*, *Lcn2*, and so on (22, 23). In addition to NF-κB binding sequences, flanking C/EBP-binding sites are also important for IκBζ-mediated control of transcription (23). Furthermore, a recent study shows that IκBζ is required for chromatin remodelling downstream of TLR stimuli (24). Recently, it was shown that T cells lacking IκBζ showed severe defects in development of Th17 cells, and IκBζ regulates IL-17 expression by cooperating with *Rorgt* (25). On the other hand, a report showed that overexpression of IκBζ induced *Ifng* in a cell line, although the mechanism was not understood (26). Nevertheless, it is unclear whether IκBζ plays any role in the activation of NK cells.

In the present study, we found that IκBζ was required for the activation of NK cells in response to IL-12 and IL-18. IL-12/IL-18–mediated gene expression including *Ifng* was profoundly impaired in *Nfkbiz*$^{-/-}$ NK cells. IκBζ was recruited to the proximal promoter region of *Ifng* and able to transactivate the *Ifng* together with IL-12. Furthermore, *Nfkbiz*$^{-/-}$ mice were highly susceptible to infection with mouse CMV (MCMV), a model virus known to be cleared by NK cells. Taken together, IκBζ plays an essential role in the activation of NK cells by triggering dynamic transcription factor recruitment and chromatin remodeling.

Results

IκBζ Expression Is Induced in Response to IL-18 in NK Cells. To investigate whether IκBζ plays a role in controlling cells other than macrophages, we first examined the expression levels of IκBζ in

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[1]To whom correspondence should be addressed. E-mail: sakira@biken.osaka-u.ac.jp.

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NK cells and T cells. We prepared splenic NK, and naïve T cells. Then, T cells were cultured in the presence of either IL-12, anti-IL-4 Ab, and anti-CD3 to develop Th1 cells or of IL-4, anti-IL-12, and anti-CD3 to develop Th2 cells. Quantitative-PCR (Q-PCR) analysis using RNAs prepared from these cells revealed that NK cells expressed the IκBζ gene higher than naïve T cells (Fig. 1*A*). Polarization to Th1 or Th2 cells did not increase the expression of *Nfkbiz*. The expression of IκBζ was highly induced in response to IL-12 and IL-18 stimulation in NK cells (Fig. 1*A*). Next, we examined kinetics of the IκBζ expression in NK cells in response to IL-12, IL-18, or both. IκBζ expression was induced at 1 h after stimulation with IL-18 alone or after costimulation with IL-12 and IL-18, and gradually decreased by 8 h after stimulation (Fig. 1*B*). In contrast, IL-12 alone slowly increased *Nfkbiz* about twofold at 8 h after stimulation. On the other hand, *Ifng* expression was increased in response to both IL-12 and IL-18, but not to either cytokine alone, and the expression peaked at 4 h after stimulation in NK cells (Fig. 1*B*). These results demonstrate that IκBζ expression is higher in NK cells than in T cells, and that this is an early induced gene in response to IL-18 stimulation in NK cells.

IκBζ Is Critical for Activation of NK Cells in Response to IL-12 and IL-18. The expression of IκBζ in NK cells prompted us to investigate a functional role of IκBζ in NK cells by using IκBζ-deficient (*Nfkbiz*$^{-/-}$) mice. Stimulation of *Nfkbiz*$^{+/-}$ splenic NK cells with IL-12, or costimulation with IL-12 and IL-18, induced production of IFN-γ (Fig. 2*A*). In contract, IFN-γ production was severely impaired in *Nfkbiz*$^{-/-}$ NK cells in response to IL-12, and IL-12 together with IL-18. These results indicated that IκBζ is indispensable for IFN-γ production in response to IL-12 and IL-18. Production of IFN-γ in response to culture with immobilized IgG1 was comparable in wild-type NK cells (Fig. 2*A*), indicating that signaling pathways from FcgRIII to induce *Ifng* expression is not affected in the absence of IκBζ. We then analyzed cytotoxic activity of NK cells to IL-12 and IL-18 stimulation by a standard ^{51}Cr release assay against YAC1 target cells. Cytotoxic activity of NK cells stimulated with IL-12 alone or costimulated with IL-12 and IL-18 was reduced in *Nfkbiz*$^{-/-}$ mice (Fig. 2*B*). IL-18 alone failed to increase cytotoxic activity even in wild-type NK cells

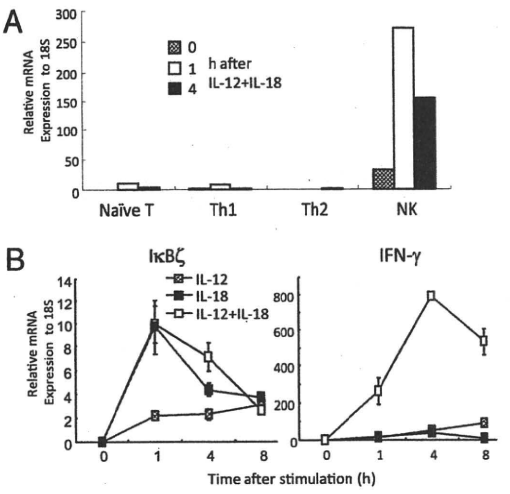

Fig. 1. Expression of IκBζ in NK cells in response to IL-12 and IL-18 stimulation. (*A*) The expression of IκBζ in naïve T, Th1, Th2 cells, and NK cells in response to IL-12 and IL-18. (*B*) NK cells were stimulated with IL-12 and/or IL-18 for the indicated periods, and the expression levels of IκBζ and IFN-γ were determined by Q-PCR analysis. Data represent means ± SD of triplicates. Similar results were obtained in three independent experiments.

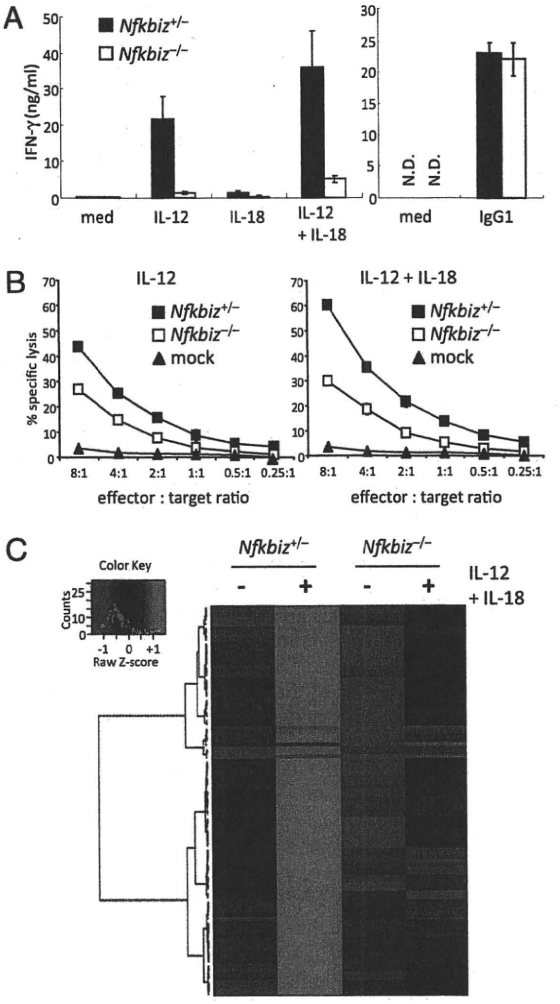

Fig. 2. Role of IκBζ in the activation of NK cells in response to IL-12 and IL-18 stimulation. (*A*) Splenic NK cells from *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice were stimulated with 1 ng/mL IL-12, 10 ng/mL IL-18, and a combination of IL-12 and IL-18 or the cells were cultured on the plates coated with IgG1 for 24 h. The concentrations of IFN-γ in the culture supernatant were measured by ELISA. Data represent means ± SD. Similar results were obtained in three independent experiments. (*B*) IL-2–induced splenic NK cells were cultivated with IL-12 together with or without IL-18 for 4 h. NK-cell cytotoxicities against YAC-1 cells were measured by a ^{51}Cr release assay at different E:T ratios. The percentages of specific cell lysis are indicated. Data represent the mean ± SD of duplicate measurements. The data shown are representative of two independent experiments. (*C*) Splenic NK cells from *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice were stimulated with IL-12 and IL-18 for 24 h, and total RNA was subjected to microarray analysis using Affymetrix mouse genome 430 2.0 microarray chips. The data were normalized by robust multichip analysis (RMA), and genes up-regulated more than twofold in wild-type NK cells after stimulation were selected. The genes were hierarchically clustered as follows: The expression values for each gene were standardized as average = 0 and SD = 1. Complete linkage method was then applied using absolute value of Pearson correlation as distance. The resulted heatmap, dendrogram, and a color key are shown. A blue line in the color key indicates the frequency distribution colors in the heatmap. For the gene lists shown in this figure, see Dataset S1.

(data not shown). These results demonstrate that IκBζ is critical for activation of NK cells in response to IL-12 and IL-18.

IκBζ Is Dispensable for NK-Cell Development. We next analyzed the differentiation of splenic NK cells in *Nfkbiz*$^{-/-}$ mice. It is known that NK-cell progenitors initially express CD122 (IL15R),

CD127 (IL7R), and c-kit (27). Upon maturation, NK cells down-regulate CD127 and c-kit. The $DX5^+CD3^-$ NK-cell population in spleen was comparable between *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice (Fig. S1). Furthermore, CD122 was highly expressed, and c-kit and CD127 expressions were comparably suppressed both in wild-type and *Nfkbiz*$^{-/-}$ splenic NK cells (Fig. S1). Collectively, IκBζ is dispensable for controlling differentiation of NK cells.

Profound Impairment in Gene Expression in *Nfkbiz*$^{-/-}$ NK Cells in Response to IL-12 and IL-18 Stimulation. Next we examined the role of IκBζ in IL-12 and IL-18–mediated gene expression in NK cells. Wild-type and *Nfkbiz*$^{-/-}$ NK cells were stimulated with IL-12 and IL-18 for 24 h, and gene expression profile was assessed by microarray analysis. In response to stimulation with IL-12 and IL-18, 96 genes were up-regulated more than twofold in wild-type NK cells. We then hierarchically clustered genes induced by IL-12 and IL-18 and found that they are roughly classified into those completely (58 genes) or partially (38 genes) dependent on IκBζ (Fig. 2*C*) (Dataset S1). Genes completely dependent on IκBζ include *Ifng*, *Il22*, *Ccr5*, and *Il18r1*. These data demonstrate that IκBζ is critical for IL-12 and IL-18–mediated gene expression in NK cells.

IκBζ Promotes STAT4 Accessibility in IFN-γ Genes in Response to IL-12 and IL-18 in NK Cells. IL-12 activates Janus kinases (JAKs) that phosphorylate STAT4 (14). STAT4 is localized in the cytoplasm in the resting cells, and phosphorylated STAT4 translocates to the nucleus for the transcriptional activation. Treatment with IL-12 greatly increased NK cells stained with anti-phospho-STAT4 (p-STAT4), and the levels of p-STAT4 positive cells were comparable between *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice (Fig. 3*A*). Furthermore, nuclear translocation of STAT4 was comparably induced in *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ NK cells in response to stimulation with IL-12 and IL-18 (Fig. 3*B*), indicating that IκBζ was dispensable for phosphorylation and nuclear translocation of STAT4. We then examined recruitment of STAT4 to *Ifng* CNSs by chromatin immunoprecipitation (ChIP) coupled with Q-PCR (ChIP-Q-PCR) analysis. We found that STAT4 was widely recruited to CNSs (−33 kb, −22 kb, −6 kb, intron 1a, +10 kb, +20 kb, and +30 kb from the TSS) of *Ifng* in response to IL-12 and IL-18 stimulation in wild-type NK cells (Fig. 4*A*). In contrast, STAT4 recruitment to the *Ifng* conserved elements was severely impaired in *Nfkbiz*$^{-/-}$ NK cells through the CNS (Fig. 4*A*). These results suggested that IκBζ promotes STAT4 accessibility to the CNS of *Ifng* gene in stimulated NK cells.

IκBζ Is Required for Change in Histone 3 Lysine 9 Acetylation in Response to IL-12 and IL-18 in NK Cells. It has been shown that histones of the *Ifng* loci were hyperacetylated even in the absence of stimulation in NK cells, compared with T cells (10). We performed ChIP analysis with anti-acetyl histone 3 lysine 9 (H3K9) antibody to assess H3K9 acetylation in *Ifng* CNS. The analysis revealed that intron regions of *Ifng* were hyperacetylated even without stimulation in wild-type and *Nfkbiz*$^{-/-}$ NK cells (Fig. 4*B*). Whereas H3K9 acetylation levels at the −6-kb region of *Ifng* were up-regulated in response to IL-12 and IL-18 in wild-type NK cells, *Nfkbiz*$^{-/-}$ NK cells failed to induce H3K9 acetylation (Fig. 4*B*). These results suggest that IκBζ is required for change in H3K9 acetylation on *Ifng* loci in response to IL-12 and IL-18 stimulation.

IκBζ Is Recruited to the *Ifng* Proximal Promoter Region. To examine the recruitment of IκBζ to the *Ifng* promoter, we examined ChIP analysis using anti-IκBζ antibody. In contrast to STAT4 recruitment or H3K9 acetylation, IκBζ was not recruited to the −6-kb region of *Ifng* locus in NK cells in response to IL-12 and IL-18 (Fig. 5*A*). In contrast, IκBζ was recruited to the proximal promoter region (−0.2 kb from the TSS) of *Ifng* (Fig. 5*A*). IκBζ failed to associate with other CNSs such as −22 kb and intron 1a (data not shown). On the other hand, STAT4 was not recruited to the proximal *Ifng* promoter (data not shown). To investigate whether IκBζ directly regulates *Ifng* through binding to the

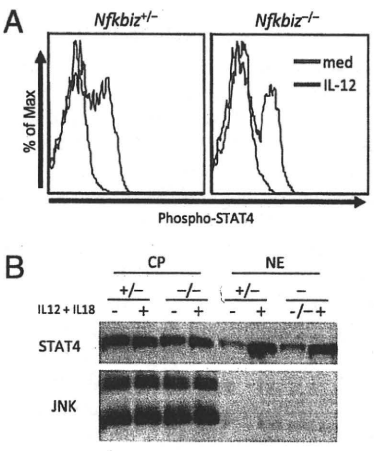

Fig. 3. Nuclear translocation of STAT4 in NK cells in the absence of IκBζ. (*A*) Splenic NK cells were stimulated with IL-12 for 30 min. Phosophorylation of STAT4 was then determined by intracellular staining of cells with anti-phospho-STAT4 Ab followed by flow cytometry. (*B*) NK cells were stimulated with IL-12 and IL-18 for 1 h. Cytoplasmic (CP) and nuclear (NE) proteins were prepared and immunoblotted using anti STAT4 Ab and anti-JNK as a control.

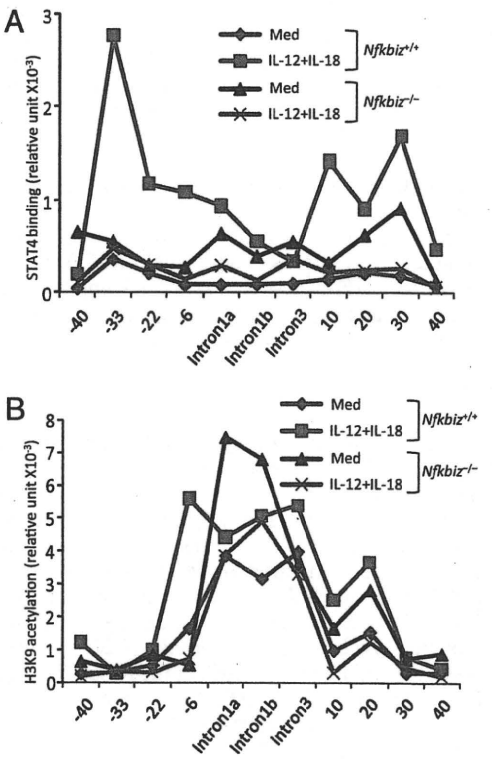

Fig. 4. Requirement of IκBζ in the recruitment of STAT4 and acetylation of H3K9 in NK cells in response to IL-12 and IL-18. Chromatin was isolated from wild-type and *Nfkbiz*$^{-/-}$ NK cells stimulated with IL-12 and IL-18 for 4 h; recruitment of STAT4 (*A*) and acetylation of H3K9 (*B*) to indicated *Ifng* gene regions was determined by ChIP-QPCR analysis. The data are representative of two independent experiments.

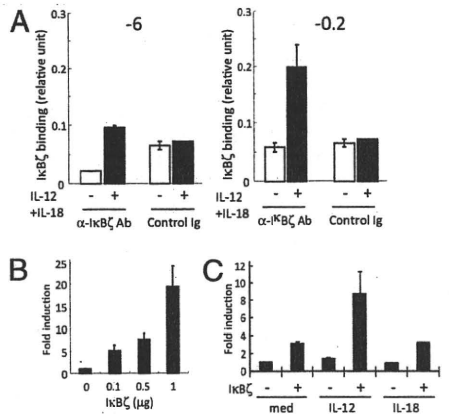

Fig. 5. Regulation of *Ifng* proximal promoter region by IκBζ in NK cells. (*A*) Chromatin was isolated from splenic NK cells stimulated with IL-12 and IL-18 for 4 h, recruitment of IκBζ to indicated *Ifng* gene regions was determined by ChIP-QPCR analysis. (*B*) A 3.6-kb fragment of the human *Ifng* promoter luciferase reporter construct was transfected to EL4 cells with increasing amounts of IκBζ construct. The luciferase activity was measured 18 h after transfection. (*C*) EL4 cells were cotransfected with the *Ifng* promoter reporter construct and IκBζ, followed by stimulation with IL-12 or IL-18. The luciferase activity was measured 18 h after stimulation.

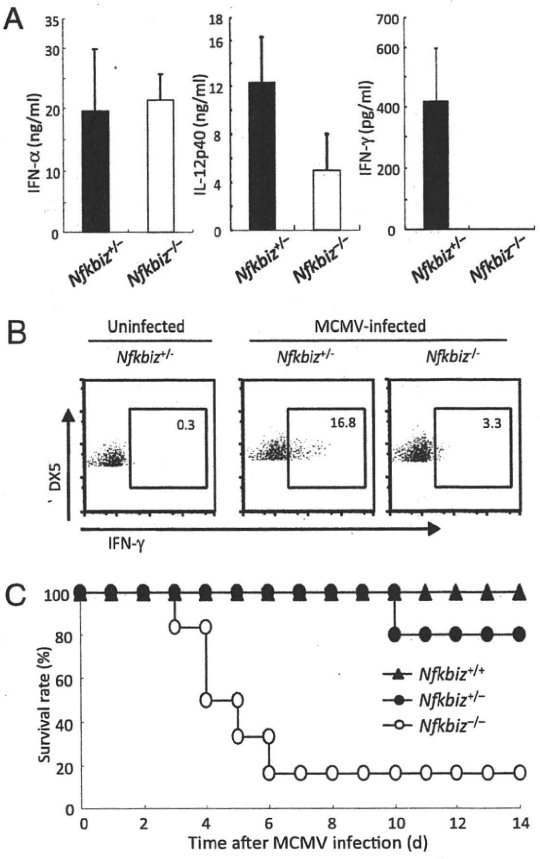

Fig. 6. Essential role of IκBζ in host defense against MCMV infection. (*A*) Wild-type and *Nfkbiz*$^{-/-}$ mice (n = 3–5) were intraperitoneally infected with 1×10^5 pfu MCMV, and the concentrations of IFN-α, IFN-γ, and IL-12p40 in the sera 36 h after infection were measured by ELISA. (*B*) The expression of IFN-γ in splenic NK cells prepared from *Nfkbiz*$^{+/-}$ and *Nfkbiz*$^{-/-}$ mice 36 h after MCMV infection was determined by intracellular staining of cells with anti-IFN-γ Ab followed by flow cytometry. (*C*) *Nfkbiz*$^{+/+}$ (n = 5), *Nfkbiz*$^{+/-}$ (n = 5), and *Nfkbiz*$^{-/-}$ (n = 6) mice were intraperitoneally infected with MCMV, and the survival of mice was monitored daily. The data are representative of two independent infection experiments.

proximal promoter region, we expressed a reporter construct with the human *Ifng* promoter region (−3.6 kb to +70 k) linked to the luciferase gene, together with IκBζ in EL4 cells. As shown in Fig. 5*B*, overexpression of IκBζ enhanced luciferase reporter activity in a dose-dependent manner. Furthermore, stimulation with IL-12, but not IL-18, synergistically activated the *Ifng* promoter with overexpression of IκBζ (Fig. 5*C*), suggesting that IκBζ expression facilitates IL-12–mediated *Ifng* promoter activation. These observations suggest that the recruitment of IκBζ to the proximal promoter region is responsible for the transcriptional activation of *Ifng*.

Essential Role of IκBζ in Host Defense Against MCMV Infection. It is known that NK cells play an important role in host defense against MCMV infection (1, 5, 28). MCMV-induced activation of innate immune cells such as dendritic cells (DCs) induces the production of type I IFNs as well as IL-12, leading to activation of NK cells to produce IFN-γ. To examine the role of IκBζ in activating NK cells in vivo in response to virus infection, we intraperitoneally inoculated MCMV into wild-type and *Nfkbiz*$^{-/-}$ mice. MCMV-induced production of IL-12p40, but not IFN-α, in the sera was partially impaired in *Nfkbiz*$^{-/-}$ mice compared with wild-type mice (Fig. 6*A*), suggesting that IκBζ plays a role in expressing IL-12p40 in innate immune cells in response to MCMV. In contrast, the production of IFN-γ in the sera was completely abrogated in *Nfkbiz*$^{-/-}$ mice (Fig. 6*A*). Furthermore, flow cytometric analysis revealed that the expression of IFN-γ in splenic NK cells was severely impaired in MCMV-infected *Nfkbiz*$^{-/-}$ mice (Fig. 6*B*), although NK-cell cytotoxicity was not altered in *Nfkbiz*$^{-/-}$ NK cells (data not shown). Although all wild-type mice and 80% of the *Nfkbiz*$^{+/-}$ mice survived, *Nfkbiz*$^{-/-}$ mice were highly susceptible and more than 80% of the *Nfkbiz*$^{-/-}$ mice died within 6 d after MCMV infection (Fig. 6*C*). Taken together, IκBζ is essential for host defense against MCMV infection by inducing IFN-γ production from NK cells.

Discussion

In the present study, we examined the role of IκBζ in NK cells and found that this nuclear protein is essential for inducing a set of genes including *Ifng* in response to IL-12 and IL-18. IκBζ was rapidly induced in response to IL-18 in NK cells, although the expression level was kept low in Th1 cells. IL-18 is a cytokine that is able to activate NK cells together with IL-12, although the precise mechanisms of the action of IL-18 has not been understood well (18). IL-18R triggers intracellular signaling pathways leading to the activation of NF-κB and MAP kinases (29). We have previously shown that IκBζ is a TLR/IL-1R–inducible gene in macrophages (22). Given that IL-18R and TLR/IL-1R share MyD88 as an adaptor molecule for the signaling, it is not surprising that IL-18 also induces IκBζ expression. IL-18 is known to augment IFN-γ production, when costimulated with IL-12.

IL-12 induces phosphorylation and nuclear translocation of STAT4, which is recruited to *cis*-regulatory regions of several genes including *Ifng* (6, 7). It has been shown that sequence elements conserved among species present up to 100 kb from the transcription start site of *Ifng* are involved in controlling the expression of *Ifng* (12). These distal elements may loop toward the *Ifng* gene for regulating its transcription (6, 7). Whereas the H3K9 acetylation as well as recruitment of STAT4 to distal conserved regions of *Ifng* was abrogated in *Nfkbiz*$^{-/-}$ NK cells, IκBζ was recruited to a proximal promoter region of *Ifng*, but not to distal sequences. These results imply that initial recruitment of

IκBζ to the proximal region is required for further chromatin remodelling and efficient STAT4 binding. A report showed that IκBζ inhibits STAT3-mediated signaling by direct interaction (30). However, given that STAT4 and IκBζ are recruited to different conserved elements on the *Ifng* promoter, it seems reasonable to assume that IκBζ and STAT4 do not control the *Ifng* expression by physically interacting with each other.

IκBζ interacts with NF-κBp50 in macrophages, and the ankyrin repeats of this protein were required for synergistically increasing transcriptional activity (22). Furthermore, IκBζ is reported to be involved in histone modifications on the *Lcn2* promoter in LPS-stimulated macrophages (24). A previous report shows that NF-κBp50–deficient mice showed increased, but not decreased, IFN-γ production in response to IL-12 and IL-18 stimulation in NK cells (31). Further, no clear NF-κB binding sequence was found in the proximal promoter region of *Ifng*. Thus, it is unlikely that NF-κBp50–IκBζ interaction plays a pivotal role in controlling IFN-γ production in NK cells. In Th17 cells, IκBζ was suggested to directly control IL-17 expression by recruiting to the *Il17* promoter regions (25). However, clear IκBζ responsive elements were not found in the proximal region of *Ifng*. Thus, it is possible that IκBζ is directly recruited to *Ifng* promoter, although further studies are required to address the mechanism of *Ifng* transactivation by IκBζ.

In contrast to the critical role of IκBζ in NK-cell activation in response to IL-12 and IL-18, *Nfkbiz*$^{-/-}$ Th1 cells developed in the presence of IL-12 and IL-18 did not show a defect in producing IFN-γ in response to antigen-receptor stimulation (25). In contrast, IκBζ is involved in the development of Th-17 cells (25). We found that IL-18 stimulation failed to up-regulate IκBζ expression in T cells. Although NK cells produced IFN-γ by stimulation with IL-12 and IL-18, Th1 cells require T-cell receptor (TCR) stimulation in addition to culture with these cytokines. In addition to the difference in IκBζ expression levels in NK cells and T cells, differential requirement of antigen-receptor signaling in producing IFN-γ may explain the role of IκBζ in NK cells but not in Th1 cells.

Although IκBζ was essential for IFN-γ production in response to IL-12 and IL-18 stimulation, *Nfkbiz*$^{-/-}$ NK cells produced IFN-γ normally in response to culture with IgG1, which is known to activate NK cells via FcgRIIIa. In contrast to IL-12 and IL-18, FcgRIII activates the intracellular signaling via recruiting ITAM-motif containing adaptors activating Syk tyrosine kinase and ZAP-70 (32). Subsequently, phospholipase C (PLC)-γ is activated to induce Ca^{2+} influx, MAP kinase activation, and NF-κB activation. The FcgRIII and TCR largely share the signaling molecules, and future studies are required for identifying the mechanisms of how activating FcgRIII and TCR induce production of IFN-γ independent of IκBζ.

NK cells are important for the elimination of MCMV (1, 5). Type I IFNs and IL-12 produced from DC subsets such as plasmacytoid DC and $CD8\alpha^+$ DC are important for the NK-cell cytotoxic activity and production of IFN-γ, respectively (3, 4). Whereas MCMV-mediated production of IFN-α in the sera was normal, IL-12p40 levels were decreased in *Nfkbiz*$^{-/-}$ mice. Because IκBζ is required for the production of IL-6 and IL-12p40 in response to TLR ligands in macrophages (22), impaired production of IL-12p40 in *Nfkbiz*$^{-/-}$ mice is likely due to the defect in TLR-mediated MCMV recognition. Although type I IFNs are known to activate NK cells in response to virus infection, it has been shown that IFN-γ production to MCMV infection was not decreased even in the absence of type I IFN production or IFNR signaling. On the other hand, we did not find a defect in MCMV-induced NK-cell cytotoxicity in *Nfkbiz*$^{-/-}$ mice. Because NK-cell cytotoxicity to MCMV infection was reported to be IL-12–independent, normal activation of NK-cell cytotoxicity to MCMV infection in *Nfkbiz*$^{-/-}$ mice suggests that IκBζ is not involved in the type I IFN signaling.

In summary, our data demonstrate that IκBζ is essential for NK-cell activation in response to IL-12 and IL-18 stimulation by inducing various genes including IFN-γ. Furthermore, induction of IκBζ is one of the important host defense mechanisms against virus infection via NK cells. Given that IκBζ looks to control different set of genes in a cell-type specific manner, further studies will clarify the mechanisms of IκBζ in controlling gene expression.

Materials and Methods

Mice, Cells, and Reagents. *Nfkbiz*$^{-/-}$ (22) and littermate control mice were used at 6–16 wk of age. Mouse experiments were conducted in accordance with institutional guidelines of animal care and use committees. Splenic NK cells were enriched via MACS separation with DX5 MicroBeads (Miltenyi Biotec). YAC-1 cells were as described previously (33). EL4 cells were obtained from ATCC (TIB-39). IL-12 and IL-2 were purchased from R&D Systems. Biotinylated rabbit anti-mouse IgG1 was purchased from Zymed. ELISA kits for IFN-γ and IL-12p40 were purchased from R&D Systems. IL-18 and ELISA kit for IFNα were purchased from PBL.

Q-PCR. Total RNA was isolated using TRIzol (Invitrogen) and reverse transcription was performed with ReverTra Ace (Toyobo) according to the manufacturer's instructions. For Q-PCR, cDNA fragments were amplified by real-time PCR Master Mix (Toyobo), Fluorescence from the taqman probe for each cytokine was detected by a 7500 real-time PCR system (Applied Biosystems). To determine the relative induction of cytokine mRNA in response to various stimuli, the mRNA expression level of each gene was normalized to the expression level of 18S rRNA.

Cytotoxicity Assay. Splenic NK cells were cultured with IL-2 (2,000 U/mL) for 7 d. IL-2–induced NK cells were stimulated with IL-12 (0.1 ng/mL) and IL-18 (1 ng/mL) for 4 h. The ^{51}Cr-labeled YAC-1 cells (1×10^4 cells/well) were cultured with indicated numbers of NK cells. After 4-h incubation, the supernatant was harvested and the ^{51}Cr radioactivity of supernatants were measured as described previously (33).

Intracellular Staining of STAT4. Antibodies for flow cytometry were purchased from BD or eBioscience. Splenocytes were stimulated with IL-12 (2 ng/mL) for 30 min and were fixed immediately with PhosFlow Lyse/Fix buffer (BD) for 10 min. Cells were washed once with PBS and stained with DX5-PE and CD3e-APC. Cells were then permeabilized by BD PhosFlow Perm Buffer III and incubated for 25 min on ice. Cells were washed with BD Pharmingen Stain Buffer and stained with Phospho-STAT4-FITC at room temperature for 30 min in the dark. Cells were washed and acquired on a FACS Calibur flow cytometer (BD) and analyzed using FlowJo (Tree Star).

Immunoblot Analysis. NK cells were lysed in a buffer containing 0.1% Nonidet-P40, 10 mM KCl, 10 mM Hepes-KOH (pH 7.8), 0.1 mM EDTA and a protease inhibitor mixture (Roche). Cell lysates were centrifuged at 5,000 rpm for 5 min, and the supernatant was harvested as cytoplasmic proteins. Pellets were lysed in a buffer containing 5 mM $MgCl_2$, 420 mM KCl, 50 mM Hepes-KOH (pH 7.8), 0.1 mM EDTA and 20% glycerol for 30 min on ice. The lysates were centrifuged at 18,500 × *g* for 15 min, and the supernatant was harvested as nuclear proteins. Cell lysates were dissolved by SDS/PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blotted with the specific antibodies to indicated proteins.

Chromatin Immunoprecipitation (ChIP). ChIP was performed by using a chip assay kit (Millipore). NK cells were stimulated with IL-12 (20 ng/mL) and IL-18 (100 ng/mL) for 4 h and were fixed with 1% formaldehyde for 10 min or 10 mM DMA for 20 min. Cells were then washed and resuspended in SDS buffer. Lysates were sonicated to obtain DNA fragments with a peak in size between 200 and 1,000 bp. Lysates precleared with protein A agarose/salmon sperm DNA were incubated with antibodies against STAT4 (Santa Cruz) or H3K9 (Abcam) and IκBζ (kindly provided by Drs. Kayama and Takeda, Osaka University, Suita, Japan), and immunoprecipitated at 4 °C overnight. The immune complexes were absorbed with beads. Beads were washed with low salt buffer, high salt buffer, LiCl wash buffer, and in TE buffer. Immune complexes were extracted with elution buffer (1% SDS 100 mM $NaHCO_3$, 10 mM DTT) and cross links were reversed by incubation overnight at 65 °C. After proteinase K treatment for 1 h, DNA was then purified via ethanol precipitation. The purified DNA was used in qPCR to assess the presence of target sequences. Quantitative RT-PCR

was performed with Thunderbird qPCR Mix (Toyobo) in an Applied Biosystem 7300. Primers used for amplifying *Ifng* loci were shown previously (10, 34).

Reporter Assay. EL4 cells were transfected with either an empty vector or the IκBζ vector, human *Ifng* reporter plasmid containing the firefly luciferase gene (1 μg) (Addgene plasmid 17598) (35) and 50 ng of pRL-TK (Promega) by using DMRIE-C reagent (Invitrogen). EL4 cells were incubated in the presence of IL-12 (2 ng/mL) and IL-18 (10 ng/mL) for 18 h after transfection. Luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega).

Microarray. Splenic NK cells were stimulated with IL-12 (1 ng/mL) and IL-18 (10 ng/mL) for 24 h. Then, total RNA was extracted with TRIzol (Invitrogen Life Technologies) and further purified using an RNeasy kit (Qiagen). Biotin-labeled cDNA was synthesized from 100 ng of total RNA using the Ovation Biotin RNA amplification and labeling system (Nugen) according to the manufacturer's protocol. Hybridization, staining, washing, and scanning of Affymetrix mouse Genome 430 2.0 microarray chips were conducted according to the manufacturer's instructions. Data analysis was performed using R.

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The Jmjd3-*Irf4* axis regulates M2 macrophage polarization and host responses against helminth infection

Takashi Satoh[1,2,8], Osamu Takeuchi[1,2,8], Alexis Vandenbon[3], Koubun Yasuda[4], Yoshiaki Tanaka[5], Yutaro Kumagai[1,2], Tohru Miyake[1,2], Kazufumi Matsushita[1,2], Toshihiko Okazaki[1], Tatsuya Saitoh[1,2], Kiri Honma[6], Toshifumi Matsuyama[6], Katsuyuki Yui[6], Tohru Tsujimura[7], Daron M Standley[3], Kenji Nakanishi[4], Kenta Nakai[6] & Shizuo Akira[1,2]

Polarization of macrophages to M1 or M2 cells is important for mounting responses against bacterial and helminth infections, respectively. Jumonji domain containing-3 (Jmjd3), a histone 3 Lys27 (H3K27) demethylase, has been implicated in the activation of macrophages. Here we show that Jmjd3 is essential for M2 macrophage polarization in response to helminth infection and chitin, though Jmjd3 is dispensable for M1 responses. Furthermore, *Jmjd3* (also known as *Kdm6b*) is essential for proper bone marrow macrophage differentiation, and this function depends on demethylase activity of Jmjd3. *Jmjd3* deficiency affected trimethylation of H3K27 in only a limited number of genes. Among them, we identified *Irf4* as encoding a key transcription factor that controls M2 macrophage polarization. Collectively, these results show that Jmjd3-mediated H3K27 demethylation is crucial for regulating M2 macrophage development leading to anti-helminth host responses.

Innate immune cells such as macrophages sense the presence of microbial infection through pattern-recognition receptors and mount anti-microbial responses[1–3]. The Toll-like receptor (TLR) family is one of the best-characterized PRR families recognizing various pathogens such as bacteria, viruses, protozoa and fungi. TLRs are crucial in evoking innate immune responses to infection by various pathogens, leading to production of inflammatory mediators, including proinflammatory cytokines, chemokines and interferons.

Macrophages are functionally polarized into M1 and M2 cells in response to infection with microorganisms and host mediators[4,5]. M1 macrophages produce large amounts of nitric oxide by expressing inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF), and are essential for clearing bacterial, viral and fungal infections[6]. Other macrophages, called alternatively activated macrophages or M2 macrophages, have an important role in responses to parasite infection, tissue remodeling, angiogenesis and tumor progression. M2 macrophages are characterized by their high expression of arginase-1 (*Arg1*), chitinase-like Ym1 (*Chi3l3*), found in inflammatory zone-1 (*Fizz1*, also called *Retnla*) mannose receptor (*Mrc1* encoding MR, also known as CD206), and chemokines such as CCL17 and CCL24 (refs. 7–11). The PRR system responsible for the recognition of helminth infection and M2 polarization has yet to be identified.

Cytokines and growth factors have been implicated in the reprogramming of M1 and M2 macrophages. Whereas interferon-γ (IFN-γ), produced by activated T cells and TLR ligands, induces M1 macrophage generation, stimulation of macrophages with interleukin-4 (IL-4) or IL-13 induces M2-type macrophages[4,5]. In addition, immune complexes, IL-10 and glucocorticoid or secosteroid hormones are also known to generate M2 macrophages. Among growth factors, treatment of bone marrow cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) leads to generation of M1 and M2 cells, respectively[12–15].

TLRs trigger intracellular signaling pathways, inducing activation of a set of transcription factors, such as NF-κB, AP-1, C/EBPβ, PU.1 and interferon-regulatory factors (IRFs)[1,16]. These transcription factors cooperatively upregulate the expression of multiple genes such as proinflammatory cytokines, leading to M1 macrophage polarization. Proteins induced by TLR signaling are known to modulate inflammatory responses. IκBζ, an IκB family member, positively regulates certain genes such as *Il6* by interacting with NF-κBp50 or inducing histone modification[17,18], whereas ATF3 negatively regulates expression[19]. In contrast, transcription factors such as STAT6 and peroxisome proliferator-activated receptor-γ (PPARγ) are involved in polarization of M2 macrophages[5,20].

[1]Laboratory of Host Defense, World Premiere Initiative Immunology Frontier Research Center, Osaka University, Osaka, Japan. [2]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan. [3]Laboratory of Systems Immunology, World Premiere International Immunology Frontier Research Center, Osaka University, Osaka, Japan. [4]Department of Immunology and Medical Zoology, Hyogo College of Medicine, Hyogo, Japan. [5]Laboratory of Functional Analysis *In Silico*, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan. [6]Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan. [7]Department of Pathology, Hyogo College of Medicine, Hyogo, Japan. [8]These authors contributed equally to this work. Correspondence should be addressed to S.A. (sakira@biken.osaka-u.ac.jp).

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