Transcriptional regulator Bhlhe40 works as a cofactor of T-bet in the regulation of IFN-γ production in iNKT cells

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Invariant natural killer T (iNKT) cells are a subset of innate-like T cells that act as important mediators of immune responses. In particular, iNKT cells have the ability to immediately produce large amounts of IFN-γ upon activation and thus initiate immune responses in various pathological conditions. However, molecular mechanisms that control IFN-γ production in iNKT cells are not fully understood. Here, we report that basic helix-loop–helix transcription factor family, member e40 (Bhlhe40), is an important regulator for IFN-γ production in iNKT cells. Bhlhe40 is highly expressed in stage 3 thymic iNKT cells and iNKT1 subsets, and the level of Bhlhe40 mRNA expression is correlated with Ifng mRNA expression in the resting state. Although Bhlhe40-deficient mice show normal iNKT cell development, Bhlhe40-deficient iNKT cells show significant impairment of IFN-γ production and antitumor effects. Bhlhe40 alone shows no significant effects on Ifng promoter activities but contributes to enhance T-box transcription factor Tbx21 (T-bet)-mediated Ifng promoter activation. Chromatin immunoprecipitation analysis revealed that Bhlhe40 accumulates in the T-box region of the Ifng locus and contributes to histone H3-lysine 9 acetylation of the Ifng locus, which is impaired without T-bet conditions. These results indicate that Bhlhe40 works as a cofactor of T-bet for enhancing IFN-γ production in iNKT cells.

natural killer T cells | basic helix-loop–helix transcription factors | Bhlhe40 | interferon-γ | T-box transcription factor Tbx21

Invariant natural killer T (iNKT) cells are a subset of innate-like T cells that represent a small percentage of lymphocytes (1). iNKT cells are well defined by their unique ability to recognize glycolipid antigens such as α-galactosylceramide (α-GC), which is bound and presented by major histocompatibility complex class I-like CD1d molecules on antigen-presenting cells (APCs) (1). Upon activation, iNKT cells act as effector immune cells through the rapid secretion of large amounts of cytokines, such as IFN-γ, interleukin-2 (IL-2), IL-4, IL-15, IL-17, IL-21, IL-22, and tumor necrosis factor-alpha (TNF-α) (1, 2) and can also stimulate other immune cells to produce cytokines, such as IL-12, from CD1d-expressing APCs (3, 4). On the other hand, iNKT cells may also act as regulatory cells of other immune cells such as macrophages, dendritic cells, T cells, and natural killer cells (5). Thus, in the immune system, iNKT cells have been considered key players for linking innate and adaptive immune responses and playing important roles in tumor immunity, autoimmune diseases, transplant tolerance, and immunity against infections (5). Populations of iNKT cells are heterogeneous and consist of multiple cell subsets characterized by distinct phenotypes [Th1-like iNKT (iNKT1; IFN-γ producing (iNKT1); IFN-γ producing (iNKT1); TH2-like iNKT (iNKT2; IL-13 producing (iNKT2)), or TH17-like iNKT (iNKT17; IL-17A and IL-22 producing iNKT cells)] (6–9). IFN-γ secreted by iNKT cells is considered one of the key molecules for the augmentation of early immune responses to tumors or infections.

Despite the studies implicating iNKT cells as important immune cells with diverse functional roles, the molecular mechanisms of IFN-γ production in iNKT cells are still not fully understood. To address this important question, we used DNA microarray analysis [expression profiles were compiled using data from RefDIC (Reference Database of Immune Cells; refdic.rcii.riken.jp/profile.cgi)] for the screening of candidate genes that might be involved in the regulation of IFN-γ production in iNKT cells and identified basic-helix-loop–helix family, member e40 (Bhlhe40) as a key regulator of iNKT cell-derived IFN-γ. Bhlhe40 belongs to the basic helix-loop–helix protein family and acts as a regulating transcription factor that controls a wide variety of biological processes, including cellular growth, proliferation, apoptosis, immune responses, and the regulation of circadian rhythms (10–13).

In this study, we show that Bhlhe40 is highly expressed in stage 3 of thymic iNKT cells and the iNKT1 subset, and its expression is higher than other lineages in resting state. The deficiency of Bhlhe40 in iNKT cells results in a remarkable decrease in IFN-γ production.

Significance

A unique characteristic of invariant natural killer T (iNKT) cells is their ability to immediately produce large amounts of interferon-gamma (IFN-γ) upon activation, which enables these cells to play critical roles in initiating immune responses in various pathological conditions. In this study, we demonstrate a previously unidentified mechanism mediated by basic helix-loop-helix transcription factor family, member e40 (Bhlhe40) for accelerating IFN-γ production in iNKT cells. Bhlhe40 is required for normal physiological functions in iNKT cells, where it positively regulates IFN-γ production. Bhlhe40 also contributes to acetylating histone H3-lysine 9 of the Ifng locus in iNKT cells. These findings may help in understanding the molecular mechanisms related to the biology of iNKT cells.

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production but does not affect cell development or proliferation, indicating that Bhlhe40 is the factor responsible for enhancing IFN-γ production in iNKT cells. In the analysis of molecular mechanisms, we show that Bhlhe40 binds to T-box transcription factor Tbx21 (T-bet) and enhances T-bet-mediated IFN-γ promoter activation. Furthermore, Bhlhe40 accumulates in the T-box region and contributes to histone H3-lysine 9 (H3-K9) acetylation of the Ifng locus. Together, our findings identify Bhlhe40 as one of the important molecules involved in the regulation of IFN-γ production in iNKT cells and provide previously unidentified insight into the molecular mechanisms that explain the characteristics of IFN-γ-producing iNKT cells.

Results

**Bhlhe40 Is Highly Expressed in Ifng-Expressing iNKT Subsets.** To investigate the role of Bhlhe40 in iNKT cells, we first examined the expression levels of Bhlhe40 mRNA in iNKT cells compared with other T lymphocytes. We found that splenic iNKT cells show a high expression of Bhlhe40 mRNA compared with CD4+ or CD8+ T cells (Fig. 1A). Bhlhe40 plays important roles in the regulation of the mammalian molecular clock in a phase-dependent manner (10). Thus, we next ascertained whether Bhlhe40 mRNA expression in iNKT cells is affected by the dark-light cycles. We compared levels of Bhlhe40 mRNA expression in iNKT and CD4+ T cells of mice exposed to dark-light cycles. No changes in Bhlhe40 mRNA expression were observed in iNKT or CD4+ T cells after exposure to dark-light cycles, suggesting that Bhlhe40 mRNA expression in iNKT cells is independent of the circadian cycle (Fig. S1). Next, we examined whether Bhlhe40 expression is induced in activated iNKT cells by comparing the expression levels of Bhlhe40 mRNA in splenic iNKT cells after stimulation with α-CD3 monoclonal antibody (mAb) alone or combined with α-CD28 mAb. Bhlhe40 mRNA was significantly up-regulated in stimulated iNKT cells in a time-dependent manner relative to its expression in resting iNKT cells (Fig. 1B). Furthermore, we evaluated Bhlhe40 mRNA expression in thymic iNKT cells. iNKT cells’ development stages can be defined through differences in the expression levels of surface markers such as NK1.1 and CD44 (14) (Fig. 1C). We found that the expression level of Bhlhe40 mRNA was higher in iNKT cells at stage 3 (NK1.1lo CD44hi) compared with stage 1 (NK1.1lo CD44lo) or stage 2 (NK1.1hi CD44hi) (Fig. 1D). Importantly, the high expression of Bhlhe40 mRNA in thymic iNKT cells at stage 3 was accompanied with high expression levels of Tbet and Ifng mRNA compared with other stages (Fig. 1D). Additionally, iNKT cells are classified based on the expression of CD4 and IL-17 receptor B (IL-17RB) into heterogeneously different subsets (9). Consistent with previous reports, we identified four subsets of iNKT cells according to CD4 and IL-17RB staining (Fig. 1E). These subsets are characterized by different cytokine patterns. Ifng mRNA was abundant in the CD4+ IL-17RB− subset, whereas Il4 and Il17a mRNA were higher in the CD4+ IL-17RB+ and CD4− IL-17RB+ subsets, respectively (Fig. S2). Interestingly, we also found that Bhlhe40 was highly expressed in the CD4+ IL-17RB+ subset, which is characterized by high levels of Ifng mRNA, compared with other subsets (Fig. 1F). Together, these data indicate that Bhlhe40 mRNA is highly expressed in Ifng-expressing stage 3 thymic iNKT cells and iNKT1 subsets. Bhlhe40 mRNA expression was inducible after T-cell receptor (TCR) stimulation and was independent of the circadian cycle.

**iNKT Cells Develop Normally in Bhlhe40−/− Mice.** To evaluate the contribution of Bhlhe40 in the development of iNKT cells, we first compared frequencies of iNKT cells between wild-type (WT) and Bhlhe40−/− mice. We found that the deficiency of Bhlhe40 did not affect the frequencies of iNKT cells in the thymus, spleen, and liver (Fig. 2A). Furthermore, the frequencies of iNKT cells during development in the thymus were similar between WT and Bhlhe40−/− mice (Fig. 2B). Next we asked whether the deficiency of Bhlhe40 might affect the maturation status of iNKT cells. To answer this question, thymic iNKT cells from WT or Bhlhe40−/− mice were used to compare the expression of Ly49 family members, which are described as being expressed on both developing and mature iNKT cells (15). Again, no major differences were observed in the expression of Ly49 family members between WT and Bhlhe40−/− iNKT cells (Fig. 2C). In addition to intrinsic factors, iNKT cell maturation is also determined extrinsically by the interaction
between receptors on NKT common precursors and corresponding ligands expressed by CD4^+CD8^+ double-positive (DP) thymocytes such as CD1d and signaling lymphocytic activation molecule family member 1 (SLAMF1) (16, 17). We also confirmed that CD1d and SLAMF1 expressing CD4^+CD8^+ DP thymocytes were comparable between WT and Bhlhe40^−/− mice (Fig. 2D). Additionally, frequencies of different iNKT subsets based on CD4 and IL-17RB expressions were also comparable between WT and Bhlhe40^−/− mice (Fig. 2E). Taken together, these data suggest that iNKT cells can develop normally even in the absence of Bhlhe40.

**Bhlhe40 Enhances IFN-γ Production in iNKT Cells.** Bhlhe40 functions as a transcriptional regulator that is involved in cellular growth, development, and immunomodulation (10–13). Bhlhe40 showed no specific roles in the development of iNKT cells, as suggested by the previously mentioned results. Thus, next we asked if Bhlhe40 might regulate iNKT cell-mediated immune responses. iNKT cells are well characterized by their ability to rapidly release large amounts of IFN-γ upon activation, which is explained by the preformed Ifng mRNA available before stimulation (18). As previously described, two Ifng-expressing iNKT subsets were identified based on CD4 and IL-17RB expression, and these two subsets expressed similar levels of preformed Ifng mRNA as compared between WT and Bhlhe40^−/− iNKT cells (Fig. S2). Surprisingly, following stimulation with a-GC, Bhlhe40^−/− iNKT cells showed significant impairment in IFN-γ production compared with WT iNKT cells (Fig. 3A). Even more interesting, Bhlhe40 deficiency has no significant effects on IL-4 production in splenic iNKT cells stimulated with a-GC (Fig. 3B). We also compared the proliferation of a-GC–stimulated WT or Bhlhe40^−/− splenic iNKT cells and found that Bhlhe40^−/− iNKT cells can proliferate normally, similar to WT iNKT cells upon stimulation (Fig. 3C). Additionally, following a-CD3/CD28 stimulation, Bhlhe40^−/− iNKT cells showed decreased levels of induced Ifng mRNA compared with WT iNKT cells (Fig. 3D), suggesting a role for Bhlhe40 in the regulation of IFN-γ expression in iNKT cells.

Fig. 2. Development of iNKT cells is independent of Bhlhe40 expression. (A) Frequencies of iNKT cells in the thymus, spleen, and liver (gated on CD19^+ ) compared between WT and Bhlhe40^−/− mice. (B) Comparison of iNKT cells’ developmental stage in the thymus between WT and Bhlhe40^−/− mice (gated on TCR^β^+CD1d−α-GC dimer^−^ thymocytes). (C) Expression of Ly49A, Ly49C, and Ly49G2 maturation markers in thymic iNKT cells isolated from WT or Bhlhe40^−/− mice (gated on TCR^β^ CD1d−α-GC dimer^+^ thymocytes). (D) Expression of SLAMF1 and CD1d on CD4^+CD8^+ DP thymocytes compared between WT and Bhlhe40^−/− mice. (E) Comparison between iNKT subsets in WT or Bhlhe40^−/− mice based on CD4 and IL-17RB expressions (gated on TCR^β^ CD1d−α-GC dimer^+^). The data shown are representative of three independent experiments.

Fig. 3. Bhlhe40 deficiency results in impaired IFN-γ production in stimulated iNKT cells. (A) ELISA measurement of Ifng in a-GC–stimulated WT or Bhlhe40^−/− iNKT cells 48 h after stimulation. (B) ELISA measurement of IL-4 in a-GC–stimulated WT or Bhlhe40^−/− iNKT cells 48 h after stimulation. (C) Cell proliferation is compared between splenic iNKT cells isolated from WT or Bhlhe40^−/− mice 48 h after stimulation with a-GC. (D) Quantitative RT-PCR analysis of Ifng mRNA expression in splenic iNKT cells isolated from WT or Bhlhe40^−/− mice after stimulation with a-CD3 and a-CD28 Ab. The Ifng mRNA level of WT iNKT cells at 0 h was considered 1. (E) Intracellular staining of Ifng- or IL-4 in splenic iNKT cells of WT or Bhlhe40^−/− mice 1 h after i.v. administration of a-GC (gated on TCR^β^ CD1d−α-GC dimer^+^ splenocytes). (F) Serum levels of Ifng- or IL-4 in WT or Bhlhe40^−/− mice i.v. injected with a-GC. Similar results were obtained in three independent experiments. *P < 0.01.
Next, we evaluated the role of Bhlhe40 in the enhancement of IFN-γ production in vivo. To do so, WT or Bhlhe40−/− mice were i.v. injected with α-GC, and 1 h after α-GC administration, splenic NKT cells were stained intracellularly to measure levels of IFN-γ or IL-4 expression. Consistent with the in vitro data, we found that α-GC administration induced higher IFN-γ expression in WT compared with Bhlhe40−/− NKT cells, whereas no differences in IL-4 expression were observed between the two groups (Fig. 3E). Previous reports have shown that NKT cells mediate a rapid release of IFN-γ and IL-4 into the serum in response to α-GC (19). Thus, we compared IFN-γ and IL-4 concentrations in the serum of WT or Bhlhe40−/− mice injected i.v. with α-GC. As expected, levels of serum IFN-γ were higher in WT compared with Bhlhe40−/− mice in response to α-GC administration, whereas IL-4 was not altered (Fig. 3F). To further confirm if decreased concentrations of serum IFN-γ were related to Bhlhe40 deficiency in NKT cells, we performed an add-back experiment. In this experiment, WT or Bhlhe40−/− NKT cells were i.v. transferred into Ja18−/− mice, and levels of serum IFN-γ were measured at different time points within 48 h after i.v. injection of α-GC. Again, high levels of IFN-γ were observed in the serum of α-GC–injected Ja18−/− mice when transferred with WT but not Bhlhe40−/− NKT cells (Fig. S3), indicating that IFN-γ in the serum of α-GC–stimulated mice was derived from iNKT cells but not from other cells, such as NK or T cells. On the other hand, levels of serum IL-4 were comparable between the two groups (Fig. S3). Together, these data suggest that although WT and Bhlhe40−/− NKT cells show similar levels of preformed Ifng mRNA, IFN-γ production was significantly impaired in Bhlhe40−/− NKT cells following stimulation, suggesting that Bhlhe40 may play an important role in the enhancement of IFN-γ production in stimulated iNKT cells.

Bhlhe40 Deficiency Impairs Antitumor Effects of NKT Cells. Several studies have reported the contribution of NKT-derived IFN-γ in the inhibition of tumor metastases in mice after α-GC stimulation (20, 21). Thus, we next aimed to evaluate the impact of Bhlhe40 deficiency on NKT-mediated antitumor effects in a lung melanoma metastasis model. Compared with the significant effects of α-GC in WT mice, we found that treatment with α-GC showed no remarkable effects in Bhlhe40−/− mice, as the numbers of B16 melanoma nodules in the lungs were similar between the α-GC and control group (Fig. 4A). To further confirm that the impaired response of α-GC in Bhlhe40−/− mice was related to NKT cells but not to other cells, we performed an add-back experiment. In a liver metastasis model, Ja18−/− mice were transferred with WT or Bhlhe40−/− NKT cells, and the effects of α-GC treatment were evaluated in comparison to WT mice. The treatment with α-GC showed significant effects in Ja18−/− mice when transferred with WT NKT cells, whereas the transfer of Bhlhe40−/− NKT cells was less effective in reducing the melanoma surface area (Fig. 4B). Together, these results indicate an impact of Bhlhe40 deficiency in NKT cell–mediated suppression of lung and liver melanoma metastasis.

Bhlhe40 Does Not Enhance Ifng Promoter Activities by Itself. Next, we aimed to gain insight into how Bhlhe40 enhances IFN-γ production in TCR-stimulated NKT cells. First, we examined the effects of Bhlhe40 on Ifng promoter activation. In Bhlhe40−/− mouse embryonic fibroblast (MEF) cells transfected with a control or Bhlhe40 expression vector, we found that the overexpression of Ifng alone in Bhlhe40−/− MEF cells has no significant effects on Ifng promoter activity (Fig. S4A). TCR signaling leads to IFN-γ production via several downstream transcription factors, including nuclear factor-kappa B kinase (NF-κB) and nuclear factor of activated T cells (NFAT) (22, 23). Thus, we next asked if Bhlhe40 may enhance IFN-γ production by supporting the functions of NF-κB or NFAT. However, overexpression of Bhlhe40 in Bhlhe40−/− MEF cells has shown no significant effects on the NF-κB responsive promoter after stimulation with phorbol 12-myristate 13-acetate (PMA) compared with Bhlhe40−/− MEF cells transfected with the control vector (Fig. S4B). Additionally, in Bhlhe40 shRNA expressing EL-4 cells, transfection with a construct encoding knockdown-resistant Bhlhe40 (Bhlhe40 shRNA) showed no marked effects on the NFAT responsive promoter (Fig. S4C). Together, these data indicate that Bhlhe40 does not mediate a direct effect on Ifng promoter activities and has no supportive roles for molecules acting downstream of TCR signaling, such as NF-κB and NFAT.

Bhlhe40 Enhances IFN-γ Expression by T-bet–Mediated Mechanisms. Because Bhlhe40 alone showed no effects on Ifng promoter activities, we hypothesized that Bhlhe40 might act as a cofactor rather than as a transcription factor for the induction of Ifng mRNA. Thus, we next searched for candidate molecules that may interact with Bhlhe40. Among these, we focused on T-bet as a major key molecule related to IFN-γ production and acting downstream of TCR signaling (24). First, we found that T-bet is
expressed at similar levels in WT and Bhlhe40−/− iNKT cells (Fig. 5A). Interestingly, in a coimmunoprecipitation assay, we found that Bhlhe40 interacts with T-bet, as seen in the lysates of Lenti-X 293T cells, followed by immunoblot analysis with α-HA-tag or α-Myc-tag Ab in whole-cell lysates. (C) Immunoprecipitation of endogenous T-bet together with Bhlhe40 in lysates of iNKT cells stimulated with α-CD3/CD28 mAbs, followed by reprobed immunoblot analysis with α-T-bet Abs. (D) Luciferase activity of the Ifng promoter-luciferase reporter plasmid in Bhlhe40−/− MEF cells transfected with control or Bhlhe40 and/or T-bet expression plasmids or control vector 24 h after transfection. The luciferase activity level of Bhlhe40−/− MEF cells without stimulation was considered 1. (E) Luciferase activity of Ifng promoter-luciferase reporter plasmid in EL-4 cells stably transfected with Bhlhe40 shRNA or control shRNA, 48 h after transfection of T-bet expression plasmid with or without Bhlhe40 shRNA’ (construct encoding knockdown-resistant Bhlhe40). The luciferase activity level of EL-4 cells transfected with the shRNA control was considered 1. Data shown are shown representative of three independent experiments. **P < 0.05.

Fig. 5. Bhlhe40 enhances IFN-γ production by a T-bet-mediated mechanism. (A) Intracellular staining of T-bet in iNKT cells (gated on TCRβ+ CD1d−α-GC dimer+ cells) from WT or Bhlhe40−/− mice. (B) Immunoprecipitation of Myc-tagged T-bet together with HA-tagged Bhlhe40 in lysates of Lenti-X 293T cells, followed by immunoblot analysis with α-HA-tag or α-Myc-tag Ab in whole-cell lysates. (C) Immunoprecipitation of endogenous T-bet together with Bhlhe40 in lysates of iNKT cells stimulated with α-CD3/CD28 mAbs, followed by reprobed immunoblot analysis with α-T-bet Abs. (D) Luciferase activity of the Ifng promoter-luciferase reporter plasmid in Bhlhe40−/− MEF cells transfected with control or Bhlhe40 and/or T-bet expression plasmids or control vector 24 h after transfection. The luciferase activity level of Bhlhe40−/− MEF cells without stimulation was considered 1. (E) Luciferase activity of Ifng promoter-luciferase reporter plasmid in EL-4 cells stably transfected with Bhlhe40 shRNA or control shRNA, 48 h after transfection of T-bet expression plasmid with or without Bhlhe40 shRNA’ (construct encoding knockdown-resistant Bhlhe40). The luciferase activity level of EL-4 cells transfected with the shRNA control was considered 1. Data shown are shown representative of three independent experiments. **P < 0.05.

Bhlhe40 Enhances Ifng Expression upon IL-12 Stimulation. In addition to TCR-dependent stimulation, iNKT cells can also be activated by cytokines such as IL-12 (1). To examine if Bhlhe40 is also involved in the enhancement of IFN-γ after IL-12 stimulation, we compared levels of Ifng mRNA expression in IL-12–stimulated WT or Bhlhe40−/− iNKT cells. We found that Ifng mRNA expression induced by IL-12 was remarkably impaired in Bhlhe40−/− compared with WT iNKT cells at different stimulation times (Fig. S5A). To unveil the related mechanism, we examined if Bhlhe40 may interact with signal transducer and activator of transcription 4 (Stat4), a key molecule that plays a critical role in IL-12–induced IFN-γ production (25). However, we could not confirm an enhancement of Ifng promoter activities in Bhlhe40−/− MEF cells transfected with Stat4 and/or Bhlhe40 expression plasmid, nor binding between Stat4 and Bhlhe40 (Fig. S5 B and C). From these results we hypothesized that Bhlhe40—together with T-bet—may induce chromatin changes that facilitate Stat4-mediated Ifng promoter activities. Thus, we next aimed to evaluate the possible involvement of a Bhlhe40/T-bet interaction in chromatin remodeling of the Ifng locus.

Impaired Histone H3-K9 Acetylation in Bhlhe40−/− iNKT Cells. To evaluate our hypothesis, we performed chromatin immunoprecipitation (ChIP) assays using PCR primers that target the Ifng locus, which includes the Ifng promoter and the conserved noncoding sequence (CNS-5 and CNS-22) previously described (26–29) (Fig. 6A and Table S1). Interestingly, we found that Bhlhe40 accumulates in the T-box region of the Ifng locus, all of which are reported as binding sites for T-bet (30) (Fig. 6B). To identify if the Bhlhe40/T-bet interaction is involved in chromatin changes in the Ifng locus, we next compared histone H3-K9 acetylation, histone H3-K4 dimethylation, and histone H3-K4 trimethylation between splenic iNKT cells isolated from WT or Bhlhe40−/− NKT clone mice by ChIP PCR assay. We found that the deficiency of Bhlhe40 has led to impaired acetylation of histone H3-K9 in the T-box region of the Ifng locus (Fig. 6C), whereas no significant differences were observed in H3-K4 dimethylation or trimethylation in iNKT cells (Fig. S6 A and B). Additionally, histone H3-K9 acetylation, H3-K4 dimethylation, and H3-K4 trimethylation in the Ifh4 locus, which is known as T-box but not a binding locus (30, 31), was not affected by the deficiency of Bhlhe40 (Fig. S6 C–F and Table S2) (32). Together, these results suggest that the binding of the Bhlhe40/T-bet complex to the T-box region is important for chromatin changes in the Ifng locus.
Next, to confirm the importance of the Bhlhe40/T-bet interaction for the chromatin changes in the Ifng locus, we compared the accumulation of Bhlhe40 in the T-box region of the Ifng locus between T-bet–positive or –negative iNKT cells. As expected, we found that Bhlhe40 accumulation was reduced in T-bet–negative iNKT cells (Fig. 7A). Additionally, histone H3-K9 acetylation of the Ifng locus in T-bet–negative iNKT cells resembles that observed in Bhlhe40−/− iNKT cells (Fig. 7B and Fig. S7). Together, these results suggest a previously unidentified role of Bhlhe40 in iNKT cells as a cofactor that binds T-bet and regulates chromatin remodeling of the Ifng locus, resulting in enhanced IFN-γ production.

**Discussion**

The immune response is a dynamic process that starts with a rapid response of innate immunity and passes through multiple phases controlled by cellular and molecular components of the immune system, ending with the development of specific and adaptive immunity. iNKT cells consist of a subset of innate-like lymphocytes that are intermediates between innate and adaptive immunity and play important roles in the initiation and regulation of immune responses to tumors and infectious organisms (1). iNKT cells have attracted attention because of their unique ability to rapidly produce large amounts of IFN-γ, which induces the cytotoxic activities mediated by natural killer and CD8+ T cells (22, 33). Activation of iNKT cells by the administration of α-GC or α-GC–pulsed dendritic cells has been considered one of the most potent and promising strategies for the eradication of tumors, as several preclinical studies in animal models in addition to clinical trials have shown encouraging results of iNKT cell-based immunotherapy (34). However, these studies still lack a clear understanding of the molecular mechanisms of cytokine production, which might be critical for the improvement of these strategies.

In this study, we identified Bhlhe40 as a key player in the molecular mechanism that explains the unique ability of iNKT cells to rapidly produce large amounts of IFN-γ. Bhlhe40 is a transcriptional regulator expressed in a wide range of cells and is involved in various physiological functions, including cellular growth and proliferation, immune responses, and circadian rhythm (11, 13). As part of the Immunological Genome Project indicated, Bhlhe40 shows high expression levels in iNKT cells (35); however, the function of Bhlhe40 remained unclear. In this study, we clarified a previously unidentified role of Bhlhe40 in the enhancement of IFN-γ production in stimulated iNKT cells. Bhlhe40 was found to interact with T-bet after stimulation, and this interaction led to an enhancement in Ifng promoter activities. The deficiency of Bhlhe40 in iNKT cells led to impaired IFN-γ production in both glycolipid antigens and α-CD3/CD28 stimulation. Additionally, the deficiency of Bhlhe40 led to impaired IFN-γ production in IL-12–stimulated iNKT cells. However, we could not confirm a direct interaction between Stat4 and Bhlhe40 in resting or IL-12–stimulated iNKT cells. Furthermore, Bhlhe40 did not enhance signals mediated by molecules that contribute to IFN-γ production, such as NFAT and NF-κB, emphasizing the fact that Bhlhe40 enhances IFN-γ production in iNKT cells through direct interaction with T-bet.

As shown in this study, Bhlhe40 was found to bind T-bet in resting iNKT cells, and this binding was enhanced following TCR-ligand stimulation. In addition to its role as a transcriptional repressor that directly binds to class B E-box element (36), Bhlhe40 also acts as a cofactor of members of the basal transcription machinery, including transcription factor II B, TATA-binding protein, and transcription factor II D, and exerts transcriptional repression (37, 38). Bhlhe40 is also reported to transactivate several targets such as survivin via binding to the Sp1 sites (39). In addition to its function as a transcriptional factor of Ifng mRNA, T-bet is also involved in chromatin remodeling (40). Previous studies have reported a role of T-bet and CBP/P300 interaction in the induction of histone H3-K9 acetylation in the Ifng locus (41). Thus, Bhlhe40 may have a role in the enhancement of functions mediated by the T-bet–CBP/P300 complex, which has to be confirmed in subsequent studies. As suggested earlier, a modest epigenetic change at the transcription start site region of the Ifng locus was observed in iNKT cells, as represented by high acetylation in the Ifng locus. In Bhlhe40−/− iNKT cells, acetylation of histone H3-K9 in the T-box region of the Ifng locus was decreased. Furthermore, in T-bet–negative iNKT cells, histone H3-K9 acetylation was

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**Fig. 6.** Bhlhe40 is involved in chromatin remodeling of the Ifng locus. (A) A scheme describes primer positions in the Ifng locus used for the ChIP PCR assay. (B) Bhlhe40 binding to the Ifng locus. (C) Histone H3-K9 acetylation on the Ifng locus was detected in WT or Bhlhe40−/− iNKT cells by ChIP PCR assay. Histone H3-K9 acetylation at #1 site was evaluated in different samples and thus is represented by a separate graph. Data shown are representative of two independent experiments.
impaired in correlation with reduced Bhlhe40 accumulation in the T-box region of the Ifng locus. T-bet is known to have an ability to recruit histone H3-K4 methyltransferase and change the histone H3-K4 methylation of the Ifng locus (42). As our data show, histone H3-K4 methylation of the Ifng locus was reduced in T-bet–negative (Fig. S7 C and D) but not Bhlhe40–/– iNKT cells (Fig. S6 A and B). Furthermore, no changes in the Ifng locus, which is not related to T-bet, were observed in either T-bet–negative (Fig. S7 F and G) or Bhlhe40–/– iNKT cells (Fig. S6 E and F). Collectively, these findings suggest that the difference in histone H3-K4 methylation of the Ifng locus between T-bet–positive and T-bet–negative iNKT cells was dependent on T-bet deficiency alone. Thus, we concluded that the Bhlhe40/T-bet interaction is important for histone H3-K9 acetylation but not for histone H3-K4 methylation of the Ifng locus. Although epigenetic changes often lead to a change in basal transcription levels, those of Ifng mRNA in iNKT cells were not affected by Bhlhe40 deficiency. Thus, these results raised the possibility that Bhlhe40 may not directly affect epigenetic modifications but could be a result of reduced gene expression. Additionally, results obtained from transfected cell lines suggest that Bhlhe40 works directly on a reporter construct that does not have a chromatin structure. As with previous concerns, this limitation should be clarified in more detail in future studies.

As suggested by other reports, the deficiency of Bhlhe40 results in decreased IFN-γ production from Th1 cells (43). Bhlhe40–/– mice showed a resistance to the induction of experimental autoimmune encephalomyelitis, which is mediated by Th1 and Th17 cells (43). On the other hand, T-bet–expressing natural killer cells and γδ-T cells are also known to rapidly produce large amounts of IFN-γ following stimulation. Therefore, it would be of great interest to investigate the role of Bhlhe40 in these cells in association with the epigenetic regulation of the Ifng locus, which will be performed in our future studies.

Previous reports have suggested a circadian fashion of Bhlhe40 expression (10). However, no significant changes in Bhlhe40 mRNA expression were observed in iNKT cells of mice subjected to dark/light cycles, suggesting that the functions of Bhlhe40 in iNKT cells are independent of the circadian rhythm. Bhlhe40 was shown to control various functions of T cells, such as turning of naïve CD8+ T cells into memory ones, cytokine productions, or tumor infiltration, in a circadian rhythm-independent manner (43–45). Similarly, in this study, we report a previously unidentified function for Bhlhe40 as an enhancer of IFN-γ production in iNKT cells that is also independent of the circadian rhythm.

In conclusion, our data demonstrate the molecular mechanism of IFN-γ production in iNKT cells, which is mediated by the interaction between Bhlhe40 and T-bet. The identification and further characterization of Bhlhe40 functions in iNKT cells will facilitate development of improved iNKT cell-based immunotherapeutic strategies.

Materials and Methods

Cells and Animals. Lenti-X 293T, B16, and EL-4 cells were purchased from Clontech Laboratory, ATCC, and RIKEN Cell Bank, respectively, and were cultured in appropriate medium supplemented with 10% (vol/vol) FBS in a 5% CO2 incubator at 37 °C.

C57BL/6 mice were purchased from Japan SLC, Inc. Bhlhe40−/− mice and J18−/− mice were generated as previously reported (11, 46). NKT clone mice were generated from NKT–NT–ES cells by nuclear transfer of iNKT cells (47), whereas Bhlhe40−/− NKT clone mice were generated by crossing Bhlhe40−/− mice and NKT clone mice in our laboratory. Mice 6 to 8 wk old were used in this study. Mice were treated with humane care according to animal procedures approved by Animal Care Committee of Hokkaido University. More detailed information is provided in SI Materials and Methods.

Quantitative Real-Time PCR. Ifng, I4, II17a, Bhlhe40, Tbet, and hypoxanthine-guanine phosphoribosyl transferase (Hprt) mRNA were analyzed by the SYBR green real-time PCR system, using the comparative Ct method and normalized by the internal control Hprt. Reagents used for the experiments are listed in SI Materials and Methods.

Flow Cytometric Analysis and Cell Sorting. Cells were stained with fluorochrome-labeled mAbs, a PE-labeled α-GC-loaded CD1d dimer (48), or an isotype-matched control Ig after preincubation with the Fc receptor blocker (2.4G2), and data were collected by FACS Calibur, FACS Canto II, or FACS Aria II (BD Bioscience).

For intracellular staining, cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Bioscience). Further information is given in SI Materials and Methods.

Cell Proliferation Assay and ELISA. iNKT cells were purified from the spleen of WT or Bhlhe40−/− mice and cocultured with 35 Gy-irradiated splenocytes from J18−/− mice in a 96-well round-bottom plate culture in appropriate medium and conditions. After 40 h of α-GC stimulation, 3H-thymidine (1 μCi per well) was added to the culture for 8 h to measure the incorporation of 3H-thymidine. IFN-γ and IL-4 production in collected supernatants were detected by enzyme-linked immunosorbent assay (ELISA). Please refer to SI Materials and Methods.

In Vivo Stimulation and Cytokine Measurement. WT or Bhlhe40−/− mice were i.v. injected with α-GC, and serum levels of IFN-γ and IL-4 were evaluated using the CBA kit (BD Biosciences). For add-back experiments, 2 × 105 splenic WT or Bhlhe40−/− iNKT cells were i.v. administrated into J18−/− mice. One hour after the iNKT cell transfer, α-GC was administered, and serum levels of IFN-γ and IL-4 were measured as described earlier. In intracellular staining experiments, splenic iNKT cells from α-GC-stimulated WT or Bhlhe40−/− mice

Fig. 7. Bhlhe40 accumulation and H3-K9 acetylation of the Ifng locus is reduced without T-bet. (A) Bhlhe40 accumulation on the ifng locus was detected in T-bet–positive or –negative iNKT cells. (B) Histone H3-K9 acetylation on the ifng locus was detected in T-bet–positive or –negative iNKT cells by ChiP PCR assay. Data shown are representative of two independent experiments.
In the lung metastasis model, B16 melanoma cells were inoculated i.v. to WT and shRNA Against Bhlhe40 mice, and stimulated with α-GC i.p. administration. At 7 d after inoculation, lung tumor nodules were evaluated by microscopic inspection. In the liver metastasis model, B16 melanoma cells were inoculated into the spleen of WT or Jα18−/− mice and transferred with WT or Bhlhe40 −/− NK cells. Then, mice received an i.p. injection with α-GC. At 10 d after inoculation, the melanoma surface area in the liver was measured. Further information is provided in SI Materials and Methods.

Luciferase Reporter Assay. Lenti-X 293T cells were transfected using poly-ethyleneimine (PEI), and EL-4 and MEF cells were transfected using the Neon system (Invitrogen). The luciferase reporter plasmid and a transfection control, beta-galactosidase control vector, were transfected simultaneously. Supernatants containing lentiviral particles were collected after 48 h posttransfection. See SI Materials and Methods for details.

shRNA Against Bhlhe40. shRNA sequences against mouse Bhlhe40 were cloned into the lentiviral plasmid. A recombinant lentiviral vector was generated by transfection of appropriate virus composition vectors to Lenti-X 293T using PEI. Supernatants containing lentiviral particles were collected after 48 h and used for the transfection to make an shRNA-expressing transfected. Please refer to SI Materials and Methods.

Commmunoprecipitation and Western Blot Assay. For the communoprecipitation assay, Lenti-X 293T cells were transfected using PEI with HA-tagged Bhlhe40 or unrelated HA-tagged protein accompanied with Myc-tagged T-bet, Eta2, or control vectors. At 48 h after transient expression, cells were harvested and lysed with lysis buffers. To analyze the interaction of endogenous Bhlhe40 and T-bet, 3× 10^6 NK cells were isolated from NKT clone mice and stimulated with α-CD3/CD28 Ab for 60 min. Cleared lysates were labeled with α-HA, α-Myc, or α-T-bet Abs and precipitated by protein G beads. Immunoprecipitated proteins were detected with α-HA Ab or α-Myc Ab for transfected samples, and α-Bhlhe40 and α-T-bet Abs were used for endogenous samples by Western blotting. Please refer to SI Materials and Methods for details.

ChIP. Lysates were prepared from the NKT cells of WT or Bhlhe40 −/− mice. Chromatin samples were sonicated by an ultrasonic sonicator. Abs used for the assay are provided in SI Materials and Methods. Genomic PCR was performed by the primers listed in Tables S1 and S2.

**Differentiation of BMDCs in Vitro.** Bone marrow progenitors were cultured with appropriate medium supplemented with granulocyte-macrophage colony stimulating factor and IL-4. On day 6, bone marrow dendritic cells (BMDCs) were collected and received 35 Gy of irradiation.

Cultivation of T-bet−Positive and −Negative iNKT Cells in Vitro. The splenic T-bet−positive iNKT cell subset (TCRγδ CD1d−α-GC dimer+ CD4+ IL17RB−) and T-bet−negative iNKT cell subset (TCRγδ CD1d−α-GC dimer+ CD4+ IL17RB+) were separated by FACS Aria II sorting. Sorted cells were separately cocultured with irradiated BMDCs with appropriate medium supplemented with α-CD3 Ab and α-CD28 Ab for 3 d. On day 3, IL-2 and IL-7 were added to the medium. After 3 wk, cells were used for experiments. Please refer to SI Materials and Methods.

**Statistical Analysis.** All data are expressed as mean ± SEM. The differences between sample groups were determined by Student’s t-test or two-sample t-test with Welch’s correction. P values less than 0.05 were considered statistically significant. NS refers to not significant values.

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