not conventional αβ T cells or NK cells developed (7). These and other studies confirmed that expression of Vα14Jα18 in mice and Vα24Jα18 in human is a unique NKT cell signature.

DISCOVERY OF THE NKT CELL LIGAND

The ligand for NKT cells was identified as α-galactosylceramide (α-GalCer), which is presented by the MHC class I-like CD1d molecule. However, unlike MHC class I molecule with polymorphic in nature, CD1d is monomorphic among species, indicating that α-GalCer can be used in any potential NKT cell therapy for all humans. The glycolipid nature of the NKT cell ligand was suggested by experiments using mice lacking the transporter associated with antigen processing (TAP), which is essential for translocation of cytoplasmic peptides generated by the ubiquitin-proteasome proteolytic pathway into the endoplasmic reticulum (ER) to make a stable complex with MHC class I molecules. The MHC peptide complex is required to select CD8 T cells, therefore, in TAP-KO mice, CD8 T cells are not generated. However, by RNase protection assays using the invariant Vα14Jα18 as a probe, we could detect significant levels of protected bands in TAP-KO mice but not in β2M-KO mice, suggesting that the ligand is not a peptide, but likely to be a glycolipid in conjunction with a β2M-associated MHC-like molecule (8). The MHC-like molecule turned out to be CD1d, which has two large hydrophobic pockets, A′ and F′, that can bind the two long fatty acid chains of the ceramide portion of α-GalCer (9). Therefore, we screened various synthetic glycolipids and found the essential structure-function relationships critical for the NKT cell recognition, such as: (1) α-linkage between the sugar moiety and the ceramide portion of α-GalCer but not β-GalCer, (2) a 2′-OH configuration on the sugar moiety different from α-ManCer, and (3) a 3′-OH on the sphingosine of α-GalCer (10).

Furthermore, by using alanine substitution to mutagenize CD1d, we also identified important amino acids on CD1d, such as Ser76, Arg79, Asp80, Glu83, and Gln153, for activation of NKT cells in mice (11). In 2007, Borg et al. succeeded in crystallizing the triple complex of α-GalCer/human Vα24Jα18/TCRVβ11/human CD1d (12). Interestingly, the Vα24Jα18 chain docks in parallel with the CD1d cleft without any direct contribution of the TCRβ chain to ligand binding. This configuration is quite different from the mode of ligand recognition by the TCRβ chain of conventional αβ T cells, in which only the TCRβ but not the TCRα chain recognizes the MHC bound peptide in a diagonal position.

Analysis of the structure also revealed that the first four amino acids (Asp94, Arg95, Gly96, and Ser97) of Jα18, which are conserved in mouse and human, are essential for binding with both CD1d and α-GalCer. The Jα18Asp94 binds with CD1dArg79, Jα18Arg95 with CD1dArg79/Ser76/Asp80 and the 3′-OH on the sphingosine, Jα18Gly96 with the 2′-OH on galactose, and Jα18Ser97 with CD1dGln150. Interestingly, the CD1d amino acid, Glu83, defined as important in functional assays with CD1d mutants, is important for binding with the TCRβ chain to make a stable complex with CD1d but has no direct contribution to the ligand binding itself. Moreover, the CD1d amino acids (Ser76, Arg79, and Asp80) important for binding with either α-GalCer or Jα18 are also well conserved among species such as mouse, rat, sheep, and human (10, 13–15). Thus, α-GalCer, identified as an NKT cell ligand in mice can also be used to activate human NKT cells.

NKT CELL-MEDIATED ADJUVANT EFFECTS ON INNATE AND ADAPTIVE IMMUNITY AGAINST CANCER

In general, tumor cells do not contain any adjuvant materials, so that it is difficult to induce proliferation of specific T cell clones to mount anti-tumor responses in patients. On this particular point, α-GalCer overcomes these problems by its intrinsic adjuvant activity, inducing clonal expansion of tumor-specific T cell cells as well as activating various innate cell types (16). In the initial anti-tumor response after stimulation with α-GalCer/DCs, NKT cells immediately produce large amounts of IFN-γ, which acts on DCs, NK cells, and neutrophils in the innate immune system to eliminate MHC negative tumor target cells and, at the same, also on CD8 cytotoxic T cells and CD4 Th1 cells to kill MHC positive tumor cells, resulting in tumor eradication (**Figure 1**) (1, 17, 18). Therefore, NKT cell-targeted therapy is expected to overcome the major problem of current anti-cancer immunotherapies – recurrent tumors – due to their targeting of only one type of effector cell (10, 19, 20). For example, in the immunotherapy using tumor peptide CTL or antibodies against PD-1 or CTLA4, the target is the CD8 killer T cell, which kills MHC positive but not negative tumor cells, resulting in tumor recurrence (21). Similarly, in the artificial cells recently developed by the forced expression of Rae1/H60 (NKG2D-L), Mult-1 (NKG2D-L), or CD70 (TNF-L), the target cells are NK cells, which will eliminate MHC negative, but not MHC positive tumor cells (22).

Tumors in general contain both MHC positive and negative cells. Therefore, for an optimal therapy, both MHC types of tumor cells should be eliminated simultaneously by activating both innate and adaptive immune responses (**Figure 1A**). Since only NKT cells, but not other immune cells, activate NK and CD8 killer T cells at the same time, thus eliminating both MHC positive and negative tumor cells, the NKT cell-targeted therapy is a promising strategy for cancer treatment (**Figures 1B,C**).

NKT CELL-MEDIATED ADJUVANT EFFECTS ON DC MATURATION

Another important NKT cell function is their ability to interact with immature DCs in the presence of α-GalCer to induce DC maturation (17). Therefore, NKT cell-targeted therapy is also useful for advanced cancer patients, who often suffer from severe immunodeficiency. DCs in these advanced cancer patients are usually immature because of the presence of immune suppressive cytokines, such as IL-10 or TGFβ, produced by tumor cells (**Figure 1A**) (23). The immature DCs are able to capture tumor antigens, but unable to activate specific T cells. However, immature DCs presenting α-GalCer are activated by NKT cells through CD40-CD40L interactions to produce IFN-γ, which induce full DC maturation (24). This leads to a robust interleukin (IL)-12 response to further activate NKT cells, followed by activation of CD8T cells and NK cells (17, 24).

The DC maturation by activated NKT cells is a prominent strategy for the enhancement of protective innate and acquired immune responses. To investigate the mechanisms of bystander potential of α-GalCer-activated NKT cells, an experimental system

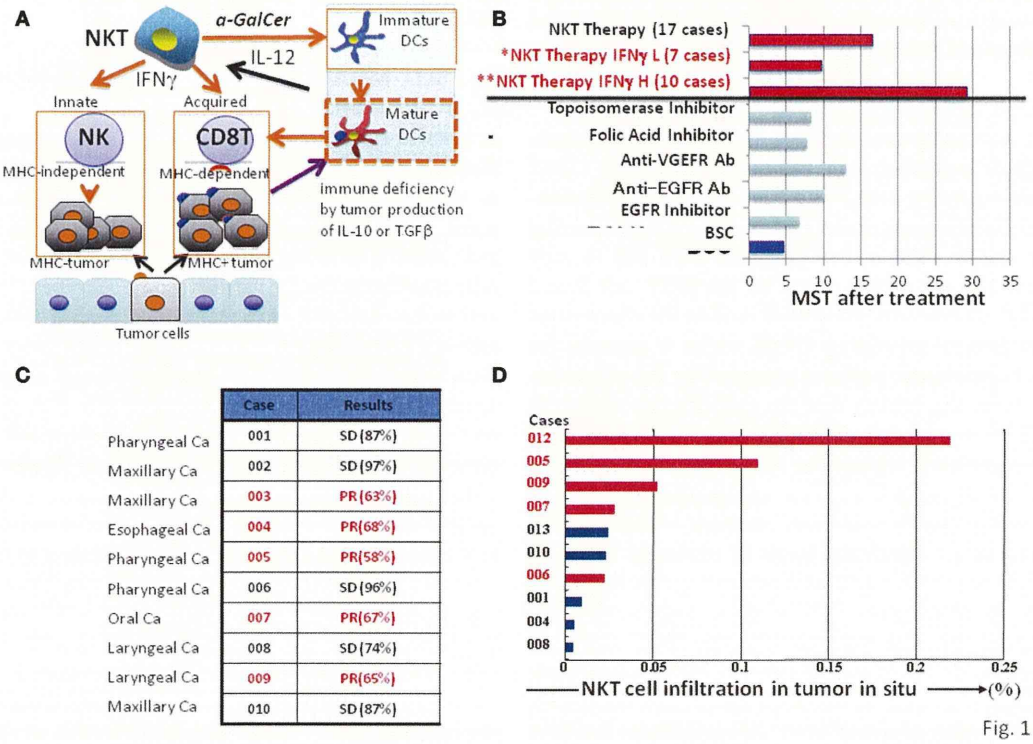

Fig. 1

FIGURE 1 | Natural killer T cell-mediated adjuvant effects on anti-tumor protective responses and clinical trial outcomes. **(A)** Mechanisms of NKT cell-targeted adjuvant cell therapy: upon NKT cell activation in patients by α-GalCer/DCs, immature DC become mature, and both MHC positive and negative tumor cells will be killed by CD8 killer T cells and NK cells, respectively. **(B)** Clinical trials of NKT cell-targeted adjuvant cell therapy on advanced non-small lung cancer: 60% of patients (**) showed significant prolonged median survival time of 29.3 months compared with best supportive care group with a MST of 4.6 months. The response to NKT cell therapy correlated with clinical efficacy (median survival time) and IFN-γ levels; patients with high (**H) levels responded significantly better than those with low (*L) levels. **(C)** Clinical trials of NKT cell-targeted adjuvant cell therapy for head and neck tumors: all 10 cases treated with the combination therapy of α-GalCer/DCs and activated NKT cells showed significant clinical efficacy (SD or PR). **(D)** Correlation between clinical efficacy (PR in red, SD in black) of head and neck tumors and NKT cell numbers in the tumor *in situ*.

using immunization with OVA-loaded TAP-deficient spleen cells loaded with OVA after permeabilization by osmotic shock was developed. In this system, OVA was used as an artificial tumor antigen to induce OVA-specific CD8 T cells to kill OVA-bearing tumor cells. Only after α-GalCer administration, IFN-γ production by NK and CD8T cells was observed (see **Figure 2A**). Under these conditions, the clonal expansion of OVA-specific CD8 T cells and strong anti-tumor responses develop in the mice, and the response requires co-administration of α-GalCer (17).

CLINICAL TRIAL OF NKT CELL-TARGETED THERAPY FOR ADVANCED LUNG CANCER AND HEAD AND NECK TUMORS

For effective NKT cell activation, α-GalCer/DC has distinct advantages to induce significant expansion of NKT cells and to inhibit *in vivo* tumor growth in a mouse model of metastatic lung cancer and liver metastasis in melanoma (25, 26). In a preclinical study, we used mouse melanoma cells, which were injected into the spleen to induce liver metastasis. Treatment of tumor-bearing mice by intravenous administration of α-GalCer/DCs (3×10^6) resulted in complete eradication of the liver metastasis within 7 days after treatment (27).

Based on the dramatic effects of α-GalCer/DCs in the preclinical studies, a clinical trial of NKT cell-targeted immunotherapy was conducted at Chiba University hospital in patients with advanced non-small cell lung cancer to evaluate the safety, feasibility, immunological responses, and clinical outcomes (28). Seventeen patients with advanced or recurrent non-small cell lung cancer refractory to the standard treatments, including surgery, chemotherapy, and radiation therapy, completed the protocol. The patient's peripheral blood mononuclear cells (PBMCs) obtained by apheresis were cultured with GMP grade GM-CSF and IL-2 for 7 days and then pulsed with α-GalCer (29). The α-GalCer-pulsed PBMCs were then intravenously administered (1×10^9 cells/m^2/injection) back into autologous patients twice with a 1-week interval followed by two courses with a 1-month interval between the second and third administration.

In the 17 patients who completed the protocol of a phase IIa clinical trial, the treatment was well-tolerated, and no severe adverse events related to the cell therapy were observed (28, 30). To monitor IFN-γ production by NKT cells from the patients, an enzyme-linked immunospot (ELISPOT) assay was performed (31). The results demonstrated that a significant increase in the number of IFN-γ-producing PBMCs was detected in 10 out of

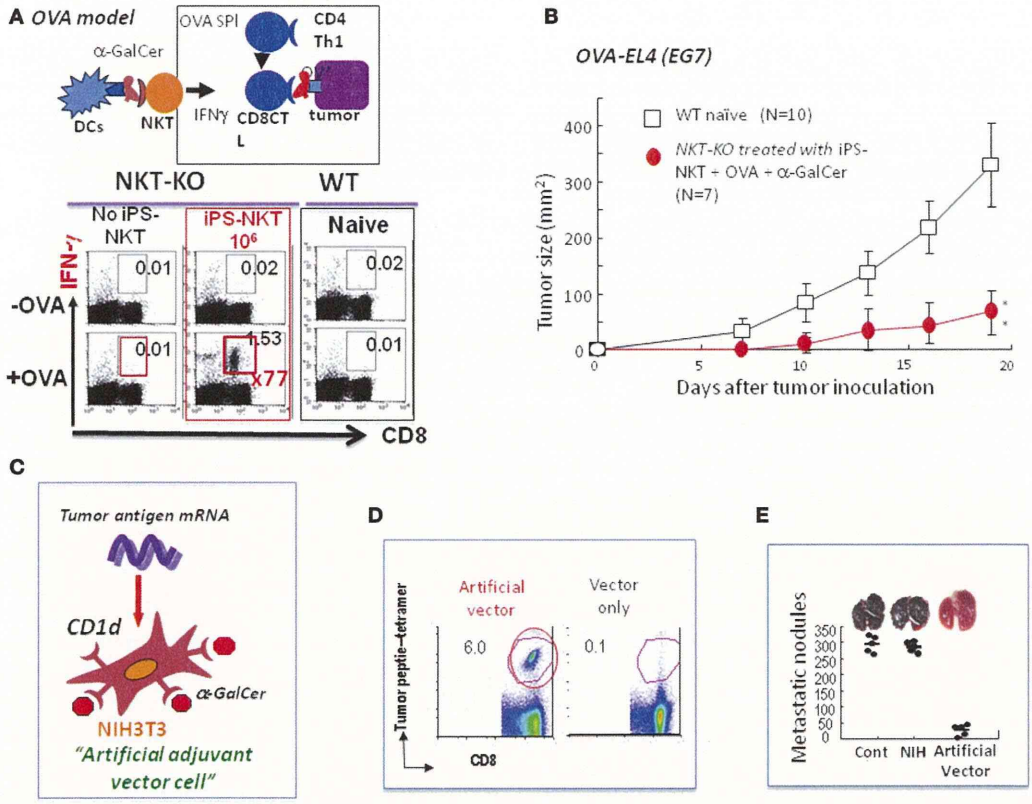

FIGURE 2 | Future directions for NKT cell-mediated cancer therapy. **(A)** Experimental model using OVA as an artificial tumor antigen to demonstrate NKT cell-mediated adjuvant activity (OVA model): the NKT-KO mice that had received iPS-derived NKT cells were immunized with OVA-loaded TAP-deficient spleen cells permeabilized by osmotic shock. A week later, the CD8 killer T cells in these immunized mice were analyzed for IFN-γ production after restimulation with OVA antigen *in vitro*. A significant increase in the number of antigen-specific IFN-γ producing CD8 killer T cells was detected in mice transferred with iPS-derived NKT cells. **(B)** Inhibition of the growth of OVA-expressing EL4 (EG7) tumor cells by NKT cell-mediated adjuvant therapy using iPS-derived NKT cells *in vivo* in the OVA model. A significant suppression of tumor growth *in vivo* was detected. **(C)** Generation of allogeneic artificial adjuvant vector cells. Artificial adjuvant vector cells were loaded with α-GalCer/CD1d and transfected with tumor mRNA. **(D)** Detection of long-term memory antigen-specific CD8 killer T cells even 1 year after a single injection of artificial adjuvant vector cells. Antigen-specific CD8 T cell responses in mice immunized with artificial adjuvant vector cells were analyzed using tetramer staining 1 year later. OVA was used in these experiments. **(E)** Suppression of melanoma lung metastasis after treatment with artificial adjuvant vector cells. Mice were intravenously injected with B16 melanoma cells to induce lung metastasis and, then 3 h later, intravenously with artificial adjuvant vector cells without tumor mRNA. The formation of metastatic nodules analyzed 2 weeks after melanoma cell injection was significantly suppressed according to the mechanisms of the activation of both NKT and NK cells but not that of CD8 killer T cells induced by artificial adjuvant vector cells carrying only α-GalCer/CD1d without tumor mRNA.

17 patients, which was correlated with a significantly prolonged median survival time (MST; 29.3 months) in comparison with the group with no increase compared to the pretreatment status in IFN-γ-producing cells (MST of 9.7 months) (**Figure 1B**) (32). The α-GalCer-reactive IFN-γ spot forming cells appeared to include both NKT cells and NK cells (31, 33), consistent with the notion that α-GalCer-activated NKT cells subsequently stimulate NK cells to produce IFN-γ (34, 35). We also investigated NKT cell infiltration in the surgically resected tumor samples and found a significant increase (25- to 60-fold) in the number of NKT cells in the tumor *in situ* (36). Because of the clinical correlation between increased IFN-γ production and prolonged overall survival, we conclude that IFN-γ may be a good biological marker for predicting clinical efficacy of this treatment. Although this prediction cannot be made prior to α-GalCer/DCs administration, the monitoring of IFN-γ production would still be valuable for patients receiving this immunotherapy. Although none of the cases showed significant tumor regression, the overall MST of all 17 patients (18.6 months) was superior to that of patients with best supportive care (4.6 months) or those treated with other types of therapies (average 10 months) in **Figure 1B** (37–40).

In the case of the head and neck tumors, we used a combination therapy with α-GalCer/DCs (10^8) and activated NKT cells (5×10^7) and completed 10 cases, including patients with pharyngeal, laryngeal, esophageal, maxillary, and oral carcinomas, who had advanced or recurrent disease after standard treatments (41). All treated patients showed either a partial response or achieved a stable disease state, indicating significant clinical efficacy (**Figure 1C**), which was associated with significant NKT cell infiltration into the tumor *in situ* (**Figure 1D**). To evaluate

clinical efficacy, a computed tomography (CT) scan was performed a few days before enrollment and also after the treatment. In some cases with partial responses, we observed that the enhanced area decreased in size, and necrosis appeared at the center of the tumor.

These encouraging clinical studies on advance lung cancers and head and neck tumors warrant further evaluation of NKT cell-targeted immunotherapy for survival benefit. In general, the immunotherapy may be more effective in patients with low tumor burden. Currently, we have been conducting α-GalCer/DC therapy for stage IIA to IIIA lung cancer patients with small tumor foci, including remaining micro-metastasis after radical surgery or after receiving the established first-line therapy in collaboration with National Hospital Organization.

FUTURE DIRECTIONS FOR NKT CELL-MEDIATED CANCER THERAPY USING iPS-DERIVED NKT CELLS

Although an NKT cell-targeted therapy has been shown to have significant clinical efficacy, only one third of patients are eligible in the case of advanced non-small lung cancer patients; the frequency of NKT cells in the other patients is too low. To overcome this problem, we established *in vitro* methods for generation of unlimited numbers of functional NKT cells, which then can be transferred into the patients whose endogenous NKT cell numbers are limited.

Induced pluripotent stem (iPS) cells were generated from mature NKT cells using *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes and then were developed into functional NKT cells *in vitro* in the presence of IL-7 and Flt3L according to the conventional protocol (42–44). The NKT cells generated *in vitro* from iPS-NKT cells were functional in the *in vivo* setting using the experimental model of OVA as an artificial tumor antigen (44). When NKT-KO mice were reconstituted with iPS-derived NKT cells followed by immunization with OVA and α-GalCer, we detected a 70-fold increase in the number of OVA-specific IFN-γ producing $CD8^+$ T cells above that seen in the control mice (**Figure 2A**). Under these conditions, the growth of the OVA-expressing EL4 (EG7) tumor cells was suppressed (**Figure 2B**). Thus, the iPS-derived NKT cells are able to function *in vivo*.

Before any clinical application of iPS-derived NKT cells, two immunological issues need to be addressed, one is whether GvHD is induced by NKT cells and the other is whether semi-allogeneic NKT cells will work *in vivo*, because of the clinical use of iPS-derived NKT cells under semi-allogeneic conditions. To address the first question, iPS-derived NKT cells on a B6 background and B6 or BALB/c CD4 T cells were injected into BALB/c RAG-KO mice. The results were very clear: only B6 CD4T cells, but not iPS-derived B6 NKT cells or BALB/c CD4 T cells, induced GvHD characterized by weight loss, diarrhea, skin disease development, or death after cell transfer. Concerning the second issue of the functional potential of semi-allogeneic NKT cells *in vivo* (129xB6) F1 NKT cells derived from cloned ES cells established by nuclear transfer of mature NKT cells into unfertilized eggs were injected into B6 NKT-KO mice and analyzed for their adjuvant activity in the OVA model. Significant proliferation of OVA-specific CD8 killer T cells was detected, even though these cells are eliminated in a few days. The ability to generate NKT cells using a simple *in vitro* culture system offers a powerful approach for the establishment of optimal NKT cell therapy. Our clinical application of the iPS-derived NKT cell therapy program has now been selected as a Center for Clinical Application Research on Specific Disease/Organ (Type B) in the Research Center Network for Realization of Regenerative Medicine, Japan.

FUTURE DIRECTIONS FOR THE NEXT GENERATION OF NKT CELL-TARGETED THERAPY

For the establishment of the next generation of NKT cell-targeted therapy, we developed artificial adjuvant vector cells to induce both innate and long-term memory CD8T cell responses against cancer. In this system, allogeneic NIH3T3 fibroblasts were used as a vector cell, into which tumor antigen mRNA and CD1d with α-GalCer were introduced. In the model experiment, we used OVA mRNA as an artificial tumor antigen together with α-GalCer/CD1d to induce the NKT cell-mediated adjuvant effects *in vivo in situ* (**Figure 2C**) (22). The allogeneic artificial vector cells were destroyed by the host immune system soon after inoculation and all materials carried by the cells were taken up by the host DCs, which immediately stimulated host NKT cells followed by induction of DC maturation and also by activation of innate NK cells and adaptive OVA-specific CD8 killer T cells. Surprisingly, long-term memory CD8 T cell responses were induced in an antigen-specific manner and persisted even 1 year after the initial single injection and suppressed OVA-expressing tumor cell metastasis (**Figures 2D,E**) (45). To test if this method could be generalized, we used TRP-2, tyrosinase related protein-2, which is a weak tumor antigen expressed by both mouse and human melanoma cells as the tumor antigen, and successfully suppressed tumor growth *in vivo*. Therefore, the artificial vector cells should be useful in the future for vaccines against various tumors.

SUMMARY

Natural killer T cells bridge innate and adaptive immunity, which enhances protective immune responses and also establishes long-term memory responses. Therefore, NKT cells have important therapeutic potential. In support of this notion, clinical trials on NKT cell-targeted therapy have demonstrated clinical safety and significant clinical efficacy in terms of prolonged median overall survival time in lung cancer patients and achieved stable disease status or partial responses in head or neck cancer patients.

The powerful treatment options for the future are to establish iPS cells that can generate unlimited numbers of NKT cells with adjuvant activity *in vitro* and suppress tumor growth *in vivo*. The other option is to establish the artificial adjuvant vector cells containing tumor mRNA and α-GalCer/CD1d, which have been shown to induce tumor-specific long-term memory CD8T cell responses and to inhibit tumor growth even 1 year after single injection. Thus, these could be therapeutic candidates for the next generation of NKT cell-targeted therapy.

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Gata3/Ruvbl2 complex regulates T helper 2 cell proliferation via repression of Cdkn2c expression

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GATA-binding protein 3 (Gata3) controls the differentiation of naive CD4 T cells into T helper 2 (Th2) cells by induction of chromatin remodeling of the Th2 cytokine gene loci, direct transactivation of *Il5* and *Il13* genes, and inhibition of *Ifng*. Gata3 also facilitates Th2 cell proliferation via additional mechanisms that are far less well understood. We herein found that Gata3 associates with RuvB-like protein 2 (Ruvbl2) and represses the expression of a CDK inhibitor, cyclin-dependent kinase inhibitor 2c (*Cdkn2c*) to facilitate the proliferation of Th2 cells. Gata3 directly bound to the *Cdkn2c* locus in an Ruvbl2-dependent manner. The defect in the proliferation of *Gata3*-deficient Th2 cells is rescued by the knockdown of *Cdkn2c*, indicating that Cdkn2c is a key molecule involved in the Gata3-mediated induction of Th2 cell proliferation. Ruvbl2-knockdown Th2 cells showed decreased antigen-induced expansion and caused less airway inflammation in vivo. We therefore have identified a functional Gata3/Ruvbl2 complex that regulates the proliferation of differentiating Th2 cells through the repression of a CDK inhibitor, *Cdkn2c*.

master transcription factor | transcriptional regulation | polycomb group complex

After antigenic stimulation in a particular cytokine milieu, naive CD4 T cells differentiate into various T helper (Th) cell subsets including Th1, Th2, and Th17 cells (1, 2). The differentiation of Th2 cells requires IL-4 stimulation, which leads to Stat6 activation and the induction of GATA-binding protein 3 (*Gata3*) transcription (3, 4). In addition, the Ras-ERK MAPK cascade controls Gata3 stability through the ubiquitin/proteasome-dependent pathway (5, 6). Gata3 is expressed in T lymphocytes, and its expression is required for the CD4 versus CD8 lineage choice and at the β-selection checkpoint in the thymus (7, 8), as well as for Th2 cell differentiation in the periphery (9–12).

It has been known that activated CD4 T cells proliferate more vigorously under the Th2 culture conditions where IL-4 is present compared with the Th1 conditions (13, 14). *Gata3*-deficient Th2 cells show a substantially reduced BrdU incorporation, indicating that Gata3 is involved in the regulation of Th2 cell expansion (15). However, no definitive analysis has yet been reported regarding the molecular mechanisms underlying the Gata3-mediated induction of Th2 cell proliferation.

T-cell proliferation following TCR signaling is stimulated by the increase in the expression levels of CDK4/6 and cyclin D (16). CDK inhibitors including the Ink4 family members *Cdkn2a* (p16, ink4a), *Cdkn2b* (p15, ink4b), *Cdkn2c* (p18, ink4c), and *Cdkn2d* (p19, ink4d) negatively regulate the activity of the cyclin D-CDK4/6 complex and block the G1-S phase transition, halting cellular proliferation in nonimmune cells (17). *Cdkn2c* has been implicated in the regulation of T-cell proliferation, supported by the observation that T cells from *Cdkn2c*-deficient mice exhibit a hyperproliferative phenotype in response to TCR stimulation (18). Although expression of *Cdkn2c* is restrained by GATA3 in mammary luminal progenitor cells, the transcriptional regulation of this gene in Th2 cells is yet to be fully elucidated (19).

We herein identified a Gata3/RuvB-like protein 2 (Ruvbl2) complex as a key regulatory mechanism of Th2 cell proliferation via repression of *Cdkn2c*. Such a regulatory mechanism differs from other cell types and might be uniquely specific for Th2-cell proliferation. Ruvbl2 regulates the recruitment of Gata3 to the *Cdkn2c* locus, and, together, they repress the expression of the *Cdkn2c*. Moreover, the defect in the proliferation of *Gata3*-deficient Th2 cells is rescued by knockdown of *Cdkn2c*, indicating that Cdkn2c is a key molecule involved in the Gata3-mediated induction of Th2 cells. Therefore, the Gata3/Ruvbl2 complex plays a pivotal role in the proliferation of differentiating Th2 cells via the repression of *Cdkn2c*.

Results

Identification of Ruvbl2 as a Molecule That Interacts with Gata3 in Th2 Cells. Gata3 is well-established as a key transcription factor involved in Th2-cell differentiation, but the nature of the functional Gata3 complexes that control the various processes required for Th2-cell generation, including their proliferation, has been unclear. To identity the functional components of the

Significance

GATA-binding protein 3 (Gata3) controls the differentiation of naive CD4 T cells into T helper 2 (Th2) cells by induction of chromatin remodeling at the Th2 cytokine gene loci. Gata3 also facilitates Th2 cell proliferation via unknown mechanisms. We have identified a functional Gata3/RuvB-like protein 2 (Ruvbl2) complex that regulates the proliferation of differentiating Th2 cells through the repression of a CDK inhibitor, cyclin-dependent kinase inhibitor 2c (*Cdkn2c*). Gata3 directly bound to the *Cdkn2c* locus in an Ruvbl2-dependent manner, and Cdkn2c-knockdown experiments indicated an important role for this molecule in the Gata3-mediated induction of Th2-cell proliferation. Ruvbl2-knockdown Th2 cells showed decreased antigen-induced expansion and caused less airway inflammation in vivo, indicating an important role for Ruvbl2 in Th2 cells in allergic reactions.

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Data deposition: The ChIP-seq data have been deposited in the DNA Data Bank of Japan, www.ddbj.nig.ac.jp (accession no. DRA001102).

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Gata3 complexes, we adopted a unique proteomics approach using affinity purification from the material obtained from the formaldehyde cross-linked Gata3 complex, in which associated molecules with low affinity can be identified (20). The 3xFlag–Gata3 complexes were precipitated efficiently by an anti-Flag mAb from the 3xFlag-tagged Gata3-expressing Th2 cell line, D10G4.1, and a mass-spectrometry analysis identified several polypeptides, including Ruvbl2 (also known as reptin or Tip49b) (Fig. 1*A*). To confirm the association of Gata3 with Ruvbl2, formaldehyde cross-linled cell extracts from the same cells were subjected to immunoprecipitation with an anti-Flag mAb. Ruvbl2 was easily detected in the cross-linked Gata3 complexes, but not in the non–cross-linked Gata3 complexes, thus indicating that there is a low-affinity association of these two molecules in D10G4.1 cells (Fig. 1*B*). The interaction between Gata3 and Ruvbl2 was also detected in non–cross-linked 293T cells in which Flag-tagged Gata3 and Myc-tagged Ruvbl2 were overexpressed (Fig. 1*C*). To address the direct molecular association of these two molecules, a GST–pull-down assay with purified recombinant Gata3 and Ruvbl2 was performed (Fig. 1*D*). The association of Gata3 with Ruvbl2 persisted in the presence of ethidium bromide, suggesting that the association is DNA-independent (Fig. S1). These results indicate that Ruvbl2 is a bona fide Gata3-interacting molecule in Th2 cells.

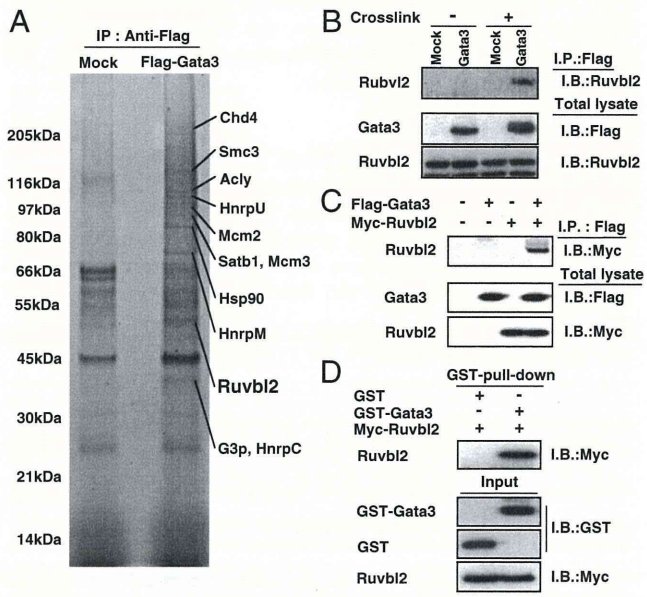

Fig. 1. Identification of Ruvbl2 as a molecule that interacts with Gata3 in Th2 cells. (*A*) Total extracts from 3xFlag–Gata3-expressing cross-linked D10G4.1 cells were subjected to affinity purification using a Flag mAb, followed by SDS/PAGE and SYPRO Ruby staining. Several specific polypeptides were identified by mass spectrometry as described in *Materials and Methods*. (*B*) The 3xFlag–Gata3-expressing D10G4.1 cells were treated with or without formaldehyde as indicated (Cross-link + or –) before extraction. The extracts were then immunoprecipitated (I.P.) with a Flag mAb, followed by immunoblotting (I.B.) with an Ruvbl2 Ab (*Upper*). Total lysates were also subjected to I.B. in parallel (*Lower*). (*C*) The 293T cells were transfected with expression plasmids encoding Flag-tagged Gata3 and Myc-tagged Ruvbl2. Two days later, the extracts were I.P. with a Flag mAb, followed by I.B. with a Myc mAb (*Upper*). The total lysates were also subjected to I.B. in parallel (*Lower*). (*D*) GST or GST–Gata3 proteins were bound to glutathione Sepharose 4B and incubated with purified Myc-tagged Ruvbl2. The bound proteins were subjected to I.B. with an anti-Myc mAb (*Upper*). The inputs represent 10% of the amount of protein used in the pull-down sample (*Lower*). Three (*C*) and two (*B* and *D*) independent experiments were performed, and similar results were obtained.

Ruvbl2 Regulates the Proliferation of Th2 Cells. To identify the function of Ruvbl2 in differentiating Th2 cells, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled naive CD4 T cells were transfected with Ruvbl2 siRNA and were cultured under Th2 conditions in vitro. The cell division was significantly inhibited in the Ruvbl2 knockdown (KD) cells, which was accompanied by decreased generation of IL-4–producing Th2 cells (Fig. 2*A*). Ruvbl2 KD had no impact on the mRNA or protein expression of Gata3 although efficient silencing of *Ruvbl2* mRNA expression was detected (Fig. S2 *A* and *B*). OX40-Cre–driven conditional *Gata3* knockout (*Gata3*-deficient) Th2 cells showed a similar phenotype as the Ruvbl2 KD Th2 cells (Fig. 2*B*). A substantial reduction in BrdU incorporation was observed in the Ruvbl2 KD Th2 cells (42.8% versus 21.6%) and also in *Gata3*-deficient Th2 cells (49.6% versus 34.5%) (Fig. 2*C*). These results indicate that Ruvbl2 and Gata3 positively regulate the proliferation of Th2 cells.

The Expression of *Cdkn2c* Is Repressed in a Gata3- and Ruvbl2-Dependent Manner. Earlier reports demonstrated that Gata3 regulates cell cycle in luminal progenitor cells and neuroblastoma cell via control of *Cdkn2c* and *Ccnd1* expression, respectively (19, 21). Thus, we next assessed the expression of *Cdkn2c* and *Ccnd1* in primary Th1 and Th2 cells from wild-type or *Gata3*-deficient mice. Although *Ccnd1* expression was not detected in primary Th1 and Th2 cells, the expression of *Cdkn2c* was lower in Th2 cells compared with Th1 cells, and the depletion of *Gata3* in Th2 cells resulted in increased expression of *Cdkn2c* (Fig. 3*A*). Moreover, the *Cdkn2c* expression was upregulated in primary Th2 cells when Ruvbl2 was silenced by siRNA (Fig. 3*B*). These results indicate that the expression of *Cdkn2c* is repressed in primary Th2 cells in a Gata3- and Ruvbl2-dependent manner.

To identify Gata3-bindng sites around the *Cdkn2c* locus, we performed a chromatin immunoprecipitation assay, followed by a massive parallel sequencing (ChIP-Seq) analysis using 3xFlag–Gata3-expressing Th2 clone cells (D10G4.1). Statistics of the tags generated for the experiment are summarized in Fig. S3*A*. The binding of 3xFlag-Gata3 at the previously validated Gata3-binding sites (Th2 cytokine and *Ccr8* loci) was confirmed (Fig. S3 *B* and *C*) (10, 11). From our ChIP-seq dataset, we identified two Gata3-binding sites around the *Cdkn2c* locus (Intron2 and +7.5-kb regions) (Fig. S3*D*, *Upper*). In the differentiating Th2 cells, Gata3 binding was detected only at the downstream region (+7.5 kb) of the *Cdkn2c* locus (Fig. 3*C*). Consistent with our results, the strongest binding of Gata3 at the +7.5-kb region of *Cdkn2c* was observed in primary Th2 cells compared with Th1 and Th17 cells in the previously reported ChIP-seq analysis for endogenous Gata3 (Fig. S3*D*, *Lower*) (11). We named the +7.5-kb region the Gata3 binding site (G3BS). To assess whether the G3BS plays a functional role in the Gata3-dependent transcriptional repression of *Cdkn2c*, a 500-bp fragment spanning the *Cdkn2c* G3BS (+7,261 ~ +7,760) (Fig. S4) was placed at the 5′-end of the *Cdkn2c* promoter (–500), and luciferase reporter assays were performed (Fig. 3*D*). The introduction of the G3BS substantially repressed the transcriptional activity of the *Cdkn2c* promoter whereas insertion of a G3BS with three mutations at the GATA consensus binding sequence did not show any effects (Fig. 3*D* and Fig. S4). These results indicate that Gata3 binds directly to the *Cdkn2c* locus and represses the mRNA expression of *Cdkn2c*.

Knockdown of *Cdkn2c* Expression Rescued the Impaired Proliferation of *Gata3*-Deficient Th2 Cells. Next, we examined whether Cdkn2c plays an important role in the Gata3-mediated induction of Th2 cell proliferation. Naive CD4 T cells from *Gata3*-deficient mice were stimulated under Th2 conditions for 2 d; then, the cells were infected with a retroviral vector containing shRNA specific for *Cdkn2c*. Four days after infection, the increased *Cdkn2c* mRNA expression in *Gata3*-deficient Th2 cells was abrogated in

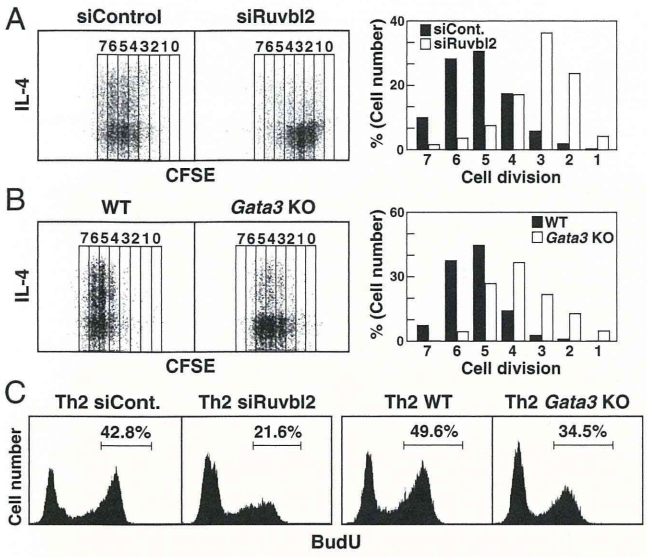

Fig. 2. Ruvbl2 regulates the proliferation of Th2 cells. (*A*) A control or Ruvbl2 siRNA was transfected into naive CD4 T cells, and the cells were labeled with CFSE. Then, the cells were stimulated with an immobilized anti-TCRβ mAb and anti-CD28 mAb under Th2 conditions for 3 d. The cells were then restimulated and subjected to intracellular staining with an APC-conjugated anti–IL-4 mAb (*Left*). The percentages of the cells in the gates representing the number of cell divisions (nos. 1 to 7) are shown (*Right*). (*B*) Naive CD4 T cells from WT or *Gata3*-deficient mice were labeled with CFSE and cultured under Th2 conditions for 3 d. The cells were then restimulated and subjected to IL-4 staining (*Left*). The percentages of the cells in the gates are shown (*Right*). (*C*) A control or Ruvbl2 siRNA was transfected into naive CD4 T cells, and the cells were stimulated with an immobilized anti-TCRβ mAb and anti-CD28 mAb under Th2 conditions for 4 d (*Left*). Naive CD4 T cells from WT or *Gata3*-deficient mice were cultured under Th2 conditions for 5 d (*Right*). Representative intracellular staining profiles for BrdU are shown with the percentages of cells in the gate. Three independent experiments were performed and similar results were obtained (*A*, *B*, and *C*).

the cells in which shCdkn2c had been introduced (Fig. 3*E*). We also found that the decreased BrdU incorporation and cell division in the *Gata3*-deficient Th2 cells compared with WT cells was restored by the introduction of shCdkn2c (Fig. 3 *F* and *G*). Interestingly, the restoration in IL-4 production was not observed by introduction of shCdkn2c in *Gata3*-deficient Th2 cells (Fig. 3*H*). These results indicate that Cdkn2c is specifically critical for the Gata3-mediated induction of the proliferation of Th2 cells.

The Transactivation Domain of Gata3 Is Required for the Association with Ruvbl2 and the Repression of *Cdkn2c* Expression. The GATA family transcriptional factors (Gata1 to -6) typically bind to a consensus motif (A/T)GATA(A/G) and regulate the specification and differentiation of numerous tissues. All GATA family members share two highly conserved C2H2-type zinc fingers, both of which are involved in DNA binding and protein–protein interactions (22, 23). Two transactivation domains are also known to be important for the function of Gata3 (24).

We examined which domains of Gata3 were important for the binding to Ruvbl2. Flag-tagged wild-type or deletion mutants of Gata3 (as depicted in Fig. S5 *A*, *Upper* and *B*, *Upper*) were cotransfected with Myc-tagged Ruvbl2 into 293T cells; then, the interaction was assessed by immunoprecipitation with a Flag mAb and subsequent immunoblotting with a Myc mAb. The association with Ruvbl2 was almost completely abrogated by the deletion of amino acids 65–178 (dTA), which is the region around the two transactivation domains of Gata3 (Fig. S5 *A* and *B*), thus indicating that the transactivation domains of Gata3 are important for binding to Rubvl2. To further examine whether these regions of Gata3 are important for the expression of *Cdkn2c*, we generated a stable Gata3 knockdown T-cell line. shRNAs against Gata3 or Ruvbl2 were introduced into a mouse T-cell line 68–41 using a lentivirus system. As expected, the expression of *Cdkn2c* was up-regulated in the Gata3 or Ruvbl2 knockdown 68–41 cells (Fig. S5*C*). We then expressed WT or dTA mutant Gata3 in these Gata3 knockdown 68–41 cells using a retrovirus system. Both the WT and dTA mutant Gata3 were substantially expressed in the Gata3 knockdown 68–41 cells (Fig. S5*D*, *Upper*). As shown in Fig. S5*D*, *Lower*, WT Gata3 repressed the *Cdkn2c* expression whereas the dTA mutant did not repress the expression of *Cdkn2c*. These results indicate that the transactivation domain of Gata3 is required for the repression of *Cdkn2c* expression.

Ruvbl2 Is Necessary for the Recruitment of Gata3 to the *Cdkn2c* Locus in Th2 Cells. To further investigate the molecular requirements for the Gata3-mediated repression of *Cdkn2c* expression in primary T cells, we used differentiating Th2 cells from *Gata3*-deficient mice. The introduction of WT Gata3 reduced the expression of *Cdkn2c* whereas the dTA mutant did not show any effect in the *Gata3*-deficient Th2 cells (Fig. 4*A*). The binding of the Gata3 dTA mutant to the *Cdkn2c* G3BS region was significantly compromised (Fig. 4*B*, *Left*). In contrast, the binding of the Gata3 dTA to the Th2 cytokine gene loci at the CGRE region was not compromised (Fig. 4*B*, *Right*), thus indicating that the Gata3 dTA mutant had a preserved DNA binding activity. In addition, a ChIP assay for endogenous Gata3 revealed that the Gata3 binding at the *Cdkn2c* G3BS region was impaired in Ruvbl2 KD Th2 cells (Fig. 4*C*). Thus, Ruvbl2 appears to regulate the binding of Gata3 to the *Cdnk2c* G3BS region in Th2 cells. Taken together, these results suggest that the association of Ruvbl2 with Gata3 is required for the binding of Gata3 to the *Cdkn2c* G3BS region.

Repressive Histone Modifications at the *Cdkn2c* Locus Induced by the Expression of Gata3 and Ruvbl2. We previously reported that the polycomb group (PcG) gene product, Bmi1, associates with Gata3 and controls the stability of the Gata3 protein in Th2 cells (25). In addition, Ruvbl2 was previously shown to be a component of the PcG complex in *Drosophila* (26, 27). Thus, we next investigated the histone modifications at the *Cdkn2c* locus, particularly histone H3-K27 trimethylation (H3-K27 Me3) and H2A-K119 monoubiquitination (H2A-K119 Ub), which are repressive marks known to be induced by PcG complexes. Interestingly, the H3-K27 Me3 and H2A-K119 Ub signals at the *Cdkn2c* locus, including the promoter, intron2, and G3BS regions, were higher in Th2 cells compared with Th1 and *Gata3*-deficient Th2 cells (Fig. 4 *D* and *E*). In addition, as shown in Fig. 4*F*, these histone modifications at the *Cdkn2c* promoter were significantly reduced in Ruvbl2 KD Th2 cells. Thus, repressive histone modifications at the *Cdkn2c* locus correlate strongly with both Gata3 and Ruvbl2 expression.

The Role of Ruvbl2 in Antigen-Induced Expansion of Th2 cells and Subsequent Induction of Allergic Airway Inflammation in Vivo. Finally, we investigated the in vivo physiological role of Ruvbl2 using a Th2 cell-dependent allergic airway inflammation model (28). Ruvbl2 KD Th2 cells or control cells generated by in vitro culture from DO11.10 Tg mice were i.v. injected into normal BALB/c mice. The mice were challenged twice by inhalation with 1% (wt/vol) OVA (Fig. S6). Before inhalation, control and Ruvbl2 KD Th2 cells were similarly detectable in the lung (Fig. 5*A*). A dramatic increase in the number of transferred Th2 cells ($KJ1^+$ cells) was induced by OVA inhalation (Fig. 5*A*, *Right*, black bars), and the increase was significantly impaired when Ruvbl2 was knocked down (Fig. 5*A*, *Right*). A significant decrease in the infiltration of inflammatory cells, including eosinophils, in the

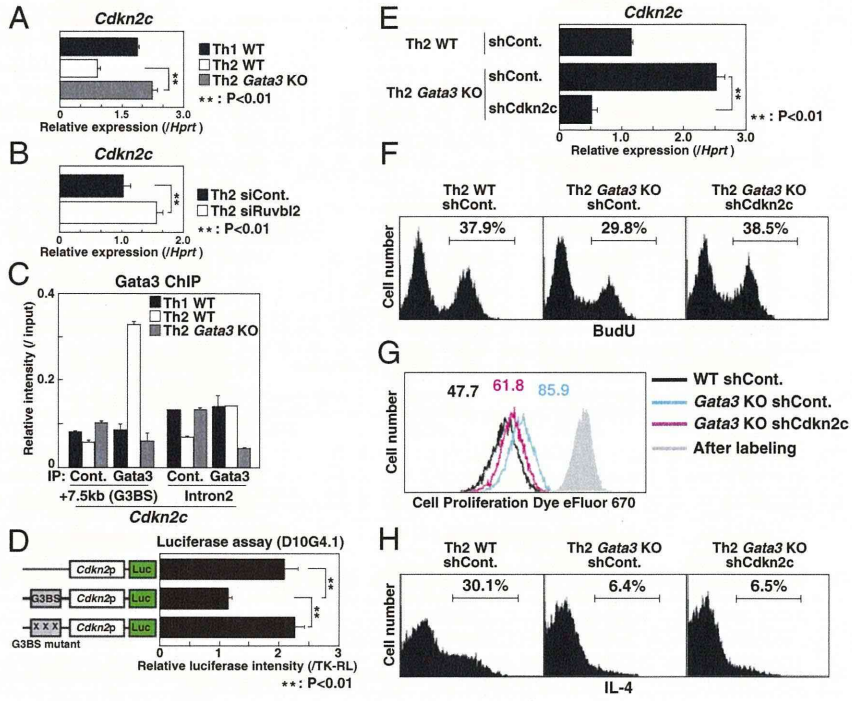

Fig. 3. The expression of *Cdkn2c* controls the Gata3-dependent proliferation of Th2 cells. (*A*) Naive CD4 T cells from either WT or *Gata3*-deficient mice were cultured under Th1 or Th2 conditions for 5 d, and the expression levels of *Cdkn2c* mRNA in Th1 WT, Th2 WT, or Th2 *Gata3* KO cells were determined by RT-qPCR. The relative expression (/*Hprt*) is shown with SDs. $^{**}P < 0.01$ by Student *t* test. (*B*) Naive CD4 T cells were transfected with control or Ruvbl2 siRNA, and cultured under Th2 conditions for 4 d; then, the expression levels of *Cdkn2c* mRNA were determined by RT-qPCR. (*C*) Naive CD4 T cells from WT or *Gata3*-deficient mice were cultured under Th1 or Th2 conditions for 3 d. The binding of Gata3 to the *Cdkn2c* locus was determined by a ChIP assay with a qPCR analysis. The relative intensities (/input) are shown with SDs. (*D*) D10G4.1 cells were transfected with the indicated reporter constructs. Two days after transfection, the cells were assayed for luciferase activity. The data indicate the mean values of three independent experiments with SDs. (*E*) Naive CD4 T cells from WT or *Gata3*-deficient mice were cultured under Th2 conditions for 2 d and then infected with a retroviral vector encoding a control shRNA or shCdkn2c bicistronically with a GFP gene. Four days later, the retrovirus-infected GFP-expressing cells were purified, and the levels of *Cdkn2c* mRNA were measured by RT-qPCR. (*F*) The BrdU incorporation in a portion of the same cultured cells used in *E* was determined. (*G*) The same cultured cells used in *E* were labeled with Cell Proliferation Dye eFluor 670 on day 3 after stimulation. Two days later, cell division was assessed by FACS. Numbers represent mean fluorescent units. (*H*) The same cultured cells used in *E* were stimulated with immobilized TCRβ mAb and subjected to IL-4 staining, followed by FACS analysis. The percentages of IL-4-producing cells are shown. Four (*A*), three (*B*, *C*, *E*, and *F*), and two (*D*, *G*, and *H*) independent experiments were performed, and similar results were obtained.

bronchioalveolar lavage (BAL) fluid was observed in the Ruvbl2 KD group in comparison with the control group (Fig. 5*B*). Histological analysis showed that mononuclear cell infiltration into the peribronchiolar regions of the lung was also modest in the Ruvbl2 KD animals (Fig. 5*C*, *Upper*). The levels of mucus hyperproduction and Goblet-cell metaplasia assessed by PAS staining were lower in the bronchioles of the Ruvbl2 KD group (Fig. 5*C*, *Lower*). These results indicate that Ruvbl2 regulates antigen-induced Th2 cell expansion and subsequent induction of allergic airway inflammation in vivo.

Discussion

We have herein identified a mechanism by which Gata3 facilitates the proliferation of differentiating Th2 cells. Gata3 associates with Ruvbl2 to form a unique repressive complex and represses the expression of a CDK inhibitor, *Cdkn2c*, via direct binding to the downstream region of the *Cdkn2c* gene (Fig. S7*B*). The discovery of the unique repressive Gata3/Ruvbl2 complex and the critical role of Ruvbl2 in the repression of *Cdkn2c* expression allow for mechanistic insight into the process of Th2-cell differentiation and proliferation.

Cdkn2c is a gene encoding p18 (ink4c), a member of the Ink4 family of cyclin-dependent kinase inhibitors. Ink4 family proteins bind to Cdk4 and Cdk6 to prevent the assembly of catalytically active cyclin D–CDK4/6 complexes and block the G1-S phase transition (17). Our results are consistent with the observation that *Cdkn2c*-deficient mice showed a hyperproliferative phenotype in response to TCR stimulation (18). In luminal progenitor cells, Gata3 was shown to directly bind to the promoter and intron of *Cdkn2c* and repress its expression to regulate the cell cycle (19). In addition, the involvement of Gata3 in the activation of Cyclin D1 (encoded by *Ccnd1*) in neuroblastoma cell lines and cell cycle entry in long-term repopulating hematopoietic stem cells were reported in previous studies (21, 29). Therefore, Gata3 appears to regulate the proliferation of various cell types through several distinct mechanisms.

Ruvbl2, a paralogue of Ruvbl1, was identified independently in multiple organisms and is implicated in many cellular pathways. Ruvbl1 and Ruvbl2 were both initially found as proteins that interacted with the TATA-box binding protein (Tbp) in *Drosophila*, yeast, and humans (30, 31). Ruvbl1 and Ruvbl2 belong to the AAA^+ (ATPase associated with multiple activities) ATPase family, have an ATPase domain with Walker A and Walker B motifs, and are conserved in different species. Ruvbl1 and Ruvbl2 are involved in the transcription of over 5% of yeast genes, many of which are directly involved in cell-cycle regulation (32). In addition to Tbp, the Ruvbl1 and Ruvbl2 have also been found to interact with various transcription factors, including c-Myc, β-catenin, and E2f, all of which are critical in the regulation of cell growth, proliferation, and apoptosis (30, 31). Previous reports have identified Ruvbl2 in various chromatin remodeling complexes, such as Tip60, Swr1, Baf, Ino80, and PcG molecule complexes. Moreover, mice heterozygous for a *Ruvbl2* mutation showed impaired T-cell development and maximal T-dependent antibody responses (33). The expression of *Ruvbl2* thus appears to play a critical role in the expansion of T cells in vivo.

Because Gata3 binding to the *Cdkn2c* locus is significantly lower in the absence of Ruvbl2, Gata3-mediated repression of *Cdkn2c* expression is dependent on Ruvbl2–Gata3 interaction (Fig. 4 and Fig. S5). With regard to the mechanisms by which Ruvbl2 controls Gata3 binding to the *Cdkn2c* locus, two possibilities should be considered. One is that the association of Gata3 with Ruvbl2 has an effect on the DNA binding activity of Gata3, and the other is that Ruvbl2 recruits chromatin-remodeling complexes altering the accessibility of the *Cdkn2c* locus to Gata3 (31). The binding of the Ruvbl2 to the *Cdkn2c* locus was comparable in Th1 and Th2 cells (Fig. S7*A*), indicating that the binding of Ruvbl2 to the *Cdkn2c* locus is Gata3-independent.