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Dok-1 is a Positive Regulator of IL-4 Signalling and IgE Response

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Interleukin-4 (IL-4) plays an essential role in the control of humoral immunity by regulating lymphocyte proliferation and differentiation, including the T helper type 2 lineage commitment of CD4$^+$ T cells as well as the isotype switching to IgE in B cells. The adaptor protein Dok-1 is known to have an essential role in the negative regulation of a variety of cytokine signalling events. However, here we have found that the loss of Dok-1 impaired the proliferative response of CD4$^+$ T cells and B cells to IL-4. Conversely, the forced expression of Dok-1 in the myeloid cell line 32D augmented the IL-4-induced proliferation, indicating a positive role for Dok-1. Tyrosine phosphorylation, and thereby the activation of Stat6 and IRS-2, is critical for IL-4 signalling; however, only the activation of Stat6, not the IRS-2-dependent phosphorylation of Akt, was perturbed in Dok-1-deficient cells stimulated with IL-4. Furthermore, mice lacking Dok-1 showed an impaired IgE response to thymus-dependent antigen. Thus, Dok-1 is a positive regulator of IL-4 signalling and IgE resonse.

Key words: Dok, IgE, Interleukin-4, signal transduction, tyrosine phosphorylation.

Abbreviations: IL-4, Interleukin-4; IgE, Immunoglobulin E.

Interleukin 4 (IL-4) is a multifunctional cytokine produced by CD4$^+$ T cells, mast cells and basophils, and exerts profound effects on T and B lymphocytes (*1*). For example, IL-4 influences the proliferation and survival of lymphocytes, T helper type 2 (Th2) lineage commitment of CD4$^+$ Th cells, and isotype switching to IgE in B cells. The receptor of IL-4 (IL-4R) is comprised of a common gamma chain (γc) and an alpha chain (IL-4Rα), which is a signalling unit of the heterodimer (*2*). Binding of IL-4 to the receptor complex promotes the activation of the non-receptor protein-tyrosine kinases (PTKs) Janus kinse (Jak) 1 and Jak3, which are constitutively associated with IL-4Rα and γc, respectively. Activated Jaks initiate several intracellular signalling events by phosphorylating specific tyrosine residues in the cytoplasmic domain of IL-4Rα (*2*). Once phosphorylated, these tyrosine residues and the surrounding peptides act as docking sites for signalling molecules. Signal transducer and activator of transcription 6 (Stat6) is recruited to activated IL-4Rα through the Src homology 2 (SH2) domain and becomes tyrosine-phosphorylated, leading to its dimerization and translocation to the nucleus, where the homodimer activates transcription of IL-4-responsive genes, including the CD23, major histocompatibility complex (MHC) class II, Gγ1, and Gε genes (*3–5*). In addition, a phosphotyrosine-binding (PTB) domain-containing adaptor protein, insulin receptor substrate 2 (IRS2), interacts with the tyrosine-phosphorylated insulin/IL-4 receptor (I4R) motif, a peptide sequence PLxxxxNPxYxSxSD, in the cytoplasmic region of IL-4Rα (*2*). The recruitment of IRS-2 results in its tyrosine phosphorylation and the subsequent activation of phosphatidylinositol-3 kinase (PI3K) and Akt (*6*, *7*). The phosphorylated I4R motif also interacts with other PTB domain-containing adaptors including Dok-2 (also known as FRIP, Dok-R or p56^{dok}) (*8*). It was previously reported that T cells from mice homozygous for the *hairless* allele (*hr*/*hr*) expressed significantly reduced levels of Dok-2 and displayed an increased proliferative response to IL-4 (*8*). However, this coincidence is not necessarily evidence of a growth inhibitory function of Dok-2 in IL-4R-mediated signalling.

The Dok family comprises at least seven members structurally characterized by amino-terminal pleckstrin homology (PH) and PTB domains, followed by carboxy-terminal SH2 target motifs (*8–16*). Therefore, they appear to be adaptor proteins, providing multiple docking sites for SH2-containing proteins upon tyrosine phosphorylation (*10*, *11*, *13*). Within the mammalian Dok family, only Dok-1, Dok-2 and Dok-3 are preferentially expressed in hematopoietic cells. However, Dok-3 appears relatively distant and does not bind to p120 rasGAP unlike Dok-1 and Dok-2 (*12*, *13*, *17*). In general, the Dok family proteins are involved in the negative regulation of signalling downstream of cell surface receptors, such as cytokine receptors or other immune receptors (*8*, *13*, *18–22*). Previous studies including ours with mice lacking Dok-1 and/or Dok-2 demonstrated that these molecules are essential for myeloid homeostasis and suppression of leukemia as well as the negative regulation of Toll-like receptor 4-mediated signalling in macrophages (*23–25*), indicating an overlapping negative function of the Dok family proteins. However, we report

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here that lymphocytes lacking Dok-1, but not those lacking Dok-2, displayed impaired responses to IL-4. Furthermore, we also found that the loss of Dok-1 impaired production of IgE in mice immunized with a T cell-dependent antigen. These findings demonstrate a positive role for Dok-1 in IL-4 signalling.

MATERIALS AND METHODS

Mice—The generation of Dok-$1^{-/-}$, Dok-$2^{-/-}$ or Dok-1/2 double-deficient (dKO) mice was described elesewhere (*18*, *24*). Mice were backcrossed to C57BL/6J for at least eight generations except for the experiments in Fig. 5, where mice were maintained with a mixed background of 129/SvJ and C57BL/6J. All mice were kept under specific pathogen-free conditions and subjected to *ex vivo* experiments at 8–12 weeks of age. Experiments and animal care were performed according to institutional guidelines.

Proliferation and Differentiation Assays—To obtain $CD4^+$ T cells or B cells, single cell suspensions were prepared from spleens and incubated with a mixture of anti-CD8, B220 and Gr-1 (BD PharMingen, San Diego, CA, USA), and anti-rat-IgG antibody-conjugated magnetic beads, or incubated with anti-Thy1.2 antibody-conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. Subsequently, $CD4^+$ T cells or B cells were negatively selected with a magnetic antibody cell sorting system (MACS, Miltenyi Biotec). Flow cytometric analysis confirmed that the purity of the MACS-purified cells was consistently above 85%. For the proliferation assay, MACS-purified $CD4^+$ T or B cells (5×10^4/200 μl) (Fig. 1) or 32D myeloid cells (2.5×10^4/200 μl) (Fig. 2) were treated at 37°C with the indicated combination of IL-4, IL-2 (Peprotec, London, UK), antibodies to CD3ε (eBioscience, San Diego, CA, USA) and phorbol myristate acetate (PMA) plus ionomycin for 48 h. These cells were then pulsed with 0.2 μCi/200 μl of ^{3}H-thymidine for 12 h and its incorporation was measured. For the *in vitro* Th cell differentiation assay, $CD4^+$ T cells (1×10^5/200 μl) were cultured at 37°C for 2, 4 or 6 days in culture plates, which had been pretreated with anti-CD3ε antibodies (10 μg/ml), in the presence of IL-2 (10 ng/ml; Peprotec), IL-12 (10 ng/ml; Peprotec), antibodies to CD28 (1 μg/ml; Southern Biotechnology, Birmingham, AL) and neutralizing antibodies to IL-4 (5 μg/ml) (Th1 polarizing conditions) or in the presence of IL-2 (10 ng/ml; Peprotec), IL-4 (100 ng/ml; Peprotec), antibodies to CD28 (1 μg/ml) and neutralizing antibodies to IFN-γ (10 μg/ml; BD PharMingen) (Th2 polarizing conditions). After additional stimulation with PMA (10 ng/ml) plus ionomycin (1 μg/ml) for 24 h, culture supernatants were removed into 96-well plates coated with mAbs to mouse IL-4 or IFNγ (BD PharMingen), and subjected to ELISA using biotinylated mAbs to each cytokine (BD PharMingen) and HRP-conjugated goat antibodies to biotin (Vector Laboratories, CA, USA).

Dok cDNAs, Transfection and Cell Cloning—Mouse cDNA encoding Dok-1 or a Dok-1 mutant lacking the PH domain (Dok-1ΔPH, 121-482) were inserted into the expression vector pAPIG-Flag bearing COOH-terminal FLAG-tag and IRES-GFP sequences. These plasmids were transfected into 32D myeloid cells, and comparable expression levels of the exogenous genes in transfected clones were confirmed by flow cytometry. Each line of cells was treated with or without 25 ng/ml IL-4 for 48 h and the proliferative response was determined as described above.

Immunoblotting and Immunoprecipitation—Splenocytes (5×10^7/200 μl) were treated with IL-4 (100 ng/ml) at 37°C for a given period, and were solubilized in lysis buffer: 10 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1.0% NonidetP-40 (NP-40), 1 mM Na_3VO_4, 2 μg/ml aprotinin, 1 mM benzamidine, 5 μg/ml chymostiatin, 5 μg/ml leupeptin, 1 μg/ml pepstatin A and 2 mM phenylmethylsulphonylfluoride (PMSF). For the immunoprecipitation, cell lysates were cleared with centrifugation and incubated with antibodies to mouse Jak1 or IL-4Rα (Santa Cruz Biotechnology, CA, USA), followed by incubation with protein G-sepharose (Amersham Biosciences, Uppsla, Sweden). The immune complexes were washed and collected as immunoprecipitates. For the immunoblotting, immunoprecipitates or cell lysates were separated by SDS/7%–PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA), which was then incubated with antibodies to phosphotyrosine (4G10), Stat6, Akt, Jak1, IL-4Rα (Santa Cruz Biotechnology), phospho-Stat6 (Tyr641), and phospho-Akt (Ser473) (Cell Signaling, Danvers, MA, USA), washed, and incubated with horseradish peroxidase-labelled (Amersham Biosciences) or alkaline phosphatase-labelled (Santa Cruz Biotechnology) antibodies to mouse IgG, rabbit IgG or goat IgG. The blots were washed and visualized with the ECL system (Amersham Biosciences) or BCIP/NBT Color Development system (Promega, Madison, WI, USA). Likewise, the 32D cells expressing exogenous Dok-1 or Dok-1ΔPH were subjected to immunoblotting with antibodies to Dok-1 (Santa Cruz Biotechnology) or Flag-tag (Sigma, St Louis, MO, USA).

Northern Hybridization—Total RNA was purified from T cells with ISOGEN (Nippon gene), separated in a 1.5% agarose gel containing 6% formaldehyde, and transferred to a Hybond N^+ membrane (Amersham Biosciences). The membrane was hybridized with radiolabelled cDNA probes for GATA-3 or GAPDH.

Immunization and Assay for Antibody Production—Mice, 7–9 weeks old, were immunized intraperitoneally with 100 μg of DNP-conjugated keyhole limpet hemocyanin (DNP-KLH) in incomplete Freud's adjuvant. The concentration of DNP-specific IgE in the serum from immunized mice was evaluated as previously described (*26*).

Flow Cytometry—MACS-purified splenic B cells were treated with IL-4 (0.03 ng/ml) at 37°C for 2 days, and then stained with FITC-conjugated antibodies to mouse CD23 (eBioscience) to be analysed with FACSCalibur (BD Biosciences). CD23 expression was quantified as mean fluorescent intensity by CellQuest software (BD Biosciences).

RESULTS

Dok-1 is a Positive Regulator of Lymphocyte Responses to IL-4—To determine whether Dok-1 and/or Dok-2

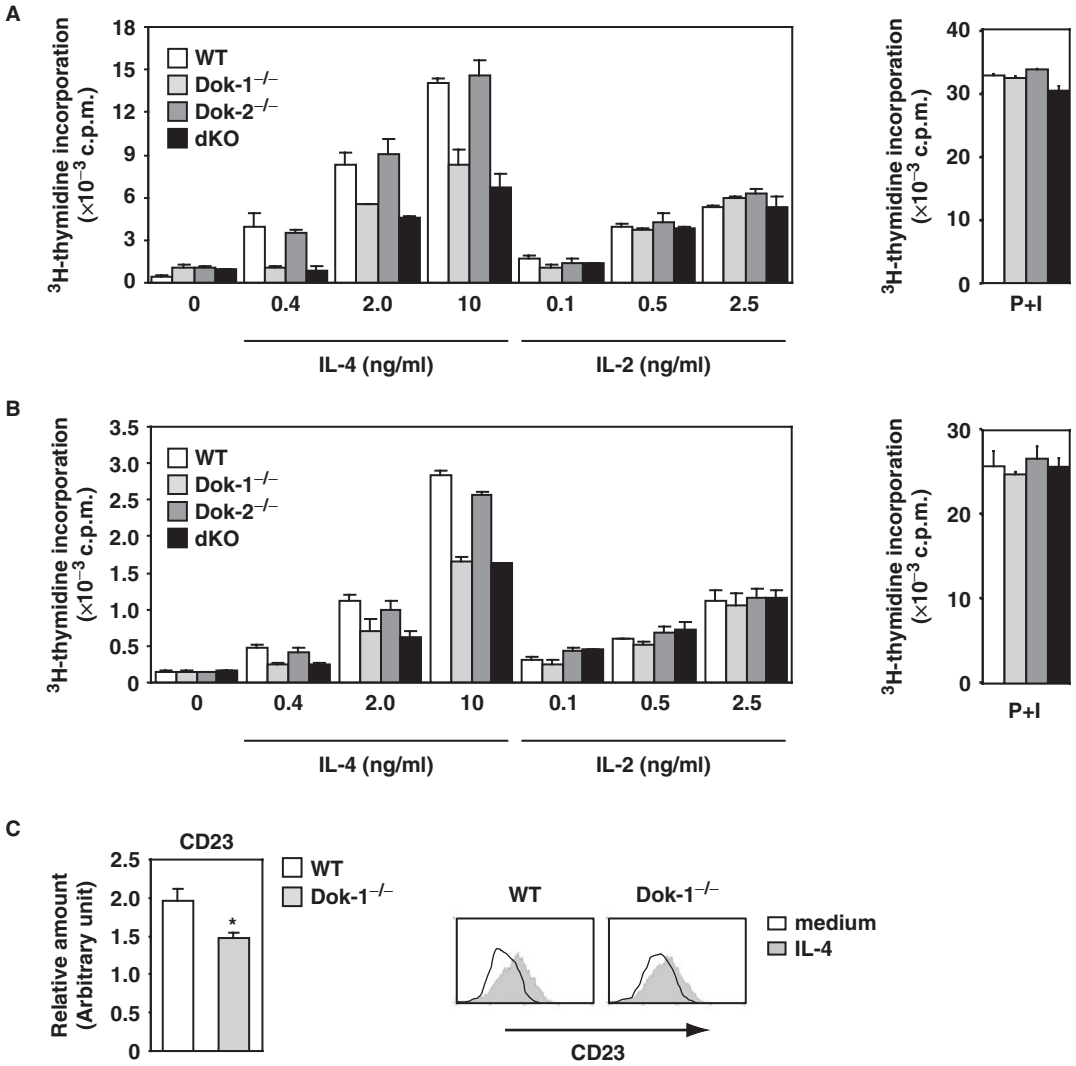

Fig. 1. **Reduced proliferation of Dok-1-deficient lymphocytes upon IL-4 treatment.** (A and B) MACS-purified splenic CD4$^+$ T cells (A) or B cells (B) were stimulated with the indicated concentration of IL-4 or IL-2 in 96-well plates pretreated (A) or untreated (B) with 1 μg/ml of antibodies to CD3ε, or stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) (P + I). The proliferative response was determined based on the incorporation of [^{3}H]-thymidine. Data are the mean ± SD of triplicate experiments. (C) Relative amount of CD23 expression on MACS-purified B cells upon treatment with IL-4 (0.03 ng/ml) for 2 days was evaluated as described in MATERIALS AND METHODS section, in which CD23 expression upon treatment with medium alone was defined as 1 in the arbitrary units (left panel). Data are the mean ± SD of triplicate experiments. *: differs from wild-type at $P < 0.02$ by Student's t test. A histogram representative of the triplicate experiments is shown (right panel).

plays a role in IL-4 signalling, we examined the proliferative response of splenic CD4$^+$ T cells prepared from wild-type (WT), Dok-1-deficient (Dok-1$^{-/-}$), Dok-2-deficient (Dok-2$^{-/-}$) or Dok-1/2 double-deficient (dKO) mice to IL-4. Although it is generally accepted that Dok-1 and Dok-2 are negative regulators of cytokine signalling, CD4$^+$ T cells lacking Dok-1, but not those lacking only Dok-2, displayed an unexpected decrease in their proliferation compared to the wild-type controls (Fig. 1A). However, these mutant T cells showed a normal response to PMA plus ionomycin or IL-2 treatment, indicating specificity (Fig. 1A). Similarly, splenic B cells prepared from mice lacking Dok-1, but not those lacking only Dok-2, demonstrated a significant decrease in proliferative responses to IL-4, but not to PMA plus ionomycin or IL-2 (Fig. 1B). These results suggest that Dok-1 plays a critical role in the proliferative responses of lymphocytes to IL-4, whereas Dok-2 is apparently dispensable. In addition to cellular proliferation, it is established that IL-4 activates B cells and induces surface expression of CD23 (*27*). Therefore, we examined its expression on B cells prepared from Dok-1-deficient mice and found that IL-4-induced surface expression of CD23 was slightly but significantly reduced in the mutant B cells (Fig. 1C). Again, these results suggest a positive role for Dok-1 in IL-4R-mediated signalling.

To confirm the positive role of Dok-1, we next employed a forced expression study with a myeloid cell

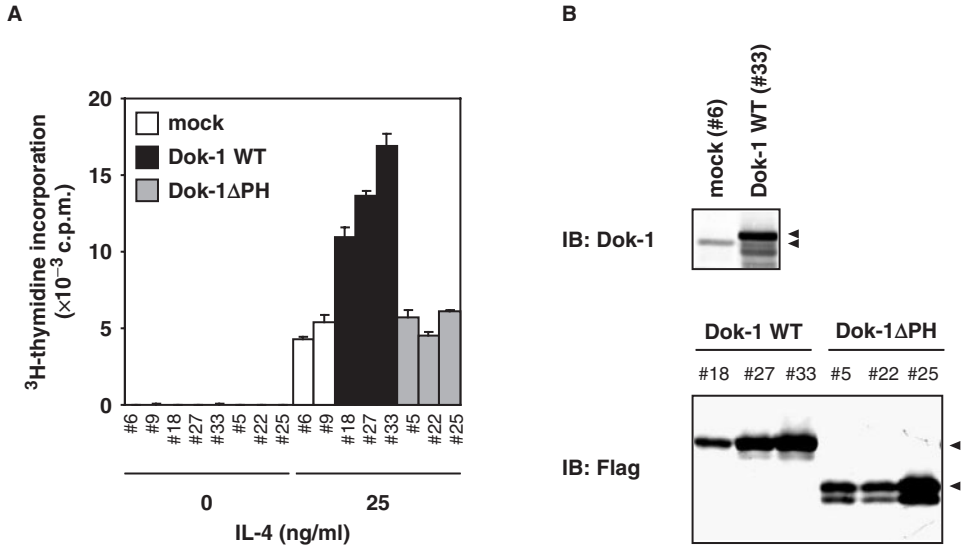

Fig. 2. **Augmented proliferation of Dok-1-overexpressing cells upon IL-4 treatment.** (A) 32D myeloid cells expressing Flag-tagged Dok-1 (Dok-1WT) or Flag-tagged Dok-1 that lacks a PH domain (Dok-1ΔPH) were treated with or without 25 ng/ml IL-4 and the proliferative response was determined by measuring the incorporation of [^{3}H]-thymidine. Data are the mean ± SD of triplicate experiments. (B) Expression levels of endogenous Dok-1, Dok-1WT or Dok-1ΔPH in the clones tested in A were evaluated by immunoblotting (IB) with antibodies to Dok-1 (upper) or Flag (lower). Arrowheads indicate positions of proteins detected by each IB. Cell clones were indicated by individual numbers.

line, 32D, which is known to express IL-4R (*28*). As expected, forced expression of Flag-tagged Dok-1, but not its mutant lacking an NH_2-terminal PH domain, induced augmented proliferation upon IL-4 (Fig. 2A). Higher expression of the exogenous Dok-1 and its mutant than that of the endogenous protein was confirmed by immunoblotting (Fig. 2B). Taken together, these findings indicate that Dok-1 is a positive regulator of IL-4R-mediated signalling.

Dok-1 Plays a Role in the Sustained Phosphorylation of Stat6 and IL-4Rα Upon IL-4 Signalling—The binding of IL-4 to the receptor complex triggers activation of Jak1 and Jak3, leading to subsequent tyrosine phosphorylation of IL-4Rα. Stat6, an essential signalling molecule for cellular proliferation and the upregulation of CD23 expression downstream of IL-4R, is recruited to phosphorylated, and thereby activated, IL-4Rα and becomes tyrosine phosphorylated (*2*, *29*, *30*). Therefore, we examined the tyrosine phosphorylation of Stat6 upon the treatment of splenocytes with IL-4. Although Stat6 was phosphorylated more rapidly in the mutant cells, its inactivation also occurred more rapidly, leading to lower levels of sustained phosphorylation (Fig. 3A). In addition to Stat6, IL-4 triggers IRS-2-dependent activation of PI3K and Akt, which is also essential for the subsequent proliferation of cells (*6*, *7*). However, the phosphorylation, and thereby activation, of Akt was not perturbed in Dok-1-deficient splenocytes, suggesting that Dok-1 is irrelevant to the IRS-2/PI3K/Akt pathway (Fig. 3B). Thus, it seems likely that the earlier inactivation of Stat6 is responsible for the impaired proliferative response of Dok-1-deficient cells to IL-4 treatment. Consistent with this, although Dok-1-deficient splenocytes showed intact phosphorylation, thereby activation, of Jak-1, the loss of Dok-1 induced rapid dephosphorylation of IL-4Rα (Fig. 3C and D). These observations indicate that Dok-1 plays an important role in the sustained phosphorylation of Stat6 and IL-4Rα, which probably facilitates a proliferative response to IL-4.

Dok-1 Appears to be Irrelevant to the Differentiation of Th Cells—There are two major subtypes of CD4$^+$ T helper cells, Th1 and Th2, which are believed to play crucial roles in the cellular and humoral immune systems, respectively. The differentiation of naïve CD4$^+$ T cells into Th1 or Th2 effectors is modulated by signals emanating from T cell receptors (TCRs) as well as cytokine receptors. For example, IFN-γ and IL-4 strongly induce differentiation into the Th1 and Th2 lineages, respectively, upon TCR-mediated stimulation *in vitro* (*31*). Stat6 is essential for Th2 differentiation (*29*, *30*, *32*). Therefore, we examined the impact of Dok-1 deficiency on the Th cell specialization; namely, CD4$^+$ T cells prepared from WT or Dok-1$^{-/-}$ mice were differentiated under Th1 or Th2 polarizing conditions *in vitro*. Unlike the proliferative responses to IL-4, there was no significant difference in IL-4 (Th2-type cytokine) production between WT and Dok-1$^{-/-}$ CD4$^+$ T cells under the Th2 polarizing conditions (Fig. 4A). Similarly, IFNγ (Th1-type cytokine) production was intact in Dok-1$^{-/-}$ CD4$^+$ T cells under Th1 polarizing conditions (Fig. 4A). Furthermore, expression of a transcription factor, GATA-3, which is a hallmark of the Th2 polarization process (*33*), confirmed unperturbed differentiation into Th2 cells in the absence of Dok-1 (Fig. 4B). These results indicate that the Dok-1 mutation does not influence Th cell differentiation *in vitro*.

Loss of Dok-1 impairs IgE Production in Mice—Cytokines produced by Th2 cells, such as IL-4, IL-5 and

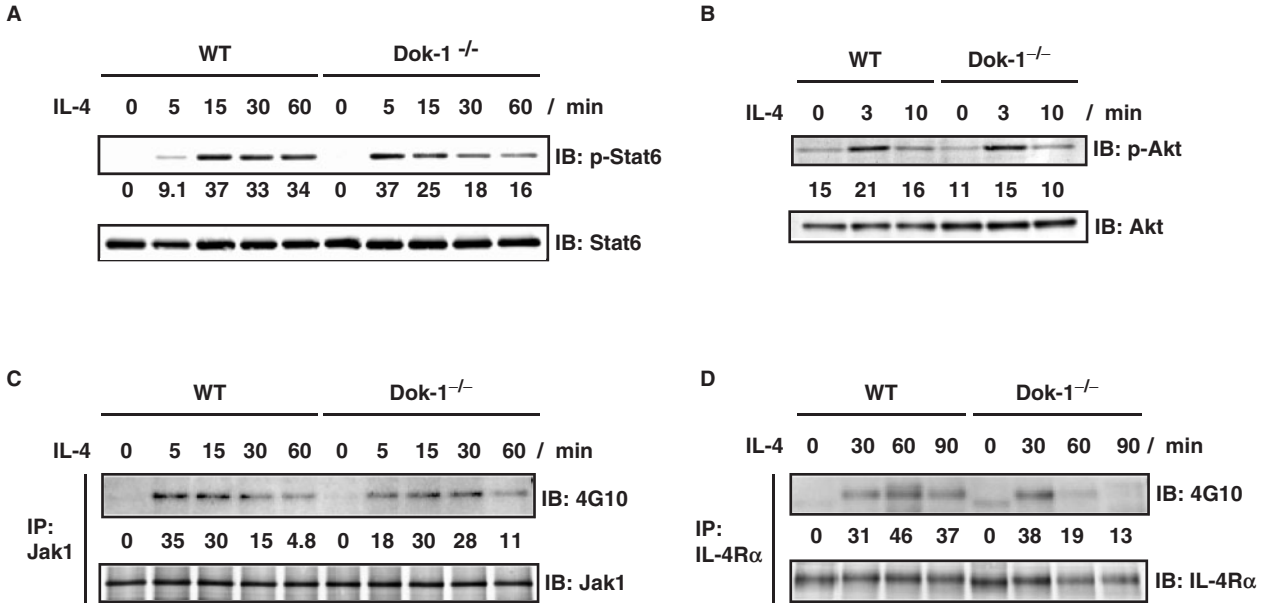

Fig. 3. **Effects of Dok-1 deficiency on IL-4-induced signalling.** Splenocytes prepared from wild-type (WT) or Dok-1$^{-/-}$ mice were stimulated with IL-4 (100 ng/ml) for the indicated period and subjected to immunoprecipitation (IP) and/or immunoblotting (IB). Densitometric measurements in the arbitrary units of the individual phosphorylated protein bands after normalization are indicated under each bands. Data representative of multiple independent experiments are shown.

IL-13, are thought to coordinate host defenses against large, extracellular pathogens. Most of the characteristic features of atopy and asthma, especially IgE synthesis, result from the combined effects of these cytokines. In particular, IL-4R/Stat6-mediated signalling is known to play an important role in the production of IgE and IgG_1 (*30*, *34*). Therefore, we examined whether Dok-1 is involved in the production of IgE to thymus-dependent antigen (DNP-KLH), even though Dok-1 deficiency does not affect antigen-specific IgG_1 production (*18*). Interestingly, the loss of Dok-1 significantly impaired IgE production as compared to the wild-type controls (Fig. 5), indicating a role for Dok-1 in IgE production *in vivo*.

DISCUSSION

Previous studies with mice lacking Dok-1 and/or Dok-2 demonstrated that the Dok proteins cooperate to act as key negative regulators of proliferative responses to cytokine treatment and antigen receptor stimulation in myeloid and lymphoid cells, respectively (*21*, *23*, *24*). However, here we have reported that both T and B lymphocytes lacking Dok-1, but not those lacking only Dok-2, displayed a significant reduction in IL-4-induced proliferation (Fig. 1A and B). Although there is a report that T cells from mice homozygous for the *hairless* allele (*hr/hr*) expressed lower levels of Dok-2 and displayed augmented proliferative responses to IL-4 (*8*), this abnormal proliferation appears not to involve Dok-2. Dok-2 is the closest homologue of Dok-1 among the Dok family and their functions largely overlap as mentioned above; however, several differences between them have been reported. For example, in macrophages, only Dok-1 is degraded upon stimulation with LPS (*25*), and only Dok-2 is increased upon stimulation with M-CSF (*19*). In platelets, only Dok-2 is phosphorylated upon stimulation with collagen, and the patterns of thrombin-induced Dok-1 and Dok-2 phosphorylation are distinct (*35*). In addition, Dok-2, but not Dok-1, is barely detectable in the B cell lineage (*8*, *13*), implying that the functions of these closely related proteins are not necessarily identical.

Immunization of Dok-1-deficient mice with DNP-KLH revealed that Dok-1 plays an essential role in T cell-dependent IgE production (Fig. 5), but not IgG_1 production (*18*). Although the IL-4 signalling pathway is thought to be important for IgE and IgG_1 production, there is a report that IL-4 is dispensable for IgG_1, but not IgE, production upon immunization (*36*). Moreover, Stat6-deficient mice immunized with anti-IgD antibodies produced normal levels of IgG_1, while IgE production was not detectable (*29*, *32*). Given that the loss of Dok-1 does not impair Th2 differentiation *in vitro* (Fig. 4), the reduced IgE response to DNP-KLH in Dok-1-deficient mice could be due to impaired IL-4 signalling in B cells. To test this, we performed *in vitro* stimulation assays of B cells with anti-CD40 plus IL-4, in which expression of ε GLT (germline transcripts for IgE heavy chain) and subsequent class switching to IgE are induced independently of T cell help. However, expression levels of both membrane-bound IgE and ε GLT were normally increased in Dok-1-deficient B cells at least in these assays (data not shown), suggesting that the impaired IgE-response of mice lacking Dok-1 to DNP-KLH is not solely due to the loss of Dok-1 in B cells. However, we could not rule out the possibility that effects of Dok-1-deficiency on T and/or B cell differentiation might be undetectable in our *in vitro* assays.

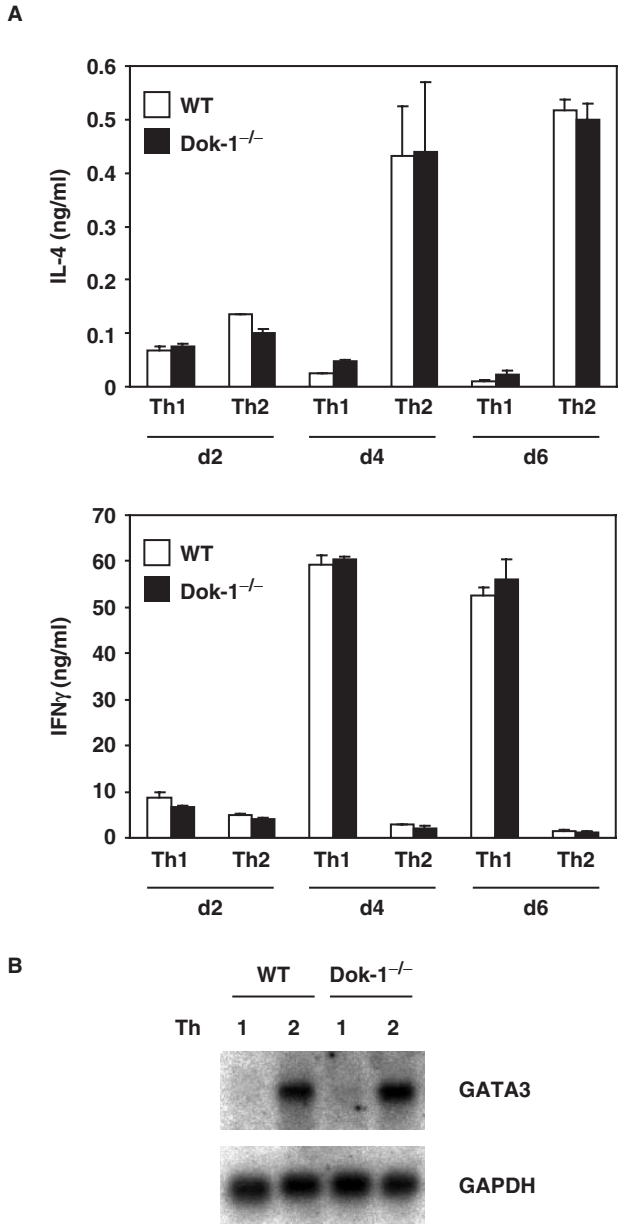

Fig. 4. **Intact Th-1/2 differentiation of Dok-1-deficient CD4⁺ T cells *in vitro*.** (A) MACS-Purified splenic CD4⁺ T cells were cultured under Th1 or Th2 polarizing conditions for the period indicated (d2, d4 or d6) and treated with PMA and ionomycin for 24 h prior to evaluation of IL-4 and IFNγ secretion as described in MATERIALS AND METHODS section. Data are the mean ± SD of triplicate experiments. (B) MACS-Purified splenic CD4⁺ T cells were cultured under Th1 or Th2 polarizing conditions for 2 days. Total RNA was prepared and subjected to Northern hybridization for GATA3 and GAPDH.

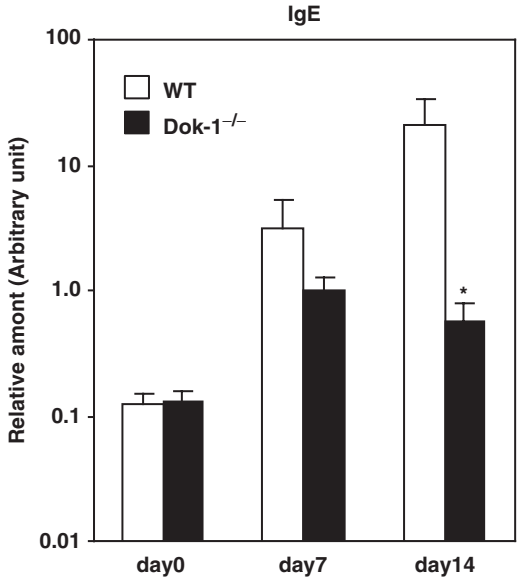

Fig. 5. **Impaired IgE response of Dok-1-deficient mice to immunization with DNP-KLH.** Relative DNP-specific IgE levels in the sera of wild-type (WT) or Dok-1$^{-/-}$ mice immunized with DNP-KLH (day 7 or day 14) was evaluated as described in MATERIALS AND METHODS section, in which the mean value of IgE levels in sera from Dok-1$^{-/-}$ mice at day 7 was defined as 1 in the arbitrary units. The preimmune sera of mice were examined as controls (day 0). Data are the mean ± SD of quadruplicate experiments. *: differs from wild-type mice at $P < 0.02$ by Student's *t*-test.

It is recently reported that PKCζ facilitates Jak1 activation upon IL-4 signalling and the loss of PKCζ dramatically impaired the activation of Jak1 upon the stimulation of CD4⁺ T cells with IL-4 (*37*), indicating a positive role for the atypical PKC. In contrast, the phosphorylation and binding to IL-4Rα of Jak1 were intact in Dok-1-deficient lymphocytes treated with IL-4 (Fig. 3C and data not shown). Therefore, unlike PKCζ, Dok-1 appears to play a role downstream of Jak1 because phosphorylation of IL-4Rα and Stat6 was significantly perturbed upon IL-4 treatment of Dok-1-deficient cells (Fig. 3A and D). Taken together, our findings would provide a unique model to understand molecular and cellular mechanisms underlying IL-4 signalling as well as IgE production in mice.

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