T Cell Receptor–induced Calcineurin Activation Regulates T Helper Type 2 Cell Development by Modifying the Interleukin 4 Receptor Signaling Complex

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Abstract

The activation of downstream signaling pathways of both T cell receptor (TCR) and interleukin 4 receptor (IL-4R) is essential for T helper type 2 (Th2) cell development, which is central to understanding immune responses against helminthic parasites and in allergic and autoimmune diseases. However, little is known about how these two distinct signaling pathways cooperate with each other to induce Th2 cells. Here, we show that successful Th2 cell development depends on the effectiveness of TCR-induced activation of calcineurin. An inhibitor of calcineurin activation, FK506, inhibited the in vitro anti-TCR–induced Th2 cell generation in a dose-dependent manner. Furthermore, the development of Th2 cells was significantly impaired in naive T cells from dominant-negative calcineurin Aα transgenic mice, whereas that of Th1 cells was less affected. Efficient calcineurin activation in naive T cells upregulated Janus kinase (Jak)3 transcription and the amount of protein. The generation of Th2 cells induced in vitro by anti-TCR stimulation was inhibited significantly by the presence of Jak3 antisense oligonucleotides, suggesting that the Jak3 upregulation is an important event for the Th2 cell development. Interestingly, signal transducer and activator of transcription (STAT)5 became physically and functionally associated with the IL-4R in the anti-TCR–activated developing Th2 cells that received efficient calcineurin activation, and also in established cloned Th2 cells. In either cell population, the inhibition of STAT5 activation resulted in a diminished IL-4–induced proliferation. Moreover, our results suggest that IL-4–induced STAT5 activation is required for the expansion process of developing Th2 cells. Thus, Th2 cell development is controlled by TCR-mediated activation of the Ca^{2+}/calcineurin pathway, at least in part, by modifying the functional structure of the IL-4R signaling complex.

Key words: crosstalk • calcineurin transgenic • Janus kinase 3 • signal transducer and activator of transcription 5 • *antennapedia* peptide

Introduction

CD4^{+} helper T cells are divided into two distinct subpopulations on the basis of their cytokine production profiles (1). Th1 cells produce IL-2, IFN-γ, and TNF-β, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (2–5). Because these cytokines play critical roles in the regulation of immune responses, the identification of the molecular mechanisms governing Th development is essential for understanding the immune responses in infectious diseases and pathogenesis of allergic and autoim-

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mune diseases. Successful development of Th2 cells requires both differentiation and expansion processes, and depends on the activation levels of IL-4R–mediated signaling (2–7). Among signaling molecules downstream of IL-4R, signal transducer and activator of transcription (STAT)[1] 6 activation is thought to be critical for the differentiation process (8–10), and phosphorylation of insulin receptor substrate (IRS)-2 is important for proliferation (11, 12). On the other hand, TCR recognition of antigen and subsequent activation of TCR-mediated signaling pathways are also indispensable for both Th1 and Th2 cell development (2–5). We recently reported that efficient activation of the p56^{lck} (Lck) and Ras/mitogen-activated protein kinase (MAPK) cascade is crucial for Th2 cell differentiation (13, 14). The activation of the Ras/MAPK cascade appears to act on Janus kinase (Jak)1 to enhance its kinase activity and improve IL-4R function. In contrast, Th1 cell differentiation and Th1 cytokine production are dependent on other MAPK pathways, including c-Jun NH$_2$-terminal kinase (JNK) and p38, respectively (15–17).

Here, we show that TCR-mediated activation of the Ca^{2+}/calcineurin (CN) pathway is required for Th2 cell development. The activation of CN in naive CD4 T cells induces transcriptional upregulation of Jak3. Moreover, STAT5 is found to be physically and functionally associated with the IL-4R complex in the anti-TCR–activated naive T cells and established cloned Th2 cells. The IL-4–induced activation of STAT5 appears to play an important role in the expansion process of the developing Th2 cells. Thus, the recruitment of Jak3 and STAT5 molecules to the functional IL-4R complex in developing Th2 cells appears to be crucial for Th2 cell development.

Materials and Methods

Animals. C57BL/6 (B6), BALB/c, and (B6 × BALB/c)F1 mice were purchased from CLEA Japan Inc. Anti-OVA–specific TCR-α/β transgenic (DO10 Tg) mice (18) were provided by Dr. Dennis Loh (Nippon Roche Research Center, Kanagawa, Japan). The dominant-negative (dn)CN Aα transgene was generated by the PCR method. Five amino acid residues in the phosphatase active site (148–152) were deleted, and the resulting construct was ligated into the BamHI cloning site of the p1017 vector (19). Overexpression of dnCN in the T cell hybridoma diminished the nuclear factor of activated T cells (NFAT) luciferase activities induced by anti-TCR mAb or PMA plus ionomycin stimulation (data not shown). A NotI fragment of this plasmid was isolated and injected into fertilized eggs of B6, and three lines of transgenic (Tg) mice were established. The Tg mouse line used in this report (no. 28) expresses ~50 copies of the transgene. All mice used in this study were maintained under specific pathogen-free conditions.

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[1]*Abbreviations used in this paper:* B6, C57BL/6; CN, calcineurin; dn, dominant negative; dnCN Tg, dnCN Aα transgenic; DO10 Tg, anti-OVA–specific TCR-α/β transgenic; HA, hemagglutinin; IRS, insulin receptor substrate; FCM, flow cytometry; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; STAT, signal transducer and activator of transcription; Tg, transgenic.

T Cell Purification and Th1/Th2 Cell Differentiation In Vitro. Naive (CD44^{low}) CD4 T cells from the spleen were prepared by panning with anti-CD8 (53-6-72) and anti-CD44 (IM7) mAbs as described (14). Contamination by memory CD44^{high} CD4 T cells and CD8 T cells was <3%. The enriched naive CD4 T cells (0.5 × 10^6) were stimulated for 2 d with immobilized anti-TCR (H57-597, 30 μg/ml) or PMA (1 ng/ml) plus ionomycin (100 or 300 nM) in the presence of IL-2 (25 U/ml) and IL-4 (100 U/ml; Th2-skewed condition) or IL-2 (25 U/ml), IL-12 (1 U/ml), and anti–IL-4 mAb (11B11, 12.5% culture supernatant; Th1-skewed condition). The cells were then cultured with only cytokines for another 3 d. In the DO10 Tg mouse system, naive (CD44^{low}) CD4 T cells were isolated by a FACSVantage™ cell sorter (Becton Dickinson), yielding purity of >98%, and 1.5 × 10^4 sorted CD44^{low} CD4 T cells were stimulated with antigenic OVA peptide (0.1–1 μM) and irradiated BALB/c APCs (10^5) in the presence of IL-4 (100 U/ml) or IL-12 (0.1 U/ml) for 5 d (14). For IL-12–induced Th1 cell generation, anti–IL-4 mAb was added. Intracellular staining of IL-4 and IFN-γ was performed as described (14).

Immunofluorescent Staining and Flow Cytometry Analysis. In general, 10^6 cells were incubated on ice for 30 min with the appropriate staining reagents using a standard method, as described previously (14). The cells were incubated with FITC-labeled anti-CD44 mAb (IM7; BD PharMingen), anti–IL-4Rα mAb (Genzyme), or anti–common γ mAb (4G3; BD PharMingen) followed by anti–rat Ig-FITC. Then, the cells were stained with anti-CD4–PE (GK1.5-PE; BD PharMingen) after blocking residual reactive sites of the anti–rat Ig-FITC with a normal rat serum. Flow cytometry (FCM) analysis was performed on a FACSort™ (Becton Dickinson), and results were analyzed using CELL-Quest™ software (Becton Dickinson).

Immunoprecipitation and Immunoblotting. Naive CD4 T cells from (B6 × BALB/c)F1 mice were activated with immobilized anti-TCR mAb and IL-4 (100 U/ml) for 2 d (induction culture). Where indicated, FK506 (100 nM) was added. Immunoprecipitation and immunoblotting were performed as described (14). The reagents used were antiserum specific for STAT6 and STAT5a (R&D systems), polyclonal Abs against Jak1, Jak3, and IRS-2 (Upstate Biotechnology), or an mAb reactive with IL-4Rα (Genzyme). Control Igs used were isotype-matched Igs (BD PharMingen). In the case of the coprecipitation assay, lysis buffer without sodium deoxycholate was used. To assess IL-4–induced tyrosine phosphorylation, stimulated cells were washed, cultured for 8 h without cytokines, and stimulated with IL-4 (100 U/ml) for 5 or 10 min at 37°C. Antiphosphotyrosine mAb (RC20; Transduction Laboratories) was used for detection.

Measurement of Phosphatase Activity of CN. Phosphatase activity was measured by the manufacturer's protocol supplied in the CN assay kit (AK-804; BIOMOL Research Laboratories). The cell lysates of naive CD4 T cells from dnCN Aα Tg (dnCN Tg) mice and littermate mice were prepared and incubated with phosphorylated substrate (RII phosphopeptide) at 37°C for 10 min. Then, the amount of released PO$_4$ was determined using the BIOMOL GREEN™ reagent.

Detection of Nuclear Translocation of NFAT. Naive CD4 T cells were stimulated with immobilized anti-TCR mAb for 2 d, and nuclear extracts were prepared with a NE-PER™ Nuclear and Cytoplasmic Extraction Reagent (No. 78833; Pierce Chemical Co.) according to the manufacturer's protocol. The amounts of NFATc1 and NFATc2 in the nuclear extracts were assessed by immunoblotting with anti-NFATc1 or anti-NFATc2 mAb (BD PharMingen) as described (20).

Proliferation Assay. IL-4–induced proliferative response was assessed as described (14). The developing Th2 cells and transfected cells were stimulated with graded doses of mouse recombinant IL-4 for 40 h, and ^{3}H was added at the 24-h time point.

Reverse Transcription PCR. Total RNA was prepared using Trizol (GIBCO BRL). Reverse transcription was done using Superscript II (GIBCO BRL). The primers for Jak3 are 5′-ATG-GCACCTCCAAGTGAGGAGAC-3′ and 5′-CCAAAGCG-GTGACATGTCTCCAG-3′.

Introduction of Jak3 Antisense Oligonucleotide. Antisense oligonucleotide of Jak3 (5′-CTCACTTGGAGGTGCCATGAGTATA-ACCGT-3′), sense oligonucleotide of Jak3 (5′-ACGGTTATA-CTCATGGCACCTCCAAGTGAG-3′), and a scrambled oligonucleotide (5′-GAGGCATGATTGACCATATCCCTCACG-GTA-3′) were coupled to 16 amino acid–long polypeptides corresponding to the third helix of the homeodomain of *antennapedia* (Penetratin™; Appligene), which has an activity of plasma membrane penetration (21), according to the manufacturer's protocol. 1 μM of each oligonucleotide was added to the in vitro differentiation culture system. No toxic effect on T cells was noted in this condition (data not shown).

Transfection of Dominant-negative STAT5 in Th2 Cloned Cells. A Th2 clone D10.G4.1 (D10 [22]) expresses substantial amounts of both IL-4Rα and STAT5a (data not shown). Hemagglutinin (HA)-tagged wild-type sheep STAT5 cDNA and Y694F mutant of STAT5 (dnSTAT5, which can inhibit the activation of both STAT5a and STAT5b) were provided by Dr. Wakao (Helix Research Institute, Chiba, Japan; reference 23). For the transient assay, pEGFP vector (2.5 μg; CLONTECH Laboratories, Inc.) was cotransfected with 10 μg of STAT5 cDNA ligated into pCR3.1 vector, and 24 h later, green fluorescent protein–positive cells were sorted by a cell sorter and used in the proliferation assay. For establishing stable transfectants, plasmid (10 μg) was transfected by electropolation, and selection was performed in a medium containing G418 (500 μg/ml; Calbiochem) and IL-2 (5 U/ml). Expression level of the transfected gene were assessed by immunoblotting with anti-HA Ab (Boehringer). In these transfectants, an equivalent expression level of IL-4Rα to that of D10 cells was detected (data not shown).

Introduction of STAT5 Phosphopeptide in Naive T Cells. A synthetic peptide containing murine STAT5b phosphopeptide (693–708, inhibiting the activation of both STAT5a and STAT5b; data not shown) and a 16 amino acid–long polypeptide corresponding to the third helix of the homeodomain of *antennapedia* (Penetratin™; RQIKIWFPNRRMKWKKAKAADGYpVKPQIKQVV) was generated. According to previous reports (21, 24–26), 30 or 100 μM of the phospho-STAT5–*antennapedia* peptide was added to the in vitro Th1/Th2 cell differentiation culture system. No significant toxic effect in T cells was noted in these conditions (data not shown).
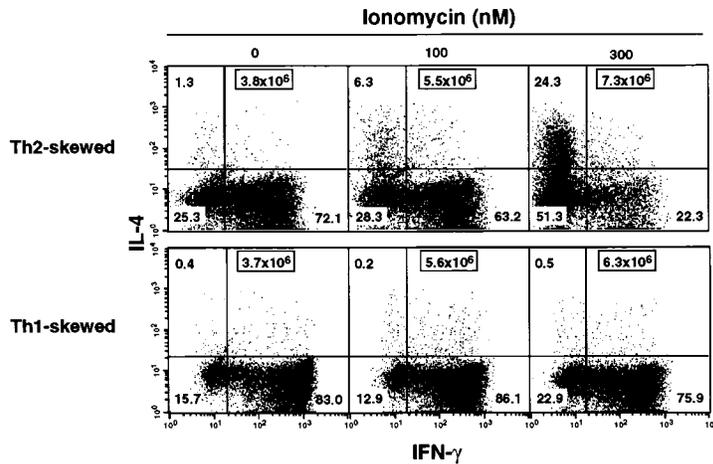
Results and Discussion

Activation of the Ca^{2+}/CN Pathway and the Generation of Th1/Th2 Cells In Vitro. The goal of this study is to clarify how TCR-induced activation of the Ca^{2+}/CN pathway regulates Th1/Th2 cell development. We initiated the study by assessing the effect of calcium ionophore on Th1/Th2 cell development using an in vitro differentiation culture system. Purified naive CD4 T cells (1.5×10^6) from normal (B6 × BALB/c)F1 mice were stimulated with a minimum dose of PMA (1 ng/ml) and various concentra-

tions of ionomycin. Th1/Th2 cell development was assessed in the presence of 100 U/ml of IL-4 (Th2 skewed) and 1 U/ml of IL-12 and anti–IL-4 (Th1 skewed). As shown in Fig. 1 A (top), IL-4–producing Th2 cells were generated in an ionomycin dose–dependent manner. The absolute number of Th2 cells generated, which was calculated from the percentages of Th2 cells and the yield of harvested cells, was increased ∼7 times with 100 nM of ionomycin, and >35 times with 300 nM ionomycin in this experiment. In contrast, the generation of Th1 cells in the Th1-skewed conditions was not dramatically enhanced, with an increase of ∼1.6 times at best in the presence of either 100 or 300 nM of ionomycin (Fig. 1 A, bottom).

Concurrently, the effect of FK506, a specific inhibitor of CN, was examined on naive T cells cultured with immobilized anti-TCR and IL-4 (Th2 skewed) and with immobilized anti-TCR, IL-12, and anti–IL-4 (Th1 skewed) in the presence of graded doses of FK506. A dose-dependent decrease in the generation of Th2 cells was observed (Fig. 1 B, top). The absolute number of Th2 cells generated in the presence of 100 nM of FK506 was ∼25 times less than that of control in this experiment. The generation of IFN-γ–producing Th1 cells was decreased significantly, but the level of the decrease was only about fourfold even in 100 nM FK506. In the presence of FK506, the proportion of cells that divided at certain times was assessed using carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled naive T cells, and a significant blocking effect on cell division was observed (data not shown). These results suggest that the generation of Th2 cells requires the activation of the Ca^{2+}/CN signaling pathway, and that Th1 and Th2 cell differentiation exhibits differential dependence on activation of the Ca^{2+}/CN pathway.

Establishment of dnCN Tg Mice. To further investigate the role of CN activation on Th2 cell development, Tg mouse lines expressing catalytically inactive CN Aα (dnCN) under the control of *lck*-proximal promoter were established. CN Aα–deficient mice established by another group (27) were found not to be suitable for our purpose, as there are three isoforms in CN Aα molecule, and CN activity was not abrogated in the CN Aα–deficient mice. In our dnCN Tg lines, no detectable impairment in T cell development in the thymus was observed, as evidenced by equivalent numbers of thymocytes, normal CD4/CD8 profiles (Fig. 2 A), and TCR Vβ usage (data not shown) compared with control littermates. Similar numbers of naive (CD44^{low}) CD4 T cells were detected in the spleen (Fig. 2 B). The expression levels of IL-4R complex (IL-4Rα and common γ) and IL-4–induced STAT6 phosphorylation in naive CD4 T cells were not affected (Fig. 2, B and C). However, the amount of CN Aα protein in the naive CD4 T cells was increased about twofold (Fig. 2 D), and there was a substantial decrease in the level of CN phosphatase activity detected in the dnCN Tg naive CD4 T cells (Fig. 2 E). Consequently, nuclear translocation of NFATc1 and NFATc2, which is thought to be a result of CN activation, was assessed in dnCN Tg naive CD4 T cells after anti-TCR activation. Significant decreases in the
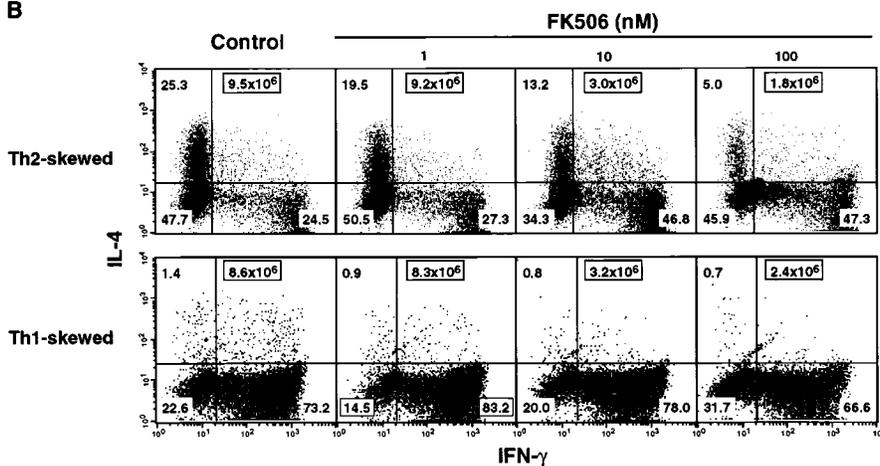
Figure 1. Activation of the Ca^{2+}/CN pathway and the generation of Th1/Th2 cells in vitro. (A) Naive (CD44^{low}) CD4 T cells from (B6 × BALB/c)F1 mice were stimulated with indicated doses of ionomycin plus PMA (1 ng/ml) in the presence of IL-4 (100 U/ml; Th2-skewed condition) or in the presence of anti–IL-4 mAb and IL-12 (1 U/ml; Th1-skewed condition). (B) Naive (CD44^{low}) CD4 T cells from (B6 × BALB/c)F1 mice were stimulated with immobilized anti-TCR mAb plus IL-4 (100 U/ml; Th2-skewed) or immobilized anti-TCR mAb plus IL-12 (1 U/ml) and anti–IL-4 (Th1-skewed) in the presence of various doses of FK506. Control represents a culture in the absence of FK506. The cultured cells were subjected to intracellular staining with anti–IL-4 and anti–IFN-γ. The numbers of the cells harvested (yield) are shown as boxed numbers. The percentages of cells in each quadrant are also shown. The estimated cell numbers of Th2 cells (calculated from the percentages of Th2 cells and the yield of harvested cells) in A (top) are 0.05×10^6, 0.35×10^6, and 1.77×10^6 from left to right. The estimated cell numbers of Th1 cells in A (bottom) are 3.07×10^6, 4.82×10^6, and 4.78×10^6. Likewise, Th2 cells in B (top) are 2.40×10^6, 1.79×10^6, 0.40×10^6, 0.09×10^6, respectively, and Th1 cells in B (bottom) are 6.30×10^6, 6.91×10^6, 2.50×10^6, and 1.60×10^6, respectively.

amount of either NFATc were detected (Fig. 2 F). Multiple bands in the NFAT immunoblot may represent differentially glycosylated molecules (28). Consistent with these results, a slight but significant decrease in the TCR-induced proliferation and IL-2 production of the dnCN Tg naive T cells was noted (data not shown).

Requirement for the Activation of CN in Th2 Cell Development. The development of Th1 and Th2 cells from naive CD4 T cells of the dnCN Tg mice was assessed using stimulation with PMA plus ionomycin or immobilized anti-TCR mAb in either Th2-skewed (Fig. 3 A) or Th1-skewed (Fig. 3 B) conditions. As expected, a transgene dose–dependent reduction of Th2 cell generation was observed in dnCN Tg mice in the Th2-skewed condition (Fig. 3 A). Contrary to the generation of Th2 cells, Th1 cell generation of dnCN Tg T cells was not significantly impaired in either Th2-skewed (Fig. 3 A) or Th1-skewed (Fig. 3 B) conditions. The absolute numbers of the cells harvested were similar in these cultures (data not shown). Next, the dnCN Tg mice (B6 background) were crossed with an OVA-specific TCR-α/β DO10 Tg mouse line (BALB/c background) in order to determine the effect of the presence of dnCN on antigenic peptide-induced Th1/Th2 cell development. As shown in Fig. 3 C (left), the peptide-induced Th2 cell development was significantly reduced in the cultures of dnCN Tg compared with littermate controls (13.1% vs. 25.2%). Similarly, the generation of Th2 cells induced by a minimal dose of antigenic peptide (0.1 μM), and exogenous IL-4 (100 U/ml) was also impaired (Fig. 3 C, middle). However, no significant reduction was detected in IL-12–induced Th1 cell differentiation (Fig. 3 C, right). The considerable inhibiting effects that result from modest expression of the dnCN Tg suggest that the activation of CN is required for the development of Th2 cells, whereas Th1 cell development shows less dependence.

CN-dependent Increase in the Expression of Jak3 in the Anti-TCR–activated CD4 T Cells. IL-4 treatment of naive T cells without TCR ligation does not induce Th2 cells (2–5), and the signal-transducing ability of the IL-4R is elevated in Th2 cells compared with that of naive T cells or Th1 cells (6, 7). Consequently, we examined whether TCR-mediated CN activation induced qualitative changes in the IL-4R signaling complex in developing Th2 cells. Naive CD4 T cells were stimulated with immobilized anti-TCR mAb and 100 U/ml of IL-4 for 2 d (induction culture), and the expression levels of Jak3, Jak1, STAT6, and IRS-2 protein and Jak3 mRNA were measured (Fig. 4 A). Interestingly, the expression levels of Jak3 protein and
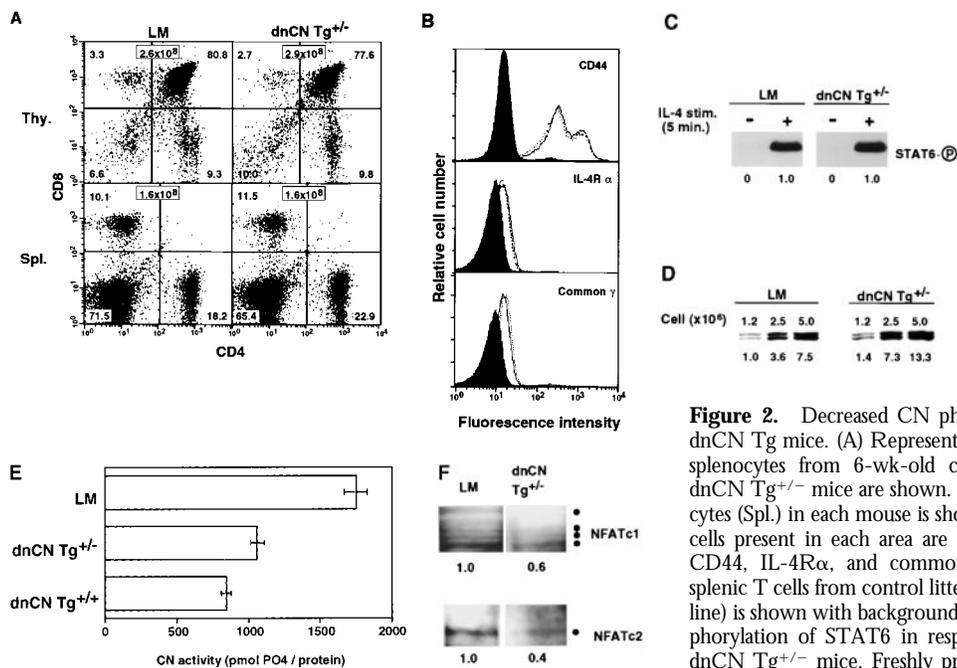
Figure 2. Decreased CN phosphatase activity in naive CD4 T cells of dnCN Tg mice. (A) Representative CD4/CD8 profiles of thymocytes and splenocytes from 6-wk-old control littermate (LM) and heterozygous dnCN Tg$^{+/-}$ mice are shown. The yield of thymocytes (Thy.) and splenocytes (Spl.) in each mouse is shown as a boxed number. The percentages of cells present in each area are also shown. (B) Cell surface expression of CD44, IL-4Rα, and common γ chain on electronically gated CD4$^+$ splenic T cells from control littermate (dashed line) and dnCN Tg$^{+/-}$ (solid line) is shown with background stainings (shaded areas). (C) Tyrosine phosphorylation of STAT6 in response to IL-4 in naive CD4 T cells from dnCN Tg$^{+/-}$ mice. Freshly prepared naive (CD44^{low}) CD4 T cells were stimulated with IL-4 (100 U/ml) for 5 min, and the tyrosine phosphorylation status of STAT6 was assessed by immunoblotting with antiphosphotyrosine mAb (RC20). Arbitrary densitometric units are shown under each band. LM, littermate; circled P, phosphorylated. (D) The amount of CN in the naive CD4 T cells from dnCN Tg$^{+/-}$ mice was assessed by immunoblottings with anti-CN Ab. Cell lysates from indicated doses of naive CD4 T cells are loaded in each lane. Arbitrary densitometric units are shown under each band. LM, littermate. (E) CN phosphatase activity in the naive CD4 T cells from control littermate (LM), dnCN Tg$^{+/-}$, and dnCN Tg$^{+/+}$ mice was assessed by a CN assay kit. Mean CN phosphatase activity with standard deviations is shown. (F) Decreased NFAT nuclear translocation in anti-TCR–activated CD4 T cells from dnCN Tg mice. Naive CD4 T cells (10^7) from littermate (LM) and dnCN Tg$^{+/-}$ mice were stimulated for 2 d with immobilized anti-TCR mAb, and nuclear extracts were prepared. NFAT translocation was examined by immunoblotting with anti-NFATc1 and anti-NFATc2 mAb. The positions where NFATc1 and NFATc2 migrate are indicated by dots. Three independent experiments were performed, and similar results were obtained. Arbitrary densitometric units (multiple bands are included for NFATc1) are shown.
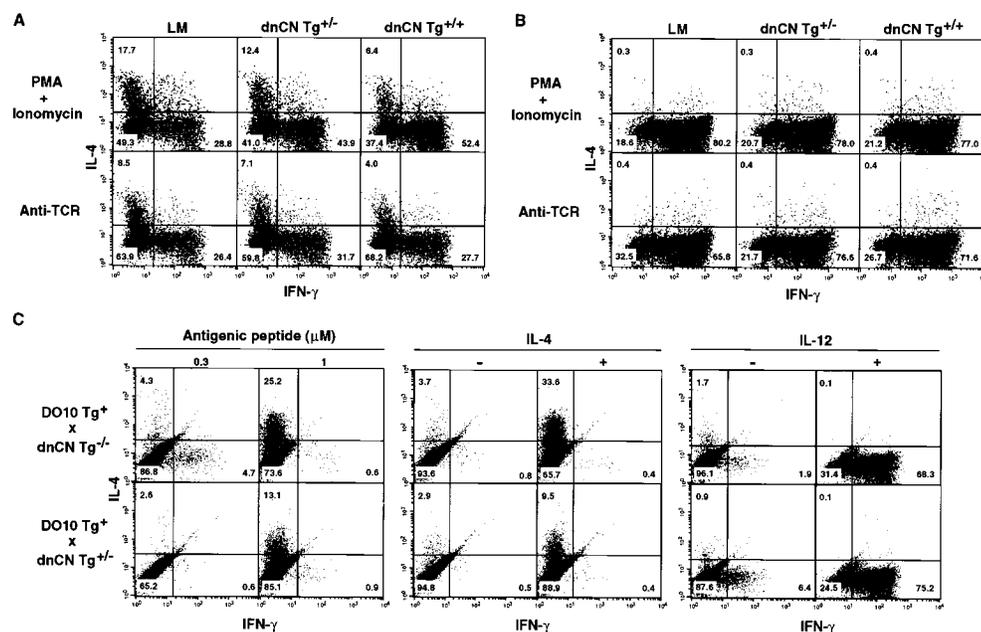

Figure 3. Requirement for CN phosphatase activity in Th2 cell development. (A) Transgene dosage–dependent decrease in the generation of Th2 cells in dnCN Tg mice. Naive CD4 T cells from heterozygous (Tg$^{+/-}$) and homozygous (Tg$^{+/+}$) dnCN Tg and control littermate (LM) mice were stimulated with PMA (1 ng/ml) plus ionomycin (100 nM) or immobilized anti-TCR mAb and IL-4 (Th2-skewed condition), and the generation of Th1/Th2 cells was assessed. The absolute numbers of the cells harvested were similar in these cultures. (B) Normal Th1 cell development in dnCN Tg mice. Naive CD4 T cells were stimulated in Th1-skewed conditions, and the generation of Th1/Th2 cells was assessed. The absolute numbers of the cells harvested were similar in these cultures. (C) FCM-sorted naive CD4 T cells from DO10 Tg$^+$ × dnCN Tg$^{-/-}$ LM and DO10 Tg$^+$ × dnCN Tg$^{+/-}$ mice were stimulated with indicated doses of antigenic peptide and APCs (left) or a minimal dose of peptide (0.1 μM) and APCs in the presence of exogenous IL-4 (middle) or IL-12 and anti–IL-4 mAb (right).

1873   Yamashita et al.

mRNA were increased about three- to fourfold in the activated T cells, and this increase was almost completely blocked by the presence of FK506. Increase in Jak3 protein was also detected in the cells stimulated with PMA plus ionomycin but not with PMA alone. In contrast, the level of Jak1 protein was not changed by TCR stimulation, and the levels of STAT6 and IRS-2 were increased significantly and partially inhibited by FK506. The cell surface expression levels of IL-4Rα were increased significantly by the induction culture with anti-TCR and IL-4, and these were not significantly affected by the presence of FK506 (Fig. 4 B). This result is consistent with our previous report, where IL-4Rα upregulation is induced by IL-4 in naive T cells (14). On the other hand, the upregulation of common γ appeared to be slightly lower in the cells cultured with FK506, but the level of upregulation was within minimal levels, and these results are therefore difficult to interpret. In addition, the number of functional binding sites of IL-4 also appears equivalent in the anti-TCR–activated T cells in the presence or absence of

FK506, as reflected by the equivalent binding of iodinated IL-4 (data not shown).

Improvement of IL-4R Function in the Anti-TCR–activated T Cells Is Dependent on CN Activation. Concurrently, IL-4R function was assessed by measurement of the IL-4–induced tyrosine phosphorylation on Jak1, Jak3, STAT6, and IRS-2. As expected, IL-4–induced phosphorylation of these signaling molecules was dramatically enhanced in the activated T cells with anti-TCR and IL-4 (14), and the enhancement was significantly inhibited by FK506, suggesting CN dependence (Fig. 4 C). Jak1 and Jak3 are known to phosphorylate to each other, and STAT6 and IRS-2 can be phosphorylated by both Jak1 and Jak3 (29). Together with the results shown in Fig. 4 A, the CN-dependent enhancement in the IL-4–induced phosphorylation of Jak1 and Jak3 may be due to the increase in the amount of Jak3 protein. The enhanced STAT6 and IRS-2 phosphorylation may be the result of the increased activation of Jak1 and Jak3, as well as increased protein levels of STAT6 and IRS-2. The improved IL-4R function in the activated T cells
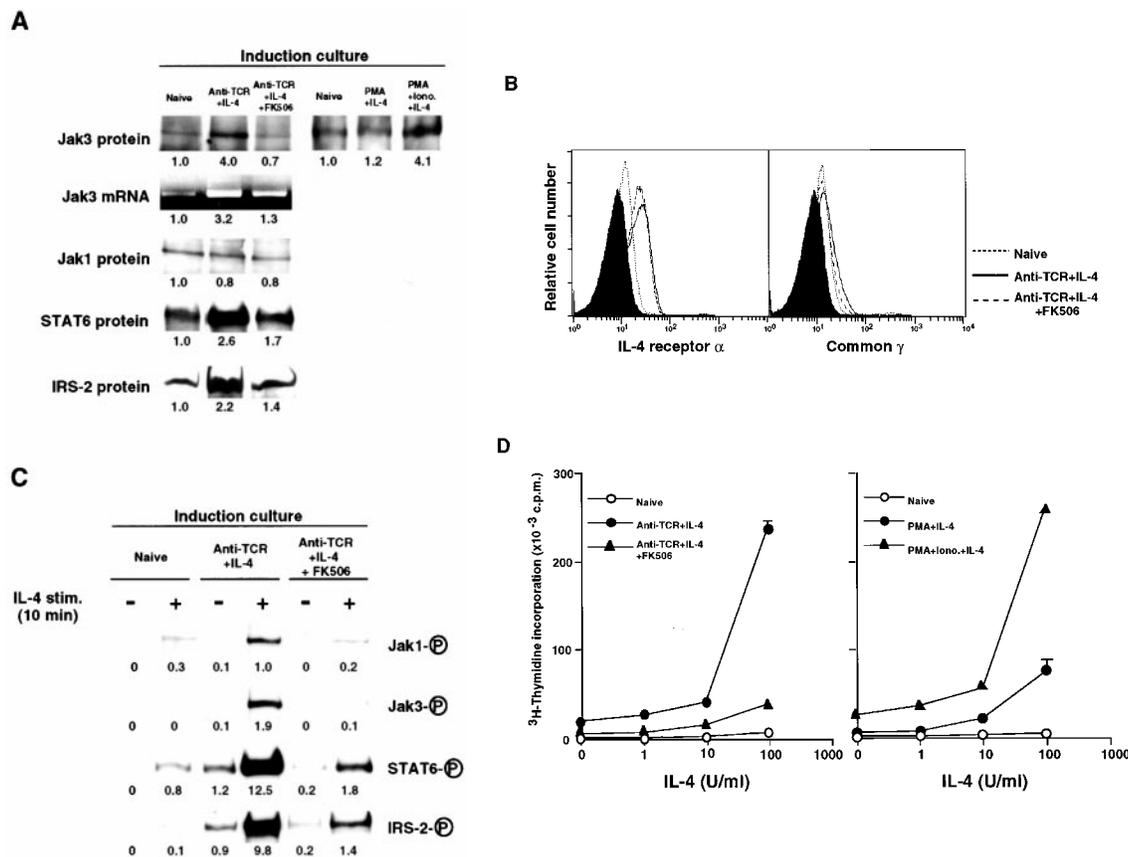

Figure 4. The increase in Jak3 expression and improvement of IL-4R function are dependent on CN activation. (A) Naive CD4 T cells were stimulated with immobilized anti-TCR mAb and IL-4 for 2 d in the presence or absence of FK506. The expression levels of the indicated molecules were assessed by immunoprecipitation and immunoblotting with specific Abs. To detect mRNA levels of Jak3, reverse transcription PCR analysis was performed with a specific primer. Arbitrary densitometric units are shown under each band. (B) Cell surface expression of IL-4Rα and common γ chain of the stimulated cells, as in A. The background stainings are shown by shaded areas. (C) Naive CD4 T cells were stimulated for 2 d as in A, and IL-4–induced phosphorylation on the indicated molecules was assessed. Arbitrary densitometric units are shown under each band. Circled P, phosphorylated. (D) The cells stimulated for 2 d as in C were cultured without anti-TCR stimulation for another 8 h, and IL-4–induced proliferative response was assessed. Mean [³H]thymidine incorporation in each culture is shown with standard deviations.

was seen in the IL-4–induced proliferative responses, where dramatic CN-dependent improvement was observed (Fig. 4 D, left). Likewise, stimulation with PMA plus ionomycin but not PMA alone induced a similar improvement in the IL-4–induced proliferation (Fig. 4 D, right).

Inhibition of Th2 Cell Development by the Presence of an Antisense DNA of Jak3. The *Drosophila antennapedia* homeodomain protein peptide (Penetratin™) is reported to penetrate mammalian plasma membranes and to work as a vector for oligonucleotides or short peptides (21, 24–26). We used this system to deliver Jak3 antisense oligonucleotide to assess the requirement for transcriptional upregulation of Jak3 in Th2 cell development. As shown in Fig. 5 A, the introduction of Jak3 antisense oligonucleotide into naive T cells inhibited the Th2 cell generation significantly, suggesting that the upregulation of Jak3 protein is an important event for the Th2 cell development. Immunoblotting with anti-Jak3 mAb revealed that the level of Jak3 protein in the activated cells with Jak3 antisense was ~60% of the control level (Fig. 5 B). The blocking effect on Th2 cell development was not observed by the presence of Penetratin™-coupled control oligo DNAs such as a Jak3 sense or a scrambled oligo DNA (Fig. 5 C).

Involvement of STAT5 Activation in IL-4–induced Proliferation and Th2 Cell Development. STAT5 is a well-known downstream signaling molecule of a wide variety of cytokine receptors, including IL-2R, IL-7R, IL-9R, and IL-15R,

and an important role for STAT5 in proliferative responses has been suggested (30–34). Although the four receptors and IL-4R share common γ and Jak3, STAT5 has not been established as a downstream signaling molecule of IL-4R. However, in the activated T cells with anti-TCR and IL-4, IL-4-stimulation (10 min) induced phosphorylation of STAT5a molecules, and this phosphorylation was significantly inhibited by FK506 in the induction culture (Fig. 6 A, left). No phosphorylation was detected in the immunoprecipitates with control Ig. The other STAT5 isoform, STAT5b, was also phosphorylated by the 10-min IL-4 stimulation (data not shown). Although significantly lower, considerable levels of IL-4–induced STAT5 phosphorylation were detected even in the activated cells with anti-TCR mAb in the presence of anti–IL-4 and IL-12 (a Th1-skewed condition; Fig. 6 A, right), suggesting that the functional association of STAT5 to the IL-4R complex is not specific for the Th2-skewed stimulation condition, but is a TCR-mediated consequence.

Simultaneously, we sought evidence for a physical association of STAT5a with IL-4R. As can be seen in Fig. 6 B, STAT5a molecules were coprecipitated with IL-4Rα in the anti-TCR–activated T cells but not in naive T cells, and this association was dependent on CN activation. Interestingly, this STAT5a association with IL-4Rα appears not to be dependent on the short-term IL-4 stimulation (10 min). Again, isotype-matched control Ig did not precipitate
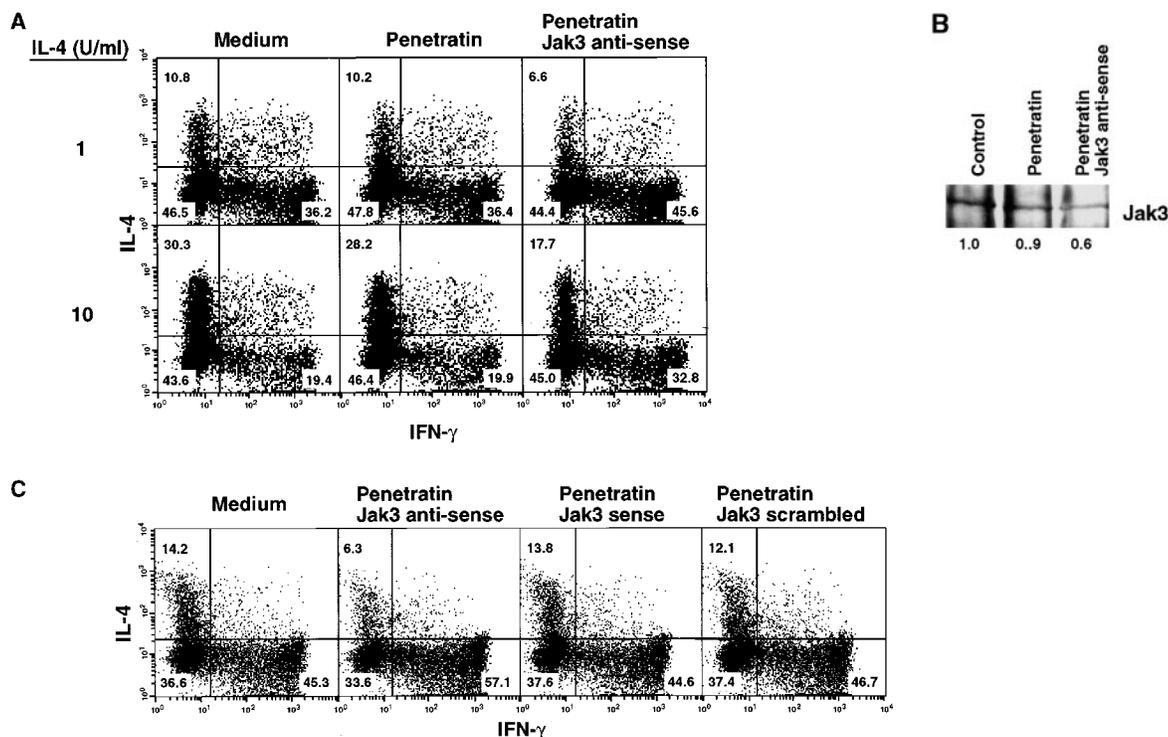

Figure 5. Inhibition of Jak3 induction by antisense oligonucleotide results in a decreased Th2 cell generation. The effect of Jak3 antisense, sense, or scrambled oligonucleotide coupled with Penetratin™ was assessed in the in vitro Th1/Th2 cell differentiation culture (Th2-skewed condition). Stimulation with immobilized anti-TCR and indicated doses of (A) IL-4 or (C) anti-TCR and 1 U/ml of IL-4 were used. The protein expression levels of Jak3 in the cells shown in A (1 U/ml of IL-4) were assessed by immunoprecipitation and immunoblotting with anti-Jak3 mAb (B).
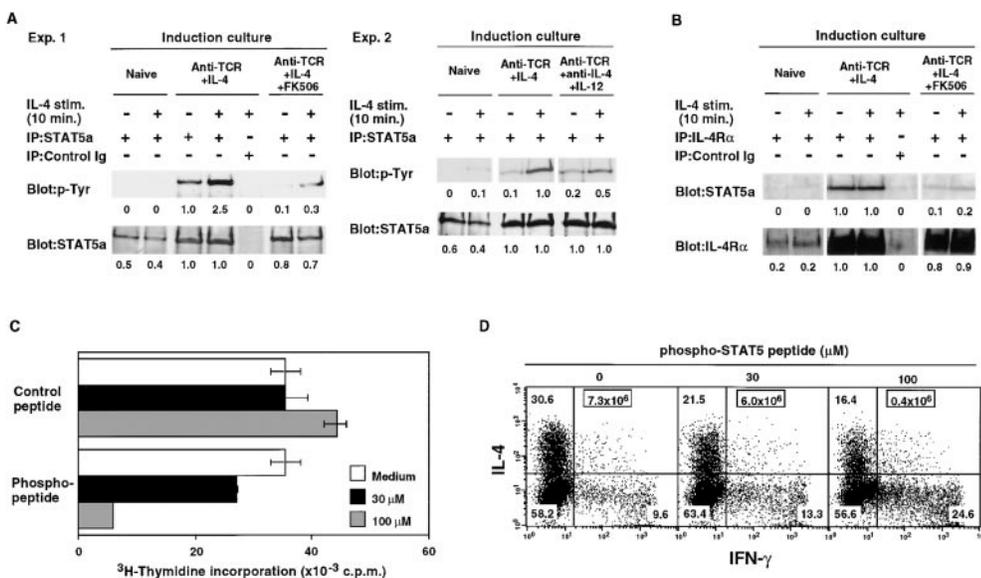
1875   Yamashita et al.

Figure 6. Requirement for STAT5 activation in IL-4–induced expansion of developing Th2 cells. (A) IL-4–induced phosphorylation of STAT5a molecules were examined in the anti-TCR–activated CD4 T cells prepared as described in the legend to Fig. 4 C. To assess the levels of STAT5 phosphorylation, immunoprecipitation (IP) with anti-STAT5a and subsequent immunoblotting (Blot) with antiphosphotyrosine (p-Tyr) were performed. An isotype-matched Ab was used for the control immunoprecipitation. For a control, immunoblotting with anti-STAT5a was also performed. In experiment (Exp.) 2, naive T cells were activated for 2 d with anti-TCR and IL-4 or with anti-TCR, anti-IL-4, and IL-12, and were subjected to STAT5 immunoprecipitation. (B) To detect STAT5a association with IL-4Rα, immunoprecipitates with anti–IL-4Rα were subjected to immunoblotting (Blot) with anti-STAT5a and anti–IL-4Rα. An isotype-matched Ab was used for the control immunoprecipitation (IP). Arbitrary densitometric units are shown under each band. (C) IL-4–induced proliferation of anti-TCR–activated developing Th2 cells as shown in A, was assessed in the presence of indicated doses of phospho-STAT5–*antennapedia* peptide or nonphosphorylated control peptide. The mean [³H]thymidine incorporation of each group is shown with standard deviations. (D) The effect of the phospho-STAT5–*antennapedia* peptide on Th2 cell development in vitro. Naive CD4 T cells from (B6 × BALB/c)F1 mice were stimulated with immobilized anti-TCR mAb and IL-4 in the presence of indicated doses of phospho-STAT5–*antennapedia* peptide. The numbers of the cells harvested are shown as boxed numbers. The percentages of cells in each quadrant are also shown. The estimated cell numbers of Th2 cells generated are 2.23×10^6, 1.29×10^6, and 0.07×10^6 from left to right.

either STAT5a or IL-4Rα. Consistent with the results by FCM analysis (Fig. 4 B), the upregulation of IL-4Rα molecule was not significantly blocked by the presence of FK506 (Fig. 6 B, bottom). These results indicate that STAT5 molecules are physically and functionally associated with the IL-4R complex in the anti-TCR–activated T cells receiving sufficient CN activation. Consistent with our results, IL-4–induced STAT5 activation has been recently reported in a certain B cell line and in PHA-activated human peripheral blood cells (35), and IL-4–induced proliferation was impaired in STAT5-deficient spleen cells (36).

To investigate the role of STAT5 activation in developing Th2 cells, we generated a fusion peptide comprising STAT5 phosphopeptides and *antennapedia* peptides (Penetratin™; phospho-STAT5–*antennapedia* peptide) to inhibit STAT5 dimerization and activation. IL-4–induced proliferation of developing Th2 cells was assessed in the presence of the phospho-STAT5–*antennapedia* peptide, and significant blocking effects were detected (Fig. 6 C). The addition of a nonphosphorylated control peptide did not affect the IL-4–induced proliferation, suggesting a phospho-STAT5 peptide–dependent phenomenon. When the phospho-STAT5–*antennapedia* peptide was added to the in vitro Th2 cell differentiation cultures (Th2-skewed condition), a similar blocking effect on the development of Th2 cells was observed (Fig. 6 D). The levels of absolute number of Th2 cells generated are at ∼60% with 30 μM and ∼3% with 100 μM of the phospho-STAT5. Thus, the IL-4–induced activation of STAT5 appeared to be crucial in the expansion of developing Th2 cells.

Constitutive Association of STAT5 with IL-4R Complex in Cloned Th2 Cells. Finally, to investigate the role of STAT5 activation for IL-4–induced proliferation in an established Th2 cell clone, an IL-4–responding Th2 clone D10 (22) was transfected with a (dn)STAT5 cDNA (23). In the parent D10 T cell clone, a dose-dependent induction of the IL-4–induced STAT5 phosphorylation was detected (Fig. 7 A). In addition, the constitutive association of STAT5a molecules with IL-4Rα was observed (Fig. 7 B). Similar to anti-TCR–activated CD4 T cells (Fig. 6 B), the STAT5a association in D10 clone did not appear to be dependent on the short-term IL-4 exposure (10 min, 10 U/ml). Analysis of transiently transfected D10 cells and three independent stable transfectants with an HA-tagged dnSTAT5 revealed a significant impairment in IL-4–induced proliferation (Fig. 7 C). The association of transfected dnSTAT5 protein in the transient transfected D10 cells was confirmed by immunoblotting with anti-HA mAb (Fig. 7 D, left). In addition, IL-4–induced phosphorylation of STAT5a molecules was not detected in the stable transfectants (Fig. 7 D, right) and transient transfectants with dnSTAT5 (data not shown). Collectively, these data suggest that IL-4–induced proliferation of D10 clone is strongly dependent on STAT5 activation.

The Strength of TCR Stimulation and Th1/Th2 Cell Development. In this report, we demonstrate that TCR-mediated activation of Ca^{2+}/CN is required for Th2 cell development, and a differential dependence on the CN activation between Th1 and Th2 cell development is revealed. Several recent reports suggested that low-dose infection or
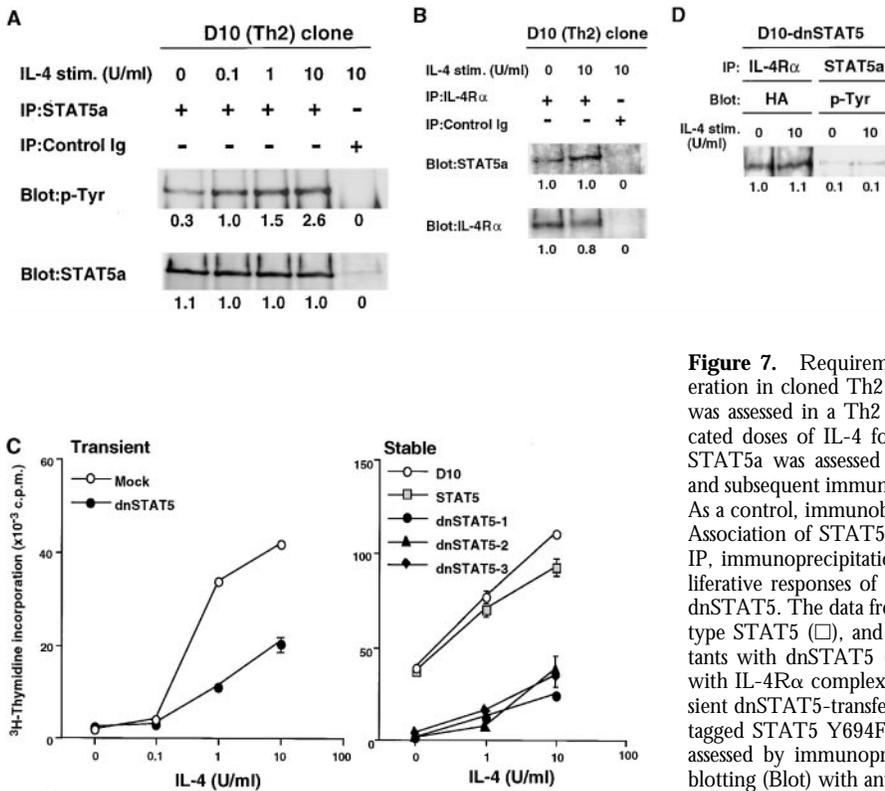
Figure 7. Requirement for STAT5 activation in IL-4–induced proliferation in cloned Th2 cells. (A) IL-4–induced STAT5a phosphorylation was assessed in a Th2 clone D10. D10 cells were stimulated with indicated doses of IL-4 for 10 min, and tyrosine phosphorylation status of STAT5a was assessed by immunoprecipitation (IP) with anti-STAT5a and subsequent immunoblotting (Blot) with antiphosphotyrosine (p-Tyr). As a control, immunoblotting with anti-STAT5a was also performed. (B) Association of STAT5a with IL-4Rα was assessed in a Th2 clone D10. IP, immunoprecipitation; Blot, immunoblotting. (C) IL-4–induced proliferative responses of transient (left) and stable (right) transfectants with dnSTAT5. The data from parent D10 (○), a stable transfectant with wild-type STAT5 (□), and a transient and three independent stable transfectants with dnSTAT5 (●, ▲, ◆) are shown. (D) dnSTAT5 association with IL-4Rα complex and lack of STAT5a phosphorylation in the transient dnSTAT5-transfected D10 cells. The association of transfected HA-tagged STAT5 Y694F mutant (dnSTAT5) molecules with IL-4Rα was assessed by immunoprecipitation (IP) with anti-IL-4Rα and immunoblotting (Blot) with anti-HA Ab. p-Tyr, antiphosphotyrosine.

low-dose antigen concentration induced Th1 responses preferentially, whereas high-dose antigen stimulation favored Th2 responses (37–39). We reported previously that strong activation of Lck and subsequent activation of the Ras/MAPK pathway was required for Th2 cell differentiation (13, 14). Taken together with the findings shown in this report, strong stimulation of naive CD4 T cells may sufficiently activate Lck and downstream signaling pathways, including the Ras/MAPK and Ca/CN pathways, and induce Th2 cell development. In contrast, weak antigenic stimulation may not activate these signaling pathways efficiently enough for Th2 cell development, but may be sufficient for Th1 development. Thus, the extent to which both Ras/MAPK and Ca^{2+}/CN pathways are activated may determine the Th1/Th2 cell lineage into which naive CD4 T cells will develop.

Functional IL-4R Complex in Developing Th2 Cells. After anti-TCR activation of naive CD4 T cells, the expression levels of various components of the IL-4R complex are upregulated, and the function of IL-4R is improved dramatically. Among them, Jak3 upregulation was most dramatic and appeared to be totally dependent on TCR-mediated CN activation. The upregulation of STAT6, STAT5a, and IRS-2 molecules was partially CN dependent. In contrast, little dependency on CN activation was observed in the upregulation of IL-4Rα (Fig. 4 B and Fig. 6 B). In fact, the upregulation of IL-4Rα is shown to be induced by IL-4 itself (14). Although of interest, the molecular mechanisms governing IL-4–induced IL-4Rα upregulation have not been well elucidated at this time. The inhibition of the Jak3

upregulation by antisense Jak3 resulted in a diminished Th2 cell development (Fig. 5), suggesting that the Jak3 upregulation is an important event for Th2 cell development. Therefore, although transcriptional upregulation of other CN/NFAT–dependent molecules may also be required for the Th2 cell development, this study strongly suggests that one of the important targets is Jak3.

Interestingly, STAT5 was found to be physically and functionally associated with IL-4R complex in the anti-TCR–activated T cells (Fig. 6). The IL-4–induced activation of STAT5 appears to be crucial for the expansion process of developing Th2 cells (Fig. 6, C and D). Interestingly, the STAT5 association is maintained in established cloned Th2 cells (Fig. 7, A and B). This may be the reason why some Th2 cell clones can proliferate to IL-4 as well as to IL-2. Although the precise molecular basis by which STAT5 associates with IL-4R complex remains unclear, the association is probably mediated by Jak3, because STAT5 association was detected only after Jak3 upregulation (Fig. 4 A and Fig. 6, A and B). A possible mechanism for the interaction of these two molecules has been proposed by other investigators (35, 40). Alternatively, STAT5 may be recruited to Jak1 associated with IL-4Rα. This possibility is also suggested in the IL-6R system (40). In addition, it is not clear whether the STAT5 association is a phosphorylation-dependent phenomenon or not. Further comprehensive biochemical analyses are required to address this issue.

In addition to STAT5 activation, it can be speculated that the CN-dependent Jak3 upregulation also facilitates IL-4–induced activation of STAT6 and IRS-1 and -2,

which are thought to be important for differentiation and expansion processes, respectively (29). In fact, the protein expression levels of STAT6 and IRS-2 were also upregulated in the anti-TCR–activated naive CD4 T cells (Fig. 4 A). Thus, successful development of Th2 cells appears to depend on cooperative events that involve IL-4–mediated activation of STAT5- as well as of STAT6- and IRS-mediated signaling pathways, and it is clearly influenced and regulated by antigen receptor–mediated signaling cascade, such as the Ras/MAPK pathway (14) and the Ca^{2+}/CN signaling pathway.

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