cleophilic displacement was effected with 2 M amine in dimethyl sulfoxide at 35°C for 2 hours. The subsequent Fmoc amino acid (10 equivalents) was coupled by using 10 equivalents of the coupling reagent PyBrop (NovaBiochem) and 18 equivalents of diisopropyl ethyl amine (three times for 2 hours). The remaining portion was synthesized by standard Fmoc chemistry. Peptoids were cleaved, purified by reversed-phase high-performance liquid chromatography, and verified by electrospray mass spectrometry. After lyophilization, peptoids were resuspended in water and the concentration was determined by tyrosine absorbance and by amino acid analysis.

25. Competitive inhibition of SH3 binding by peptoids was assayed by mixing the indicated concentration of inhibitor peptide with a Grb2 SH3 domain (~50 nM), expressed as a fusion to a protein that is endogenously biotinylated in *Escherichia coli* (PINPOINT vector, Promega, Madison, WI) in STE [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA], in a total volume of 1.5 ml for 30 min. Glutathione agarose beads (15 μl) that had been bound with ~250 nmol of a GST fusion to a tandem repeat of the Sos derived peptide $(PPPVPPRR)_2$ were added to the above mixture and incubated at 4°C for 1 hour. The agarose beads were washed with 3 × 1 ml of STE and boiled in 30 μl of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) buffer, and the eluate was subjected to SDS-PAGE. The amount of biotinylated SH3 fusion retained on the beads was measured by blotting these gels onto nitrocellulose, blocking in TBST [100 mM tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20) with 1% milk, probing with streptavidin–horseradish peroxidase conjugate, and developing with Pierce chemiluminescent SuperSubstrate. Under these reaction conditions, the K_i is calculated to be approximately 1/10th of the observed IC_{50}. The observed IC_{50} for peptide 45, $\sim 5 \times 10^{-7}$ M, is therefore close to the lower limit of detection for this assay, given the concentration of biotinylated SH3 used in the assay (5×10^{-8} M).

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Defective T Cell Differentiation in the Absence of *Jnk1*

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The c-Jun NH_2-terminal kinase (JNK) signaling pathway has been implicated in the immune response that is mediated by the activation and differentiation of CD4 helper T (T_H) cells into T_H1 and T_H2 effector cells. JNK activity observed in wild-type activated T_H cells was severely reduced in T_H cells from *Jnk1*$^{-/-}$ mice. The *Jnk1*$^{-/-}$ T cells hyperproliferated, exhibited decreased activation-induced cell death, and preferentially differentiated to T_H2 cells. The enhanced production of T_H2 cytokines by *Jnk1*$^{-/-}$ cells was associated with increased nuclear accumulation of the transcription factor NFATc. Thus, the JNK1 signaling pathway plays a key role in T cell receptor–initiated T_H cell proliferation, apoptosis, and differentiation.

When activated by antigen-presenting cells (APCs), T_H cells undergo clonal proliferation and produce interleukin 2 (IL-2). The activated T_H cells may then become T_H1 or T_H2 effector cells (*1*), which mediate inflammatory or humoral responses, respectively. The polarization of T_H cell differentiation is, at least in part, determined by the cytokine environment (*1*). IL-12, produced by activated APCs, induces T_H1 development of naïve T_H cells. IL-4, made by T cells, is required for T_H2 differentiation. Thus, early production of IL-4 or IL-12 determines T_H cell lineage commitment and the type of immune response that occurs. Although most attention has focused on the effect of polarizing cytokines on T_H cell differentiation, signals from the T cell receptor (TCR)–CD3 complex and from the costimulatory factor CD28 may also affect cytokine production by mechanisms not yet understood (*2*).

JNK, also known as stress-activated protein kinase, phosphorylates the transcription factor c-Jun and increases AP-1 transcription activity (*3, 4*). Other substrates include JunD, ATF2, ATFa, Elk-1, Sap-1, and NFAT4 (*3, 4*). Signals from both the TCR-CD3 complex and CD28 are required for JNK and AP-1 activation in T cells, and these signals may be integrated in such a way as to mediate T cell activation and the induction of IL-2 transcription (*5*). Although JNK is implicated in IL-2 gene transcription, JNK may also act to stabilize IL-2 mRNA (*6*). AP-1 has also been reported to be important for the regulation of T_H1 and T_H2 cytokine genes (*7, 8*).

To understand the role or roles of JNK in T_H cell activation and differentiation, we generated *Jnk1*-deficient mice through homologous recombination in embryonic stem cells (*9*) (Fig. 1A). Targeted disruption of the *Jnk1* gene resulted in a null allele, as confirmed by mRNA (*10*) and protein expression analysis of embryonic fibroblast (Fig. 1B) and T cell (*11*) extracts. *Jnk1*-deficient mice were fertile and of normal size. Lymphocyte development appeared normal, with typical ratios of T cells to B cells, CD4 to CD8, and naïve to memory T cells in the periphery (*10*). The absence of apparent developmental defects of *Jnk1*$^{-/-}$ lymphocytes might be the result of redundancy, because *Jnk1* and *Jnk2* are coexpressed in lymphoid tissues (*4*). Therefore, we tested whether JNK1 and JNK2 are activated similarly during the course of T_H cell activation.

Purified CD4 T cells from wild-type or knockout mice were stimulated by antibodies to CD3 (anti-CD3) with or without anti-CD28 (*12, 13*), and JNK activity was measured using c-Jun as the substrate. During the first 48 hours, induced JNK activity was greatly reduced in the *Jnk1*$^{-/-}$ T_H cells; moreover, anti-CD28 could not enhance kinase activity (Fig. 1C). Essentially no JNK activity was detected in *Jnk1*$^{-/-}$ T_H cells stimulated for only 5 min, despite the same JNK2 protein expression (*11*). Thus, JNK1 appeared to account for most of the JNK activity in newly activated T cells. After 60 hours of stimulation, JNK activity in the *Jnk1*$^{-/-}$ cells was similar to that in wild-type cells, and in each case this activity was presumably derived from JNK2. In fact, JNK2 represents most JNK activity in T_H1 effector cells (*14*).

To investigate the role or roles of JNK1 in T_H cell activation and IL-2 production, we stimulated T cells with concanavalin A (Con A), anti-CD3, or anti-CD3 plus anti-CD28 (*12, 13*). Relative to wild-type cells, *Jnk1*$^{-/-}$ spleen cells produced the same amount of IL-2 (Fig. 2A) and CD4 T cells produced the same amount of IL-2 mRNA (*10*) 24 hours after stimulation, despite the lack of JNK activation (Fig. 1C), similar to *Jnk2*- and c-*Jun*-deficient T cells (*14, 15*). Although JNK may therefore not be required for IL-2 expression, it is also possible that JNK1 and JNK2 are redundant for IL-2 regulation. Despite normal IL-2 production, *Jnk1*$^{-/-}$ splenocytes and CD4 T cells (*10*) displayed enhanced proliferation (*12, 13*) (Fig. 2B). In addition, *Jnk1*-deficient T_H cells had a

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moderate reduction of activation-induced cell death (from 46 to 27%) (*13*), suggesting that JNK1 may be involved in the regulation of T cell apoptosis. Decreased apoptosis by *Jnk1*$^{-/-}$ T cells could therefore contribute to the increased proliferation of these cells.

To test whether JNK1 is involved in T_H cell differentiation, we measured cytokine production by purified CD4 T cells after stimulation for 4 days by immobilized anti-CD3 with or without anti-CD28 (*12, 13*). Production of interferon-γ (IFN-γ) T_H1 cytokine by *Jnk1*$^{-/-}$ T_H cells appeared normal in response to anti-CD3, but there was a consistent lack of enhancement by anti-CD28 (Fig. 2C). Wild-type cells produced small amounts of IL-4, IL-5, and IL-10 T_H2 cytokines in response to immobilized anti-CD3, whereas the *Jnk1*$^{-/-}$ T_H cells secreted remarkably large amounts of these T_H2 cytokines (Fig. 2C). Addition of anti-CD28 enhanced T_H2 cytokine production in both wild-type and *Jnk1*$^{-/-}$ T_H cells (Fig. 2C). These observations indicate that *Jnk1*$^{-/-}$ CD4 T cells were hyperresponsive to anti-CD3 and produced T_H2 cytokines even in the absence of costimulation.

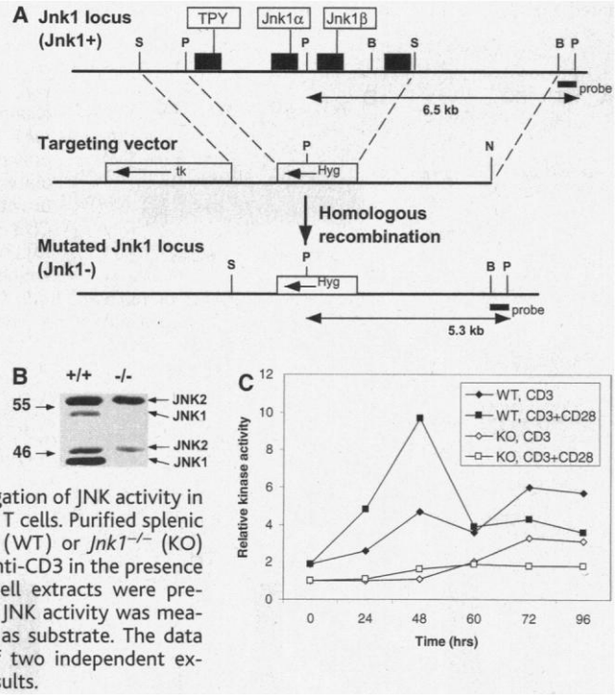

Fig. 1. Generation of *Jnk1*-deficient mice. (**A**) Schematic representation of the genomic *Jnk1* locus, the targeting vector, and the mutated *Jnk1* locus. Restriction enzyme sites (B, Bam HI; N, Not I; P, Pst I; S, Spe I) and the probe for Southern blot analysis are indicated. (**B**) Protein immunoblot analysis confirmed the absence of expression of the JNK1 protein. Extracts of embryo fibroblasts were examined by immunoblot analysis with a monoclonal antibody to JNK1 and JNK2. The 55-kD and 46-kD forms of JNK1 and JNK2 are indicated. (**C**) Abrogation of JNK activity in activated *Jnk1*-deficient CD4 T cells. Purified splenic CD4 T cells from wild-type (WT) or *Jnk1*$^{-/-}$ (KO) mice were stimulated with anti-CD3 in the presence or absence of anti-CD28. Cell extracts were prepared at different times and JNK activity was measured with the use of c-Jun as substrate. The data shown are representative of two independent experiments with the same results.

Differentiating *Jnk1*$^{-/-}$ T_H cells produced more IL-4 than did wild-type cells. To assess whether *Jnk1*$^{-/-}$ T_H cells become different effector cells (that is, polarized toward T_H2), we isolated wild-type and *Jnk1*$^{-/-}$ $CD44^{lo}CD45RB^{hi}$ naïve CD4 T cells and cultured them in vitro under neutral conditions, using immobilized anti-CD3, IL-2, and irradiated APCs derived from the wild-type mice (*16*). After restimulation with anti-CD3, wild-type effector cells made significant amounts of IFN-γ (361 U/ml in one representative experiment) but no detectable IL-4, whereas *Jnk1*$^{-/-}$ T_H cells made much more IL-4 (33 U/ml) and much less IFN-γ (53 U/ml). Consistent with these observations, larger amounts of IL-4 mRNA were detected (*17*) (Fig. 3A). Thus, *Jnk1*$^{-/-}$ CD4 T cells differentiated preferentially into T_H2 cells, whereas the wild-type cells of the same genetic background became T_H1 cells.

To test the possibility that the hyperproduction of T_H2 cytokines by *Jnk1*$^{-/-}$ T_H cells was caused by an intrinsic deficiency in their ability to become T_H1 cells, we differentiated the CD4 T cells under the above conditions with the addition of IL-12 and anti–IL-4 to promote T_H1 differentiation, or IL-4 and anti–IFN-γ for T_H2 development (*16*). When restimulated with anti-CD3 for 24 hours, the *Jnk1*$^{-/-}$ T_H1 effector cells secreted amounts of IFN-γ similar to wild-type cells (Fig. 3B), which indicated that the *Jnk1*$^{-/-}$ T_H cells could differentiate to T_H1 cells and produce T_H1 cytokines. However, the *Jnk1*$^{-/-}$ T_H1 population also made IL-4, IL-5, and IL-10 after anti-CD3 restimulation (Fig. 3B), suggesting a failure to down-regulate these cytokines by the IL-12– and IFN-γ–rich environment. This T_H2 cytokine production by the *Jnk1*$^{-/-}$ T_H1 population was independent of IL-4, because anti–IL-4 was present during the differentiation process. The *Jnk1*$^{-/-}$ *Jnk1*$^{-/-}$

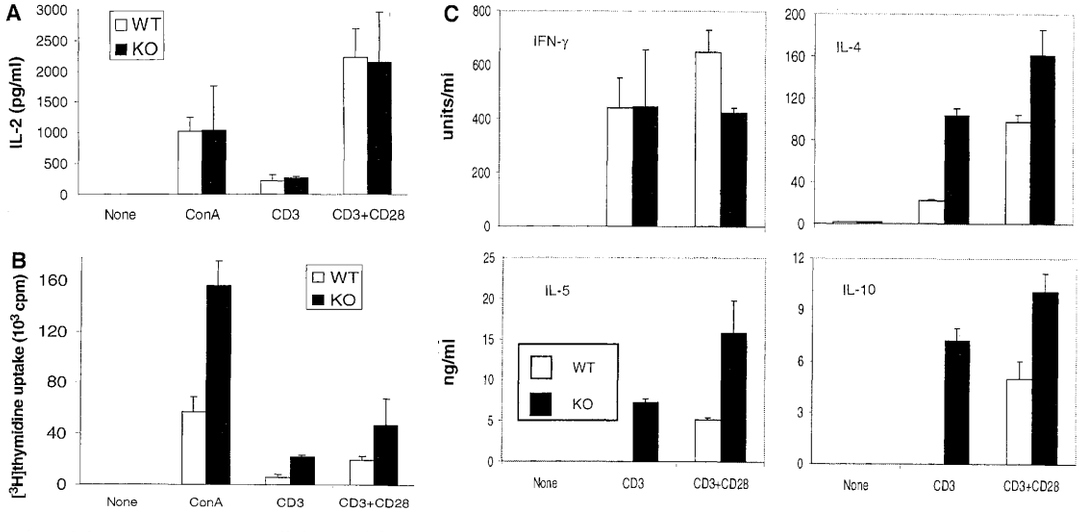

Fig. 2. Increased proliferation and T_H2 cytokines in *Jnk1*$^{-/-}$ T cells. (**A**) Normal IL-2 production by activated *Jnk1*$^{-/-}$ spleen T cells. Spleen cells from 2-month-old wild-type (WT) or *Jnk1*$^{-/-}$ (KO) mice were stimulated as indicated for 24 hours, and the amount of IL-2 in the cell supernatant was measured by ELISA. A representative of three independent experiments using different mice is shown. (**B**) Increased proliferation by *Jnk1*-deficient T cells. Wild-type or *Jnk1*$^{-/-}$ splenocytes were stimulated as in (A) for 3 days, and [^{3}H]thymidine was included during the last 8 hours to measure cellular proliferation. (**C**) Splenic CD4 T cells from 6- to 8-week-old wild-type (WT) or *Jnk1*$^{-/-}$ (KO) mice were stimulated as shown (*12*); 4 days later, amounts of T_H cytokines were measured by ELISA. The data shown are representative of 10 independent experiments, each using different mice.

T_H2 effector cells also secreted greatly increased amounts of T_H2 cytokines: Relative to the same number of wild-type cells, the *Jnk1*$^{-/-}$ T_H2 effector cells produced almost 10 times as much IL-4, five times as much IL-5, and moderately increased amounts of IL-10 (Fig. 3B). The same results were obtained when purified naïve CD4 T cells were differentiated (*10*). The increased amount of T_H2 cytokines in *Jnk1*$^{-/-}$ T_H cells was also not the result of differential survival of T_H2 versus T_H1 cells in *Jnk1*$^{-/-}$ mice, as their patterns of apoptosis were similar (*10*).

To test whether the exaggerated T_H2 cytokine production by the polyclonally activated *Jnk1*$^{-/-}$ T_H cells in vitro reflects a feature of the in vivo antigen-specific immune response, we immunized age- and sex-matched mice with keyhole limpet hemocyanin (KLH) precipitated in alum, an adjuvant promoting T_H2 responses. When the draining lymph node cells were restimulated with KLH in vitro, relative to wild-type cells, *Jnk1*$^{-/-}$ cells produced at least four times as much IL-5 (Fig. 3C). This finding is indicative of an enhanced T_H2 response, which correlates well with the earlier in vitro results with anti-CD3 activation (Fig. 3B); as expected, neither wild-type nor knockout T cells made IFN-γ under these immunization conditions.

These results showed that *Jnk1*$^{-/-}$ T_H effector cells, in vitro or ex vivo, made elevated T_H2 cytokines. We also found that 24 and 48 hours after treatment with anti-CD3, relative to wild-type cells, *Jnk1*$^{-/-}$ CD4 T_H precursor cells made more IL-4 mRNA and slightly more IL-5 mRNA (*10*). Because IL-4 is necessary and sufficient to generate T_H2 cells, the preference of naïve *Jnk1*$^{-/-}$ T_H cells to differentiate to the T_H2 lineage and make IL-5 and IL-10. under neutral conditions (Fig. 3A) could be caused by increased IL-4 production in the early activation phase.

To identify factors that may be responsible for the exaggerated IL-4 production by *Jnk1*-deficient cells, we treated purified CD4 T cells with anti-CD3 for 48 hours and prepared nuclear extracts. Several transcription factors involved in IL-4 transcription or required for T_H2 differentiation were assayed by immunoblot analysis (*18, 19*). There was no difference in the amounts of the transcription factors GATA-3 or Stat-6 in the nuclei of wild-type or *Jnk1*$^{-/-}$ cells, both of which were diminished by anti–IL-4 treatment in culture (Fig. 4A). Thus, GATA-3, like Stat-6, is probably responsive to IL-4 under these conditions. There was a moderate increase in the amount of JunB protein (Fig. 4A), a member of the Jun family that is increased in T_H2 cells and important for IL-4 transcription (*7*). NFATc is essential for IL-4 production and T_H2 differentiation (*20*). Anti-CD3–activated wild-type cells had little NFATc in the nucleus, whereas costimu-

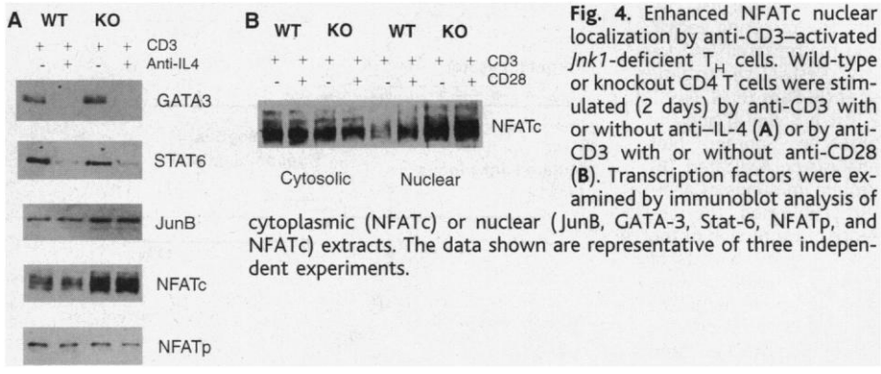

Fig. 4. Enhanced NFATc nuclear localization by anti-CD3–activated *Jnk1*-deficient T_H cells. Wild-type or knockout CD4 T cells were stimulated (2 days) by anti-CD3 with or without anti–IL-4 (**A**) or by anti-CD3 with or without anti-CD28 (**B**). Transcription factors were examined by immunoblot analysis of cytoplasmic (NFATc) or nuclear (JunB, GATA-3, Stat-6, NFATp, and NFATc) extracts. The data shown are representative of three independent experiments.

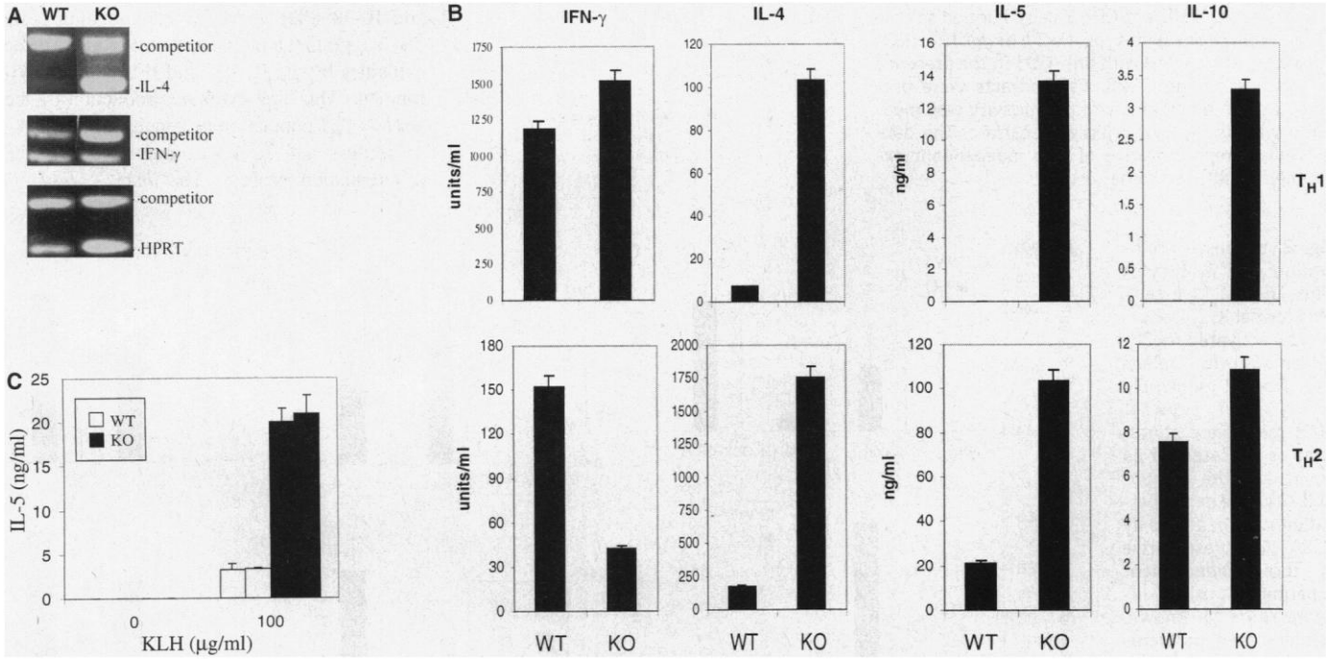

Fig. 3. *Jnk1*$^{-/-}$ T_H precursor cells preferentially differentiate to the T_H2 lineage, and differentiated *Jnk1*$^{-/-}$ T_H1 and T_H2 effector cells make increased amounts of T_H2 cytokines. (**A**) Spleen and lymph node naïve CD4 T cells of wild-type or *Jnk1*$^{-/-}$ mice were differentiated under neutral conditions (*16*) and then restimulated with anti-CD3 for 24 hours. The cytokine mRNAs produced after restimulation were analyzed by competitive RT-PCR and cytokines by ELISA. (**B**) Wild-type (WT) or knockout (KO) splenic CD4 T cells were differentiated and restimulated in vitro as in (A), except that IL-12 and anti–IL-4 were added for T_H1 differentiation, and IL-4 and anti–IFN-γ for T_H2 differentiation. (**C**) Age- and sex-matched wild-type (WT) or knockout (KO) animals were immunized with KLH peptide precipitated in alum; 9 days later, the draining lymph node cells were treated with or without KLH peptide for 4 days. The amount of IL-5 produced was determined by ELISA.

lation with anti-CD28 enhanced NFATc nuclear accumulation (Fig. 4B), in keeping with the finding that T_H2 cytokine induction in wild-type T_H cells requires costimulation (Fig. 2C). In contrast, anti-CD3 treatment alone led to an increase in nuclear NFATc in *Jnk1*$^{-/-}$ T_H cells and a decrease in cytoplasmic NFATc (Fig. 4, A and B), consistent with the high T_H2 cytokine production by CD3-activated *Jnk1*$^{-/-}$ cells (Fig. 2C). The enhanced accumulation of nuclear NFATc in *Jnk1*$^{-/-}$ T_H cells was observed in cells 8, 24, and 48 hours after stimulation, but was not observed in nonactivated cells (*10*). NFATc accumulation was specific because the amount of nuclear NFATp, a proposed negative regulator of T_H2 cytokine genes (*21*), was the same in wild-type and *Jnk1*$^{-/-}$ cells (Fig. 4A). Enhanced nuclear accumulation of NFATc in *Jnk1*$^{-/-}$ T cells was not blocked by anti–IL-4 (Fig. 4A); hence, increased IL-4 production and NFATc nuclear localization is intrinsic to T cell receptor signaling and is not secondary to IL-4 production. Because NFATc can bind to the IL-4 promoter and is required for IL-4 production and T_H2 differentiation (*20, 22*), the greatly enhanced amount of nuclear NFATc could account for the increased IL-4 production in CD3-activated *Jnk1*-deficient mice.

The mechanism by which JNK1 negatively regulates NFATc nuclear accumulation remains to be resolved. The isoform NFAT4 is phosphorylated and negatively regulated by JNK, leading to nuclear exclusion (*23*). This regulation appears to be specific to the NFAT4 isoform; evidence for JNK regulation of NFATc was not reported (*23*). An indirect mechanism may therefore account for the altered regulation of NFATc in *Jnk1*$^{-/-}$ T_H cells. NFATc and NFATp can bind to the IL-4 promoter NFAT sites (*22*). Both *Jnk1* and *NFATp* knockout mice have enhanced T cell proliferation and T_H2 cytokine production (*21, 24*), precisely the opposite of the NFATc knockout. It is therefore possible that these two NFAT factors antagonize each other in the regulation of the IL-4 gene. The apparent similarity between *NFATp*$^{-/-}$ and *Jnk1*$^{-/-}$ phenotypes supports a functional linkage between JNK1 and NFAT.

Our results further reveal a novel mechanism by which TCR signaling negatively regulates T_H2 cytokines through JNK1. Positive and negative regulation of JNK1 activity may affect the decision of T_H cells to differentiate into T_H1 or T_H2 effectors, and therefore may affect the type of immune response that is initiated. The function of JNK1 demonstrated in this study is distinct from that of JNK2, which is required for IFN-γ production in T_H1 cells (*14*). Moreover, the related p38 mitogen-activated protein kinase pathway is T_H1 specific and drives IFN-γ transcription (*25*). Together, these pathways potentiate the T_H1 response and provide a potential target for pharmaceutical intervention.

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9. The murine *Jnk1* locus was isolated from a 129/Sv mouse genomic library (Stratagene) using the human *Jnk1* cDNA as a probe. An internal 5.5-kb genomic fragment containing four exons was replaced by a PGK-hyg (hygromycin phosphotransferase) cassette. The knockout vector was electroporated into W9.5 ES cells, and 15 targeted clones were identified by Southern blot analysis of genomic DNA, three of which gave germ line transmission of the disrupted allele. Heterozygous (+/–) mice were intercrossed to generate homozygous wild-type and mutant mice, which were independently bred; their age- and sex-matched offspring were used for experiments.
10. C. Dong, D. D. Yang, M. Wysk, A. J. Whitmarsh, R. J. Davis, R. A. Flavell, unpublished data.
11. The data are shown at *Science* Online (www.sciencemag.org).
12. Total spleen cells or purified CD4 T cells were stimulated as triplicates with Con A (2.5 μg/ml), plate-bound anti-CD3 with or without anti-CD28 (plates were precoated with antibody at 10 μg/ml). IL-2 production was measured by enzyme-linked immunosorbent assay (ELISA; Pharmingen) 24 hours after stimulation. Proliferation was assayed after 3 days of treatment by adding [^{3}H]thymidine to the culture for the last 8 hours. At day 4, the supernatant of stimulated cells was removed and T_H cytokine production was measured by ELISA. In activation-induced cell death experiments, CD4 T cells were stimulated with Con A for 4 days, extensively washed, and restimulated with immobilized anti-CD3 for 48 hours. Apoptosis was determined by staining the cells with 7-aminoactinomycin D (7-AAD) and Annexin V (Pharmingen); dead cells were scored as Annexin$^+$ 7-AAD$^+$. The data are shown at *Science* Online (www.sciencemag.org).
13. CD4 T cells were isolated from 6- to 8-week-old mice by depletion of major histocompatibility class II$^+$, CD8$^+$, and NK1.1$^+$ cells using magnetic beads. The CD44^{lo}CD45RB^{hi} naïve cells were further purified by a FACS sorter (Becton Dickinson). APCs were prepared from spleen by complement-mediated lysis of Thy1$^+$ T cells.
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16. Naïve or total CD4 T cells (10^6 cells/ml) were differentiated in the presence of irradiated APCs (10^6 cells/ml), immobilized anti-CD3, and IL-2 (30 U/ml). IL-12 (3.5 ng/ml) and anti–IL-4 (4 μg/ml) were added for T_H1 differentiation, and IL-4 (1000 U/ml) and anti–IFN-γ (4 μg/ml) for T_H2 differentiation. After 4 days, the cells were extensively washed, counted, and restimulated with plate-bound anti-CD3 for 24 hours. Cytokine production was measured by ELISA (Pharmingen) in triplicates or by competitive reverse transcription polymerase chain reaction (RT-PCR) (*17*).
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19. Nuclear and cytosolic fractions of T_H cells were prepared [E. Schreiber, P. Matthias, M. M. Muller, W. Shaffner, *Nucleic Acids Res.* **17**, 6419 (1989)], and amounts of protein were determined by Bio-Rad protein assay to ensure equal protein loading for the analysis. Antibodies to GATA-3, Stat-6, JunB, and NFATp were from Santa Cruz Biotechnology and anti-NFATc was from Affinity Bioreagents.
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Eight Calves Cloned from Somatic Cells of a Single Adult

Yoko Kato, Tetsuya Tani, Yusuke Sotomaru, Kazuo Kurokawa, Jun-ya Kato, Hiroshi Doguchi, Hiroshi Yasue, Yukio Tsunoda*

Eight calves were derived from differentiated cells of a single adult cow, five from cumulus cells and three from oviductal cells out of 10 embryos transferred to surrogate cows (80 percent success). All calves were visibly normal, but four died at or soon after birth from environmental causes, and postmortem analysis revealed no abnormality. These results show that bovine cumulus and oviductal epithelial cells of the adult have the genetic content to direct the development of newborn calves.

Nuclear transfer is an efficient technique for assessing the developmental potential of a nucleus and for analyzing the interactions between the donor nucleus and the recipient cytoplasm. In amphibians, successful nuclear transfer was first reported by Briggs and King who used blastula cells for nuclear transfer to oocytes, which proceeded to develop into