JNK2 is required for efficient T-cell activation and apoptosis but not for normal lymphocyte development

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Background: The Jun N-terminal kinase (JNK) signaling pathway has been implicated in cell proliferation and apoptosis, but its function seems to depend on the cell type and inducing signal. In T cells, JNK has been implicated in both antigen-induced activation and apoptosis.

Results: We generated mice lacking the JNK2 isozymes. The mutant mice were healthy and fertile but defective in peripheral T-cell activation induced by antibody to the CD3 component of the T-cell receptor (TCR) complex — proliferation and production of interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) were reduced. The proliferation defect was restored by exogenous IL-2. B-cell activation was normal in the absence of JNK2. Activation-induced peripheral T-cell apoptosis was comparable between mutant and wild-type mice, but immature ($CD4^+CD8^+$) thymocytes lacking JNK2 were resistant to apoptosis induced by administration of anti-CD3 antibody *in vivo*. The lack of JNK2 also resulted in partial resistance of thymocytes to anti-CD3 antibody *in vitro*, but had little or no effect on apoptosis induced by anti-Fas antibody, dexamethasone or ultraviolet-C (UVC) radiation.

Conclusions: JNK2 is essential for efficient activation of peripheral T cells but not B cells. Peripheral T-cell activation is probably required indirectly for induction of thymocyte apoptosis resulting from administration of anti-CD3 antibody *in vivo*. JNK2 functions in a cell-type-specific and stimulus-dependent manner, being required for apoptosis of immature thymocytes induced by anti-CD3 antibody but not for apoptosis induced by anti-Fas antibody, UVC or dexamethasone. JNK2 is not required for activation-induced cell death of mature T cells.

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Background

Mitogenic, inflammatory and stress signals are transduced by evolutionarily conserved pathways that modulate expression of genes controlling cell proliferation, survival and death. These signals activate members of the mitogen activated protein (MAP) kinase family, which can translocate to the nucleus to phosphorylate various transcription factors [1,2]. Several MAP kinase subgroups differing in their response and substrate specificities, including the extracellular-signal-regulated kinases (ERKs), the Jun N-terminal kinases (JNKs) and the p38s, have been described. All MAP kinases are regulated upstream by MAP kinase kinases and MAP kinase kinase kinases. Unlike the ERK pathway, which is activated most effectively by growth factors [1], the JNK and p38 pathways [3] are stimulated primarily by proinflammatory and stress stimuli [4–7], although they also respond to mitogens [8,9]. Activated JNKs phosphorylate the transcription factors c-Jun and ATF2, which participate in activation and formation of the AP-1 complex [6,10–12]. The JNKs are activated through phosphorylation by specific MAP kinase kinases — JNKK1 (also known as SEK1 or MKK4) and JNKK2 (also known as SEK2 or MKK7) [10,13–15]. The JNK subgroup consists of three members — JNK1, JNK2 and JNK3 [5,7,16] — each with multiple isoforms generated through alternative splicing [17]. Combinatorial use of various JNKs, JNKKs and JNKK activating MAP kinase kinase kinases, such as MEKK1, MEKK2, MEKK3, ASK1, TAK1 and MLK [9,18–23], can lead to JNK activation in response to multiple stimuli and differential regulation of substrate proteins.

T-cell activation involves several steps including induction of immediate early genes, followed by induction of interleukin-2 (IL-2), whose secretion leads to autocrine and paracrine T-cell proliferation [24]. In T lymphocytes, the JNKs are synergistically activated by costimulation of the T-cell receptor (TCR) and the CD28 auxiliary receptor or by a combination of pharmacological agents, phorbol myristate acetate (PMA) and Ca^{2+} ionophore [25–27]. By contrast, no synergy is observed in ERK activation. The synergistic JNK activation response is unique to T and B cells [26–28]. Anergic T cells have been shown to exhibit a defective JNK activation response [29,30].

The importance of JNK activity for T-cell activation is suggested by its correlation with IL-2 induction [26,27] and the role of the AP-1 transcription factor, which is composed of Jun and Fos subunits. In non-stimulated T cells, the basal levels of AP-1 proteins are low but T-cell activation results in *jun* and *fos* gene induction [26,31]. This process partially depends on JNK activation [26] and, consistent with the defect in JNK activation, anergic T cells exhibit a deficient AP-1 response [32]. In addition to pre-existing factors, such as c-Jun, ATF2 and Elk-1, involved in c-*jun* and c-*fos* induction, the JNKs phosphorylate newly synthesized c-Jun or JunD and enhance their ability to activate transcription [33]. Full IL-2 induction also requires stabilization of its mRNA [34], a process in which the JNKs are also involved [35].

The role of AP-1 and the JNK pathway in T lymphocyte development and activation has been under intense investigation [35–39]. Chimeras generated with c-*Jun*$^{-/-}$ embryonic stem (ES) cells exhibit poor restoration of thymocyte number, but contain peripheral B and T cells that can be activated efficiently [36]. Chimeras generated using SEK1$^{-/-}$ ES cells give different results [37–39]. Nishina *et al.* [37,38] found poor thymocyte restoration, whereas Swat *et al.* [39] found normal numbers of all thymocyte subpopulations. Swat *et al.* [39] also found normal activation of T cells lacking SEK1, which contrasts with the findings of Nishina *et al.* [37,38]. As SEK1-deficient embryos die at midgestation, the developmental function of this JNK-activating kinase [13] is irreplaceable during embryogenesis, whereas it seems to be dispensable for T-cell differentiation and activation [39,40].

To examine the role of the JNK pathway in T-cell differentiation and activation, we focused on the functions of individual JNKs. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 expression is restricted to the brain [41]. The predominant isoform of JNK2 exhibits higher affinity to c-Jun than the predominant JNK1 isoform [7]. We generated JNK2-null mice, which were viable and fertile and had no overt developmental abnormalities. Although the differentiation of T and B lymphocytes was not altered, T-cell activation was impaired. Activated peripheral T cells produced less IL-2, IL-4 and interferon-γ (IFN-γ), as a consequence of reduced JNK activity. Moreover, activation-induced thymocyte death was affected, as double-positive (DP) CD4^{+}CD8^{+} thymocytes lacking JNK2 were relatively resistant to apoptosis induced by injecting antibodies to the CD3 component of the TCR complex. In contrast, B cells were efficiently activated in the absence of JNK2.

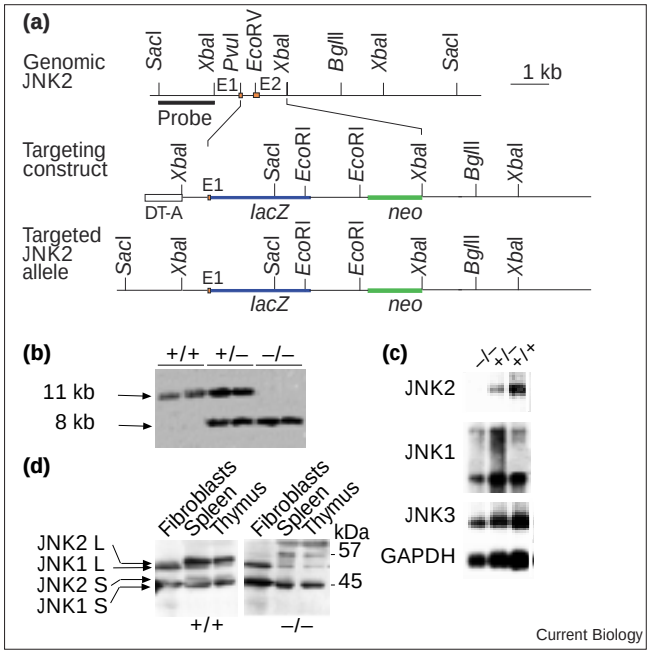
Results

Generation of mice with a disrupted JNK2 gene

The JNK2 gene was inactivated by homologous recombination using a targeting construct that lacked parts of the kinase domain (Figure 1a). Heterozygous mice appeared phenotypically normal and were intercrossed to produce mice homozygous for the mutation (Figure 1b). Homozygous mutants were viable, fertile and exhibited no macroscopic abnormalities at birth. The expected 4.5 kb JNK2 transcript was present in wild-type but not in JNK2$^{-/-}$ mice (Figure 1c). No truncated JNK2 mRNA could be detected in any tissue from JNK2$^{-/-}$ mice and there was no compensatory increase in the levels of either JNK1 or JNK3 mRNA (Figure 1c).

JNK isoforms migrating at 46 and 55 kDa are generated by alternative splicing of each primary JNK transcript [17]. Wild-type splenocytes and thymocytes express high levels of the 55 kDa JNK2 isoform but extremely low levels of the 46 kDa isoform (Figure 1d). Primary embryonic fibroblasts express lower levels of the 55 kDa JNK2 isoform and barely detectable levels of the 46 kDa isoform (Figure 1d). Cells derived from JNK2$^{-/-}$ mice, however, did not express either of the JNK2 isoforms (Figure 1d). The major JNK1 isoform is the 46 kDa one although fibroblasts express more of the 55 kDa isoform than do

Figure 1



Targeted mutation of the murine JNK2 gene in ES cells. (a) Structure of the genomic DNA encoding a portion of the protein kinase domain of JNK2 (top). The targeting vector (middle) replaced parts of exons 1 and 2 (E1, E2) with a β-galactosidase (*lacZ*) gene fused in frame with E1 and a *neo* cassette driven by the Rous sarcoma virus (RSV) promoter. DT-A, diptheria toxin A gene. The structure of the targeted allele following homologous recombination is depicted below. (b) Genotype analysis by Southern blotting of DNA isolated from offspring of heterozygous intercrosses. The probe indicated in (a) was used. (c) JNK1, JNK2, JNK3 and GAPDH mRNA expression. (d) Western blot analysis of JNK protein expression in primary embryonic fibroblasts, splenocytes and thymocytes from wild-type and homozygous mutant cells. The positions of the long (L) and short (S) isoforms of the JNK proteins are indicated.

splenocytes and thymocytes. Both JNK1 isoforms were present at similar levels in wild-type and JNK2$^{-/-}$ cells (Figure 1d). No truncated JNK2 polypeptide reactive with our antiserum was detected. These results indicate that the targeted mutation of the JNK2 gene results in complete absence of a functional gene product.

JNK2 is not required for development of primary B or T lymphocytes

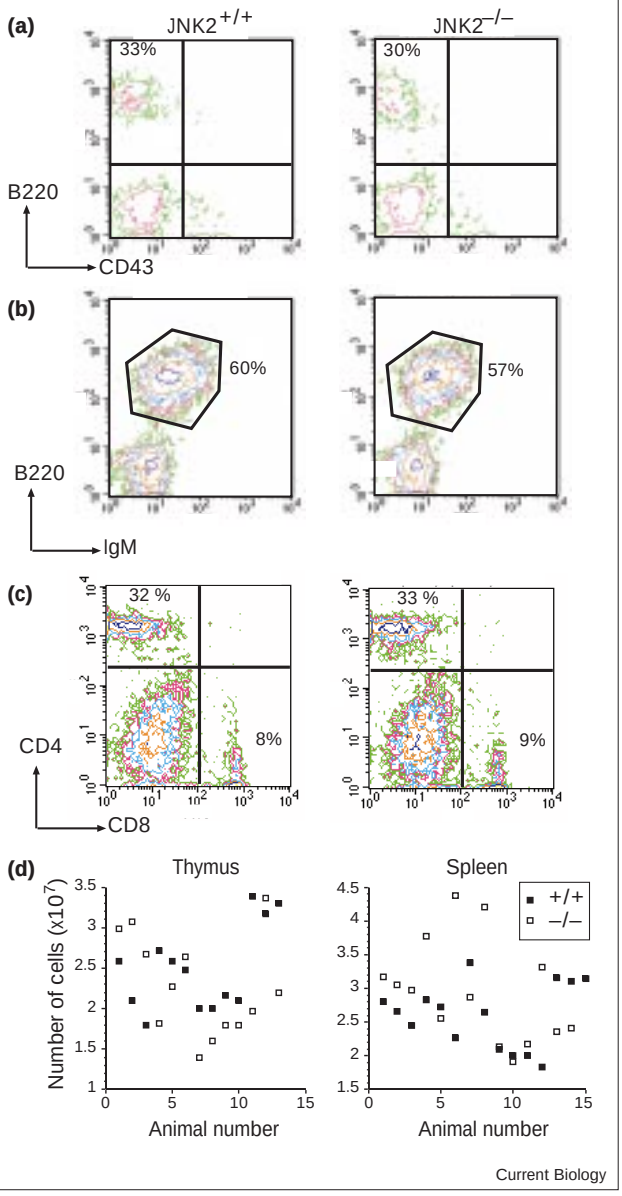
JNK signaling has been implicated in development of both B and T lymphocytes [37,38]. Development of B and T cells was not impaired in mice lacking JNK2. The frequencies of B220^{+}CD43^{-} and B220^{+}IgM^{+} mature B cells in bone marrow and spleen of JNK2$^{-/-}$ mice were indistinguishable from those of wild-type mice (Figure 2a,b). Using CD4 and CD8 as markers for differentiation stages of thymocytes, we found that JNK2$^{-/-}$ mice had normal numbers and proportions of CD4^{-}CD8^{-}, CD4^{+}CD8^{+}, CD4^{+}CD8^{-} and CD4^{-}CD8^{+} thymocytes (Figures 2d,3). Finally, JNK2$^{-/-}$ splenic T-cell populations were phenotypically similar to those of wild-type mice, as assessed by staining with antibodies to CD4, CD8, CD3, heat-stable antigen (HSA), TCRαβ, TCRγδ, Thy 1.2, CD44 and CD25 (Figure 2c; data not shown). The number of spleen cells in JNK2$^{-/-}$ mice was normal (Figure 2d). Therefore JNK2 is not required for normal development of B or T lymphocytes.

JNK2$^{-/-}$ thymocytes are resistant to cell death induced by anti-CD3 antibody *in vivo*

To determine whether JNK2 is essential for induction of cell death in thymocytes, we injected the potent pan-T-cell activator, anti-CD3 antibody, intraperitoneally into wild-type and JNK2$^{-/-}$ mice and analysed the mice for depletion of DP CD4^{+}CD8^{+} thymocytes. Wild-type mice injected with anti-CD3 antibody exhibited nearly complete depletion of the DP cell compartment 48 hours post-injection (Figure 3a), and the thymi were dramatically reduced in size (0.1–0.05 × that of untreated mice; data not shown). In contrast, thymi of JNK2$^{-/-}$ mice injected with anti-CD3 antibody were about 0.4 × the size of thymi from untreated mutant mice 48 hours post-injection (data not shown) and the DP compartment was only partially depleted (Figure 3a). Time-course analysis of apoptosis revealed that, while wild-type thymocytes began to die by 24 hours post-injection (only 52% of all cells were DP thymocytes), the JNK2$^{-/-}$ DP thymocyte population was unaltered (85% of total) at this point (Figure 3a). Thus, the absence of JNK2 confers resistance to DP thymocytes against cell death induced by administration of antibody to the CD3 component of the TCR *in vivo*.

Induction of JNK activity, that is, JNK-mediated c-Jun phosphorylation, was examined next (Figure 3b). JNK activity in wild-type thymic extracts was detected 24 hours after anti-CD3 antibody injection, that is, at the time point when cell death was first observed. Hence, JNK activation coincides with thymocyte cell death induced by anti-CD3 antibody. As expected, injection of anti-CD3 antibody did not increase JNK activity in JNK2$^{-/-}$ thymic extracts (Figure 3b).

Figure 2

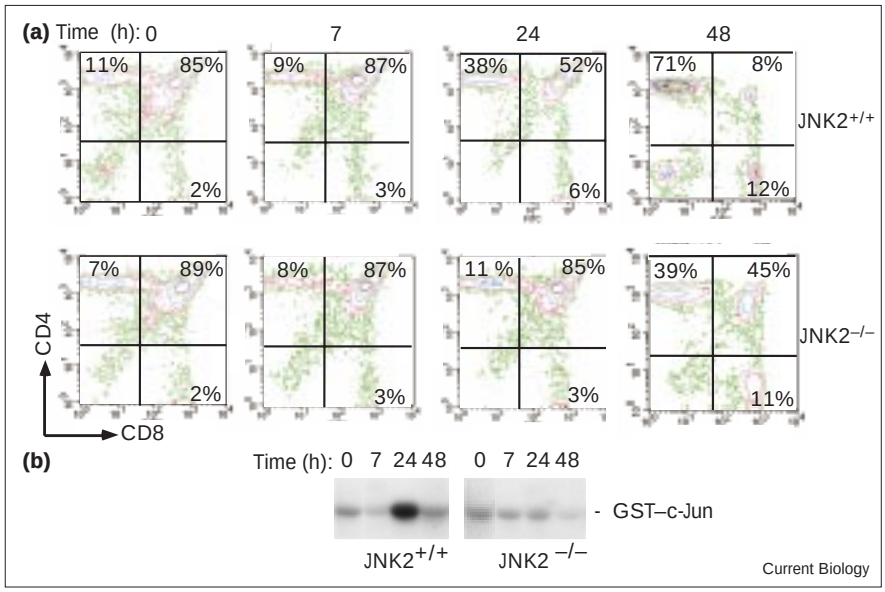


JNK2 is not required for normal development of B and T lymphocytes. Flow cytometric analysis of cells from (a) bone marrow and (b,c) spleen of wild-type and JNK2$^{-/-}$ 12-week-old mice. The results shown are representative of six mice per group. (d) The numbers of thymic and splenic cells from 12-week-old mice are shown, each point corresponding to an individual mouse.

Susceptibility of JNK2$^{-/-}$ thymocytes to cell death induced by anti-CD3 antibody and other apoptotic stimuli *in vitro*

To determine whether the resistance of JNK2$^{-/-}$ DP thymocytes to apoptosis induced by injection of anti-CD3

Figure 3



Lack of JNK2 results in increased resistance of immature thymocytes to cell death induced by anti-CD3 antibody. **(a)** Wild-type and JNK2$^{-/-}$ mice were injected with phosphate-buffered saline (PBS) or anti-CD3 antibody, and thymi were removed for flow cytometric analysis after the indicated time periods. The results are representative of nine wild-type and eight JNK2$^{-/-}$ mice per group. **(b)** JNK activity in thymocytes from mice injected with anti-CD3 antibody was assayed using a glutathione-S-transferase fusion protein coupled to amino acids 1–79 of c-Jun (GST–c-Jun) as a substrate.

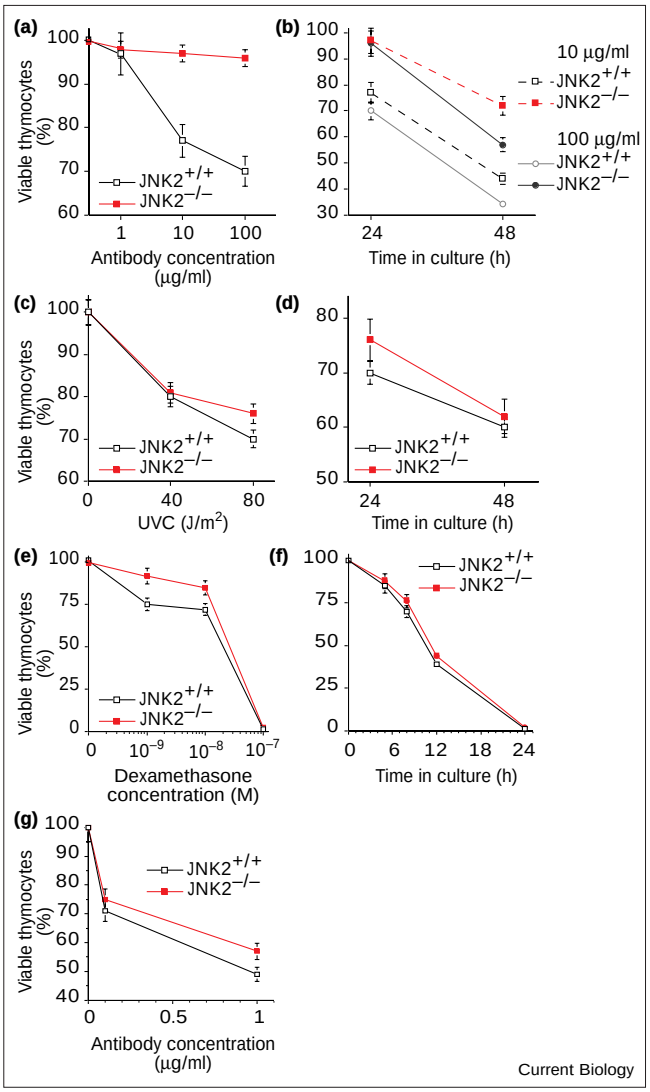
antibody is a cell-autonomous defect, we compared the susceptibility of JNK2$^{-/-}$ and wild-type thymocytes to cell death induced by direct incubation with anti-CD3 antibody in the presence of an antibody that activates the CD28 auxiliary receptor. Although JNK2$^{-/-}$ thymocytes directly incubated with a range of anti-CD3 antibody concentrations were markedly resistant to apoptosis (Figure 4a,b), the difference in survival of cells of the two genotypes was not as dramatic as after administration of anti-CD3 antibody *in vivo* (Figure 3). JNK2$^{-/-}$ thymocytes were, however, consistently more resistant than their wild-type counterparts to incubation with a low dose (10 μg/ml) of anti-CD3 antibody (97% versus 77%, and 72% versus 44% viability at 24 and 48 hours, respectively; Figure 4a,b). At a higher dose of anti-CD3 antibody (100 μg/ml), 96% of JNK2$^{-/-}$ thymocytes were viable at 24 hours compared with 70% of wild-type thymocytes (Figure 4a,b). After 48 hours, JNK2$^{-/-}$ thymocytes were still more resistant than wild-type thymocytes (Figure 4b). Thus, JNK2 appears to be necessary for apoptotic responses induced by anti-CD3 antibody *in vivo* and *in vitro*.

We also induced thymocyte death by incubation with either an agonistic anti-Fas antibody or the synthetic glucocorticoid dexamethasone, or by exposure to ultraviolet-C (UVC) radiation. In these cases, JNK2$^{-/-}$ thymocytes were not significantly more resistant to apoptosis than their wild-type counterparts (Figure 4c–g). No difference in Fas or CD3 expression was detected between JNK2$^{-/-}$ and wild-type thymocytes (data not shown). Thus, the absence of JNK2 did not confer marked resistance to apoptosis mediated by anti-Fas antibody, dexamethasone or UVC radiation, suggesting that JNK2 is specifically involved in thymocyte apoptosis induced by anti-CD3 antibody. In addition, the marked resistance of JNK2$^{-/-}$ thymocytes to cell death induced by *in vivo* delivery of anti-CD3 antibody might not be fully cell autonomous and could well depend on factors produced outside the DP thymocyte compartment.

Activation and proliferation, but not cell death, is impaired in JNK2$^{-/-}$ peripheral T lymphocytes

As production of cytokines by peripheral T cells that subsequently induce expression of cell-death mediators has been suggested as a mechanism for DP cell killing induced by anti-CD3 antibody *in vivo* [42], we examined the *in vitro* activation of JNK2$^{-/-}$ peripheral T cells by antibodies to CD3 and CD28. Purified splenic T cells were cultured on plates coated with anti-CD3 antibody, in the absence or presence of anti-CD28 antibody. Although both wild-type and JNK2$^{-/-}$ T cells proliferated in response to this treatment, T cells lacking JNK2 consistently exhibited markedly reduced proliferation rates, especially at lower/limiting concentrations of activating stimuli (Figure 5a–c). Wild-type T cells proliferated vigorously in response to plate-bound anti-CD3 antibody (10 μg/ml), whereas JNK2$^{-/-}$ T cells showed significantly reduced proliferation rates (Figure 5a). When T-cell proliferation was analyzed at lower concentrations of anti-CD3 antibody (1 μg/ml) in the presence of various anti-CD28 antibody concentrations, wild-type T cells exhibited enhanced proliferation rates in a dose-dependent manner, in contrast to JNK2$^{-/-}$ T cells which failed to respond to the costimulatory antibody (Figure 5b). The difference between T cells of the two genotypes became less significant at high doses of anti-CD3 and anti-CD28 antibodies,

Figure 4



Susceptibility of JNK2-deficient thymocytes to cell death caused by direct incubation with anti-CD3 antibody, and other apoptotic stimuli *in vitro*. (a,b) Thymocytes from wild-type (open symbols) and JNK2$^{-/-}$ (filled symbols) mice were cultured on 24-well plates coated with anti-CD3ε antibody for (a) 24 h or (b) the indicated periods in the presence of soluble anti-CD28 antibody, and their viability determined. (c,d) Cell death was induced by 40 and 80 J/m^2 of UVC radiation, and cell viability was determined after (c) 24 h or (d) 24 h and 48 h post 80 J/m^2 UVC irradiation. (e–g) Thymocyte viability was determined (e) after treatment with various concentrations of dexamethasone for 24 h, or (f) with 10^{-7} M dexamethasone for various time periods, or (g) after 24 h incubation with various concentrations of anti-Fas antibody. The results are representative of three independent experiments, each using three pairs of mice.

however (Figure 5c). These data indicate that the proliferation defect depends on the level of costimulation.

Analysis of cytokine production after 36 hours in culture revealed that wild-type T cells produced significant amounts of IL-2, IL-4 and IFN-γ, whereas JNK2$^{-/-}$ T cells produced barely detectable levels of these cytokines (Figure 5d–l). As the cytokines produced by activated T cells are used in a paracrine/autocrine manner for further proliferation, we also analyzed cytokine production after 24 hours. At this point, JNK2$^{-/-}$ T cells produced significant amounts of IL-2, which nevertheless were lower than those produced by wild-type cells, especially at low levels of costimulation (Figure 5m). The impaired production of IL-2, IL-4 and IFN-γ by JNK2$^{-/-}$ T cells correlates with the inefficient activation and proliferation of these cells and most likely is the cause for the proliferation defect, as treatment with anti-CD3 antibody resulted in equal levels of IL-2 receptor α chain expression in wild-type and JNK2$^{-/-}$ T cells (data not shown). Indeed, when purified T cells from wild-type and JNK2$^{-/-}$ mice were stimulated with low doses of anti-CD3 and anti-CD28 antibodies in the presence of exogenous IL-2, no differences in their proliferation capacity were detectable (Figure 5n).

Inefficient proliferation of JNK2$^{-/-}$ mature T cells was a direct consequence of impaired activation as we have found that JNK2$^{-/-}$ peripheral T cells are not more susceptible to apoptosis induced by anti-CD3 antibody (Figure 5o; data not shown). Collectively, these findings indicate that the lack of JNK2 results in inefficient activation of peripheral T cells in response to anti-CD3-antibody injection, which probably results in insufficient signals being relayed to the thymus to induce the death of DP cells.

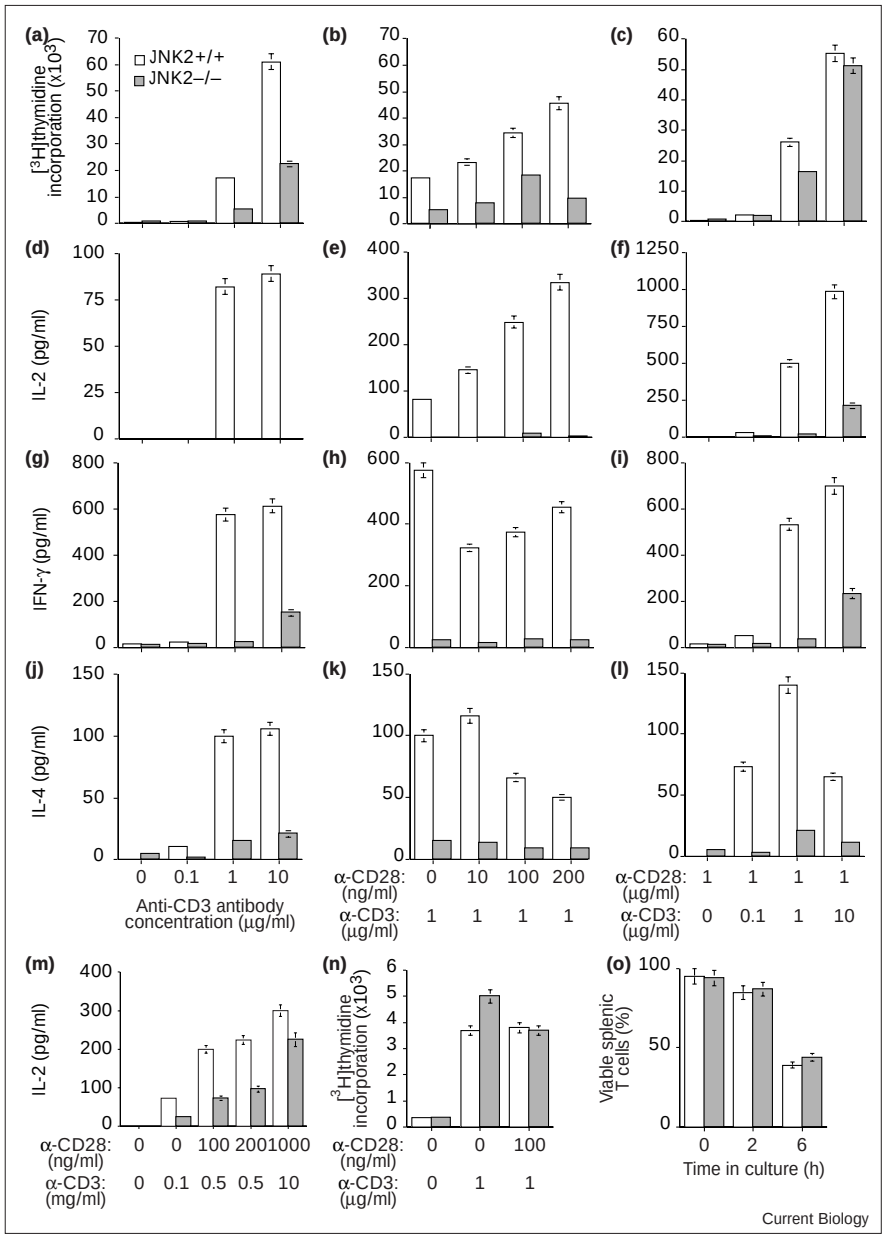
We examined whether DNA-binding activity of the transcription factor AP-1 is affected in JNK2-deficient T lymphocytes. Nuclear extracts from wild-type and JNK2$^{-/-}$ peripheral T cells activated with anti-CD3 antibody exhibited similar levels of AP-1-binding activity (Figure 6a). Furthermore, addition of a pan-Jun antibody resulted in further retardation of the complex. Moreover, c-Jun, JunB and JunD were all found at similar levels in AP-1 complexes from nuclear extracts of both wild-type and JNK2$^{-/-}$ T cells (data not shown).

We next investigated whether the total amount of JNK activity is reduced in JNK2$^{-/-}$ T cells. Splenic T cells were stimulated for various times with either anti-CD3 antibody alone or in combination with anti-CD28 antibody or with PMA and ionomycin. Induction of JNK activity was observed in both wild-type and JNK2$^{-/-}$ T cells, although the activity was lower in the latter (Figure 6b). Western blotting indicated that JNK1 expression was not upregulated in JNK2$^{-/-}$ T cells (Figure 6b).

Lack of JNK2 does not affect B-cell activation

CD40 signaling in B cells results in JNK activation [43]. However, JNK2$^{-/-}$ B cells proliferated normally in

Figure 5



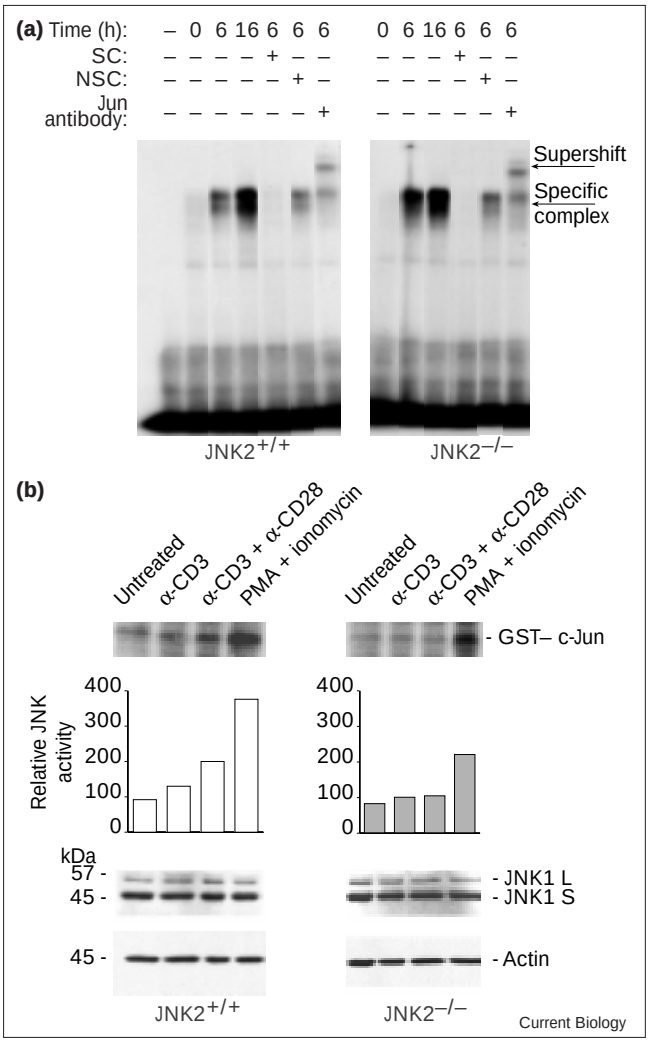
IL-2, IL-4 and IFN-γ production by T cells lacking JNK2 and inefficient proliferation after stimulation with antibodies to CD3 and CD28. (a–c) Purified T cells from spleens of 8–10-week-old mice were cultured (a) in the presence of the indicated concentrations of plate-bound anti-CD3ε antibody (α-CD3), or (b) 1 μg/ml plate-bound anti-CD3ε antibody and the indicated concentrations of soluble anti-CD28 antibody (α-CD28), or (c) 1 μg/ml soluble anti-CD28 antibody and the indicated concentrations of plate-bound anti-CD3ε antibody. Proliferation rates were determined at 72 h. (d–l) IL-2 (d–f), IFN-γ (g–i) or IL-4 (j–l) production was measured by enzyme-linked immunosorbent assay (ELISA) after 36 h stimulation of purified T cells as described above. (m) IL-2 production was measured after 24 h of stimulation. (n) Proliferation rates of purified T cells cultured in the presence of 50 U/ml IL-2 plus anti-CD3 and/or anti-CD28 antibodies. (o) Cell death of activated T cells induced by anti-CD3 antibody. Activated enriched splenic T cells were cultured on plates coated with anti-CD3ε antibody and the rates of cell death were measured as described above. The results are representative of three independent experiments, each using three matched pairs of mice.

response to lipopolysaccharide (LPS), IL-4, anti-CD40 antibody or IL-4 plus anti-CD40 antibody (Figure 7a). Moreover, JNK2$^{-/-}$ B cells upregulated CD23 upon CD40 stimulation (data not shown). JNK activation is also implicated in B-cell differentiation within germinal centers [44]. We thus examined germinal center formation following immunization with dinitrophenyl ovalbumin (DNP–OA) in JNK2$^{-/-}$ mice and found normal formation of germinal centers (Figure 7b). Germinal center B cells of both genotypes were positive for peanut agglutinin (PNA) expression (Figure 7b). Thus, JNK2 deficiency does not affect gross B-cell proliferation and differentiation.

Discussion

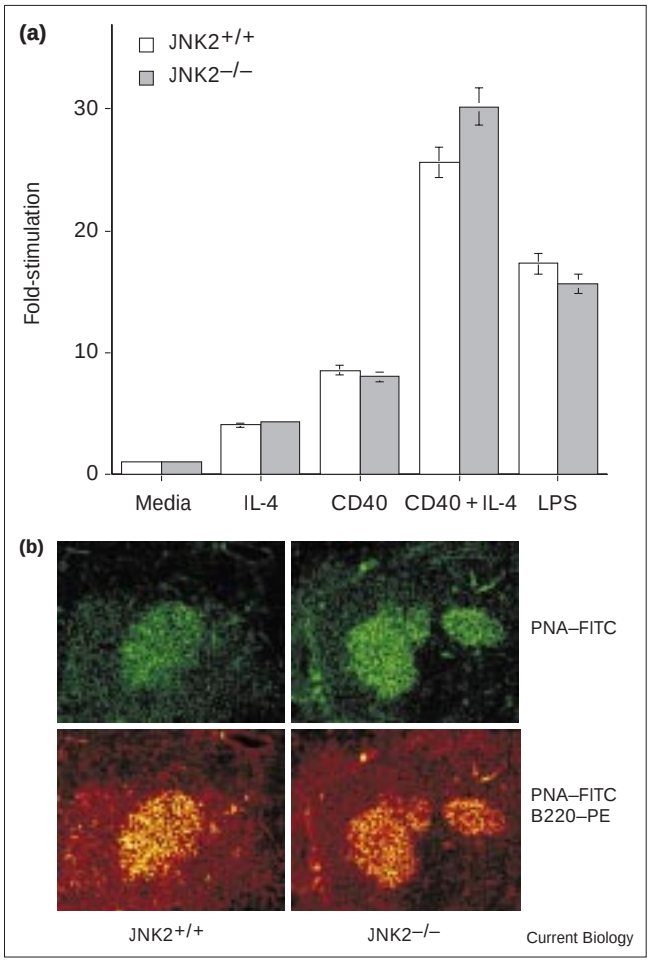
We generated JNK2$^{-/-}$ mice and found that JNK2 is not essential for embryonic development. Mice lacking JNK1 or JNK3 also complete embryogenesis (K.S., M.K. and E.F.W., unpublished observations; [41]), suggesting that the loss of any individual JNK can be compensated for during embryogenesis. Nevertheless, the results described here indicate that JNK2 is required for efficient T-cell but not B-cell activation, thus defining at least one cell-type-specific function for JNK2. Moreover, within the peripheral T-cell compartment, lack of JNK2 resulted in impaired T-cell activation but did not alter sensitivity to apoptotic

Figure 6



AP-1-binding activity and JNK activity in JNK2-deficient T cells. (a) AP-1-binding activity was measured using nuclear extracts from enriched splenic T cells that were untreated (–), or stimulated with anti-CD3 antibody for the indicated times. The specific protein–DNA complex is indicated. SC, 100× specific competitor; NSC, 100× non-specific competitor. (b) JNK activity in enriched splenic T cells that were untreated, or treated with anti-CD3ε antibody (α-CD3, 10 μg/ml), anti-CD3ε (1 μg/ml) plus anti-CD28 (1 μg/ml) antibodies (α-CD3 + α-CD28), or PMA (50 μg/ml) plus ionomycin (1 μg/ml). T cells were stimulated for 10 min and whole cell extracts were prepared and assayed for JNK activity. The same extracts were analyzed for their content of JNK1 and actin by western blotting with specific antibodies.

Figure 7



B-cell activation is not impaired in the absence of JNK2. (a) Proliferation rates of splenic B cells in the presence of various activating stimuli. (b) Germinal center formation. Histological sections taken from spleens of wild-type and JNK2$^{-/-}$ mice after immunization with DNP–OA were stained with fluorescein-isothiocyanate-labeled peanut agglutinin (PNA–FITC) and phycoerythrin-conjugated anti-B220 antibody (B220–PE). B cells localize to areas of PNA–FITC staining (yellow).

stimuli. In immature DP thymocytes, however, JNK2 is required for maximal sensitivity to certain death-inducing stimuli, such as anti-CD3 antibody, but not others, such as anti-Fas antibody, UVC radiation and glucocorticoids.

The most dramatic difference between wild-type and JNK2$^{-/-}$ mice was revealed after *in vivo* injection of the pan-T-cell activator, anti-CD3 antibody — JNK2$^{-/-}$ DP thymocytes were significantly resistant to induction of cell death compared with their wild-type counterparts. JNK2$^{-/-}$ thymocytes, however, were only partially resistant to death caused by direct incubation with anti-CD3 antibody. By contrast, in peripheral T cells, the loss of JNK2 had no effect on activation-induced cell death and, instead, resulted in impaired activation. It should be emphasized, however, that this impairment is not absolute because JNK2$^{-/-}$ T cells proliferated after extensive TCR stimulation. Thus, JNK2 is required for T-cell activation only at limiting levels of costimulation. While this manuscript was under review, Yang *et al.* [45] reported that they found only a marginal difference in proliferation rates and IL-2 production between wild-type and JNK2$^{-/-}$ total spleen cells (which contain accessory cells) activated with high concentrations of the pharmacological agents concanavalin A and

PMA/ionomycin. It appears that the differences between wild-type and JNK2$^{-/-}$ T cells become less significant with increased intensity of the activating stimulus. Nevertheless, our results indicate that the defect in proliferation of JNK2$^{-/-}$ T cells is due to their inability to produce sufficient IL-2 in response to low-level stimulation via the TCR and the CD28 auxiliary receptor. As both JNK1 and JNK2 are regulated in the same manner [26], it is likely that, in the absence of JNK2, there is a partial compensation by the different JNK1 isoforms. In support of this explanation, activated JNK2$^{-/-}$ T cells exhibit a partial reduction but not a complete loss of total JNK activity. Nevertheless, these results indicate that JNK2 plays a major role in cytokine induction in response to T-cell activation.

IL-2 production, the hallmark of T-cell activation, is under control of the JNK signaling pathway [26,27,38]. The results here confirm the importance of JNK2 as an essential component of the signaling pathway leading to efficient IL-2 induction in normal T cells. Recently, Yang *et al*. [45] reported that *in vitro* differentiation of purified CD4^{+} cells into TH1 effector cells is defective in the absence of JNK2. TH1 differentiation is associated with production of both IL-2 and IFN-γ [46]. Although our observation that there is a reduction in IFN-γ production by JNK2$^{-/-}$ T cells is consistent with that of Yang *et al*. [45], it should be noted that JNK2$^{-/-}$ T cells also produce lower levels of IL-4, a hallmark of TH2 differentiation [46]. Thus, it is unlikely that JNK2 (or JNK1 for that matter) is specifically involved in production of effector T cells rather than being generally required for efficient activation of all T-cell types. Most likely, JNK activation is required for optimal production of many cytokines, whose genes are responsive to AP-1.

How does inefficient peripheral T-cell activation result in increased resistance of DP thymocytes to administration of anti-CD3 antibody *in vivo*? It was recently shown that activation of the peripheral T-cell compartment causes selective depletion of DP thymocytes [42]. Presumably, activation of T cells results in direct or indirect (for example, via macrophages) production of cytokines that kill DP thymocytes. As JNK2$^{-/-}$ peripheral T cells produce less IL-2, IL-4 and IFN-γ, it is likely that this activation defect is largely responsible for the marked decrease in killing of DP thymocytes following administration of anti-CD3 antibody *in vivo*. Interestingly, thymocytes from mice that are deficient in the α and β subunits of the IL-2 receptor or IL-2 exhibit decreased sensitivity to cell death induced by incubation with anti-CD3 antibody *in vitro*. [47–49]. As JNK2$^{-/-}$ thymocytes are also partially resistant to activation-induced cell death caused by direct incubation with anti-CD3 antibody, it is possible that their marked resistance to administration of anti-CD3 antibody *in vivo* is a combination of this cell-autonomous defect and the decreased production of cell-death-inducing cytokines by JNK2$^{-/-}$ cells in the periphery.

The involvement of the JNK signaling pathway in apoptosis seems to depend on the cell type and the death-inducing stimulus. In DP thymocytes, the loss of JNK2 does not significantly alter their ability to undergo apoptosis induced by anti-Fas antibody, UVC and glucocorticoid *in vitro*, although it confers resistance to apoptosis caused by direct treatment with anti-CD3 antibody. The latter effect, however, is unique to thymocytes, as peripheral JNK2$^{-/-}$ T cells are not more resistant to cell death induced by anti-CD3 antibody than their wild-type counterparts. Also, wild-type and JNK2$^{-/-}$ B cells are equally sensitive to apoptosis caused by IgM crosslinking (data not shown). Thus, if JNK is involved in apoptosis of mature (B and T) lymphocytes, the loss of JNK2 must have been efficiently compensated for by JNK1.

The importance of the JNKK (SEK)–JNK–c-Jun/ATF2 pathway in lymphocyte development and activation is becoming increasingly clear with the generation of individual knockout mice. Studies with mutant SEK1 (JNKK1) lymphocytes indicate that this JNK-activating kinase is not required for lymphocyte development but is essential for maintenance of a normal peripheral compartment [39]. Our data further suggest that JNK2 is not required for lymphocyte development but is essential for efficient T-lymphocyte activation. Moreover, although T lymphocytes lacking c-Jun do not show an activation defect [36], JNK2 is required for efficient T-cell activation. The precise definition of the JNK substrates that are involved in T-lymphocyte development and activation should emerge with the continuing analysis of individual and compound knockout mouse strains.

Conclusions

The results presented here provide strong genetic evidence that the MAP kinase JNK2 is required in a cell-type-specific and signal-specific manner for activation of mature T cells and apoptosis of immature DP thymocytes. The loss of JNK2 does not affect the apoptotic response of mature T cells to anti-CD3 antibody, but renders immature DP thymocytes resistant to this cell-death-inducing stimulus. The resistance of DP thymocytes lacking JNK2 to anti-CD3 antibody treatment is largely dependent on peripheral T-cell activation, however. Moreover, the requirement for JNK2 of immature DP thymocytes to undergo apoptosis is stimulus-dependent; JNK2 is required for the apoptotic response to anti-CD3 antibody, but not to Fas ligation, UVC radiation or glucocorticoid treatment.

Materials and methods

Generation of JNK2$^{-/-}$ mice by gene targeting

The JNK2 gene-targeting construct was made by subcloning a *Pvu*I–*Xba*I fragment containing sequences encoding the first 32 amino acids of exon 1 in frame with the *lacZ* gene in the pGNA vector. The diptheria toxin A gene was used as a negative selection marker together with the MCI promoter in pGNA containing the JNK2 sequences. D3 and R1 ES cells were electroporated with *Pme*I-linearized JNK2

targeting construct and screened for homologous recombination events. *Sac*I-digested ES cell DNA was probed with a 2 kb *Xba*I–*Sac*I fragment (Figure 1a), which detects an 11 kb fragment from the wild-type allele and an 8 kb fragment from the targeted allele. Two independent targeted ES cell clones were injected into blastocysts of C57BL/6 mice. Heterozygous mice were interbred to obtain JNK2$^{-/-}$ mice, which were genotyped by PCR using the following primers: 5′-TCCAGTACAGCGCGGCTGAA-3′ and 5′-GCAGCAGCCCTCAGGATCCT-3′, which detect the mutant allele, and 5′-TCTGACGTCCTGGGCTGGAC-3′ and 5′-GCAGCAGCCCTCAGGATCCT-3′, which detect the wild-type allele. Northern analysis was performed according to standard protocols using polyA$^+$ RNA isolated from brain of wild-type, JNK2$^{+/-}$ and JNK2$^{-/-}$ mice, and hybridized with cDNA fragments specific for mouse JNK1, JNK2, JNK3 and GAPDH.

Western blot analysis and JNK assay

Western blots [7] using 100 μg whole cell extracts from primary embryonic fibroblasts, thymocytes and splenic T cells were probed with anti-JNK1(333.8) or anti-JNK(666.8) antibodies (Pharmingen). Kinase assays were performed using 60 μg whole cell extracts and GST–c-Jun(1–79) as a substrate [7], and quantified by Phosphorimager analysis (Molecular Dynamics). Relative JNK activity is represented by pixel values obtained by quantification of the intensity of the signal. Parallel samples were analyzed for protein expression by immunoblotting with anti-JNK1 and anti-actin antibodies (Sigma).

Flow cytometric analysis

Single-cell suspensions of thymus, spleen and bone marrow were prepared, stained by standard procedures and analyzed on a FACScan flow cytometer (Becton Dickinson) using CellQuest software.

Proliferation assays and ELISA

T cells from spleens of wild-type, JNK2$^{+/-}$ and JNK2$^{-/-}$ mice were purified by isolating the CD4$^+$ and CD8$^+$ populations by FACS. Purified cells were plated in 96-well plates precoated with anti-CD3ε antibody for 2 h at 37°C in the absence or presence of soluble anti-CD28 antibody. After 60 h, cultures were pulsed for 10–12 h with 1 μCi [^{3}H]thymidine per well and cells were subsequently harvested and analyzed using standard procedures. T lymphocytes stimulated with anti-CD3ε antibody for 24 and 36 h were harvested, and the supernatants used for measurement of IL-2 , IFN-γ and IL-4 levels by ELISA (R&D Systems); 50 U/ml IL-2 was added to cells for proliferation assays performed in the presence of exogenous IL-2. JNK2$^{+/-}$ cells gave similar results to wild-type cells and there was no effect of gene dosage resulting from less JNK2 mRNA transcript. B-cell proliferation was analyzed by stimulation of total spleen cells with IL-4 (50 U/ml), soluble anti-CD40 antibody (1 μg/ml), IL-4 plus anti-CD40 antibody or LPS (10 μg/ml). The magnitude of stimulated [^{3}H]thymidine or bromodeoxyuridine (BrdU) incorporation over background values are indicated as fold-stimulation. All experiments were performed in triplicate.

In vivo *thymocyte death assay*

Wild-type and JNK2$^{-/-}$ mice (12 weeks old) were injected with either PBS or 50 μg purified anti-CD3ε antibody intraperitoneally. After various time periods, mice were sacrificed and thymocytes subjected to flow cytometric analysis after staining with appropriate antibodies.

In vitro *apoptosis assays*

Freshly isolated thymocytes were plated in each well of a 24-well dish (5×10^5 cells/ml). For cell death induced by anti-CD3ε antibody, wells were pre-coated with various concentrations of the antibody for 1 h at 37°C and thymocytes were subsequently cultured in the presence of 1 μg/ml soluble anti-CD28 antibody for the indicated periods. Apoptosis was also induced by addition of various concentrations of anti-Fas antibody (clone Jo-2, Pharmingen), dexamethasone (Fluka) or by UVC irradiation using a Stratagene crosslinker. After incubation for the indicated periods at 37°C, the cells were harvested and stained with FITC-conjugated Annexin-V (Pharmingen) and propidium iodide and analyzed with a FACScan cytometer as described above. Thymocytes cultured in medium alone served as controls to measure spontaneous rate of cell death. Viability of thymocytes is expressed as percentage of viable thymocytes treated with apoptotic stimuli over viability of untreated thymocytes. Enriched splenic T cells were treated with 3 μg/ml concanavalin-A for 48 h, washed in 10 mg/ml α-methylmannosidase and cultured for additional 48 h in the presence of 50 U/ml IL-2. The cells were then washed and plated (1×10^6 cells/well) on 24-well plates coated with anti-CD3ε antibody (20 μg/ml), and cell death was measured at the indicated times. Individual treatments were done in triplicate, and at least three experiments were performed, each using three matched pairs of wild-type and JNK2$^{-/-}$ mice.

Nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared from 7×10^6 enriched splenic T cells as described [38]. The double-stranded oligonucleotide used in the mobility shift assay was from the human osteocalcin AP-1 site (5′-GGGTGACTCACCGGGTGAA-3′). For supershift experiments, 2 μg anti-Jun antibody (Santa Cruz, SC-44) was added to the reactions.

Germinal center formation and immunohistochemistry

Mice were injected intraperitonially with 100 μg DNP–OA in complete Freund's adjuvent on day 0 and boosted on day 21; 10 days after secondary immunization, spleens were snap frozen and processed for cryosections. Cryosections were stained with FITC-conjugated peanut agglutinin (Vector) and PE-conjugated anti-B220 antibody (Pharmingen).

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