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Antioxidant Treatment of Thymic Organ Cultures Decreases NF-κB and TCF1(α) Transcription Factor Activities and Inhibits αβ T Cell Development[1]

Vladimir Ivanov,*†[2] Matthias Merkenschlager,* and Rhodri Ceredig*

*INSERM Unité 184, Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France, and the †Institute of Gene Biology, Russian Academy of Sciences, Moscow B334, Russia

Abstract. Using electrophoretic mobility shift assays (EMSA), we have recently shown that nuclear extracts of 14-day mouse fetal thymocytes contain abundant NF-κB transcription factor activity. To determine the functional role of NF-κB in early thymocyte development, we have exposed fetal thymus organ cultures to inhibitors of NF-κB activation, namely the antioxidants *N*-acetyl-L-cysteine and butylated hydroxyanisole. Both compounds caused a dose-dependent arrest of thymocyte differentiation toward αβ, but not γδ, T cells. This was associated with a profound decrease in nuclear content of NF-κB and TCF1(α) transcription factor activity, as determined by EMSA. In contrast, NF-Y was affected less strongly, and cyclic AMP-response-element-binding protein levels remained essentially unchanged by antioxidants. To test the idea that αβ T cell development is correlated with NF-κB and TCF1(α) activity, we conducted additional experiments in a submersion culture system in which the generation of αβ T cells can be manipulated. Standard submersion culture supports γδ but not αβ T cell development. Under these conditions, EMSA showed that transcription factor activities were similar to those seen in the presence of antioxidants. Importantly, when the generation of αβ T cells in submersion culture was restored by elevating oxygen concentrations, there was a dramatic increase in TCF1(α) activity, and both NF-κB and NF-Y returned to control levels. Taken together, these results strongly suggest that NF-κB and TCF1(α), presumably in concert with other transcription factors, play an important role in the development of αβ T cells. *Journal of Immunology,* 1993, 151: 4694.

Transcription factors are key regulators of differential gene activity which in turn specifies cellular development and function (1). Using EMSA,[3] we have begun to study transcription factor activity during the early development of T lymphocytes from hemopoietic precursors in the fetal thymus (2). Thymocyte development was chosen as a system because it proceeds in phenotypically well defined, contingent stages (3). In addition, organ culture of fetal thymi allows normal development to occur in vitro, greatly facilitating the experimental manipulation of this process (4).

At day 10 of embryonic development, the mouse thymus is a cluster of epithelial cells which becomes colonized by hemopoietic precursor cells. By day 14, surface expression of γδ TCR can be detected (on about 3% of cells), but only truncated (1.0 kb) transcripts are found for the TCR β chain. Two days later, cells expressing both CD4 and CD8, so-called DP thymocytes, appear and express low levels of αβ TCR in association with CD3. This population is subject to positive and negative selection by thymic stromal elements,

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[2] Present address: University of Miami School of Medicine, Department of Microbiology and Immunology, Miami, FL 33136.
Address correspondence to Dr. Rhodri Ceredig, INSERM, Faculté de Médecin, 11 Rue Humann, 67085, Strasbourg Cedex, France.

[3] Abbreviations used in this paper: BHA, butylated hydroxyanisol; CREB, cyclic AMP-response-element-binding protein; DN, double negative; DP, double positive; EMSA, electrophoretic mobility shift assay; FTOC, fetal thymus organ culture; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor κB; SP, single positive; TCF1(α), T cell-specific factor 1(α); PE, phycoerythrin; SN, supernatant. TCR, T cell receptor.

giving rise to mature SP CD4 or CD8 cells with high levels of $\alpha\beta$ TCR (reviewed in reference 3). Most previous studies on transcription factors in developing T lymphocytes have focused on the regulation of $\alpha\beta$ TCR gene expression (5, 6). For example, TCF1(α)/LEF1 was identified as essential participant in TCR α-chain transcription, and is also involved in CD4 and *lck* gene transcription (7–10). Another important factor controlling $\alpha\beta$ TCR (and several other genes in thymocytes) is the CREB (11–14). Little is known about transcriptional control of early stages of thymocyte development before $\alpha\beta$ TCR expression. We have recently observed that the 14-day fetal thymus contains abundant NF-κB activity, as determined by using EMSA (2). We reasoned that if NF-κB is important for early T cell development, then blocking NF-κB activity should have potent effects on thymocyte differentiation. Antioxidant compounds including NAC and BHA have recently been identified as inhibitors of nuclear NF-κB activity in a number of cell lines (15–17). We therefore decided to add NAC and BHA to FTOC, and indeed observed decreased levels of NF-κB and also TCF1(α) activity. Simultaneous phenotypic analysis showed a profound block in $\alpha\beta$ but not $\gamma\delta$ T cell development. Similar changes in transcription factor activity and phenotype of developing thymocytes were observed in a submersion culture system. Importantly, experimental rescue of $\alpha\beta$ T cell generation (by elevated oxygen concentration, reference 18) restored both NF-κB and TCF1(α) activity.

Materials and Methods

Culture of fetal thymi

Thymus lobes were removed from approximately 100 (C57BL/6 × SJL) F_2 day 14 embryos and placed on Nucleopore polycarbonate filters (0.8 μm pore size) floating on 5 ml DMEM, supplemented as described (19), in 60-mm Petri dishes (day 0). Four groups of two filters each with 25 lobes were set up containing 0 (Control), 20, 50, or 100 mM NAC (Sigma Chemical Co., St. Louis, MO). NAC was kept as a 1 M stock solution in DMEM brought to pH 7.4 by NaOH. Before being placed on filters, lobes were incubated for 2 h in 5 ml medium containing the corresponding concentrations of NAC. After 24 to 48 h, lobes were then transferred to fresh medium without NAC. For experiments with BHA, FTOC were treated for 24 h with concentrations of BHA ranging from 0.5 to 4.0 mM. In some experiments, day 14 fetal thymi were cultured submerged in medium. These cultures were supplemented with 10 U/ml recombinant IL-2 where indicated (19, 20). Submersion cultures were maintained either in a standard atmosphere of 5% CO_2 in air (ambient O_2 concentration of 21%) or in a mixture of 60% O_2, 10% CO_2, and 20% N_2 as described recently (18). To facilitate gas exchange, these latter cultures were conducted in permeable Petri dishes (Petri-perm, Heraeus, Germany).

Cellular proliferation and IL-2 production

Groups of FTOC were treated for 24 h with NAC before being transferred to non-NAC-containing DMEM. After 9 days, cells were harvested and duplicate cultures containing 5, 2.5, 1.0, 0.5, and 0.25 × 10^5 cells set up in round-bottom microtiter plates. Cells were stimulated with 1 ng/ml PMA (Sigma) and 500 ng/ml Ionomycin (Calbiochem Corp., La Jolla, CA) as described previously (19). After 24 h, 100 μl of SN was collected, replaced with 100 μl of fresh medium, and after 48 h cells were pulsed for 5 h with 1 μCi [^{3}H]-thymidine before harvesting and counting. The SN were assayed for IL-2 content using CTLL cells as previously described (19) along with r-IL-2 (Cetus) as IL-2 control.

Immunofluorescence staining and flow cytometry

At the times indicated, between 2 and 8 lobes from each group were pooled, and cell suspensions prepared by mincing with cataract knives, and then gently passing them through a 26-gauge hypodermic needle. Viable cells were counted in a hemocytometer. Cells were washed in medium and 3 × 10^4 to 2 × 10^5 cells incubated for 30 min at 4°C with saturating concentrations of FITC-labeled anti-CD8 and PE-conjugated anti-CD4 mAb (Becton-Dickinson, Mountain View, CA) diluted in 50 μl DMEM or in medium alone (unstained samples). For three-color analysis, cells were stained with biotinylated anti-CD3 mAb 500A2 (Fig. 4*A*), anti-TCR β-chain H57 mAb (Fig. 4*B*), anti-$\gamma\delta$ TCR mAb GL3 (Fig. 3*C*), or anti-CD25, IL-2Rα chain, mAb PC61 (Fig. 3) followed by streptavidin-cychrome (PharMingen, San Diego, CA), and subsequently incubated with anti-CD4-PE and anti-CD8-FITC mAb. Controls were stained with all reagents except the biotinylated mAb. After washing, cells were analyzed unfixed (except for those at 21 days which were fixed in 1% paraformaldehyde in PBS) on a FACScan flow cytometer (Becton Dickinson) and compensations set using singly and doubly labeled samples of normal adult thymocytes. Viable cells were identified by a combination of forward and side scatter signals, and 5 to 20 × 10^3 cells were acquired per sample.

Oligonucleotides and electrophoretic mobility shift assay

Synthetic oligonucleotides were end-labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (22). Sequences of double-stranded oligonucleotides used in this study are shown in Table I. Nuclear extracts were prepared from fresh

Table I
Oligonucleotides with binding sites for transcription factors

Sequence[a]	Binding Sites and Factors	Gene Promoters or Enhancers
5′ 3′		
AGCTTGGGGACTTTCCAGCCG	κB/NF-κB	HIV
AGCTTG**CTC**ACTTTCCAGCCG	mκB (mutated site)[b]	
AGCTTGATGAGTCAGCCG	TRE/AP1	Collagenase
AGCTCCATGACGTCATGG	CRE/CREB	TCRα
GCTTCAAAGGGCGCC	Tα2/TCF-1(α)	TCRα
AAAAAAGAACAAAGGGCCTAGATT	CD4 = 2/TCF1(α)	CD4
AAAAAAGAA**TCC**AGGGCCTAGATT	mCD4 = 2 (mutated site)	
GTCTGAAACATTTTTCTGATTGGTTAAAAGTTGAGTGCT	Y-box/NF-Y	MHCII(Eα)

[a] Known binding sites of transcription factors are underlined.
[b] Mutated nucleotides are shown in bold.

fetal, newborn, or adult (C57BL/6 × SJL) F_2 mouse thymocytes using the techniques described by Dignam et al. (23) and Schreiber et al. (24). Buffers for extraction of nuclear proteins contained the protease inhibitors PMSF (0.5 mM) and 5 μg/ml each of leupeptin, aprotinin, pepstatin, chymostatin, and antipain. Proteins were quantitated according to Bradford (25). Binding reactions were conducted by incubating the end-labeled DNA (20,000 cpm) with 1 or 2 μg of nuclear proteins and 2 μg of poly(dI-dC) in a buffer containing 10 mM HEPES, pH 7.9, 60 mM KCl, 4% Ficoll, 1 mM EDTA. After 30 min at room temperature, the reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.25 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 10 V/cm for 1.5 h at room temperature. For competition experiments, a 100- to 200-fold molar excess of unlabeled oligonucleotide was added to the reaction mixture before the labeled DNA. A polyclonal rabbit antiserum to p50 subunits of NF-κB (26) was kindly provided by Dr. A. Israel, Pasteur Institute, Paris. A mouse mAb to NF-Y (27) was kindly provided by Dr. R. Mantovani, Institut de Chimie Biologique, Strasbourg. Nuclear extracts were preincubated with 1.0 μl of antiserum or mAb for 15 min at 20°C before addition of labeled oligonucleotide probes. Thereafter, labeled probes were added and the incubation continued for a further 30 min. Gels were dried and exposed overnight at −80°C with intensifying screens.

Results

Transcription factor TCF1(α), NF-Y, NF-κB, and CREB activities in fetal thymus ontogeny

Using EMSA, we have determined the binding activities of the transcription factors TCF1(α), NF-Y, NF-κB, and CREB from day 14 of fetal development until birth (day 20). NF-Y, a highly conserved transcription factor which recognizes CCAAT motifs in a variety of genes (28) was used as a reference. The specificity of transcription factor binding activities was confirmed by addition of excess unlabeled homologous or mutated oligonucleotides as competitors (Fig. 1). A specific antiserum to the p50 subunit of NF-κB (26) completely inhibited the formation of the lower band (b2) and partially inhibited the upper band (b1), inducing a supershift of band b1 (Fig. 1*A*). Together with our previous UV cross-linking experiments (2), these results identify the lower band (b2) as a homodimer of p50-p50 NF-κB, and the upper (b2) band as a complex containing the p50-p65 heterodimer. A supershift seen with mAb to NF-Y (but not with control ascites or anti-p50 antiserum) also demonstrated the presence of canonical NF-Y factor in fetal thymus (Fig. 1*A*). As observed previously (2), nuclear extracts from day 14 fetal thymus contained abundant NF-κB activity, which decreased at days 16 and 17 to rise again toward birth (Fig. 1*B*). The kinetics of TCF1(α) activity during thymus development were different, with low levels at day 14 and a rise by day 16. CREB and NF-Y activities remained relatively unchanged throughout ontogeny (Fig. 1*B*).

NAC blocks thymocyte differentiation along the αβ T cell lineage

To make thymocytes accessible to experimental manipulation, we employed air/liquid interface cultures of day 14 fetal thymus (FTOC), a system which supports normal thymocyte differentiation (4). Cultures were exposed to doses of NAC (20, 50, or 100 mM) known to inhibit NF-κB activation in other systems (15, 17), and then allowed to develop in the absence of NAC. At the indicated times, we analyzed cell recovery, and the expression of CD4 and CD8 (Fig. 2). 20 mM NAC had no effect on cell recovery, whereas 50 mM NAC resulted in growth arrest which was reversible 2 days after removal of NAC. No cell loss from FTOC was caused by 50 mM NAC in several different experiments. In contrast, initial loss of cells was seen from FTOC exposed to 100 mM NAC (Fig. 2*A*), but the sur-

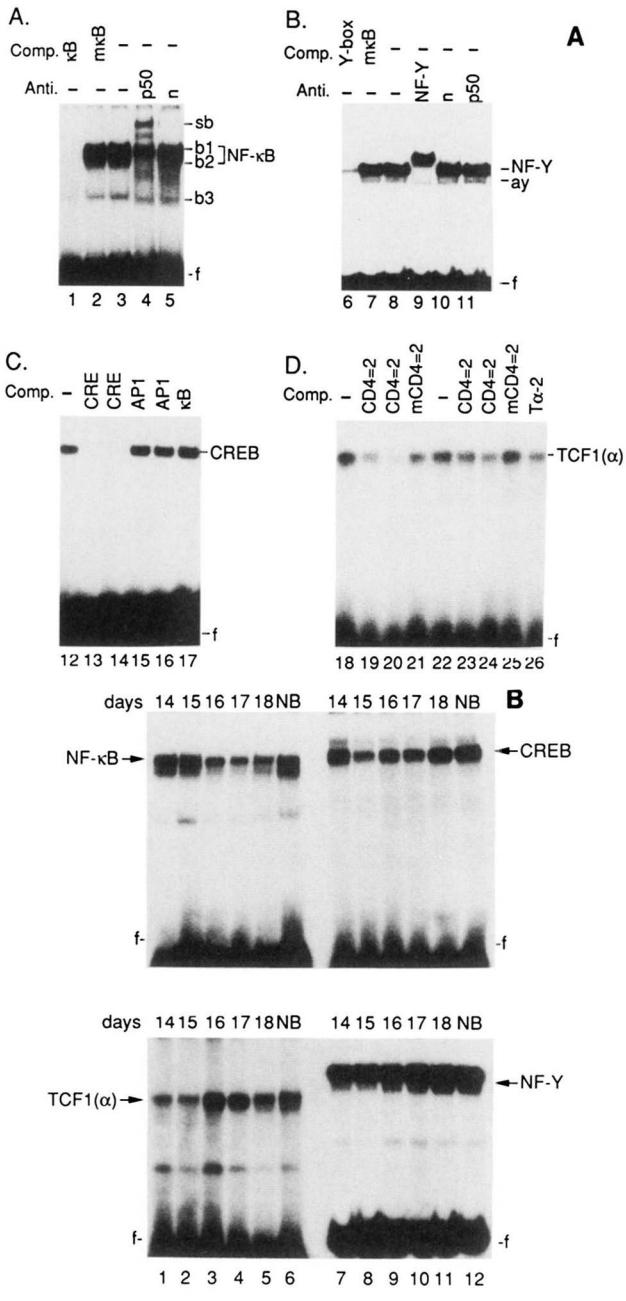

FIGURE 1. *A*, Presence and specificity of (*A*) NF-κB, (*B*) NF-Y, (*C*) CREB, and (*D*) TCF-1(α) transcription factor binding activity in nuclear extracts of mouse fetal thymocytes. *B*, Ontogeny of transcription factor binding activities in nuclear extracts of mouse thymocytes. *A*, Nuclear extracts were prepared from day 15 (*A*, *B*, and *C*), newborn (*D*, *lanes 18* to *21*), or adult (*D*, *lanes 22* to *26*) thymocytes. EMSA used κB (*A*), Y-box (*B*), CRE (*C*), or CD4=2 (*D*) oligonucleotides (compare sequences in Table I). Unlabeled homologous or mutated oligonucleotides (*Comp.*) were added at 100-fold (*lanes 13, 15, 19, 23*) or 200-fold excess (all other lanes with *Comp.*). Antibodies (*Anti.*) used were a polyclonal rabbit antiserum to p50 subunits of NF-κB (25) (*A*, *lane 4*, and *B*, *lane 11*), preimmune rabbit serum (*A*, *lane 5*), a mouse mAb to NF-Y (26) (*B*, *lane 9*), or control ascites (*B*, *lane 10*). Antibodies inhibited the formation of nuclear protein/oligonucleotide complexes and/or produced supershifts in EMSA. For (*A*), two bands of retardation for NF-κB were designated *b1* and *b2*, *sb* = supershift, *b3* represents a nonspecific band. For (*B*), *NF-Y* corresponds to the major, specific band and *ay* to a minor CCAAT-binding protein. In all panels, *f* refers to free probe. *B*, Thymi were obtained from embryos at days 14 to 18 of development or from newborn (*NB*) mice. EMSA conducted as described in Figure 1*A*. The day 14 fetal thymus contains abundant NF-κB activity which decreases on days 16 and 17 relative to CREB and NF-Y. There is an increase in TCF1(a) activity between days 15 and 16.

viving cells were still able to proliferate and differentiate after removal of NAC (see below).

Control cultures (Fig. 2*B*) generated abundant $CD4^+$ $CD8^+$ DP progeny (52% of thymocytes were DP by day 4). Addition of 20 mM NAC had no effect on the phenotypic differentiation, but exposure to 50 mM NAC drastically reduced the fraction of DP thymocytes (3% DP cells at day 4 and 21% by day 8). An almost complete block of development was seen in the presence of 100 mM NAC (Fig. 2*B*).

To analyze the phenotypic properties of the DN cells in NAC-treated FTOC, we conducted three-color analysis with anti-CD25 (IL-2Rα chain), anti-CD4, and anti-CD8 mAb. In these experiments, FTOC were treated for 24 h with 0, 20, 40, 60, 80, or 100 mM NAC, and the recovery of CD4- and CD8-expressing cells was similar to that shown in Figure 2*B*. As shown in Figure 3, DN cells in freshly isolated day 15 fetal thymus lobes contained subpopulations of $CD25^{weak}$ and $CD25^{bright}$ cells (R. Ceredig, manuscript in preparation). Forty-eight hours after removal of NAC, it could be seen that $CD25^+$ cells were depleted from the 60, 80, and 100 mM groups. After 72 h, DN cells in the 60 and 80 mM groups contained $CD25^{weak}$ and $CD25^{bright}$ cells. By 120 h, $CD25^{weak}$ cells had reappeared among DN cells from the 100 mM group.

Three-color FACS analysis also showed that after 12 days in FTOC, CD3 was expressed normally by CD4- and CD8-defined subsets in the control as well as in 20 mM NAC (Fig. 4*A*). Exposure to 50 and 100 mM NAC caused a strong decrease in CD3 expression, particularly among the CD4 SP subset and CD4 CD8 DN thymocytes. By comparison, CD3 expression by CD8 SP cells was only slightly affected, and there was essentially no effect on CD3 expression by residual DP cells. Interestingly, changes in CD3 expression correlated with differential effects of NAC on αβ vs γδ TCR expression. Most strikingly, NAC markedly reduced the frequency of αβ T cells in all thymocyte populations. Among CD8 SP cells (but not CD4 SP or DN cells) this reduction was compensated by a rise in the proportion of γδ TCR positive cells. In this context, we have recently shown that during normal thymus ontogeny, $\gamma\delta TCR^+$ cells occur within the CD8 SP as well as the DN thymocyte

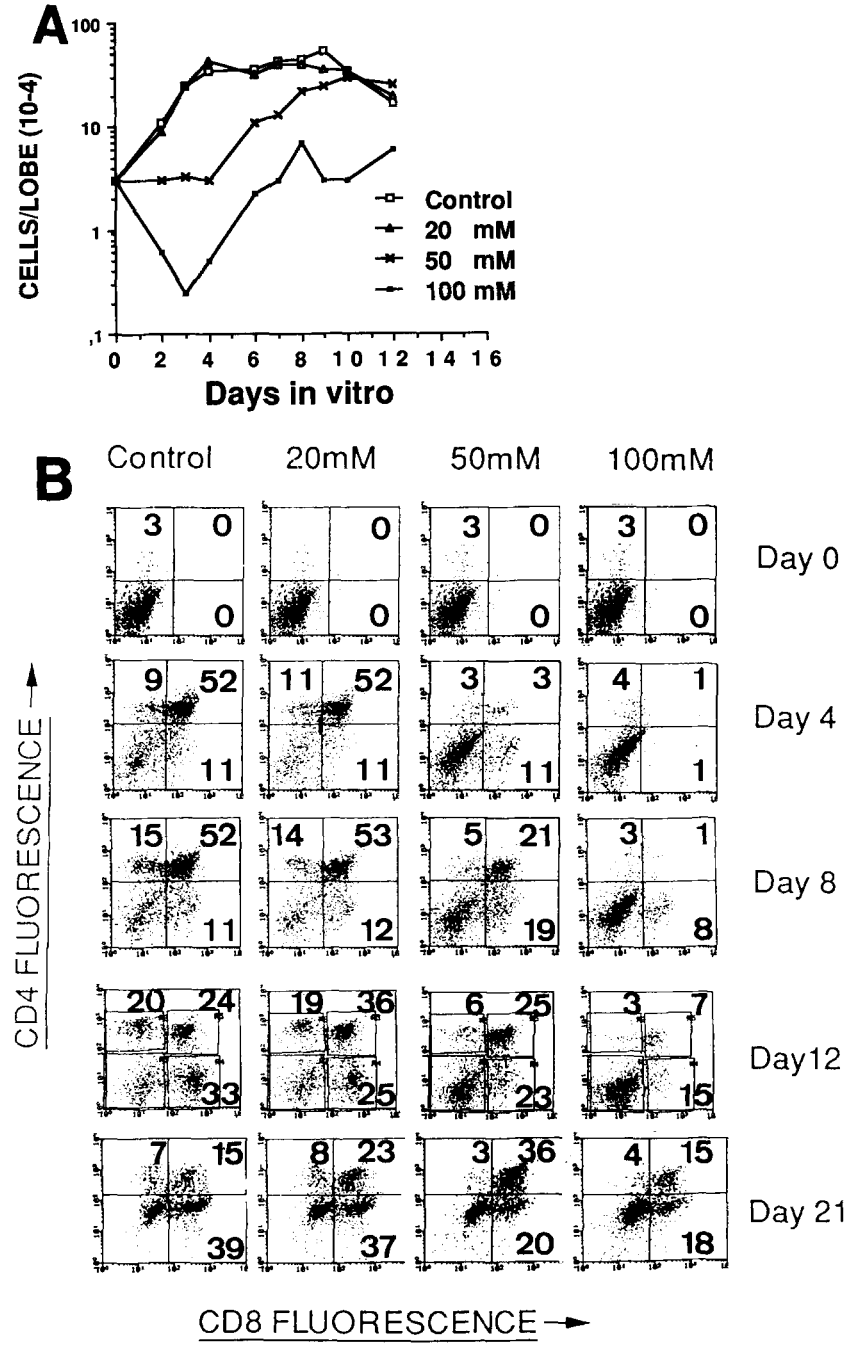

FIGURE 2. Kinetics of cell recovery (*A*) and thymocyte differentiation (*B*) in FTOC cultured for the first 48 h with 0 (*Control*), 20, 50 or 100 mM NAC. Each panel in Figure 3*B* represents CD8 (*horizontal axis*) vs CD4 (*vertical axis*) expression (logarithmic scale). The *vertical* and *horizontal lines* in each panel enclosed 99% of unstained cells in the lower left quadrant (not shown), and the percentage of cells in each quadrant is indicated.

population (29). Taking into account cell numbers, however, lobes from control cultures contained 5×10^4 γδ TCR^+ cells, whereas there were 3×10^4 from those pretreated with 100 mM NAC. Therefore, γδ TCR cell numbers remain relatively unchanged in NAC-pretreated lobes.

To analyze the functional properties of NAC-pretreated FTOC, lobes were pretreated for 24 h with NAC, and 9 days later cells were harvested and stimulated with a mitogenic combination of PMA and Ionomycin. As shown in Table II, cells harvested from such lobes were fully capable of proliferating and producing IL-2 after mitogen activation.

Effects of other antioxidant compounds on thymocyte differentiation

For comparison, we used another antioxidant compound, BHA, a reagent known to inhibit the activation of mouse thymocyte and peripheral T cells in response to Ag and mitogens (30). Recently, BHA at a concentration of 100 to 500 μM, was also shown to inhibit NF-κB activity in human T cell and monocyte lines (16). FTOC were exposed to BHA for 24 h, after which cultures were transferred to fresh medium. Five days later, thymocytes were harvested

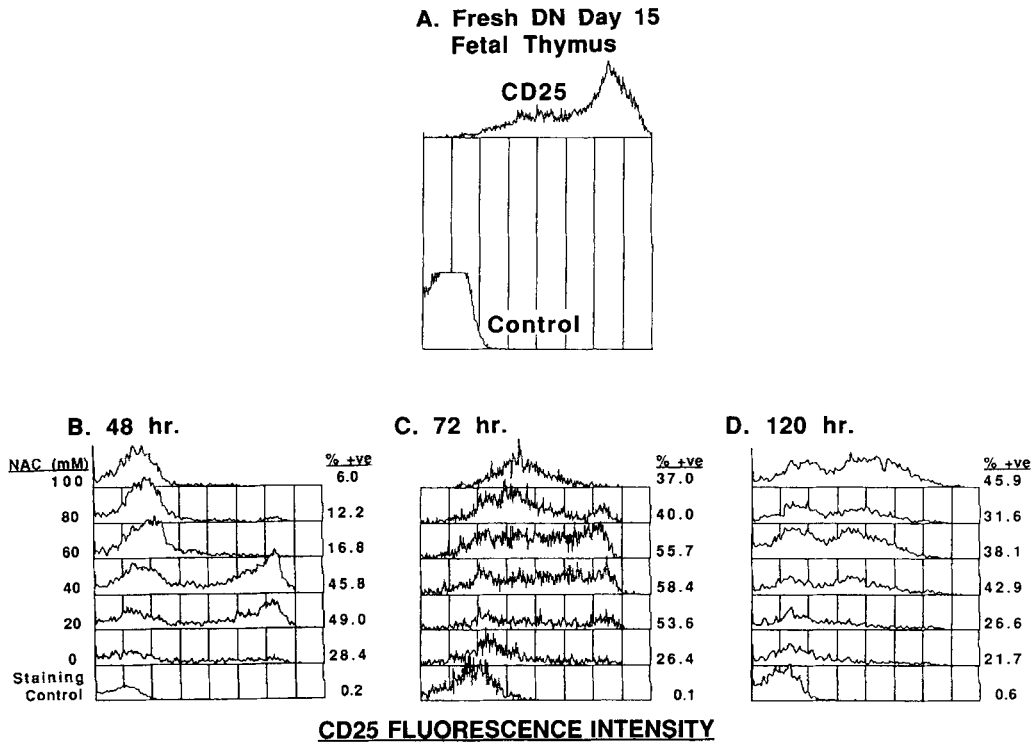

FIGURE 3. Kinetics of CD25 (IL-2Rα) expression by DN cells from NAC-pretreated FTOC. The *top panel* (*A*) represents CD25 expression by DN cells from freshly isolated day 15 fetal thymus lobes. Below (*panels B, C,* and *D*) are the CD25 profiles of DN cells from FTOC pretreated for 24 h with the indicated concentrations of NAC and analyzed at (*B*) 48, (*C*) 72, and (*D*) 120 h after removal of NAC. In all instances, cells were stained with anti-CD25, anti-CD4, and anti-CD8, and the histograms represent the CD25 fluorescence profiles of gated DN cells.

and stained for FACS analysis. At 4.0 mM BHA, essentially all (92%) thymocytes were CD4 CD8 DN (Fig. 5). BHA concentrations below 1.0 mM did not affect thymocyte development. Summarizing four independent experiments, BHA was about 25 times more potent than NAC; 4 mM BHA and 100 mM NAC had comparable effects (Fig. 5).

Transcription factor activities in thymocytes exposed to antioxidants

Nuclear extracts were prepared from thymocytes cultured for different times in NAC, and equal amounts (2 μg) of nuclear proteins were subjected to EMSA. As shown in Figure 6, control thymocytes and cultures treated with 20 mM NAC contained abundant NF-κB, CREB, and NF-Y activities. The only detectable effect at this concentration was a slight decrease of TCF1(α) (Fig. 6*A*). At 50 mM NAC, NF-κB and especially TCF1(α) were substantially reduced relative to control levels, whereas NF-Y and CREB activities decreased only slightly (Fig. 6*A*). TCF1(α) and NF-κB were essentially undetectable as soon as 24 h after exposure to 100 mM NAC. A decrease of NF-Y activity was also observed, but CREB levels remained essentially unchanged, declining only slightly after 2 days in 100 mM NAC (Fig. 6*B*). Thus, reduced NF-κB and TCF1(α) levels appeared to correlate with the inhibition of αβ (but not γδ) thymocyte differentiation in FTOC (Figs. 3 and 4). Importantly, both NF-κB and TCF1(α) returned to levels higher than those seen before treatment following transfer of FTOC to medium without NAC (Fig. 6, *A* and *B*, *lanes R-5*). Recovery of NF-κB and TCF1(α) was more rapid in the 20 and 50 mM than in the 100 mM group, in which recovery of NF-κB preceded TCF1(α). Previous experiments suggested that NF-κB was affected by antioxidants (15–17), whereas our results indicate more complex effects of NAC on transcription factor activities, in particular TCF1(α).

Thymocyte development and transcription factor activity in submersion culture

To test the hypothesis that abundant NF-κB and TCF1(α) is associated with the development of αβ T cells, we employed a culture system in which differentiation toward αβ vs γδ T cells can be manipulated by nonpharmacologic means. We have previously reported that submersion culture of day 14 fetal thymi yields mainly CD4 CD8 DN and CD8 SP γδ, but not αβ T cells (20, 21), a phenotypic constellation similar to that described here for FTOC exposed to antioxidants. Recently, Watanabe and Katsura (18) showed that the differentiation of αβ T

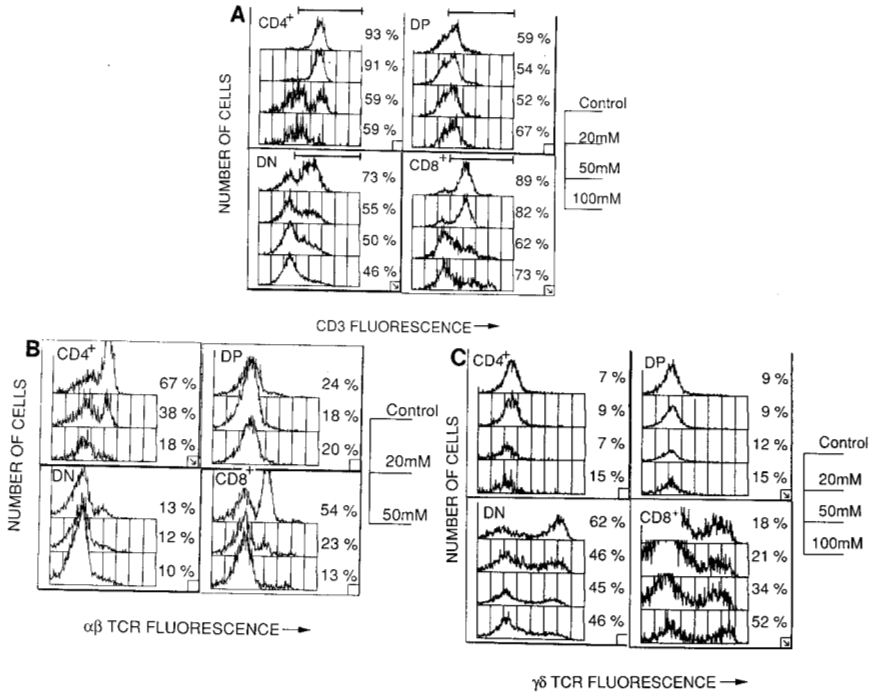

FIGURE 4. Three-color FACS analysis of FTOC cultured in the presence of NAC. Expression of CD3 (*A*), αβ TCR (*B*), and γδ TCR (*C*) by CD4 CD8 DN (*lower left*), CD4 CD8 DP (*upper right*), CD4 SP (*upper left*), and CD8 SP (*lower right*). Overlays represent controls, and cultures exposed to 20, 50, and 100 mM NAC after 12 days in culture. The percentages of stained cells are indicated.

Table II
Proliferation and IL-2 production by NAC-treated FTOC

Treatment Group[a]	Proliferation[b] ([^{3}H]Thymidine cpm)	IL-2 Production[c] (U/ml)
Control	1.2×10^5	10
20 mM NAC	1.2×10^5	10
40 mM NAC	2.0×10^5	13.2
60 mM NAC	1.5×10^5	11.2
80 mM NAC	1.0×10^5	2.8
100 mM NAC	7.4×10^4	2.8

[a] Groups of FTOC were treated for 24 h with the indicated concentrations of NAC and then cultured for 9 days in medium. Lobes were then harvested and 10^5 cells stimulated with 1 ng/ml PMA and 500 ng/ml Ionomycin. Twenty-four hours later, SN was harvested, and after 48 h, cellular proliferation was measured by [^{3}H]thymidine incorporation.

[b] Values represent the mean [^{3}H]thymidine incorporation from duplicates of 10^5 FTOC cells stimulated as above and pulsed for 5 h.

[c] IL-2 content of SN from 10^5 FTOC cells stimulated as above. Values determined using CTLL cells. In this assay, 50% maximum thymidine incorporation with 1 U/ml r-IL-2 gave 7.5×10^4 cpm from 10^4 CTLL cells pulsed for 5 h.

cells in submersion culture is restored under elevated O_2 concentrations.

Submersion cultures were set up in ambient or 60% O_2, and thymocytes were analyzed by FACS (Fig. 7*A*) and EMSA (Fig. 7*B*). FTOC generated 16.0×10^4 thymocytes per lobe, whereas submersion cultures yielded 1.5×10^4 at standard, and 6.5×10^4 at high oxygen concentration. After 1 wk in control FTOC, day 14 thymocyte precursors had differentiated, resulting in substantial populations of CD4 CD8 DP, CD4 SP, and CD8 SP cells. Half of the thymocytes (80×10^3 per lobe) expressed detectable levels of αβ TCR. Fourteen percent of cells, mainly CD4 and CD8 SP, were αβ TCR bright (Fig. 7*A*, *panel A*). In marked contrast, the majority of thymocytes in standard submersion culture remained CD4 CD8 DN, and only 21% of all cells (3×10^3 per lobe) expressed αβ TCR, 3% at high levels (Fig. 7*A*, *panel B*). Increasing the oxygen concentration to 60% restored the generation of CD4 CD8 DP and SP thymocytes and most importantly, the expression of αβ TCR: 41% of thymocytes (26×10^3 per lobe) were stained, 19% brightly (Fig. 7*A*, *panel C*). The pattern of αβ TCR expression by thymocyte subpopulations was normal, with low levels on DP and high levels on SP cells (compare histogram overlays in *panels A* and *C* of Fig. 7*A*).

EMSA with equal amounts of nuclear extract from FTOC and submersion cultures at low- and high-oxygen concentration showed (Fig. 7*B*) that NF-κB and TCF1(α) was abundant in FTOC, but barely (NF-κB) or not at all (TCF1(α)) detectable in standard submersion culture. Both NF-κB and TCF1(α) activities were restored under elevated oxygen concentration (Fig. 7*B*). NF-Y activity was less dependent on the culture conditions, whereas CREB increased in submersion culture particularly at high oxygen concentration. Taken together with the data on antioxidant-treated FTOC, these results confirm the correlation between NF-κB and TCF1(α) activities and thymocyte differentiation toward the αβ T cell lineage.

Discussion

Nuclear NF-κB activity is abundant in the fetal thymus as early as day 14 (2, this report), when most thymocytes are still devoid of TCR. Little is known about the interactions between T cell precursors and thymic stromal cells at this stage, but adhesion molecules (such as the LFA-1/ICAM1

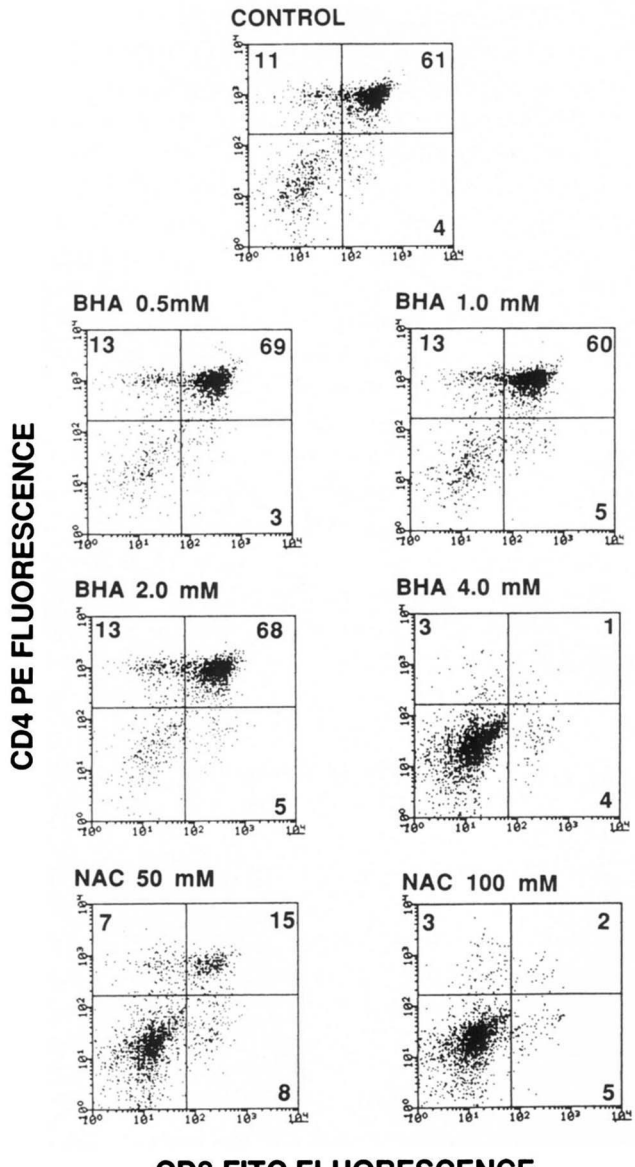

FIGURE 5. Inhibition of thymocyte differentiation by BHA. FTOC were exposed to the indicated concentrations of BHA and NAC. Cultures were transferred to fresh medium after 24 h and harvested and stained for CD4 and CD8 expression 5 days later (see legend to Fig. 3).

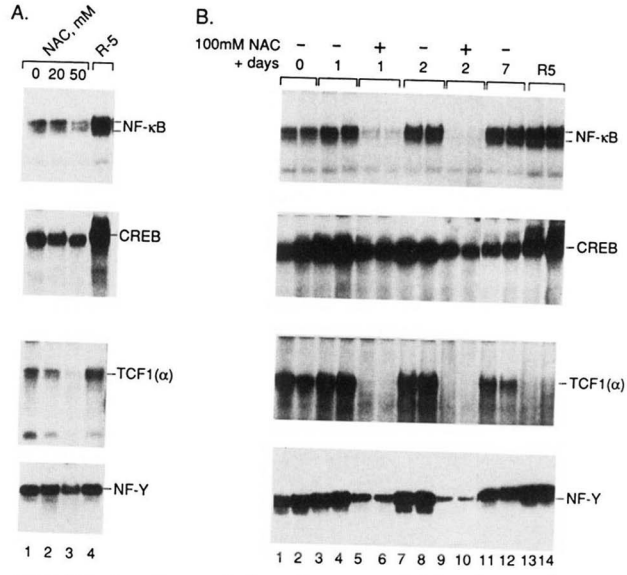

FIGURE 6. Transcription factor activities in nuclear extracts from FTOC cultured in NAC. (*A*) 0, 20, or 50 mM and (*B*) 100 mM NAC. In (*A*), three groups of 60 thymi were cultured for 2 days in the indicated doses of NAC. Nuclear proteins were isolated from 30 lobes (*lanes 1* to *3*), and the remaining thymi were transferred to filters on fresh medium, and allowed to recover for a further 5 days (*R5*) (*lane 4*). In (*B*), duplicate lanes are shown for thymi cultured for 0, 1, 2 or 7 days (*lanes 1* to *12*) with (+), *lanes 5, 6,/9,10* or without (–), *lanes 1, 2, 3, 4, 7, 8, 11, 12*, NAC. As in (*A*), lobes that had been cultured for 2 days with NAC were transferred to fresh medium and allowed to recover for 5 days (*R5, lanes 13, 14*). Nuclear extracts were analyzed by EMSA for the indicated transcription factor binding activities.

and CD2/LFA-3 system, references 31–33) and lymphokines (e.g., IL-1 and other factors released by thymic stromal cells, reference 34) presumably act as important mediators at this stage prior to TCR expression. NF-κB is inducible by IL-1 in T-cell and pre-B cell lines in vitro (35, 36), and it seems likely that this and other stroma-derived factors regulate NF-κB activity also in the early fetal thymus. Targets for NF-κB include cytokine and receptor genes (IL-2, IL-6, TNF-α, GM-CSF, and the IL-2 receptor α-chain), major histocompatibility complex-related genes (MHC class I, and the class II associated invariant chain) (37, 38), and the murine T cell receptor β2 intron enhancer (39). To address the functional significance of this transcription factor for early thymocyte development, we attempted to inhibit nuclear NF-κB activity in fetal thymus organ cultures using antioxidant compounds (15, 16). Phenotypic analysis showed that both NAC and BHA inhibited thymocyte differentiation in a dose-dependent manner, resulting in the accumulation of CD4 CD8 DN cells. Detailed analysis of DN cells from NAC-pretreated FTOC showed that there was a dose-dependent inhibition of CD25 expression. The data presented in Figure 3 suggests that NAC affected thymocyte differentiation at a stage before CD25 expression by DN cells. This inhibition affected mostly the αβ TCR pathway and could not be attributed to MHC Class I or Class II expression which was not decreased in NAC-treated FTOC (unpublished data). Functional analysis revealed that cells from NAC-pretreated FTOC were fully capable of proliferation and IL-2 production after mitogen stimulation.

As expected, EMSA experiments demonstrated that antioxidants affected NF-κB. In addition, we have demonstrated here that TCF1(α) activity was at least equally sensitive to antioxidants as NF-κB. Effects on NF-Y were also detected, whereas changes in CREB activity were relatively minor. Removal of NAC from FTOC resulted in the rapid

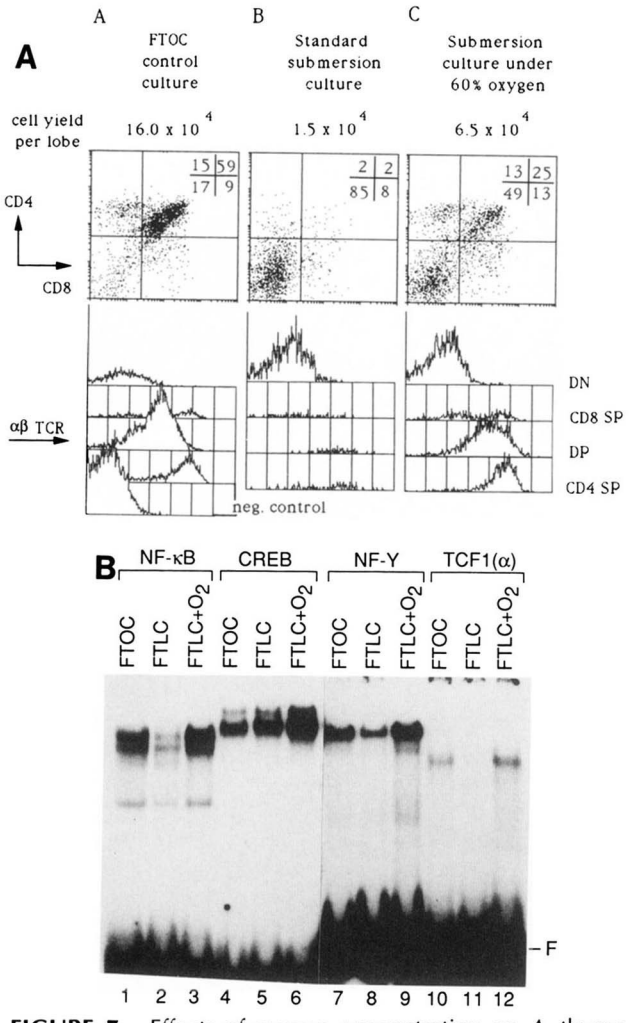

FIGURE 7. Effects of oxygen concentration on *A*, thymocyte differentiation, and *B*, transcription factor activity. *A*, Day 14 fetal thymus lobes were cultured as FTOC (*panel A*) or submerged in medium at ambient oxygen concentration (*panel B*) or at 60% O_2 (*panel C*). After 1 wk, thymocytes were harvested, counted, and stained with CD4-PE, CD8-FITC, and H-57-biotin (αβ TCR) followed by streptavidin-cycrome (PharMingen) for three-color immunofluorescence analysis. The cytogram displays (*top panels*) show (on log scales) the expression of CD4 and CD8; histogram overlays below represent αβ TCR staining of the populations (log scale). In the negative control, the biotinylated first antibody (αβTCR) was omitted. *B*, EMSA was performed for the detection of NF-κB, NF-Y, CREB, and TCF-1(α) from nuclear extracts of fetal thymus lobes cultured as FTOC or as submersion cultures at ambient (FTLC) or at 60% oxygen (FTLC+O_2). Two micrograms of nuclear protein was used in each lane.

recovery of NF-κB and TCF1(α) activities, and also restored thymocyte development.

NF-κB resides in the cytoplasm in an inactive form complexed to the inhibitor, IκB (40). Cellular activation results in the dissociation of IκB proteins from NF-κB with consequent translocation of active NF-κB to the nucleus (37, 41). The precise mechanisms by which antioxidants block NF-κB and/or possibly TCF1(α) activation remain unclear, but may be related to the scavenging of oxygen radicals (42), as reactive oxygen intermediates appear to participate in the activation of the cytoplasmic precursor of NF-κB.

In earlier studies, we had shown that thymus lobes submerged in liquid culture generated primarily γδ T cells (20, 21). Recently, Watanabe and Katsura demonstrated that elevated oxygen concentrations could restore αβ T cell development in such submerged cultures (18). This system provided a test for the correlation of NF-κB and TCF1(α) with the generation of αβ T cells. Here, we show that the rescue of αβ T cell development in submersion cultures by high oxygen indeed correlates with increased levels of NF-κB and TCF1(α) activity. The signaling pathway whereby high concentrations of oxygen activate transcription factors is unclear, but elevated levels of reactive oxygen intermediates generated in cells cultured at high oxygen concentration might play a role. The experiments confirm that the inhibition of thymocyte differentiation in NAC- and BHA-treated FTOC and the concomitant decrease in transcription factor activities are not simply due to toxic effects of the drugs: viability of cells in submersion culture is excellent, yet we observed a dramatic decrease in NF-κB and TCF1(α) which was fully reversible by increasing the O_2 concentration.

Cells of the αβ and γδ T lineages differ in several important respects including anatomical distribution, developmental pathways, and timing of appearance (43, 44). Transcripts and surface expression of γδ TCR begins at about day 13 to 14, at which time the thymus contains a truncated 1.0-kb TCR β transcript. The abundant NF-κB activity present in the fetal thymus at this time may play a role in the generation of these TCR transcripts (2, 39). Expression of α TCR transcripts, which is controlled by several transcription factors including TCF1(α) (5) begins 2 days later, at day 16 (3). Presence of α TCR transcripts is accompanied by full-length (1.3-kb) TCRβ transcripts and surface expression of αβ TCR, along with high level expression of CD4 and CD8, begins at this time (3). This pattern of gene expression is in good agreement with our finding of increased TCF1(α) activity around this time. The difference in ontogeny of γδ vs αβTCR expressing T-cells suggests that the two lineages may be independently regulated, by for example different sets of transcription factors.

In conclusion, we have shown that experimental manipulations which affect NF-κB and TCF1(α) activity abrogate αβ T cell development, but leave the differentiation of γδ T cells relatively unaffected. Although only correlative at present, but supported by two independent experimental systems, our results suggest that successful development of αβ T cells requires conditions under which the transcription factors NF-κB and TCF1(α), and probably others, are active.

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