

Table 4. Uterine Adenocarcinomas in ENNG-initiated Rats with Exposure to High-dose OP*

Group	No. of rats examined	Incidence of endometrial lesions						
		Hyperplasia			total	Adenocarcinoma		
		+	++	+++		differentiation**		
						G1	G2	G3
1. Control	23	2	8	7	4	4	0	0
2. OP-treated (Adulthood)	26	1	8	5	12***	9 (G1 and/or G2)		
3. Control	23	3	7	5	6	6	0	0
4. OP-treated (PNDs 1-5)	28	1	3	5	18***	17	1	0
5. OP-treated (PNDs 1-15)	22	2	2	1	8	1***	3	4***

*: Groups 1-2: Katsuda *et al.*, *Jpn J Cancer Res* 2002; 93: 117-124.

Groups 3-5: Yoshida *et al.*, *Carcinogenesis* 2002; 23: 1745-1750.

** : Histological grades of uterine adenocarcinomas by tumor differentiation.

G1: well differentiated; G2: moderately differentiated; G3: poorly differentiated.

***: Significantly different from the control value ($p < 0.05$).

end of the experiment, however, development of uterine adenocarcinomas was significantly increased in animals exposed to t-OP during adulthood, but no tumors developed in OVX rats. This finding suggests that high-dose t-OP has tumor-promoting effects on the ENNG-treated endometrium of rats, possibly due to direct action on the uterus, as indicated by the uterotrophic effect of OP⁶⁴ (Table 4).

Uterine carcinogenesis in Donryu rats treated neonatally with a high-dose of t-OP has also been investigated. Female pups were subcutaneously administered 100 mg/kg/day t-OP every other day for the first 5 days after birth (PNDs 1-5), or the first 2 weeks (PNDs 1-15). Thereafter, they received a single intra-uterine injection of 20 mg/kg ENNG at 11 weeks of age and were observed until 15 months of age. PNDs 1-5 OP-treated animals showed normal development of the female reproductive system, including uterine gland genesis before weaning and normal estrous cycling immediately after vaginal opening. However, the treatment accelerated the occurrence of persistent estrus after 6 weeks of age, and increased the number of well-differentiated uterine adenocarcinomas at the end of the experiment (15 months of age), as compared with controls. This indicates that PNDs 1-5 OP-treatment resulted in delayed modulation of the hypothalamus-pituitary-ovarian hormonal control system, and thus increased the serum E2:P ratio, leading to promotion of uterine carcinoma development. On the other hand, PNDs 1-15 OP-treatment demonstrated immediate and irreversible influences on the control system, called "androgenization", and induced suppression of uterine gland genesis as well as abnormal uterine development manifested by prolonged persistent estrus immediately after vaginal opening, similar to our previous report³⁴. In addition, at the end of the experiment, uterine tumor malignancy as assessed by morphological and biological properties was clearly increased, although there was no significant alteration in the total incidence of adenocarcinomas. The total incidence of hyperplasias was significantly lowered, probably related to suppression of uterine gland genesis (Table 4). That study

provided evidence that neonatal exposure during PNDs 1-5 or 1-15 to high-dose t-OP enhances uterine carcinogenesis in ENNG-initiated rats, and that the type of uterine tumor is changed by the period of neonatal treatment³⁷.

Concerning the histogenesis of endometrial adenocarcinomas in Donryu rats, the tumors are considered to arise from hyperplasias of the luminal or glandular epithelium, especially the latter²⁸. In humans, it has been pointed out that the presence or absence of hyperplasia as the background is important for the biological behavior of endometrial adenocarcinomas. High-dose OP treatment at PNDs 1-15 induced luminal epithelial hyperplasia in the uteri of rats at 8 weeks of age³⁴, and finally increased development of undifferentiated adenocarcinomas, although the incidence of hyperplasias was decreased³⁷. Carthew *et al.* also reported that tamoxifen induced uterine adenocarcinomas, including biologically malignant examples, in rats in the absence of endometrial hyperplasia, when given on days 2-5 after birth⁵⁴. These results are very interesting in consideration of the histogenesis of uterine adenocarcinomas.

As mentioned above, estrogen and related compounds are reported to increase the risk of endometrial adenocarcinoma development in women. Estrogens occur naturally within the normal body, and are mainly metabolized in the liver by two separate pathways, producing either catechol estrogens (2- or 4-hydroxylated products) or 16 α - or 16 β -hydroxylated products. 2-Hydroxylation of estradiol or estrone to a catechol is a major metabolic pathway, and the catechol estrogens 2-OHE2 and 2-OHE1 have much weaker hormonal potency than their parent hormones, and lack carcinogenic potency when given to adult animals. On the other hand, 4-hydroxyestradiol (4-OHE2) and the two 16 α -hydroxylated forms, 16 α -OHE1 and 16 α -OHE2, retain potent hormonal activity by acting on classical estrogen receptor and also are tumorigenic⁶⁵. In fact, induction of preneoplastic and neoplastic lesions by estrogen and its steroid metabolites (16 steroids) were studied with our two-stage mouse uterine carcinogenesis

model, and 2-OHE1 or 2-OHE2 exerted promoting, but not progressing, effects, while 16 α - and 16 β -OHE1 caused both promotion and progression⁶⁶.

It is known that indole-3-carbinol binds to the Ah receptor, similar to TCDD, and induces cytochrome p450 metabolic enzymes mainly in the liver. It has been reported that this chemical shows a chemopreventive effect on spontaneous endometrial adenocarcinoma development in Donryu rats when given orally, the effect being speculated to be due to enhanced 2-hydroxylation⁶⁷. We also assessed the effect of indole-3-carbinol on uterine carcinogenesis using our two-stage rat uterine carcinogenesis model. Contrary to expectation, however, the incidences of endometrial carcinomas were increased. In rats given indole-3-carbinol, elevated liver weights and centrilobular enlargement of hepatocytes were also observed, the results indicating an effect on estrogen metabolism in the liver, and further studies are now under way, to clarify the discrepancy (Yoshida *et al.* unpublished data).

Effects of Low-doses of EDCs

The concentrations of EDCs including OP in the environment are very low, and the main exposure route is oral, rather than cutaneous, in humans. In general, the toxicokinetics of chemicals including EDCs in animals is known to be influenced by the method of administration. It has been reported that low doses of estrogens and EDCs such as OP might be removed from the blood during the first passage through the liver, when given orally^{68,69}. For risk assessment of EDCs, it is very important to investigate oral dose effects at human exposure levels and thus we have also focused on relatively low doses of OP (t-OP or n-OP) by oral administration. Female Donryu rats initiated by intrauterine administration of ENNG were given diets containing 100 or 1000 ppm t-OP (about 5 or 50 mg/kg/day) or 100 ppm n-OP (about 5 mg/kg/day) from 11 weeks of age to 15 months of age. Although the concentrations are higher than those in the environment, no significant increase in the incidences of uterine adenocarcinomas was observed in any treated group at the end of the experiment, and also there was no difference in tumor malignancy among the groups (Yoshida *et al.* unpublished data).

As detailed above, exposure to high doses of estrogens or EDCs in the fetal or new born period exerts irreversible androgenization of the female reproductive organs, because of heightened sensitivity. In addition, "delayed" influences on these organs may occur after puberty or sexual maturation. Therefore, relatively long-term comprehensive studies on the endocrinological and morphological aspects may be necessary for determination of prenatal and/or neonatal effects of low doses of EDCs regarding toxicity/carcinogenicity in the female genital organs. Low doses of EDCs such as NP or bisphenol A (BPA) were given orally to pregnant rats, and offspring were observed until 15 months of age, to investigate the prenatal and neonatal effects on growth and development of the female reproductive system

and uterine carcinogenesis. In the reproductive toxicity studies reported by others, high doses of NP caused estrogenic effects on pubertal development in male and female rats^{70,71}. However, maternal or neonatal exposure to relatively low doses demonstrated no adverse influence on the reproductive tract⁷². In our study with NP, dams were administered 0.1, 10 and 100 mg/kg daily by gavage from gestation day 2 up to the day before weaning of their offspring. Then, all female pups at 11 weeks of age were administered a single dose of 20 mg/kg ENNG into a uterine horn, and observed until 15 months of age. The low level, 0.1 mg/kg, was selected as a dose relevant to human daily intake (1 mg/kg) of isoflavones, uterotrophic activity of NP being reported to be 10 times stronger than that of daidzein, one of the major isoflavones, and the middle-dose, 10 mg/kg, was selected as near the no observed effect level in a multi-generation reproductive study using rats⁷¹. None of the treated groups demonstrated any alteration in reproductive ability. In their offspring also, uterine growth and development, vaginal opening and hormonal secretion until puberty were not changed and there were no effects on estrous cyclicity and morphology of the reproductive organs after maturation, or on uterine carcinogenesis in animals initiated with ENNG⁷³.

BPA, a volume chemical used in the manufacture of polycarbonate plastics and found in canned foods, lacquered containers and composite dental sealant, is one of the most representative EDCs with weak estrogenic activity, and uterotrophic potential has been demonstrated in the immature rat assay⁷⁴. A study conducted by the National Toxicity Program (NTP) in the USA demonstrated that maternal exposure to high doses of BPA at 0.5 or 1.0% in the diet (approximately daily intakes of 875 and 1750 mg/kg/day) reduced the number of live pups per litter and litters per pair in first generation mice⁷⁵, although pre- and/or postnatal high-dose BPA exposure did not have any apparent adverse effects on pubertal development in female rats or reproductive functions in rats and mice⁷⁶⁻⁷⁸. Recently, however, perinatal treatment with BPA at much lower doses has been described to influence male reproductive organ parameters such as weight of the testis, prostate, preputial gland and epididymis, and the efficiency of sperm production in rodents⁷⁹⁻⁸¹, and neonatal treatment advanced puberty in mice⁸², although there are also some reports of no treatment-related effects at low dose levels when given to pregnant mice and rats⁸³⁻⁸⁵, and to rats in a three-generation reproductive toxicity study⁸⁶. To further assess the risk, we also investigated effects of maternal exposure to low-doses of BPA, including a human exposure-level, on growth and development of the female reproductive system, and also uterine carcinogenesis in Donryu rats. Dams were administered BPA (0, 0.006 and 6 mg/kg/day) daily by gavage from gestation day 2 up to the day before weaning (PND 21). The concentration of 0.006 mg/kg was selected as consistent with the 63 ppb defined as the average daily intake from canned food in human beings, and 6 mg/kg was selected as appropriate to simulate the maximum dose level

Table 5. Proliferative Uterine Endometrial Lesions in Rats Given Low-doses of BPA*

Dose	Incidence of lesions (%)			
	Hyperplasia			Adenocarcinoma**
	+	++	+++	
0 mg/kg/day	21	21	13	33
0.006 mg/kg/day	20	20	17	33
6 mg/kg/day	13	47	17	20

*: Yoshida *et al.*, *J Reprod Dev* 2004; 50: 349–360.

** : All adenocarcinomas in the three groups were well differentiated and limited to the uterus.

(80 ppm) detected in plastic plates⁸⁷. The treatment did not exert any influences on the reproductive system of female offspring in either treated group, in terms of prepubertal uterine growth and gland-genesis, vaginal opening and gonadotropin secretion. After maturation also, no effects were evident with regard to estrous cyclicity, age-matched sequential changes of the reproductive organs, and uterine carcinogenesis until 15 months of age (Table 5). The results demonstrated that maternal exposure to BPA at human exposure-levels did not have any adverse effects on the female reproductive organs of offspring in rats⁸⁸.

For determination of effects of EDCs on offspring by maternal treatment, biotransfer of the chemicals from dam to offspring is crucial, because the impact is fundamentally related to the serum EDC level⁴⁰. However, data for transfer of the test chemical via the placenta or milk to offspring, or toxicokinetics of low-dose EDCs are very limited⁸⁹. In one of our studies, NP at 10 and 100 mg/kg doses was transferred from dams to their offspring via the milk, but the compound could not be detected in their serum or liver⁷³. Furthermore, BPA levels in the milk of dams, and those in the serum and liver in offspring were comparable between control and treated groups, although the serum level of BPA in dams receiving 6 mg/kg was significantly elevated⁸⁸.

Quite recently, it was reported that cadmium has potent estrogen-like activity *in vivo*⁹⁰. Thus, exposure to low-dose cadmium (a single ip injection at a dose of 5 μ g/kg) increased uterine net weight accompanied by proliferation of the endometrium, promoted growth and development of the mammary glands, and induced hormone-regulated genes in ovariectomized rats. In utero exposure to the metal (i.p. injections of 0.5 or 5 μ g/kg on days 12 and 17 gestation) mimicked also the effects of estrogens, and female offspring experienced an earlier onset of puberty and an increase in the epithelial area and the number of terminal end buds in the mammary gland. The amounts of cadmium used in the study were environmentally relevant, because the WHO-recommended Provisional Tolerable Weekly Intake Level is 7 μ g/kg/week. Although the administration route was intraperitoneal, not oral, the ability of environmentally relevant amounts of cadmium to mimic the effects of estradiol is very important and the metal may represent a new class of EDC.

Species Differences in Toxicologic/carcinogenic Effects of EDCs, Effects of EDCs based on the Molecular Biology and Extrapolation of the Effects to Humans

It is well known that the toxicokinetics of chemicals in animals are influenced by many factors, including species, strain, sex, age, dosage and/or administration method, as mentioned above. In particular, species differences are very important for the risk assessment in humans. Species differences in occurrence of toxicologic/carcinogenic effects of EDCs may be an indication of variation in endogenous hormonal factors, in addition to susceptibility to exogenous agents. Mice are generally more sensitive to estrogens than rats, and uterine adenocarcinomas can be induced in mice by estrogen alone, but not in rats. In mice, perinatal exposure to estrogens was found to induce ovary-independent proliferation of the vaginal epithelium, which could not be abolished by ovariectomy⁹¹. On the other hand, the vagina in rats neonatally exposed to high-dose t-OP became atrophic immediately after ovariectomy³⁴. Adenocarcinoma development in the ENNG-initiated endometrium of Donryu rats exposed to high-dose t-OP was also ovary-dependent⁶⁴. In mice, however, E2 promoted uterine adenocarcinoma development ovary-independently⁵⁶. Differences in the reaction to estrogens or EDCs with estrogenic activity may help to explain species differences in toxicity/carcinogenicity, though further studies on this point, focusing on metabolism of estrogens or EDCs and localization of ER expression, are needed.

The prenatal and/or neonatal periods are more sensitive to estrogens, and also EDCs, than the adult period. As mentioned above, it has been pointed out that tamoxifen increases the risk of endometrial cancer in women. In rodents, TAM can induce uterine carcinomas when given to newborn animals, but not adults. Similarly, 2-OHE2 has weak estrogenic, but not carcinogenic effects, although 4-OHE2 is a potent estrogenic catechol causing uterine tumors in adult mice. However, both catechols induced tumors when given on days 1–5 of neonatal life, although carcinogenic activity of 4-OHE2 is stronger than that of 2-OHE2⁹². Further studies on age-dependent differences in the mechanism of EDCs' effects on the female genital organs are also needed.

Various methods such as DNA micro-array techniques based on gene expression levels have recently been used for the evaluation of hazardous effects of various chemicals, because they should reveal very early changes. Up till now, however, there have only been a few reports concerning EDCs. The fact that changes in ontogenic expression of ER alpha and not of ER beta occur in the fetal female rat reproductive tract, provides fundamental information critical for clarifying species-specific physiological roles of ER subtypes during fetal development and for investigating the tissue-specific mechanisms underlying prenatal responses to estrogen and E2 agonists⁹³. Genome-wide analysis of early gene expression has furthermore suggested a basis for the

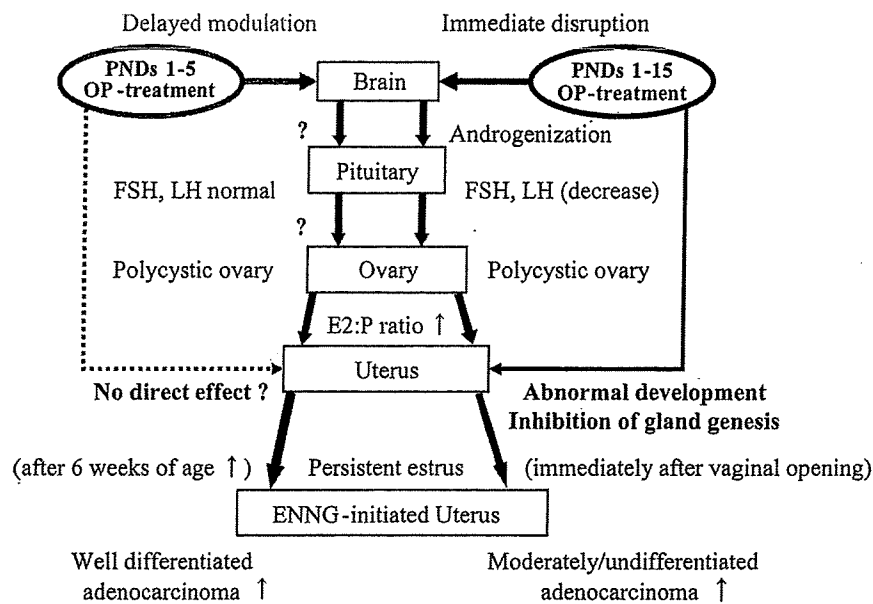


Fig. 3. Hypothesis of the scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP.

drastic uterotrophic effects following estrogen administration⁹⁴. Although DNA micro-array techniques presently demonstrate problems with reliability and reproducibility, future precise analysis should facilitate understanding of the mechanisms underlying effects of estrogenic EDCs⁹⁵.

Almost all EDCs exist at only very low concentrations in the environment, but humans may be exposed for long periods. In many animal studies, various toxicologic effects of EDCs on the female genital organs were demonstrated when very high doses were given, but no obvious effects were detected with low-doses. In humans also, low doses may show no adverse effects, because of homeostasis, although there are notable exceptions in animal and human studies. In fish, the ova-testis is known to be a good indicator of estrogenic effects of EDCs in females, but in female rodents, we are still lacking a consensus regarding equivalent reliable endpoint markers. Thus, more comprehensive studies of the endocrinological, morphological and also biomolecular aspects are necessary in animal studies using rodents for extrapolation of EDCs' effects to humans.

Conclusion

It is well known that the prenatal and/or neonatal period is particularly sensitive to various chemicals, including EDCs, in humans and rodents. Inappropriate exposure may exert irreversible influence, resulting in androgenization of the female genital system. In addition, it has also been reported that a delayed influence may be exerted. Neonatal exposure to a high dose of t-OP (100 mg/kg s.c. injection

every other day from PND 1 to PND 15) induced various long-term persistent irreversible effects on the female reproductive system of Donryu rats, such as lower gonadotropin levels at prepuberty, inhibition of uterine gland genesis, persistent estrus shown by vaginal cytology and polycystic ovaries. Neonatal treatment of high-dose EDCs having estrogenic activity can thus affect gonadotropin secretion during the developmental period of sexual maturation with direct masculinization of the hypothalamic function. Abnormal differentiation in the developing rat uteri may be induced via abnormal ER expression and subsequent alteration of cell proliferating activity. However, exposure limited to the first 5 days after birth to 100 mg/kg t-OP caused "delayed" influence which was characterized by accelerated appearance of atrophic ovary, manifested by an early-occurring and long-term continuing persistent estrus status after puberty, whereas no abnormalities could be found with regard to growth and development of the reproductive organs and the hypothalamo-pituitary-gonadal control system up to maturation. The hypothetic scheme for "androgenization effects" or "delayed modulation effects" on the hypothalamo-pituitary-gonadal system in rats exposed neonatally to high-dose OP is shown in Fig. 3.

On the other hand, the most notable effect on the female reproductive system when normal cycling rats were exposed to a high-dose of t-OP for a short time (28 days), was disappearance of the estrous cycle, and no clear changes were detected in other parameters such as uterine weight and morphology. These results indicate that the vaginal smear is the most sensitive parameter for the detection of effects of EDCs in normal cycling rats.

Well or moderately differentiated adenocarcinomas

were increased in Donryu rats initiated by ENNG, when high dose t-OP was given subcutaneously during adulthood. Neonatal exposure to a high dose of t-OP also showed promoting effects on uterine adenocarcinoma development in a two-stage rat uterine carcinogenesis model using Donryu rats, with slight to higher malignancy with more prolonged treatment.

For the risk assessment of EDCs to human health, it is very important to investigate the effects of low doses at actual human exposure levels, because the concentrations of agents, including alkylphenols and BPA, in the environment are very low. In addition, the main exposure route to EDCs is oral, not subcutaneous, in humans. Thus, we have focused on effects of maternal exposure to low doses of EDCs, such as NP and BPA, by the oral route, which have shown no effects on growth and development of the female reproductive system or uterine carcinogenesis. Transfer of low doses of BPA from dams to offspring via the placenta and/or milk was not unequivocal, although NP was transferred when relatively high doses were given.

These results indicate that dietary exposure to low doses of EDCs might not induce any adverse effects on the female genital system in mammals, including humans, because of the effects of homeostasis and clearance from the blood stream on first passage through the liver.

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Regulation of naive T cell function by the NF- κ B2 pathway

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T cell activation involves the orchestration of several signaling pathways, including that of the 'classical' transcription factor NF- κ B components NF- κ B1–RelA. The function of the 'nonclassical' NF- κ B2–RelB pathway is less clear, although T cells lacking components of this pathway have activation defects. Here we show that mice deficient in NF- κ B-inducing kinase have a complex phenotype consisting of immunosuppression mediated by CD25⁺Foxp3⁺ memory CD4⁺ cells and, in the absence of those cells, hyper-responsive naive CD4⁺ T cells, which caused autoimmune lesions after adoptive transfer into hosts deficient in recombination-activating genes. Biochemical studies indicated involvement of a cell-intrinsic mechanism in which NF- κ B2 (p100) limits nuclear translocation of NF- κ B1–RelA and thereby functions as a regulatory 'brake' for the activation of naive T cells.

The transcription factor NF- κ B is key in the regulation of many inflammatory processes of immune cells¹. The NF- κ B family consists of five subunits: NF- κ B1 (p105–p50), NF- κ B2 (p100–p52), RelA (p65), RelB and c-Rel. Hetero- or homodimers of these subunits can be translocated into the nucleus to bind to κ B sequences of neighboring 'target' genes, thus regulating the transcription of genes required for cell activation, survival and development². Two pathways of NF- κ B have been defined in immune cells³: the 'classical' pathway, which is initiated by complexes of NF- κ B1 and RelA, and an alternative or 'nonclassical' pathway, which is initiated by complexes of NF- κ B2 and RelB.

For T cells, stimulation via the T cell receptor (TCR) and costimulatory molecules such as CD28 leads to NF- κ B activation through a variety of intracellular signaling molecules⁴. Initially, TCR-CD28 signaling via many adaptor molecules leads to activation of protein kinase C- θ (PKC- θ)⁵. Thereafter, CARMA1–Bcl-10–MALT1 proteins 'downstream' of PKC- θ activate I κ B kinase (IKK) complexes, including IKK α , IKK β and the adaptor protein IKK γ (also called NEMO)^{6,7}. Activated IKK complexes then phosphorylate I κ B, releasing it from its constitutively bound state with cytoplasmic NF- κ B complexes (mainly NF- κ B1–RelA) that normally prevents NF- κ B complexes from translocating to the nucleus. Ubiquitination and degradation of I κ B by IKK complexes allows components of the classical NF- κ B pathway, especially p50–RelA, to be transported into the nucleus, thus promoting transcription of essential target genes required for survival, cytokine and chemokine production, upregulation of adhesion molecules, organogenesis and apoptosis in the immune system⁸. The classical NF- κ B pathway is especially important for the synthesis of interleukin 2 (IL-2) as well as IL-2 receptor (IL-2R, also called CD25) in T cells^{9,10}.

As for the alternative, nonclassical NF- κ B pathway, signals from specific cytokine receptors (such as the lymphotoxin- β receptor) activate NF- κ B-inducing kinase (NIK) as well as PKC- θ and CARMA1–Bcl-10–MALT1, which in turn activate homodimers of IKK α that are required for the cleavage of p100 to p52 (refs. 11–13). Complexes of p52 and RelB then translocate into the nucleus to regulate transcription of a different set of genes.

Studies of gene-knockout mice have demonstrated that individual members of the NF- κ B family have distinct roles *in vivo* in T cell function. Thus, T cell proliferation and T helper type 2 cytokine production is reduced in NF- κ B1-deficient (*Nfkb1*^{-/-}) mice, and these mice show increased susceptibility to experimental autoimmune encephalomyelitis, typhlocolitis and infection with *Leishmania major* but are resistant to asthma^{14–17}. RelA-deficient (*Rela*^{-/-}) mice have a phenotype that is embryonically lethal, but studies with fetal liver chimeras indicate that *Rela*^{-/-} T cells are functionally defective^{18,19}. RelB-deficient (*Relb*^{-/-}) mice are viable but develop systemic inflammation and severe anemia around 2–3 months of age, and the T cell and B cell functions of these mice are suppressed²⁰. NF- κ B2-deficient (*Nfkb2*^{-/-}) mice have B cell defects as well as T cell hyperplasia and hyperactivation of dendritic cells^{21,22}. Finally, mice deficient in c-Rel (*Rel*^{-/-}) have B cell defects, impaired T cell proliferative responses and reduced susceptibility to experimental autoimmune encephalomyelitis^{23,24}. Nevertheless, precise information about the functions of the individual NF- κ B family members on T cell function is still unclear.

The importance of NIK for NF- κ B activation has been demonstrated in studies of NIK-deficient (*Map3k14*^{-/-}) mice and also mice with alymphoplasia (*Map3k14*^{aly/aly} or 'aly/aly' mice), which carry a mutation of *Map3k14*. Functionally, NIK is key in regulating the

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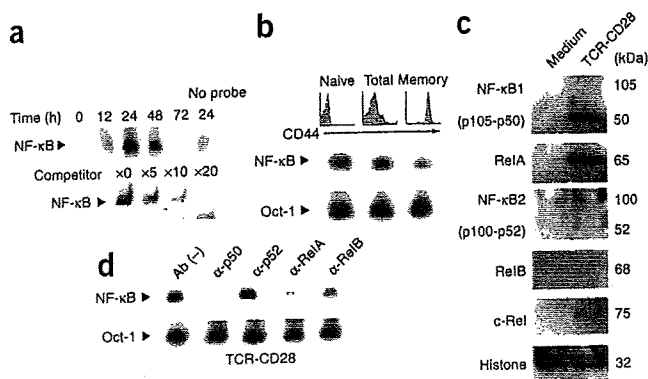


Figure 1 NF- κ B activation in CD4⁺ T cells. (a) EMSA of NF- κ B activity in nuclear extracts from CD4⁺ T cells from lymph nodes of B6 mice stimulated (times, above lanes) with crosslinked anti-TCR (1 μ g/ml) and anti-CD28 (20 μ g/ml). Below, EMSA with competitor (concentration, above lanes). (b) EMSA of NF- κ B and Oct-1 activity in nuclear extracts of total, naive CD44^{lo} or memory CD44^{hi} CD4⁺ T cells stimulated for 24 h by TCR-CD28 ligation as in a. Top, CD44 expression on the cells before culture, determined by flow cytometry. (c) Immunoblot for NF- κ B subunits in nuclear extracts of naive CD4⁺ T cells cultured in medium alone or activated for 24 h by TCR-CD28 ligation. (d) Antibody supershift assay of nuclear extracts from purified naive CD4⁺ T cells obtained from B6 lymph nodes; cells were stimulated for 24 h by TCR-CD28 ligation. Ab (-), no antibody; α -, antibody to. Results are representative of three to five independent experiments.

processing of p100 to p52 through IKK α both in hematopoietic cells and osteoclasts^{25–27}. *Map3k14*^{-/-} and *aly/aly* mice lack lymph nodes, and, at least for *aly/aly* mice, T cells show defective proliferation and IL-2 production in response to stimulation with antibody to CD3 (anti-CD3)^{13,28,29}. In addition, NIK may be involved in the maintenance of central tolerance in the thymus³⁰. Moreover, *aly/aly* mice as well as *Relb*^{-/-} mice show signs of autoimmune disease^{20,31,32}. Despite those findings, the mechanism for the regulation of peripheral T cell activation through NIK has not been established.

Much of the data on the function of NIK has come from studies of T cell populations that have not been separated into individual subsets based on their activation status. Expression of certain surface markers, notably CD44, distinguishes mature T cells as those that are immunologically naive (naive T cells) versus those that have been primed through contact with environmental antigens (memory T cells)^{33,34}. In mice, low or intermediate expression of CD44 (CD44^{lo} or CD44^{int}) indicates a naive differentiation status, whereas CD44^{hi} cells have differentiated into memory cells. Here we examine the functions of NIK, both *in vivo* and *in vitro*, in purified subsets of naive and memory CD4⁺ cells.

RESULTS

NF- κ B1 in CD4⁺ cell activation

To define the kinetics of NF- κ B activation during the course of normal T cell activation, we analyzed the transcriptional activity of NF- κ B in stimulated CD4⁺ T cells from normal C57BL/6 (B6) mice by electrophoretic mobility-shift assay (EMSA) using an NF- κ B-binding DNA probe. After total CD4⁺ T cells were stimulated with plate-bound monoclonal antibodies (mAbs) to TCR and CD28, activation of NF- κ B, measured in nuclear extracts of the cells, reached a peak after 24 h and then decreased to undetectable amounts by 72 h (Fig. 1a). We confirmed the specificity of NF- κ B activation by using unlabeled NF- κ B-binding DNA as a competitor to diminish the signal.

To determine NF- κ B activation in naive and memory CD4⁺ T cells, we stimulated enriched subsets of normal B6 CD44^{lo} (naive) and CD44^{hi} (memory) CD4⁺ T cells by TCR-CD28 ligation for 24 h, followed by EMSA to detect transcriptional activity of NF- κ B in the nuclear extracts; we used total CD4⁺ cells as a control. Nuclear translocation of NF- κ B was much more prominent for naive CD4⁺ cells than for memory cells; in contrast, transcriptional activity of an internal control (Oct-1) was the same for both subsets of CD4⁺ T cells (Fig. 1b).

To determine the extent of nuclear translocation of individual NF- κ B family members in each subset, we treated naive CD4⁺ T cells for 24 h with mAbs to TCR and CD28, then purified nuclear extracts and did immunoblot analysis with NF- κ B protein subunit-specific antibodies. Nuclear extracts had substantial amounts of both p50 and RelA, a small amount of c-Rel protein and a conspicuous absence of p52 or RelB proteins (Fig. 1c). Consistent with those findings, analysis of the nuclear extracts after incubation with antibodies specific for each NF- κ B protein subunit ('supershift assay') showed that the mobility shift of the NF- κ B DNA probe was greatest with anti-p50 or anti-RelA but only minimal with anti-p52 or anti-RelB (Fig. 1d), suggesting a predominance of p50-RelA dimers. That indicated, therefore, that early activation of NF- κ B in naive CD4⁺ cells reflects nuclear translocation of NF- κ B1 (p50)-RelA, with little or no contribution from NF- κ B2 (p52)-RelB.

NF- κ B2 in CD4⁺ T cell activation

The findings reported above failed to explain the T cell defects seen in NIK-deficient *aly/aly* and *Relb*^{-/-} mice^{13,20,29}. Total CD4⁺ T cells have been used in studies published before; thus, it was unclear whether the abnormalities noted occur at the level of specific CD4⁺ T cell subsets. To examine that issue, we compared the functions of total CD4⁺ cells and enriched subsets of naive and memory CD4⁺ cells. As anticipated from prior studies^{13,20,29}, the proliferative responses of total CD4⁺ cells treated for 3 d *in vitro* with mAbs to TCR and CD28 were much lower (50–70% reduction) for *Relb*^{-/-}, *aly/aly* and *Map3k14*^{-/-} mice than for heterozygous littermates or wild-type B6 mice (Fig. 2a). We obtained similar findings with mixed-lymphocyte reactions, in which proliferative responses were elicited by exposure to allogeneic (BALB/c) spleen cells (Fig. 2b). The decreased response of *Relb*^{-/-}, *aly/aly* and *Map3k14*^{-/-} CD4⁺ cells was only mildly improved after removal of CD25⁺CD4⁺ cells; that is, cells with T regulatory function (T_{reg} cells)^{35–37} (Fig. 2a–c). That finding was unexpected because removing CD25⁺ T_{reg} cells from control CD4⁺ cell samples, thus leaving CD25⁻CD4⁺ cells, led to enhanced responses (Fig. 2c, left versus right). Notably, CD25⁺CD4⁺ cells are nearly all CD44^{hi}, but about 50% of CD44^{hi} cells are CD25⁻. In wild-type mice, CD25⁻CD44^{hi}CD4⁺ cells have little or no T regulatory function and are generally considered to be memory (or 'memory-phenotype') cells. As shown for *aly/+* cells in Figure 2c, left, depleting normal control cell samples of both CD25⁺ and CD44^{hi} cells, thus leaving enriched naive CD25⁻CD44^{lo}CD4⁺ cells, led to much lower proliferative responses than those noted after removal of CD25⁺ cells alone. These findings indicated that in wild-type mice, total CD44^{hi} cells are a mixture of two functionally distinct populations: an inhibitory population of CD25⁺ T_{reg} cells, and a helper population of CD25⁻ T memory cells, which probably release stimulatory cytokines (discussed below). The situation with respect to these cell subtypes and functions is radically different for NIK-deficient cells.

For NIK-deficient *aly/aly* cells, poor TCR-mediated proliferative responses were generally improved only slightly after selective removal

of CD25⁺ cells (classical T_{reg} cells). In contrast, removal of both CD25⁺ and CD44^{hi} cells led to a substantial increase in the response; thus, the remaining CD25⁻CD44^{lo}CD4⁺ naive aly/aly T cells demonstrated a hyper-responsive proliferation compared with that of a comparable population of CD4⁺ cells from control mice (Fig. 2a,b,d versus Fig. 2d–f). We noted the hyper-responsiveness of CD44^{lo}CD4⁺ aly/aly cells in samples from *Map3k14*^{-/-} and *Relb*^{-/-} mice, and it was apparent for both mixed-lymphocyte reactions and TCR-CD28–induced proliferation (Supplementary Fig. 1 online). We also noted hyper-responsiveness for the main subset of CD44^{int} cells, which like CD44^{lo} cells are considered immunologically naive (that is, they have not experienced foreign antigen stimulation; Fig. 2f); in contrast, CD44^{hi}CD4⁺ cells were hypo-responsive compared with control cells (Fig. 2g and Supplementary Fig. 1). In these and all subsequent experiments, samples were enriched for ‘memory’ CD44^{hi} cells by removal of CD25⁺ cells (which included activated T cells as well as T_{reg} cells) and CD62L⁺ cells.

The main conclusion from the experiments reported above is that in contrast to wild-type control cells, naive CD4⁺ cells in NIK-deficient and *Relb*^{-/-} mice are poised to hyper-respond to TCR-mediated signals and the hyper-proliferative response is ‘suppressed’ by CD25⁻CD44^{hi} memory T cells (called ‘CD44^{hi}CD4⁺ memory T cells’ here) when total CD4⁺ T cells are assayed. As discussed below, the properties of CD44^{hi}CD4⁺ memory T cells in wild-type and aly/aly mice are very different: CD44^{hi}CD4⁺ memory T cells from wild-type mice function as helper T cells for primed naive cells, whereas CD44^{hi}CD4⁺ memory T cells from aly/aly mice function as ‘suppressor’ cells.

To quantitatively evaluate the suppressor function of aly/aly CD44^{hi}CD4⁺ memory T cells, we monitored the dilution of carboxy-fluorescein diacetate succinimidyl diester (CFSE) in naive CD44^{lo}CD4⁺ ‘reporter’ cells preincubated with the fluorescent dye. Early (48 h) proliferative responses of CFSE-labeled control (aly/+) CD44^{lo}CD4⁺ cells were appreciably enhanced by the addition of a similar number (5×10^4) of syngeneic aly/aly CD44^{hi}CD4⁺ memory T cells (Supplementary Fig. 2 online). In contrast, the proliferation of aly/aly CD44^{lo}CD4⁺ cells was reduced considerably by the addition of syngeneic aly/aly CD44^{hi}CD4⁺ memory T cells (Supplementary Fig. 2). We obtained similar results when we incubated CD44^{hi}CD4⁺ memory T cells from wild-type B6 or aly/aly mice (both Thy-1.2⁺) with CFSE-labeled CD44^{lo}CD4⁺ cells from B6.PL mice (Thy-1.1⁺; Supplementary Fig. 2). For CD44^{hi}CD4⁺ memory T cells from wild-type mice, the ‘helper’ effect of these cells correlated with increased IL-2 in the mixed cultures (Supplementary Fig. 2), presumably reflecting IL-2 synthesis by the added CD44^{hi}CD4⁺ memory T cells. In contrast, the inhibitory influence of aly/aly CD44^{hi}CD4⁺ memory T cells correlated with a decrease in IL-2 in the cultures (Supplementary Fig. 2). To directly test the function of IL-2 in this experimental situation, we

added exogenous IL-2 and found that it was sufficient to overcome the inhibitory effect of adding aly/aly CD44^{hi}CD4⁺ memory T cells (Supplementary Fig. 2).

Because the CD44^{hi}CD4⁺ memory T cell samples in the experiments reported above were depleted of typical CD25⁺ T_{reg} cells, the substantial suppressive influence of aly/aly CD25⁻CD44^{hi}CD4⁺ memory T cells was very unexpected. These cells could have had high expression of Foxp3, a transcription factor that in normal mice is selectively expressed mainly in CD25⁺ T_{reg} cells^{38–40}. That was not the case, however, as we found that Foxp3 protein was undetectable in both wild-type and aly/aly CD25⁻CD44^{hi}CD4⁺ memory T cells even after TCR stimulation (Supplementary Fig. 3 online). Detection of Foxp3 protein was restricted to CD25⁺CD4⁺ typical T_{reg} cells, and the numbers of Foxp3⁺ cells were much lower (70–80% reduction) for aly/aly than for wild-type, consistent with published findings^{30,41}.

Mechanism of suppression

The findings reported above indicated that aly/aly naive and memory T cell subsets differ considerably in their capacity to synthesize and/or use IL-2. To test that hypothesis, we evaluated both cell subsets for IL-2 synthesis and IL-2R α (CD25) expression after TCR-CD28 ligation (Fig. 3). For naive CD4⁺ T cells, there was somewhat less IL-2, as measured by enzyme-linked immunosorbent assay (ELISA), in the

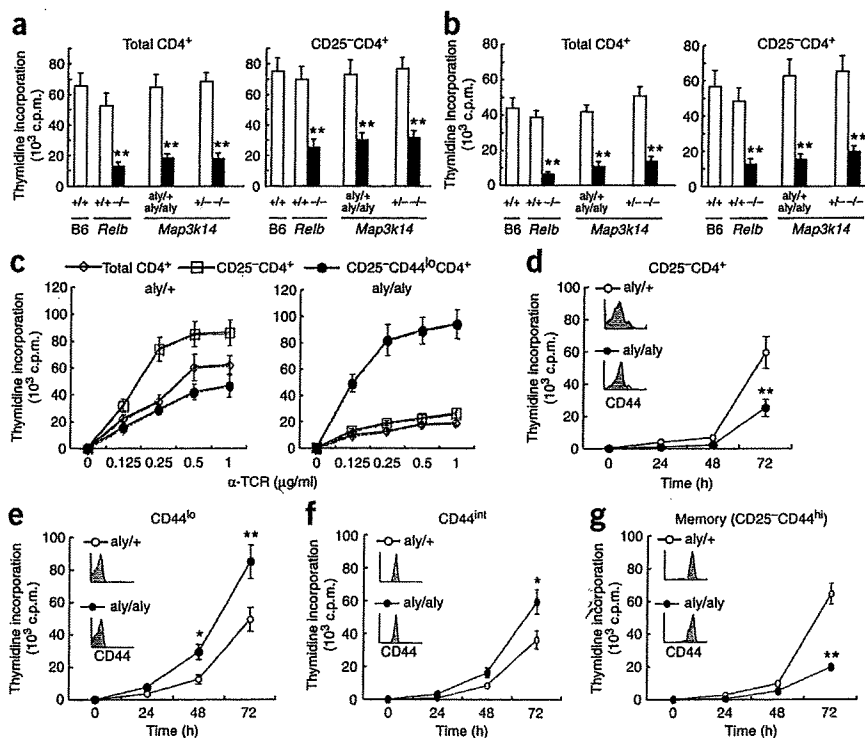


Figure 2 T cell responses of mice deficient in NF- κ B2-RelB. (a,b) Proliferation assays of total CD4⁺ and enriched CD25⁻CD4⁺ cell populations from spleens of *Relb*^{-/-}, aly/aly, NIK-deficient (*Map3k14*^{-/-}) and control (B6, *Relb*^{+/+}, aly/+, and *Map3k14*^{+/+}) mice stimulated for 72 h by TCR-CD28 ligation as in Figure 1 (a) or by culture for 96 h together with irradiated T cell-depleted spleen cell samples from BALB/c mice (b). (c) Proliferation assays of total, CD25⁻ and CD25⁻CD44^{lo} CD4⁺ T cells from aly/+ and aly/aly mice; cells were stimulated for 72 h with 0–1 μ g/ml (horizontal axes) of mAb to TCR (α -TCR) and 20 μ g/ml of mAb to CD28. (d–g) Proliferation assays of CD25⁻CD4⁺ cells and enriched subsets of CD44^{lo}, CD44^{int} and CD44^{hi} CD4⁺ T cells (all CD25⁻) separated by FACSsort and stimulated for 72 h with TCR-CD28 ligation. Insets, CD44 expression on the cells before culture. *, $P < 0.05$, and **, $P < 0.005$, aly/aly mice versus control mice. Data are means \pm s.d. of triplicate samples and are representative of three independent experiments.

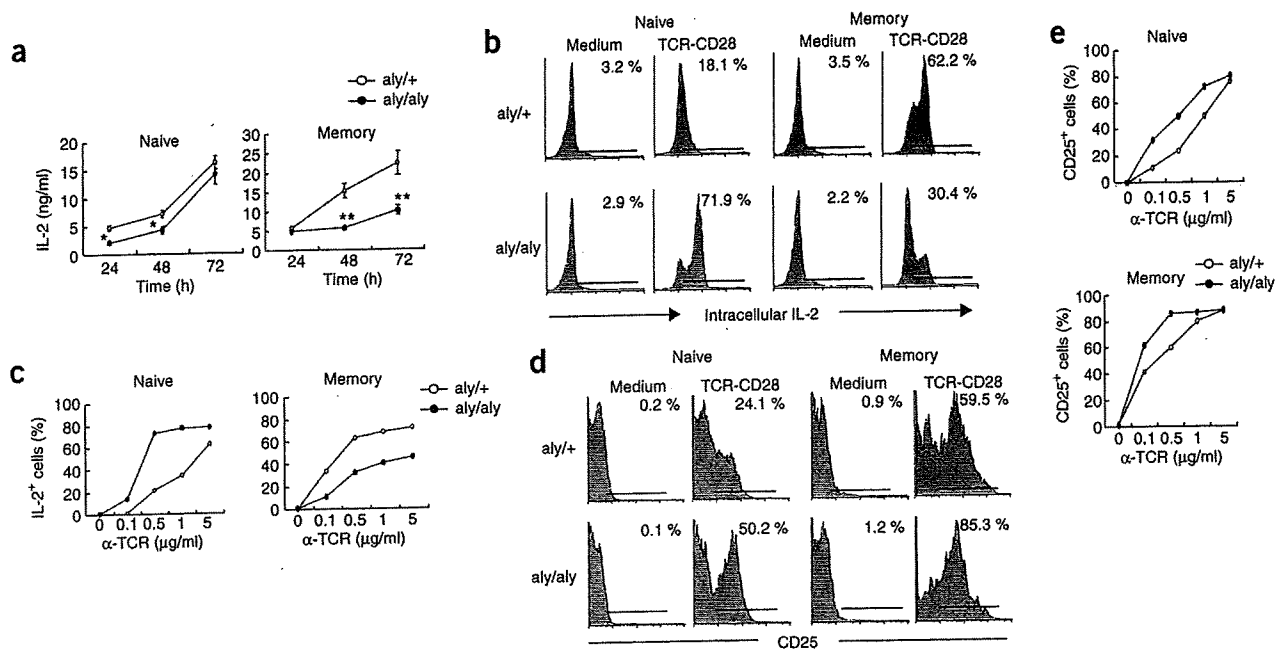


Figure 3 IL-2 secretion and IL-2R synthesis by aly/aly CD4⁺ subsets. (a) ELISA of IL-2 in culture supernatants of aly/aly and aly/+ naive CD44^{lo} and memory CD44^{hi}CD4⁺ T cells stimulated (time, horizontal axes) by TCR-CD28 ligation (as in Fig. 1a). Data are means \pm s.d. of triplicate samples and are representative of four independent experiments. *, $P < 0.05$, and **, $P < 0.005$, aly/aly mice versus control mice. (b,c) Flow cytometry for intracellular IL-2 in aly/aly and aly/+ naive CD44^{lo} and memory CD44^{hi}CD4⁺ T cells stimulated for 24 h with mAb to TCR (0.1–5 μ g/ml; horizontal axes) and mAb to CD28 (20 μ g/ml; 'TCR-CD28'). (b) Representative data for percent IL-2⁺ cells after stimulation with 'graded' concentrations of mAb to TCR. (c) Mean percent of IL-2⁺ cells after stimulation with 'graded' concentrations of mAb to TCR. (d,e) Flow cytometry for CD25 expression on CD4⁺ T cells of aly/aly and aly/+ mice from b,c. (d) Numbers above horizontal lines indicate percent CD25⁺ cells after stimulation at 0.5 μ g/ml of mAb TCR. (e) Percent CD25⁺ cells after stimulation with 'graded' concentrations of mAb to TCR. Results are representative of three to five independent experiments.

culture supernatants of aly/aly cells than of wild-type (aly/+) cells (Fig. 3a). That result was unexpected given the enhanced proliferative responses of naive aly/aly cells; however, intracellular staining showed that there was much more IL-2 protein in the cytoplasm of aly/aly cells than wild-type cells (Fig. 3b,c). Likewise, induction of CD25 cell surface expression was much greater on aly/aly cells than on wild-type cells (Fig. 3d,e). Hence, the reduced IL-2 in the culture supernatants of naive aly/aly cells presumably reflected enhanced IL-2 consumption through binding to the increased CD25 expressed on the cell surface. From these results we concluded that the increased proliferative responses of aly/aly naive cells correlated with increased IL-2 and IL-2R protein synthesis.

For CD44^{hi}CD4⁺ memory T cells, there was much less IL-2 synthesis in cells from aly/aly mice than in cells from control (aly/+) mice, as assessed by both the amount of IL-2 secreted into the culture supernatant (Fig. 3a) and the amount detected inside the cells by intracellular staining (Fig. 3b,c). However, the results were very different for IL-2R; compared with wild-type CD44^{hi}CD4⁺ memory T cells, aly/aly CD44^{hi}CD4⁺ memory T cells had enhanced CD25 cell surface expression, similar to that seen in the aly/aly naive cells (Fig. 3d,e), and the cells also demonstrated enhanced CD69 expression (data not shown). Hence, the reduced proliferative response of aly/aly CD44^{hi}CD4⁺ memory T cells correlated with poor IL-2 synthesis despite high IL-2R induction. The suppressive effect of aly/aly CD44^{hi}CD4⁺ memory T cells on naive CD4⁺ T cells therefore might reflect the possibility that aly/aly CD44^{hi}CD4⁺ memory T cells deplete the cultures of IL-2 because of enhanced expression of CD25. In agreement with that interpretation, proliferative responses of both wild-type and aly/aly naive CD4⁺ cells were considerably reduced by

depleting the cultures of IL-2 with mAb to IL-2 (Supplementary Fig. 2). Moreover, in mixed cultures of naive and memory CD4⁺ T cells (Supplementary Fig. 2), poor IL-2 synthesis by these cells combined with high IL-2R expression led to IL-2 depletion, which is the likely mechanistic explanation of the suppressive property of aly/aly memory cells.

The finding that total aly/aly CD4⁺ cells were hyporesponsive to TCR-CD28 ligation after selective depletion of CD25⁺ cells suggested that the CD25⁺ T_{reg} cells in aly/aly mice are functionally defective. Alternatively, the aly/aly T_{reg} cells might have 'normal' suppressive activity but function poorly because their relative numbers are much lower than those in wild-type mice (Supplementary Fig. 3). In support of the last idea, the capacity of purified CD25⁺CD4⁺ cells to suppress proliferation of wild-type naive CD4⁺ T cells was almost as efficient for aly/aly CD25⁺ cells as it was for wild-type CD25⁺ cells (Supplementary Fig. 4 online). Likewise, aly/aly and wild-type CD25⁺ cells were comparable in their low synthesis of IL-2 but high synthesis of both IL-10 and transforming growth factor- β (Supplementary Fig. 4).

In vivo responses

To examine responses *in vivo*, we first compared normal and aly/aly CD4⁺ subsets for their capacity to undergo homeostatic proliferation in syngeneic irradiated mice. As described before⁴², the paucity of T cells in irradiated mice allows adoptively transferred CD4⁺ T cells to proliferate in response to major histocompatibility complex class II-restricted self peptides. Homeostatic proliferation of CFSE-labeled naive CD44^{lo}CD4⁺ cells was greater for aly/aly cells than for normal aly/+ cells (Fig. 4a). In contrast, homeostatic proliferation

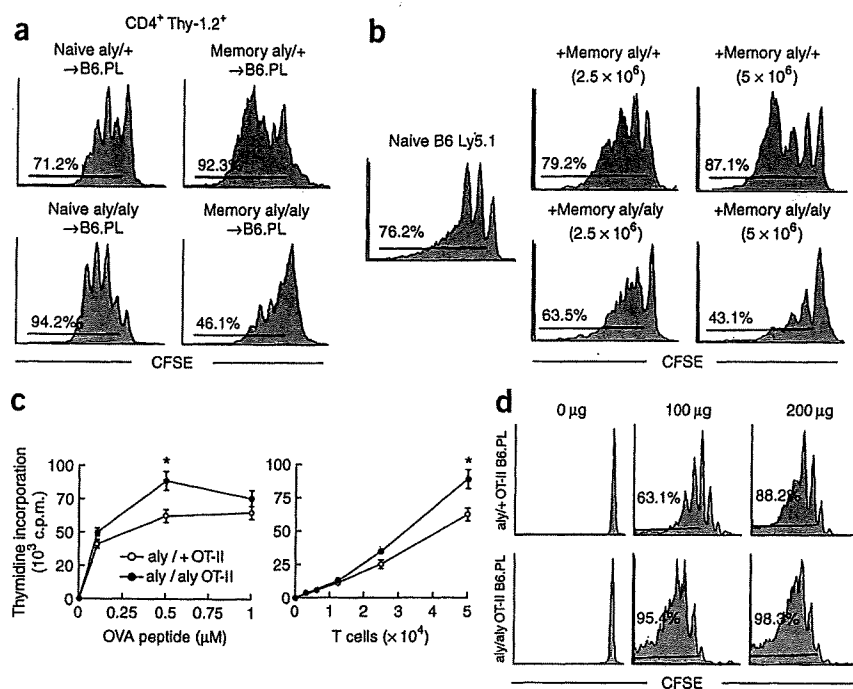


Figure 4 Proliferative responses of naive and memory aly/aly CD4⁺ subsets *in vivo*. (a) Flow cytometry of CFSE-labeled naive CD44^{lo} or memory CD44^{hi} CD4⁺ T cells (5×10^6) from aly/aly and aly/+ mice (both Thy-1.2) transferred 7 d previously into irradiated (700 rads) B6.PL (Thy-1.1) mice. (b) Flow cytometry of CFSE-labeled naive CD4⁺ T cells (5×10^6) from B6 Ly5.1 mice transferred together with unlabeled aly/+ or aly/aly memory cells (2.5×10^6 or 5×10^6 ; both Ly5.2) into irradiated (700 rads) B6 mice. Ly5.1⁺CD4⁺ T splenocytes were evaluated 7 d after transfer. (c) Proliferative assay of naive CD44^{lo}CD4⁺ T cells from either aly/aly OT-II or aly/+ OT-II mice cultured for 72 h *in vitro* with irradiated (1,500 cGy) T cell-depleted B6 spleen cell samples (5×10^5 cells) in the presence of 'graded' concentrations of OVA peptide (left) or with 'graded' numbers of T cells and 0.5 μ M OVA peptide (right). Data are means \pm s.d. of triplicate cultures. *, $P < 0.05$, aly/aly OT-II versus aly/+ OT-II cells. (d) Flow cytometry of CFSE-labeled naive CD4⁺ T cells (5×10^6) from aly/aly or aly/+ OT-II B6.PL mice (both Thy-1.1⁺) transferred intravenously into B6 (Thy-1.2⁺) mice; 1 d later, OVA peptide (0–200 μ g) was injected intraperitoneally into recipient mice. Thy-1.1⁺V β 5.2⁺CD4⁺ T splenocytes were analyzed 3 d after peptide injection. Numbers above horizontal lines (a,b,d) indicate percentages of divided cells from the fourth division. Results are representative of two (b) or three (a,c,d) independent experiments.

of CD44^{hi}CD4⁺ memory T cells was much less for aly/aly cells than for normal aly/+ cells. Likewise, homeostatic proliferation of wild-type naive CD4⁺ cells (CFSE labeled) *in vivo* was enhanced by the addition of wild-type memory CD4⁺ cells (not CFSE labeled) but was inhibited by the addition of aly/aly CD44^{hi}CD4⁺ memory T cells (Fig. 4b). There was also hyper-responsiveness of naive aly/aly CD4⁺ cells *in vivo* for antigen-specific CD4⁺ cells. Thus, compared with control aly/+ naive OT-II cells, aly/aly naive CD44^{lo}CD4⁺ OT-II cells demonstrated enhanced proliferative responses to stimulation with specific ovalbumin (OVA) peptide *in vitro* (Fig. 4c) and to a limiting dose of OVA peptide (100 μ g/mouse) *in vivo* (Fig. 4d).

NF- κ B expression in naive versus memory CD4⁺ cells

To understand the hyper-responsiveness of naive aly/aly cells, it was important to determine the expression of individual NF- κ B subunits in wild-type and NIK-deficient CD4⁺ cells. For naive CD44^{lo}CD4⁺ cells from control aly/+ mice, confocal microscopy after TCR-CD28 ligation for 24 h showed increased synthesis of NF- κ B1 and RelA in the cytoplasm and translocation of both subunits to the nucleus (Fig. 5a); the two subunits were in close proximity, as indicated by

their merged fluorescence. We obtained similar results for naive aly/aly cells, although in this case the fluorescence of NF- κ B1 and RelA was stronger in the nucleus than in the cytoplasm, suggesting increased nuclear translocation (Fig. 5a). In support of that conclusion, immunoblot analysis of nuclear extracts showed substantially more p50 and RelA in aly/aly cells than in aly/+ cells (Fig. 5b). In contrast, NF- κ B2 and RelB were almost undetectable in the nuclei of both aly/aly and aly/+ cells, despite high expression of both proteins in the cytoplasm of aly/+ cells (Fig. 5a). These data confirmed that at 1 d after TCR-CD28 ligation, nuclear translocation of NF- κ B was restricted mainly to NF- κ B1 (p50)-RelA dimers (Fig. 1). There was also enhanced nuclear translocation of p50 and RelA in naive RelB^{-/-} cells, as demonstrated by both confocal and immunoblot analyses (Supplementary Fig. 5 online). Thus, hyper-responsiveness of naive aly/aly and RelB^{-/-} CD4⁺ T cells correlated with increased nuclear translocation of NF- κ B1 (p50)-RelA compared with that of wild-type naive T cells.

Through I κ B-like ankyrin repeats, NF- κ B2 p100 can bind other NF- κ B family members and thereby prevent their nuclear translocation³. The hyper-responsiveness of naive aly/aly CD4⁺ cells, therefore, might reflect reduced p100. To test that possibility, we evaluated cytoplasmic and total cell lysates of naive aly/aly CD4⁺ cells; both showed a substantial reduction in p100 protein relative to that in aly/+ cells (Fig. 5c). We confirmed that result by fluorescence resonance energy transfer (FRET) analysis, which showed that association of p52-p100 with RelA or p50 in aly/aly cells was undetectable (Fig. 5d). In contrast, control naive aly/+ cells showed substantial intracytoplasmic association of p52-p100 with both RelA and p50 (Fig. 5d). For the control aly/+ and wild-type B6 cells, we confirmed the association of RelA and p50 with p52-p100 by immunoprecipitation of cytoplasmic extracts with anti-p52-p100 followed by immunoblot with NF- κ B subunit-specific antibodies. After TCR-CD28 stimulation, p52-p100 protein (mostly p100) was associated with RelB, RelA and p50 but not with c-Rel (Fig. 5e and Supplementary Fig. 5). Also, immunoprecipitation of [³⁵S]methionine-labeled cells with anti-RelA demonstrated a notable p100-RelA complex in wild-type cells but not in aly/aly cells (Supplementary Fig. 5). Based on those observations, the hyper-responsiveness of naive aly/aly CD4⁺ cells can be attributed to reduced p100, which enhances nuclear translocation of p50-RelA and NF- κ B-mediated gene transcription and cell activation.

For aly/+ CD44^{hi}CD4⁺ memory T cells, there was increased production and nuclear translocation of both NF- κ B1 and RelA after TCR-CD28 ligation, although less than for naive aly/+ cells (Fig. 5a). Nuclear translocation of the alternative NF- κ B2 and RelB proteins was very prominent for aly/+ CD44^{hi}CD4⁺ memory T cells and correlated with much more p52 in total cell lysates (Fig. 5c). In notable contrast, aly/aly CD44^{hi}CD4⁺ memory

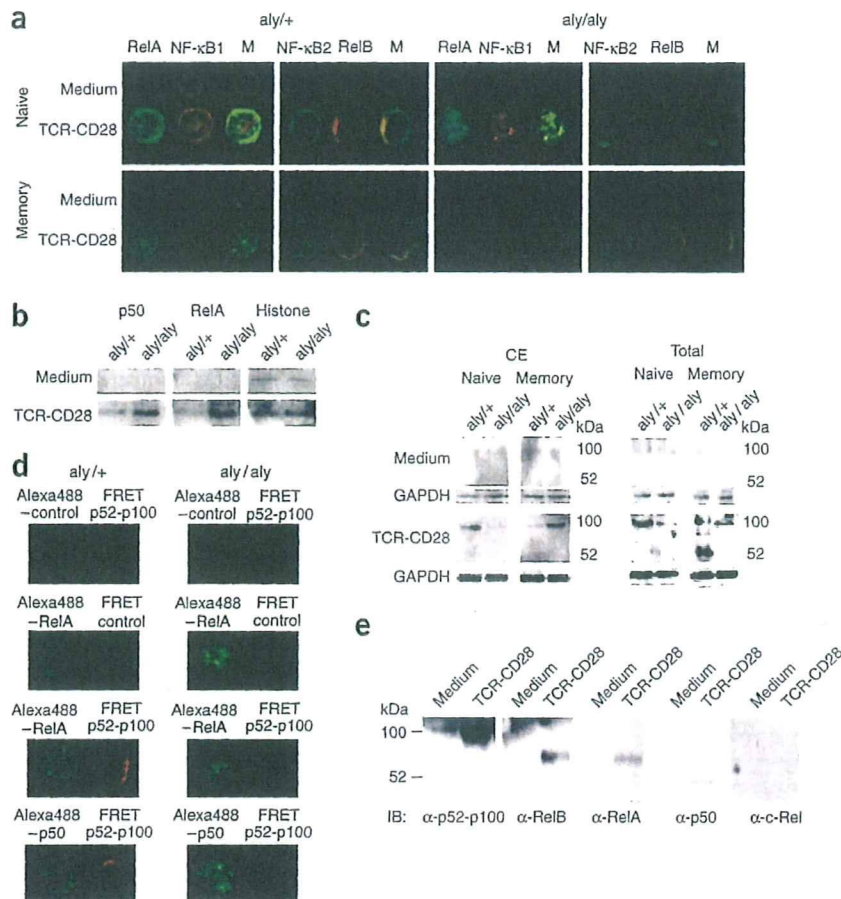


Figure 5 Molecular interactions between NF- κ B subunits during T cell activation. (a) Confocal microscopy of naive CD44^{hi} and memory CD4⁺ T cells from *aly/aly* and *aly/+* mice stimulated for 24 h with TCR-CD28 ligation (as in Fig. 1), fixed in 3% paraformaldehyde on a glass slide and stained with anti-p50, anti-RelA, anti-p52 and anti-RelB followed by Alexa Fluor 488-labeled (green) or Alexa Fluor 568-labeled (red) anti-mouse or anti-rabbit IgG. M, merged image. Original magnification, $\times 630$. (b) Immunoblot to detect p50 and RelA in nuclear extracts from naive *aly/aly* and *aly/+* CD4⁺ T cells after 24 h of TCR-CD28 ligation. Histone serves as a control. (c) Immunoblot to detect NF- κ B2 (p52-p100) in total and cytosolic extracts (CE) of stimulated naive and memory CD4⁺ T cells from *aly/aly* and *aly/+* mice. Glyceraldehyde phosphate dehydrogenase (GAPDH) serves as a control. (d) FRET analysis of *aly/aly* and *aly/+* naive CD4⁺ T cells stimulated for 24 h with TCR-CD28 ligation, fixed in 3% paraformaldehyde on a glass slide and stained with Alexa Fluor 488-labeled (Alexa488) anti-p50 or anti-RelA and Alexa Fluor 546-labeled anti-p52. The binding of p52 to RelA or p50 is detected as a FRET signal (red). Control, no first antibody. Original magnification, $\times 630$. (e) Immunoprecipitation with anti-NF- κ B2 p100-p52 and immunoblot (IB) for p100-p52, RelB, p50, RelA and c-Rel in cytosolic extracts of naive B6 CD4⁺ T cells stimulated for 24 h with TCR-CD28 ligation. Results are representative of three to five independent experiments.

T cells demonstrated no detectable nuclear translocation of RelA, NF- κ B1, NF- κ B2 or RelB (Fig. 5a). For NF- κ B2, p100 was apparent in the cytoplasm of *aly/aly* CD44^{hi}CD4⁺ memory T cells, as shown by both confocal microscopy (Fig. 5a) and immunoblot of cytoplasmic extracts (Fig. 5c); processing of p100 to p52 was undetectable.

Kinetics of p100 synthesis and p52 nuclear translocation

The data reported above apply to early (day-1) responses to TCR-CD28. For naive *aly/aly* CD4⁺ cells, kinetics experiments showed that p100 in protein cytoplasmic extracts was undetectable for up to 72 h, which correlated with above-normal nuclear translocation of NF- κ B1-p50 throughout this time (Fig. 6a,b). In contrast, for *aly/+* naive CD4⁺ cells, the amount of p100 in cytoplasmic extracts was high at 24 and 48 h, which correlated with only moderate amounts of NF- κ B1-p50 in nuclear extracts. We also noted that nuclear p50 in *aly/+* naive cells fell to undetectable amounts by 72 h and was 'replaced' by low but detectable amounts of p52-RelB. We noted the delayed nuclear translocation of p52-RelB only for *aly/+* and not *aly/aly* cells, and this paralleled a decrease in cytoplasmic p100, perhaps reflecting p100-to-p52 processing; delayed nuclear translocation of p52-RelB in *aly/+* cells was also apparent by confocal microscopy (Fig. 6c). These observations strengthen the view that via direct protein-protein interaction, p100 in the cytoplasm serves to inhibit nuclear translocation of p50-RelA in *aly/+* naive CD4⁺ cells and thereby acts as a 'brake' for gene transcription. The data also indicate that after several days, NF- κ B activation in *aly/+* naive CD4⁺ cells involves a 'switch' from NF- κ B1- to NF- κ B2-dependent pathways.

Induction of autoimmune disease

NIK-deficient and *Relb*^{-/-} mice develop CD4-dependent, slow-onset (> 14 weeks), multiorgan autoimmune disease, which can be 'adoptively transferred' to hosts deficient in recombination-activating gene 2 (*Rag2*^{-/-} hosts)^{20,31,32}. Given the data reported above, autoimmune disease might be enhanced by the removal of CD44^{hi}CD4⁺ memory T cells. To investigate that possibility, we adoptively transferred enriched subsets of *aly/+* and *aly/aly* CD4⁺ T cells into *Rag2*^{-/-} hosts. For both control naive and memory *aly/+* CD4⁺ T cells, cell numbers recovered from the spleen after adoptive transfer were modest (about 5×10^5 cells/mouse; Fig. 7a), infiltration of lymphocytes in the lungs and lacrimal glands was minimal (Fig. 7b) and evidence of autoimmune disease in those tissues was undetectable (Fig. 7c). We obtained very different results after injecting *aly/aly* CD4⁺ cells. For those, injection of either total CD4⁺ or CD25⁻CD4⁺ cells resulted in relatively low recovery of cells from the spleen (Fig. 7c), thus correlating with the hyporesponsiveness of the *aly/aly* cells (as demonstrated above). As for autoimmune disease induction, both populations produced mild but detectable lymphocyte infiltration of lungs and lacrimal glands, with such pathology being slightly more prominent with CD25⁻ cells (Fig. 7b,c). The effects were much more prominent, however, after injection of enriched naive CD4⁺ cells (that is, samples depleted of both T_{reg} cells and memory CD4⁺ T cells): cell recoveries were considerably enhanced in the spleen (presumably reflecting enhanced homeostatic proliferation) and there was substantial lymphocytic infiltration and prominent pathology in lung and lacrimal glands. These results demonstrated that the hyper-responsiveness of purified naive *aly/aly* CD4⁺ cells applies not only to short-term proliferative

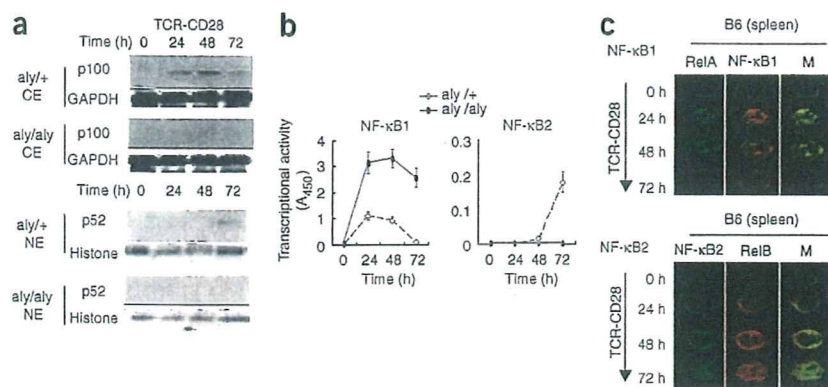


Figure 6 Kinetics of NF- κ B1 and NF- κ B2 expression in naive CD4⁺ cells after TCR ligation. (a) Immunoblot to detect p100 and p52 in cytosolic extracts (CE) and nuclear extracts (NE) of aly^{+/+} and aly/aly naive CD4⁺ cells after 0–72 h of TCR-CD28 ligation. GAPDH and histone serve as controls. Results are representative of two independent experiments. (b) Nuclear expression of NF- κ B1 and NF- κ B2. Relative activities were measured with the nuclear extracts of naive T cells from aly^{+/+} and aly/aly mice stimulated for 0–72 h with TCR-CD28 ligation. Data represent means \pm s.d. of triplicate wells. (c) Confocal analysis of NF- κ B subunits in naive CD4⁺ cells from B6 mice stimulated for 0–72 h with TCR-CD28 and then stained with anti-p50, anti-RelA, anti-p52 and anti-RelB, followed by secondary Alexa Fluor 488–labeled (green) or Alexa Fluor 568–labeled (red) anti-mouse or anti-rabbit IgG. M, merged images. Original magnification, \times 630. Results are representative of two independent experiments.

responses and related cytokine production but also to induction of autoimmune disease after transfer into *Rag2*^{-/-} hosts.

DISCUSSION

For T cells, primary immune responses generally require the classical NF- κ B1 pathway⁴³; whether T cell activation also requires the non-classical NF- κ B2 pathway is less clear. Nevertheless, studies of NIK-deficient aly/aly mice have led to the conclusion that ‘optimal’

activation of mature T cells requires signaling via NIK as well as PKC- θ ¹³. Because NIK controls processing of p100 to p52, it would seem to follow that T cell activation is partly dependent on nonclassical NF- κ B2. However, the T cell defects in aly/aly mice also correlate with reduced spleen cell expression of p50, RelA and c-Rel^{13,29}, suggesting an indirect effect on the classical NF- κ B1 pathway.

Here we have shown that the relative contributions of NF- κ B1 and NF- κ B2 to T cell activation are crucially dependent on whether the cells are immunologically naive. We have made two main points in this context. The poor immune response of total aly/aly T cells (equally true for NIK-deficient *Map3k14*^{-/-} and *Relb*^{-/-} cells) does not reflect a positive requirement for NF- κ B2 but instead reflects the inhibitory function of a unique population of ‘suppressor’ cells in the total cell population. However, when depleted of those suppressor cells, the aly/aly naive T cell samples showed their cell-intrinsic ‘defect’ of hyper-reactivity after TCR stimulation. Thus, the aly, NIK-deficient and *Relb*^{-/-} phenotype is actually a combination of a cell-extrinsic suppressor function in a subset of CD44^{hi}CD4⁺ NIK-deficient T cells and a cell-intrinsic hyperactivation response in naive CD4⁺ T cells that lack NIK function.

For wild-type mice, it is well established that primary responses of T cells can be suppressed by a population of CD25⁺Foxp3⁺CD4⁺ T_{reg} cells and are enhanced when T_{reg} cells are eliminated^{38–40}. For aly/aly T cells, the enhancing effect of removing CD25⁺CD4⁺ T_{reg} cells was much less pronounced, probably because the proportion of the NIK-deficient CD25⁺CD4⁺ T_{reg} cells (specifically Foxp3⁺ cells) is only 20–30% of the number of such cells in wild-type mice. However, the aly/aly CD25⁺CD4⁺ T_{reg} cells are suppressor cells functionally, as after enrichment they suppressed the proliferation of naive T cells nearly as well as T_{reg} cells from control mice. In addition, cytokine production by T_{reg} cells from aly/aly and control mice was comparable. Hence, except for an overall reduction in

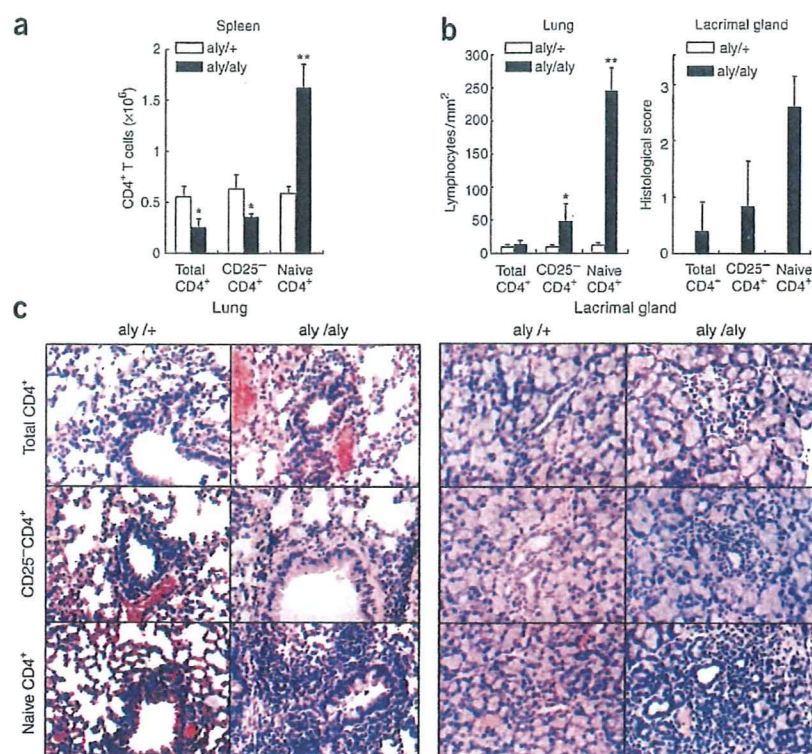


Figure 7 Induction of autoimmune disease by subsets of aly^{+/+} and aly/aly CD4⁺ cells. Enriched subsets of total CD4⁺, CD25⁺CD4⁺ or naive (CD25⁻CD44^{lo}) CD4⁺ cells from aly^{+/+} and aly/aly mice were transferred into *Rag2*^{-/-} hosts (5×10^6 cells/mouse); mice were killed 4 weeks after transfer. (a) Total number of CD3⁺CD4⁺ splenocytes. Data are means \pm s.d. of four to five mice. *, $P < 0.05$, and **, $P < 0.005$, aly/aly versus aly^{+/+} cells in each group. (b) Histopathological analysis of lung and lacrimal gland sections. Left, number of lymphocytes/mm² of lung; right, pathological score of inflammatory lesions of lacrimal glands. Data are means \pm s.d. of four to five mice. (c) Histology of lungs and lacrimal gland sections stained with hematoxylin and eosin. Original magnification, \times 100. Results are representative of four to five mice in each group.

total numbers, the 'classical' CD25⁺CD4⁺ T_{reg} cells in aly/aly mice showed no obvious abnormality.

The situation with memory CD4⁺ cells was very different. Wild-type memory CD25⁺CD44^{hi}CD4⁺ T cells functioned like 'typical' antigen-primed T cells: they rapidly generated large amounts of IL-2 after TCR ligation *in vitro* and provided efficient help to naive CD4⁺ T cells. In contrast, all of the data presented here indicate the conclusion that aly/aly memory CD4⁺ T cells function as suppressor cells. In every test, both *in vivo* and *in vitro*, elimination of both CD25⁺ ('typical' T_{reg} cells) and CD25⁺CD44^{hi}CD4⁺ memory T cells was required for reversal of the poor proliferative response of aly/aly CD4⁺ cells. The origin of such 'naturally' occurring CD44^{hi}CD4⁺ memory cells in either wild-type or NIK-deficient mice is unclear; such cells may arise through contact with various environmental antigens and/or be stimulated by self antigens.

The mechanism of suppression by aly/aly memory CD4⁺ cells has yet to be resolved. After TCR ligation, suppression correlated with a combination of low IL-2 synthesis and enhanced CD25 expression, which suggests that the cells could function mostly through CD25-mediated consumption of IL-2. That idea fits with the finding that suppression by aly/aly memory CD4⁺ T cells *in vitro* could be overcome by the addition of exogenous IL-2. However, simple consumption of IL-2 is unlikely to explain the strong inhibitory influence of aly/aly memory CD4⁺ T cells *in vivo*, which was apparent for both homeostatic proliferation and autoimmune disease induction. By analogy with 'typical' T_{reg} cells, suppression via transforming growth factor- β or IL-10 production could be involved³⁵⁻³⁷; however, at least *in vitro*, production of these cytokines was no higher for aly/aly memory CD4⁺ T cells than for control memory cells. Hence, resolving the mechanisms of suppression by aly/aly memory CD4⁺ cells must await further investigation.

Why memory CD4⁺ cells in aly/aly mice show strong CD25 expression but poor IL-2 synthesis after TCR stimulation is also still unclear. Nevertheless, it is notable that despite enhanced synthesis of CD25, aly/aly memory CD4⁺ T cells demonstrated very little nuclear translocation of p50, RelA, p52, RelB (as presented here) or c-Rel (data not shown), indicating that CD25 upregulation in aly/aly memory cells involves an NF- κ B-independent pathway, such as AP-1 and/or NF-AT^{44,45}. Because nuclear translocation of p50-RelA was high in naive aly/aly T cells, the limited translocation of these subunits in memory aly/aly T cells in comparison was unexpected although consistent with the reduced p50 and RelA reported for aly/aly total spleen cells²⁹. Based on those preliminary data, poor nuclear translocation of p50 and RelA in memory aly/aly CD4⁺ cells correlates with reduced synthesis in the cytoplasm, suggesting that p50 and RelA synthesis in memory CD4⁺ cells is partly controlled by a NIK-dependent pathway. In addition, as in stimulated osteoclast precursors²⁶, the low amounts of p50 and RelA in aly/aly memory CD4⁺ cells may be 'held' in the cytoplasm through association with p100; thus, cytoplasmic p100 in aly/aly cells is much higher in memory cells than naive cells.

The hyper-responsiveness of naive aly/aly CD4⁺ cells was associated with increased synthesis of both IL-2R (CD25) and IL-2, relative to that of wild-type naive cells; detection of an increase in IL-2 synthesis required intracellular staining, presumably because of efficient absorption of extracellular IL-2 by the increased CD25 expressed on the cell surface. Other signs of T cell activation, such as CD69 expression and interferon- γ synthesis, were nearly normal (data not shown), indicating that the hyper-responsiveness was centered on the IL-2-IL-2R axis. As for NF- κ B, it is particularly notable that the increased proliferative responses of naive aly/aly CD4⁺ cells were associated with considerable

increases in nuclear translocation of p50 and RelA, apparent in both nuclear extracts and by confocal microscopy. Therefore, this indicates that NIK functions by preventing nuclear translocation of p50 and RelA. The simplest possibility is that NIK regulates autocrine synthesis of p100 through continuous p100-p52 processing, thus allowing nuclear translocation of p52-RelB dimers and transcription of the gene encoding p100 (ref. 26); steady-state production of p100 then keeps p50 and RelA in the cytoplasm. We favor that hypothesis because there was much less p100 in aly/aly naive cells than in control cells; that result was also confirmed by FRET analysis, which showed no apparent association of p100 with p50 or RelA. Hence, we attribute the hyper-responsiveness of aly/aly naive CD4⁺ cells (as well as *Map3k14*^{-/-} (NIK-deficient) and *Relb*^{-/-} cells) to reduced p100 synthesis, which leads to unregulated nuclear translocation of p50-RelA and enhanced IL-2 and IL-2R synthesis. For *Relb*^{-/-} cells, p100 was also reduced (data not shown), presumably because expression of the gene encoding autocrine p100 is controlled by p52-RelB heterodimers²⁶.

For naive CD4⁺ cells from wild-type mice, despite prominent synthesis in the cytoplasm, nuclear translocation of p52-RelB was undetectable within the first 2 d after TCR-CD28-induced activation, indicating no involvement of the nonclassical NF- κ B2 pathway. However, by day 3 after TCR ligation, substantial nuclear translocation of p52-RelB was evident, which paralleled a decrease in p50-RelA. That result provides direct support for the hypothesis that NF- κ B2-RelB is involved in the later stages of the primary response⁴⁶⁻⁴⁸, perhaps by 'substituting' for the classical NF- κ B1 pathway. One point to emphasize here is that nuclear translocation of NF- κ B2-RelB presumably has only a positive effect on gene transcription and thus cannot account for the regulatory effect of NIK on TCR responsiveness. As mentioned above, we envisage that NIK acts as a 'brake' for the NF- κ B1 pathway simply by maintaining unprocessed p100 in the cytoplasm, thereby impeding entry of p50-RelA into the nucleus.

In summary, our data here have shown that the T cell defects reported for NIK-deficient mice¹³ and *Relb*^{-/-} mice²⁰ reflect a dominant form of immunoregulation in which otherwise hyper-responsive NIK-deficient naive T cells are suppressed by a subset of NIK-deficient CD25⁺ memory T cells. Only when the suppressor cells are eliminated is the cell-intrinsic phenotype of NIK-deficiency demonstrated. At face value, these results may seem at odds with the observation that aly/aly mice develop multiorgan autoimmune disease. That syndrome, associated with a reduction in classic CD25⁺ T_{reg} cells, may be triggered by poor negative selection in the thymus because of reduced expression of various self antigens in the thymic medulla³⁰. Given such a self-tolerance defect, it is unexpected that autoimmune disease in aly/aly mice is relatively mild, which suggests that the 'nontolerant' T cells in these mice are generally very well controlled, perhaps by the inhibitory memory subset we have described here. The fulminating autoimmune disease seen when purified naive aly/aly CD4⁺ cells were transferred adoptively is consistent with that idea.

METHODS

Mice. B6 and 129 mice were obtained from the Jackson Laboratory. *Map3k14*^{-/-} (NIK-deficient), *Map3k14*^{aly/aly} (aly/aly) and *Relb*^{-/-} mice were provided by R. Ulevitch (The Scripps Research Institute, La Jolla, California) and M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, California), and OT-II mice⁴⁹ (C57BL/6-Tg(TcraTcrb)425Cbn/J) were obtained from S. Webb (The Scripps Research Institute, La Jolla, California); aly/aly.OT-II mice were generated by crossing of aly/aly mice with OT-II mice. *Rag2*^{-/-} mice were obtained from Taconic. All mice were maintained in specific pathogen-free conditions in our animal facility and the experiments were approved by an animal ethics board of The Scripps Research Institute (La Jolla, California) or Tokushima University (Tokushima, Japan).

Antibodies. Antibodies specific for p50 (C-19, NLS and D-17), p52 (K-27 and C-5), RelA (A-20, C-20 and F-6), RelB (C-19), c-Rel, histone and glyceraldehyde phosphate dehydrogenase were purchased from Santa Cruz Biotechnology and were used for immunoprecipitation, immunoblot analysis, EMSA and confocal microscopy.

Cell purification. For purification of CD4⁺ subsets, lymph node or spleen cells were treated for 45 min at 37 °C with cytotoxic mAbs specific for CD8 (3.168.8) and CD24 (J11D) plus guinea pig complement (Rockland)⁵⁰. After being washed, CD25⁺CD4⁺ T cells, NK1.1⁺ T cells and cells positive for major histocompatibility complex class II first underwent depletion by negative selection with DynaBeads (Dyna). Samples were enriched for CD44^{lo} and CD44^{hi} cells by negative selection with anti-CD44 (IM7) or anti-CD62L (Mel-14) and were positively 'panned' with anti-CD4 (RL172). In addition, samples were enriched for CD44^{lo}, CD44^{int} and CD44^{hi} CD4⁺ and CD25⁺CD4⁺ T cells by cell sorting with a FACSvantage (Becton Dickinson).

Culture conditions. Cells were cultured in 0.2 ml of RPMI medium supplemented with 50 μ M 2-mercaptoethanol, L-glutamine and 10% FCS in 96-well tissue culture plates coated with mAbs specific for TCR β (H57-597) and CD28 (37.51; eBiosciences).

Flow cytometry. A FACSort (Becton Dickinson) was used for flow cytometry and data were analyzed with FlowJo FACS Analysis software (Tree Star). Analysis of cell division with CFSE (Molecular Probes)⁴², intracellular cytokine production (IL-2) with a BD Cytofix/Cytoperm kit (BD Biosciences)⁵¹ and intracellular Foxp3 expression with an Intracellular Foxp3 Detection Kit (eBioscience) was done according to the manufacturers' instructions.

Proliferation assay. Cell proliferation was evaluated by [³H]thymidine incorporation or by counting of divisions by CFSE dilution of labeled cells. In most experiments, 5 \times 10⁴ enriched CD4⁺ T cells were stimulated for 24–72 h with plate-bound mAbs to TCR (0.1–1 μ g/ml) and CD28 (20 μ g/ml). OT-II T cells (0.625 \times 10⁴ to 5 \times 10⁴ cells/well) were cultured together with irradiated (3,000 cGy) Thy-1.2⁺-depleted syngeneic spleen cell samples (5 \times 10⁵ cells) and were stimulated with OVA peptide, amino acids 323–339 (Sigma Genosys). Stimulated cells were then pulsed with 0.5 μ Ci [³H]thymidine per well for the last 8 h of culture. For CFSE labeling, purified CD4⁺ T cells were resuspended with 0.1% BSA in PBS at a density of 5 \times 10⁶ cells/ml and were labeled for 10 min at 37 °C with 0.3 μ M CFSE. CFSE-labeled cells were 'quenched' with PBS containing 5% FCS and were washed twice. Cell division at 48–72 h was analyzed by flow cytometry.

EMSA. Nuclear extracts of stimulated CD4⁺ T cells were prepared as described⁵². Cells were washed twice with PBS and were resuspended in 100 μ l ice-cold lysis buffer, were vortexed and were centrifuged for 5 min at 5,000g. Nuclear pellets were resuspended in 100 μ l extraction buffer containing 20 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.4 mM NaCl, 2.5% glycerol and a mixture of protease inhibitors. After centrifugation for 30 min at 12,000g, nuclear extracts (0.5–5 μ g) in the supernatant were incubated for 20 min at 25 °C with biotin-labeled κ B oligonucleotide probe (5'-AGTTGAGGG GACTTCCCAGGC-3') and Oct-1 oligonucleotide probe (5'-TGTCGAATG CAAATCACTAGAA-3'). Protein-DNA complexes were resolved by nondenaturing 4–6% PAGE in 0.5 \times TBE and were transferred to nylon membranes (Pierce). After crosslinking of transferred DNA to the membranes, biotin-labeled DNA was detected with a LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions. For supershift assay, NF- κ B subunit-specific antibodies were added before the formation of DNA-protein complexes at 25 °C for 15 min.

Immunoprecipitation and immunoblot analysis. Nuclear extracts, cytoplasmic extracts and total cell lysates from CD4⁺ T cells (2.5–10 μ g) were separated by 10% SDS-PAGE and were blotted onto polyvinylidene difluoride membranes. Blotted membranes were incubated with antibodies specific for NF- κ B subunits, followed by incubation with goat anti-mouse, or donkey anti-rabbit coupled to horseradish peroxidase, and proteins were made visible with the SuperSignal West Pico Chemiluminescent Substrate (Pierce). For immunoprecipitation, purified proteins (10–50 μ g) 'captured' with the antibody were

incubated with immobilized protein G and were precipitated with a Seize Classic Mammalian Immunoprecipitation Kit (Pierce). Precipitated proteins were analyzed by immunoblot with the NF- κ B subunit-specific antibodies described above and a Rabbit IgG TrueBlot set (eBioscience). Positive controls for the detection of NF- κ B subunits were confirmed by immunoblot analysis (Supplementary Fig. 6 online) using Jurkat or A431 cell lysates (BD Transduction Laboratories).

ELISA. The amount of IL-2 and IL-10 proteins in culture supernatants was determined by ELISA. Production of transforming growth factor- β was measured with the DuoSet ELISA Development System (R&D Systems). For this, 96-well flat-bottomed plates were precoated with capture antibodies, and diluted samples or standard recombinant cytokines were added to each well. After plates were washed, biotinylated antibodies were added and then wells were incubated with horseradish peroxidase-labeled, affinity-purified anti-rat immunoglobulin G (IgG). A solution of *o*-phenyldiamine (Sigma) was added to each well as a substrate. The optical density at 490 nm was measured with a microplate reader (Molecular Devices).

Confocal microscopy. Cells were deposited onto poly-L-lysine-coated glass slides, were fixed with 3% paraformaldehyde in PBS, were made permeable for 2 min with 0.2% Triton X-100 in PBS and were preblocked for 1 h with 1% BSA and 2.5% FCS in PBS. Cells were stained for 1 h with 1 μ g/ml of the appropriate primary antibodies. After being washed three times with 0.001% Triton X-100 in PBS, cells were stained for 30 min with secondary Alexa Fluor 488-conjugated donkey anti-mouse or goat anti-rabbit IgG (heavy plus light) and then were washed with PBS. Coverslips were applied with Fluoromount-G (Molecular Probes). Cells were visualized with a BioRad 1024 laser-scanning confocal microscope (BioRad Laboratories). Each optical section was acquired sequentially with 488-nm and 568-nm laser lines to excite Alexa Fluor 488 (green) and Alexa Fluor 568 (red) fluorescence, respectively. Merged images are presented as yellow. For FRET, Zenon Rabbit IgG Labeling Kits or Zenon Mouse IgG Labeling Kits (Molecular Probe) were used to label antibodies with Alexa Fluor 488 as the 'donor dye' or Alexa Fluor 546 as the 'acceptor dye'⁵³. Red color indicates that the acceptor dye was activated by the donor dye, as the two dyes were in close proximity.

NF- κ B transcription activity assay. The transcriptional activity of NF- κ B subunits of the nuclear extracts from naive T cells was analyzed with NF- κ B Family Transcription Factor Assay Kit (Chemicon). Nuclear extracts were incubated with biotinylated double-stranded oligonucleotide probe containing the consensus sequence (5'-GGGACTTTC-3') for NF- κ B on a streptavidin-coated plate. Captured complexes, including active NF- κ B protein, were incubated with the primary antibody for NF- κ B subunit and horseradish peroxidase-conjugated secondary antibody and tetramethylbenzidine substrate. The absorbance of the samples was measured with a spectrophotometry microplate reader set at 450 nm.

Pulse-chase assay. Purified naive CD4⁺ T cells from *aly/+* and *aly/aly* mice were cultured for 4 h in methionine-free RPMI 1640 medium (Sigma) supplemented with ³⁵S-labeled methionine (50 μ Ci/ml) on plates coated with mAbs to TCR and CD28. Purified total extracts were immunoprecipitated with rabbit anti-RelA. Radiolabeled proteins in the immunoprecipitate were resolved by reduced SDS-PAGE; the dried gel was exposed to autoradiography film in a phosphorimaging cassette.

Cell transfer. CFSE-labeled naive or memory CD4⁺ T cells (5 \times 10⁶) from *aly/aly*, *aly/+* or B6.Ly5.1 mice were transferred intravenously into irradiated (700 cGy) B6.PL (Thy-1.1⁺) or B6 mice. On day 7 after transfer, spleen cells were analyzed to measure homeostatic proliferation via CFSE dilution by flow cytometry. For analysis of *in vivo* antigen-specific T cell responses, 5 \times 10⁶ CFSE-labeled naive CD4⁺ T cells from *aly/aly* B6.PL and *aly/+* OT-II B6.PL mice were transferred intravenously into B6 mice; OVA peptide of amino acids 323–339 (0–200 μ g) was injected intraperitoneally into the mice; 3 d later, proliferation of the donor cells in spleen and lymph node was analyzed by flow cytometry. For induction of autoimmune lesions in *aly/aly* mice, 5 \times 10⁶ enriched total CD4⁺, CD25⁺CD4⁺ or naive CD4⁺ cells from *aly/+* or *aly/aly* mice were transferred intravenously into *Rag2^{-/-}* mice.

Histological analysis. All organs of *Rag2*^{-/-} mice that had received cell transfer were removed, were fixed with 4% phosphate-buffered formaldehyde, pH 7.2, and were prepared for histological examination. Sections were stained with hematoxylin and eosin. The disease incidence and severity in pancreata and lacrimal glands was determined by the histological score of inflammatory lesions as described⁵⁴. For the inflammatory lesions of lungs, lymphocytes per mm² were counted. Histological findings were estimated by three independent, well-trained pathologists 'blinded' to sample identity.

Statistics. Student's *t*-test was used for statistical analyses.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Novel Role for RbAp48 in Tissue-Specific, Estrogen Deficiency-Dependent Apoptosis in the Exocrine Glands

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Although tissue-specific apoptosis in the exocrine glands in estrogen-deficient mice may contribute to the development of autoimmune exocrinopathy, the molecular mechanism responsible for tissue-specific apoptosis remains obscure. Here we show that RbAp48 overexpression induces p53-mediated apoptosis in the exocrine glands caused by estrogen deficiency. RbAp48-inducible transfectant results in rapid apoptosis with p53 phosphorylation (Ser9) and α -fodrin cleavage. Reducing the expression of RbAp48 through small interfering RNA inhibits the apoptosis. Prominent RbAp48 expression with apoptosis was observed in the exocrine glands of C57BL/6 ovariectomized (OVX) mice but not in OVX estrogen receptor $\alpha^{-/-}$, p53 $^{-/-}$, and E2F-1 $^{-/-}$ mice. Indeed, transgenic expression of the RbAp48 gene induced apoptosis in the exocrine glands but not in other organs. These findings indicate that estrogen deficiency initiates p53-mediated apoptosis in the exocrine gland cells through RbAp48 overexpression and exerts a possible gender-based risk of autoimmune exocrinopathy in postmenopausal women.

Estrogenic action has been suggested to be responsible for the strong female preponderance of many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome (50, 51). Recent evidence suggests that apoptosis plays a key role in the physiology and pathogenesis of various autoimmune diseases (2, 7, 19, 35, 42). We have demonstrated that estrogenic action influences target epithelial cells through Fas-mediated apoptosis in a murine model for Sjögren's syndrome (13). Recently, we found that tissue-specific apoptosis in the exocrine glands spontaneously occurring in estrogen-deficient mice may contribute to the development of autoimmune exocrinopathy (14). We speculate that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens. It has been reported that the antiestrogen tamoxifen (TAM) induces cell death in the human breast cancer cell line MCF-7 (17). We observed a time- and concentration-dependent increase in apoptosis of mouse and human salivary gland cells ([MSG] mouse primary culture; [HSG] human cell line) treated with TAM but not in other cell lines (HT-29, Colo201, and Jurkat) (14).

Apoptosis can be initiated by many different factors, but activation of caspases, which are a special class of proteolytic enzymes, is always involved in this process. Activation of caspases may be achieved by several molecular pathways; the best known stimuli triggering the caspase cascade are stimulation of Fas or TNF receptors, release of cytochrome *c* from the cellular mitochondria, and exposure to granzymes, which are secreted by cytotoxic T cells (3, 12, 31, 37, 54). Detailed research on the mechanisms controlling caspase activity will pro-

vide better insight into the pathogenesis of autoimmune diseases with special reference to estrogen deficiency. In this study, we have focused on the molecular mechanisms responsible for tissue-specific apoptosis caused by estrogen deficiency and identified RbAp48 as a novel apoptosis-inducing gene exclusively in the exocrine glands. Retinoblastoma (Rb) protein is a multifunctional protein that binds to transcription factors and kinases to regulate both cell growth and apoptosis (11). Although recent data suggest that loss of Rb can cause apoptosis through derepression of basally inhibited extrinsic apoptotic pathway genes (20), no mechanism has provided a molecular explanation for RbAp48 in the induction of apoptosis.

MATERIALS AND METHODS

Cell culture and gene transfection. HSG, MSG, HT29, Colo201, HeLa, HepG2, SH-SY5Y, NEC14, THP-1, Jurkat, Raji, U937, and W138 cells were cultured in Dulbecco's modified Eagle medium (DMEM) or RPMI 1640 medium containing 10% fetal bovine serum at 37°C. HSG and MSG cells have been described elsewhere (38, 40). The following were used for cell cultures: 10⁻⁷ M TAM (Wako Pure Chemical, Osaka, Japan), 10⁻⁹ M β -2-estradiol (E2; Wako), 10⁻⁷ M staurosporin (Wako), paclitaxel (Wako), 1 μ M etoposide (Wako), 1 μ M ICI182780 (Wako), 25 ng/ml anti-Fas monoclonal antibody (MAb) (MBL, Nagoya, Japan), and 10 ng/ml recombinant human gamma interferon (R&D Systems, Minneapolis, MN). The RbAp48 gene inserted into the pCMV (2N3T) vector, a gift from D. Trouche, was transfected into the cells using FuGENE6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). The RbAp48-stable cell line (RH0) from HSG cells in which RbAp48 expression was regulated by isopropyl-1-thio- β -D-galactopyranoside (IPTG), was established using a LacSwitch II Inducible Mammalian Expression System (Stratagene, La Jolla, CA). Briefly, the repressor vector (pCMVLacI) and RbAp48-inserted operator vector (pOPRV1/MCS) were cotransfected into HSG cells with FuGENE6, and the RbAp48 expression of hygromycin and G418-resistant transfectants was controlled by IPTG. For infection of adenovirus vector, RbAp48 gene-transfected MSG cells from p53 $^{-/-}$ or wild-type mice were infected with 100 multiplicities of infection of adenovirus vector including the p53 gene obtained from Toren Finkel (National Institutes of Health). MSG and mouse mammary glands (MMG) were removed, placed in DMEM containing 10% fetal calf serum (FCS) and 10 mM HEPES (pH 7.4), and rapidly minced. The mate-

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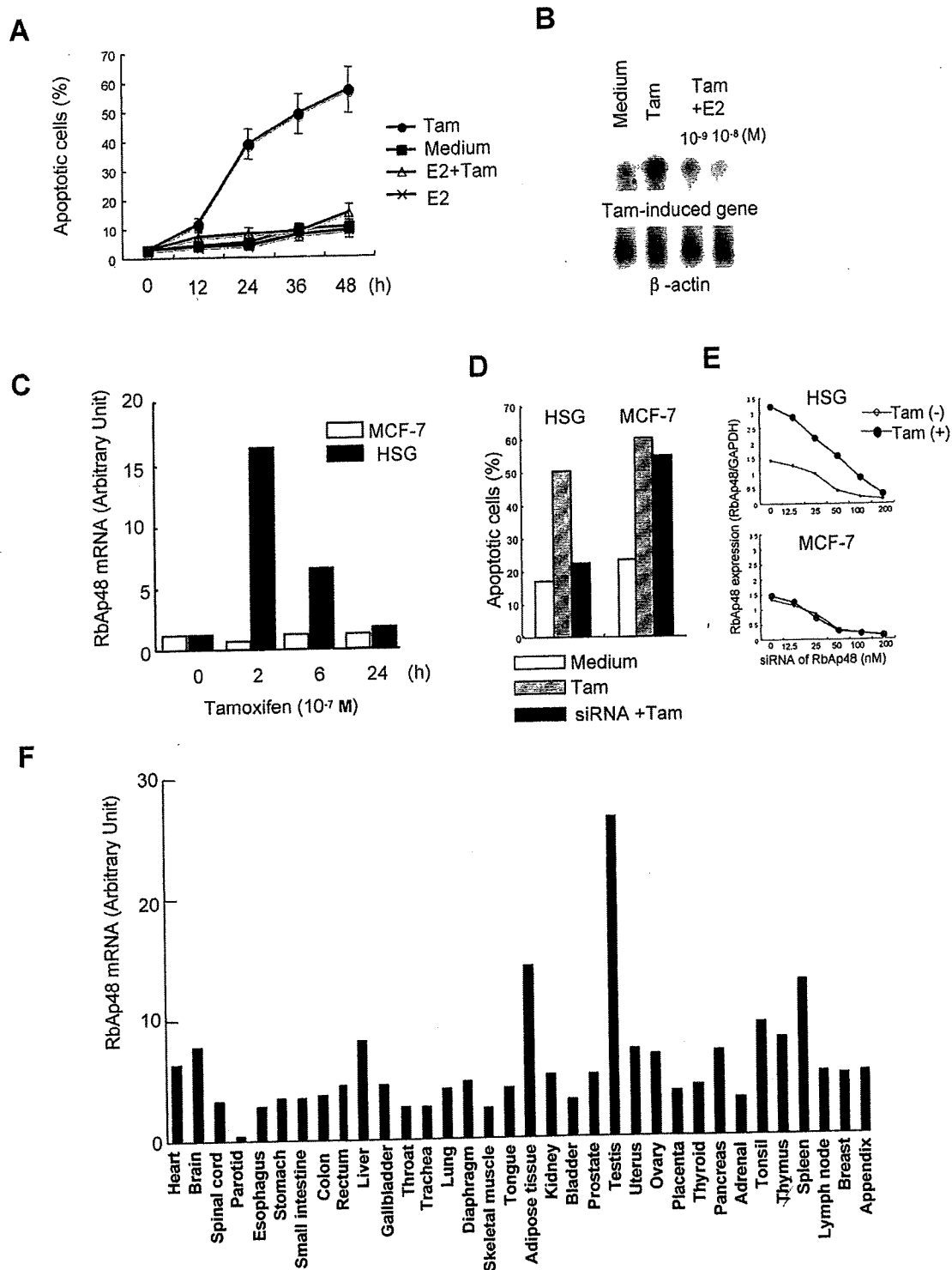


FIG. 1. Identification of the RbAp48 gene in salivary gland cell apoptosis. (A) A time-dependent increase in apoptotic HSG cells stimulated with TAM (10^{-7} M) was observed, and E2 (10^{-9} M) treatment inhibited apoptosis. Apoptotic cells were detected by flow cytometry using FITC-conjugated annexin V. (B) TAM-induced gene fragments cloned by differential display PCR were used as a probe by Northern blotting with mRNA from HSG cells treated with TAM (10^{-7} M) or E2 (10^{-9} M and 10^{-8} M). β -Actin mRNA was detected as an internal control. Each blot is representative of three independent experiments. (C) Analysis of RbAp48 mRNA expression was performed using total RNA from TAM-stimulated HSG and MCF-7 cells for 0 to 24 h. The graph is representative of three independent experiments. (D) The inhibitory effects of siRNA on TAM-induced apoptosis in HSG cells, with siRNA (15 nM) of the RbAp48 construct but not in MCF-7 cells. After transfection of siRNA and a fluorescence-labeled control gene, the cells were incubated with TAM (10^{-7} M) for an additional 24 h. Apoptosis was estimated by flow cytometric analysis using PE-conjugated annexin V. The graph is representative of three independent experiments. (E) A dose-dependent inhibition of siRNA (0 to 200 nM) of TAM-induced RbAp48 expression in HSG cells, not MCF-7 cells, was observed. The graph is representative of two independent experiments. (F) RbAp48 mRNA expression in HSG cells, not MCF-7 cells, was observed. The graph is representative of two independent experiments. Message levels (arbitrary units) were analyzed using human tissue total RNA-blotted membrane. Message levels (arbitrary units) were quantified by BAS-2000II and expressed as the ratio of RbAp48 mRNA/ β -actin mRNA. The graph is representative of two independent experiments.