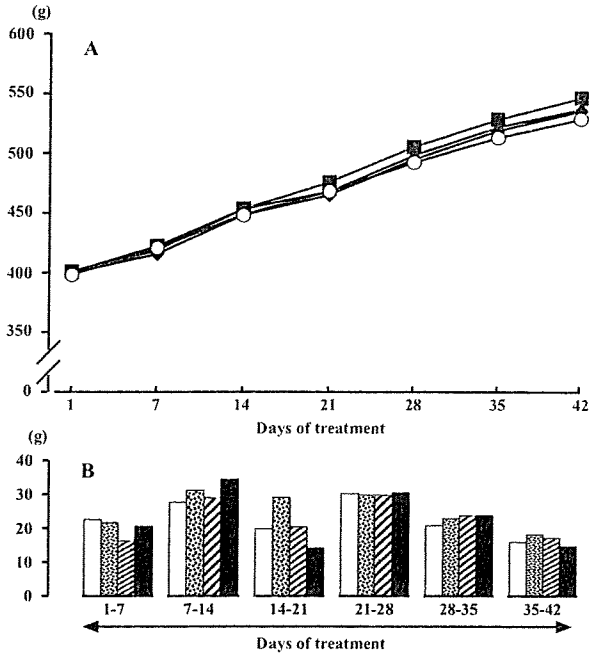

Fig. 3. Changes in body weight (A) and its gain (B) in male rats treated orally with 4-methylbenzoic acid for 42-days at dose level of 0 (○ and open column), 100 (▫ and dashed column), 300 (▲ and hatched column) or 1,000 mg/kg/day (♦ and closed column) in the reproduction/developmental toxicity screening test. Each value represents the average for 13 animals.

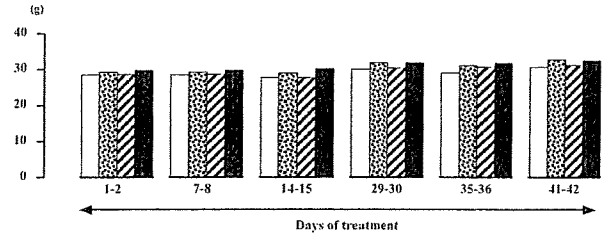

Fig. 4. Changes in food consumption of male rats treated orally with 4-methylbenzoic acid for 42-days at dose level of 0 (open column), 100 (dashed column), 300 (hatched column) or 1,000 mg/kg/day (closed column) in the reproduction/developmental toxicity screening test. Each column represents the average for 13 males.

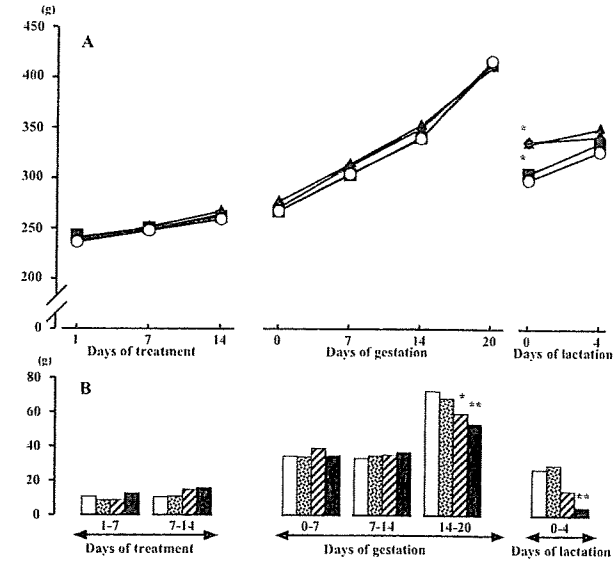

Fig. 5. Changes in body weight (A) and its gain (B) in female rats treated orally with 4-methylbenzoic acid at a dose level of 0 (○ and open column), 100 (▫ and dashed column), 300 (▲ and hatched column) or 1,000 mg/kg/day (♦ and closed column) in the reproduction/developmental toxicity screening test. Administration of the compound was started 2 weeks prior to mating, and was continued through mating period and gestation period until 3 days after delivery. Each value during the pre-mating treatment period represents the average for 13 animals. That during the gestation and lactation periods represents the average for 9-13 dams. * and ** indicate significant difference from control at $p < 0.05$ and 0.01, respectively.

control. Males in the groups given 300 mg/kg or less did not show such abnormalities at any part of their epididymis (Table 6). In the testis, no abnormal findings related to the doses of the compound were observed.

Reproductive performances in the reproduction/developmental study

Except one female in the control and one female in the 100 mg/kg treated groups, all the females revolved on a regular 4-day estrous cycle until mating (data not shown). Mating performance and dam data are shown in Table 7. None of indices for mating performance were different between the compound treated group and the control group. In addition, the number of corpora lutea, which represented the number of oocytes shed for impregnation, was not different between these groups. Thus, the compound did not affect the estrous cycle, ovulation or mating, at any dose level.

Although all of the females that had copulated in the 100 mg/kg and less treated groups became pregnant, one such female and four such females in the 300 mg/kg and 1,000 mg/kg treated groups, respectively, did not become pregnant (Table 7). The Fisher's direct probability test indicated that there was a significant difference in the fertility between the control and the 1,000 mg/kg treated groups, which indicated that the compound disrupted fertility at 1,000 mg/kg.

As shown in Table 7, pregnant females delivered live fetuses without differences in gestation length between the control and the compound treated groups. However, the implantation index and the number of pups born were significantly decreased in the 300 mg/kg or more treated groups. Furthermore, the numbers of pups alive on Days 0 and 4 of lactation were significantly smaller in the 1,000 mg/kg treated group than those in the control. The other dam data, such as the birth index, the live birth index and the viability index on Day 4 of lactation, were not affected by the treatment. In addition, pup body weights at birth and on Day 4 of lactation and the sex ratios on these days were not affected by the treatment, in any group.

At necropsy of females, the uterus of the female that failed to become pregnant in the 300 mg/kg treated group showed ballooning and accumulation of cloudy fluid. Histopathology of the uterus revealed lumen dilatation and cellular infiltration of neutrophils in the epithelium and endometrial stroma, with edema in the endometrial stroma. In the 1,000 mg/kg treated group, however, no abnormality was observed in the reproductive organs of the four females that failed to become pregnant, either, at the gross necropsy or in the histopathological examination of the ovary, while a moderate increase in atretic follicles was observed in one of the females. Including this case, there were no histopathological findings in the ovary related to doses of the compound (data not shown).

No abnormalities in the morphology or behavior of pups was noted in any group, except the following occasional cases in a single dam of the 1,000 mg/kg treated group: temporary cyanosis at birth and dilatation of renal pelvis at necropsy on Day 4 of lactation in one male pup.

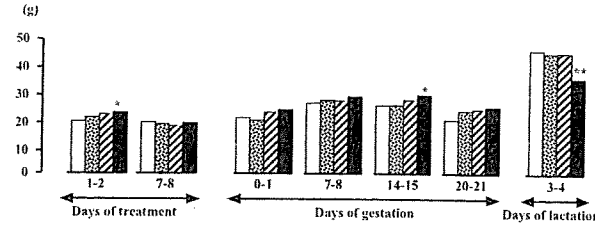

Fig. 6. Changes in food consumption of female rats treated orally with 4-methylbenzoic acid at dose levels of 0 (open column), 100 (dashed column), 300 (hatched column) or 1,000 mg/kg/day (closed column) in the reproduction/developmental toxicity screening test. Administration of the compound was started 2 weeks prior to mating, and was continued through mating period and gestation period until 3 days after delivery. Each value during the pre-mating treatment period represents the average for 13 females. That during the gestation and lactation periods represents the average for 9-13 dams. * and ** indicate significant difference from control at $p < 0.05$ and 0.01, respectively.

Table 5. Weights of testes and epididymides in rats treated orally with 4-methylbenzoic acid for 42-days in reproduction/developmental toxicity screening test

Dose (mg/kg)	0	100	300	1,000
Number of animals	13	13	13	13
Body weight (g)	527.9 ± 37.8	549.9 ± 42.8	541.6 ± 26.6	542.4 ± 30.7
Absolute weight				
Testes (g)	3.37 ± 0.24	3.29 ± 0.19	3.29 ± 0.22	3.31 ± 0.17
Epididymides (g)	1.28 ± 0.08	1.27 ± 0.08	1.24 ± 0.07	1.13 ± 0.09**
Relative weight (g/100 g)				
Testes	0.64 ± 0.07	0.60 ± 0.06	0.61 ± 0.04	0.61 ± 0.05
Epididymides	0.24 ± 0.03	0.23 ± 0.02	0.23 ± 0.01	0.21 ± 0.02**

Values represent average ± S.D.
**, significant difference from control at $p < 0.01$.

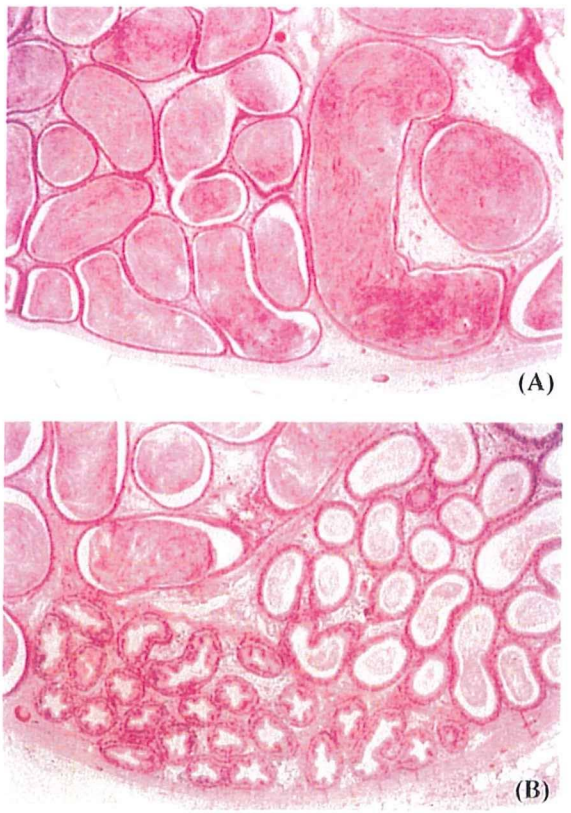

Fig. 7. Representative photographs of microscopic cross section of the cauda epididymis in male rats treated orally with 4-methylbenzoic acid at dose levels of 0 (A) or 1,000 mg/kg/day (B) in the reproduction study conducted under the OECD test guideline 421. Note that a few sperm are found in the lumen of the compound treated rats (B), and the epithelial height of the lumen becomes high. In contrast, the epithelial height of the lumen in the vehicle treated rats (A) becomes low by filling sperm in the lumen. Hematoxylin-eosin stain, x36.

DISCUSSION

Although the results obtained from the present studies are limited to short-term oral toxicity of 4-methylbenzoic acid, they indicate some toxicological properties of the compound. Namely, inconsistent effects of the compound on the epididymis indicate different toxicological potencies of the compound in male animals between the 28-day study and the reproduction/developmental study. While oral daily administration with 1,000 mg/kg of the compound reduced epididymal weight and caused oligo/azoospermia in the cauda epididymal lumen in the reproduction/developmental study, the same dose of the compound did not cause such effects in the 28-day study. Thus, the compound exerts adverse effects only on adult males when given for 42 days. Except that, repeated administration of 1,000 mg/kg of the compound led to similar results in males in both studies, such as a temporary salivation after dosing and no effects on body weight increase or food consumption. Therefore, the inconsistencies may be caused by differences in the timing or duration of administration.

The oligo/azoospermia seemed to develop at the distal part of the epididymis, since no abnormalities were observed in the caput epididymis and since there were normal lumens in the cauda epididymis. As reported by Robaire *et al.* (2006), spermatozoa enter the epididymis with testicular fluid and progress downward toward the vas deferens by smooth muscle contraction. In the reproduction/developmental study, the oligo/azoospermia developed where mature spermatozoa are stored. Spermatozoa are found in seminiferous tubules at the time of puberty, approximately 6 weeks of age in the rat, and it takes at least 10 or 15 days to reach the cauda epididymis (Sommer *et al.*, 1996; Robaire *et al.*, 2006). We have confirmed previously that spermatozoa are found in the cauda epididymis at 56 days of age at the youngest in the Sprague-Dawley derived inbred rats, which attain puberty at a comparable range of ages to Sprague-Dawley rats (Sato *et al.*, 2002). Since the compound was administered for 4 weeks in the 28-day study, from 5 weeks of age, the compound could influence epididymal spermatozoa for a short period. The storage period of spermatozoa in the cauda epididymis is estimated to be one week. In contrast, the compound was administered from 10 weeks of age in the reproduction/developmental study and could influence spermatozoa in all segments of the epididymis for 42 days. The inconsistent adverse effects of the compound on the cauda epididymis between the studies could be explained by either, the timing or the duration, of the administration.

Although the results obtained from the reproduction/developmental study seemed to suggest epididymal spermatozoa as the toxicological target of the compound, 4-methylbenzoic acid could influence spermatozoa function during their transit to the cauda epididymis. It has been reported that spermatozoa become functionally mature during transit to the distal part of the epididymis, while they are protected from oxidative stress and harmful xenobiotics by the blood-epididymis barrier and different types of antioxidant enzymes in each segment of the epididymis (Tengpwski *et al.*, 2007; Kim *et al.*, 2004; Robaire *et al.*, 2006). Therefore, detailed investigation of the effects of the compound on production, function and transition of spermatozoa is required.

Table 6. Histopathological findings of testis and epididymis in rats treated orally with 4-methylbenzoic acid for 42 days in the reproduction/ developmental toxicity screening test

Dose	0 mg/kg					100 mg/kg					300 mg/kg					1,000 mg/kg				
Grade	-	±	+	++	+++	-	±	+	++	+++	-	±	+	++	+++	-	±	+	++	+++
Testis	(13)					(13)					(13)					(13)				
Atrophy, focal, seminiferous tubule, bilateral	12	1	0	0	0	12	1	0	0	0	13	0	0	0	0	13	0	0	0	0
Multinucleated giant cell, seminiferous tubule	13	0	0	0	0	13	0	0	0	0	13	0	0	0	0	12	1	0	0	0
Epididymis	(13)					(13)					(13)					(13)				
A few number of sperm, lumen, cauda, bilateral	13	0	0	0	0	13	0	0	0	0	13	0	0	0	0	**0	11	2	0	0
																		##		
Cell debris, lumen, cauda, bilateral	12	1	0	0	0	13	0	0	0	0	13	0	0	0	0	8	5	0	0	0
Spermatid granuloma, cauda, unilateral	13	0	0	0	0	13	0	0	0	0	12	0	1	0	0	13	0	0	0	0

-, Negative; ±, Very slight; +, Slight; ++, Moderate; +++, Severe

** and ##, significant difference from control at $p < 0.01$ for incidence and grades of the findings, respectively.

Table 7. Mating performances and dam data of rats treated orally with 4-methylbenzoic acid in the reproduction/developmental toxicity screening test

Dose (mg/kg)	0	100	300	1,000
Mating performance				
Copulated pairs/Co-housed pairs (%)	13/13 (100)	13/13 (100)	12/13 (92.3)	13/13 (100)
Pregnant females/Copulated pairs (%)	13/13 (100)	13/13 (100)	11/12 (91.7)	9/13 (69.2)*
Pairing days until copulation [a]	3.0 ± 3.4	2.4 ± 1.3	2.4 ± 1.3	3.2 ± 3.3
Number of estrus revolved until copulation [a]	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.3
Dam data				
Number of pregnant females with live pups	13	13	11	9
Gestation length in days [a]	22.3 ± 0.5	22.5 ± 0.5	22.3 ± 0.5	22.8 ± 0.4
Number of corpora lutea [a]	16.2 ± 1.3	15.7 ± 1.3	16.2 ± 1.5	15.9 ± 1.3
Number of implantation sites [a]	16.0 ± 1.3	15.4 ± 1.0	14.0 ± 2.4	12.1 ± 5.3
Implantation index [a, b]	99.1 ± 2.2	98.2 ± 3.5	86.5 ± 12.6**	75.9 ± 32.6*
Day 0 of lactation				
Number of pups born [a]	15.2 ± 1.4	14.1 ± 1.8	12.5 ± 2.1**	10.2 ± 5.1**
Delivery index [a, c]	94.7 ± 5.2	91.4 ± 8.5	90.1 ± 8.4	82.9 ± 19.5
Number of pups alive	14.3 ± 1.7	14.1 ± 1.8	12.3 ± 2.2	10.0 ± 5.0*
Birth index [a, d]	89.7 ± 10.4	91.4 ± 8.5	88.0 ± 9.1	81.5 ± 19.8
Live birth index [a, e]	94.8 ± 10.5	100 ± 0	97.6 ± 4.2	98.2 ± 3.7
Sex ratio [a, f]	54.1 ± 14.3	51.9 ± 12.8	46.6 ± 13.8	59.8 ± 17.7
Day 4 of lactation				
Number of pups alive [a]	14.3 ± 1.7	13.9 ± 1.8	12.3 ± 2.2	9.7 ± 5.3*
Viability index [a]	100 ± 0	99.0 ± 3.7	100 ± 0	88.1 ± 33.1
Sex ratio [a, f]	54.1 ± 14.3	52.3 ± 12.3	46.6 ± 13.8	54.4 ± 9.5
Pups weight on Day 0 of lactation (g/pup)				
Male [a]	6.8 ± 0.4	7.0 ± 0.5	7.0 ± 0.5	6.7 ± 0.3
Female [a]	6.5 ± 0.4	6.7 ± 0.6	6.6 ± 0.5	6.4 ± 0.5
Pups weight on Day 4 of lactation (g/pup)				
Male [a]	10.7 ± 1.0	11.2 ± 1.1	11.8 ± 1.2	11.1 ± 0.9
Female [a]	10.3 ± 1.0	10.7 ± 1.1	11.2 ± 1.3	10.5 ± 0.9

[a] Average ± S.D.; [b] (Number of implantation sites/Number of corpora lutea) × 100, %; [c] (Number of pups born/Number of implantation sites) × 100, %; [d] (Number of live pups on Day 0 of lactation/Number of implantation sites) × 100, %; [e] (Number of live pups on Day 0 of lactation/Number of pups born) × 100, %; [f] (Number of live male pups/Number of live pups) × 100, %
* and **, significant difference from control at $p < 0.05$ and 0.01, respectively.

The reproduction/developmental study revealed that the compound reduced fertility of animals within a relatively short period of dosing, since the four males that failed to impregnate in the 1,000 mg/kg treated group had copulated on Days 16, 19, 19 and 28 of treatment. Furthermore, the compound increased preimplantation loss and decreased implantation index at a lower dose level than that caused oligo/azoospermia. It is not clear whether the reduced fertility and the increase in the preimplantation loss are consequent effects of the compound on the epididymal spermatozoa or are effects on such as fertilization, early embryonic development or implantation, although the results obtained from the reproduction/developmental study indicate no adverse effects on female reproductive function until mating.

Because mating was done within the same dose groups

in the reproduction/developmental study, it was impossible to determine the target sex of the compound. The reproductive toxicities of several compounds related to 4-methylbenzoic acid have been studied. A feeding administration of *p*-nitrobenzoic acid (CAS No. 62-23-7) (NTP, 1994) and m-nitrobenzoic acid (CAS No. 121-92-6) has been found to reduce the number of offspring in continuous breeding studies in CD-1 mice (NTP/NIEHS, 1997). Crossover mating conducted in these studies revealed that female reproduction is more sensitive than male reproduction. On the other hand, 3-methylbenzoic acid (CAS No. 99-04-7) and 4-hydroxybenzoic acid (CAS No. 99-96-7) did not show any reproductive toxicity in studies conducted under a protocol similar to the reproduction/developmental study (Nagao *et al.*, 1997; Yamamoto *et al.*, 1999), although estrogenic potency of 4-hydroxybenzoic acid was observed in a uterotrophic assay (Lemini *et al.*, 1997). Detailed investigation, such as crossover mating, quantification and qualification of spermatozoa, may elucidate the characteristics of the reproductive toxicity of 4-methylbenzoic acid.

In the reproduction/developmental study, maternal body weight was reduced during the latter period of gestation at the dose levels of 300 mg/kg or more, whereas it did not affect female body weight, at any dose level, in the 28-day study. It is clear, however, that the decrease in the maternal body weight was not caused by the direct toxicity of the compound. It was caused by a small litter size resulting from a reduced number of implantations, as discussed above, since fetal weight in the uterus greatly contributes to maternal body weight during the latter period of gestation. Comparable or greater food consumption during this period and greater body weight after parturition in dams of these groups also indicate no adverse effects of the compound on the maternal animals. Decreases in food consumption and body weight gain during the lactation period in the 1,000 mg/kg treated group might be physiological changes due to a smaller demand of nutrition for the small litter size.

In the males, urine specific gravity was decreased in the 300 mg/kg or more treated groups. The change, however, was reflected the increase in urine volume due to increased water consumption, and did not accompany morphological alterations or functional impairments of their kidneys. Therefore, the change was judged as a physiological response. Except for the effects on the epididymis in the reproduction/development study, no systemic effect of the compounds was observed in the males of both studies.

In the females, a slight increase in food consumption was observed in the 1,000 mg/kg treated group at the beginning of dosing in both studies. In the 28-day study, increase in AST activity and decrease in serum total protein concentration were observed in the 1,000 mg/kg treated females. Since increase in AST activity has been reported as an effect of 3-methylbenzoic acid (CAS No. 99-04-7) in the study noted above (Yamamoto *et al.*, 1999), 4-methylbenzoic acid may slightly alter female AST activity. Thus, the compound affected females at the dose level of 1,000 mg/kg. However, all these changes did not accompany structural changes and changes in organ weights. Therefore, repeated dosing of the compound may affect females at the dose level of 1,000 mg/kg, but may not affect adversely, at any dose level.

From these results, the no-observed-effect-level (NOEL) for reproductive toxicity is considered to be 100 mg/kg, whereas 1,000 mg/kg did not show any effect on neonates. That for repeated dose toxicity is considered to be 300 mg/kg for male and female rats in the both studies, but toxic effects on the epididymis differed between the studies. Thus, 4-methylbenzoic acid has a potential for reproductive toxicity and deserves further study, taking the exposure state into consideration, as discussed above, since it has been reported that the reproduction/developmental study provides screening information but does not provide a complete characterization and evaluation of reproductive or developmental toxicity (Gelbke *et al.*, 2004).

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REFERENCES

Gelbke, H.P., Fleig, H., Meder, M. and German Chemical Industry Association. (2004): SIDS reprotoxicity screening test update: testing strategies and use. Regul. Toxicol. Pharmacol., **39**, 81-86.

Kim, E., Nishimura, H., Iwase, S., Yamagata, K., Kashiwabara, S. and Baba, T. (2004): Synthesis, processing, and subcellular localization of mouse ADAM3 during spermatogenesis and epididymal sperm transport. J. Reprod. Dev. **50**, 571-578.

Lemini, C., Silva, G., Timossi, C., Luque, D., Valverde, A., González-Martínez, M., Hernández, A., Rubio-Póo, C., Chávez Lara, B. and Valenzuela F. (1997): Estrogenic effects of p-hydroxybenzoic acid in CD1 mice. Environ. Res., **75**, 130-134.

Mineshita, T., Toteno, I., Yui, Y., Furukawa, S. and Wakabayashi, K. (1978): Acute toxicity study of p-toluic acid, The Clinical Report, **12**, 1893-1904.

Nagao, T., Kuwagata, M., Kato, H. and Miyahara, T. (1997): Combined repeat dose and reproductive/developmental toxicity

screening test of hydroxybenzoic acid in rats. Toxicity Testing Reports of Environmental Chemicals **5**, 251-257.

National Toxicology Program (1994): NTP Toxicology and Carcinogenesis Studies of p-Nitrobenzoic Acid (CAS No. 62-23-7) in F344/N Rats and B6C3F1 Mice (Feed Studies). Natl. Toxicol. Program Tech. Rep. Ser. **442**,1-306.

National Toxicology Program / National Institute of Environmental Health Sciences (1997): Reproductive toxicology. m-Nitrobenzoic acid. Environ. Health Perspect., **105** (Suppl. 1), 325–326.

OECD (1997a): OECD Guideline for the Testing of Chemicals 407, "Repeated Dose 28-day Oral Toxicity Study in Rodents" adopted on 27 July 1995.

OECD (1997b): OECD Guideline for the Testing of Chemicals 421, "Reproduction/Developmental Toxicity Screening Test" adopted on 27 July 1995.

OECD (2004): The 2004 OECD list of high production volume chemicals.

Robaire, B., Hinton, B. T. and Orgebin-Crist, M-C. (2006); The epididymis. In Knobil and Neill's Physiology of Reproduction, 3rd ed. (Neill, J. D. ed.) pp.1071-1148, Academic Press, USA and UK.

Sato, M., Ohta, R., Kojima, K., Shirota, M., Koibuchi, H., Asai, S., Watanabe, G. and Taya, K. (2002): A comparative study of puberty and plasma gonadotropin and testicular hormone levels in two inbred strains of Hatano rats. J. Reprod. Dev., **48**, 111-119.

Sommer, R. J., Ippolito, D. L., and Peterson, R.E.(1996): In utero and lactational exposure of the male Holtzman rat to 2,3,7,8-tetrachlorodibenzo-p-dioxin: decreased epididymal and ejaculated sperm numbers without alterations in sperm transit rate. Toxicol. Appl. Pharmacol., **140**, 146-153.

Tengowski, M.W., Feng, D., Sutovsky, M. and Sutovsky, P. (2007); Differential expression of genes encoding constitutive and inducible 20S proteasomal core subunits in the testis and epididymis of theophylline- or 1,3-dinitrobenzene-exposed rats. Biol. Reprod., **76**,149-163.

Yamamoto, Y., Ito, Y., Nosa, A., Ito, M., Akagi, H. and Hoshi, F. (1999): Combined repeat dose and reproductive/developmental toxicity screening test of 3-methylbenzoic acid in rats. Toxicity Testing Reports of Environmental Chemicals **7**, 302-308.

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Skin sensitization potency of isoeugenol and its dimers evaluated by a non-radioisotopic modification of the local lymph node assay and guinea pig maximization test

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ABSTRACT: **Allergic contact dermatitis is the serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with spicy, carnation-like scent, is used in many kinds of cosmetics and is a well-known moderate human sensitizer. It was previously reported that the dimerization of eugenol yielded two types of dimer possessing different sensitization potencies. This study reports the differences in skin sensitization potencies for isoeugenol and two types of dimer, β-O-4-dilignol and dehydrodiisoeugenol (DIEG), as evaluated by the non-radioisotopic local lymph node assay (non-RI LLNA) and guinea pig maximization test. In the guinea pig maximization test, isoeugenol, β-O-4-dilignol and DIEG were classified as extreme, weak and moderate sensitizers, respectively. As for the results of non-RI LLNA, the EC3 for isoeugenol, β-O-4-dilignol and DIEG were calculated as 12.7%, >30% and 9.4%, respectively. The two types of isoeugenol dimer showed different sensitizing activities similar to the case for eugenol dimers. A reduction of sensitization potency achieved by dimerization may lead to developing safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, the dimerization of isoeugenol may yield a promising candidate as a cosmetic ingredient with low sensitization risk. The data may also provide useful information for the structure-activity relationship (SAR) in skin sensitization. Copyright © 2007 John Wiley & Sons, Ltd.**

KEY WORDS: isoeugenol; isoeugenol-dimer; local lymph node assay; guinea pig maximization test

Introduction

Allergic contact dermatitis is a serious unwanted effect arising from the use of consumer products such as cosmetics. Isoeugenol is a fragrance chemical with a spicy, carnation-like scent, and is used in many kinds of cosmetics (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). However, isoeugenol is a well-known moderate human sensitizer of human potency class II, and its sensitizing activity was confirmed in mice, guinea pigs and humans (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). Eugenol is also known as a human sensitizer and is used in various consumer products similar to isoeugenol (Rastogi *et al.*, 1998; Schnuch *et al.*, 2004). There are many publications concerning the sensitization potential of eugenol monomer, however, the sensitization potentials of eugenol dimers which are formed through its auto-oxidation process were not known. Recently the sensitization potential of eugenol dimers was investigated and it was found that the dimerization of eugenol, a moderate human skin sensitizer, yielded two types of dimer possessing different sensitization potencies, namely 2,2′-dihydroxyl-3,3′-dimethoxy-5,5′-diallyl-biphenyl as a weak sensitizer and 4,5′-diallyl-2′′-hydroxy-2,3′-dimethoxy phenyl ether as an extreme sensitizer (Takeyoshi *et al.*, 2004).

Eugenol and isoeugenol are both important cosmetic ingredients with whitening and antibacterial effects (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). A reduction of sensitization potency achieved by dimerization may lead to the development of safer cosmetic ingredients. As in the case of eugenol, dimerization of isoeugenol yields two types of isoeugenol dimer. These isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and anti-bacterial effects similar to the monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for a cosmetic ingredient with low sensitization risk.

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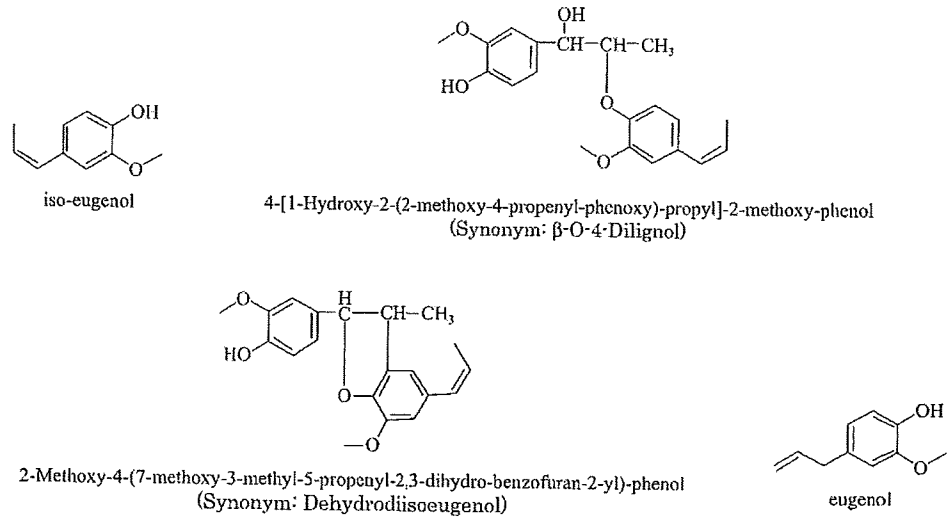

Figure 1. Chemical structures of isoeugenol, isoeugenol dimers and eugenol used in this study

This study employed two types of sensitization assay, i.e. a conventional guinea pig maximization test and non-radioisotopic local lymph node assay (non-RI LLNA) which can provide quantitative outcomes (Takeyoshi *et al.*, 2004) for evaluating the sensitization potency of related compounds. The possibility of a reduction of sensitization potency by dimerization of isoeugenol monomer was investigated.

Materials and Methods

Chemicals and Reagents

Isoeugenol and its dimers, β-O-4-dilignol and dehydrodiisoeugenol, were provided by Kanebo cosmetics, Inc. (Fig. 1, Kanagawa, Japan). Eugenol was obtained from Aldrich Chemicals Inc. All chemicals were confirmed to have >98% purity according to the HPLC analysis and were dissolved in olive oil for the GPMT, or in acetone:olive oil (AOO; 4:1) for the non-RI LLNA. 5-Bromo-2′-deoxyuridine (BrdU; Nacalai Tesque, Kyoto, Japan) was dissolved in physiological saline at a concentration of 10 mg ml^{-1}.

Animals

Female Hartley guinea pigs and CBA/JN strain mice were obtained from SLC Japan Ltd (Shizuoka, Japan) and Charles River Japan Ltd (Kanagawa, Japan), respectively. The animals were housed in animal rooms maintained at a temperature of 22 ± 3 °C, and at a relative humidity of 55 ± 15%. The rooms were ventilated at a frequency of 10–15 cycles h^{-1}, and lighted artificially for 12 h daily. Animals were allowed free access to laboratory diet (RC-4 for guinea pigs and MF for mice, Oriental Yeast Co., Tokyo, Japan) and tap water.

Guinea Pig Maximization Test (GPMT)

Guinea pigs were allocated randomly to three groups (10 animals/group). The test was conducted according to a method described previously (Magnusson and Kligman, 1969). Guinea pigs received a series of intradermal injections of eugenol, or of its dimers, in the shoulder region to induce sensitization. After 6–8 days, sensitization was boosted by a 48 h occluded patch of the same compound placed over the injection sites. Then 14 days later, the animals were challenged on a shaved flank by a 24 h occluded patch containing the same compound. All induction and challenge concentrations were set at 5% (maximum non-irritant concentration) in olive oil for all compounds as a result of preliminary dose finding tests. All compounds elicited apparent irritation at 10% in preliminary tests for intradermal injection and topical application, and then it was decided to use induction and challenge concentrations of 5% for all compounds to compare the sensitization potency of these three compounds. Chemicals were classified by the sensitization rate for each chemical, 0%–8%: weak, 9%–28%: mild, 29%–64%: moderate, 65%–80%: strong, 81%–100%: extreme.

Non-radioisotopic Local Lymph Node Assay (Non-RI LLNA)

The assay was conducted according to a method described previously (Takeyoshi *et al.*, 2001). Mice were

allocated randomly to 12 groups (4 animals/group). A 25 µl volume of test chemicals at concentrations of 3%, 10% or 30% was applied to the dorsum of both ears of the mice, daily for 3 consecutive days. The concentration ranges of each test chemical were decided according to the sensitization potencies classified by the results of GPMT. A single intraperitoneal injection (5 mg per mouse) of BrdU was then given on day 4. On day 5, the draining auricular lymph nodes were removed, weighed and stored at −20 °C until analysis using an enzyme-linked immunosorbent assay (ELISA) to measure the BrdU incorporation. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit (Roche Applied Science Corp., Indianapolis, IN, USA; Cat. No. 1647229). The lymph nodes were crushed, passed through a 70 µm nylon mesh, and the lymph node cells were suspended in 15 ml of physiological saline individually. The cell suspension (100 µl) was added to the wells of a flat-bottom microplate in triplicate. After centrifugation (300 *g*, 10 min), the supernatants were removed. A 200 µl volume of Fix-Denat solution was added to each well, and then the plate was allowed to stand for 30 min at room temperature. After removing the Fix-Denat solution, diluted anti-BrdU antibody solution (100 µl, Boehringer Mannheim Corp.) was added to each well, and after rinsing three times with washing buffer (phosphate-buffered saline), 100 µl of substrate solution containing tetramethylbenzidine (TMB) was added and allowed to react for 15 min at room temperature. Absorbance at 370 nm was determined with a microplate reader (SpectraMAX™, Molecular Devices Inc., Sunnyvale, CA, USA) at a reference wavelength of 492 nm. The absorbance was defined as the BrdU labeling index and the stimulation index (SI) which is the ratio of individual labeling index of each animal against the mean labeling index for the concurrent vehicle control group was calculated. Then the estimated concentration of a chemical required to induce a stimulation index of 3 relative to vehicle-treated controls (EC3 value) was derived by linear interpolation as described previously (Basketter *et al.*, 2000). The EC3 value was calculated by interpolating between two points on the SI axis, one immediately above, and one immediately below, the SI value of 3. The vehicle-treated control value (SI = 1) cannot be used for the latter. Where the data points lying immediately above and below the SI value of 3 have the coordinates (a, b) and (c, d) respectively, then the EC3 value may be calculated using the following equation:

$$EC3 = c + [(3 - d)/(b - d)](a - c)$$

Statistical Analysis

The mean labeling index was calculated for vehicle control group. The stimulation index (SI) defended as a relative labeling index of the test group to the vehicle control value was then calculated. Data were analysed using a one-way analysis of variance (one-way ANOVA). If the one-way ANOVA produced a significant difference, the differences between the concurrent vehicle control group and each of the experimental groups were analysed using the Dunnett's multiple comparison tests (Bruning and Kintz, 1997).

Results

Guinea Pig Maximization Test (GPMT)

In the guinea pig maximization test for isoeugenol and its dimers, the sensitization response rates were as follows: isoeugenol 100%, β-O-4-dilignol 0%, dehydrodiisoeugenol 50% and eugenol 20%. According to convention (Magnusson and Kligman, 1969), isoeugenol was therefore classified as an extreme skin sensitizer; β-O-4-dilignol as a weak skin sensitizer, dehydrodiisoeugenol as a moderate skin sensitizer and eugenol as a mild sensitizer (Table 1).

Non-RI LLNA

The results are shown in Table 2. Dose dependent increases of the stimulation indices were noted in the animals tested with isoeugenol, dehydrodiisoeugenol and eugenol, and stimulation indices for 3%, 10% and 30% isoeugenol were 1.52 ± 0.49, 2.43 ± 0.45 and 6.73 ± 0.88, respectively. Stimulation indices for 3%, 10% and 30% β-O-4-dilignol were 1.02 ± 0.27, 1.19 ± 0.30 and 1.05 ± 0.20, respectively. Stimulation indices for 3%,

Table 1. Results of the guinea pig maximization test for isoeugenol, isoeugenol dimers and eugenol

Chemical name	Sensitization rate (%)	Grade[a]	Classification[a]
Isoeugenol	100	V	Extreme
β-O-4-Dilignol	0	I	Weak
Dehydrodiisoeugenol	50	III	Moderate
Eugenol	20	II	Mild

[a] Classifications were made according to the criterion of Magnusson and Kligmann (1969).

Table 2. Results of non-radioisotopic local lymph node assay with isoeugenol, isoeugenol dimers and eugenol used in this study

	Isoeugenol		β-O-4-Dilignol		Dehydrodiisoeugenol		Eugenol	
% tested	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0%	1.00	0.10	1.00	0.12	1.00	0.11	1.00	0.10
3%	1.52	0.49	1.02	0.27	1.95	0.42	0.75	0.13
10%	2.43	0.45	1.19	0.30	3.09	0.31[a]	1.46	0.20
30%	6.73	0.88[a]	1.05	0.20	5.37	0.50[a]	3.83	0.68[a]
EC3 (%)	12.7		>30		9.4		22.8	

Results represent mean values and standard errors in four mice
The stimulation index (SI) was calculated by dividing the mean value obtained in each treatment group by that of the control group.
[a] Significantly different from the concurrent vehicle control (0%) at $P < 0.05$ (Dunnett's test).

10% and 30% dehydrodiisoeugenol were 1.95 ± 0.42, 3.09 ± 0.31 and 5.37 ± 0.50, respectively. Stimulation indices for 3%, 10% and 30% eugenol were 0.75 ± 0.13, 1.46 ± 0.20 and 3.83 ± 0.68, respectively. Concentrations of 10% or greater exposure of dehydrodiisoeugenol, 30% of isoeugenol and eugenol caused a significant increase in SIs compared with concurrent vehicle controls (Dunnett's multiple comparison tests, $P < 0.05$). Whereas β-O-dilignol did not show any lymph node cell proliferative activity in all concentrations tested. The EC3 values for isoeugenol, dehydrodiisoeugenol and eugenol were calculated using a standard method and found to be 12.7, 9.4 and 22.8, respectively. With regard to the test groups given β-O-dilignol, SI values did not exceed a value of 3 even in the highest dose group, and the EC3 value for this chemical was estimated as >30% for the purposes of comparison.

Discussion

Allergic contact dermatitis is regarded as a serious side effect of chemical products. Eugenol and isoeugenol are components of clove oil and nutmeg oil, and are fragrance chemicals with a spicy scent. They are extensively used in perfumes and deodorants, moreover they are expected to make whitening and antibacterial effects in the cosmetic and dermatological fields (Yamazaki *et al.*, 1998; Yamazaki *et al.*, 2000). However, these chemicals are known to be human contact sensitizers. Isoeugenol is a much stronger sensitizer than eugenol and classified as a moderate allergen, human class 2 (Basketter *et al.*, 2000). Meanwhile eugenol is classified as a weak allergen, human class 3. Isoeugenol has been reported to induce positive responses in the standard LLNA, other murine predictive tests and guinea pig prediction tests (Barratt and Basketter, 1992; Basketter *et al.*, 2005; Hilton *et al.*, 1996). The decreased sensitization potency of eugenol by dimerization with the addition of a hydroxyl group was previously reported (Takeyoshi *et al.*, 2004). Isoeugenol is an important cosmetic ingredient similar to eugenol, but isoeugenol is a stronger human sensitizer than eugenol. Therefore the possibility of reducing the sensitization potency of isoeugenol by dimerization was examined as well as a case of eugenol.

The guinea pig maximization test is a conventional skin sensitization test method, and it has been widely used for regulatory purposes. However, this method is not suitable for quantitative sensitization potency prediction because GPMT requires an independent dose selection for intradermal and topical applications. The LLNA is recognized as a reliable sensitization test, and it is currently accepted by many authorities for regulatory purposes. From the point of view of animal welfare, the LLNA is a preferable test method and it is the first choice sensitization test for the REACH program in the EU. In addition, LLNA can give a quantitative endpoint and it would be suitable for sensitization potency prediction. However, the standard LLNA requires the use of radioisotopes for detecting the lymph node cell proliferative response, and it requires specialized test facility and handling procedures. Meanwhile, non-RI LLNA employed in this study is totally based on the standard LLNA protocol except for using BrdU instead of ^{3}H-thymidine, and it can provide sensitizing potency information as well as standard LLNA (Takeyoshi *et al.*, 2004, 2005). So these two types of major skin sensitization tests were conducted.

The present investigations have shown that this chemical also tested positive in the non-RI LLNA and the GPMT. The derivation of EC3 values provides an opportunity to compare these forms of the assay with respect to sensitivity. In one series of investigations, the EC3 values for isoeugenol and eugenol were recorded as values of approximately 1.3% and 13%, respectively (Basketter *et al.*, 2000). In this study using the non-RI LLNA reported here, the EC3 values for isoeugenol and eugenol were calculated as 12.7% and 22.8%, respectively. The EC3 values obtained in the non-RI LLNA for these chemicals were somewhat higher than those in the standard LLNA. However, the EC3 in non-RI LLNA

for isoeugenol was lower than that for eugenol, thus the quantitative relationship of EC3 values are regarded as the same as the standard LLNA, and the values would be a parameter for the relative sensitization potency. The GPMT was also conducted with the same test chemicals. The sensitization rate for isoeugenol and eugenol were 100% and 20%, respectively. Thus isoeugenol was classified as an extreme sensitizer according to the classification criterion of this method. Similarly eugenol was classified as a moderate sensitizer. The results of non-RI LLNA and guinea pig maximization tests for isoeugenol and eugenol were consistent with the results reported previously.

In this study two types of isoeugenol dimer were tested with different bond-structures in addition to their monomer. Dehydrodiisoeugenol, one of the isoeugenol dimers, showed a 50% sensitization rate in the guinea pig maximization test and was classified as a moderate sensitizer. In the non-RI LLNA, this chemical induced dose dependent lymph node cell proliferation, and its EC3 value was estimated as 9.4%. Meanwhile, the other type of isoeugenol dimer, β-O-dilignol, turned out to have a 0% sensitization rate in the guinea pig maximization test, and was classified as a weak sensitizer. This chemical caused no lymph node cell proliferation, and its EC3 value was estimated as >30%.

Dehidrodiisoeugenol is a dimer in which the monomers are covalently bonded each other, so it would not be easily broken down to monomers in the skin. β-O-Dilignol is a dimer in which the monomers are bonded with ether bond. β-O-Dilignol may be broken down easier than dehydrodiisoeugenol. However, β-dilignol exerts less sensitization potency than its monomer, isoeugenol. It shows that the degradation of β-dilignol in the skin is not significant in this study.

In this study, dimerization of isoeugenol also yielded dimers with different sensitization potencies. Isoeugenol and eugenol are both important cosmetic ingredients, so the reduction of sensitization potency achieved by the dimerization may lead to the development of safer cosmetic ingredients. Isoeugenol dimers are not currently used for fragrance chemicals. However, these two isoeugenol dimers still retain their anti-oxidative and antibacterial effects similar to its monomer (unpublished data). Accordingly the dimerization of isoeugenol may yield a promising candidate for cosmetic ingredients with a low sensitization risk. This trial may contribute to a novel strategy to develop safer cosmetic ingredients.

In addition, when these two types of isoeugenol dimer were applied to the DEREK prediction system (Lhasa Limited, Leeds, UK), both dimers were predicted as 'PLAUSIBLE'. More data accumulation would be necessary, but the case studies for eugenol and isoeugenol dimers would provide valuable information to improve the prediction accuracy of SAR systems. A reduction of false-positives in SAR systems would contribute to effective screening of the promising cosmetic and pharmaceutical candidate chemicals.

References

Barratt MD, Basketter DA. 1992. Possible origin of the skin sensitization potential of isoeugenol and related compounds. (I). Preliminary studies of potential reaction mechanisms. *Contact Dermatitis* **27**: 98–104.

Basketter DA, Andersen KE, Liden C, Van Loveren H, Boman A, Kimber I, Alanko K, Berggren E. 2005. Evaluation of the skin sensitizing potency of chemicals by using the existing methods and considerations of relevance for elicitation. *Contact Dermatitis* **52**: 39–43.

Basketter DA, Blaikie L, Dearman RJ, Kimber I, Ryan CA, Gerberick GF, Harvey P, Evans P, White IR, Rycroft RJ. 2000. Use of the local lymph node assay for the estimation of relative contact allergenic potency. *Contact Dermatitis* **42**: 344–348.

Bruning JL, Kintz BL. 1997. *Computational Handbook of Statistics*, 4th edn. Addison-Wesley: Boston, USA.

Hilton I, Dearman RJ, Fielding I, Basketter DA, Kimber I. 1996. Evaluation of the sensitizing potential of eugenol and isoeugenol in mice and guinea pigs. *J. Appl. Toxicol.* **16**: 459–464.

Magnusson B, Kligman AM. 1969. The identification of contact allergens by animal assay. The guinea pig maximization test. *J. Invest. Dermatol.* **52**: 268–276.

Rastogi SC, Johansen JD, Frosch P, Menne T, Bruze M, Lepoittevin JP, Dreier B, Andersen KE, White IR. 1998. Deodorants on the European market: quantitative chemical analysis of 21 fragrances. *Contact Dermatitis* **38**: 29–35.

Schnuch A, Lessmann H, Geier J, Frosch PJ, Uter W; IVDK. 2004. Contact allergy to fragrances: frequencies of sensitization from 1996 to 2002. Results of the IVDK*. *Contact Dermatitis* **50**: 65–76.

Takeyoshi M, Iida K, Shiraishi K, Hoshuyama S. 2005. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. *J. Appl. Toxicol.* **25**: 129–134.

Takeyoshi M, Noda S, Yamazaki S, Kakishima H, Yamasaki K, Kimber I. 2004. Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the local lymph node assay. *J. Appl. Toxicol.* **24**: 77–81.

Takeyoshi M, Yamasaki K, Yakabe Y, Takatsuki M, Kimber I. 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2′-deoxyuridine (BrdU) incorporation. *Toxicol. Lett.* **119**: 203–208.

Yamazaki S, Suzuki K, Ikemoto T, Kakishima H. 1998. Analysis of contact dermatitis by cytokines III, sensitization and cross-reaction of phenolic compounds. *Yakugaku Zassi* **118**: 324–331.

Yamazaki S, Suzuki K, Ikemoto T, Kakishima H. 2000. Analysis of contact dermatitis by cytokines IV, skin sensitization of biphenyl compounds. *Yakugaku Zassi* **120**: 1221–1225.

Immunity
Article

The Cytokine RANKL Produced by Positively Selected Thymocytes Fosters Medullary Thymic Epithelial Cells that Express Autoimmune Regulator

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SUMMARY

The thymic medulla provides a microenvironment where medullary thymic epithelial cells (mTECs) express autoimmune regulator and diverse tissue-restricted genes, contributing to launching self-tolerance. Positive selection is essential for thymic medulla formation via a previously unknown mechanism. Here we show that the cytokine RANK ligand (RANKL) was produced by positively selected thymocytes and regulated the cellularity of mTEC by interacting with RANK and osteoprotegerin. Forced expression of RANKL restored thymic medulla in mice lacking positive selection, whereas RANKL perturbation impaired medulla formation. These results indicate that RANKL produced by positively selected thymocytes is responsible for fostering thymic medulla formation, thereby establishing central tolerance.

INTRODUCTION

The thymus provides multiple microenvironments that sequentially support the development and selection of T lymphocytes. Thymocytes that express clonotypic and diverse T cell antigen receptors (TCR) are generated in the thymic cortex where cortical thymic epithelial cells (cTECs) present a set of self-peptides by virtue of proteasomes containing β5t and lysosomal proteases including cathepsin L (Finkel et al., 1989; Honey et al., 2002; Murata et al., 2007). TCR interaction with self-peptide-loaded MHC molecules expressed by cortical cells, including cTECs and hematopoietic cells, determines the life and death of immature thymocytes, and a fraction of cortical thymocytes survive TCR signals for further development (Kisielow et al., 1988; Daniels et al., 2006) and relocate to the thymic medulla chiefly via CCR7-mediated chemotaxis (Ueno et al., 2004; Kwan and Killeen, 2004). This process in the thymic cortex, referred to as positive selection, enriches T lymphocytes that demonstrate modest reactivity to self-peptides and potential responsiveness to foreign antigens. Entering the thymic medulla, positively selected semimature thymocytes further interact with self-peptides that are expressed by medullary thymic epithelial cells (mTECs) and dendritic cells (DCs). mTECs express a diverse set of genes representing essentially all tissues of the body, at least partly because of a nuclear molecule called autoimmune regulator (Aire) (Derbinski et al., 2001; Anderson et al., 2002), whereas thymic DCs, which efficiently present various self-peptides, are predominantly localized in the medulla and at least in part derived from circulation (Bonasio et al., 2006). The interaction of positively selected thymocytes in the thymic medulla with a diverse set of self-peptides, including tissue-restricted antigens presented by mTECs and DCs, is essential for establishing self-tolerance (Gallegos and Bevan, 2004).

Thymic medulla formation is dependent on the differentiation of mTECs from their endodermal precursor cells that are generated at the third pharyngeal pouch (Rossi et al., 2006; Bleul et al., 2006; Hamazaki et al., 2007), and the development of mTECs is regulated by the NF-κB activation pathway that includes transcription factor RelB and signal transducer TRAF6 (Burkly et al., 1995; Boehm et al., 2003; Akiyama et al., 2005). A recent study showed that $CD4^+CD3^-$ lymphoid tissue inducer (LTi) cells are involved in initiating embryonic mTEC development by producing the cytokine RANK ligand (RANKL) (Rossi et al., 2007).

Importantly, generation of the thymic microenvironment is also regulated by the development of thymocytes. This regulation is referred to as thymic crosstalk, and the signals produced by positively selected thymocytes are crucial for thymic medulla formation (Shores et al., 1991; van Ewijk et al., 1994). Mice deficient for the generation of mature thymocytes because of the lack of

positive selection exhibit defective medulla development (Philpott et al., 1992; Negishi et al., 1995), and reconstitution of the thymus with positively selected thymocytes restores formation of the medullary region (Surh et al., 1992; Nasreen et al., 2003). Thymus deficient for positive selection contains scattered and small clusters of mTECs, suggesting that positively selected thymocytes are responsible for the development of mTECs (Naspetti et al., 1997; Gray et al., 2006). Nonetheless, whether positive selection regulates the number or functional maturation of mTECs remains unclear. Molecular signals that regulate positive-selection-mediated medulla formation are also unknown.

The present study was aimed at identifying molecules that participate in positive-selection-mediated medulla formation in the thymus. We show that the interaction between RANKL produced by positively selected thymocytes and RANK and osteoprotegerin (OPG, a decoy receptor for RANKL) expressed by mTECs played a crucial role in positive-selection-mediated medulla formation by regulating the cellularity of Aire-expressing mTECs. We also present evidence that medulla formation in adult mice was chiefly regulated by positively selected thymocytes rather than by other intrathymic RANKL-expressing cells, including LTi cells. Thus, this study demonstrates that RANKL produced by positively selected thymocytes plays a major role in increasing the number of mTECs and forming thymic medulla that contains Aire-expressing mTECs.

RESULTS

Positive Selection Fosters Medulla Formation by Affecting mTEC Cellularity

It was previously shown that positive selection is crucial for thymic medulla formation and that mice lacking positive selection because of a deficiency of either TCRα or TCR-associated tyrosine kinase ZAP70 exhibit defective medulla formation in the thymus (Philpott et al., 1992; Negishi et al., 1995; also shown in hematoxylin-eosin-stained sections of Figure 1A). However, in agreement with previous findings (Naspetti et al., 1997; Gray et al., 2006), the scattered distribution of small mTEC clusters identified by mTEC-specific antibody ER-TR5 and mTEC-binding lectin UEA1 was detectable in the thymus of TCRα-deficient (*Tcra*$^{-/-}$) or ZAP70-deficient (*Zap70*$^{-/-}$) mice (Figure 1A). Flow cytometry analysis of thymic stromal cells showed that the numbers of mTEC identified as CD45$^-$I-A$^+$UEA1$^+$ or CD45$^-$I-A$^+$Ly51$^-$ were markedly reduced in TCRα-deficient or ZAP70-deficient mice (approximately 5%–10% of normal numbers), whereas the numbers of cTEC identified as CD45$^-$I-A$^+$UEA1$^-$ or CD45$^-$I-A$^+$Ly51$^+$ were not considerably reduced (Figures 1B and 1C). These results indicate that positive selection affects the number of mTECs but not of cTECs.

The major function of mTECs so far identified is to establish central tolerance by expressing a diverse set of tissue-restricted genes and by attracting positively selected cortical thymocytes for interaction with mTECs and DCs in the medulla (Derbinski et al., 2001; Anderson et al., 2002; Ueno et al., 2004; Kwan and Killeen, 2004; Gallegos and Bevan, 2004; Bonasio et al., 2006). Molecules involved in this mTEC function include Aire, a nuclear factor associated with promiscuous gene expression (Anderson et al., 2002), and CCL21, a chemokine that attracts positively selected thymocytes that express CCR7 (Ueno et al.,

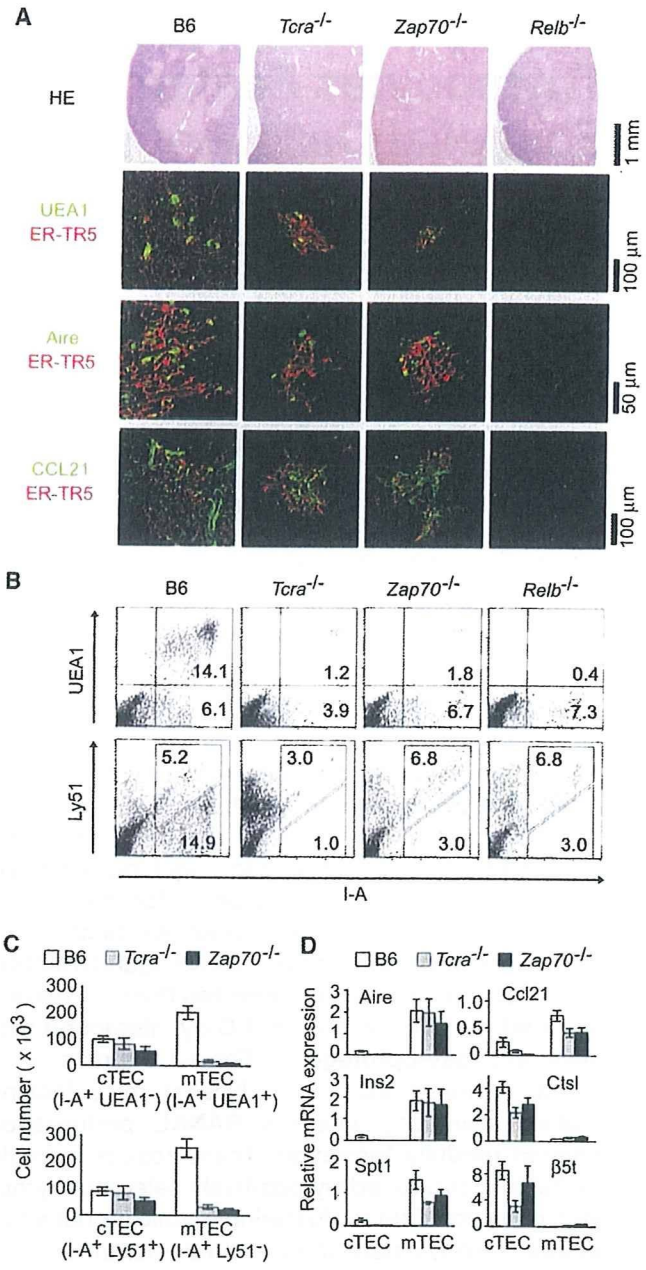

Figure 1. Positive Selection of Thymocytes Affects Number of mTECs

(A) Thymus lobes from 4- to 6-week-old young adult B6, *Tcra*$^{-/-}$, *Zap70*$^{-/-}$, or *Relb*$^{-/-}$ mice were stained with hematoxylin and eosin (HE) or with mTEC-specific monoclonal antibody ER-TR5 (red) and UEA1, antibody specific for Aire, or antibody specific for CCL21 (green).

(B) Two-color flow cytometry profiles for I-A and UEA1 (top) and I-A and Ly51 (bottom) of CD45$^-$ nonleukocytes prepared from thymuses of 4- to 6-week-old mice. Numbers indicate frequency of cells within indicated areas.

(C) Numbers of cTECs and mTECs per mouse in indicated mice. Averages and standard errors (B6, n = 4; *Tcra*$^{-/-}$, n = 4; *Zap70*$^{-/-}$, n = 3) are shown.

(D) Quantitative RT-PCR analysis of indicated genes in isolated cTECs and mTECs. mRNA expression was normalized to GAPDH mRNA, and those in CD45$^-$ total thymic stromal cells were arbitrarily set to 1. Averages and standard errors of at least three independent measurements are shown.

2004). Aire and CCL21 were detectable in mTEC clusters of TCRα-deficient or ZAP70-deficient mice, unlike in the thymus of RelB-deficient (*Relb*$^{-/-}$) mice where mTEC development was defective (Figure 1A). Quantitative mRNA analysis showed that mTECs isolated from TCRα-deficient or ZAP70-deficient mice indeed expressed Aire and CCL21 (Figure 1D). The generation of CD80-expressing mature mTECs was also detectable in TCRα-deficient or ZAP70-deficient mice (data not shown). The expression of Aire-dependent tissue-restricted genes, such as insulin 2 and salivary protein 1, was detectable in mTECs of TCRα-deficient or ZAP70-deficient mice, indicating that mTECs in these mice were capable of Aire-dependent promiscuous gene expression (Figure 1D). cTECs from TCRα-deficient or ZAP70-deficient mice expressed cathepsin L and β5t (Figure 1D), the molecules essential for thymocyte development in the cortex (Honey et al., 2002; Murata et al., 2007). These results indicate that mTECs and cTECs generated without positive selection express molecules that represent their functional maturity.

Small numbers of mTECs including Aire-expressing mTECs were detectable even in RAG2-deficient mice that lacked CD4$^+$CD8$^+$ (double-positive, DP) thymocytes (Derbinski et al., 2001; Rossi et al., 2007; also shown in Figure S1 available online), in agreement with the notion that functionally mature mTECs are generated without positive selection. Together, these results indicate that positive selection promotes the increase in the number of mTECs rather than the functional maturation of mTECs and thereby nurtures the formation of thymic medulla.

TNFSF Expressed in Thymocyte Subsets and TNFRSF Expressed in TEC Subsets

In order to explore the molecular mechanisms mediating the increase in mTEC cellularity caused by positive selection, we surveyed oligonucleotide microarray data from our previous study where we compared gene-expression profiles between positively selected TCR-transgenic thymocytes and wild-type thymocytes (Nitta et al., 2006). We noticed that genes encoding tumor necrosis factor superfamily (TNFSF) members, such as lymphotoxin (LT) α, LTβ, and RANKL, were strongly expressed in positively selected TCR-transgenic thymocytes (Table S1). We therefore analyzed the expression of all TNFSF genes in thymocyte subsets fractionated from normal adult thymus according to the expression of CD4 and CD8. As shown in Figure 2A and in agreement with microarray data, LTα, TNFα, LTβ, OX40L, CD40L, FasL, CD30L, and RANKL were expressed at significantly ($p < 0.05$) higher amounts in CD4$^+$CD8$^-$ and/or CD4$^-$CD8$^+$ (single-positive, SP) thymocytes than in DP thymocytes. LTα, TNFα, LTβ, CD30L, and RANKL expression was high in both CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes, whereas OX40L, CD40L, and FasL expression was high in CD4$^+$CD8$^-$ thymocytes but not in CD4$^-$CD8$^+$ thymocytes. Other TNFSF members showed no significant difference ($p \geq 0.05$) in the expression between DP and SP thymocyte subsets.

We then analyzed the expression of TNF receptor superfamily (TNFRSF) genes in mTECs and cTECs isolated from normal adult thymus. We found that the genes for OX40, CD40, Fas, CD30, 4-1BB, TRAILR2, RANK, OPG, BAFFR, BCMA, RELT, and Eda2r were expressed at significantly ($p < 0.05$) higher amounts in mTECs than in cTECs (Figure 2B). In contrast, the expression of CD27, TWEAKR, GITR, and TNFRH3 was significantly ($p < 0.05$) higher in cTECs than in mTECs. Other TNFRSF members showed no significant difference ($p \geq 0.05$) in the expression between mTECs and cTECs.

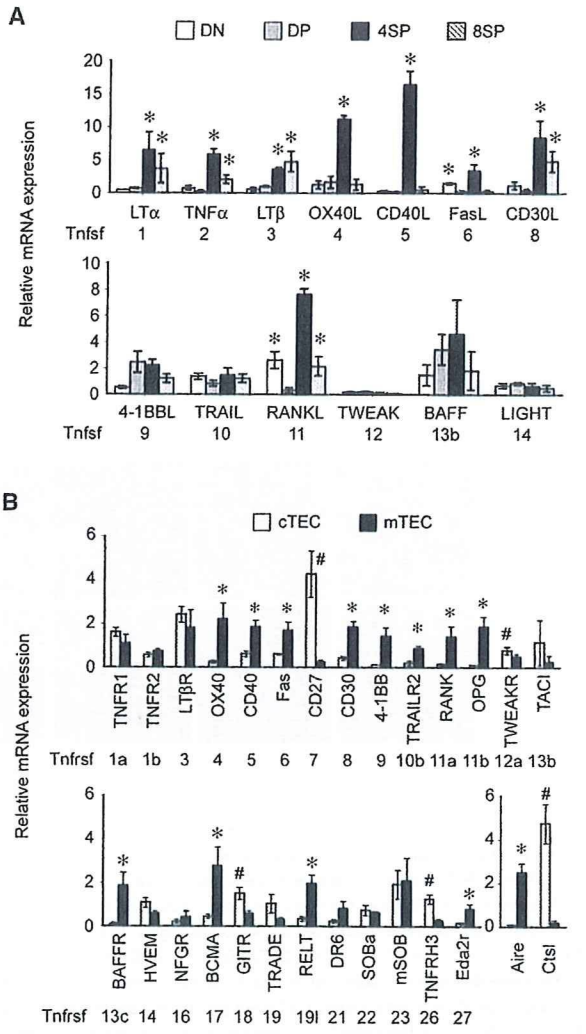

Figure 2. Expression of TNFSF Genes in Thymocytes and TNFRSF Genes in Thymic Epithelial Cells

(A) Quantitative RT-PCR analysis of sorted CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (4SP), and CD4$^-$CD8$^+$ (8SP) thymocytes. mRNA expression of TNFSF genes was normalized to GAPDH mRNA, and those in total thymocytes were arbitrarily set to 1. Bar graphs show means ± standard errors of at least three independent measurements. Asterisks indicate significant ($p < 0.05$) increase compared to the amounts in DP thymocytes. mRNA expression of genes encoding Tnfsf7 (CD27L), Tnfsf13 (APRIL), Tnfsf15 (TL1), and Tnfsf18 (GITRL) was not detectable in total thymocytes or thymocyte subpopulations examined.

(B) Quantitative RT-PCR analysis of sorted CD45$^-$I-A$^+$UEA1$^-$ cTECs and CD45$^-$I-A$^+$UEA1$^+$ mTECs. mRNA expression of TNFRSF genes was normalized to GAPDH mRNA, and those in CD45$^-$ total thymic stromal cells were arbitrarily set to 1. Bar graphs show means ± standard errors of at least three independent measurements. Asterisks indicate significant ($p < 0.05$) increase in mRNAs in mTECs compared to those in cTECs, whereas sharps indicate significant ($p < 0.05$) increase in mRNA amounts in cTECs compared to those in mTECs. mRNA expression of Aire and cathepsin L (Ctsl) indicates successful isolation of mTECs and cTECs, respectively. mRNA expression of Tnfrsf25 (DR3) was not detectable in CD45$^-$ thymus cells, cTECs, or mTECs.

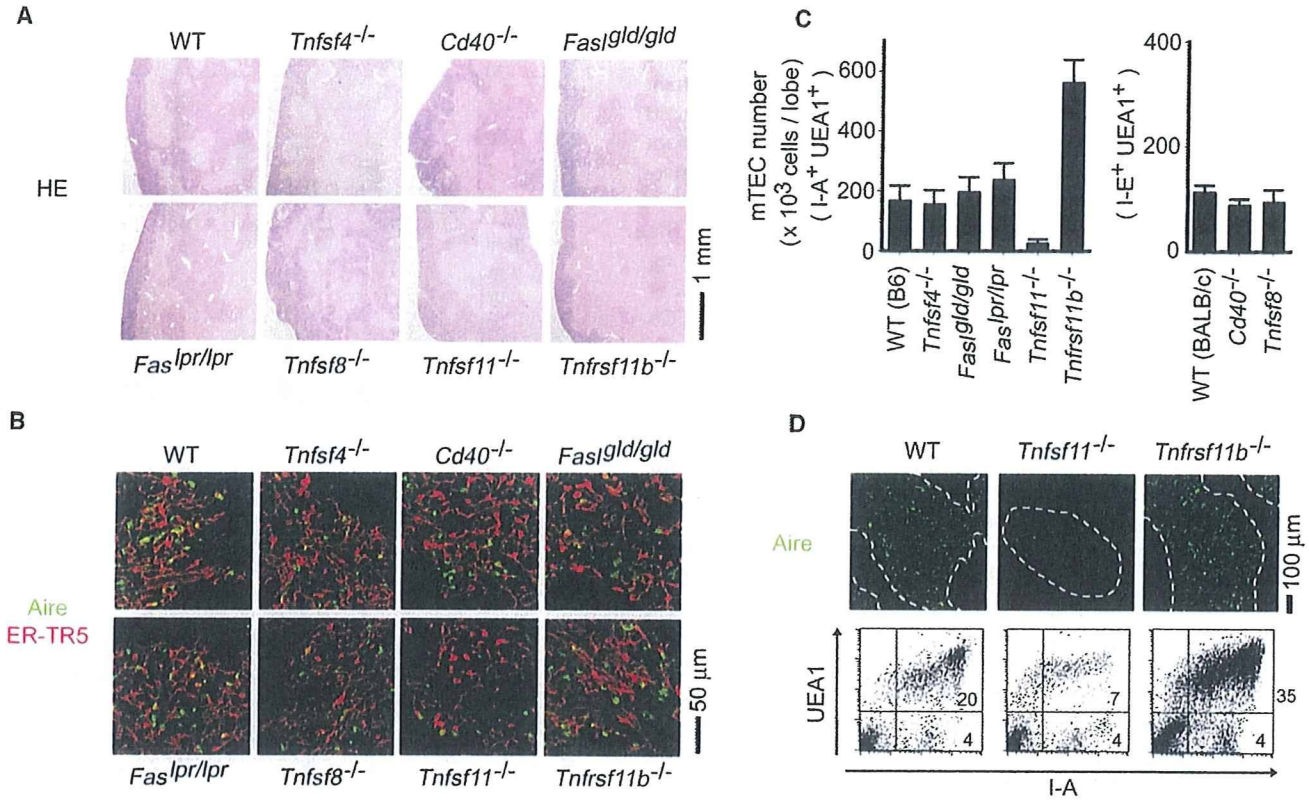

Figure 3. RANKL and OPG Affect Number of mTECs

(A and B) Thymus sections from indicated mice at 3–9 weeks old were analyzed by HE staining (A) and two-color immunostaining with antibody specific for Aire (green) and ER-TR5 (red) (B). B6 mice were used as wild-type (WT) control.

(C) Numbers of mTECs per thymus lobe were calculated by flow cytometry analysis of $CD45^-I\text{-}A^{b+}UEA1^+$ cells for mice of B6 background (left) and $CD45^-I\text{-}E^{d+}UEA1^+$ cells for mice of BALB/c background (right). Averages and standard errors are shown. B6, n = 6; *Tnfsf4*$^{-/-}$, n = 4; *Fasl*$^{gld/gld}$, n = 3; *Fas*$^{lpr/lpr}$, n = 3; *Tnfsf11*$^{-/-}$, n = 2; *Tnfrsf11b*$^{-/-}$, n = 3; BALB/c, n = 3; *Cd40*$^{-/-}$, n = 3; *Tnfsf8*$^{-/-}$, n = 3.

(D) Representative low-magnification images of Aire immunostaining in medullary region identified by ER-TR5 staining and marked by dotted lines, indicating that Aire-expressing cells are present in all three groups but are fewer and more detectable in the thymus of *Tnfsf11*$^{-/-}$ and *Tnfrsf11b*$^{-/-}$ mice, respectively, than in B6 wild-type (WT) mice. Representative two-color flow cytometry profiles for I-A and UEA1 of $CD45^-$ nonleukocytes of indicated mice are also shown. Numbers in quadrants indicate frequency of cells in boxes.

Consequently, we found that in the following five TNFSF ligand-receptor combinations, namely, between OX40L and OX40, between CD40L and CD40, between FasL and Fas, between CD30L and CD30, and among RANKL, RANK (signaling receptor for RANKL), and OPG (nonsignaling soluble decoy receptor for RANKL; Theill et al., 2002), ligands were more strongly expressed in SP thymocytes than in DP thymocytes and receptors were more strongly expressed in mTECs than in cTECs.

RANKL and OPG Influence mTEC Cellularity

We next examined mTEC development and medulla formation in mice deficient for one of the five TNFSF ligand-receptor combinations. We found that the deficiency of OX40L (*Tnfsf4*$^{-/-}$), CD40 (*Cd40*$^{-/-}$), FasL (*Fasl*$^{gld/gld}$), Fas (*Fas*$^{lpr/lpr}$), or CD30L (*Tnfsf8*$^{-/-}$) did not significantly ($p \geq 0.05$) affect the number of mTECs, the generation of Aire-expressing mTECs, or medulla formation (Figures 3A–3C). However, mice deficient for RANKL (*Tnfsf11*$^{-/-}$) exhibited a significant ($p < 0.05$) reduction in the number of mTECs (Figure 3C). In the thymus of RANKL-deficient mice, the number of Aire-expressing mTECs was also decreased as shown by immunohistological analysis (Figure 3D). Nonetheless, the development of Aire-expressing mTECs and the formation of thymic medulla were detectable in the thymus of RANKL-deficient mice (Figures 3A–3C). In contrast, mice deficient for OPG (*Tnfrsf11b*$^{-/-}$), a soluble decoy receptor for RANKL (Theill et al., 2002) and the receptor more strongly expressed in mTECs than in cTECs (Figure 2B), developed a significantly ($p < 0.05$) large number of mTECs and exhibited large thymic medulla with many Aire-expressing mTECs (Figures 3A–3D).

These results indicate that among TNFSF members that are more strongly expressed in SP thymocytes than in DP thymocytes, RANKL plays a major role in increasing the number of mTECs, and its decoy receptor, OPG, regulates mTEC cellularity and medulla formation.

RANKL Is Produced by Positively Selected Thymocytes

The expression of RANKL in normal adult mice was detectable in bulk $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes but not DP thymocytes, as determined by quantitative mRNA analysis (Figures 2A and 4A). RANKL expression was detectable in $CD69^{hi}$ semimature and $CD69^{lo}$ mature $CD4^+CD8^-$ SP thymocytes isolated from normal B6 mice, both of which expressed $TCR\alpha\beta^{hi}$

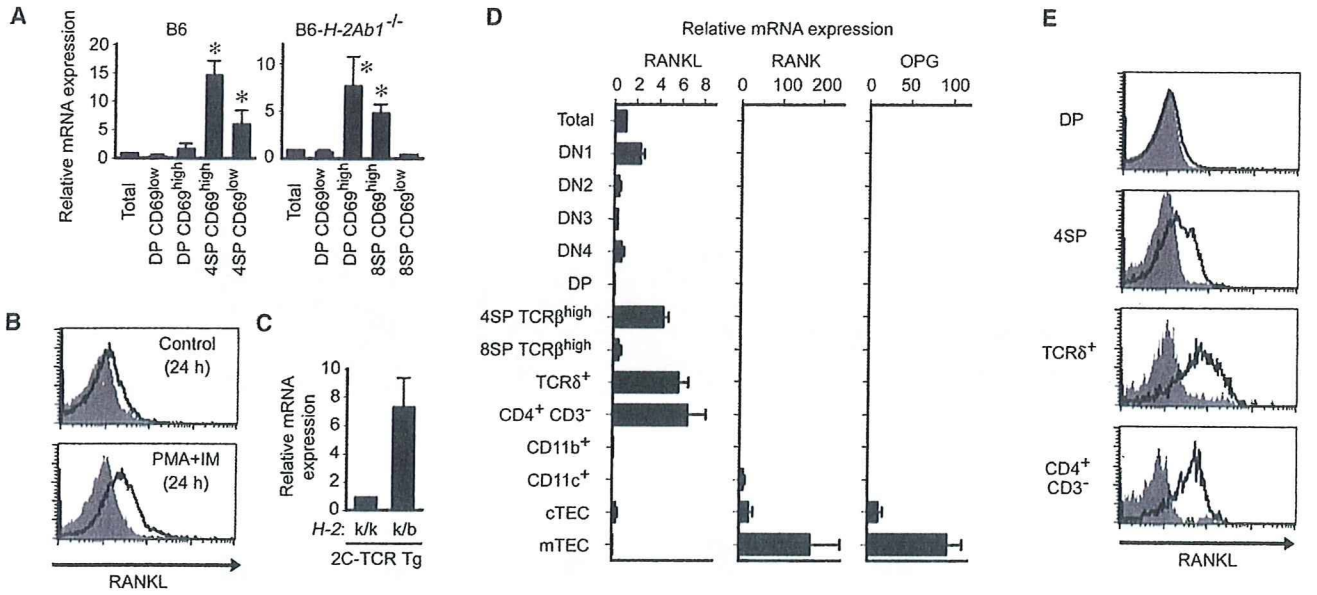

Figure 4. Expression of RANKL, RANK, and OPG in Thymus Cell Subpopulations

(A) mRNA expression of RANKL in total, $CD4^+CD8^+CD69^{lo}$, $CD4^+CD8^+CD69^{hi}$, $CD4^+CD8^-CD69^{hi}$, and $CD4^+CD8^-CD69^{lo}$ thymocytes isolated from 4-week-old B6 mice (n = 4), and total, $CD4^+CD8^+CD69^{lo}$, $CD4^+CD8^+CD69^{hi}$, $CD4^-CD8^+CD69^{hi}$, and $CD4^-CD8^+CD69^{lo}$ thymocytes isolated from MHC class II-deficient 4-week-old B6-*H-2Ab1*$^{-/-}$ mice (n = 3).

(B) Flow cytometry analysis of RANKL expression by TCR-stimulated thymocytes. Thymocytes from 3-week-old MHC class I and class II double-deficient B6-*H-2Ab1*$^{-/-}$*B2m*$^{-/-}$ mice were cultured in the absence or presence of phorbol 12-myristate 13-acetate (PMA, 0.2 ng/ml) and ionomycin (0.2 μg/ml) for 24 hr. RANKL staining profiles (solid lines) and control staining profiles (shaded lines) are shown. Representative profiles of three independent experiments are shown.

(C) mRNA expression of RANKL in $CD4^+CD8^+$ thymocytes isolated from positively selecting *H-2*$^{k/b}$ 2C-TCR-transgenic mice and null selecting *H-2*$^{k/k}$ 2C-TCR-transgenic mice (n = 3).

(D) mRNA expression of RANKL, RANK, and OPG in indicated thymus cell subpopulations isolated from 4- to 5-week-old B6 mice. DN1, DN2, DN3, and DN4 represent $CD4^-CD8^-CD25^-CD44^+$, $CD4^-CD8^-CD25^+CD44^+$, $CD4^-CD8^-CD25^+CD44^-$, and $CD4^-CD8^-CD25^-CD44^-$ thymocyte subpopulations. $CD4^+CD3^-CD8^-B220^-CD11c^-$ thymocyte subpopulation was used as $CD4^+CD3^-$ cells (Rossi et al., 2007). $CD45^-I\text{-}A^+UEA1^-$ cTECs and $CD45^-I\text{-}A^+UEA1^+$ mTECs were used. mRNA expression in total thymus cells are normalized to 1. Averages and standard errors (n = 3) are shown. Note that $CD4^+CD8^-TCR\beta^{hi}$ cells, $TCR\delta^+$ cells, and $CD4^+CD3^-$ cells are the three major cells that express RANKL mRNA in the thymus. RANKL expression detectable in DN1 ($CD44^+CD25^-CD4^-CD8^-$) cells may be due to $TCR\gamma\delta^+$ cells that are contained in DN1 population.

(E) Cell-surface RANKL detection (solid lines) and control staining (shaded lines) were examined in indicated subpopulations of 5-week-old B6 thymocytes incubated for 16 hr without stimulation. Representative profiles of three independent experiments are shown.

(Figure 4A). RANKL was also detectable in $CD69^{hi}$ semimature $CD4^-CD8^+$ SP thymocytes, rather than $CD69^{lo}$ mature $CD4^-CD8^+$ SP thymocytes, isolated from MHC class II-deficient mice (Figure 4A), indicating that RANKL is expressed in semimature thymocytes that are destined to become either $CD4^+CD8^-$ or $CD4^-CD8^+$ SP thymocytes. The $CD69^{hi}$ subpopulation of DP thymocytes, which represent semimature thymocytes that recently received positive-selection-inducing TCR signals (Bendelac et al., 1992), expressed a significantly ($p < 0.05$) higher amount of RANKL than the majority of DP thymocytes, which were $CD69^{lo}$, in MHC class II-deficient mice (Figure 4A). Stimulation of preselected DP thymocytes isolated from MHC class I- and MHC class II-deficient mice (*H-2Ab1*$^{-/-}$*B2m*$^{-/-}$) with plate-bound antibody specific for TCR or with phorbol ester plus ionomycin, which mimics positive-selection-inducing TCR signals (Takahama and Nakauchi, 1996), elevated cell-surface expression of RANKL protein (Figure 4B, data not shown). Indeed, DP thymocytes isolated from positively selecting 2C-TCR-transgenic mice expressed a significantly ($p < 0.05$) higher amount of RANKL than did DP thymocytes isolated from null selecting 2C-TCR-transgenic mice (Figure 4C). These results indicate that RANKL is produced in semimature thymocytes that recently received positive-selection-inducing TCR signals. These results also suggest that the amount and kinetics of RANKL expression may be unequal between cells destined to become $CD4^+CD8^-$ T lymphocytes and those destined to become $CD4^-CD8^+$ T lymphocytes.

$TCR\gamma\delta^+$ Cells or Id2-Dependent LTi Cells Are Dispensable for Medulla Formation in Adult Mice

To address whether cells other than positively selected thymocytes might produce RANKL in the thymus, we examined the expression of RANKL as well as its receptors, RANK and OPG, in various cell subpopulations isolated from adult thymus (Figure 4D). We found that in addition to positively selected SP thymocytes, RANKL expression in the thymus was highly detectable in $TCR\gamma\delta^+$ cells and $CD4^+CD3^-$ LTi cells, as assessed by quantitative mRNA analysis (Figure 4D) and by cell-surface protein analysis (Figure 4E). The detection of RANKL in SP thymocytes was specific, as indicated by the fact that no signals were detected in SP thymocytes isolated from RANKL-deficient mice (Figure S2). RANKL expression was detectable in CD4SP thymocytes from embryonic day 18.5 (E18.5) mice and 1-day-old newborn mice (Figure S3), suggesting that newly generated

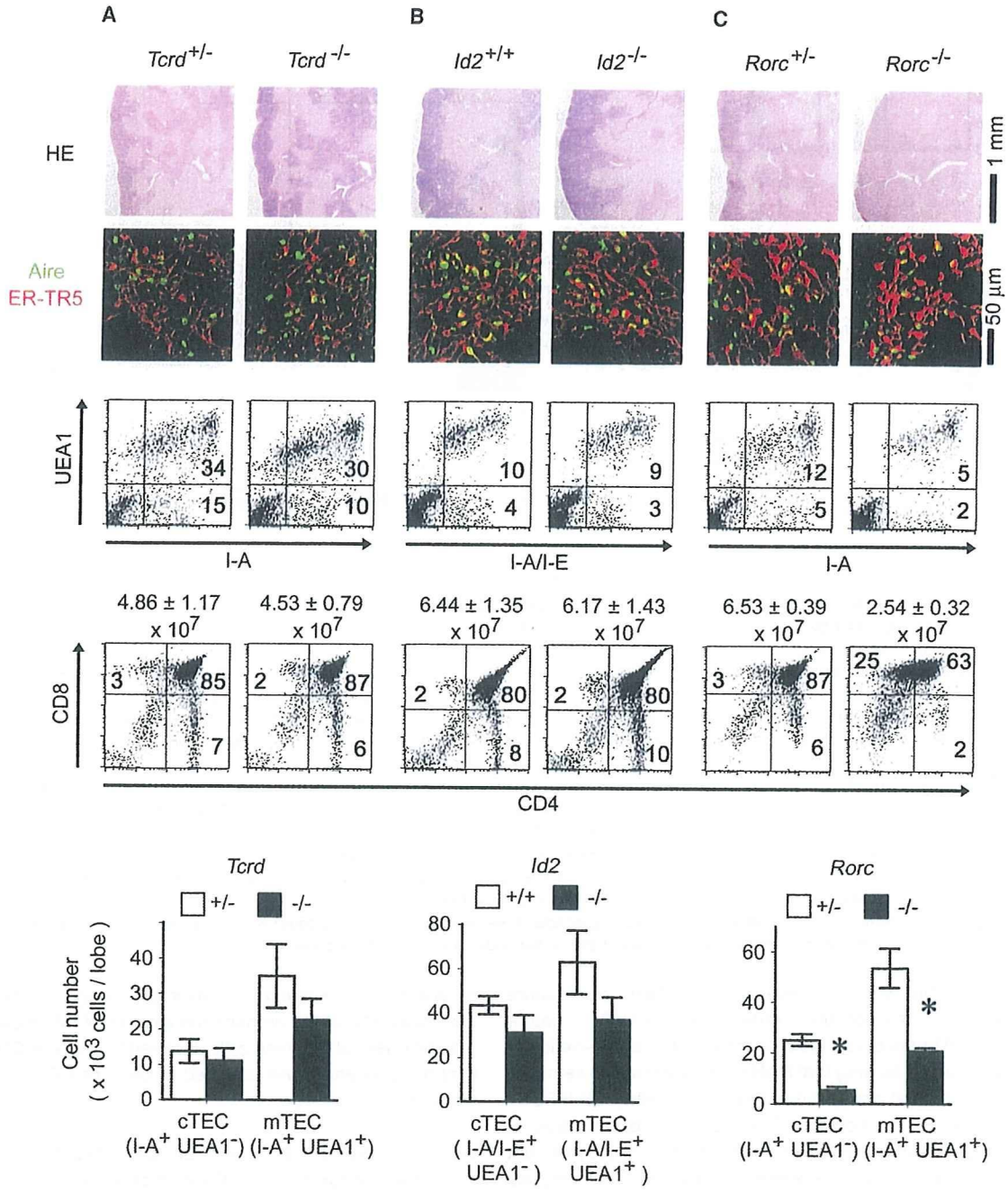

Figure 5. Thymic Epithelial Cells in TCRδ-, Id2-, or RORγt-Deficient Mice

Thymuses from age- and genetic background-matched *Tcrd*$^{+/-}$ and *Tcrd*$^{-/-}$ mice (B6 background [A]), *Id2*$^{+/+}$ and *Id2*$^{-/-}$ mice (ICR background [B]), or *Rorc*$^{+/-}$ and *Rorc*$^{-/-}$ mice (B6 background [C]) were subjected to section analysis and flow cytometry analysis as indicated. A monoclonal antibody specific for both I-A and I-E (clone M5/114) was used to detect class II MHC molecules in mice of ICR background. Averages and standard errors of thymocyte numbers per thymus lobe are also shown. Bar graphs show averages and standard errors of the numbers of CD45$^-$I-A$^+$UEA1$^-$ cTECs and CD45$^-$I-A$^+$UEA1$^+$ mTECs in indicated mice. *Tcrd*$^{+/-}$, n = 4; *Tcrd*$^{-/-}$, n = 5; *Id2*$^{+/+}$, n = 5; *Id2*$^{-/-}$, n = 6; *Rorc*$^{+/-}$, n = 3; *Rorc*$^{-/-}$, n = 3. Asterisks indicate significant difference ($p < 0.01$).

SP thymocytes can be the source of RANKL in the thymus. The detection of RANKL in CD4$^+$CD3$^-$ LTi cells of adult thymus was in agreement with previously reported results (Rossi et al., 2007). On the other hand, among thymic cell subpopulations examined, RANK and OPG were most prominently and almost exclusively detected in mTECs (Figure 4D).

In contrast to mice deficient for positive selection (Figure 1), we found no significant ($p \geq 0.05$) impairment in the number of mTECs or the development of thymic medulla containing Aire-expressing mTECs in adult mice lacking TCRδ (*Tcrd*$^{-/-}$) (Figure 5A) or adult mice deficient for Id2 (*Id2*$^{-/-}$) (Figure 5B; Figure S4), in which LTi cells in embryonic intestine are barely

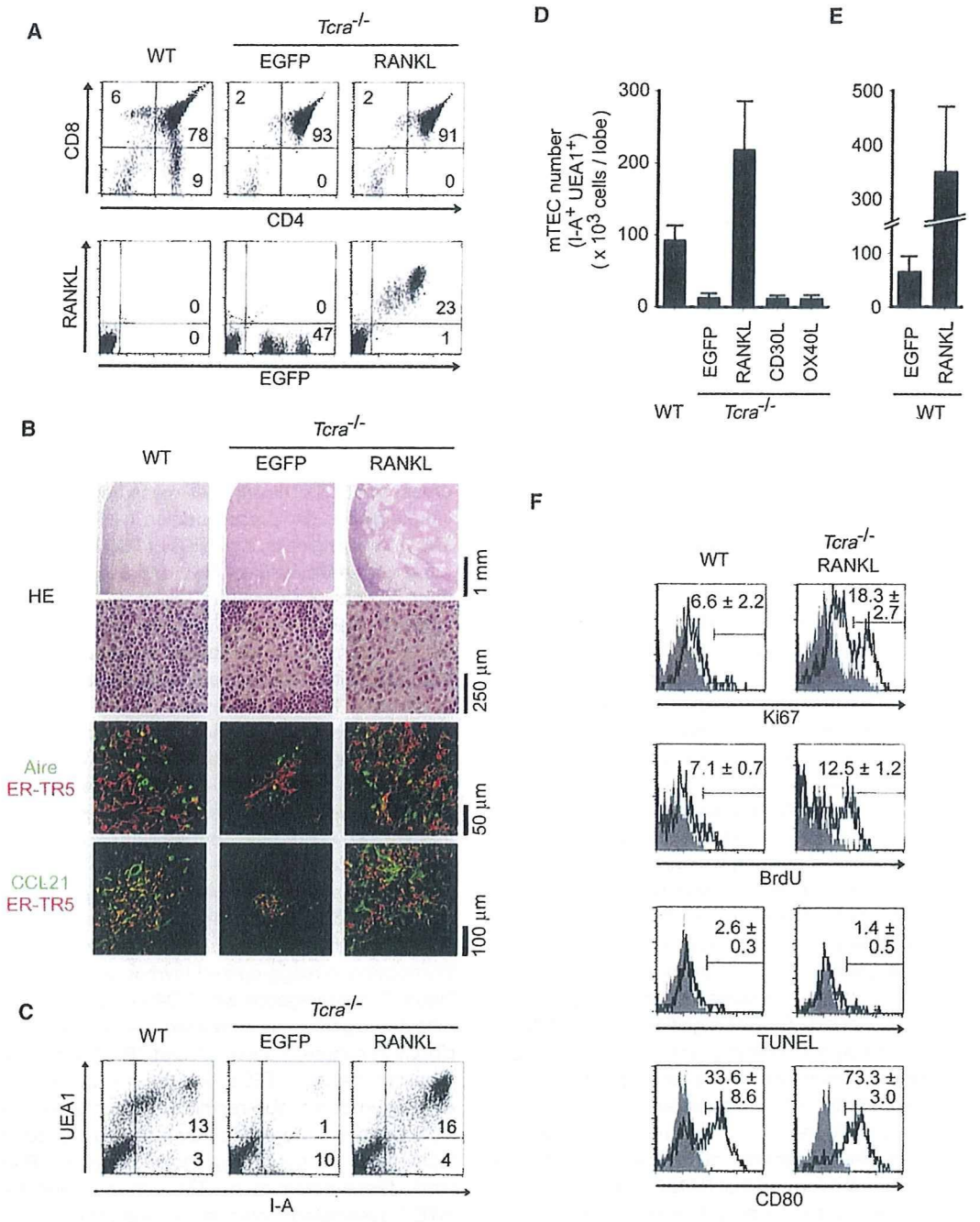

Figure 6. RANKL Expression Restores Medulla Formation without Positive Selection

Sca1$^+$ bone marrow cells from *Tcra*$^{-/-}$ mice were infected with retrovirus expressing RANKL along with EGFP or EGFP alone, and transplanted into lethally irradiated *Tcra*$^{-/-}$ mice. Mice were analyzed 4–5 weeks after transplantation. Frequencies of EGFP$^+$ cells in thymocytes ranged from 23% to 73%.

(A) Flow cytometry profiles of ungated thymocytes from adult B6 mice (WT), *Tcra*$^{-/-}$ mice expressing EGFP alone, or *Tcra*$^{-/-}$ mice expressing RANKL along with EGFP. Numbers indicate frequency of cells within indicated areas.

(B) Thymus section analysis. High-magnification HE-stained images show that thymic medullary areas generated in RANKL-expressing *Tcra*$^{-/-}$ mice are devoid of lymphoid cells, and this finding was supported by the failure to detect CD4- or CD8-expressing cells in those areas (not shown).

(C) Representative results of flow cytometry analysis for I-A$^+$UEA1$^+$ mTECs in CD45$^-$ nonleukocytes.

(D) Numbers (averages and standard errors) of CD45$^-$I-A$^+$UEA1$^+$ mTECs per thymus lobe in B6 mice (WT) or *Tcra*$^{-/-}$ mice reconstituted with *Tcra*$^{-/-}$ bone marrow cells that retrovirally expressed EGFP alone or EGFP along with RANKL, CD30L, or OX40L. WT, n = 6; EGFP, n = 4; RANKL, n = 3; CD30L, n = 3; OX40L, n = 3.

(E) Numbers (averages and standard errors) of CD45$^-$I-A$^+$UEA1$^+$ mTECs per thymus lobe in B6 mice (WT) reconstituted with bone marrow cells that expressed EGFP alone or EGFP along with RANKL, indicating that retroviral expression of RANKL increases the number of mTECs even in normal mice. EGFP, n = 4; RANKL, n = 3.

detectable (Yokota et al., 1999), indicating that unlike positively selected thymocytes, TCR$\gamma\delta^+$ cells and Id2-dependent LTi cells are dispensable for mTEC development and medulla formation in adult mice. However, the number of mTECs appeared slightly, although not significantly ($p \geq 0.05$), reduced in TCRδ-deficient mice or Id2-deficient mice (Figures 5A and 5B), suggesting that TCR$\gamma\delta^+$ cells and Id2-dependent LTi cells may partially contribute to the optimal cellularity of mTECs.

Similar to Id2-deficient mice, Aire-expressing mTECs were detectable in RORγt-deficient (*Rorc*$^{-/-}$) mice (Figure 5C) lacking LTi cells (Eberl et al., 2004). Unlike Id2-deficient mice, however, RORγt-deficient mice exhibited reduced number of mTECs and small medulla (Figure 5C). The deficiency in RORγt causes not only loss of LTi cells but also reduction in the numbers of DP and SP thymocytes because of reduced survival of DP thymocytes (Sun et al., 2000; also shown in Figure 5C). Accordingly, the number of cTECs was significantly ($p < 0.01$) reduced in RORγt-deficient mice, unlike Id2-deficient mice (Figures 5B and 5C). Thus, the reduced number of mTECs in RORγt-deficient mice may be due to the reduced number of DP and SP thymocytes, including positively selected thymocytes, rather than the loss of LTi cells.

RANKL Neutralization Perturbs mTEC Cellularity

The above-mentioned results suggest that RANKL produced by positively selected thymocytes plays a major role in expanding mTEC cellularity to form thymic medulla. We then examined whether RANKL expressed by developing thymocytes is essential for medulla formation in normal adult mice. To do so, normal B6 mice were reconstituted with B6 bone marrow hematopoietic progenitor cells that were infected with a retrovirus that expressed a soluble fusion protein of RANK and human immunoglobulin Fc portion (RANK-Fc) along with enhanced green fluorescence protein (EGFP). RANK-Fc fusion protein used in this study specifically binds to RANKL (Figure S5A) and neutralizes RANKL-mediated signals (Hsu et al., 1999). Thymocyte development, including the generation of SP thymocytes, was apparently not affected in RANK-Fc-expressing mice (Figure S5B). However, the number of mTECs was significantly ($p < 0.05$) reduced and thymic medulla containing Aire-expressing mTECs became underrepresented in B6 mice expressing RANK-Fc (Figures S5C–S5E). In contrast, RANK-Fc expression did not affect the number of cTECs (Figure S5E). These results indicate that in vivo blockade of RANKL perturbs mTEC cellularity and medulla formation in normal mice and that RANKL is essential for increasing mTEC cellularity and forming thymic medulla.

RANKL Expression Restores Thymic Medulla without Positive Selection

Finally, we addressed whether RANKL expression in developing thymocytes might be sufficient for increasing mTEC cellularity and forming thymic medulla even without positive selection. To do so, bone marrow hematopoietic progenitor cells from TCRα-deficient (*Tcra*$^{-/-}$) mice were infected with a retrovirus that expressed RANKL along with EGFP and were transferred into TCRα-deficient mice. In these mice, positive-selection-mediated generation of mature SP thymocytes remained defective because of the lack of TCR expression by DP thymocytes, whereas RANKL expression was detectable in the majority of EGFP$^+$ thymocytes (Figure 6A). We found that the number of mTECs was significantly ($p < 0.01$) restored and thymic medulla containing Aire-expressing and CCL21-expressing mTECs was obviously formed in RANKL-expressing TCRα-deficient mice but not in control EGFP-expressing TCRα-deficient mice (Figures 6B–6D). Thymic medulla formed in RANKL-expressing TCRα-deficient mice was not colonized with thymocytes because of the lack of positive selection (Figure 6B). Unlike RANKL expression, the expression of CD30L or OX40L did not restore the number of mTECs (Figure 6D). These results indicate that forced RANKL expression was sufficient in vivo for increasing mTEC cellularity and forming thymic medulla even without positive selection.

In order to better understand how RANKL expression increases mTEC cellularity, we measured Ki67 expression in proliferating cells, BrdU incorporation in DNA synthesizing cells, and TUNEL in dying cells, in mTECs of TCRα-deficient mice reconstituted with retrovirally RANKL-expressing TCRα-deficient bone marrow cells. As shown in Figure 6F, RANKL expression elevated the frequency of Ki67-expressing cells and BrdU-incorporated cells in mTECs compared to those in mTECs isolated from wild-type mice. The frequency of TUNEL$^+$ cells was only slightly reduced by RANKL expression (Figure 6F). RANKL expression also elevated the frequency of CD80-expressing mature cells in mTECs (Figure 6F). These results indicate that RANKL expression in bone-marrow-derived cells causes elevated proliferation of mTECs without positive selection, suggesting the possibility that RANKL contributes to medulla formation by promoting the proliferation of mTECs.

We finally addressed whether RANKL produced by SP thymocytes could directly promote the increase in mTEC cellularity. To do so, fetal thymus stromal cells were cultured with isolated SP thymocytes in reaggregated thymus organ culture. As shown in Figure 7, reaggregation with CD4SP thymocytes but not DP thymocytes significantly increased the number of mTECs but not cTECs in culture (Figures 7A and 7B). The number of CD80-expressing mature mTECs and the mRNA amounts of Aire and Aire-dependent tissue-restricted self-antigens (insulin 2 and salivary protein 1) were also increased by the addition of CD4SP thymocytes but not DP thymocytes (Figures 7B and 7C). Importantly, these increases in mTEC cellularity and the expression of mTEC-associated molecules were significantly ($p < 0.05$) diminished by the addition of RANK-Fc (Figure 7), which competitively antagonized RANKL (Figure S5). These results indicate that SP thymocytes are sufficient and RANKL is essential for fostering mTECs.

DISCUSSION

The present results show that positive selection promotes the increase in the number of mTECs and thereby fosters the

(F) Ki67 expression, BrdU incorporation, TUNEL, and CD80 expression were detected in CD45$^-$I-A$^+$UEA1$^+$ mTECs. BrdU (2 mg) was intravenously administered 24 hr before analysis. Shaded profiles indicate control staining. Numbers indicate averages and standard errors (from at least three independent measurements) of frequency of cells within indicated areas.