pathway is not limited by other proteins and is independently capable of achieving sufficient inhibition of T cell activation.

Laptm5$^{-/-}$ spleen T cells expressed higher amounts of surface TCR, as well as CD3ζ proteins, than did wild-type T cells only after CD3 stimulation, suggesting that LAPTM5 regulates CD3ζ degradation in a ligand-dependent manner. Consistent with this interpretation, LAPTM5 colocalized with CD3ζ in normal spleen T cells only after CD3 stimulation. Furthermore, *Laptm5*$^{-/-}$ DP thymocytes, which are undergoing positive and negative selection and thereby are stimulated through their TCR, expressed higher amounts of surface TCR and cellular CD3ζ, whereas *Laptm5*$^{-/-}$ SP thymocytes, which have already undergone selection, expressed identical amounts of surface TCR and CD3ζ as wild-type SP thymocytes. Direct TCR stimulation is apparently no longer required in the 2B4 T hybridoma cell line where overexpressed LAPTM5 was able to interact with CD3ζ and mediate its degradation without the requirement for CD3 stimulation. However, our data in normal T cells and thymocytes indicate that LAPTM5-mediated CD3ζ degradation is regulated by TCR stimulation, presumably as a feedback mechanism to prevent prolonged TCR signaling.

Deficiency in c-Cbl results in enhanced TCR signaling and positive selection of thymocytes because of the elevated surface TCR expression on DP thymocytes (Naramura et al., 1998). Paradoxically, peripheral T cells lacking c-Cbl respond rather poorly to CD3 stimulation in terms of both proliferation and total tyrosine phosphorylation. Because c-Cbl is expressed at a much lower amount in mature T cells as compared to that in thymocytes (Naramura et al., 1998), it is likely that the absence of c-Cbl increases the TCR signaling on DP thymocytes and allows for positive selection of thymocytes with lower TCR responsiveness to become mature T cells. *Laptm5*$^{-/-}$ DP thymocytes also expressed increased TCR on DP thymocytes, but, in contrast to c-Cbl deficiency, *Laptm5*$^{-/-}$ mature T cells still exhibited enhanced responses to TCR stimulation. The similar phenotype in thymocytes but opposite responses by mature T cells observed in mice lacking LAPTM5 or c-Cbl could be attributable to the differential expression patterns of these molecules. *Laptm5* is expressed at higher amounts in the spleen than in the thymus (Scott et al., 1996). Therefore, it is conceivable that the positively selected *Laptm5*$^{-/-}$ thymocytes can maintain their hyperresponsiveness in the periphery relative to wild-type T cells, which express higher amounts of LAPTM5. In this regard, it will be interesting to inactivate *Laptm5* specifically in thymocytes or peripheral T cells and investigate T cell function in vivo. Studies thus far have revealed that multiple proteins, including SLAP, c-Cbl, Cbl-b, and perhaps SLAP-2 (Holland et al., 2001; Pandey et al., 2002; Loreto et al., 2002), are involved in the regulation of surface TCR expression. SLAP and c-Cbl are predominantly expressed and function in DP thymocytes, whereas Cbl-b mainly functions in mature T cells. However, although c-Cbl and Cbl-b predominantly function in thymocytes and mature T cells, respectively, they appear to functionally compensate for each other because Cbl-b and c-Cbl double deficiency exhibits a much more severe defect both in mature T cells and in thymocytes than seen in mice singly deficient for either gene (Naramura et al., 2002; Huang et al., 2006). LAPTM5 functions both in the thymus and in the periphery, presumably downstream of SLAP and/or Cbl, and it promotes CD3ζ degradation. Therefore, TCR metabolism is regulated by multiple differentially expressed proteins that have distinct as well as overlapping functions to allow precise control of cell-surface TCR expression in response to the different external signals encountered during T cell development and activation.

It has recently been shown that SLAP functions as an adaptor for the E3 ubiquitin ligase c-Cbl and that these two proteins together mediate the ubiquitination and subsequent degradation of CD3ζ (Myers et al., 2005, 2006). Interestingly, LAPTM5 contains a UIM that overlaps with the second PY motif. We found that LAPTM5-mediated TCR downmodulation in 2B4 cells required the UIM. It is tempting to speculate that LAPTM5 might bind the ubiquitinated CD3ζ and promote its degradation in the lysosomal compartment. Moreover, we found that the PY motifs of LAPTM5 are critical for TCR downmodulation. Because PY motifs are known to interact with WW domains involved in protein-protein interactions, it is conceivable that additional proteins with WW domains might also be involved in CD3ζ degradation. Recently, LAPTM5 was found to interact in 293T cells with Nedd4, a HECT-type E3 ubiquitin ligase that plays multiple roles in protein transport, through the PY motifs of LAPTM5 and the WW domains of Nedd4 (Pak et al., 2006). In addition, LAPTM5 UIM was shown to bind ubiquitinated GGA3, an adaptor protein involved in endosomal and lysosomal sorting. The interaction of LAPTM5 with both Nedd4 and GGA3 appears to be essential for its lysosomal localization (Pak et al., 2006). Moreover, in a functional-proteomics mapping of a TGF-β signaling system, LAPTM5 was shown to interact with Smurf2, a ubiquitin ligase for Smads, and inhibit TGF-β signaling in HepG2 cells (Colland et al., 2004). Although the physiological significance of LAPTM5 interactions with Nedd4, GGA3, and Smurf2 remains to be established, these results suggest that LAPTM5 contains multiple functional domains that control its localization and interaction with other proteins. Intriguingly, we reproducibly detected much lower amounts of CD3ζ than LAPTM5 when the LAPTM5 was immunoprecipitated. Because LAPTM5 interacts with multiple proteins, it is possible that LAPTM5 antibodies pulled down multiple proteins, with only a fraction being CD3ζ chain. Alternatively, the small amount of CD3ζ detected in the LAPTM5 immunoprecipitates may be due to LAPTM5 overexpression in 2B4 cells.

Identification of LAPTM5 as a new regulator that mediates CD3ζ degradation and inhibits T cell activation raises many questions. How do LAPTM5 and other molecules involved in TCR metabolism, including SLAP and Cbl family proteins, cooperate to regulate TCR metabolism? Are there any human immunodeficiencies or autoimmune diseases that are associated with dysregulated LAPTM5 function? It is interesting to note that *Laptm5* gene is inactivated by either chromosomal deletion or promoter methylation in four cases of human multiple myeloma (Hayami et al., 2003), raising the possibility that LAPTM5 may also have a tumor-suppressor function in humans.

EXPERIMENTAL PROCEDURES

Generation of *Laptm5*-Gene-Targeted Mice

A targeting vector was constructed to replace exon 1 and a part of the promoter region with the neomycin gene. The C57BL/6-derived Bruce4 ESCs were transfected with the linearized targeting vector, and 2 days after

transfection, were cultured in the presence of 250 μg/ml G418 and 1 μM of GANC (only for the first two days). ESC colonies were expanded and subjected to genomic PCR to identify homologous recombinants with the use of primers flanking 5′ and 3′ homology regions and neo primers. The sequences are as follows: 5′ primer, s157459, 5′-TGTGTCTATGTGTTCGTGTG-3′; 3′ primer, as169968, 5′-CGCATCAGTCTACCTAAACA-3′; neos, 5′-TCGCCTTCTAT CGCCTTCTT-3′; and neoas, 5′-ATAGCCGAATAGCCTCTCCA-3′. PCR was performed at 95°C for 2 min, followed by 30 cycles of amplification at 95°C for 10 s, 60°C for 20 s, and 68°C for 10 min with LA-Taq polymerase (TAKARA Bio., Japan). Mouse genotypes were determined by PCR using primers Laptm5s161549 (5′-ATGGAGATGTGTGAGAAGCC-3′), Laptm5as161994 (5′-TGCCTGCTTCTCCCTACCAA-3′), and neos at 95°C for 2 min, followed by 30 cycles of amplification at 95°C for 5 s, 60°C for 10 s, and 72°C for 1 min with Taq polymerase (TOYOBO, Japan). Chimeric mice were bred with C57BL/6 mice to obtain heterozygotes, which were then crossed to obtain homozygotes. The *Laptm5*$^{-/-}$ mice are in C57BL/6 background. Mice were maintained in specific-pathogen-free conditions, and all experiments were approved by the Animal Facility Committee of the RIKEN Yokohama Institute.

Antibodies and Other Reagents

Polyclonal rabbit antibodies were raised against a peptide (PPKTPEGDPAPP-PYSEV) located near the C terminus of mouse LAPTM5. The specificity of the LAPTM5 antibodies was verified by both intracellular staining and western-blot analysis of lysates derived of 293T cells transfected with a *Laptm5* expression vector. The following commercial antibodies were used in these studies: FITC-anti-CD4 (RM4-5), PE-anti-CD8 (Ly-2), APC-anti-CD3ε (2C11), PE-anti-TCRβ (H57-597), PE-B220 (RA3-6B2), FITC-anti-CD43, FITC-anti-IgM, PE-anti-IgD, and APC-B220 for FACS staining (purchased from BD Biosciences); anti-mouse CD3ζ (H146, CEDARLANE), anti-CD3ε (M-20, Santa Cruz), anti-human CD3ζ (6B10.2, Santa Cruz), anti-phosphotyrosine (4G10, Upstate), anti-GFP (IE4, Stressgen), anti-Flag (M5, Sigma), anti-Actin (Sigma), anti-CD3δ (M-20, Santa Cruz), anti-CD3γ (C-20, Santa Cruz), and anti-phospho Lck (pY505, BD Biosciences) for immunoblot analyses. The following phospho antibodies were purchased from Cell Signaling: phospho ZAP70 (Try319), phospho LAT (Tyr171), phospho Erk, phospho JNK, and phospho p38 MAP kinase. For intracellular staining, anti-CD3ζ (H146, CEDARLANE), anti-LAMP1 (1D4B, BD Biosciences), FITC-anti-rabbit IgG (Santa Cruz), Alexa555-anti-rat IgG (Molecular probes), Streptavidin-Alexa647 (Molecular probes), and PE-anti-IFN-γ (XMG1.2, BD Biosciences) were used. For CFSE assay, CellTrace CFSE Cell Proliferation Kit (Molecular Probes) was used. For T cell stimulation, anti-CD3ε (2C11, NA/LE, BD Biosciences), anti-CD28 (37.51, eBioscience), IL-2 (R&D), anti-hamster IgG (Cappel), PMA (Sigma), and ionomycin (Sigma) were used.

Purification of Spleen T Cells, Proliferation, Cytokine Production, and Ca^{2+} Influx

Spleen T cells were isolated via negative sorting with the IMag T cell purification kit (BD Biosciences). 2B4 is a T cell hybridoma and has been described previously (D'Oro et al., 2002). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and 100 U/ml of penicillin and streptomycin. For proliferation assay, cells were cultured for 1 or 2 days and pulsed for the last 6 hr with 1 μCi/well of [^{3}H] thymidine. Thymidine uptake was analyzed as described previously (Masuda et al., 2007). IL-2 and IFN-γ production were measured by ELISA with a kit (BD Biosciences). Ca^{2+} influx experiments were performed as previously described (Hikida et al., 2003). In brief, purified spleen T cells were suspended at 10^7 cells/ml and incubated with 1.2 μM of Indo 1-AM (Dojindo) at 37°C for 45 min in the dark. After washing, the cells were resuspended at 10^6 cells/ml, stimulated with anti-CD3 followed by anti-hamster IgG, and immediately analyzed with a LSR flow cytometer (BD Biosciences).

TCR Internalization and Recycling

TCR internalization was analyzed as described previously (Naramura et al., 2002). In brief, spleen cells were labeled with phycoerythrin (PE)-conjugated hamster anti-mouse TCRβ and crosslinked with goat antibodies against hamster IgG at 4°C. Cells were then cultured at 37°C for various times to allow TCR internalization. At each time point, noninternalized antibodies were removed by washing cells twice in ice-cold acidic buffer (PBS containing 1% BSA, pH 3.0), followed by immediate neutralization in PBS containing 1% BSA and 0.5% NaN_3, pH 7.4. Both treated and untreated cells were then stained with FITC-anti-CD4 or FITC-anti-CD8 and analyzed for PE fluorescence on gated CD4$^+$ and CD8$^+$ cells by flow cytometry. The percentage of TCR internalization was calculated as described previously (Naramura et al., 2002).

For analysis of the recycling of internalized TCR, spleen cells were labeled as described above and cultured for 30 min to allow for TCR internalization. Cells were then washed twice with cold acidic buffer, followed by immediate neutralization to remove noninternalized antibodies, and cultured at 37°C again for various times to allow for the recycling of the internalized TCR. At each time point, half of the cells were treated with cold acidic buffer followed by neutralization, stained with FITC-anti-CD4 or anti-CD8, and analyzed for PE fluorescence on gated CD4$^+$ and CD8$^+$ cells. The percentage of TCR recycling was calculated as previously described (Myers et al., 2005). For both TCR internalization and recycling, the acid stripping of cells did not induce massive cell death (Figure S8).

Immunoblot Analysis

Cells were lysed directly in SDS sample buffer and sonicated for 2 min. The lysate was resolved in a 5%–20% gradient gel and transferred to immobilon-P membranes (MILLIPORE). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies and then horseradish peroxidase (HRP)-conjugated secondary antibodies and developed with an enhanced chemiluminescence light (ECL) reagent.

Construction of Laptm5-IRES-GFP Vector and Retroviral Infection

A 1.1 kb *Laptm5* cDNA fragment was cloned into pMX-IRES-GFP retroviral vector (Nosaka et al., 1999). Various LAPTM5 constructs were prepared as described recently (Pak et al., 2006) by site-directed mutagenesis with a kit (Invitrogen). These retroviral constructs were transfected into the PHOENIX packaging cell line, and the virus supernatant was prepared as described previously (Nosaka et al., 1999). Virus transduction of the 2B4 T cell hybridoma and normal spleen T cells was carried out in the presence of 10 μg/ml of DOTAP reagent (Roche).

Immunofluorescence

Spleen T cells were first treated with 10 mM NH_4Cl for 2 hr and then stimulated with plate-bound anti-CD3 for 6 hr. Cells were then fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences); stained with anti-LAPTM5, anti-LAMP1, and anti-CD3ζ (H146); washed three times; and stained with biotinylated anti-Hamster IgG. After they were washed three times, the cells were further stained with FITC-anti-rabbit IgG to detect LAPTM5, Alexa 555-anti-rat IgG to detect LAMP1, and avidin-Alexa 647 to detect CD3ζ. After they were washed three times, the cells were attached to glass slides via a cytospin and fixed with ProLong Gold antifade reagent containing DAPI. For intracellular staining of thymocytes, cells were first stained with FITC-anti-CD4 and APC-anti-CD8, fixed and permeabilized with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit, and stained with anti-CD3ζ followed by PE-anti-hamster IgG.

Coimmunoprecipitation of LAPTM5 and CD3ζ

2B4 cells (2×10^7) were first cultured in the presence of the lysosome inhibitor NH_4Cl for 2 hr and then lysed on ice for 60 min in a buffer containing 1% digitonin (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA) in the presence of a protease inhibitor cocktail for mammalian cells (Sigma). The lysate was centrifuged for 20 min at 20,000 × g, and the supernatant was precleared with 100 μl of protein G sepharose beads (Amersham) three times at 1 hr intervals and immunoprecipitated with anti-LAPTM5 or control rabbit IgG-conjugated protein G beads at 4°C for 3 hr. The beads were then washed ten times with the lysis buffer and resuspended in SDS sample buffer. The samples were boiled for 5 min and subjected to immunoblot analyses.

Assay for T Cell Activation In Vivo

For the ex vivo assay, mice were first immunized intradermally in both hind footpads with 80 μg of KLH (Sigma) in complete Freund's adjuvant (CFA, Difco). Seven days after immunization, whole lymph node cells (2×10^5 cells) were prepared from popliteal draining lymph nodes and cultured in the presence of different doses of KLH. For proliferation assay, cells were cultured

for 3 days and pulsed with 1 μM of [^{3}H] thymidine for the last 6 hr. For cytokine production, cells were cultured for 2 days and supernatants were harvested. IL-2 concentration was determined by ELISA (eBioscience). For the DTH assay, mice were sensitized with 1.25 mg/ml mBSA (Sigma) in CFA subcutaneously at the base of the tail. Seven days after sensitization, mice were challenged with 0.3 mg/ml of mBSA in a 20 μl volume into one footpad, and an equal volume of PBS was injected into another footpad. Footpad swelling was measured by a caliper. Footpad swelling was determined as follows: (cm) = [footpad thickness of mBSA-injected footpad (cm)] − [footpad thickness of PBS-injected footpad (cm)] as described (Nakae et al., 2002).

SUPPLEMENTAL DATA

Supplemental Data include eight figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/1/33/DC1/.

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Repressor of GATA regulates T_H2-driven allergic airway inflammation and airway hyperresponsiveness

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Background: Studies of human asthma and of animal models of allergic inflammation/asthma highlight a crucial role for T_H2 cells in the pathogenesis of allergic asthma. Repressor of GATA (ROG) is a POZ (BTB) domain–containing Kruppel-type zinc finger family (or POK family) repressor. A repressive function to GATA3, a master transcription factor for T_H2 cell differentiation, is indicated.

Objective: The aim of this study was to clarify the regulatory roles of ROG in the pathogenesis of T_H2-driven allergic diseases, such as allergic asthma.

Methods: We examined allergic airway inflammation and airway hyperresponsiveness (AHR) in 3 different mouse models, which use either ROG-deficient ($ROG^{-/-}$) mice, ROG transgenic mice, or adoptive transfer of cells.

Results: In $ROG^{-/-}$ mice T_H2 cell differentiation, T_H2 responses, eosinophilic airway inflammation, and AHR were enhanced. In ROG transgenic mice the levels of eosinophilic airway inflammation and AHR were dramatically reduced. Furthermore, adoptive transfer of T_H2 cells with increased or decreased levels of ROG expression into the asthmatic mice resulted in reduced or enhanced airway inflammation, respectively.

Conclusion: These results indicate that ROG regulates allergic airway inflammation and AHR in a negative manner, and thus ROG might represent another potential therapeutic target for the treatment of asthmatic patients. (J Allergy Clin Immunol 2008;122:512-20.)

Key words: *Repressor of GATA, POK family, repressor, airway inflammation, airway hyperresponsiveness, GATA3, T_H2, allergic asthma*

Abbreviations used
AHR: Airway hyperresponsiveness
APC: Antigen-presenting cell
BAL: Bronchoalveolar lavage
H&E: Hematoxylin and eosin
MDC: Macrophage-derived chemokine
OVA: Ovalbumin
PAS: Periodic acid–Schiff
PLZF: Promyelocytic leukemia zinc finger
ROG: Repressor of GATA
TARC: Thymus and activation-regulated chemokine
TCR: T-cell receptor
Tg: Transgenic

Asthma is a major public health problem that has increased markedly in prevalence in the past 2 decades.[1] Asthma is characterized by a chronic inflammatory disease of the lower airways that causes airway hyperresponsiveness (AHR) to a wide variety of specific and nonspecific stimuli.[2,3] The cardinal features of acute asthma are airway inflammation predominated by eosinophils, hypersecretion of mucus, and AHR. A critical role for T_H2 cells in the pathogenesis of allergic asthma has been demonstrated in the studies of human asthma, as well as in animal models of allergic airway inflammation.[4-10]

It is well established that $CD4^+$ effector T_H cells can be categorized into 3 subsets: T_H1, T_H2, and T_H17 cells. T_H1 cells produce large amounts of IFN-γ and direct cell-mediated immunity against intracellular pathogens. T_H2 cells produce IL-4, IL-5, and IL-13 and are involved in humoral immunity and allergic reactions. Recently, another T_H subset, T_H17, was identified.[11] T_H17 cells produce IL-17 and are involved in the pathogenesis of autoimmune diseases.[12-14] In addition, several transcription factors that control the differentiation of these T_H subsets were identified. Among them, GATA3 appears to be a key transcription factor for T_H2 cell differentiation,[15,16] T-bet for T_H1,[17] and retinoid-related orphan receptor γ t for T_H17 cell differentiation.[18]

GATA3 is selectively induced in developing T_H2 cells after T-cell receptor (TCR) stimulation in the presence of IL-4, and the ectopic expression of GATA3 resulted in the induction of T_H2 cell differentiation in the absence of signal transducer and activator of transcription 6.[19] GATA3 acts as a transcriptional factor for *IL5* and *IL13* genes.[20-22] In addition to the promoter regions, GATA3 also binds to various regulatory regions for T_H2 cytokine expression, including the conserved GATA3 response element,[23] the 3′ site of IL-4,[24] the IL-4/IL-13 intergenic region (conserved noncoding sequence 1),[25] and the 3′ end of the radiation 50 gene.[26] We reported that the histone modifications at the T_H2 cytokine gene loci are primarily mediated through GATA3 in T_H2 and T_C2 (type 2 cytotoxic T) cells.[23,27,28]

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The inhibition of GATA3 activity in dominant-negative GATA3 transgenic mice results in a reduction in T_H2 cytokine production and less severe allergic inflammation in a murine model of asthma.[29] Moreover, allergic airway inflammation and AHR have been reported to be compromised by the intranasal administration of antisense GATA3.[30] More recently, in allergen-challenged transgenic mice overexpressing GATA3, airway smooth muscle hyperplasia and subepithelial fibrosis were reported.[31]

Repressor of GATA (ROG) is a POZ (BTB) domain–containing Kruppel-type zinc finger family (or POK family) repressor and is highly homologous to another POK family protein, promyelocytic leukemia zinc finger (PLZF).[32] ROG is also identified as PLZF-like zinc finger protein,[33] testis zinc finger protein,[34] and Fanconi anemia zinc finger.[35] The BTB/POZ domain mediates homodimerization and heterodimerization and recruits corepressor molecules, including histone deacetylases.[36] Two POK family proteins, B-cell lymphoma 6 and PLZF, are known to be implicated in the oncogenic activity in non-Hodgkin's lymphomas[37] and acute promyelocytic leukemia,[38] respectively. Overexpression of ROG exhibited repression of GATA3-induced transactivation of the IL-4 and IL-5 promoters in the M12 B-cell line and the EL-4 T-cell line.[32] We previously reported that the level of ROG is significantly higher in CD8 T cells than in CD4 T cells and that ROG might confer CD8 T cell–specific repression of histone hyperacetylation and activation of the *IL4* gene locus.[27] T cells from ROG-deficient mice showed an increased proliferative response.[33,39] However, the biologic role of ROG in the T_H2 immune responses and the T_H2-dependent diseases has not been investigated.

Here we have established ROG-deficient ($ROG^{-/-}$) mice on either a BALB/c or C57BL/6 background and also ROG-transgenic mice on a C57BL/6 background and used these animals to investigate the role of ROG in T_H1/T_H2 cell differentiation and in the pathogenesis of T_H2-dependent allergic airway inflammation. Our results indicate that ROG negatively regulates T_H2-dependent airway allergic inflammation.

METHODS

Mice

The animals, including $ROG^{-/-}$ mice, used in this study were backcrossed to either BALB/c or C57BL/6 10 times. $ROG^{-/-}$ x DO11.10 transgenic (Tg) mice (anti-ovalbumin [OVA]–specific TCRαβ Tg),[40] $ROG^{-/-}$ x OT-I or OT-II Tg mice (anti-OVA–specific TCRαβ Tg),[41,42] and ROG Tg x OT-I or OT-II Tg mice were used at 6 to 8 weeks of age. BALB/c, C57BL/6, and BALB/c *nu/nu* mice were purchased from Clea, Inc (Tokyo, Japan). All mice used in this study were maintained under specific pathogen-free conditions. Animal care was conducted in accordance with the guidelines of Chiba University.

Immunofluorescent staining and flow cytometric analysis

In general, one million cells were stained with antibodies, as indicated, according to a standard method.[43,44]

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously with an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, Calif) under standard conditions.[45,46] The primers for TaqMan probes for the detection of ROG (exon 2-3), IL-4, IL-5, IL-13, eotaxin 2, RANTES, TNF-α, macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), and hypoxanthine-guanine-phosphoribosyl transferase were purchased from Applied Biosystems.

ELISA

Cytokine production was assessed by means of ELISA, as described previously.[44]

In vitro T_H1/T_H2 cell differentiation cultures

A detailed protocol is described in the Methods section available in the Online Repository at www.jacionline.org.

OVA sensitization, inhalation, and analysis of airway inflammation

A detailed protocol is described in the Methods section in the Online Repository.

Statistical analysis

The significance between 2 groups was determined by using the 2-tailed Student *t* test. Comparisons for all pairs were performed with the Kruskal-Wallis test.

RESULTS

Enhanced OVA-induced eosinophilic inflammation and AHR in $ROG^{-/-}$ mice

We generated $ROG^{-/-}$ mice, in which *ROG* mRNA was not expressed in the thymus and peripheral T cells (see Fig E1, *C*, in this article's Online Repository at www.jacionline.org). T-cell development in $ROG^{-/-}$ mice appeared to be normal because no apparent difference in the cellularity and CD4/CD8 ratio in the thymus and spleen compared with that seen in wild-type control animals was observed (see Fig E1, *D*). The cell-surface expression of TCRβ, CD3ε, CD25, CD69, CD44, CD62L, common γ (Cγ), IL-2Rβ, IL-4Rα, and IL-7Rα was normal in $ROG^{-/-}$ splenic CD4 and CD8 T cells (see Fig E1, *E*).

The bronchoalveolar lavage (BAL) fluid of OVA-immunized, OVA-inhaled $ROG^{+/+}$, and $ROG^{-/-}$ mice was collected 48 hours after the last OVA challenge to assess the role of ROG in allergic airway inflammation (see Fig E2 in this article's Online Repository at www.jacionline.org). Significantly increased infiltration of eosinophils and lymphocytes in the antigen-challenged $ROG^{-/-}$ group was detected, whereas the OVA-immunized and PBS-challenged $ROG^{-/-}$ mice did not have airway eosinophilia or show signs of abnormal cellular infiltrates in the BAL fluid (Fig 1, *A*). Evaluation of histologic changes in the lungs of $ROG^{-/-}$ mice by means of hematoxylin and eosin (H&E) staining revealed that the levels of OVA-induced inflammatory mononuclear cell infiltrates in the peribronchiolar region were higher in $ROG^{-/-}$ mice in comparison with the infiltrates in wild-type littermates (Fig 1, *B*, upper panel). No inflammatory cell infiltration was detected in untreated $ROG^{-/-}$ mice (data not shown) or OVA-immunized and PBS-challenged $ROG^{-/-}$ mice. Significant augmentation of eosinophil infiltration was revealed by means of LUNA staining in $ROG^{-/-}$ mice (Fig 1, *B*, lower panel), and as demonstrated by means of periodic acid–Schiff (PAS) staining, enhanced hypermucus production was detected in $ROG^{-/-}$ mice (Fig 1, *C*, and see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). To assess the extent of AHR, we measured the methacholine-induced airflow obstruction

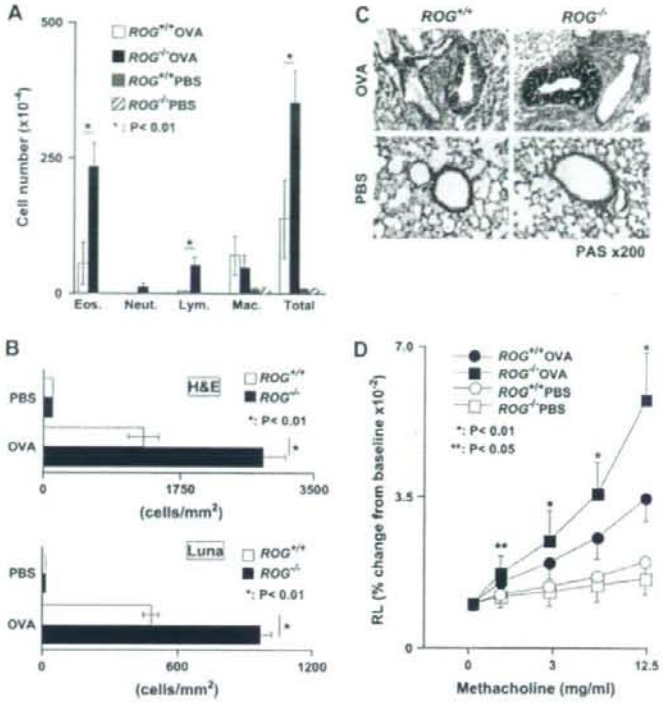

FIG 1. Increased OVA-induced airway inflammation and AHR in $ROG^{-/-}$ mice. **A**, $ROG^{+/+}$ and $ROG^{-/-}$ mice were sensitized with OVA and underwent inhalation with OVA. Infiltrated leukocytes in BAL fluid were assessed. The absolute cell number of eosinophils *(Eos.)*, neutrophils *(Neut.)*, lymphocytes *(Lym.)*, and macrophages *(Mac.)* in the BAL fluid are shown with SDs. Seven to 8 mice per group were used. Three independent experiments were performed, and similar results were obtained. $*P < .01$, Student *t* test. **B**, Semiquantitative analysis of peribronchiolar leukocyte and eosinophil infiltration in the lung. $*P < .01$, Student *t* test. **C**, Hypermucus production was detected in $ROG^{-/-}$ mice. **D**, AHR was assessed as airway resistance *(RL)*. Mean values (5 mice per group) are shown with SDs. $*P < .01$ and $**P < .05$, Kruskal-Wallis test.

in an invasive assay for lung resistance at 24 hours after the last OVA challenge. The mice were anesthetized, tracheostomized, and mechanically ventilated, and lung resistance was measured directly. As expected, lung resistance was also significantly increased in $ROG^{-/-}$ mice in comparison with that seen in $ROG^{+/+}$ mice (Fig 1, *D*). Neither PBS-challenged wild-type nor PBS-challenged $ROG^{-/-}$ mice had AHR. Approximately 2-fold enhancement of AHR was seen at all doses of methacholine in $ROG^{-/-}$ mice in a whole-body plethysmograph (see Fig E3, *B*). Similar enhancement in the levels of eosinophilic inflammation and AHR was observed in $ROG^{-/-}$ mice with a C57BL/6 background (data not shown). These results indicate that the extent of OVA-induced airway inflammation and AHR is enhanced in $ROG^{-/-}$ mice.

Enhanced OVA-induced T_H2 immune response in $ROG^{-/-}$ mice

Proliferative responses induced by anti-TCR mAb stimulation (see Fig E4, *A*, in this article's Online Repository at www.jaci online.org) or antigenic peptide stimulation (see Fig E4, *C*) were significantly higher in $ROG^{-/-}$ CD4 and CD8 T cells. The rate of cell division after antigen stimulation was also moderately higher in $ROG^{-/-}$ mice (see Fig E4, *B*). An assessment of the capability of $ROG^{-/-}$ CD4 T cells to differentiate into T_H1/T_H2 cells *in vitro* indicated moderate enhancement in T_H2 cell differentiation under T_H2 conditions (34.9% vs 44.9% and 27.8% vs 37.6%; Fig 2, *A*) and under neutral conditions (3.0% vs 8.7% and 15.3% vs 26.7%; see Fig E5 in this article's Online Repository at www.jacionline.org), whereas T_H1 cell differentiation was equivalent (Fig 2, *A*).

We then examined the expression levels of IL-4, IL-5, IL-13, eotaxin 2, RANTES, and TNF-α in the BAL fluid cells of OVA-sensitized and OVA-challenged $ROG^{-/-}$ mice shown in Fig 1. Quantitative RT-PCR analysis was performed with RNA isolated from the infiltrates in the BAL fluid. The expression levels of IL-4, IL-5, IL-13, eotaxin 2, and RANTES were higher in $ROG^{-/-}$ mice than in $ROG^{+/+}$ mice, whereas the TNF-α level was comparable (Fig 2, *B*). Moreover, cytokine levels in the BAL fluid, as measured by means of ELISA, showed increased production of IL-5 and IL-13 in $ROG^{-/-}$ BAL fluid (Fig 2, *C*). At the same time, mediastinal lymph nodes were harvested and stained for intracellular IFN-γ and IL-4. Modest but reproducibly increased numbers of IL-4–producing cells were

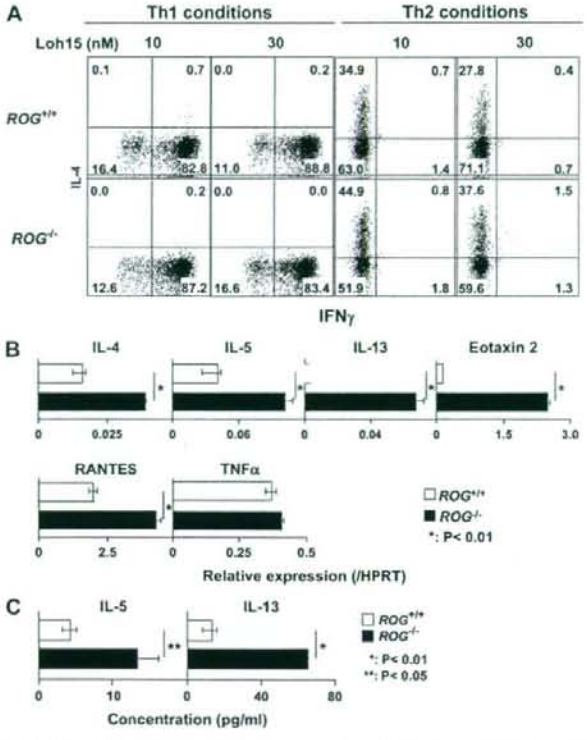

FIG 2. Increased T_H2 cytokine production in the airways of $ROG^{-/-}$ mice. **A**, The ability to differentiate into T_H1/T_H2 cells from $ROG^{-/-}$ x DO11.10 Tg mice was assessed. The results are representative of 5 experiments. **B**, mRNA levels of IL-4, IL-5, IL-13, eotaxin 2, RANTES, and TNF-α in BAL fluid cells were determined by using quantitative RT-PCR. $*P < .01$, Student *t* test. **C**, The amount of IL-5 and IL-13 in the BAL fluid was determined by means of ELISA. The mean values with SDs (3-5 mice per group) are shown. $*P < .01$ and $**P < .05$, Student *t* test.

observed (see Fig E6 in this article's Online Repository at www.jacionline.org). We measured the mRNA expression levels of TARC and MDC, which are known to be the chemokines for T_H cell recruitment by using mRNA from the allergic lung, and no significant difference was noted between $ROG^{+/+}$ and $ROG^{-/-}$ mice (data not shown). Thus the enhanced OVA-induced airway inflammation and AHR observed in $ROG^{-/-}$ mice could be due to the enhanced T_H2 responses in the airways of $ROG^{-/-}$ mice.

Attenuated OVA-induced eosinophilic inflammation and airway AHR in ROG Tg mice

To further investigate the regulatory role of ROG in T cells, we generated ROG Tg mice under the control of a T-cell specific lck proximal promoter (see Fig E7, *A* and *B*, in this article's Online Repository at www.jacionline.org). Quantitative RT-PCR analysis revealed that *ROG* mRNA was expressed 10-fold higher in thymocytes and splenic CD4 and CD8 T cells in ROG Tg mice (see Fig E7, *C*). The cellularity and CD4/CD8 ratio in the thymus and spleen in ROG Tg mice did not differ from those in wild-type mice (see Fig E7, *D*). The cell-surface expression of TCRβ, CD3ε, CD25, CD69, CD44, CD62L, Cγ, IL-2Rβ, IL-4Rα, and IL-7Rα on the splenic CD4 and CD8 T cells from ROG Tg mice was normal (see Fig E7, *E*).

BAL fluid of OVA-immunized and OVA-inhaled wild-type and ROG Tg mice was analyzed to evaluate the extent of T_H2-dependent airway inflammation in ROG Tg mice. The number of total infiltrated cells, lymphocytes, and eosinophils was found to be reduced in ROG Tg mice: the most striking difference between wild-type and ROG Tg mice was the difference in the number of eosinophils (Fig 3, *A*). Histologic examination revealed very low-level infiltrates in the peribronchiolar regions in the lungs of ROG Tg mice (Fig 3, *B*, upper panel). The decreased eosinophilic infiltration in ROG Tg mice was confirmed by means of LUNA staining (Fig 3, *B*, lower panel). Goblet cell metaplasia and mucus hyperproduction were also reduced in ROG Tg mice (Fig 3, *C*, and see Fig E8, *A*, in this article's Online Repository at www.jacionline.org). Lung resistance, as measured by using a direct invasive method, showed no increase in the AHR in ROG Tg mice compared with the increase in wild-type mice (Fig 3, *D*). ROG Tg mice did not have significant AHR, and the sensitivity to methacholine was almost equivalent to that seen in PBS-challenged control mice (see Fig E8, *B*). From these results, we conclude that overexpression of ROG in T cells results in the inhibition of OVA-induced airway inflammation and AHR.

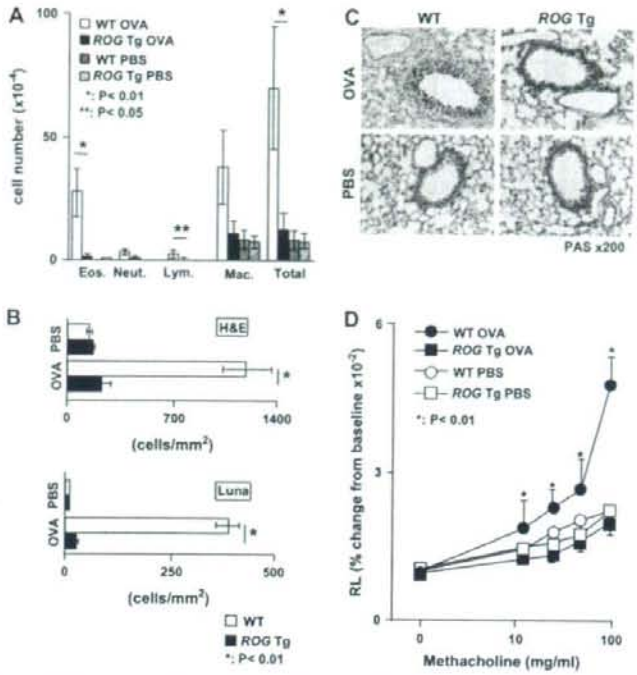

FIG 3. Inhibition of OVA-induced airway inflammation and AHR in ROG Tg mice. **A**, Decreased infiltration of eosinophils in BAL fluid in asthmatic ROG Tg mice. Five mice per group were used. Two independent experiments were performed, and similar results were obtained. $*P < .01$ and $**P < .05$, Student *t* test. **B**, Semiquantitative analysis of peribronchiolar leukocyte infiltrates from H&E-stained *(upper panel)* and LUNA-stained *(lower panel)* samples. $*P < .01$, Student *t* test. **C**, Reduced mucus production in ROG Tg mice sensitized and challenged with OVA. **D**, AHR was assessed in an invasive assay system. Mean values (6 mice per group) are shown with SDs. $*P < .01$, Kruskal-Wallis test. *WT*, Wild-type.

Attenuated T_H2 responses induced by ROG Tg CD4 T cells

Assessment of T_H1/T_H2 cell differentiation of ROG Tg mice showed reduced T_H2 cell differentiation in CD4 T cells from ROG Tg mice compared with that seen in wild-type mice (39.3% vs 27.0% and 30.2% vs 14.8%), whereas T_H1 cell (80.2% vs 77.8% and 64.6% vs 64.4%) differentiation was equivalent (Fig 4, *A*). As expected, proliferative responses of both CD4 and CD8 T cells were reduced in ROG Tg mice (see Fig E9, *A* and *B*, in this article's Online Repository at www.jacionline.org). Consequently, we examined the expression levels of IL-4, IL-5, IL-13, eotaxin 2, RANTES, and TNF-α in the BAL fluid cells of the OVA-sensitized and OVA-challenged ROG Tg mice shown in Fig 3. The expression levels of mRNA for IL-4, IL-5, IL-13, eotaxin 2, and RANTES in Tg mice were clearly lower than those in wild-type mice (Fig 4, *B*). Reduced production of IL-5 and IL-13 in BAL fluids from ROG Tg mice was also confirmed by means of ELISA (Fig 4, *C*). We measured the mRNA expression levels of TARC and MDC using allergic lung samples, and no significant decrease was noted in ROG Tg mice (data not shown). These results indicate that the extent of T_H2 cell differentiation was reduced in ROG Tg CD4 T cells and that T_H2-dependent immune responses in the airway in an allergic asthma model were attenuated.

Decreased airway inflammation in mice after adoptive transfer of ROG-overexpressing T cells

Next we examined whether the retrovirus-mediated overexpression of ROG into effector T_H2 cells would affect downregulation of T_H2 cell–mediated inflammatory responses. As shown in Fig 5, *A*, a modest decrease in the numbers of IL-4–producing T_H2 cells was detected in the retrovirus-mediated ROG-overexpressing cell cultures. One million IL-4–producing cells from each culture were transferred into BALB/c *nu/nu* mice. At 48 and 96 hours after cell transfer, mice were challenged with OVA, and infiltration of inflammatory cells was determined. As shown in Fig 5, *B*, the induction of airway infiltration of leukocytes, including eosinophils, was essentially not observed in the mice adoptively transferred with ROG-introduced T_H2 cells compared with the mice with wild-type T_H2 cells. Consequently, RNA was isolated and quantitative RT-PCR analysis was performed to assess the expression of T_H2 cytokines and chemokines in the infiltrates in BAL fluid. The expression levels of IL-4, IL-5, IL-13, and eotaxin 2 in the mice receiving ROG-introduced cells were lower than those in mice receiving mock-introduced cells, whereas RANTES and TNF-α levels were comparable (Fig 5, *C*). Lung resistance, as measured by using a direct invasive method, showed no obvious increase in the AHR in mice transferred with ROG-introduced cells compared with those with

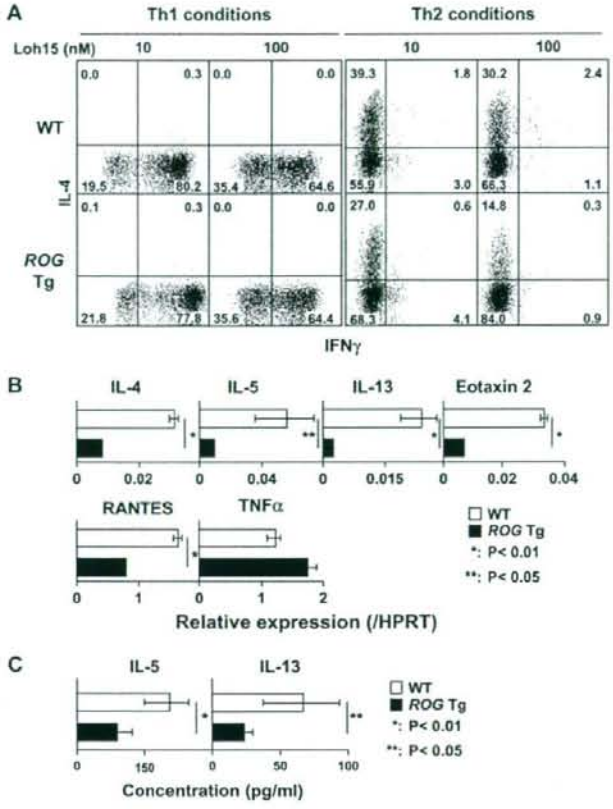

FIG 4. Decreased T_H2 cell differentiation and T_H2 cytokine production in the airways of ROG Tg mice. **A**, The ability to differentiate into T_H1/T_H2 cells *in vitro* in ROG Tg x OT-II transgenic mice was assessed. The results are representative of 5 experiments. **B**, mRNA levels of IL-4, IL-5, IL-13, eotaxin 2, RANTES, and TNF-α in BAL fluid cells of wild-type and ROG Tg mice were determined by means of quantitative RT-PCR. $*P < .01$ and $**P < .05$, Student *t* test. **C**, The amount of IL-5 and IL-13 in the BAL fluid was determined by means of ELISA. The mean values with SDs (4 mice per group) are shown. $*P < .01$ and $**P < .05$, Student *t* test. *WT*, Wild-type.

mock-introduced cells (Fig 5, *D*). These results indicate that the expression levels of ROG in T_H2 cells can affect the OVA-induced airway inflammation.

Modulation of airway inflammation by adoptively transferred *ROG*$^{-/-}$ and ROG Tg T cells

Finally, we addressed whether OVA-primed T cells with altered ROG expression are able to modulate OVA-induced allergic inflammation. After immunization with OVA, splenic CD3 T cells from ROG Tg mice or *ROG*$^{-/-}$ mice (C57BL/6 background) were prepared and transferred into C57BL/6 mice that had been also immunized with OVA twice. Two and 4 days after cell transfer, mice were treated with OVA by means of inhalation, and the inflammatory infiltrates in the BAL fluid were analyzed. As shown in Fig 6, *A*, the adoptive transfer of CD3 T cells from ROG Tg mice significantly reduced the levels of infiltration of leukocytes, especially eosinophils. In contrast, the adoptive transfer of CD3 T cells from *ROG*$^{-/-}$ mice resulted in increased levels of eosinophilic infiltrates. When primed CD3 T cells from ROG Tg mice were used, moderate but significantly decreased numbers of inflammatory mononuclear cells and eosinophils were observed by means of histologic analyses with H&E and LUNA staining (Fig 6, *B*), respectively. A significantly decreased number of PAS-stained cells in the mice receiving ROG Tg T cells was seen, whereas an increased number of these cells was noted in mice receiving *ROG*$^{-/-}$ T-cell transfer (Fig 6, *B*, right panel).

DISCUSSION

Murine models of allergic asthma have been used to dissect the underlying pathogenesis of human asthma. In this study we demonstrate an important role for ROG in the regulation of T_H2-dependent allergen-induced airway inflammation and AHR by using newly established *ROG*$^{-/-}$ and ROG Tg mice with a BALB/c or C57BL/6 background and with a retrovirus-mediated ROG gene-introduction system. T_H2-dependent airway inflammation was enhanced in *ROG*$^{-/-}$ mice (Fig 1) and was dramatically inhibited in ROG Tg mice (Fig 3). We observed moderate enhancement in T_H2 cell differentiation in *ROG*$^{-/-}$ mice, with a marginal effect in T_H1 cell differentiation (Fig 2). In ROG Tg

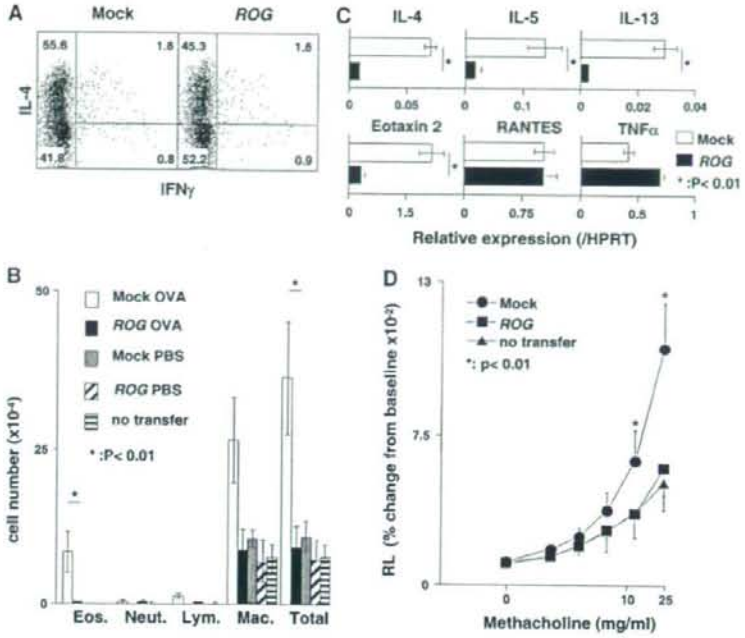

FIG 5. Decreased airway inflammation in mice after adoptive transfer of ROG-overexpressing T cells. **A**, IFN-γ/IL-4 profiles of the transferred *in vitro* generated T_H2 cells. **B**, Decreased infiltration of eosinophils in BAL fluid in mice receiving ROG-overexpressing T cells. Five mice per group were used. Two independent experiments were performed, and similar results were obtained. $*P < .01$, Student *t* test. **C**, mRNA levels of IL-4, IL-5, IL-13, eotaxin 2, RANTES, and TNF-α in the BAL fluid cells from adoptive transfer mice were determined by using quantitative RT-PCR. $*P < .01$, Student *t* test. **D**, One day after the last OVA inhalation, AHR was assessed, and results are presented as airway resistance *(RL)*. Mean values (5-6 mice per group) are shown with SDs. $*P < .01$, Kruskal-Wallis test.

mice T_H2 cell differentiation was affected, whereas T_H1 cell differentiation was normal (Fig 4). Moreover, retrovirus-mediated ROG-overexpressing T_H2 cells also did not induce airway inflammation in an adoptive transfer model (Fig 5). From these results, we conclude that ROG in T cells plays an important regulatory role in T_H2-dependent inflammatory responses in the airway.

The preferential effect in T_H2 cells might be consistent with the notion that ROG negatively regulates GATA3,[32] which is highly expressed in T_H2 cells.[15,16] ROG regulates in a negative fashion the proliferative responses of CD4 and CD8 T cells after TCR stimulation with $ROG^{-/-}$ and ROG Tg mice (see Figs E4 and E9). Furthermore, the rate of cell division of CD4 and CD8 T cells was moderately higher in $ROG^{-/-}$ mice (see Fig E4, *B*). The levels of enhancement were quite comparable in the previously reported $ROG^{-/-}$ mice.[33,39] Taken together, ROG can exert control of at least 2 different processes in T cells during the induction of T_H2 responses and inflammation: (1) a general process, proliferation of T cells after TCR stimulation, and (2) a T_H2-specific process, the extent of differentiation into T_H2 cells through the regulation of GATA3. In the first process the involvement of the regulation of nuclear factor of activated T cells, cytoplasmic 2 (nuclear factor of activated T cells 1)–initiated suppression of the nuclear factor κB pathway is suggested.[32]

We observed greater effects in cytokine expression and eosinophil numbers in the BAL fluid than in those of IL-4–producing T_H2 cells in *in vitro* intracellular cytokine staining experiments (Figs 1-5). This could be due to the involvement of ROG-mediated regulation of the expansion of T cells *in vivo* in the case of the BAL fluid results. The expression of ROG controls antigen-induced proliferative responses of T cells (see Figs E4 and E9), and therefore the expansion of OVA-specific T cells after OVA inhalation might account for the results seen with the BAL fluid samples (cytokine expression and eosinophil number). In contrast, no T-cell expansion would be expected to occur after restimulation with anti-TCR mAb for 6 hours in the presence of monensin *in vitro*.

In a previous study by Kang et al,[39] no significant changes in the generation of IL-4–producing cells was observed in their $ROG^{-/-}$ mice. However, Piazza et al[33] used an independent line of $ROG^{-/-}$ mice and showed an increased T_H2 cytokine mRNA expression in T_H2 cells, which is consistent with our results. Piazza et al[33] showed a normal expression of the mixed lineage leukemia 2 (*MLL2*), a neighboring gene of ROG, in $ROG^{-/-}$ mice. Normal expression of *MLL2* was confirmed in our $ROG^{-/-}$ mice (data not shown). Kang et al[39] demonstrate that the deficiency of ROG resulted in a marginal effect on the severity of experimental autoimmune encephalomyelitis, a T_H cell–mediated disease. We used a T_H2-dependent experimental asthma model in which we observed a substantial effect. Thus some apparent differences in the results obtained in these studies could be due to the difference in the experimental model systems used. In any event, with our newly established $ROG^{-/-}$ mice and ROG Tg mice, which have been extensively

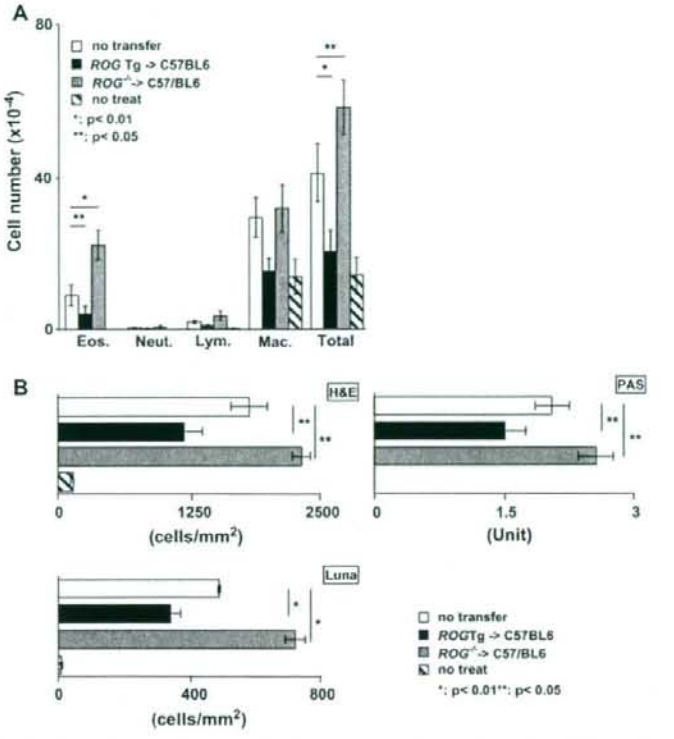

FIG 6. Modulation of airway inflammation by adoptive transfer of $ROG^{-/-}$ and ROG Tg T cells. **A**, Donor mice ($ROG^{-/-}$ and ROG Tg mice on a C57Bl/6 background) and recipient mice (C57BL/6) were sensitized with OVA. Splenic CD3 T cells were prepared from donor mice and transferred into recipient mice. Infiltrated leukocytes in BAL fluid are shown. Five mice per group were used. $*P < .01$ and $**P < .05$ between groups, Student *t* test. **B**, Semiquantitative analysis of peribronchiolar leukocyte infiltration (H&E stain), peribronchiolar eosinophil infiltration (LUNA stain), and the abundance of PAS-positive mucus containing cells (PAS stain). $*P < .01$ and $**P < .05$ between groups, Student *t* test.

backcrossed on either C57BL/6 or BALB/c backgrounds, we demonstrate clearly that ROG regulates T_H2 cell differentiation and T_H2-dependent inflammation in the airway.

T_H2-dependent airway inflammation was not significantly induced in nude mice after adoptive transfer of effector T_H2 cells expressing increased levels of ROG (Fig 5). In addition, the transfer of ROG Tg T_H2 cells into normal mice that were OVA sensitized and challenged resulted in the inhibition of the T_H2-dependent airway inflammation (Fig 6). This might raise the possibility of the therapeutic potential of the ROG-overexpressing T cells. Adoptive transfer of ROG Tg T cells can compete with antigen-presenting dendritic cells in the airway with the host T_H2 cells that induce T_H2-dependent airway inflammation. Indeed, our preliminary results indicate that ROG Tg CD4 T cells compete efficiently with wild-type CD4 T cells to reduce the wild-type CD4 T-cell proliferation induced by OVA peptide pulsed on dendritic cells (unpublished observation). It was recently reported that during clonal expansion antigen-specific T cells could compete for the limited number of peptide/MHC complex sites on dendritic cells if the number of T cells is abundant.[47]

In summary, our study highlights a role for ROG in the development of eosinophilic inflammation and AHR and suggests that ROG could consequently be another possible therapeutic target for the treatment of allergic asthma.

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Key messages

- **ROG regulates the pathogenesis of T_H2-driven allergic airway inflammation and AHR.**
- **ROG might be a potential therapeutic target for the treatment of asthmatic patients.**

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METHODS

Mice

OT-I Tg mice are OVA-specific, MHC class I–restricted TCRαβ transgenic mice with a C57BL/6 background.[E1] The TCR recognizes a specific OVA peptide (SIIN, OVA 257-264).

OT-II Tg mice are OVA-specific, MHC class II–restricted TCRαβ transgenic mice with a C57BL/6 background.[E2] The TCR recognizes a specific OVA peptide (Loh 15, OVA 323-339). DO11.10 Tg mice are OVA-specific, MHC class II–restricted TCRαβ transgenic mice with a BALB/c background.[E3] The TCR recognizes a specific OVA peptide (Loh 15, OVA 323-339). BALB/c *nu/nu* mice lack the thymus, and no functional T cells are present.

Genetic background of mice used in the study

It has been recognized that BALB/c mice show more prominent T_H2 responses than C57BL/6 mice. To investigate the T_H2-dependent responses *in vivo*, we used $ROG^{-/-}$ mice with a BALB/c background (Figs 1 and 2). We have obtained similar results in $ROG^{-/-}$ mice with a C57BL/6 background (data not shown). We have ROG Tg mice only with a C57BL/6 background, and therefore we used C57BL/6 background mice in the experiments using ROG Tg mice (Figs 3 and 4) and with adoptive transfer of ROG Tg T cells (Fig 6).

Antigen-presenting cells used in *in vitro* stimulation and T_H1/T_H2 differentiation

Thy1.2-positive cells were eliminated from splenocytes with anti-Thy1.2 mAb (53-2.1) and magnetic bead sorting (MACS sorting, Mitenyi Biotec, Bergish Gladbach, Germany). Then Thy1.2-negative cells were irradiated (3500 rad) and used as antigen-presenting cells (APCs; 2×10^5; Figs 2, *A*; 4, *A*; 4, *B* and *C*; 5; and 9, *B*).

In vitro T_H1/T_H2 cell differentiation cultures

Naive ($CD44^{low}$) splenic DO11.10 Tg CD4 T cells (2×10^4) or OT-II Tg CD4 T cells (2×10^4) prepared by means of cell sorting were stimulated with indicated doses of antigenic OVA peptide (OVA 323-339) and irradiated (3500 rad) BALB/c or C57BL/6 APCs (Thy1.2-negative APCs, 2×10^5) in the presence of IL-2 (25 U/mL) and IL-4 (100 U/mL; T_H2 condition); IL-2 (25 U/mL), IL-12 (100 U/mL), and anti-IL-4 mAb (T_H1 condition); or IL-2 (25 U/mL) only (neutral condition).[E4]

In vitro ROG-overexpressing T_H2 cell cultures

Freshly isolated KJ1-positive CD4 T cells from DO11.10 Tg mice were stimulated with immobilized anti-TCR plus anti-CD28 mAb for 2 days. Then the *ROG* gene was introduced by using a retrovirus vector containing the *ROG-IRES-hNGFR* gene, and 4 days after infection, *hNGFR*-positive infected cells were enriched by sorting. Infected cells were stimulated with OVA peptide plus APCs in the presence of IL-2 for 5 days. Then the stimulated cells (1×10^6) were transferred into BALB/c *nu/nu* mice, as previously described.[E5]

Measurement of AHR

AHR responses were assessed by using methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics, Inc, Wilmington, NC), as described previously.[E6] Airway function was also assessed by measuring the changes in lung resistance and dynamic compliance in response to increasing doses of inhaled methacholine, as described previously.[E7,E8]

Analysis of lung histology

The lung samples taken on day 25 were sectioned and stained with H&E reagents, PAS reagents, and LUNA reagents, as previously described.[E6] PAS-positive cells were defined as the average of the score. The numeric scores for the abundance of PAS-positive mucus-containing cells in each airway were determined as follows: 0, less than 5% PAS-positive cells; 1, 5% to 25%; 2, 25% to 50%; 3, 50% to 75%; and 4, more than 75%.[E9]

Supplemental discussion

RANTES (CCL5) belongs to the CC chemokine family and induces leukocyte migration by binding to specific receptors in the G protein–coupled receptor family.[E10] RANTES is produced predominantly by CD8 T cells, epithelial cells, fibroblasts, and platelets and is associated with airways inflammation.[E11-E15] The modulation of RANTES expression by different levels of ROG was detected (Figs 2, *B*, and 4, *B*), whereas no difference was observed in the expression of RANTES in the transfer experiments by using BALB/c nude mice (Fig 5, *C*). The reason for this discrepancy remains unclear at this time, but this could be due to the absence of endogenous CD8 T cells in the host BALB/c *nu/nu* mice. A critical role of CD8 T cells in the OVA-induced airway inflammation has been reported.[E16] RANTES could be involved in the modulation of airway inflammation by CD8 T cells.

The absolute number of macrophages in the BAL fluid is enhanced after OVA challenge (Fig 1, *A*). No remarkable change in the number of macrophages between $ROG^{+/+}$ and $ROG^{-/-}$ mice was detected (Fig 1, *A*), and moderate decreases in the number of macrophages were observed in ROG Tg and in cell-transfer experiments with ROG-overexpressing cells (Fig 3, *A*; 5, *B*; and 6, *A*). Thus no clear link can exist between the expression levels of ROG and the level of infiltration of macrophages into the lung.

Kang et al[E17] demonstrate that $ROG^{-/-}$ T cells express more CD25 and CD69. We did not detect the difference in the expression of CD25 and CD69 in freshly prepared $ROG^{-/-}$ T cells (see Fig E1). Kang et al measured the expression of CD25 and CD69 after anti-TCR stimulation, whereas we examined the expression in freshly prepared CD4 T cells in Fig 1, *E*. Thus the apparent discrepancy between our results and theirs could be due to the cells analyzed in their studies compared with ours (activated vs not activated).

We showed a modulation effect of ROG Tg T cells in an OVA-induced airway inflammation model (Fig 6). The ROG-overexpressing cells showed the decreased ability to induce the airway inflammation (Fig 5). The expression levels of ROG in the retrovirus-transduced ROG-overexpressing T_H2 cells were modest compared with those of the ROG Tg T cells. Thus we performed the modulation experiment using ROG Tg T cells (Fig 6).

We measured the mRNA expression levels of TARC and MDC, which are known to be the chemokines for T_H cell recruitment, and no significant difference was noted in $ROG^{-/-}$ or ROG Tg mice compared with those in wild-type mice. It is known that macrophages, natural killer cells, and B cells constitutively secrete MDC,[E18] and TARC is secreted by airway epithelium.[E19] Thus ROG might not be involved directly in the regulation of the secretion of these chemokines.

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E3 Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. Science 1990;250:1720-3.

E4 Yamashita M, Kimura M, Kubo M, Shimizu C, Tada T, Perlmutter RM, et al. T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation. Proc Natl Acad Sci U S A 1999;96:1024-9.

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E17 Kang BY, Miaw SC, Ho IC. ROG negatively regulates T-cell activation but is dispensable for Th-cell differentiation. Mol Cell Biol 2005;25:554-62.

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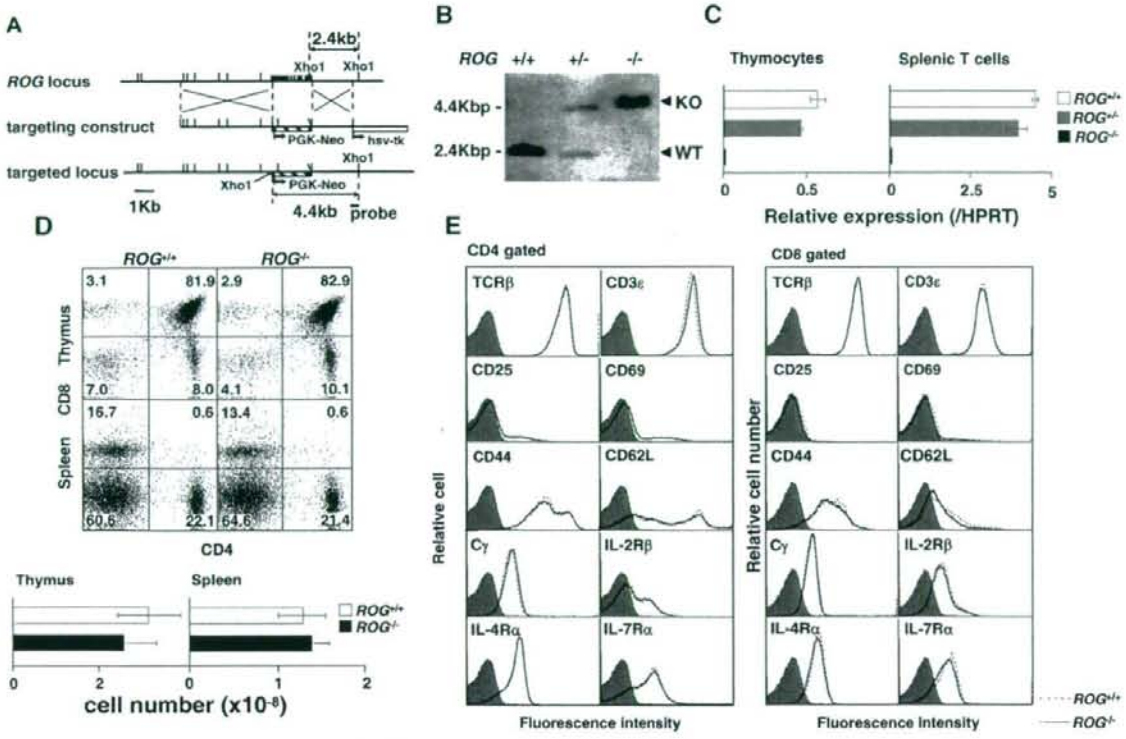

FIG E1. Phenotypic characterization of thymocytes and peripheral CD4 and CD8 T cells in *ROG*$^{-/-}$ mice. **A**, Schematic representation of wild-type and mutant loci of the *ROG* gene together with the targeting vector. **B**, Representative Southern blot analysis with *Xho*I-digested DNA. **C**, ROG mRNA levels in *ROG*$^{+/+}$, *ROG*$^{+/-}$, and *ROG*$^{-/-}$ thymocytes and splenic T cells were determined by means of quantitative RT-PCR. **D**, Representative CD4/CD8 profiles of thymocytes and splenocytes of *ROG*$^{-/-}$ mice. The yields of thymocytes and splenocytes are shown with standard deviations *(lower panel)*. **E**, The phenotypic features of splenic CD4 and CD8 T cells from *ROG*$^{-/-}$ mice. Each histogram depicts the expression of the indicated maker antigens on electronically gated splenic CD4 or CD8 T cells. Background staining is shown in *shaded areas*.

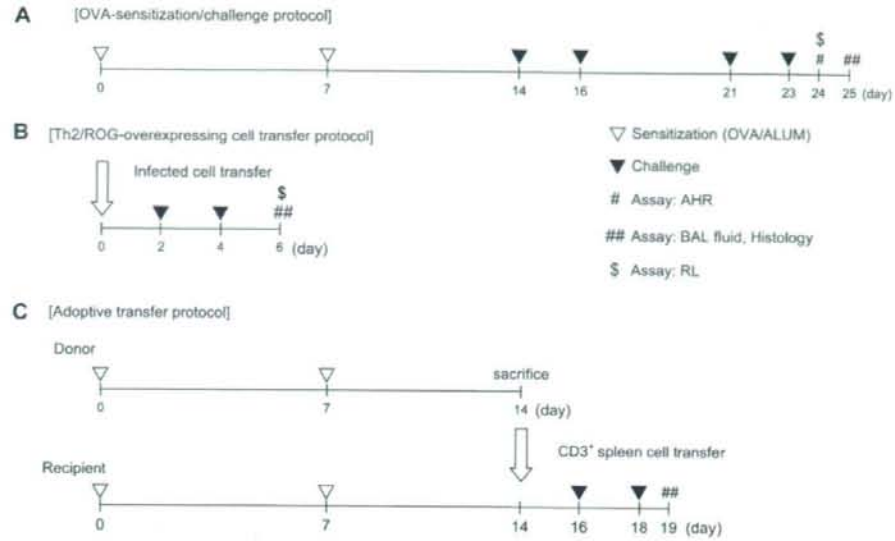

FIG E2. Experimental protocols for antigen sensitization, challenge, and assay in the different mouse models. **A**, BALB/c and C57BL/6 mice were injected intraperitoneally with 250 μg of OVA in 4 mg of aluminum hydroxide gel on days 0 and 7. Mice were then treated with aerosolized OVA in saline (10 mg/mL) by means of inhalation on days 14, 16, 21, and 23 (challenge). On day 24, AHR and lung resistance *(RL)* were assessed. On day 25, BAL fluid cells were prepared. Samples for histologic analysis were prepared on day 25. **B**, On day 0, retrovirus-mediated ROG-overexpressing cells (1×10^6) were transferred into BALB/c *nu/nu* mice. After cell transfer, the mice were treated with aerosolized OVA in saline (10 mg/mL) by means of inhalation on days 2 and 4. No OVA immunization was performed. RL was measured and BAL fluid was collected on day 6. **C**, Donor mice ($ROG^{-/-}$ and ROG Tg mice on a C57Bl/6 background) and recipient mice (C57BL/6) were sensitized intraperitoneally with 250 μg of OVA with alum on days 0 and 7. On day 14, splenic CD3 T cells were prepared from donor mice and transferred into recipient mice. The recipient mice were treated with aerosolized OVA in saline (10 mg/mL) by means of inhalation on days 16 and 18. BAL fluid was collected on day 19. Samples for histologic analysis were prepared on day 19.

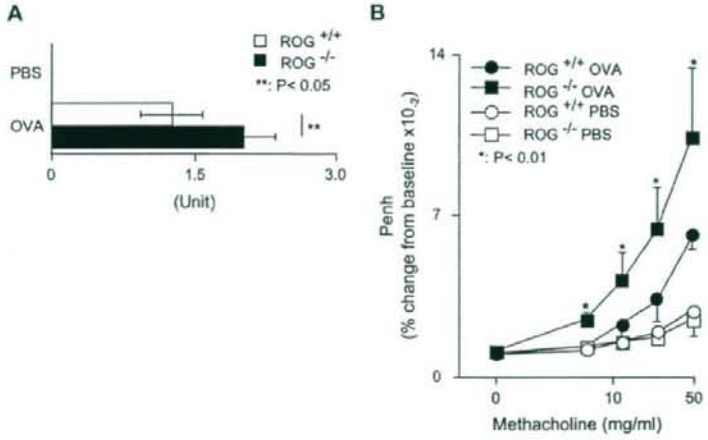

FIG E3. ROG$^{+/+}$ and *ROG*$^{-/-}$ mice were sensitized with OVA and underwent inhalation challenge of OVA or PBS. **A**, Semiquantitative analysis of the abundance of PAS-positive mucus-containing cells. **P* < .01, Student *t* test. **B**, One day after the last OVA inhalation, AHR in response to increasing doses of methacholine was measured in a whole-body plethysmograph. Mean enhanced pause *(Penh)* values (6 mice per group) are shown with SDs. Three independent experiments were performed, and similar results were obtained. **P* < .01, Kruskal-Wallis test.

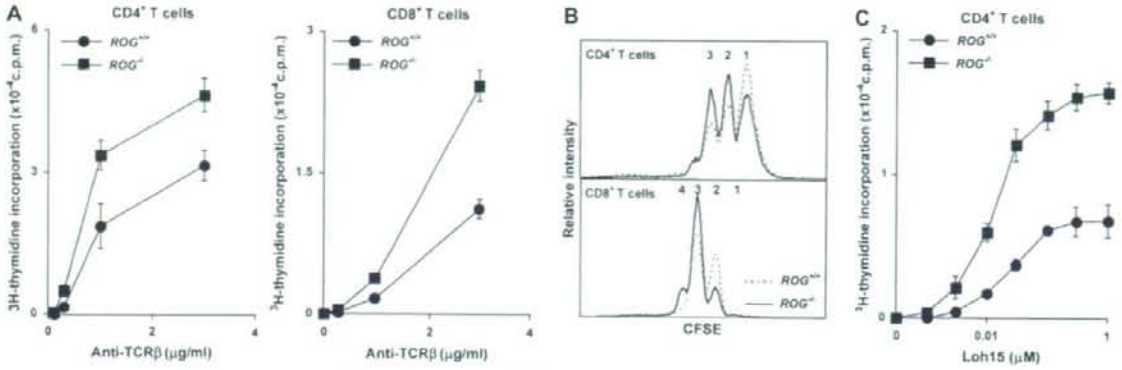

FIG E4. Enhanced proliferative responses in $ROG^{-/-}$ T cells. **A**, Splenic CD4 and CD8 T cells from $ROG^{-/-}$ mice were stimulated with indicated doses of immobilized anti-TCRβ mAb (3 μg/mL) for 2 days, and proliferative response was assessed. Mean tritiated thymidine incorporation in each culture is shown with SDs. **B**, Splenic CD4 T cells from $ROG^{-/-}$ x DO11.10 Tg mice or CD8 T cells from $ROG^{-/-}$ x OT-I Tg mice were labeled with carboxyfluorescein succinimidyl ester and were stimulated with antigenic peptide (0.1 μmol/L) plus irradiated APCs (Thy1.2-negative cells) in the presence of IL-2 (25 U/mL) for 48 hours. **C**, Splenic CD4 T cells from $ROG^{-/-}$ x DO11.10 Tg mice were stimulated with indicated doses of antigenic peptide and irradiated BALB/c APCs (Thy1.2-negative cells) for 2 days, and antigenic peptide–induced proliferative response was assessed. Mean tritiated thymidine incorporation in each culture is shown with SDs.

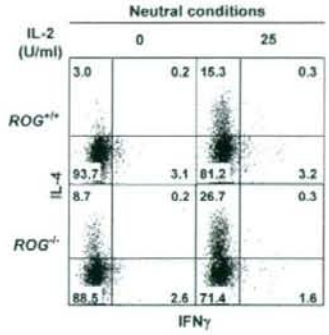

FIG E5. Increased T_H2 cell differentiation of $ROG^{-/-}$ T cells under neutral conditions *in vitro*. Naive ($CD44^{low}$) CD4 T cells from $ROG^{-/-}$ x DO11.10 Tg mice were purified by means of cell sorting and stimulated with indicated doses of antigenic peptide plus irradiated APCs (Thy1.2-negative cells) under neutral conditions for 5 days. The results are representative of 5 experiments.