Runx3 Is Required for Full Activation of Regulatory T Cells To Prevent Colitis-Associated Tumor Formation

Manabu Sugai, Koji Aoki, Motomi Osato, Yukiko Nambu, Kosei Ito, Makoto M. Taketo and Akira Shimizu

*J Immunol* 2011; 186:6515-6520; Prepublished online 22 April 2011; doi: 10.4049/jimmunol.1001671
http://www.jimmunol.org/content/186/11/6515

References

This article cites 31 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/186/11/6515.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Runx3 Is Required for Full Activation of Regulatory T Cells To Prevent Colitis-Associated Tumor Formation

Manabu Sugai,†,‡ Koji Aoki,† Motomi Osato,§,¶,†† Yukiko Nambu,*,# Kosei Ito,** Makoto M. Taketo,† and Akira Shimizu*‡

Inflammation is increasingly recognized as an essential component of tumorigenesis, which is promoted and suppressed by various T cell subsets acting in different ways. It was shown previously in Runx3-deficient mice that differentiation of CD8 T and NK cells is perturbed. In this study, we show that Runx3 is also required for proper differentiation and function of regulatory T cells. In Runx3-deficient mice, T cells were unable to inhibit inflammation and to suppress tumor development. As expected, recombination activating gene 2-deficient mice bearing Runx3-deficient lymphocytes spontaneously developed colon tumors. However, tumor formation was completely blocked by transfer of either regulatory T cells or CD8 T cells derived from wild-type mice to mutant mice or by housing mutant mice in a specific pathogen-free condition. These results indicate that Runx3-deficient lymphocytes and microorganisms act together to induce inflammation and consequently induce the development of colon tumors.

The Journal of Immunology, 2011, 186: 6515–6520.

C

olitis-associated cancer is the most serious complication of inflammatory bowel disease (IBD) (1). Accumulating evidence indicates that immune responses have positive and negative roles in tumor formation (2) and maintenance; chronic inflammatory disease increases the risk of cancer development (3–5), whereas the suppression of immune responses against tumors by enhancing regulatory T cell (Treg) activity allows tumor cells to survive (6). Thus, to maintain a tumor-free status, it is important for the host to respond appropriately during the course of inflammation and tumor formation. TGF-β is essential for the maintenance of inflammatory homeostasis, and the loss of the TGF-β1 signaling pathway results in severe inflammation and malignant tumor formation (7), especially in adenomatous polyposis coli (ApC) mutant mice (8). T cells are known to be among the targets of the TGF-β1 signaling pathway because T cell-specific deletion of Smad4 results in spontaneous gastrointestinal cancer (9). It has been demonstrated that various CD4 T cell subsets differ in their ability to inhibit or enhance IBD

and that these T cell subsets act cooperatively in immune surveillance against tumors. The functional balance of various T cell subsets therefore plays a central role in maintaining the integrity of the epithelial barrier and inhibiting tumor formation in the gastrointestinal tract.

Acting downstream of TGF-β1 signaling, Runx proteins are the interacting and functional partners of R-Smad proteins (10–13). The loss of Runx proteins in lymphocytes can therefore affect the severity of inflammation in the gastrointestinal tract and the incidence of tumor formation. Among the three Runx proteins, Runx3 is involved in the differentiation of immune cells, including CD8 and NK cells (14–17), both of which have cytotoxic activity against tumors. Furthermore, Runx3-deficient mice have defects in a subset of dendritic cell (18) and B cell functions (19). Thus, Runx3−/− mice are severely compromised immunologically. The functions of Runx3 are not limited to lymphoid tissues, and this protein is also involved in the regulation of epithelial homeostasis, acting within the epithelium of the gastrointestinal tract. Ito and colleagues (20) have demonstrated that Runx3−/− gastric epithelial cells are resistant to the growth-inhibitory and apoptosis-inducing action of TGF-β1, resulting in hyperplasia of the gastric mucosa. In addition, Runx3 was shown to attenuate β-catenin/T cell factor functions in intestinal tumorigenesis (21). These data indicate that Runx3 is a tumor suppressor acting in the gastrointestinal epithelium. This notion is strongly supported by the analysis of human tumors. Contrary to these results, Groner and colleagues (22) have demonstrated that Runx3−/− mice develop spontaneous IBD and gastric hyperplasia. Because Runx3 was expressed at a high level in lymphoid and myeloid cells, these authors concluded that the colitis and gastric lesions in Runx3−/− mice result from the loss of Runx3 in leukocytes (22). However, both groups performed the experiments in mice that were deficient in Runx3 in all tissues, including epithelium and lymphocytes. Therefore, their conclusion needs further investigation. To determine whether hyperplastic changes in the intestines in Runx3−/− mice are caused by a defect of epithelial cells or blood cells, Ito et al. (21) generated mice whose leukocytes, but not epithelial cells, were Runx3−/− by transplanting bone marrow cells from Runx3−/− mice into irradiated wild-type (WT) mice. These mice showed no symptoms of hyperplasia or dysplasia in the intestines 1 y after
transplantation (21). This observation supports the direct involvement of epithelial cells in tumor formation in Runx3−/− mice. However, we cannot rule out the involvement of WT-derived radio-resistant lymphocytes in maintaining the health of intestinal epithelial cells. To exclude this possibility, we used recombination activating gene 2 knockout (Rag2−/−) mice as recipients because Rag2 is a essential factor for lymphocyte generation.

In this study, we examined whether the loss of functioning Runx3 in lymphocytes contributes to these phenotypes. We found that loss of Runx3 in T cells resulted in suppression of Treg function and that this suppression was the primary cause of colitis but not gastritis observed in Runx3−/− mice. In addition, we assessed tumor formation in the colon. All mice bearing Runx3−/− lymphocytes, but not WT mice, developed tumors in the large intestine or cecum when they were housed in a conventional mouse facility. However, tumor formation was completely blocked by housing them in a specific pathogen-free (SPF) condition, indicating that microorganisms are involved in this process. Furthermore, no tumor formation was observed when CD8 T cells or Tregs of WT origin were transferred into mutant mice. These results, in addition to previous observations, suggest that Runx3 is a suppressor of gastrointestinal tract tumors acting in lymphocytes and epithelial cells.

Materials and Methods

**Mice**

WT, Runx3−/−, and Rag2−/− mice with the C57BL/6 genetic background were maintained in an SPF mouse facility. For some experiments, mice that received transfers of fetal liver cells (FLCs) were maintained in a conventional mouse facility. Procedures involving animals and their care were conducted according to the guidelines for animal treatment of the Institute of Laboratory Animals, Kyoto University.

**Fetal liver transfer**

Single-cell suspensions of 2 × 10^6 to 4 × 10^6 whole fetal liver mononuclear cells harvested from Runx3−/− and WT embryos at E14.5 were injected intravenously into sublethally irradiated (4 Gy) male Rag2−/− recipient mice. Mice were sacrificed at least 10 wk after transplantation, and cell compartments were analyzed by flow cytometry or used for in vitro culture. All Rag2−/− mice, which were used for a series of experiments comparing the effects of Runx3, were age-matched, and FLCs from littermates were used.

**Histologic analysis**

The colon and cecum were removed from mice after euthanasia and dissected free from the anus to a point distal to the cecum and the small intestine. Contents were removed and cleaned with PBS prior to fixation in 4% paraformaldehyde and routine paraffin embedding. Sections were then cut and stained with H&E.

**Cell preparation**

Naive CD4 T, CD8 T, and Tregs were prepared by magnetic cell sorting using appropriate isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). For some experiments, a CD62L−CD44−CD25−CD4+ population was isolated to provide naive CD4 T cells by flow cytometry with a FACSArray (Becton Dickinson, Mountain View, CA).

**Cell cultures**

In all experiments, the percentages of CD62L−CD44−T cells or CD44−CD25+ T cells were >95%. Naive CD4−/− T cells were activated with plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), anti-IFN-γ (10 µg/ml), and anti–IL-4 (10 µg/ml) in the presence or absence of TGF-β1 (3 ng/ml).

**Flow cytometric analysis**

The following Abs were used for staining: FITC anti-mouse Foxp3 (eBioscience, San Diego, CA); biotin anti-mouse CD25 (BD Pharmingen, San Diego, CA); allophycocyanin anti-mouse CD4 (BD Pharmingen); PE anti-mouse CD6 (BD Pharmingen); and PerCP–streptavidin (Molecular Probes, Eugene, OR). All analyses were performed with FACSCalibur or FACSArray (Becton Dickinson).

**Results**

Runx3 deficiency in lymphocytes leads to the development of spontaneous colitis but not gastritis

To determine whether Runx3 expression in lymphocytes affects the homeostasis of gastrointestinal epithelial cells, FLCs from

![FIGURE 1. Representative photographs of the colon of Rag2−/− mice 10 wk after transfer of FLCs from WT and Runx3−/− mice. Samples taken from Lymph-Runx3−/− or Lymph-WT mice were fixed, sectioned, and stained. Specimens were stained with H&E. Scale bars, 400 µm. A, Normal appearance of the colon in a mouse that received WT FLCs. B, Moderate colitis in a mouse that received Runx3−/− FLC. C, Colitis grades are shown as the colitis index. Colitis was scored from 0 to 5 in a blinded fashion. Histologic features were graded as follows: 0, no inflammation; 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; 3, mild to moderate inflammatory cell infiltrates, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; 4, marked inflammatory cell infiltrates, often associated with ulceration, with marked epithelial hyperplasia and mucin depletion; 5, marked transmural inflammation with severe ulceration and loss of intestinal glands. Runx3−/−, Lymph-Runx3−/− mice: WT, Lymph-WT mice.](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org/)

Retroviral infection

Retrovirus was produced by transfecting the ecotropic Plat-E packaging cell line (23) with pMSCV-IRES-hCD4 retroviral vector (24). After stimulation with plate-bound anti-CD3 and soluble anti-CD28 for 24 h, purified naive CD4 T cells were spin-infected with the virus-containing supernatant in the presence of 4 mg/ml polybrene for 1.5 h at 2500 rpm and 32°C and cultured for 48 h.

After removing virus-containing supernatants, cells were reactivated with plate-bound anti-CD3 (5 µg/ml), soluble anti-CD28 (1 µg/ml), anti–IFN-γ (10 µg/ml), and anti–IL-4 (10 µg/ml) in the presence or absence of TGF-β1 (3 ng/ml).

**RT-PCR and real-time PCR**

Total RNAs were extracted from cultured T cells or sorted cultured T cells using TRIzol (Life Technologies-BRL, Gaithersburg, MD). Oligonucleotide-primed cDNAs were prepared with reverse transcriptase. For semiquantitation, 50 ng cDNA was serially diluted and subjected to PCR amplification. All PCR products were resolved electrophoretically in 2% agarose gel and visualized by ethidium bromide staining. For real-time PCR, RT-PCR was performed using 2 × SYBER PCR Master Mix (Qiagen). Specific primer pairs used for real-time PCR were as follows: Runx3 forward (5′-ACCAGCAGACCATCTGCAG-3′) and Runx3 reverse (5′-CGATGGTGTCGCTGTGA-3′); Foxp3 forward (5′-GATGTTCCGC-TACTCCAGAAAATGTCTGT-3′) and Foxp3 reverse (5′-CCACTCGACAAAGCA-CTTG-3′); granzyme B forward (5′-CTCCACGTGCTTCTCCAA-3′) and granzyme B reverse (5′-AGGATCCTGCTTTCTGTAGT-3′); Hprt forward (5′-CTGTTGCATACAGGCGAATCTTG-3′) and Hprt reverse (5′-GATTCACCTGGCGTCATCTTAGC-3′). Relative expression of mRNA was normalized to Hprt mRNA levels within each sample.

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017
Runx3<sup>-/-</sup> embryos and control littermates were transferred into irradiated Rag2<sup>-/-</sup> mice, and inflammatory status was assessed 10 wk after FLC transplantation ("Lymph-Runx3<sup>-/-</sup>" or "Lymph-WT" mice; Runx3<sup>-/-</sup> and WT indicate the genotypes of transferred FLCs). As shown in Fig. 1A, 1B, Lymph-Runx3<sup>-/-</sup> mice exhibited spontaneous colitis, but the colitis was not as severe as previously described (22) (Fig. 1C). To assess whether the severity of colitis depended on the time course of the disease, we examined the colitis index (25) over a more extended period. The colitis index did not change during the course of our observations from 10 to 65 wk after fetal liver cell transfer (FLT) (Fig. 1C and data not shown). These results indicate that Runx3 deficiency in lymphocytes is not sufficient to induce severe colitis, as previously demonstrated (22), at least in this experimental setting. In addition, no Lymph-Runx3<sup>-/-</sup> mice had gastric hyperplasia. Accordingly, functions of Runx3 in other cell types, but not in lymphocytes, also contribute to the inflammatory status in the gastrointestinal tract.

**Runx3 is required for proper Treg function**

Because Tregs are important in preventing an excessive inflammatory reaction in the gastrointestinal tract, we examined Foxp3-expressing natural Tregs (nTregs) in the thymus, spleen, and lymph nodes of Lymph-Runx3<sup>-/-</sup> mice. The percentages and numbers of Foxp3<sup>+</sup> cells were essentially the same in the different genotypes (Fig. 2A and data not shown), indicating that nTreg

---

**FIGURE 2.** Runx3 is required for Treg function. A, FACS analysis of spleen cells, thymocytes, and lymph node cells from Lymph-Runx3<sup>-/-</sup> (Runx3<sup>-/-</sup>) and Lymph-WT (Runx3<sup>+/+</sup>) mice. All plots are gated on CD4<sup>+</sup>CD8<sup>-</sup> cells. Numbers indicate the percentages of Foxp3<sup>+</sup> cells. Data are representative of six independent experiments. B, CD4<sup>+</sup>CD25<sup>+</sup> cells were cultured with anti-CD3 and IL-2 for 48 h. RNA was prepared, and expression of granzyme B was determined by RT-PCR. GAPDH was used as an internal control for RT-PCR. Data are representative of three independent experiments. Fivefold serial dilutions of cDNAs were amplified for the indicated transcripts. C, Suppression assays. CD4<sup>+</sup>CD25<sup>-</sup> responder T cells (2 x 10<sup>4</sup> cells) were placed in 96-well round-bottom plates with CD4<sup>+</sup>CD25<sup>+</sup> cells and APCs (2 x 10<sup>5</sup> cells, T cell-depleted spleen cell populations irradiated with 2000 rad) at 37˚C in 7% CO<sub>2</sub> and were stimulated for 72 h with mAb to CD3 (1 μg/ml). After 72 h of incubation, cultures were pulsed with [3H]thymidine (1 mCi) 6 h before collection. Teff cells from WT mice were cultured with APC plus anti-CD3 with the indicated ratios of Tregs derived from Lymph-Runx3<sup>-/-</sup> or Lymph-WT mice. The p values were calculated by Student t test. *p < 0.05. D, Foxp3 staining of CD4<sup>+</sup> T cells after culture with the indicated agents. Numbers shown in the upper-right corners indicate the percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> cells. Data are representative of 12 independent experiments. E, In each experiment shown in D, cells were classified into competent and defective groups with respect to iTreg differentiation. “iTreg normal” (black bar), iTreg competent group in which 20% cells expressed Foxp3; “iTreg defective” (dotted bar), <5% cells expressed Foxp3. F, Expression of Foxp3 was determined by real-time PCR using RNA from cultures shown in D (Runx3<sup>+/+</sup> and Runx3<sup>-/-</sup>-1, iTreg defective). Hprt was used for normalization. G, Real-time PCR analysis of granzyme B (GZMB) and Foxp3 expression using cDNAs from retroviral expression of Runx3 in Runx3<sup>-/-</sup> T cells. Hprt was used for normalization. Runx3<sup>+/+</sup>, Lymph-WT mice or cells derived from these mice; Runx3<sup>-/-</sup>, Lymph-Runx3<sup>-/-</sup> mice or cells derived from these mice.
The colitis index were examined around 65 wk after FLT transfer. 1, legend of Fig. 1. The SPF facility was free from the following pathogens: 1, Citrobacter rodentium; 2, Corynebacterium kutscheri; 3, Mycoplasma pulmonia; 4, Pasteurella pneumotropica; 5, Salmonella spp.; 6, Pseudomonas aeruginosa; 7, Clostridium piliforme; 8, ectromelia virus; 9, LCM virus; 10, mouse hepatitis virus; 11, Sendai virus; 12, ectoparasites; 13, intestinal protozoa; 14, pinworm; 15, Pneumocystis carinii; 16, Helicobacter bilis; 17, Helicobacter biliis. The conventional facility was free from the following pathogens: 1, Citrobacter rodentium; 2, Corynebacterium kutscheri; 3, Mycoplasma pulmonia; 4, Pasteurella pneumotropica; 5, Salmonella spp.; 6, Pseudomonas aeruginosa; 7, Clostridium piliforme; 8, mouse hepatitis virus; 9, Sendai virus; 10, intestinal protozoa; 11, dermatophytes; 12, Staphylococcus aureus. Co-transferred cells, cells cotransferred with FLCs; FL Genotype, genotypes of the FLCs transferred into Rag2−/− mice; Runx3−/−, Lymphe-WT mice; Wild type, Lymphe-WT mice.

FIGURE 5. Representative tumors of Lymphe-Runx3−/− mice in large intestine (A–D) and cecum (E, F). Enlarged pictures of tumor regions are shown at right (B, D, F). Specimens were stained with H&E. Black scale bars, 1 mm (A, C, E); white scale bars, 100 μm (B, D, F). N, normal regions; T, tumors.
functionally defective, which may be a cause of colitis induced in Lymph-Runx3−/− mice.

Runx3-deficient CD8 T cells, Tregs, and microorganisms are required for the development of colitis-associated tumors in lymph-Runx3−/− mice

Our previous experiments (20, 21) indicated that loss of Runx3 in epithelial cells of the gastrointestinal tract causes hyperplasia of the gastric mucosa and the formation of intestinal tumors. In those experiments, we excluded the possibility of the involvement of lymphocytes in colitis and tumor formation by transplanting bone marrow cells from Runx3−/− mice into irradiated WT mice (21). However, this procedure is not suitable for assessing the functions of Tregs because ~5% of the lymphocytes survive after irradiation, and the remaining Tregs affect the result. To examine further the possible role of Tregs in colitis, we transferred Runx3−/− FLCs into Rag2−/− mice, which have no lymphocytes because of a defect in VDJ recombination. As demonstrated earlier, Runx3-deficient Tregs are one of the causes of colitis in Runx3−/− mice. In our previous report (21), we also demonstrated that small intestinal adenomas developed in Runx3−/− mice at around 65 wk of age, with an incidence rate of 54%. We therefore examined the involvement of Runx3−/− lymphocytes in intestinal tumor formation using Lymph-Runx3−/− mice. Unexpectedly, no tumor formation was observed in these mice. Because the colonic lumen contains abundant commensal bacteria and the composition of this population plays an essential role in colitogenesis, the same assays were performed after housing Lymph-Runx3−/− mice in a conventional mouse facility. In this case, all Lymph-Runx3−/− mice developed tumors in the large intestine or cecum (Fig. 4A; 100% in Lymph-Runx3−/− mice compared with 0% in Lymph-WT mice). The colitis index was also increased in Lymph-Runx3−/− mice but not in Lymph-WT mice (Fig. 4B). A representative tumor in Lymph-Runx3−/− mice is shown in Fig. 5. Notably, it contained abundant stromal cells, such as lymphocytes, fibroblasts, macrophages, and smooth muscle fibers, whereas changes in the epithelial cells were milder and different from typical adenomas observed in Runx3+/− mice (Fig. 5). These data suggest that loss of Runx3 activity in lymphocytes contributes to the development of IBD and subsequent colon tumorigenesis. However, the mechanisms of tumorigenesis appear different between Lymph-Runx3−/− and Runx3−/− mice. To examine further the involvement of CD8 T cells and lymphocytes in the development of intestinal tumors, CD8 T cells or Tregs of WT origin were cotransferred with Runx3−/− FLCs. As shown in Fig. 4A, tumors were not formed in the colon in either case. These data indicate that loss of Runx3 in Tregs and CD8 T cells also contributes to tumorigenesis, in addition to colitogenic microorganisms in the colon.

Discussion

Our results show that inactivation of Runx3 in lymphocytes can induce colitis and consequently tumorigenesis in the large intestine. Thus, the inflammatory phenotype observed in Lymph-Runx3−/− mice is limited to the large intestine and cecum. However, Groner and colleagues (22) demonstrated that Runx3−/− mice developed spontaneous IBD and gastric hyperplasia. This difference suggests several possibilities. In comparison with Runx3−/− mice (22), Lymph-Runx3−/− mice show a weaker inflammatory phenotype. Thus, it is conceivable that other defects, but not lymphocyte defects, caused by the loss of Runx3 contribute to the severity of inflammation. If the gastric mucosa is more resistant to irritating stimuli than the intestinal mucosa, differences in the phenotype can be explained by this difference in inflammatory severity between these mice. Another possibility is that the gastric epithelium requires more Runx3 activity than the intestinal epithelium to maintain epithelial homeostasis; thus, Lymph-Runx3−/− mice do not show gastric hyperplasia because Runx3 expression is not perturbed within the gastric epithelium in Lymph-Runx3−/− mice. In addition, differences in the targeting strategy will affect the severity of gastrointestinal inflammation, which will change the expression levels of splicing variants of the Runx3 gene (22). The exact mechanisms of these processes are yet to be clarified.

Runx factors may be involved in Treg functions (29–31). Runx1 makes a greater contribution to Treg function than the two other Runx family proteins because Treg-specific deletion of Runx1 results in gastritis (30). Akdis and colleagues (29) found that Runx1 and Runx3 have some functional redundancy in Tregs in the human. In this report, we have shown that Runx3-deficient Tregs have a defect in Treg function in vivo and in vitro. In addition, we have found that colitis worsens when Lymph-Runx3−/− mice, but not lymph-WT mice, are housed in a conventional mouse facility. This indicates that commensal microorganisms and Tregs work together to establish a mutual relationship. Taking these observations into account, we propose that both Runx1 and Runx3 are required for the proper functioning of Tregs in becoming tolerant to intestinal bacteria and establishing a good commensal relationship with various bacterial species. However, how to regulate the balance between inhibiting infection and preventing an excessive inflammatory reaction remains to be clarified. In addition, it is unclear why iTreg differentiation fluctuated in different samples of Runx3-deficient cells (Fig. 2D, middle and lower columns, 2E). It is likely that fluctuations in compensatory expression or activity of other members of the Runx family in Runx3−/− T cells affect the capacity of these cells to differentiate into iTregs. This possibility requires investigation in future studies.

Previous reports indicated that Runx3 is required for the proper differentiation and function of CD8 T cells. We therefore examined the involvement of CD8 T cells in tumor formation and found that these cells prevented colitis-associated tumor formation in our experimental setting. In addition to the essential role of CD8 T cells in tumor immunity, recent data indicate that several subsets of CD8 T cells have the capacity to inhibit immune reactions. In this regard, we have not found any obvious anti-inflammatory effect of CD8 T cells. To reveal the exact functions of CD8 T cells in preventing tumor formation in Lymph-Runx3−/− mice remains an important issue for investigation.

Acknowledgments

We thank K. Nakano for technical assistance, K. Ikuta (Institute for Virus Research, Kyoto University) for discussion, T. Kitamura (Institute of Medical Science, University of Tokyo) for扁平E packaging cells, S. Teramukai (Translational Research Center, Kyoto University) for advice on statistical analysis, and F. Alt (Department of Genetics, Harvard Medical School) for Rag2−/− mice.

Disclosures

The authors have no financial conflicts of interest.

References

6520 TUMOR SUPPRESSIVE FUNCTION OF Runx3 IN T LYMPHOCYTES


