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Original Article

Dietary riboflavin deficiency promotes N-nitrosomethylbenzylamine-induced esophageal tumorigenesis in rats by inducing chronic inflammation

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Abstract: Epidemiological studies in high-incidence areas of esophageal cancer in China suggest that environmental carcinogen N-nitrosomethylbenzylamine (NMBA) and riboflavin (RBF) deficiency may be the main risk factors for esophageal cancer. However, it is not clear that the combination induces cancer. Here, experiment (Exp) 1 evaluated the effects of NMBA and RBF deficiency individually or in combination on esophageal tumorigenesis. Male F344 rats were randomly assigned to 4 groups into a 2 (no NMBA *vs*. NMBA) × 2 (normal RBF *vs*. RBF-deficient) factorial design, including normal RBF (6 mg/kg, R_6), RBF-deficient (0 mg/kg, R_0), normal RBF combined with NMBA (R_6N), and RBF-deficient combined with NMBA (R_0N) groups. The Exp 2 explored the effects of RBF deficiency at different doses combined with NMBA (0.6 mg/kg, $R_{0.6}N$; 0.06 mg/kg, $R_{0.06}N$) on esophageal tumorigenesis. Results showed that R_0N enhanced the incidence of esophageal intraepithelial neoplasia (EIN, 53.3%, $P = 0.06$), including carcinoma *in situ*, whereas R_6N mainly induced the occurrence of esophageal benign hyperplasia (38.9%) and EIN (16.7%). RBF deficiency promotes EIN in a dose-dependent manner, and $R_{0.06}N$ significantly increases the incidence of EIN (57.9%, $P < 0.05$). Gene expression profiling demonstrated that inflammatory cytokines were highly expressed in R_0N EIN tissues, whereas R_6N EIN tissues had a proliferation and differentiation gene signature (fold-change > 1.5). Furthermore, RBF deficiency aggravated oxidative DNA damage (8-OHdG) and double-strand breaks (γH2AX) ($P < 0.05$). Our results suggest that RBF deficiency causes chronic inflammation-associated genomic instability contributes to NMBA-induced esophageal tumorigenesis.

Keywords: Esophageal cancer, rat model, esophageal intraepithelial neoplasia, inflammatory cytokines, 8-OHdG, γH2AX

Introduction

Esophageal cancer is the eighth most common and sixth most lethal cancer worldwide, having a 5-year survival rate between 15-25% [1]. Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer and accounts for approximately 90% of esophageal cancer cases [2, 3]. Development and progression of ESCC involves multiple stages, individually known as low-grade intraepithelial neoplasia, high-grade intraepithelial neoplasia, subsequent invasive ESCC, and final metastasis [4]. Esophageal intraepithelial neoplasia is graded according to the proportion of epithelial dysplasia involved, with the lower third to two-thirds being referred to as low-grade intraepithelial neoplasia, and above two-thirds or carcinoma *in situ* being referred to as high-grade intraepithelial neoplasia [5-7]. At the initial stage, squamous epithelial cells exhibit nuclear atypia and abnormal maturation. This stage is known as dysplasia, and is believed to precede ESCC [6]. From a genomic perspective, little is known about the evolution from dysplasia to ESCC, especially

how and at which stage the key carcinogenic events are acquired.

Epidemiological and etiological studies have shown that ESCC has a striking geographic distribution worldwide, suggesting that environmental factors play crucial roles in esophageal carcinogenesis [8]. The major risk factors for ESCC are chronic alcohol consumption, tobacco use, nutritional deficiency, and exposure to environmental carcinogens, such as N-nitrosomethylbenzylamine (NMBA) [9, 10]. NMBA has been widely used to induce esophageal tumors in rodents [11-13]. A typical esophageal tumorigenesis chemoprevention study showed that weekly administration of low doses of NMBA for 15 weeks (cumulative dose = 7.5 mg/kg) produces a 100% incidence of squamous papilloma, but without ESCC development [14]. Combination with other risk factors, such as nutritional deficiency (zinc deficiency, vitamin E deficiency), alcohol, and arecoline, can greatly increase the incidence of ESCC [12, 15-17]. Multiple risk factors are involved in the development of ESCC.

Riboflavin (RBF) is the precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide, which participate in a wide range of biological oxidation-reduction reaction. RBF deficiency may result in disease, such as cancer [18]. The blood concentration of RBF in Kazak ESCC patients is lower than that in healthy controls [19]. Our early studies demonstrated that plasma RBF levels of ESCC patients are significantly lower than that of healthy controls in the Chaoshan area of Guangdong Province [20]. Moreover, we observed that enhanced subcutaneous tumorigenicity in NU/NU mice following injection of RBF-depleted HEK293T cells [21].

In this study, we established a rat models to evaluate the effects of NMBA and RBF deficiency individually or in combination on esophageal tumorigenesis. Furthermore, we reveal the potential molecular mechanisms of RBF deficiency combined with NMBA inducing esophageal tumors.

Materials and methods

Animals and diets

Five-week-old male F344 rats were obtained from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rats were housed in a controlled environment and were allowed access to water ad libitum. The normal RBF diets were AIN-93M purified diet, which contains RBF 6 mg/kg (R_6). RBF-deficient diets were made with the same formula, but contained RBF 0 mg/kg (R_0), 0.6 mg/kg ($R_{0.6}$), or 0.06 mg/kg ($R_{0.06}$) (Trophic Animal Feed High-Tech Co., Ltd., Nantong, China) [22, 23]. The concentration of RBF in the diets was analyzed by reversed-phase high-performance liquid chromatography (HPLC, Agilent 1200 system). N-nitrosomethylbenzylamine (NMBA, CAS NO. 937-40-6) was purchased from Shi Ruikai Technology Co., Ltd. (Wuhan, China).

Experimental design

This study was approved by the Institutional Animal Care and Use Committee of Shantou University. The Exp 1 design is shown in **Figure 1A**. Sixty-nine male F344 rats were randomly assigned to 4 groups (n = 15-18/group) into a 2 (no NMBA *vs*. NMBA) × 2 (normal RBF *vs*. RBF-deficient) factorial design, including normal RBF (R_6), RBF-deficient (R_0), normal RBF combined with NMBA (R_6N) and RBF-deficient combined with NMBA groups (R_0N). For NMBA treatment, the rats received subcutaneous 0.1 mL injections of 20% dimethyl sulfoxide (DMSO) containing NMBA (0.35 mg/kg body weight) 3 times per week for 5 weeks. Control rats received an equivalent volume of 20% DMSO (vehicle control). All rats were euthanized by general anesthesia with an overdose of diethyl ether at week 25 to assess the development of esophageal lesions.

The Exp 2 design is shown in **Figure 1B**. Forty-three male F344 rats were randomized into 3 groups (n = 12-19/group), including R_6N, $R_{0.6}N$ and $R_{0.06}N$ groups. All rats were euthanized at 35 week to assess the development of esophageal lesions.

Serum free RBF

Analysis of serum free RBF was performed using HPLC as described previously [24]. Briefly, proteins were removed from plasma by acid precipitation, and an aliquot of the resulting supernatant was analyzed by reversed-phase HPLC. Impurities were separated from RBF isocratically, and the target material was detected fluorometrically (excitation 450 nm; emission 520 nm).

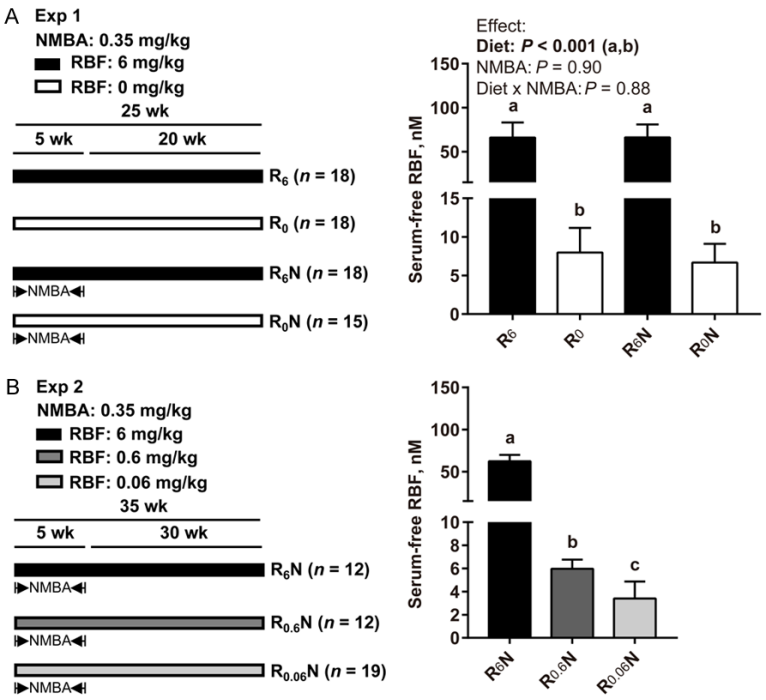

Figure 1. Establishment of a rat model induced by RBF deficiency combined with NMBA. (A, *left*) Exp 1. Sixty-nine male F344 rats were randomly assigned to 4 groups (n = 15-18/group) into a 2 (no NMBA *vs*. NMBA) × 2 (normal RBF *vs*. RBF-deficient) factorial design, including R_6, R_0, R_6N, and R_0N groups. NMBA (0.35 mg/kg body weight) subcutaneous injection 3 times per week for 5 weeks. Rats were euthanized at 25 weeks. (B, *left*) Exp 2. Forty-three male rats were randomly assigned to 3 groups (n = 12-19/group), including R_6N, $R_{0.6}N$, and $R_{0.06}N$ groups. Rats were euthanized at 35 weeks. (A, B, *right*) Serum free RBF levels in rats (nM). Values are means ± SD. Exp 1 different letters (a and b for diet effect) are significantly different by two-way ANOVA and LSD test (P < 0.05). Exp 2 different letters (a, b and c) are significantly different from each other by one-way ANOVA and LSD test (P < 0.05).

Histopathological analysis

Tissue was fixed in 4% paraformaldehyde for 6 hours. After paraffin embedding, serial 4-μm tissue sections were cut for staining. Hematoxylin and eosin (HE)-stained sections were used for histological diagnosis.

Immunohistochemical staining and quantitative analysis

Immunohistochemistry (IHC) was performed using a 2-step protocol (PV-9000 Polymer Detection System; ZSGB-BIO, China) according to the manufacturer's instructions and has been described in our previous studies [25]. Then, the esophageal sections were incubated with primary antibody for pan-Cytokeratin (1:250, Abcam, ab7753, UK), Ki67 (prediluted, Abcam, ab21700, UK), 8-hydroxy-2'-deoxyguanosine (8-OHdG) (1:16,000, Abcam, ab62623, UK), γH2AX (1:2,000, Novus Biologicals, NB-100-384, USA) overnight at 4°C. Cells with a brown reaction product were defined as positive. Stain-positive cells were analyzed by a Perkin-Elmer Vectra system (PerkinElmer, PerkinElmer Vectra, USA). The percentage of positive cells of each group was calculated as the number of positive cells divided by the total number of basal cells.

RNA extraction and gene chip analysis

Comparative transcriptomic analysis of esophageal intraepithelial neoplasia (EIN) tissues *vs*. adjacent normal tissues in the R_6N and R_0N groups (n = 2 rats/group) was performed using GeneChip® Rat Transcriptome Array 1.0 (also known as the Clariom™ D Assay; Thermo Fisher Scientific Inc., 902634). (GEO accession numbers: GSE11-1207). HE-stained tissue was observed under a microscope, and the tissue of interest was isolated with a manual tissue arrayer and placed in a 1.5-mL microcentrifuge tube. The RNA extraction and gene chip analysis were performed by Shanghai Biotechnology Corporation (Shanghai, China) and in accordance with relevant protocols.

Expression data analysis

Raw data was normalized using the quantile normalization of the robust multi-array average (RMA) method in Expression Console (software provided by Affymetrix). Fold change was used to identify differentially-expressed mRNAs. Significantly up-regulated genes were defined by a fold change > 1.5, and significantly down-regulated genes were defined by a fold change < 0.66.

Gene ontology and pathway analysis

To explore the biological functions of the identified differentially-expressed genes, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were con-

ducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [26, 27]. Significant GO terms and KEGG pathways were selected as the enriched terms based on values with $P < 0.05$.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

All primer sequences are shown in Supplementary Table 1. PCR amplifications were performed using SYBR Green Premix (TaKaRa Bio Inc., RR420B) according to the manufacturer's instructions, and qRT-PCR analysis and data collection were carried out on an ABI 7500 qPCR system. For each mRNA, qRT-PCR reactions were performed in triplicate, with β-actin as the internal control gene for normalization. The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method [28].

Enzyme-linked immunosorbent assay (ELISA)

The level of serum inflammatory cytokines S100A8, S100A9, and tumor necrosis factor alpha (TNF-α) were assayed using an ELISA kit (CSB-EL020641RA, CSB-EL020642RA and CSB-E11987r; Cusabio, China). ELISAs were performed according to the manufacturer's instructions. The optical density value of each well was detected using a microplate reader set to 450 nm.

Routine blood tests

Collect rat post-glomus venous plexus blood and send it to the Department of Clinical Laboratory of the Cancer Hospital of Shantou University Medical College. Red blood cells, white blood cells, platelets and other indicators were detected by automatic biochemical analyzer (LH780, BECKMAN-COULTER).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software (SPSS, Chicago, IL). Data are presented as the means ± SD. Exp 1 data were tested by two-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) post hoc test. Exp 2 data were tested by one-way ANOVA followed by LSD post hoc test. Data having unequal variances were log-transformed to achieve equal variances. Differences in tumor incidence were assessed by Fisher's exact test. Differential expression of mRNA between esophageal intraepithelial neoplasia and normal tissue were tested by Student's *t*-test. Statistical tests were two-sided and were considered significant at $P < 0.05$.

Results

RBF deficiency combined with NMBA increases the incidence of esophageal tumors

In Exp 1, serum free RBF levels in RBF-deficient diet-fed rats (R_0 and R_0N groups) were significantly lower than that in normal RBF diet-fed rats (R_6 and R_6N groups) at 25 weeks ($P < 0.001$, **Figure 1A**). There was no interaction between diet and NMBA on the reduction of serum free RBF levels. RBF-deficient (R_0) diet causes weight loss in rats, a representative picture is shown in Supplementary Figure 1A. At week 5, R_0 diet significantly reduced the rat body weight (Supplementary Figure 1B, Exp 1, $P < 0.001$) and no interaction between diet and NMBA was observed. In Exp 2, the serum free RBF concentration was significant differences among the three groups at 35 weeks ($P < 0.05$, **Figure 1B**), resulting in the R_6N group > $R_{0.6}N$ group > $R_{0.06}N$ group. Similarly, there were significant differences among the three groups in rat body weight at week 10 (Supplementary Figure 1B, Exp 2, $P < 0.05$). The above results indicate that a RBF-deficient diet establishes RBF deficiency in our model.

Representative images of esophageal papilloma (R_6N) and carcinoma *in situ* (R_0N) are shown in **Figure 2A**. In Exp 1, no benign hyperplasia (including hyperplasia and papilloma) occurred in either R_6 or R_0 group (0/18, 0%, respectively). R_0 group induced a case of esophageal intraepithelial neoplasia (1/18, 5.6%), while R_6 group did not induce. There was no significant difference between the R_6 group and R_0 group in esophageal tumors ($P > 0.05$). The results indicate that RBF deficiency alone is not sufficient to cause esophageal tumors.

The incidence of pathological damage in the esophagus is summarized in **Figure 2B** (Exp 1). In combination with NMBA treatment, R_6N mainly elicited an incidence of 38.9% benign hyperplasia and 16.7% esophageal intraepithelial neoplasia. R_0N elicited an incidence of 53.3% esophageal intraepithelial neoplasia,

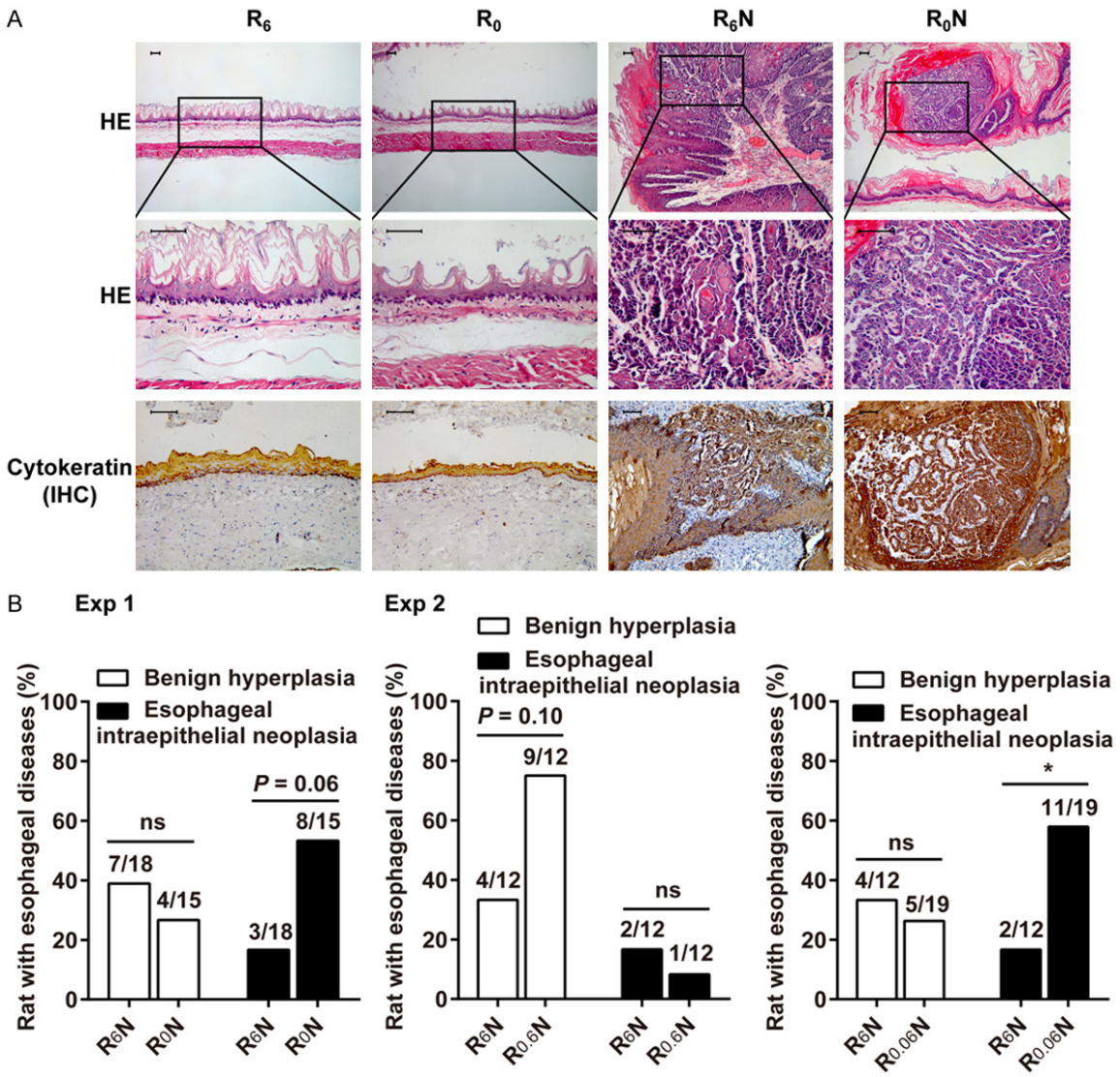

Figure 2. RBF deficiency combined with NMBA induces esophageal tumors in rats. A. HE staining and IHC for cytokeratin were performed on rat esophageal tissues. Representative images of esophageal papilloma (R_6N) and carcinoma *in situ* (R_0N). Scale bars, 50 μm. B. Incidence of benign hyperplasia and esophageal intraepithelial neoplasia in Exp 1 (n = 15-18/group) and Exp 2 (n = 12-19/group). Difference in incidence were assessed by Fisher's exact test ($P < 0.05$). *Means differ, $P < 0.05$.

including carcinoma *in situ*. The incidence of esophageal intraepithelial neoplasia in the R_0N group tends to be higher than that in the R_6N group ($P = 0.06$). Three rats in R_0N group died before the end of study and were not included in the statistics. It may die from esophageal tumors. Other important organs, such as the heart, lung, liver, kidney, stomach, duodenum, jejunoileum, and colon tissues showed no histopathological lesions. Representative pictures are shown in Supplementary Figure 1C. Taken together, RBF deficiency promotes NMBA-induced esophageal tumorigenesis.

RBF deficiency promotes the incidence of esophageal tumors in a dose-dependent manner

In Exp 2, the incidence of pathological damage in the esophagus is summarized in **Figure 2B** (Exp 2). The results show that R_6N treatment elicited a 33.3% incidence of benign hyperplasia and 16.7% incidence of esophageal intraepithelial neoplasia. Mild RBF deficiency, when combined with NMBA ($R_{0.6}N$), elicited an incidence of 75.0% benign hyperplasia and 8.3% esophageal intraepithelial neoplasia. The inci-

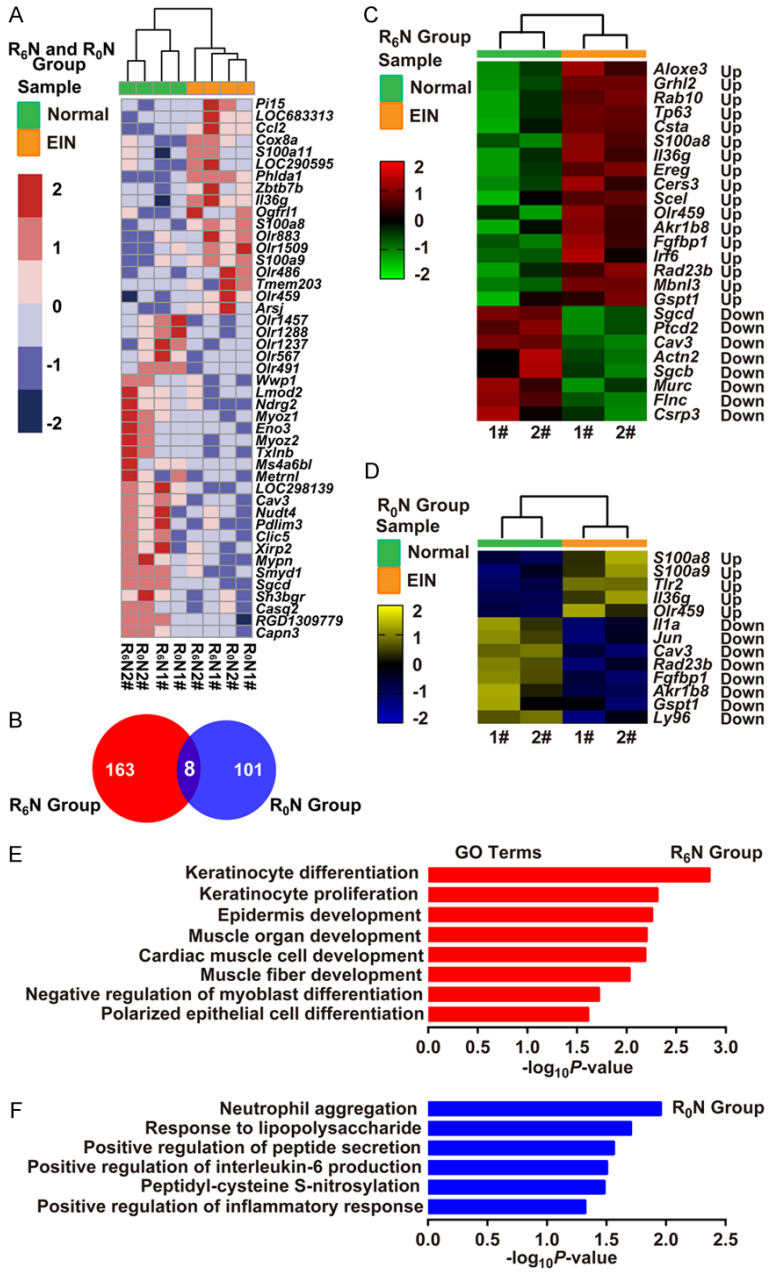

Figure 3. RBF deficiency induces an inflammatory gene expression profile in rat esophageal tumors. (A) Heat map showing gene expression profiling of the EIN *vs.* Normal tissues in the R_6N and R_0N groups (n = 2/group). (B) Venn diagram showing the unique and shared differentially-expressed genes between the R_6N and R_0N groups. (C, D) Heat map showing the differential gene expression profiling of EIN *vs.* Normal tissues in the R_6N (C) and R_0N (D) groups, respectively (n = 2/group). Log_2-fold change ratios are shown in the results. (E, F) Bar diagrams showing the biological processes in the R_6N (E) and R_0N (F) groups. Predicted GO categories ranked by enrichment score [$-log_{10}$ (*P*-value)]. Up, Up-regulated expression; Down, Down-regulated expression; 1# and 2#, identifier of rats.

dence of benign hyperplasia in the $R_{0.6}N$ group tends to be higher than that in the R_6N group (P = 0.10). Severe RBF deficiency ($R_{0.06}N$) elicited an incidence of 26.3% benign hyperplasia and 57.9% esophageal intraepithelial neoplasia, including a case of carcinoma *in situ*. The incidence of esophageal intraepithelial neoplasia in the $R_{0.06}N$ group was increased above that in the R_6N group ($P < 0.05$). Therefore, RBF deficiency promotes esophageal tumorigenesis in a dose-dependent manner.

RBF deficiency induces an inflammatory gene expression pattern in rat esophageal tumors

Hierarchical clustering analyses revealed that R_0N esophageal intraepithelial neoplasia (EIN) tissues had distinct gene expression patterns when compared with R_6N EIN tissues, although the expression profile exhibited a 2-branch partition with the 4 EIN samples clustered together and well separated from their matched normal controls (**Figure 3A**, **3B**). Overall, a total of 163 genes (128 up- and 35 downregulated) were identified to be specifically differentially expressed in the R_6N group; a total of 101 genes (36 up- and 65 downregulated) were identified to be specifically differentially expressed in the R_0N group. The 2 sets of genes shared 8 common genes (**Figure 3B**). Among them, *S100a8*, *Il36g,* and *Olr459* were upregulated, and *Cav3* was downregulated in the 2 groups; *Akr1b8, Fgfbp1*, *Rad23b,* and *Gspt1* were upregulated in the R_6N group, but downregulated in the R_0N group (**Figure 3C**, **3D**).

GO and KEGG pathway annotation of differentially-expressed genes showed the R_6N group was enriched in cell proliferation and differen-

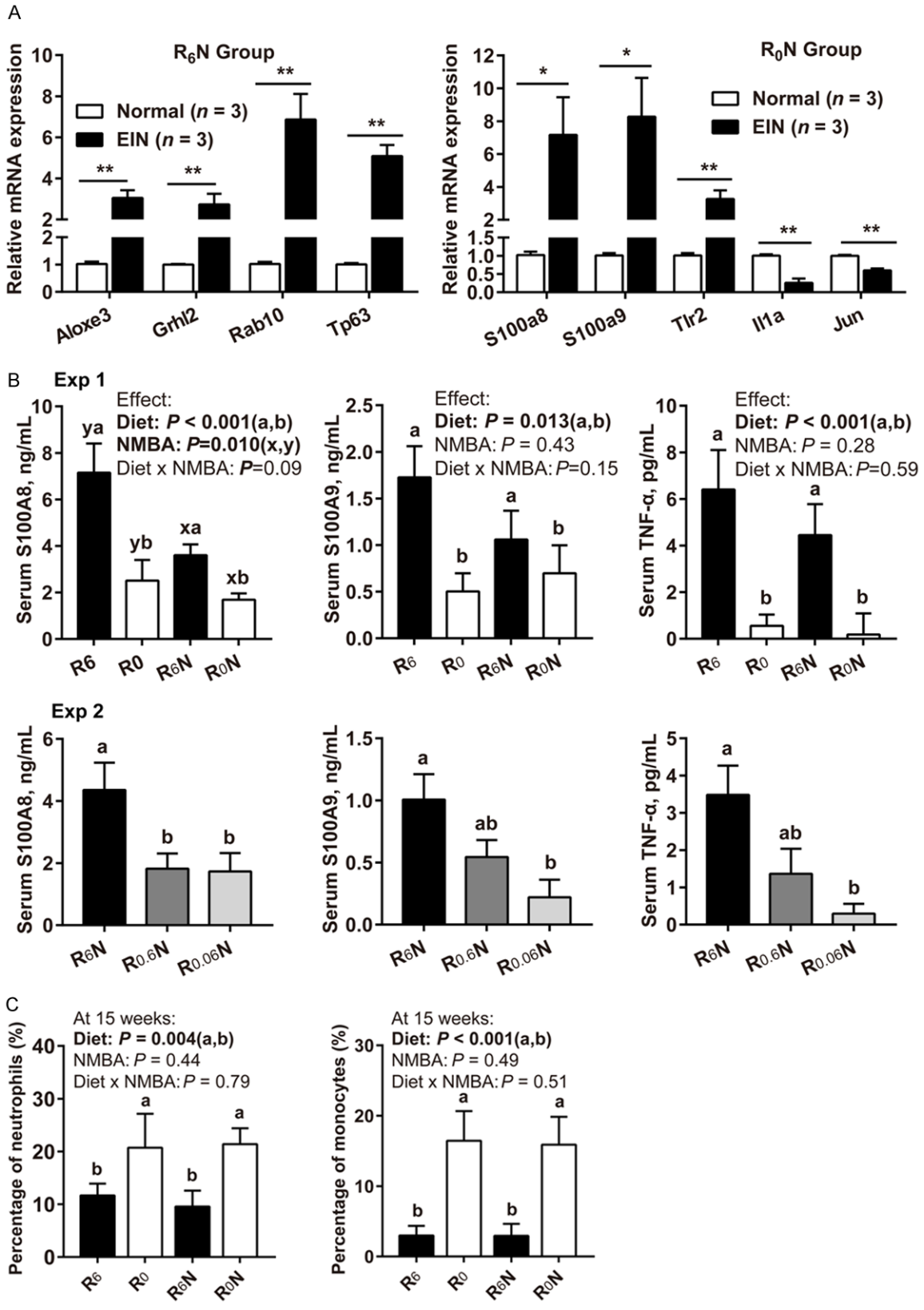

Figure 4. Effect of RBF deficiency on the levels of inflammatory markers in rat esophageal neoplasia tissues and peripheral blood. A. Validation of microarray data by qRT-PCR (EIN *vs.* Normal tissues, normalized to β-actin, *n* = 3/group). Data were analyzed by Student's *t*-test. B. Detection of inflammatory marker expression levels in rat serum

by ELISA (n = 6/group). C. At 15 weeks, the percentage of peripheral blood neutrophils, and monocytes in the total white blood cells (n = 15-18/group). Values are means ± SD. Exp 1 different letters (a and b for diet effect; x and y for NMBA effect) are significantly different by two-way ANOVA and LSD test ($P < 0.05$). Exp 2 different letters (a and b) are significantly different from each other by one-way ANOVA and LSD test ($P < 0.05$). *Means differ, $P < 0.05$. **Means differ, $P < 0.01$.

tiation terms (**Figure 3E**). The upregulated genes related to these terms were *Aloxe3, Grhl2*, *Rab10, Tp63*, *Csta*, *Ereg*, *Cers3, Scel, and Irf6*, with fold-changes ranging from 1.67- to 18.9-fold (**Figure 3C**). Interestingly, differentially-expressed genes in the R_0N group were mainly annotated into inflammation-related terms (**Figure 3F**). The upregulated genes related to these terms were *S100a8*, *S100a9, Tlr2*, and *Il36g*, with fold-changes ranging from 1.55- to 4.3-fold. The down-regulated genes were *Il1a, Jun*, and *Ly96* (**Figure 3D**). By quantitative real-time RT-PCR analysis, we validated that 4 proliferation-related genes (*Aloxe3*, *Grhl2*, *Rab10,* and *Tp63*) were highly expressed in EIN of the R_6N group (**Figure 4A**, left). For RBF deficiency, inflammation-related genes *S100a8*, *S100a9,* and *Tlr2* were highly expressed in EIN of the R_0N group, while *Il1a* and *Jun* showed low expression (**Figure 4A**, right).

Surprisingly, the serum inflammatory cytokines S100A8, S100A9, and TNF-α were actually reduced in tumors developing under RBF deficiency (**Figure 4B**). In Exp 1, the serum concentrations of S100A8, S100A9, and TNF-α in the R_0 diet-fed rats (R_0 and R_0N groups) were significantly lower than that in the R_6 diet-fed rats (R_6 and R_6N groups) at week 25 (**Figure 4B**, Exp 1, $P < 0.05$). There was no interaction between diet and NMBA in the reduction of serum S100A8, S100A9, and TNF-α. In Exp 2, the serum concentrations of S100A8, S100A9, and TNF-α were significantly lower in group $R_{0.06}N$ than in group R_6N at week 35 (**Figure 4B**, Exp 2, $P < 0.05$). Routine blood tests found that R_0 diet significantly elevated peripheral blood neutrophils, and monocytes at week 15 (**Figure 4C**, $P < 0.01$), no interaction between diet and NMBA was observed. The total number of white blood cells and percentage of lymphocytes, eosinophils, basophils did not change (Supplementary Figure 2A, 2B, $P > 0.05$). Taken together, these results illustrated that RBF deficiency induces an inflammatory gene expression pattern in esophageal tumors.

RBF deficiency combined with NMBA promotes DNA damage in esophageal epithelium

Representative images are shown in **Figure 5A**. The percentage of Ki67-positive cells in the NMBA treatment group (R_6N and R_0N groups) were significantly higher than that in the NMBA untreated group (R_6 and R_0 groups) (**Figure 5B**, $P < 0.001$). There was no interaction between diet and NMBA in the elevation of Ki67-positive cells. Considering that prolonged inflammation may lead to DNA damage, we detected oxidative DNA damage biomarker 8-OHdG and double-strand breaks biomarker γH2AX [29-32]. Both diet and NMBA elevated the percentage of nuclear staining of 8-OHdG and γH2AX (**Figure 5C**, **5D**, $P < 0.05$). However, we did not find any interaction between diet and NMBA in the elevation of 8-OHdG and γH2AX positive cells. These results revealed that NMBA, not RBF deficiency, is the main cause of esophageal cell proliferation. Both NMBA and RBF deficiency promote DNA damage.

Discussion

Several epidemiological studies have shown that RBF deficiency is a risk factor for esophageal cancer. Supplementation of RBF through nutritional intervention or RBF-fortified salt can effectively reduce the incidence of esophageal cancer in high-risk areas of China [33-35]. In animal studies, supplementing marginally deficient corn or wheat diets with various combinations of nicotinic acid, RBF, zinc, magnesium, molybdenum, and selenium significantly reduces NMBA-induced esophageal tumors [36, 37]. In this study, our data demonstrate that RBF deficiency promotes NMBA-induced esophageal tumorigenesis. Mechanistically, RBF-deficient diet increases inflammatory markers, forming an inflammatory microenvironment in esophageal tumor tissue. Chronic inflammation-related DNA damage (as demonstrated by accumulation of DNA oxidative damage and DNA double-strand breaks) may trigger esophageal tumorigenesis.

The proposed mechanism of NMBA carcinogenicity is through microsomal activation to form benzaldehyde and an electrophilic metabolite that methylates DNA, producing O^6- and ^{7}-methylguanine adducts [38]. The occurrence of O^6-methylguanine is thought to play a primary role in inducing DNA mutations [39, 40]. *Tp53* and *Ha-Ras* gene mutations are present in the majority of NMBA-induced rat esophageal pap-

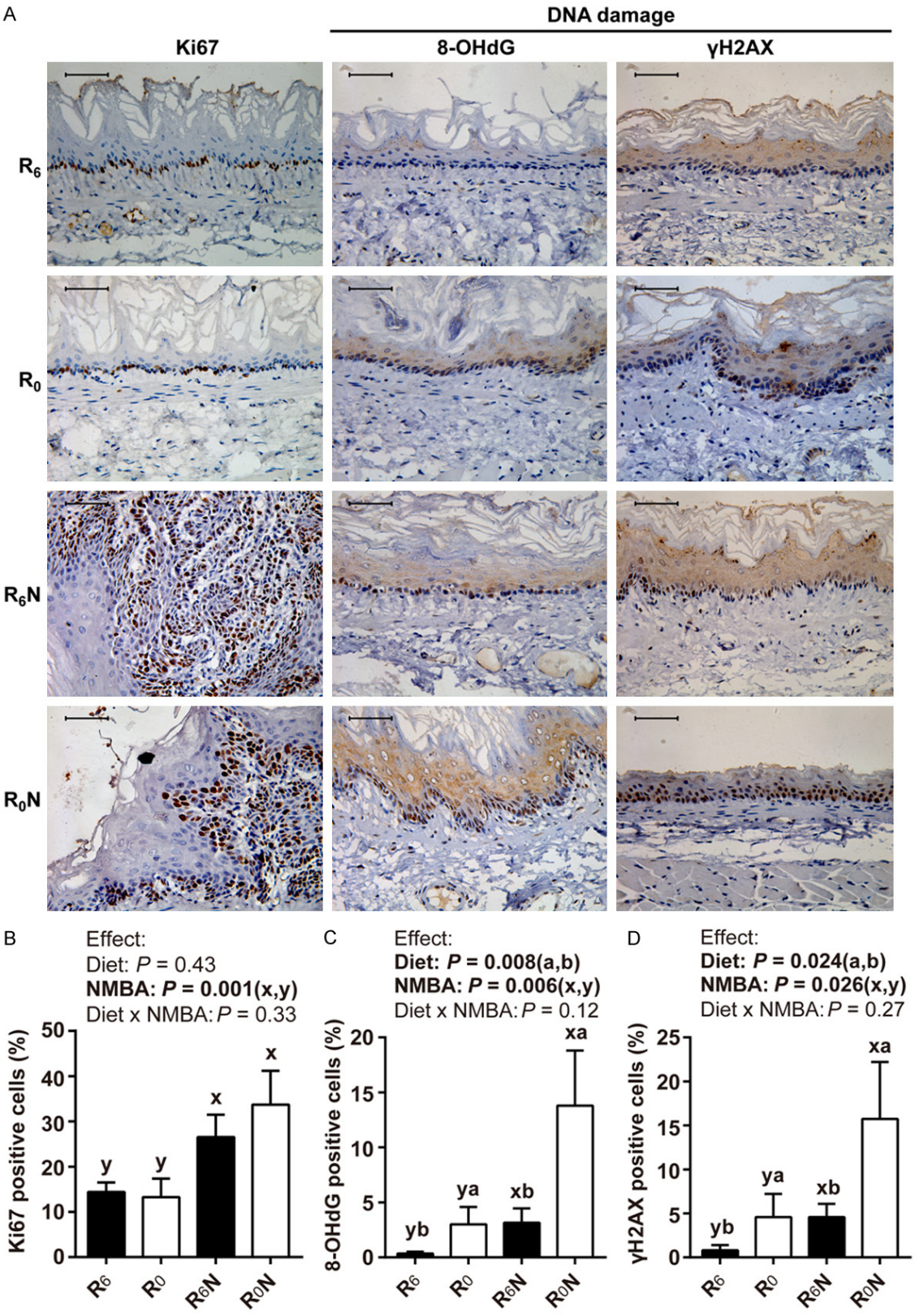

Figure 5. RBF deficiency combined with NMBA promotes DNA oxidative damage and double-strand breaks in the rat esophagus. A. Representative pictures of Ki67, 8-OHdG, and γH2AX immunohistochemistry in esophageal epithelium. Scale bars, 50 μm. B-D. Expression level of Ki67, 8-OHdG, and γH2AX was quantified by immunohistochemical staining, respectively (n = 15-18/group). Values are means ± SD. Different letters (a and b for diet effect; x and y for NMBA effect) are significantly different by two-way ANOVA and LSD test ($P < 0.05$).

illomas [41, 42]. In addition, dysregulation of TGF-alpha and EGFR expression play an important role in NMBA-induced rat esophageal tumorigenesis [43]. Our studies show that R_6N esophageal intraepithelial neoplasia has a proliferation, differentiation, and development gene signature. The overall Ki67 (a cell proliferation marker) staining in esophagus of NMBA treatment group were significantly higher than that in NMBA untreated group. In summary, the primary carcinogenic mechanism of NMBA is to cause gene mutations, cell proliferation, and abnormal differentiation. DNA damage and double-strand breaks are not severe in epithelial cells of the esophagus, which may be responsible for the low tumorigenicity of NMBA.

Flavins play a central role in whole-body energy metabolism. RBF deficiency leads to low efficiency of growth energy utilization in rats [44, 45]. Recently, multiple studies highlighted the pro-inflammatory effects of RBF-deficient. The expression and release of inflammatory cytokines are elevated in RBF-deficient adipocytes [46, 47]. Chronic inflammation and low efficiency of growth energy utilization may be the main cause of weight loss in RBF-deficient rats. RBF deficiency elicits a pro-inflammatory gene expression profile in T lymphoma Jurkat cells [48]. Accordingly, our research shows that RBF deficiency induces an inflammatory gene expression pattern in esophageal tumors. The elevated inflammatory cytokines cause activation of NF-κB signaling pathway in ESCC cells [28]. NF-κB family members modulate the transcription of genes that regulate inflammation, proliferation, and immune regulation [49, 50]. We speculate that RBF deficiency increases inflammatory cytokines and activates NF-κB, resulting in the release of cytokines, which in turn causes the activation of NF-κB. The positive feedback regulatory mechanism maintains chronic inflammation.

In humans, esophageal intraepithelial neoplasia and ESCC share high frequency of mutations and large-scale chromosome aberrations, indicating that the genetic stability of esophageal intraepithelial neoplasia has collapsed [51, 52]. Chronic inflammation-related DNA damage (genomic instability) may trigger the initiation of esophageal squamous dysplasia [53]. In this study, RBF deficiency is a trigger of chronic inflammation that provides a microenvironment conducive to tumor initiation. Normal RBF combined with NMBA mainly induces the occurrence of benign esophageal hyperplasia. RBF deficiency in combination with NMBA accumulates DNA oxidative damage and DNA double-strand breaks, resulting in chronic inflammation-associated DNA damage, and promotes NMBA-induced esophageal tumorigenesis. The underlying molecular mechanisms still need to be revealed.

In conclusion, the present study demonstrates that RBF deficiency causes chronic inflammation-associated genomic instability, which may contribute to NMBA-induced esophageal tumorigenesis in rats. Our study suggests that RBF deficiency in the diet is a likely etiologic agent of chronic inflammation in the esophagus that contributes to ESCC development in humans. RBF supplementation in high incidence area of esophageal cancer has important clinical significance in reducing the incidence of esophageal cancer.

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Disclosure of conflict of interest

None.

Abbreviations

DAVID, the Database for Annotation, Visualization and Integrated Discovery; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ESCC, esophageal squamous cell carcinoma; EIN, esophageal intraepithelial neoplasia; Exp, experiment; GO, Gene Ontology; γH2AX, the specific phosphorylation at serine 139 of the histone H2AX; HE, hematoxylin and eosin; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HPLC, high performance liquid chromatogra-

phy; IHC, immunohistochemistry; KEGG, Kyoto Encyclopedia of Genes and Genomes; NMBA or N, N-nitrosomethylbenzylamine; RBF or R, riboflavin; wk, week.

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References

[1] Pennathur A, Gibson MK, Jobe BA and Luketich JD. Oesophageal carcinoma. Lancet 2013; 381: 400-412.

[2] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136: E359-386.

[3] Siegel R, Ma J, Zou Z and Jemal A. Cancer statistics, 2014. CA Cancer J Clin 2014; 64: 9-29.

[4] Wang JD, Ma J, Wang FY, Peng LB, Wang X, Shi SS, Ma HH, Lu ZF, Lu GM and Zhou XJ. Amplification of the telomerase RNA component gene as a new genetic marker for disease progression and prognosis in esophageal squamous cell carcinoma. Dis Esophagus 2013; 26: 737-745.

[5] Dawsey SM, Lewin KJ, Liu FS, Wang GQ and Shen Q. Esophageal morphology from Linxian, China. Squamous histologic findings in 754 patients. Cancer 1994; 73: 2027-2037.

[6] Taylor PR, Abnet CC and Dawsey SM. Squamous dysplasia--the precursor lesion for esophageal squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev 2013; 22: 540-552.

[7] Shimizu M, Nagata K, Yamaguchi H and Kita H. Squamous intraepithelial neoplasia of the esophagus: past, present, and future. J Gastroenterol 2009; 44: 103-112.

[8] Abnet CC, Arnold M and Wei WQ. Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 2018; 154: 360-373.

[9] Yang CS. Research on esophageal cancer in China: a review. Cancer Res 1980; 40: 2633-2644.

[10] Tan HZ, Lin WJ, Huang JQ, Dai M, Fu JH, Huang QH, Chen WM, Xu YL, Ye TT, Lin ZY, Lin XS, Cai JX, Dong YH, Luo HY, Chen SH, Huang YL, Yang J, Lin AX, Yuan XQ, Chen SY, Wang KS, Zhuang CY, Wang SC, Lin LL, Zou XF, Song ZH, Fang XH, Chen T, Zhang JH, Li KQ, Chen LH, Lin XP, Lin JM, Lin JN, Lin PL, Chen JT, Lin KM, Hong XC, Wang LD, Xu LY, Li EM and Zhang JJ. Updated incidence rates and risk factors of esophageal cancer in Nan'ao Island, a coastal high-risk area in southern China. Dis Esophagus 2017; 30: 1-7.

[11] Peiffer DS, Wang LS, Zimmerman NP, Ransom BW, Carmella SG, Kuo CT, Chen JH, Oshima K, Huang YW, Hecht SS and Stoner GD. Dietary consumption of black raspberries or their anthocyanin constituents alters innate immune cell trafficking in esophageal cancer. Cancer Immunol Res 2016; 4: 72-82.

[12] Taccioli C, Chen H, Jiang Y, Liu XP, Huang K, Smalley KJ, Farber JL, Croce CM and Fong LY. Dietary zinc deficiency fuels esophageal cancer development by inducing a distinct inflammatory signature. Oncogene 2012; 31: 4550-4558.

[13] Sun J, Liu J, Pan X, Quimby D, Zanesi N, Druck T, Pfeifer GP, Croce CM, Fong LY and Huebner K. Effect of zinc supplementation on N-nitrosomethylbenzylamine-induced forestomach tumor development and progression in tumor suppressor-deficient mouse strains. Carcinogenesis 2011; 32: 351-358.

[14] Stoner GD and Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. Carcinogenesis 2001; 22: 1737-1746.

[15] Xu M, Yang H, Zhang Q, Lu P, Feng Y, Geng X, Zhang L and Jia X. Alpha-tocopherol prevents esophageal squamous cell carcinoma by modulating PPARgamma-Akt signaling pathway at the early stage of carcinogenesis. Oncotarget 2017; 8: 95914-95930.

[16] Wu IC, Chen YK, Wu CC, Cheng YJ, Chen WC, Ko HJ, Liu YP, Chai CY, Lin HS, Wu DC and Wu MT. Overexpression of ATPase Na+/+ transporting alpha 1 polypeptide, ATP1A1, correlates with clinical diagnosis and progression of esophageal squamous cell carcinoma. Oncotarget 2016; 7: 85244-85258.

[17] Tatematsu K, Koide A, Morimura K, Fukushima S and Mori Y. The enhancing effect of ethanol on the mutagenic activation of N-nitrosomethylbenzylamine by cytochrome P450 2A in the rat oesophagus. Mutagenesis 2013; 28: 161-169.

[18] Rivlin RS. Riboflavin and cancer: a review. Cancer Res 1973; 33: 1977-1986.

[19] Ainiwaer J, Tuerhong A, Hasim A, Chengsong D, Liwei Z and Sheyhidin I. Association of the plasma riboflavin levels and riboflavin transporter (C20orf54) gene statuses in Kazak esophageal squamous cell carcinoma patients. Mol Biol Rep 2013; 40: 3769-3775.

[20] Li SS, Xu YW, Wu JY, Tan HZ, Wu ZY, Xue YJ, Zhang JJ, Li EM and Xu LY. Plasma riboflavin level is associated with risk, relapse, and sur-

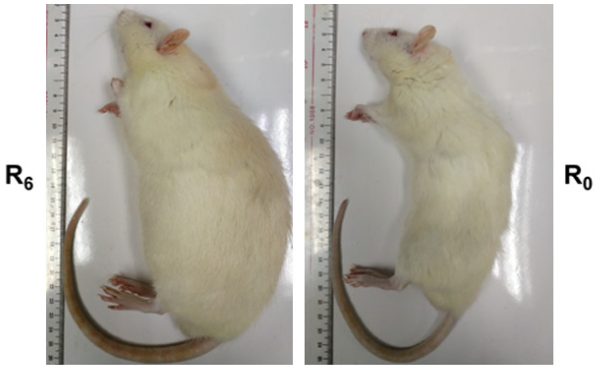
vival of esophageal squamous cell carcinoma. Nutr Cancer 2017; 69: 21-28.
[21] Long L, He JZ, Chen Y, Xu XE, Liao LD, Xie YM, Li EM and Xu LY. Riboflavin depletion promotes tumorigenesis in HEK293T and NIH3T3 cells by sustaining cell proliferation and regulating cell cycle-related gene transcription. J Nutr 2018; 148: 834-843.
[22] Tanabe R, Haraikawa M, Sogabe N, Sugimoto A, Kawamura Y, Takasugi S, Nagata M, Nakane A, Yamaguchi A, Iimura T and Goseki-Sone M. Retention of bone strength by feeding of milk and dairy products in ovariectomized rats: involvement of changes in serum levels of 1alpha, 25(OH)2D3 and FGF23. J Nutr Biochem 2013; 24: 1000-1007.
[23] Fotschki B, Juskiewicz J, Jurgonski A, Rigby N, Sojka M, Kolodziejczyk K, Mackie A and Zdunczyk Z. Raspberry pomace alters cecal microbial activity and reduces secondary bile acids in rats fed a high-fat diet. J Nutr Biochem 2017; 46: 13-20.
[24] Petteys BJ and Frank EL. Rapid determination of vitamin B(2) (riboflavin) in plasma by HPLC. Clin Chim Acta 2011; 412: 38-43.
[25] He JZ, Wu ZY, Wang SH, Ji X, Yang CX, Xu XE, Liao LD, Wu JY, Li EM, Zhang K and Xu LY. A decision tree-based combination of ezrin-interacting proteins to estimate the prognostic risk of patients with esophageal squamous cell carcinoma. Hum Pathol 2017; 66: 115-125.
[26] Da WH, Sherman BT and Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4: 44.
[27] Wei HD, Sherman BT and Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009; 37: 1.
[28] Long L, Pang XX, Lei F, Zhang JS, Wang W, Liao LD, Xu XE, He JZ, Wu JY, Wu ZY, Wang LD, Lin DC, Li EM and Xu LY. SLC52A3 expression is activated by NF-kappaB p65/Rel-B and serves as a prognostic biomarker in esophageal cancer. Cell Mol Life Sci 2018; 75: 2643-2661.
[29] Mantovani A, Allavena P, Sica A and Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-444.
[30] Geric M, Gajski G and Garaj-Vrhovac V. Gamma-H2AX as a biomarker for DNA double-strand breaks in ecotoxicology. Ecotoxicol Environ Saf 2014; 105: 13-21.
[31] Dabrowska N and Wiczkowski A. Analytics of oxidative stress markers in the early diagnosis of oxygen DNA damage. Adv Clin Exp Med 2017; 26: 155-166.
[32] Anuja K, Roy S, Ghosh C, Gupta P, Bhattacharjee S and Banerjee B. Prolonged inflammatory microenvironment is crucial for pro-neoplastic growth and genome instability: a detailed review. Inflamm Res 2017; 66: 119-128.
[33] Taylor PR, Li B, Dawsey SM, Li JY, Yang CS, Guo W and Blot WJ. Prevention of esophageal cancer: the nutrition intervention trials in Linxian, China. Linxian Nutrition Intervention Trials Study Group. Cancer Res 1994; 54: 2029s-2031s.
[34] Dawsey SM, Wang GQ, Taylor PR, Li JY, Blot WJ, Li B, Lewin KJ, Liu FS, Weinstein WM, Wiggett S, et al. Effects of vitamin/mineral supplementation on the prevalence of histological dysplasia and early cancer of the esophagus and stomach: results from the dysplasia trial in Linxian, China. Cancer Epidemiol Biomarkers Prev 1994; 3: 167-172.
[35] He Y, Ye L, Shan B, Song G, Meng F and Wang S. Effect of riboflavin-fortified salt nutrition intervention on esophageal squamous cell carcinoma in a high incidence area, China. Asian Pac J Cancer Prev 2009; 10: 619-622.
[36] van Rensburg SJ, Hall JM and du Bruyn DB. Effects of various dietary staples on esophageal carcinogenesis induced in rats by subcutaneously administered N-nitrosomethylbenzylamine. J Natl Cancer Inst 1985; 75: 561-566.
[37] van Rensburg SJ, Hall JM and Gathercole PS. Inhibition of esophageal carcinogenesis in corn-fed rats by riboflavin, nicotinic acid, selenium, molybdenum, zinc, and magnesium. Nutr Cancer 1986; 8: 163-170.
[38] Fong LY, Lin HJ and Lee CL. Methylation of DNA in target and non-target organs of the rat with methylbenzylnitrosamine and dimethylnitrosamine. Int J Cancer 1979; 23: 679-682.
[39] Bedell MA, Lewis JG, Billings KC and Swenberg JA. Cell specificity in hepatocarcinogenesis: preferential accumulation of O6-methylguanine in target cell DNA during continuous exposure to rats to 1,2-dimethylhydrazine. Cancer Res 1982; 42: 3079-3083.
[40] Doniger J, Day RS and DiPaolo JA. Quantitative assessment of the role of O6-methylguanine in the initiation of carcinogenesis by methylating agents. Proc Natl Acad Sci U S A 1985; 82: 421-425.
[41] Liston BW, Gupta A, Nines R, Carlton PS, Kresty LA, Harris GK and Stoner GD. Incidence and effects of Ha-ras codon 12 G-->A transition mutations in preneoplastic lesions induced by N-nitrosomethylbenzylamine in the rat esophagus. Mol Carcinog 2001; 32: 1-8.
[42] Wang D, Weghorst CM, Calvert RJ and Stoner GD. Mutation in the p53 tumor suppressor gene in rat esophageal papillomas induced by N-nitrosomethylbenzylamine. Carcinogenesis 1996; 17: 625-630.
[43] Wang QS, Sabourin CL, Bijur GN, Robertson FM and Stoner GD. Alterations in transforming

growth factor-alpha and epidermal growth factor receptor expression during rat esophageal tumorigenesis. Mol Carcinog 1996; 15: 144-153.
[44] Olpin SE and Bates CJ. Lipid metabolism in riboflavin-deficient rats. Br J Nutr 1982; 47: 589-596.
[45] Duerden JM and Bates CJ. Effect of riboflavin deficiency on reproductive performance and on biochemical indices of riboflavin status in the rat. Br J Nutr 1985; 53: 97-105.
[46] Mazur-Bialy AI and Pochec E. Vitamin B2 deficiency enhances the pro-inflammatory activity of adipocyte, consequences for insulin resistance and metabolic syndrome development. Life Sci 2017; 178: 9-16.
[47] Mazur-Bialy AI and Pochec E. Riboflavin reduces pro-inflammatory activation of adipocyte-macrophage co-culture. Potential application of vitamin B2 enrichment for attenuation of insulin resistance and metabolic syndrome development. Molecules 2016; 21.
[48] Liu D and Zempleni J. Low activity of LSD1 elicits a pro-inflammatory gene expression profile in riboflavin-deficient human T Lymphoma Jurkat cells. Genes Nutr 2014; 9: 422.
[49] Hayden MS and Ghosh S. Shared principles in NF-kappaB signaling. Cell 2008; 132: 344-362.
[50] Sun SC. The noncanonical NF-kappaB pathway. Immunol Rev 2012; 246: 125-140.
[51] Chen XX, Zhong Q, Liu Y, Yan SM, Chen ZH, Jin SZ, Xia TL, Li RY, Zhou AJ, Su Z, Huang YH, Huang QT, Huang LY, Zhang X, Zhao YN, Yun JP, Wu QL, Lin DX, Bai F and Zeng MS. Genomic comparison of esophageal squamous cell carcinoma and its precursor lesions by multi-region whole-exome sequencing. Nat Commun 2017; 8: 524.
[52] Liu X, Zhang M, Ying S, Zhang C, Lin R, Zheng J, Zhang G, Tian D, Guo Y, Du C, Chen Y, Chen S, Su X, Ji J, Deng W, Li X, Qiu S, Yan R, Xu Z, Wang Y, Guo Y, Cui J, Zhuang S, Yu H, Zheng Q, Marom M, Sheng S, Zhang G, Hu S, Li R and Su M. Genetic alterations in esophageal tissues from squamous dysplasia to carcinoma. Gastroenterology 2017; 153: 166-177.
[53] Lin R, Zhang C, Zheng J, Tian D, Lei Z, Chen D, Xu Z and Su M. Chronic inflammation-associated genomic instability paves the way for human esophageal carcinogenesis. Oncotarget 2016; 7: 24564-24571.

Supplementary Table 1. Primer sequences for qRT-PCR

Primer Name	Sequence (5' to 3')
Tp63-qF	TGTGTCGGAGGAATGAACCG
Tp63-qR	CTTCGTACCATCGCCGTTCT
Rab10-qF	ACAAGTGTGACATGGACGACA
Rab10-qR	TACAGGGGTCTTTCGGAGGAT
Grhl2-qF	GATGATGAGCGAGAAGGCAGCA
Grhl2-qR	GCGATTTCAGCATCAGAGCATC
Aloxe3-qF	GGTGAACACCACCTGTAGCA
Aloxe3-qR	CGTGCCCTGATGTCCTTTGA
S100a8-qF	GGAATCACCATGCCCTCTACA
S100a8-qR	CTGTCTTTATGAGCTGCCACG
S100a9-qF	ACACCCTGAACAAGGCGGAA
S100a9-qR	CTGGTTTGTGTCCAGGTCCTC
Tlr2-qF	TCTTAGGCGCCCTGTGTTAC
Tlr2-qR	TCCTGCTCGCTGTAGGAAAC
Jun-qF	GAAAGCGCAAAACTCCGAGC
Jun-qR	TGCGTTAGCATGAGTTGGCA
Il1a-qF	ACTCAGCTCTTTGTGAGTGCT
Il1a-qR	TGAGGTCGGTCTCACTACCT

A



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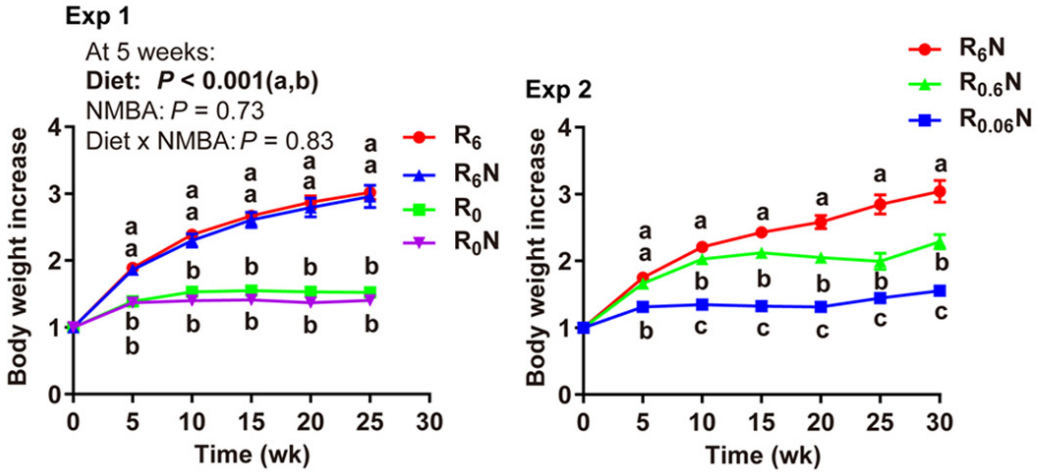
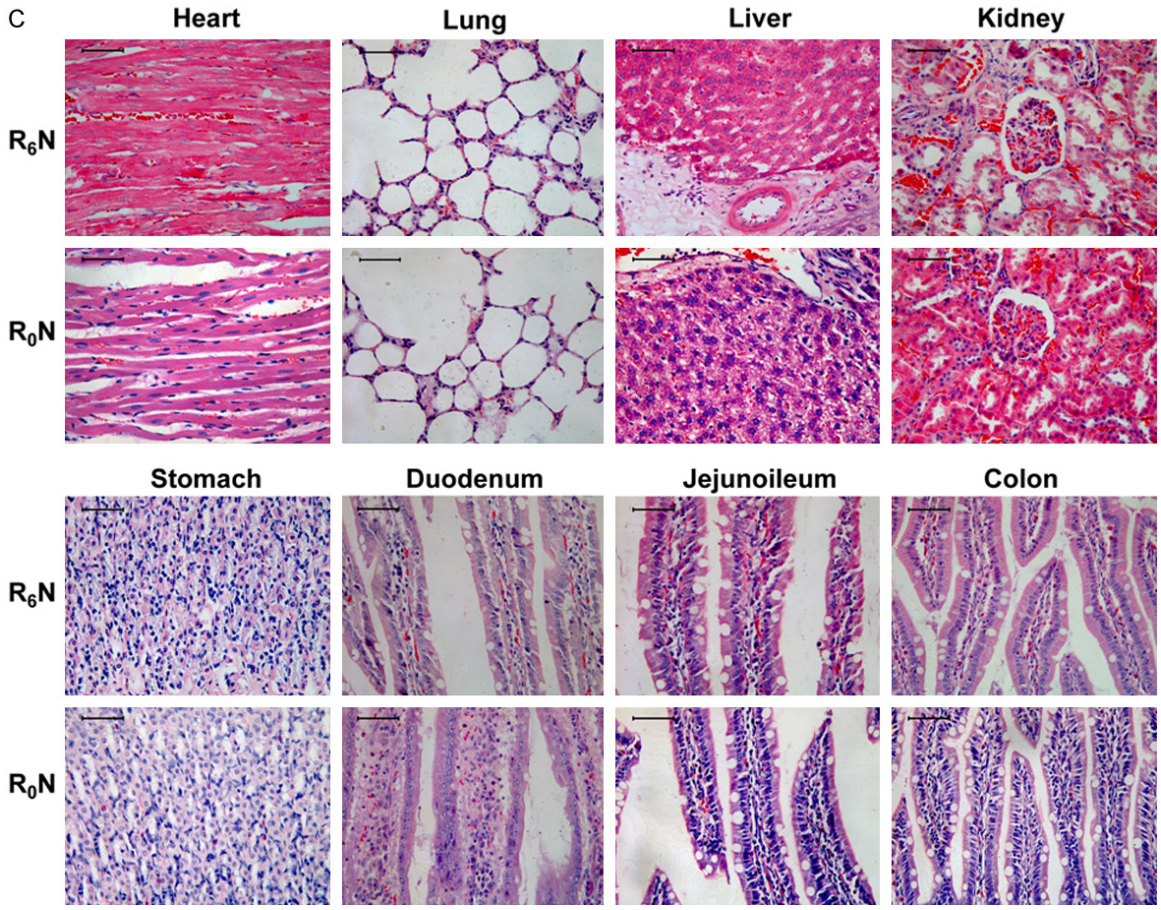
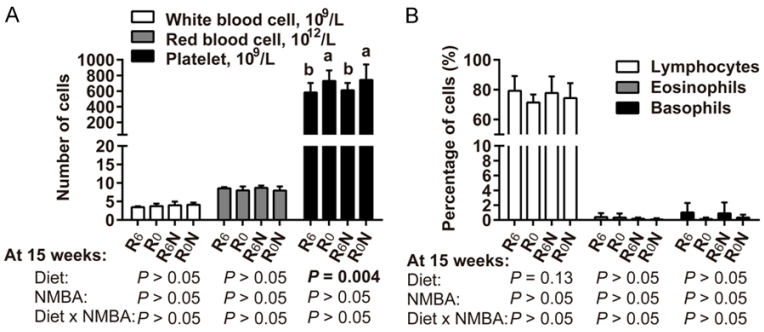




Supplementary Figure 1. Effect of RBF deficiency combined with NMBA on body weight and histopathological changes of organs in rats. A. Representative pictures of rat body weight. B. Rat body weight increase. Exp 1, *n* = 15-18/group; Exp 2, *n* = 12-19/group. Values are means ± SD. Exp 1 different letters (a and b for diet effect) are significantly different by two-way ANOVA and LSD test ($P < 0.05$). Exp 2 different letters (a, b and c) are significantly different from each other by one-way ANOVA and LSD test ($P < 0.05$). C. HE staining of rat heart, lung, liver, kidney, stomach, duodenum, jejunoileum, and colon. Scale bars, 50 μm.



Supplementary Figure 2. Effect of RBF deficiency combined with NMBA on the total number and percentage of white blood cells. A. Number of various cells in peripheral blood. B. The percentage of lymphocytes, eosinophils, and basophils in the total white blood cells, respectively. *n* = 15-18/group. Values are means ± SD. Different letters (a and b for diet effect) are significantly different by two-way ANOVA and LSD test ($P < 0.05$).