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**Antibodies and reagents.** The following antibodies and reagents were used: anti-mouse CD3 and anti-mouse CD28 antibodies (BD Biosciences); Mercury TransFactor ELISA kit; biotinylated anti-IL-4 mAb, anti-IL-13 pAb, mouse IL-4, mouse eotaxin, human IL-4, and IL-13 ELISA Kits (R&D Systems); anti-NF- $\kappa$ B p65 and anti-NF- $\kappa$ B p50 polyclonal antibodies and rabbit anti-Nrf2 polyclonal antibody (Santa Cruz Biotechnology, Inc.); rabbit anti-rat IgG-HRP conjugate (DakoCytomation); BIOXYTECH GSH/GSSG-412 kit (Oxis International Inc.); diaminobenzidine (Vector Laboratories); Diff-Quick reagent (Baxter Dade); complete protease inhibitor cocktail tablets (Roche); SuperScribe II reverse transcriptase, RNeasy mini kits, TOPO 2.1, KpnI, SacI, and NotI restriction endonucleases (Invitrogen); assay on-demand kits, fluorogenic probes, and TaqMan universal PCR master mix (Applied Biosystems); consensus sequence for the octamer transcription factor 1 (OCT1), PGL3 basic reporter construct, and Dual-Luciferase Reporter Assay system (Promega); acetylcholine, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), BSA, FCS, ketamine, OVA, pepsin, normal rabbit serum, normal rabbit IgG, sodium pentobarbital, succinylcholine, xylazine, N-acetyl L-cysteine, collagenase IV, and bovine pancreatic DNase I (Sigma-Aldrich); PMA and A23187 (Calbiochem); ECL chemiluminescence detection kit (GE Healthcare); PVDF membrane (Bio-Rad Laboratories); red cell lysis buffer (eBioscience); CD4 $^{+}$  T cell isolation kit (Miltenyi Biotec); Cell stainer (Costar); and anti-lamin B1 antibody (Zymed Laboratories).

**GSH and GSSG analysis.** The concentrations of GSH and GSSG in the lung tissues were measured using a BIOXYTECH GSH/GSSG-412 kit. To measure GSSG, 10 mg of lung tissue was homogenized with a 300- $\mu$ l solution containing 10  $\mu$ l 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate and 290  $\mu$ l of 5% cold metaphosphoric acid and centrifuged for 10 min at 1,000 g. The supernatant was diluted (1:15) with GSSG buffer. 200  $\mu$ l of the diluted supernatant was mixed with an equal volume of chromogen and GSH reductase enzyme solution and incubated at room temperature for 5 min. To this, 200  $\mu$ l NADPH was added and the change in absorbance was recorded at 412 nM for 3 min. To measure GSH, 10 mg of the lung tissue was homogenized with 350  $\mu$ l of 5% cold metaphosphoric acid solution and centrifuged for 5 min at 1,000 g. The remaining procedure was similar to the one described for measuring GSSG. Different concentrations of GSSG were used as the standard.

**Isolation of CD4 $^{+}$  T cells and macrophages from the lungs.** To isolate lung CD4 $^{+}$  T cells, mice were killed, and the pulmonary cavities were opened. The blood circulatory system in the lungs was cleared by perfusion through the right ventricle with 3 ml of saline containing 50 U of heparin/ml. Lungs were aseptically removed and cut into small pieces in cold PBS. The dissected tissue was then incubated in PBS containing 150 U/ml collagenase IV and 50 U/ml bovine pancreatic DNase I for 1 h at 37°C. The digested lungs were further disrupted by gently pushing the tissue through a nylon screen. The single-cell suspension was then washed and centrifuged at 500 g for 5 min. The pellet was resuspended in PBS and passed through a cell stainer to remove the coagulated proteins and centrifuged for 5 min at 500 g. To lyse the contaminating RBCs, the cell pellet was incubated for 5 min at room with red cell lysis buffer. Cells were then washed with PBS containing 2% FBS and counted.

CD4 $^{+}$  T cells were isolated by negative selection using CD4 $^{+}$  T cell isolation kit. 10 $^{7}$  cells isolated from the lungs were first incubated with a biotin-antibody cocktail containing anti-CD8  $\alpha$ , anti-CD11b, anti-CD45R, anti-DX5, and anti-Ter119 for 10 min, and then with antibiotin microbeads for 15 min at 4°C. The cells were then washed with 20 volumes of buffer and passed through MACS MS column. The magnetically labeled non-CD4 $^{+}$  T cells were depleted by retaining them on MACS MS column, while the eluents containing the unlabeled CD4 $^{+}$  T cells were collected. An aliquot of cells was analyzed by immunofluorescence and flow cytometry using anti-CD4 antibodies. After gating on scatter characteristics to exclude dead cells and debris, the purity of cells was 90–92% CD4 $^{+}$  T lymphocytes. RNA was isolated from the purified CD4 $^{+}$  T cells using RNeasy mini columns.

Alveolar macrophages were obtained from the OVA-challenged (24 h after first OVA challenge) *Nrf2* $^{+/+}$  and *Nrf2* $^{-/-}$  mice ( $n = 15$ ) by saline lavage (3  $\times$  1 ml). The BAL fluid collected from each mice group was pooled separately and centrifuged at 500 g for 5 min at 4°C. The cell pellets were suspended in RPMI 1640 medium and cultured in 6-well plate for 2 h in CO<sub>2</sub> incubator (1). The nonadherent cells were removed with the supernatant. The wells were washed two times with sterile PBS. The adherent macrophages were then lysed with RLT buffer and the RNA was isolated using RNeasy mini columns. Real-time RT-PCR was used to determine the expression of three well-characterized *Nrf2*-regulated genes (*GCLm*, *GCLc*, and *HO-1*) in the isolated CD4 $^{+}$  T cells and macrophages by following the procedure described in the previous paragraph. The fold change was obtained by comparing the message level of antioxidant genes in the CD4 $^{+}$  T and macrophages of wild-type mice over their levels in the knockout counterparts.

The expression of *Nrf2* mRNA in the lung CD4 $^{+}$  T cells and macrophages was determined by RT-PCR using the mouse *Nrf2* 5'-TCTCCTCGCTG-GAAAAAGAA-3' and 3'-AATGTGCTGGCTGTGCTTTA-5' primers. Total RNA (500 ng) was reverse transcribed into cDNA in a volume of 50  $\mu$ l, containing 1  $\times$  PCR buffer (50 mM KCl and 10 mM Tris, pH 8.3), 5 mM MgCl<sub>2</sub>, 1 mM each dNTPs, 125 ng oligo (dT)<sub>15</sub>, and 50 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies) at 45°C for 15 min and 95°C for 5 min using gene amp PCR System 9700 (Perkin Elmer Applied Biosystems). Separate but simultaneous PCR amplifications were performed with aliquots of 1  $\mu$ l cDNA at a final concentration of 1  $\times$  PCR buffer, 4 mM MgCl<sub>2</sub>, 400  $\mu$ M dNTPs, and 1.25 U *Taq* Polymerase (Life Technologies) in a total volume of 50  $\mu$ l using 240 nM each of forward and reverse primers.

**Assay of T lymphocyte activation.** Spleens were aseptically removed from OVA-challenged (48 h after second challenge) *Nrf2* $^{+/+}$  and *Nrf2* $^{-/-}$  mice and mechanically dissociated in cold PBS, followed by depletion of erythrocytes with lysis buffer containing NH<sub>4</sub>Cl. Splenocytes were suspended in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM Hepes, and 20  $\mu$ M 2-ME. 10 $^{6}$  splenocytes/ml were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere and stimulated for 24 h with 5  $\mu$ g/ml OVA or anti-mouse CD3 plus 0.5  $\mu$ g/ml anti-mouse CD28 antibodies. After 24 h of incubation, cell-free culture supernatants were collected and stored at -70°C until cytokine analyses were performed.

To determine whether *Nrf2* played a T cell-intrinsic role in regulating Th2 cytokine gene expression, we isolated CD4 $^{+}$  T cells by negative immuno-magnetic selection from single cell spleen suspensions of control wild-type and *Nrf2* $^{-/-}$  mice. Equal numbers of viable cells (10 $^{6}$ /ml) were incubated for 24 h in complete medium alone or stimulated with 2  $\mu$ g/ml of plate bound anti-CD3 plus 2  $\mu$ g/ml of soluble anti-CD28 or 1  $\mu$ M calcium ionophore A23187 plus 20 ng/ml PMA. Cell supernatants were collected and analyzed for IL-4 or IL-13 secretion by ELISA.

**Construction of *Nrf2* expression vector and IL-4 and IL-13 promoter constructs.** An *Nrf2*-overexpressing construct was made with the ubiquitin C promoter. *Nrf2* cDNA lacking a stop codon was cloned in TOPO 2.1 vector and sequenced. The *Nrf2*-TOPO construct was digested with KpnI and NotI

to release the *Nrf2* cDNA. The cDNA was purified and ligated with pUB6/V5-His vector digested with KpnI and NotI. The recombinant clones were further screened and confirmed by sequencing. To test whether Nrf2 is able to bind to ARE and activate luciferase activity, the Nrf2 construct was transfected into Hepa cells stably transfected with *HO-1* ARE. Luciferase activity was measured after 36 h. For the IL-4 and IL-13 promoter constructs, human genomic DNA was used as a template with PCR primers designed to amplify sequences 270 and 312 bp upstream, respectively, and 65 bp downstream of the transcription start sites. PCR primers contained restriction sites for KpnI and SacI to facilitate subsequent ligation. After sequencing to ensure accurate replication, PCR products were ligated into the KpnI and SacI sites of the luciferase-based reporter construct pGL3 Basic.

**Transfection in Jurkat cell line.** To test the possibility that Nrf2 might act as a transcriptional repressor of Th2 cytokines, we first electroporated the Jurkat T cell line ( $20 \times 10^6$  cells/0.5 ml of OPT-MEMI) with *Nrf2*-overexpressing vector (20  $\mu\text{g}/20 \times 10^6$  cells) or pUB6 control vector (20  $\mu\text{g}/20 \times 10^6$  cells) using a BioRad electroporator (at 300V and 1050 capacity) and analyzed effects of Nrf2 overexpression on endogenous IL-13 gene expression. The cells were then mixed with OPT-MEMI ( $2 \times 10^6$  cells/2 ml/ well of 6-well plates) and incubated for 4 h at 37°C in a CO<sub>2</sub> incubator. 10% FBS (final concentration) was added to each well and incubated for 14 h. Cells were centrifuged, resuspended in 10<sup>6</sup> cells/ml OPTI-MEMI with or without 0.5  $\mu\text{g}/\text{ml}$  of the calcium ionophore A23187 (final) and 10 ng/ml PMA (final), and cultured at 37°C for 18 h in a CO<sub>2</sub> incubator. The cultures were centrifuged at 500 g for 5 min at 4°C. The supernatants were collected and IL-4 and IL-13 cytokines were assayed using the human Quantikine ELISA kits. The Jurkat T cells used in these experiments do not secrete abundant IL-4 protein because of poorly understood posttranscriptional defects. To ensure that Nrf2 was overexpressed and activate downstream target genes, cell pellets were homogenized with RLT buffer and the RNA was isolated using the RNeasy mini columns. The levels of *Nrf2* and the classical *Nrf2*-regulated genes *NQO1* and *GCLm* mRNA were analyzed using real time RT-PCR using the assay on demand kits containing the respective primers for human *Nrf2*, *GCLc*, and *NQO1* genes.

To test the possibility that Nrf2 was acting to repress Th2 cytokine gene transcription, Nrf2 or empty expression vectors were cotransfected into Jurkat T cells together with reporter constructs containing the human IL-4 or IL-13 promoters driving the firefly luciferase gene. Cells were transfected and stimulated as above although in a scaled down version ( $5 \times 10^6$  cells, 5  $\mu\text{g}$  reporter construct, up to 5  $\mu\text{g}$  expression vector or control). Both approaches yielded similar transfection efficiencies (unpublished data). 18 h after transfection, cells were lysed and firefly luciferase gene expression was analyzed by luminometry using a Monolight 3010 Luminometer and assay buffers according to the manufacturer's instructions (Promega).