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Nrf2 Suppresses Allergic Lung Inflammation by Attenuating the Type 2 Innate Lymphoid Cell Response

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The Keap1–Nrf2 system plays a pivotal role in the oxidative stress response by inducing a number of cytoprotective genes. Under stress, damaged epithelial cells release cytokines that activate type 2 innate lymphoid cells (ILC2s), which mediate the allergic immune response. In this article, we investigated the role of the Keap1–Nrf2 pathway in ILC2 homeostasis and allergic inflammation using Nrf2 knockout mice. ILC2s from Nrf2-deficient mice showed a transient, upregulated IL-33 response and underwent hyperproliferation in response to a combined stimulation of IL-33 with IL-2, IL-7, or TSLP. This enhanced proliferation was correlated with an increased activation of downstream signals, including JAK1, Akt, and Erk1/2. In contrast, activating Nrf2 with a chemical inducer (CDDO-Im) decreased the viability of the wild-type but not of the Nrf2-deficient ILC2s. This effect on viability resembled that exerted by the corticosteroid dexamethasone; however, unlike the latter, the Nrf2-dependent cell death was mediated by neither caspase 3–dependent apoptosis nor necroptosis. Using a mouse intratracheal IL-33 administration allergy model, we found that the activation of Nrf2 by CDDO-Im in vivo decreased the number of pulmonary ILC2s and eosinophils. These findings indicated that Nrf2 is an important regulator of the allergic response by determining the survival and death of ILC2s, and these findings suggest that Nrf2 activation is a potential therapeutic strategy for steroid-resistant allergy alleviation. *The Journal of Immunology*, 2019, 202: 1331–1339.

Type 2 innate lymphoid cells (ILC2s) are a major source of the type 2 signature cytokines IL-5 and IL-13, which drive allergic inflammation. IL-5 released from ILC2 promotes eosinophil accumulation, and IL-13 stimulates airway goblet cells to vigorously secrete mucous (1–4). ILC2s residing in the lung have beneficial host–defense roles, including eradicating parasitic helminth infections and reversing the airway epithelial barrier damage after influenza virus infection (5–8). Allergen exposure and the subsequent allergic inflammation cause ILC2 recruitment, and the chronic or dysregulated activation of these cells is implicated in pulmonary disease, such as asthma and chronic obstructive pulmonary disease (9). ILC2s do not express Ag receptors but do possess receptors for epithelial cell–derived cytokines, including IL-25, IL-33, and thymic stromal lymphoprotein (TSLP). Accordingly, these cytokines can initiate the activation of ILC2s, and two lymphopoietic cytokines, IL-2 and IL-7, further augment their proliferation. Activated ILC2s can be at least partially attenuated by corticosteroids, and dexamethasone

induces their apoptotic cell death (10, 11). However, little is known about how ILC2 homeostasis is maintained.

Oxidative stress results from an imbalance between oxidative and reductive status and is induced by a variety of insults associated with inflammation and allergy. A broad spectrum of genes involved in oxidative defense mechanisms share an antioxidant-responsive element in their regulatory region. NF erythroid 2–related factor 2 (Nrf2) is a major transcription factor that recognizes antioxidant-responsive element and regulates antioxidant or antielectrophiles gene, including *GST*, NAD(P)H:quinone oxidoreductase 1 (Nqo-1), and hemeoxygenase-1 (*HO-1*) (12, 13). At a steady-state, Nrf2 is kept sequestered in the cytoplasm by its inhibitor Kelch-like ECH-associated protein 1 (Keap-1), but it translocates to the nucleus upon sensing oxidative stress, where it transactivates a number of genes. Allergen-induced asthma is tightly associated with oxidative stress; notably, Nrf2-deficient mice exhibit exacerbated asthma as a result of increased type 2 cytokine secretion in the airways, and the pharmacological activation of Nrf2 alleviates

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; BM, bone marrow; Fer-1, ferrostatin-1; HO-1, hemeoxygenase-1; ILC2, type 2 innate lymphoid cell; KO, knockout; Nec-1, necrostatin-1; NH, natural helper; Nqo-1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF erythroid 2–related factor 2; PAS, periodic acid–Schiff; ROS, reactive oxygen species; TSLP, thymic stromal lymphoprotein; WT, wild-type.

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OVA-induced allergic asthma (14, 15). However, the contribution of Nrf2 to ILC2 homeostasis remains largely unknown.

In this study, we investigated the role of Nrf2 in allergic lung inflammation in terms of ILC2s in mice. Nrf2 activation decreased the number of pulmonary ILC2s both in vitro and in vivo, leading to an attenuation of allergic lung inflammation. Nrf2 activation induced ILC2 cell death without affecting the apoptosis-related caspases or the survival signals mediated by Akt/Erk. We conclude that Nrf2 activation is a potential therapeutic strategy for overcoming the allergic inflammation mediated by ILC2s.

Materials and Methods

Mice

All mice were bred in-house and maintained under specific pathogen-free conditions at the Miyagi Cancer Center Research Institute mouse facility. The mice used for experiments were 8–10-wk-old female C57BL/6N (wild-type [WT]) mice purchased from CLEA Japan (Tokyo, Japan) and previously developed Nrf2 knockout (KO) mice (16). All animal procedures were performed in accordance with protocols approved by the Miyagi Cancer Center Animal Committee.

Reagents

Recombinant murine cytokines (IL-2, IL-25, IL-33, TSLP) were purchased from BioLegend (San Diego, CA) or R&D Systems (Minneapolis, MN). CDDO-Im (Tocris Bioscience, Bristol, U.K.), 15d-PGJ₂ (Cayman Chemical, Ann Arbor, MI), dexamethasone (Wako Chemicals, Osaka, Japan), Z-VAD-FMK (Medical & Biological Laboratories, Tokyo, Japan), necrostatin-1 (Nec-1) (Sigma-Aldrich, St. Louis, MO), Necrox-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and ferrostatin-1 (Fer-1) (Sigma-Aldrich) were purchased.

In vivo induction of ILC2

Mice were anesthetized using a mixture of three anesthetic agents (medetomidine, midazolam, and butorphanol) and challenged intratracheally with recombinant mouse IL-33 (1 µg) in 50 µl of PBS administered through an intubated 24-gauge plastic cannula. *Alternaria alternata* extracts (10 µg; Stallergenes Greer, Lenoir, NC) in 50 µl of PBS was intratracheally administered. CDDO-Im was administered i.p. at 20 µmol/kg on two consecutive days before analysis. For ILC2 sorting, recombinant mouse IL-25 and IL-33 (each 0.5 µg/mouse) were administered i.p. for four consecutive days. The mice were sacrificed 24 h after the last challenge. The lung lobes and bone marrow (BM) were used for experiments.

Lung digestion

The excised bilateral lung lobes were minced by surgical scissors and digested in RPMI 1640 containing 10% heat-inactivated FBS, 1 mg/ml Collagenase/Dispase (Sigma-Aldrich), and 50 µg/ml DNase I (Sigma-Aldrich) at 37°C with gentle mixing for 45 min. The dissociated lung cell suspensions were filtered through 70-µm nylon mesh, washed with PBS, and treated with a RBC lysis solution before further analyses. For ILC2 sorting, the excised bilateral lung lobes from $n = 3$ –5 mice per group were pooled.

Flow cytometry and cell sorting

The monoclonal anti-murine fluorochrome-conjugated Abs used were from BioLegend, including a lineage mixture (anti-CD3e, anti-Ly-6G/Ly-6C [Gr-1], anti-CD11b, anti-CD45R [B220], and anti-Ter-119 Abs), and anti-CD278 (ICOS), anti-ST2 (IL-33Rα), anti-CD127 (IL-7Rα), and anti-CD25 (IL-2Rα). The anti-Siglec-F Ab was from BD Pharmingen (San Diego, CA). Dead cells were excluded with 7-aminoactinomycin D (7-AAD) (Calbiochem, San Diego, CA) and Zombie NIR (BioLegend). Nonspecific Ab binding was blocked with an anti-CD16/32 Ab (BioLegend). To detect phosphorylated STAT1 or STAT5, ILC2s were fixed, permeabilized, and stained with anti-phospho-STAT1 (Ser727) (BioLegend) or anti-phospho-STAT5 (Tyr694) Abs (Thermo Fisher Scientific, Waltham, MA). Data were acquired with a FACSCanto II flow cytometer (BD Biosciences) or a SA3800 cell analyzer (Sony) and analyzed using FlowJo software (TreeStar, Ashland, OR). CountBright Absolute Counting Beads (Thermo Fisher Scientific) were used to determine ILC2 and eosinophil cell numbers. To sort ILC2s from lung cell suspensions, lineage-negative cells were first enriched using a MACS-Lineage Cell Depletion Kit (Miltenyi Biotec, Germany) and then stained with a lineage mixture, including anti-CD25, anti-CD278 (ICOS), and anti-IL-33Rα (IL-1RL1, ST2) Abs. Cells were sorted using a FACSARIA II

(BD Biosciences, San Jose, CA), and lung ILC2s were determined as lineage negative, ICOS⁺, CD25⁺, and IL-33Rα⁺ cells in this study. In all experiments, the monitored ILC2 purity was >95%. Typical postsorting yields of at least 1×10^5 cells per group were used for further experiments.

Lung histology

Mouse whole lung specimens were fixed by 10% formalin for longer than 24 h and embedded in paraffin. Lung slices (3 µm) were stained with H&E or with a periodic acid–Schiff (PAS) staining kit (Muto Pure Chemicals, Tokyo, Japan).

ILC2 culture in vitro

Sorted lung ILC2s were cultured (37°C, 5% CO₂) at an initial density of 3000 cells/well (96-well round-bottom plates) in RPMI 1640 supplemented with 10% heat-inactivated FBS (Corning, Corning, NY) and penicillin/streptomycin/10 mM HEPES/1 mM sodium pyruvate/1×non-essential amino acids (Wako Chemicals). The cells were stimulated with either rIL-33 or with a combination of IL-2/IL-33 or TSLP/IL-33 at 10 ng/ml each (BioLegend). The cells were harvested on day 3, 6, or 9 of culture and then counted by the trypan blue staining method using a Luna II Automated Cell Counter (Logos Biosystems). To evaluate proliferative responses, purified ILC2s were labeled with 5 µM CFSE (Thermo Fisher Scientific) for 15 min, and then 5×10^5 of these cells were stimulated with a combination of IL-2/IL-33 for 3 d. The Thy-1.2⁺ cells were then analyzed for CFSE staining. To assay apoptosis, lung ILC2s directly sorted into 96-well round-bottom plates at a density of 2000 cells per well were stimulated with IL-2/IL-33 or TSLP/IL-33 (each at 10 ng/ml). On day 4, CDDO-Im, dexamethasone, or 15d-PGJ₂ was added to each well at various concentrations, and on day 6, the cells were stained with FITC–Annexin V (BioLegend) and 7-AAD in the Annexin V-binding buffer (BioLegend). Cell death was determined by a FACSCanto II flow cytometer (BD Biosciences) or an SA3800 cell analyzer (Sony).

Western blotting

Cytoplasmic protein fractions were extracted from sorted lung ILC2s and resolved by SDS-PAGE. Western blotting was performed with the following primary Abs against phospho-p38 MAPK (Thr180/Tyr182): p38 MAPK, phospho-NF-κB p65 (Ser536), NF-κB p65, phospho-Erk1/2 (Thr202/Tyr204), Erk1/2, phospho-Akt (Ser473), Akt, phospho-JAK1 (Tyr1022/1023), JAK1, Bcl-xL, Caspase-3, (Cell Signaling Technology, Danvers, MA), Bcl-2, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear HDAC2 and Nrf2 were analyzed using anti-HDAC2 (Santa Cruz Biotechnology) and anti-Nrf2 (Cell Signaling Technology) Abs, respectively. Each protein was detected by the appropriate HRP-conjugated secondary Abs and exposed by LAS-4000 mini (Fujifilm Corporation, Tokyo, Japan).

ELISA

Cell culture supernatants from in vitro ILC2 cultures or bronchoalveolar lavage fluid from asthmatic WT or Nrf2-KO mice were collected, and IL-5 or IL-13 was measured using ELISA kits (eBioscience) according to the manufacturer's instructions.

Real-time PCR

RNA was extracted from sorted lung ILC2s or from dissociated lung cells using an RNeasy Micro or RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The eluted RNA was reverse transcribed using the Primescript II First Strand cDNA Synthesis Kit (Takara, Shiga, Japan). The primers used for SYBR Green PCR were *Nqo-1*: 5'-AGCGTTCGGTATTACGATCC-3', 5'-AGTACAATC-AGGGCTCTTCTCG-3'; *HMOX-1*: 5'-CCTGAACCTTTGAAACCAG-CAG-3', 5'-TGCTTTTACAGGCCAGTTTGTG-3'; and *Hprt-1*: 5'-TCCTCCTCAGACCGCTTTT-3', 5'-CCTGGTTCATCATCGCTAATC-3'.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Statistical significance was assessed by Student *t* test, and $p < 0.05$ was considered to be significantly different.

Results

Nrf2 suppresses ILC2 proliferation in response to IL-33, either alone or in combination with IL-2

IL-33 signaling is a prerequisite for ILC2 activation in the peripheral tissues in vivo, and together with auxiliary help from IL-25, it promotes ILC2 development from the common lymphoid progenitors (17, 18). We hypothesized that Nrf2 contributes to

allergic inflammation by activating ILC2s. To examine this possibility, we first induced ILC2s *in vivo* in WT and Nrf2-KO mice by the i.p. administration of IL-25/IL-33 for four consecutive days (Fig. 1A). The lung ILC2s in this study were defined as viable cells that were lineage-negative ICOS⁺ CD25⁺ IL-33Rα⁺ (Supplemental Fig. 1A) (2, 3). ILC2s from WT and Nrf2-KO mice had similar frequencies among the lineage-negative cells in the lung, and the expression level of four typical markers (ICOS, CD25, CD127, and IL-33Rα) did not show any significant difference between the two mouse lines (Supplemental Fig. 1B, 1C). We next characterized the proliferation of lung ILC2s. Pulmonary ILC2 activation is initiated by IL-33, but the ILC2 proliferation is largely affected by IL-2, which binds to the common γ-chain receptor (2, 4, 19). We investigated whether the ILC2s' proliferative response to IL-33 in the presence or absence of IL-2 differed between the WT and Nrf2-KO mice. On day 3 after the IL-33 stimulation alone, WT ILC2s formed small clusters, suggesting a marginal proliferative response, whereas the Nrf2-KO cells formed larger clusters (Fig. 1B). This enhanced proliferation of Nrf2-KO compared with WT ILC2s was also observed after the IL-2/IL-33 combination stimulation, after which the Nrf2-KO ILC2s again formed larger clusters than the WT ILC2s. These microscopic observations were confirmed by cell counting, which showed a modest and transient increase in Nrf2-KO ILC2 numbers in response to IL-33 on day 3 (Fig. 1C), whereas no apparent proliferation was seen for ILC2s stimulated with IL-25 (data not shown). Stimulation with IL-2 and IL-33 in combination induced a robust increase in ILC2s, and the cell numbers of Nrf2-KO ILC2s on day 3 and day 6 were significantly higher than those of WT ILC2s. To further evaluate the proliferation potential, we labeled ILC2s with CFSE and stimulated them for 3 d. The stimulation potency of the IL-2/IL-33 combination was stronger in Nrf2-KO than WT ILC2s (WT mean fluorescence intensity = 1356, Nrf2-KO mean fluorescence intensity = 1164, Fig. 1D). We next investigated whether Nrf2 affected the ILC2-derived Th2 cytokine production *in vitro*. In response to IL-33 alone, small but significantly increased IL-5 and IL-13 secretions were observed in the Nrf2-KO compared with WT ILC2s (Fig. 1E). Combinatorial stimulation with IL-2/IL-33 induced secretion of significant amounts of Th2 cytokines, with the Nrf2-KO ILC2s showing a modest but significant increase in IL-13 release compared with WT ILC2s, without affecting the IL-5 production. Under these stimulation conditions, the cell viability did not differ between ILC2s from WT and Nrf2-KO mice (Fig. 1F). These results indicated that Nrf2-deficient ILC2s have an enhanced proliferation capability in response to IL-33 and IL-2, suggesting that Nrf2 has a suppressive role on ILC2 proliferation *in vitro*.

Nrf2 activation decreases the ILC2 cell numbers in vitro

The finding that Nrf2-KO ILC2s showed enhanced proliferation compared with WT ILC2s led us to hypothesize that Nrf2 suppresses their proliferation. To test this possibility, we used a novel Nrf2 activator, CDDO-Im. Sorted lung ILC2s cultured in the presence of IL-2/IL-33 for 3 d were further treated with CDDO-Im. Forty-eight hours after 10 nM CDDO-Im treatment, two Nrf2 target genes, *Nqo-1* and *HO-1*, were highly induced in the WT ILC2s (Fig. 2A), although these transcriptional activations were completely absent from the Nrf2-KO ILC2s. Under the same experimental conditions, CDDO-Im treatment decreased the live ILC2 frequency by ~14% in WT mice (DMSO: 63.4 ± 0.97 versus CDDO-Im: 54.4 ± 3.56), whereas it decreased the live ILC2 frequency in Nrf2-KO mice by only ~6% (DMSO: 59.9 ± 0.20 versus CDDO-Im: 56.4 ± 1.52) (Fig. 2B). Accordingly, 10 nM CDDO-Im treatment strongly decreased the live ILC2 numbers in

WT mice, without affecting those of Nrf2-KO mice (Fig. 2C). We also found that the number of Nrf2-KO ILC2s decreased in the presence of 100 nM CDDO-Im, indicating that this concentration was toxic to the cells. To investigate whether IL-2/IL-33 stimulation induced reactive oxygen species (ROS) production, we measured the intracellular ROS level in WT and Nrf2-KO ILC2s. Rather unexpectedly, intracellular ROS were not detected under the IL-2/IL-33 stimulation condition (Fig. 2D). Treating the cells with NAC, a potent ROS scavenger, showed that neither WT nor Nrf2-KO ILC2s were decreased by the ROS inhibition (Fig. 2E). To assess whether the cell death of ILC2s from other tissues was also regulated by Nrf2, we examined BM ILC2s and found that they were also susceptible to CDDO-Im, albeit with less sensitivity than the lung ILC2s (Fig. 2F). These results indicated that Nrf2 activation reduced the lung ILC2 numbers *in vitro* by a mechanism that was independent of ROS accumulation.

Nrf2-mediated ILC2 reduction is mediated neither by apoptosis nor by the attenuation of Akt/Erk-dependent survival

We next investigated whether Nrf2 activation increased the death of ILC2s. Dexamethasone is a corticosteroid that is highly effective for treating asthma and is cytotoxic to ILC2s; however, stimulating ILC2s with IL-2/IL-33 or TSLP/IL-33 induces a resistance to this drug. To examine whether Nrf2 activation could overcome this steroid tolerance of ILC2s, cells preincubated with IL-2/IL-33 or TSLP/IL-33 were further exposed to CDDO-Im or dexamethasone. Unexpectedly, the live WT and Nrf2-KO ILC2s pretreated with IL-2/IL-33 were less sensitive to dexamethasone in terms of frequency reduction than were the same cells treated with TSLP/IL-33 (Fig. 3A). However, the numbers of live ILC2s from both WT and Nrf2-KO mice were significantly reduced by dexamethasone treatment irrespective of IL-2/IL-33 or TSLP/IL-33 pretreatment (Fig. 3B). In addition, CDDO-Im treatment in the presence of Nrf2 reduced the frequency and numbers of live ILC2s to levels similar to those seen with dexamethasone treatment (Fig. 3A, 3B). To further elucidate the mechanism of the ILC2 reduction mediated by CDDO-Im compared with that by dexamethasone, we treated ILC2s with either agent for 6 h and then assayed them for apoptosis- and survival-related molecules by Western blot. First, we confirmed that Nrf2 accumulated in the nucleus of WT ILC2s treated with CDDO-Im for 6 h (Supplemental Fig. 2B). Cleaved caspase-3, a key executor of apoptosis, was positive in the dexamethasone-treated ILC2s but not in the CDDO-Im-treated ones (Fig. 3C, Supplemental Fig. 2A). To assess the prosurvival/proliferation signaling pathways of the ILC2s, JAK1, Akt, and Erk1/2 were examined. ILC2s from Nrf2-KO mice showed a higher basal activation of all the three kinases (Fig. 3D). However, none of these activations were affected by the CDDO-Im treatment in WT or Nrf2-KO ILC2s. The phosphorylations of p38 MAPK and NF-κB p65, the two major IL-33 downstream signals, were unaltered by dexamethasone or CDDO-Im treatment (Supplemental Fig. 2A). Bcl-2 was upregulated in IL-2/IL-33-pretreated ILC2s from Nrf2 KO mice, and CDDO-Im did not induce Bcl-2, irrespective of the presence of Nrf2. TSLP/IL-33 treatment slightly activated Bcl-2 expression, but it more strongly induced Bcl-xL; however, neither dexamethasone nor CDDO-Im induced the Bcl-xL expression. We also assessed two transcriptional factors downstream of cytokine receptor signaling, STAT1 and STAT5, both of which have pivotal roles in ILC2 activation or inhibition (6, 8, 20). However, the phosphorylation levels of STAT1 and STAT5 were not affected by CDDO-Im treatment in the presence or absence of Nrf2 (Fig. 3E). These results indicated that neither apoptosis nor the attenuation of cell-survival signals

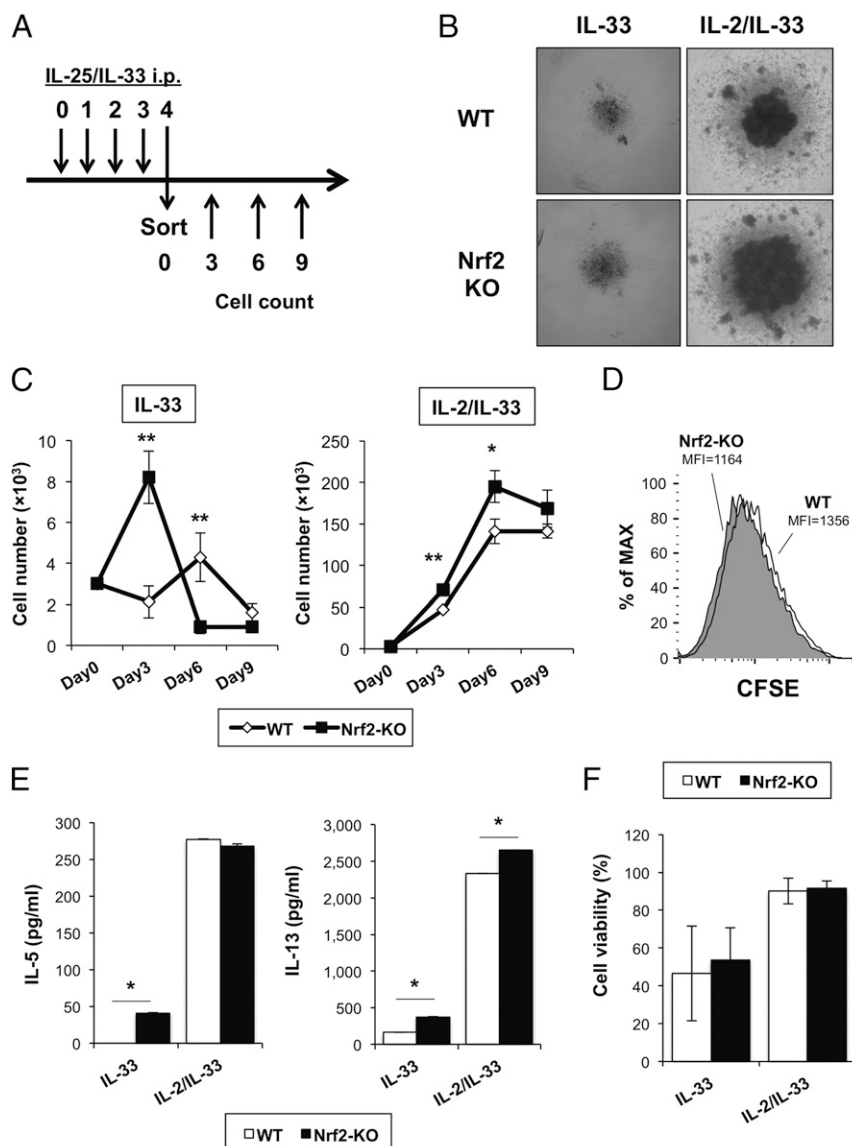


FIGURE 1. Nrf2 suppress ILC2 proliferation in vitro. **(A)** WT or Nrf2-KO mice were i.p. administered IL-25 and IL-33 (each 0.5 μ g) for four consecutive days, and the ILC2s (lineage negative, ICOS⁺, CD25⁺, IL-33R α ⁺) were sorted from the lungs of these mice ($n = 3$ –5 per group). Cultured ILC2s were counted by trypan blue staining every 3 d. **(B)** Microscopic observation of ILC2 proliferation at 6 d of culture. Proliferating cells typical of at least 100 wells are shown. **(C)** Sorted ILC2 (3000 cells per well) were cultured with IL-33 only or with a combination of IL-2/IL-33 (each 10 ng/ml). After 3, 6, or 9 d of culture, ILC2s were counted by trypan blue staining. **(D)** Proliferation of sorted ILC2s cultured with IL-2/IL-33 was analyzed by FACS after CFSE labeling. **(E)** Culture supernatants after 3 d of culture were subjected to ELISAs to measure the IL-5 or IL-13 production. **(F)** ILC2 viability was assessed by trypan blue staining. Data shown are representative of three independent experiments. Error bars show SE. * $p < 0.05$, ** $p < 0.01$.

was responsible for the reduction in ILC2 cell numbers by Nrf2 activation.

Neither RIPK1-dependent necroptosis nor oxidative stress-induced necrosis was responsible for the Nrf2 activation-induced ILC2 death

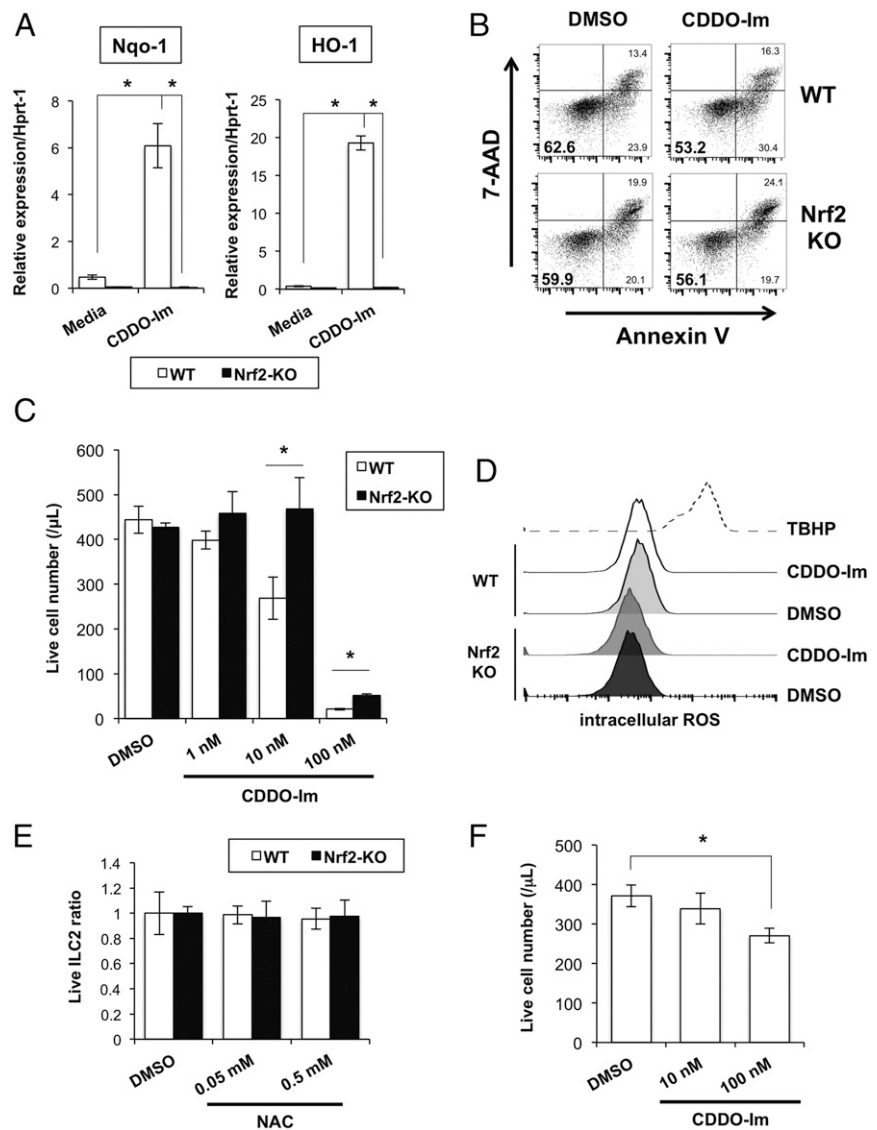
To further investigate the mechanism of Nrf2 activation-induced ILC2 death, we next examined whether necroptosis or oxidative stress was involved (Fig. 4A). In a cell death inhibition assay, IL-2/IL-33-pretreated ILC2s from WT mice were sensitive to CDDO-Im and to another Nrf2 activator 15d-PGJ₂; the ILC2 cell numbers decreased similarly by the two reagents (Fig. 4A, 4B, bars at left). A pan-caspase inhibitor Z-VAD-FMK did not restore the live ILC2 numbers that were reduced in response to the Nrf2 activators (Fig. 4B). In addition, neither a necroptosis inhibitor Nec-1 nor a selective inhibitor of oxidative stress-induced necrosis NecroX-2 could ameliorate the ILC2 reduction under the Nrf2 activation conditions (Fig. 4B). We then attempted to rescue the ILC2 reduction by combined treatments of Z-VAD-FMK and Nec-1, Z-VAD-FMK and NecroX-2, Nec-1 and NecroX-2, or all three inhibitors (Fig. 4B); nevertheless, none of the combinations prevented cell death. We also examined whether Nrf2 regulated ferroptosis, an iron-dependent cell death pathway for which Nrf2

might activate responsible genes, such as *GPX-4*, *HO-1*, and *FTL* (21, 22). Nevertheless, a potent ferroptosis inhibitor, Fer-1, did not restore the Nrf2-mediated ILC2 reduction (Fig. 4C). Together, these results suggest that the ILC2 death induced by the Nrf2 activation is not mediated by canonical apoptosis, necroptosis, or ferroptosis.

Nrf2 activation suppresses the allergic lung inflammation induced by ILC2s

Numerous studies have shown an association of IL-33 with allergic diseases, including asthma. To evaluate whether the Nrf2 activation alleviated allergic lung inflammation in vivo, we introduced recombinant mouse IL-33 into the trachea of mice by aspiration and observed the ILC2-mediated response. After three intratracheal treatments with IL-33, the mice were given i.p. CDDO-Im injections for the last two consecutive days before analysis (Fig. 5A). Within this relatively short time period, we did not observe any significant increase in the IL-5⁺ or IL-13⁺ population within the lineage-positive cells, indicating that the Th2 cells were negligible in this process (Supplemental Fig. 3). First, we confirmed that CDDO-Im caused a significant induction of *Nqo-1* in WT lung cells and that this induction was completely absent in Nrf2-KO lung cells, ensuring that the expected activation

FIGURE 2. ILC2 cell numbers decrease by Nrf2 activation using CDDO-Im. Sorted lung ILC2s were cultured with IL-2/IL-33 for 3 d, treated with CDDO-Im, and then analyzed by real-time PCR or FACS. **(A)** Relative mRNA levels of Nqo-1 or HO-1. **(B)** Lung ILC2s were sorted from WT or Nrf2-KO mice ($n = 3-5$ per group), cultured with or without CDDO-Im for 48 h, and then analyzed for apoptosis by FACS using Annexin V and 7-AAD. Numbers in the plots indicate the frequency of live cells (left lower), cells in early apoptosis (right lower), and cells in necrosis/late apoptosis (upper right). **(C)** Absolute numbers of live ILC2s. **(D)** Analysis of the intracellular ROS in sorted lung ILC2s cultured with IL-2/IL-33. **(E)** Lung ILC2s from WT or Nrf2-KO mice were cultured with IL-2/IL-33 for 6 d and then treated with NAC for 24 h. Live ILC2s were counted by FACS using Annexin V and 7-AAD and expressed relative to the value for cells treated with DMSO. **(F)** BM ILC2s were sorted from WT or Nrf2-KO mice ($n = 3-5$ per group) and cultured with or without CDDO-Im for 48 h. The absolute numbers of live ILC2s are shown. Data shown are representative of three independent experiments. Error bars show SE. $*p < 0.05$.



of Nrf2-mediated transcriptions would occur (Fig. 5B). Surprisingly, CDDO-Im administration severely decreased the lineage-negative ICOS⁺ population, indicative of ILC2s, in WT mice (Fig. 5C). In sharp contrast, this reduction in the ILC2 population was minimal in Nrf2-KO mice given the same treatment (Fig. 5C). Accordingly, the *in vivo* pulmonary ILC2s in WT mice, defined as lineage-negative ICOS⁺ CD127⁺ IL-33R α ⁺, showed a striking decrease in numbers by the CDDO-Im treatment. As expected, the same ILC2 population was left intact in the CDDO-Im-treated Nrf2-KO mice (Fig. 5D, left). We also examined the local eosinophil accumulation, one of the hallmarks of allergic inflammation *in vivo*. CDDO-Im effectively decreased the eosinophil accumulation in the WT lung tissues to ~50% of the level in control mice, and this significant decrease was completely absent in the Nrf2-KO lung (Fig. 5D, right). The infiltration of lymphocytes and eosinophils around airway bronchi was also decreased in WT mice treated with CDDO-Im (Fig. 5E, upper). Furthermore, mucus production by the bronchial epithelial cells was greatly diminished in WT mice by the Nrf2 activation, as judged by PAS staining (Fig. 5E lower). Again, a reduction in mucous-positive cells by Nrf2 activation was not apparent in the Nrf2-KO lung. Finally, we confirmed that the Nrf2 activation exerted similar effects on lung ILC2s in an allergic lung inflammation model using extracts of *A. alternata*, which is a

highly allergenic fungus (Fig. 5F, 5G). Taken together, these results suggested that the Nrf2 activation *in vivo* was highly effective in suppressing the allergic lung inflammation associated with ILC2s and eosinophil accumulation.

Discussion

Intensive study in recent years revealed that the ILC2s are a major player in allergic inflammation and a source of Th2 cytokines (19, 23, 24). ILC2s contribute to symptoms in an airway disease model and link innate immunity to the acquired immune response. However, little is known about the factors governing the ILC2 biology. In this study, we demonstrated that Nrf2 is a negative regulator of ILC2s. IL-33, IL-25, and TSLP are epithelial-derived cytokines that are released upon exposure to external stimuli and damage and are associated with asthma. We observed robust ILC2 expansion upon combined stimulation with IL-33 and IL-2, IL-7, or TSLP, indicating that these lymphopoietic cytokine receptor-mediated signals are required for full ILC2 growth. In our study, however, Nrf2 deficiency did not affect the IL-2R α , IL-33R α , or IL-7R α expression on ILC2s (Supplemental Fig. 1C). The receptors for IL-2 and IL-7 share the common γ -chain, and IL-7R α is shared between IL-7 and TSLP. All of these cytokines activate JAK1. Accordingly, JAK1 was highly phosphorylated in Nrf2-KO ILC2s upon IL-2/IL-33 or TSLP/IL-33 stimulation; thus, the

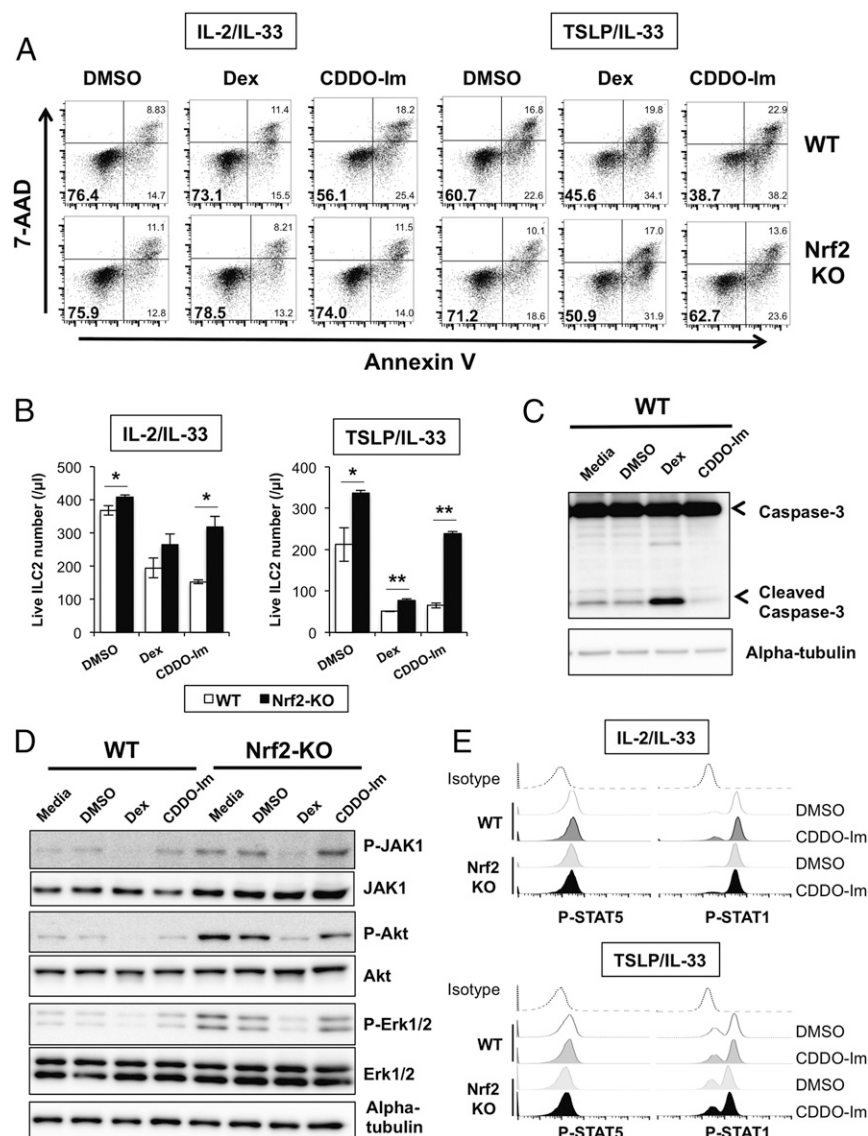


FIGURE 3. Nrf2 activation decreases the number of ILC2s stimulated with TSLP/IL-33 by a caspase-independent cell death and Akt/Erk-independent pathway. Sorted lung ILC2s from WT or Nrf2-KO mice ($n = 3-5$ per group) were cultured with IL-2/IL-33 or TSLP/IL-33 for 3 d, treated with CDDO-Im, and then analyzed by FACS or Western blotting. **(A)** ILC2 apoptosis was assessed by FACS using Annexin V and 7-AAD. Numbers in the plots indicate the frequency of live cells (left lower), cells in early apoptosis (right lower), and cells in necrosis/late apoptosis (upper right). **(B)** Absolute numbers of live ILC2s counted by FACS. **(C and D)** Sorted lung ILC2s were cultured with TSLP/IL-33 for 6 d, treated with dexamethasone or CDDO-Im for 6 h, and then analyzed by Western blotting. α -tubulin was used as a loading control. **(E)** WT or Nrf2-KO ILC2s were treated with DMSO or CDDO-Im for 6 h, and then the phosphorylation of STAT1 or STAT5 was assessed by FACS. Data shown are representative of three independent experiments. Error bars show SE. * $p < 0.05$, ** $p < 0.01$.

enhanced proliferation of Nrf2-KO ILC2s can be attributed to the increased activation of JAK1 and its downstream molecules Akt and Erk1/2. Notably, the same combined stimulation of IL-33 with IL-2, IL-7, IL-9, or TSLP induces a dexamethasone resistance of ILC2s and NH (natural helper) cells through MEK-Erk activation (11), and the modest steroid-resistant phenotype of the Nrf2-KO ILC2s in this study corresponded well with these signaling states. Likewise, STAT5 phosphorylation is also implicated in dexamethasone resistance (10, 11). STAT5 and dexamethasone cooperatively induce the upregulation of IL-7R α , which further induces JAK-STAT and Erk activations (11). However, neither STAT5 nor Erk1/2 was affected by the CDDO-Im stimulation, and the above mechanism is unlikely to be involved in the Nrf2 activation-induced ILC2 cell death. Moreover, IL-2/IL-33 stimulation induced an upregulation of Bcl-2 in Nrf2-KO ILC2s, suggesting that Bcl-2 may play a role in ILC2 survival. However, this was not the case for ILC2s after the TSLP/IL-33 pretreatment, in which Bcl-xL, another antiapoptotic factor, was induced irrespective of Nrf2, suggesting that Bcl-xL may be related to the basal TSLP/IL-33-mediated ILC2 survival but not with the Nrf2-induced activation. HDAC2 reduction is reported to affect the susceptibility to steroid resistance in lung, but no reduction in HDAC2 expression in the nucleus of lung ILC2s

from WT or Nrf2-KO mice was observed in our experimental conditions (25). The mechanism by which steroid resistance is induced by IL-33/IL-2- and IL-33/TSLP-activated JAK-STAT pathways under Nrf2 deficiency awaits further study.

ILC2 death induction is of particular interest because it is tightly linked to allergic disease control. We found that the activation of Nrf2 by either of two reagents, CDDO-Im and 15d-PGJ₂, successfully decreased the TSLP/IL-33-stimulated ILC2 cell numbers. These two Nrf2-activating reagents were as effective as, or even more potent than, dexamethasone in decreasing ILC2s. In this regard, Nrf2 activation has an advantage over dexamethasone treatment in that it suppresses both IL-2- and TSLP-pretreated ILC2s. We further examined the underlying mechanisms for the Nrf2-activation-induced death. Unexpectedly, none of the apoptosis and/or necroptosis inhibitors we tried could rescue the Nrf2 activation-induced ILC2 reduction. Nrf2 activation did not induce caspase-3-dependent canonical apoptosis or the caspase-independent apoptosis involving the release of apoptosis-inducing factor from mitochondria (data not shown). Furthermore, we found that the cell contact-dependent apoptosis occurring through the Fas and Fas ligand pathway was not involved in the cell death, given that Fas ligand was not expressed on the ILC2s from WT or Nrf2-KO mice (data not shown). Necroptosis also seems unlikely to

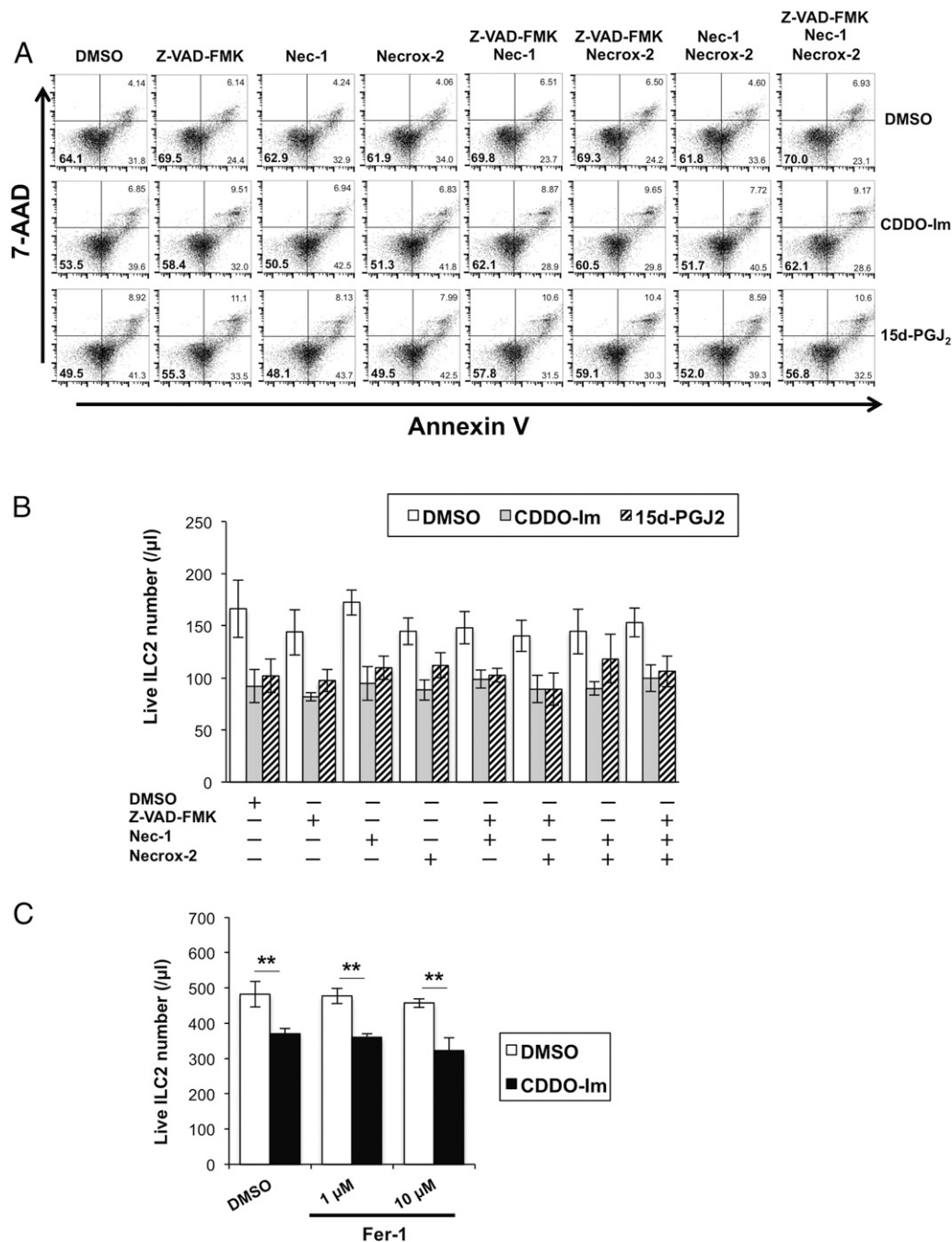
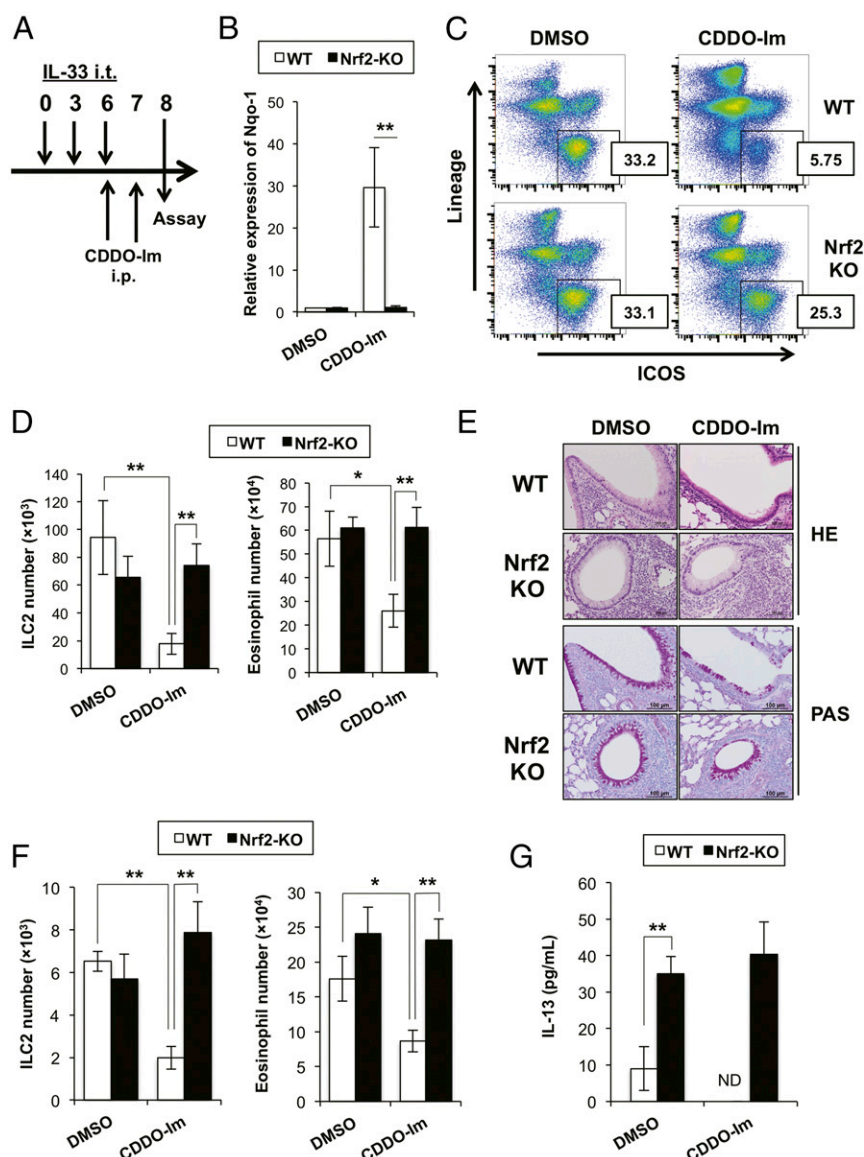


FIGURE 4. ILC2 reduction by Nrf2 activation does not occur by a canonical cell death mechanism. Sorted lung ILC2s from WT mice ($n = 3-5$) were cultured with TSLP/IL-33 for 3 d and then treated with or without Z-VAD-FMK, Nec-1, and/or Necrox-2 for 1 h. The cells were then treated with or without CDDO-Im or 15d-PGJ₂ for 48 h and analyzed by FACS. **(A)** ILC2 apoptosis was assessed by FACS using Annexin V and 7-AAD. Numbers in the plots indicate the frequency of live cells (left lower), cells in early apoptosis (right lower), and cells in necrosis/late apoptosis (upper right). **(B)** Absolute numbers of live ILC2s counted by FACS. **(C)** Sorted lung ILC2s from WT mice were cultured with TSLP/IL-33 for 3 d and then treated with or without Fer-1 for 1 h. The cells were then treated with or without CDDO-Im for 48 h and analyzed by FACS. The absolute numbers of live ILC2s are shown. Data shown are representative of two independent experiments. Error bars show SE. $**p < 0.01$.

govern the ILC2 cell death, because a recent paper suggests that Nrf2 actually prevents necroptosis in alcoholic liver damage (26). We suspected that ferroptosis, an iron-dependent nonapoptotic cell death caused by cellular lipid peroxide accumulation (27, 28), was involved in the ILC2 cell death because a number of anti-ferroptotic genes are among Nrf2's target genes (13, 29). However, the lung ILC2s tested in this study did not support this hypothesis. Thus, the mechanism responsible for ILC2 cell death awaits further analysis. In contrast, we discovered the possible existence of a cell type- and environment-specific ILC2 death. Recently, Ricardo-Gonzalez et al.

(30) reported that tissue-resident ILC2s have tissue-specific transcriptional profiles and that the maturation of ILC2s depends on tissue-derived signals. This tissue-dependent characterization of ILCs is consistent with our finding that the lung ILC2s were more susceptible to Nrf2 activation than were the BM ILC2s. Lung ILC2s may have a different transcriptional profile from that of other ILC2s, and the microenvironment in the lung may affect their sensitivity to Nrf2. In support of this idea, Cheon et al. (31) reported that lung ILC2s under 80% O₂ show a highly enhanced expression of two Nrf2 target genes *Nqo-1* and *HO-1*, suggesting that the Nrf2

FIGURE 5. Nrf2 activation suppresses lung allergic inflammation caused by ILC2 and eosinophils. **(A)** WT or Nrf2-KO mice were intratracheally treated with recombinant mouse IL-33 (1 μ g) on day 0, 3, and 6 to induce lung ILC2s and eosinophils. DMSO or CDDO-Im (20 μ mol/kg) was then injected i.p. for two consecutive days before analysis. Bilateral lung lobes from each mouse were used separately to determine the ILC2 numbers (DMSO: $n = 4-6$ versus CDDO-Im: $n = 9-10$). **(B)** Analysis of the relative mRNA level of Nqo-1 in dissociated lung cells. **(C)** Flow cytometric analysis of lung ILC2s gated as the lineage-negative ICOS⁺ population. Numbers in the plots are the percentage of total cells. Data shown are representative of three independent experiments. **(D)** Absolute numbers of ILC2s and eosinophils in the lung. **(E)** Histological analysis of mouse lungs by H&E and PAS staining. **(F)** WT or Nrf2-KO mice were treated intratracheally with *A. alternata* extracts (10 μ g) on days 0, 3, and 6 to induce lung ILC2s and eosinophils. DMSO ($n = 5-6$) or CDDO-Im (20 μ mol/kg) ($n = 8-9$) was then injected i.p. for two consecutive days, and the bilateral lung lobes from each mouse were analyzed separately. Data show the absolute numbers of ILC2s and eosinophils in the lung. **(G)** ELISA for IL-13 in the bronchoalveolar lavage fluid of WT or Nrf2-KO mice after the intratracheal administration of *A. alternata* extracts. Graph shows the pooled data of three independent experiments. Error bars show SE. * $p < 0.05$, ** $p < 0.01$.



pathway is activated under an oxidative stimulus. The detailed profiling of lung ILC2s will help elucidate this issue. The murine lung ILC2s in this study were far more resistant to steroids than were the NH cells in fat-associated lymphoid clusters (10). In this study, sorted and in vitro cultured ILC2s acquired the steroid-resistant phenotype under IL-2/IL-33 culture conditions, and TSLP/IL-33-stimulated ILC2s were partially resistant. The IL-25/IL-33 treatment used for the in vivo ILC2 induction may have conferred an inflammatory phenotype to the lung ILC2s, whereas the same stimulation leaves the NH cells in fat-associated lymphoid clusters with a naive phenotype. Similarly, human ILC2s in the blood are less resistant to cell death than are human lung ILC2s, and this difference is attributed to the milieu; lung ILC2s may often be exposed to TSLP, which is secreted from the inflamed environment. In this study, lung ILC2s cultured with IL-7 alone showed a tolerant phenotype and exhibited only low death rates by the CDDO-Im treatment, possibly due to an uninflamed environmental condition that mimicked a relatively naive condition (Supplemental Fig. 4). Together, these observations suggest that Nrf2 activation may be only minimally cytotoxic to naive ILC2s.

Oxidative stress has been postulated to be involved in the pathogenesis of asthma, and a defect in antioxidant responses has been speculated to exacerbate the severity of asthma. The disruption of Nrf2 in mice enhances their susceptibility to Ag-induced severe airway inflammation and asthma (14). A later study showed that Nrf2 reduces the development and pathogenesis of allergic asthma through its cytoprotective function in lung epithelial cells (15). Thus, Nrf2 activators are expected to serve as useful therapeutic agents for asthma, with less adverse effects than dexamethasone. Apart from Nrf2, other possible intervention targets for ILC2s include a PKC inhibitor C-20, which reduces the ILC2 cell numbers and activation and decreases airway inflammation (32). C-20 suppresses *IRF4* and *NFAT1* expression, thus attenuating ILC2 homeostasis and function, and miR-19, which targets both A20 (a negative regulator of NF- κ B) and SOCS1 (an inhibitor of the JAK-STAT pathway), thereby regulates ILC2s' effector function (33). However, Nrf2 activation has several advantages over these targets. First, Nrf2 has multiple effects on inflammation's control over the airway epithelium and ILC2s. Nrf2 activation suppresses the release of IL-33 from the affected epithelia, which attenuates airway inflammation (34). OVA-induced allergic asthma is also alleviated through an

Nrf2-dependent cytoprotective effect on the epithelial tight junctions of the airway by preserving ZO-1 and E-cadherin (15). Second, several well-known Nrf2 activators, including a natural compound sulforaphane, have been identified, some of which are currently being developed as potential therapeutics. In conclusion, we found that Nrf2 is a negative regulator of ILC2 activation and cell death. Agents that promote Nrf2 activation may manage allergic lung inflammation in cases that have been difficult to cure. Nrf2 activation is expected to be a powerful therapeutic strategy for allergic diseases, including steroid-resistant severe asthma.

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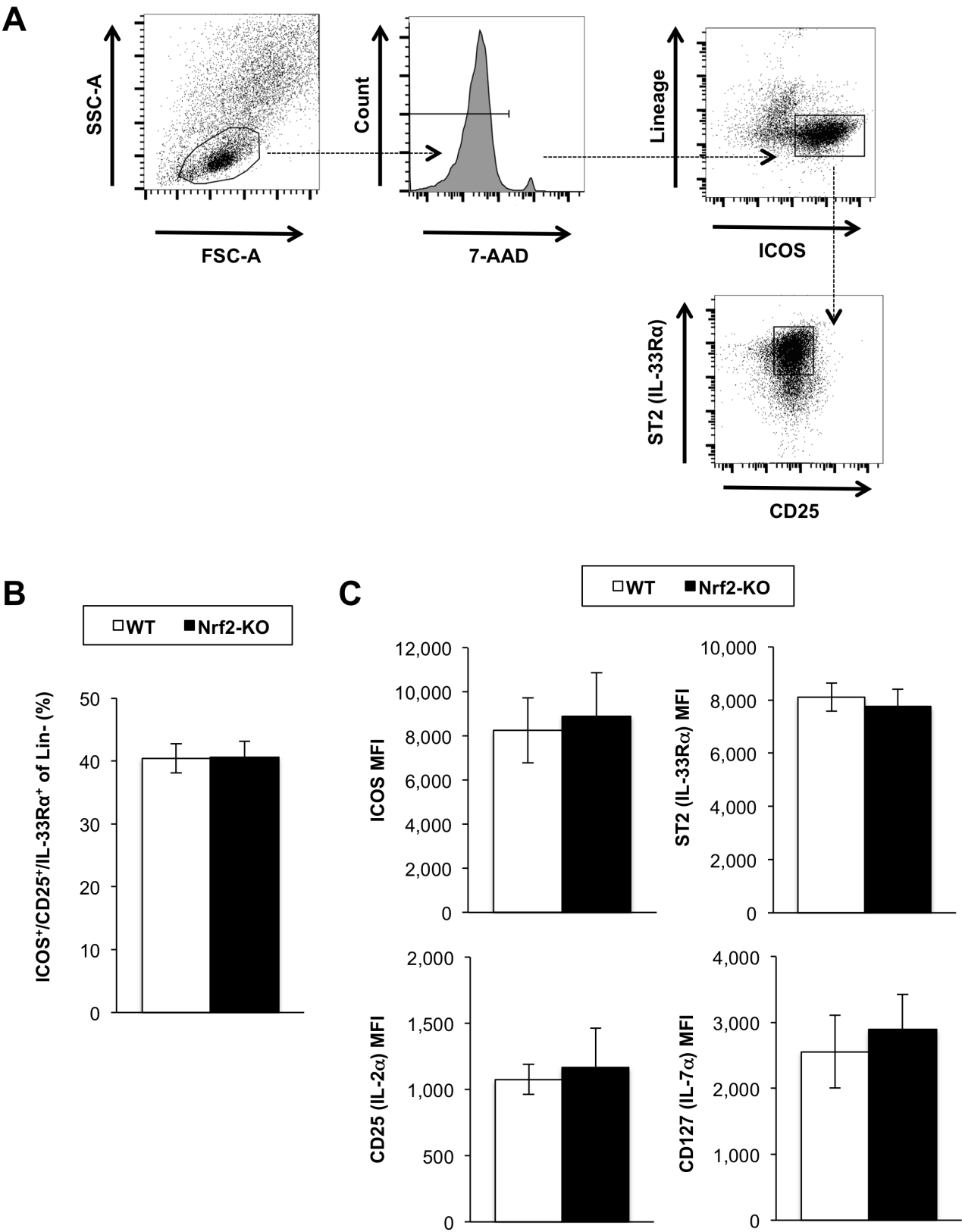
Disclosures

The authors have no financial conflicts of interest.

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Suppl. Figure 1

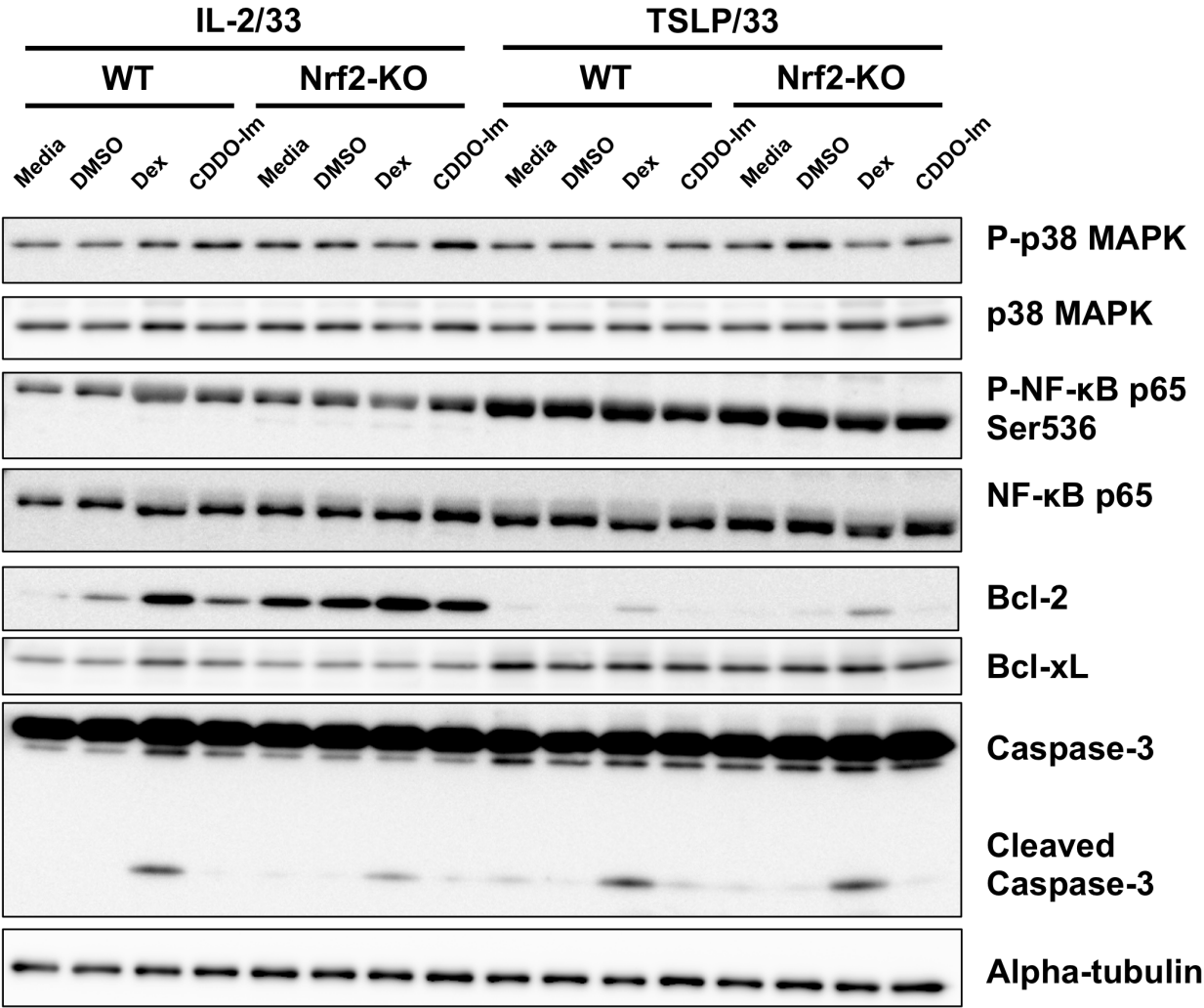


Gating strategy for isolating ILC2s in the lung.

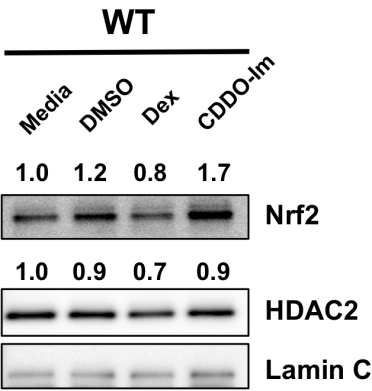
(A) WT or Nrf2-KO mice were intraperitoneally inoculated with IL-25 and IL-33 (each 0.5 μ g) for 4 consecutive days, and ILC2s (Lineage⁻, ICOS⁺, CD25⁺, IL-33R α ⁺) were sorted from the lungs of these mice. (B) Frequency of the ICOS⁺, CD25⁺, IL-33R α ⁺ population in Lineage⁻ cells of the lung. (C) Mean fluorescence intensity (MFI) of ICOS, CD25, or CD127. Pooled data from three independent experiments are shown, with error bars indicating SE (n=13).

Suppl. Figure 2

A



B

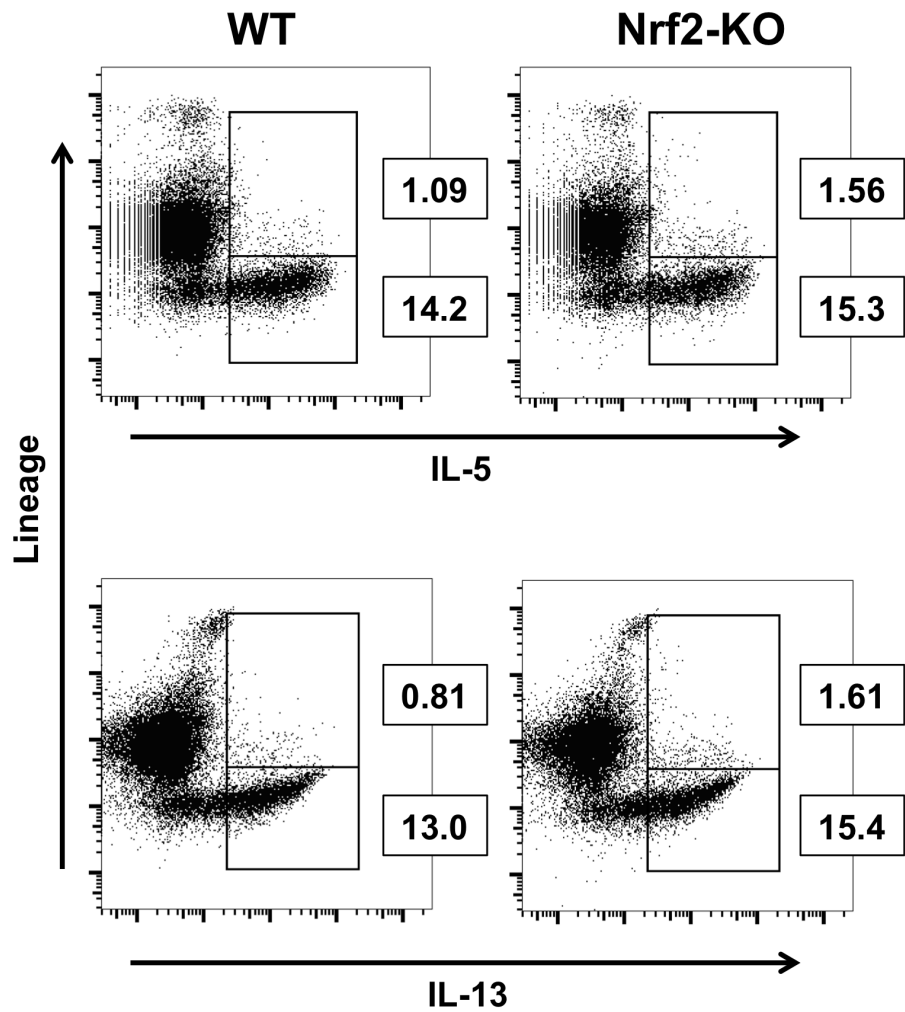


Signal analysis of IL-2/IL-33 or TSLP/IL-33 stimulation in lung ILC2s.

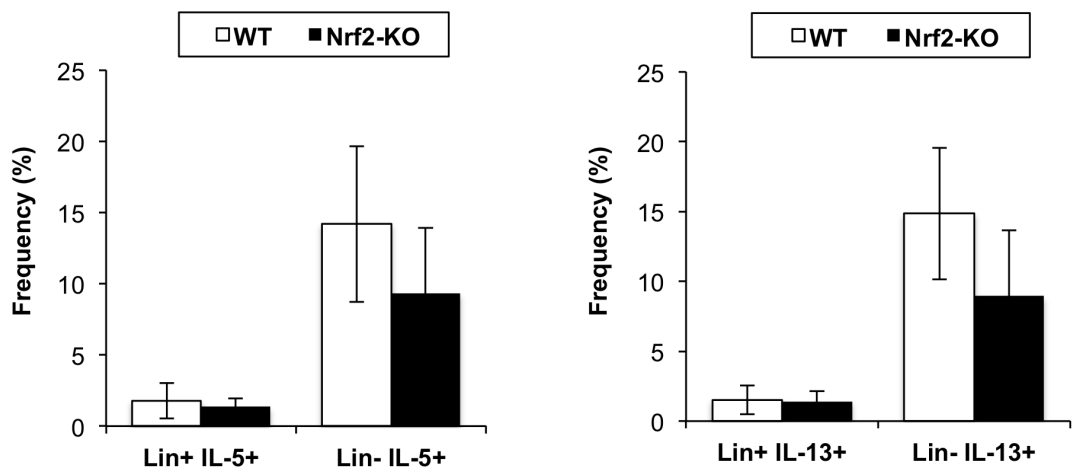
(A) Western-blot analysis of NF-κB p65, p38 MAPK, Bcl-2, Bcl-xL, or caspase-3 in lung ILC2s stimulated with IL-2/IL-33 or TSLP/IL-33 and then treated with dexamethasone or CDDO-Im for 6 h. (B) Western-blot analysis of Nrf2 or HDAC2 in the nuclear fractions of WT lung ILC2s. Cells were stimulated with IL-2/IL-33, and then treated with dexamethasone or CDDO-Im for 6 h. The numbers show the expression levels relative to that observed in cells with no stimulation (media).

Suppl. Figure 3

A



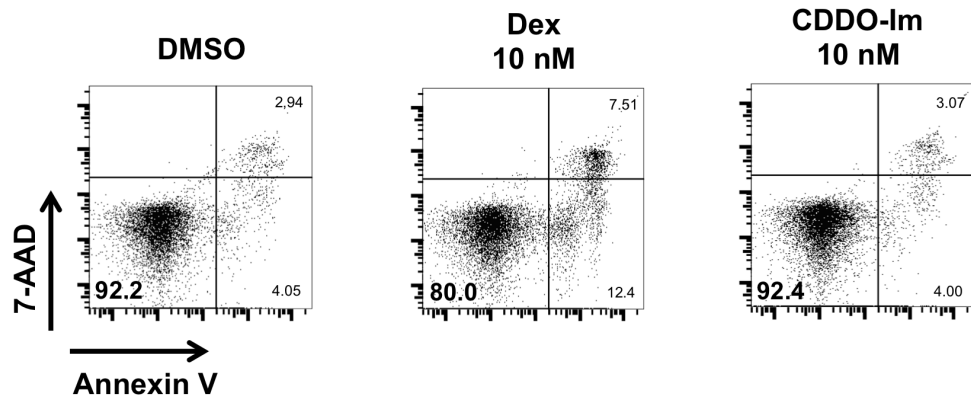
B



Intratracheal administration of recombinant mouse IL-33 did not induce Th2 cells.

WT or Nrf2-KO mice (n=3-5/group) were intratracheally treated with recombinant mouse IL-33 (1 μ g) on day 0, 3, and 6. (A) Intracellular staining of IL-5 and IL-13 in lung cells. The cells were gated for Th2 cells (Lineage⁺) or ILC2s (Lineage⁻), and the numbers in the plots are the percentage of total cells. (B) Frequency of cytokine-producing Th2 cells or ILC2s. For the intracellular cytokine measurement, lung cells were stimulated with 25 ng/ml phorbol 12-myristate 13 acetate (PMA) and 500 ng/mL ionomycin for 4 h, and with brefeldin A for the last 2 h. Cells were fixed in 2% paraformaldehyde-PBS, and cytokine staining was performed in permeabilization buffer with antibodies against IL-5 and IL-13.

Suppl. Figure 4



ILC2s cultured with IL-7 did not undergo CDDO-Im-induced cell death.

Sorted WT lung ILC2s were cultured in the presence of IL-7, and treated with dexamethasone or CDDO-Im (each 10 nM) for an additional 48 h. Apoptosis of ILC2s was assessed by FACS using annexin-V and 7-AAD.