

Letter to the Editor

Pulmonary alveolar proteinosis in adenosine deaminase-deficient mice

To the Editor:

Adenosine deaminase (ADA) is a ubiquitous enzyme important for the metabolism of adenosine and deoxyadenosine. Inherited defects in the function of ADA lead to severe immune abnormalities with increased susceptibility to lethal infections. Current treatments for ADA deficiency include allogeneic hematopoietic stem cell transplantation, autologous gene therapy, and enzyme replacement therapy with bovine ADA conjugated to polyethylene glycol (PEG-ADA).

Recently, we identified the accumulation of a surfactant-like substance, suggestive of pulmonary alveolar proteinosis (PAP), in ADA-deficient patients.¹ PAP is a rare lung disorder characterized by impaired pulmonary surfactant homeostasis resulting in progressive respiratory failure.² To further establish and understand the role of ADA deficiency in the development of PAP, we studied ADA-deficient (ADA-KO) mice (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for additional information), which display many of the metabolic, immune, and systemic abnormalities observed in ADA-deficient patients.³ Previous reports found increased bronchial airways mucus, inflammatory cells accumulation, and structural abnormalities in the lungs of ADA-KO mice, although PAP was not described.^{4,5} All our ADA-KO mice died by age 18 to 21 days, as previously noted by others.³ At age 18 days, we found that ADA-KO mice suffered from severe respiratory distress and hypoxia with significantly reduced ($P = .001$) arterial blood oxygen saturation ($81.0\% \pm 3.8\%$) compared with normal littermate control mice ($96.0\% \pm 3.5\%$). Hematoxylin and eosin staining of noninflated lung sections from 16- to 18-day-old mice showed that alveoli of all ADA-KO mice examined were filled with a granular homogeneous eosinophilic substance, not present in littermate control mice ([Fig 1, A](#)). The granular substance in the alveoli of ADA-KO mice was periodic acid-Schiff positive and resistant to diastase pretreatment ([Fig 1, B](#)), typical for surfactant.⁶ Electron microscopy analysis also demonstrated the granular substance as well as many alveolar macrophages (AMs) in the lungs of ADA-KO mice ([Fig 1, C](#)). Additional analysis of the alveolar substance was performed by measuring cholesterol and protein in the bronchoalveolar lavage fluid (BALF). Increased cholesterol concentrations were found in the BALF from ADA-KO mice than from littermate control mice ($617.9 \pm 184.4 \mu\text{g/mL}$ vs $397.9 \pm 68.6 \mu\text{g/mL}$, respectively, $P = .012$). Significantly increased protein concentrations were also found in supernatants of the BALF from ADA-KO mice than from littermate control mice ($575.4 \pm 176.0 \mu\text{g/mL}$ vs $221.0 \pm 78.6 \mu\text{g/mL}$, respectively, $P = .005$). Collectively, our data show excessive periodic acid-Schiff positive lipoproteinaceous substance, characteristic of a surfactant, in the alveoli of ADA-KO mice, similar to that found in patients and mice suffering from PAP.^{2,6} Importantly, our study demonstrates for the first time that ADA-KO mice develop PAP, reminiscent to our findings in ADA-deficient patients.

Next, we used the ADA-KO mice model to investigate the pathogenesis of PAP in ADA deficiency. Infections have been associated with PAP,² and few of our ADA-deficient patients had lung infections concomitant with PAP.¹ However, the ADA-KO

mice that we studied were rederived from frozen embryos and were maintained in a pathogen-free environment, and repeated microbial assessments did not identify an infectious etiology, indicating that infections are not the cause of PAP in ADA deficiency. Autoantibodies against granulocyte-macrophage colony-stimulating factor or its receptor can also lead to abnormal function of AMs and PAP,² and some ADA-deficient patients and mice can produce autoantibodies despite their severe immunodeficiency.⁷ However, the appearance of PAP rapidly after birth in all ADA-KO mice and the prevention of PAP within days of treatment (as described below), a period that is shorter than the biological half-life of antibodies, suggest that autoantibodies are not the culprit for PAP in these mice.

PAP has often been attributed to AM abnormalities, including impaired ability of these cells to clear the surfactant²; therefore, we focused our investigations on AMs from ADA-KO mice. The BALF from ADA-KO mice contained significantly more AMs than did the BALF from littermate control mice ($610.9 \pm 176.5 \times 10^3$ cells/mL vs $174.7 \pm 56.0 \times 10^3$ cells/mL, respectively, $P = .0024$), similar to previous reports,⁴ suggesting that the initial development and chemotaxis of AMs to the lungs was not compromised. The AMs isolated from the BALF of ADA-KO mice were significantly larger than the AMs isolated from the BALF of littermate control mice (average cell diameter, $16.37 \pm 4.79 \mu\text{m}$ vs $12.51 \pm 2.24 \mu\text{m}$, respectively, $P < .0001$). Electron microscopy analysis showed that AMs from ADA-KO mice were foamy and filled with inclusion bodies, cholesterol crystals, and many concentric "lamellar-like" structures typical of phospholipids (see [Fig E1](#) in this article's Online Repository at www.jacionline.org), similar to our findings in ADA-deficient patients.¹ The AMs isolated from the BALF of ADA-KO mice, but not the AMs isolated from the BALF of littermate control mice, also stained with Oil-Red-O, which labels neutral lipids such as cholesterol (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

Adenosine and adenosine receptors (AdoR) have diverse roles in different cells and tissues. We found that adenosine concentrations in AMs isolated from the BALF in ADA-KO mice were significantly increased compared with adenosine concentrations in AMs isolated from the BALF in littermate control mice ($87.4 \pm 12.3 \text{ pmol}/10^5$ cells vs $<20.0 \text{ pmol}/10^5$ cells, respectively, $P < .001$), concordant with previous studies, which identified increased adenosine concentrations in the BALF of these mice.⁴ Accumulation of purine metabolites has been shown previously to induce cells' apoptosis, including AMs.^{4,8} Indeed, flow cytometry analysis demonstrated increased Annexin V expression in AMs isolated from the BALF of ADA-KO mice ([Fig 2, A](#)) with normal propidium iodide intercalation ([Fig 2, B](#)). Moreover, electron microscopy revealed that many, albeit not all, AMs in lung sections from ADA-KO mice have typical features of apoptosis, including nuclear chromatin fragmentation and bleb formation ([Fig 2, C](#)). In contrast, the ability of adherent AMs from ADA-KO mice to engulf IgG-coated beads was not different than that of AMs from littermate control mice (see [Fig E3](#) in this article's Online Repository available at www.jacionline.org). Adenosine elicits its actions on cells by activating different AdoR, and previous reports demonstrated increased AdoR-1, AdoR-2B, and AdoR-3 in the lungs of ADA-KO mice.⁹ Removal of AdoR-1,

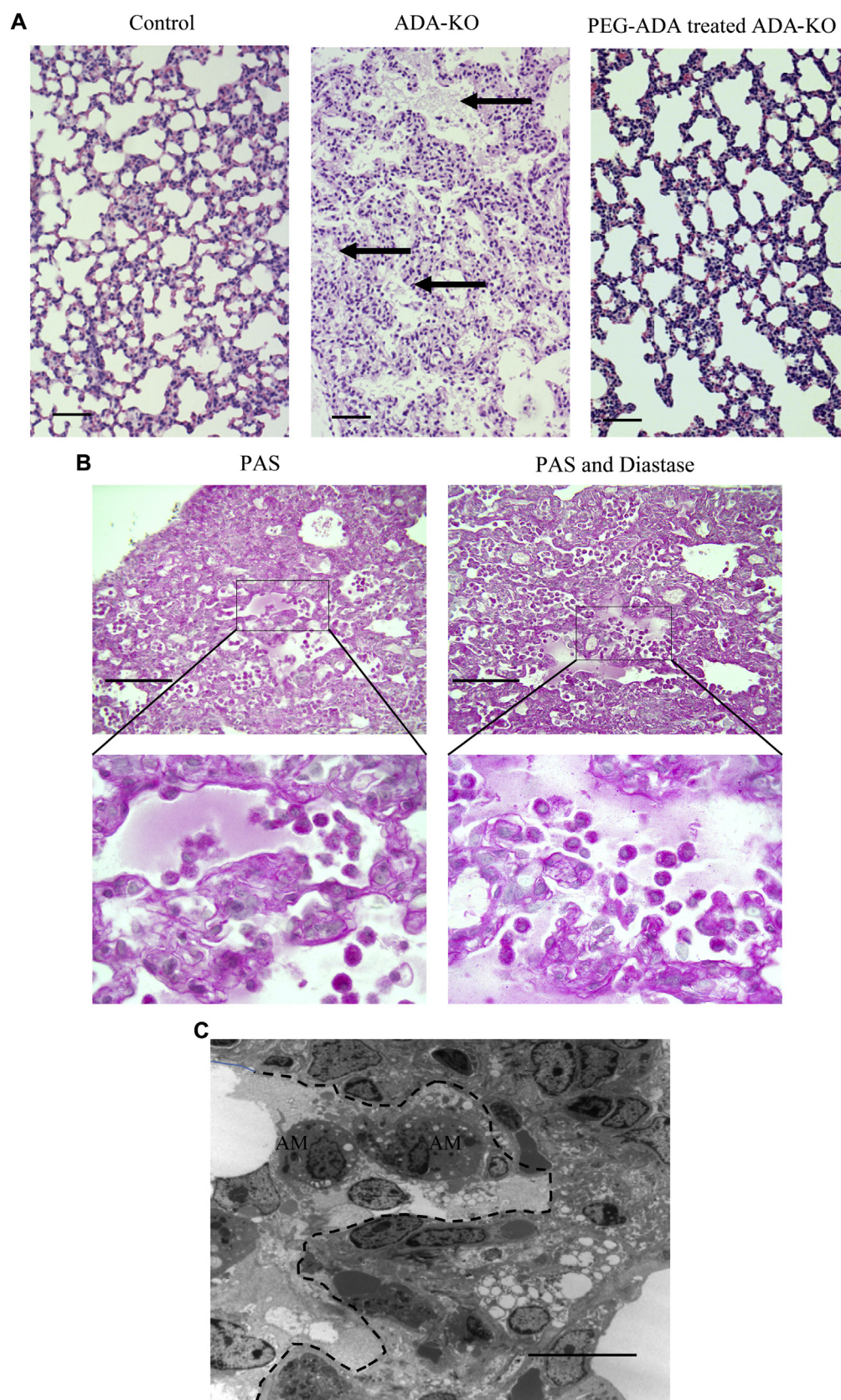


FIG 1. Surfactant accumulation in the alveoli of 16- to 18-day-old ADA-KO mice, which is prevented by PEG-ADA treatment. **A**, Hematoxylin and eosin demonstrates a granular eosinophilic substance (*arrows*) in the alveoli of ADA-KO mice (*middle*), not present in littermate control mice (*left*) or ADA-KO mice treated with

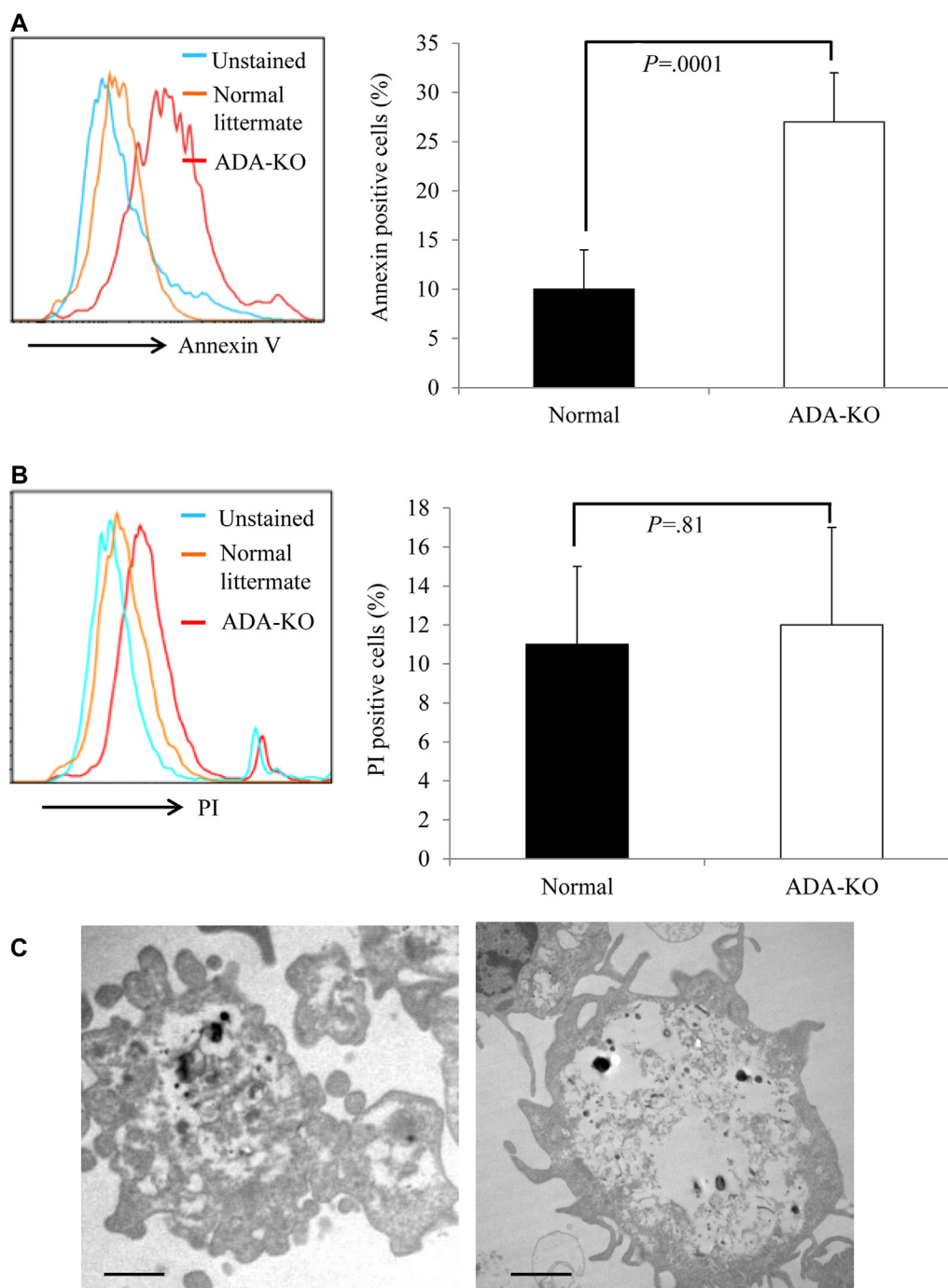


FIG 2. Increased apoptosis of AMs from ADA-KO mice. Representative flow cytometry analysis (*left*) and bar graph (*right*, mean $\pm$ SD) of AMs from 16- to 18-day-old ADA-KO and littermate control mice assessed for Annexin V (**A**) and propidium iodide (**B**) intercalation. Results are representative of 3 independent experiments. **C**, Electron microscopy analysis of AMs from ADA-KO mice demonstrating nuclear chromatin fragmentation and bleb formation typical of apoptosis. Figures are representative of 40 images; $\times 15,000$ magnification. Bar = 3 μ m.

PEG-ADA (*right*); $\times 20$ magnification. **B**, Periodic acid–Schiff (PAS)-stained substance (*left*) in the lungs of ADA-KO mice is diastase resistant (*right*); $\times 40$ magnification. Enlargements are $\times 100$ magnification. Bar = 200 μ m. **C**, Electron microscopy demonstrates accumulation of a granular substance and AMs in the alveoli of ADA-KO mice ($\times 3000$ magnification, bar = 10 μ m). Figures representative from N = 5 mice of each genotype.

which is expressed on AMs, as well as AdoR-2A was shown previously to exacerbate lung disease in ADA-KO mice. In contrast, blocking the AdoR-2B led to reduced AMs in the BALF of ADA-KO mice while removal of AdoR-3 diminished pulmonary inflammation and mucus accumulation in the lungs of ADA-KO mice, suggesting that different AdoR might have opposing roles in mediating lung damage in ADA deficiency.⁹ Collectively, these previous reports and our study reveal diverse AM abnormalities in ADA-KO mice, although the precise contributions of adenosine and AdoR changes as well as PAP to AM dysfunction and impaired surfactant clearance require additional studies.

To further study the role of adenosine metabolism in PAP development, we restored ADA activity in the blood of ADA-KO mice by using PEG-ADA. Injecting ADA-KO mice with 2.5 units/g body weight, but not 1 or 0.5 units/g body weight, PEG-ADA or equivalent volumes of PBS, twice a week from age 7 days, prevented the respiratory distress and hypoxia ($94.0\% \pm 4.3\%$ oxygen saturation) in the blood of 16- to 18-day-old ADA-KO mice and normalized adenosine concentrations in AMs (<20.0 pmol/ 10^5 cells). Similar to previous reports,³ PEG-ADA treatment from age 7 days prevented structural lung changes, such as enlarged alveoli, in ADA-KO mice. Indeed, the number of alveoli (57.4 ± 9.6 alveolar spaces/field) in PEG-ADA-treated ADA-KO mice increased significantly ($P = .004$) than in untreated ADA-KO mice (30.6 ± 4.8 alveolar spaces/field) and was practically identical to that of littermate control mice (60.6 ± 10.3 alveolar spaces/field). PEG-ADA also prevented the accumulation of surfactant and macrophages in the lungs of ADA-KO mice (Fig 1, A) and normalized the number of Oil-Red-O-stained AMs in the BALF of ADA-KO mice (see Fig E2, B). Hence, correction of adenosine metabolism in ADA-KO mice prevented the development of PAP. Importantly, our results also suggest that early initiation of PEG-ADA should be considered for ADA-deficient patients suffering from PAP, particularly when rapid hematopoietic stem cell transplantation cannot be performed. Our study also has several significant limitations. While we focused on AMs, future studies are needed to assess the role of pneumocytes and other lung cell abnormalities in the development of PAP in ADA deficiency. In addition, although we found that phagocytosis of IgG-coated beads by ADA-deficient AMs was normal, surfactant uptake was not directly determined. Moreover, AM function differs between humans and mice; hence, further studies with AMs isolated from ADA-deficient patients or developed from induced pleuri-potent stem cells of ADA-deficient patients will be required.

In conclusion, we establish here that ADA-KO mice develop PAP, in association with adenosine, surfactant, and AM abnormalities, and that early restoration of blood ADA activity can prevent PAP and early lethality in ADA-deficient mice.

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METHODS

ADA-KO mice

ADA-KO mice (FVB, 129-*Ada*^{tm1Mw}-*TgN*[PLADA]) purchased from Jackson Laboratories (Bar Harbor, Me) and normal littermate control mice, maintained in a pathogen-free environment, received 2.5, 1, or 0.5 U/g body weight PEG-ADA (ADAGEN; ENZON Pharmaceuticals, Piscataway, NJ) or equivalent volume of PBS by intraperitoneal injections twice a week from age 7 days. Oxygen saturation in blood samples from the mice carotid artery was analyzed by using the ABL800 FLEX blood gas analyzer (Radiometer, Copenhagen, Denmark). All procedures were approved by the institute's Animal Care Committee and performed in accordance with the guidelines of the Canadian Council for Animal Care.

Histologic evaluation of lung tissue

Noninflated lung tissue was fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or periodic acid-Schiff as previously described.^{E1} Some sections were pretreated with diastase before periodic acid-Schiff staining. The numbers of alveoli in the hematoxylin and eosin lung sections were quantified in randomly chosen 2.8 mm² fields. Sections were visualized with the Nikon Ellipse TE2000-EE histology/fluorescence microscope and photographed by using the Hamamatsu AG camera. Analysis was performed by using Volocity software (PerkinElmer, Woodbridge, Ontario, Canada).

Electron microscopy

For electron microscopy, ultrathin sections were cut from lung tissue fixed in 2.5% glutaraldehyde, postfixed in 1% OsO₄, dehydrated, and embedded in Embed 812-araldite, as previously described.^{E2} Sections were stained in uranyl acetate and lead citrate and examined in a JEOL JEM 1011 transmission electron microscopy (JEOL USA, Peabody, Mass). Images were recorded with a CCD camera (AMT Corp, Danvers, Mass).

BALF

BALF samples were obtained by lavaging the lungs 4 times with 300 μ L cold PBS. The BALF was spun at 400g for 15 minutes at 4°C to separate the supernatant from the cells. Cells were counted by using a hemacytometer (Hausser Scientific, Horsham, Pa) or cytospun onto slides and stained with hematoxylin and eosin or Oil-Red-O as previously described.^{E3} Some cells were frozen rapidly in liquid nitrogen. Adenosine was extracted from frozen samples by using 0.4 N perchloric acid, separated, and quantitated by using high-pressure liquid chromatography and tandem-MS.^{E4}

Flow cytometry

Cells from the BALF were evaluated by using flow cytometry (FACScaliber from Becton Dickinson [BD], Franklin Lakes, NJ) and analyzed by using FlowJo software (Tree Star, Ashland, Ore) as previously described.^{E5} AMs were identified by using anti-CD11b, CD11c and Mac-3 antibodies

(Ebioscience, San Diego, Calif). Apoptosis was determined by using Annexin V-fluorescein isothiocyanate Apoptosis Detection Kit (BD).

Phagocytosis

The ability of 5×10^4 AMs isolated from the BALF by adherence to phagocytize 4×10^6 polystyrene beads was determined as previously described.^{E6} Briefly, 3.5- μ m beads (Bangs Laboratory, Fishers, Ind) preincubated with human 1 mg/mL IgG for 45 minutes were incubated with AMs for 30 minutes at 37°C. Cells were then washed extensively with serum-free media and cold PBS. Rabbit anti-human IgG conjugated to Cy3 (Jackson, Bar Harbor, Me) was added for 5 minutes, followed by PBS wash, 4% paraformaldehyde fixation, and nuclear counterstaining with DAPI (Vector Laboratories, Burlington, Calif). The slides were viewed and images were analyzed as described above for lung tissue. The phagocytic index was calculated by dividing the number of engulfed beads by the number of cells. The phagocytic index for ADA-proficient was assigned a value of 1.

Cholesterol and protein concentrations in the BALF

Total cholesterol and protein concentrations in the BALF were determined by using the fluorescent cholesterol assay kit (Caymen Chemicals, Ann Arbor, Mich) and the bicinchoninic acid assay (Thermo Fischer Scientific, Rockford, Ill), respectively, in accordance with the manufacturer's protocols.

Statistical analysis

Values are expressed as mean $\pm$ SD. The statistical significance of differences between the 2 groups was determined by using the 2-tailed Student *t* test by using Microsoft Excel software (Microsoft, Redmond, Wash). Differences among 3 or more groups were determined by using ANOVA with Tukey *post hoc* comparison adjustment for multiple comparisons using Stata software (StataCorp LP, College Station, Tex). A *P* value of less than .05 was considered statistically significant.

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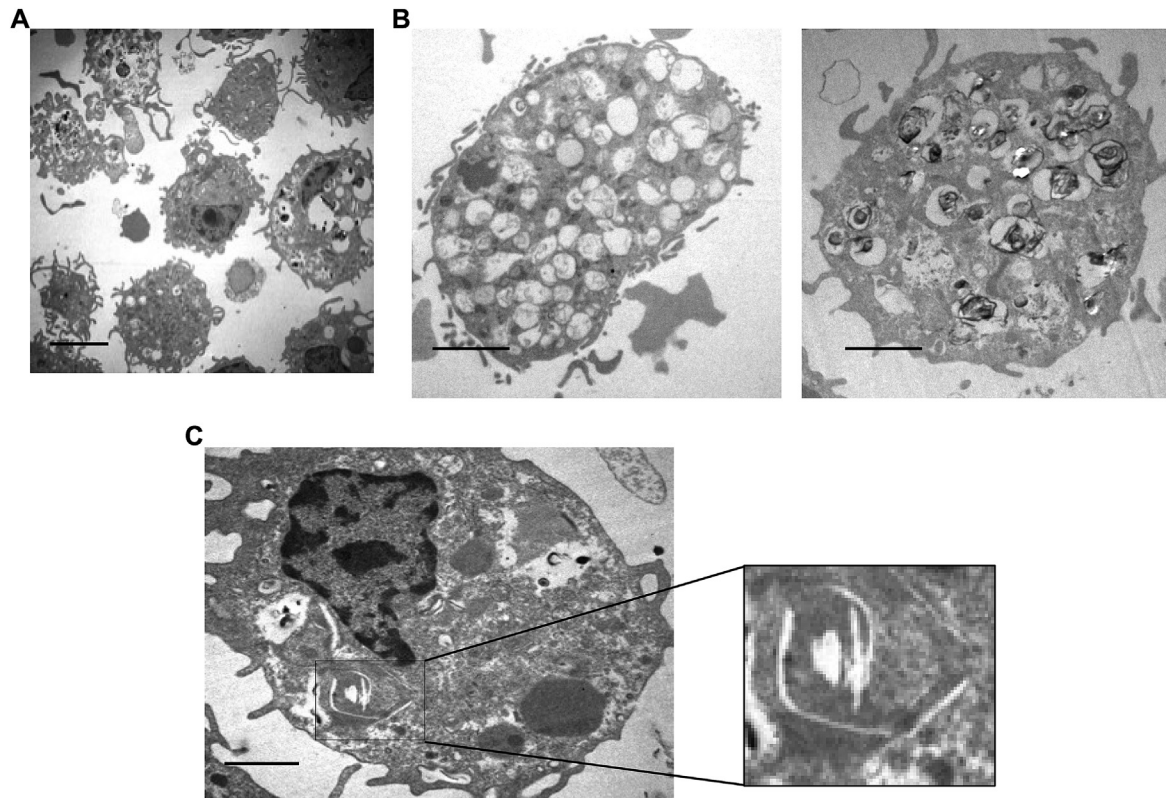


FIG E1. Alveolar macrophages from ADA-KO mice are foamy and contain inclusion bodies, lamellar structures, and cholesterol crystals. Electron microscopy of AMs from 16- to 18-day-old ADA-KO mice. **A**, AMs are foamy and filled by inclusion bodies ($\times 5,000$ magnification, *bar* = 10 μm). **B**, AMs from ADA-KO mice contain many concentric “lamellar-like” structures ($\times 10,000$ magnification, *bar* = 5 μm). **C**, Cholesterol crystals ($\times 20,000$ magnification, *bar* = 1 μm). Figures are representative of 40 independent images (N = 5 mice).

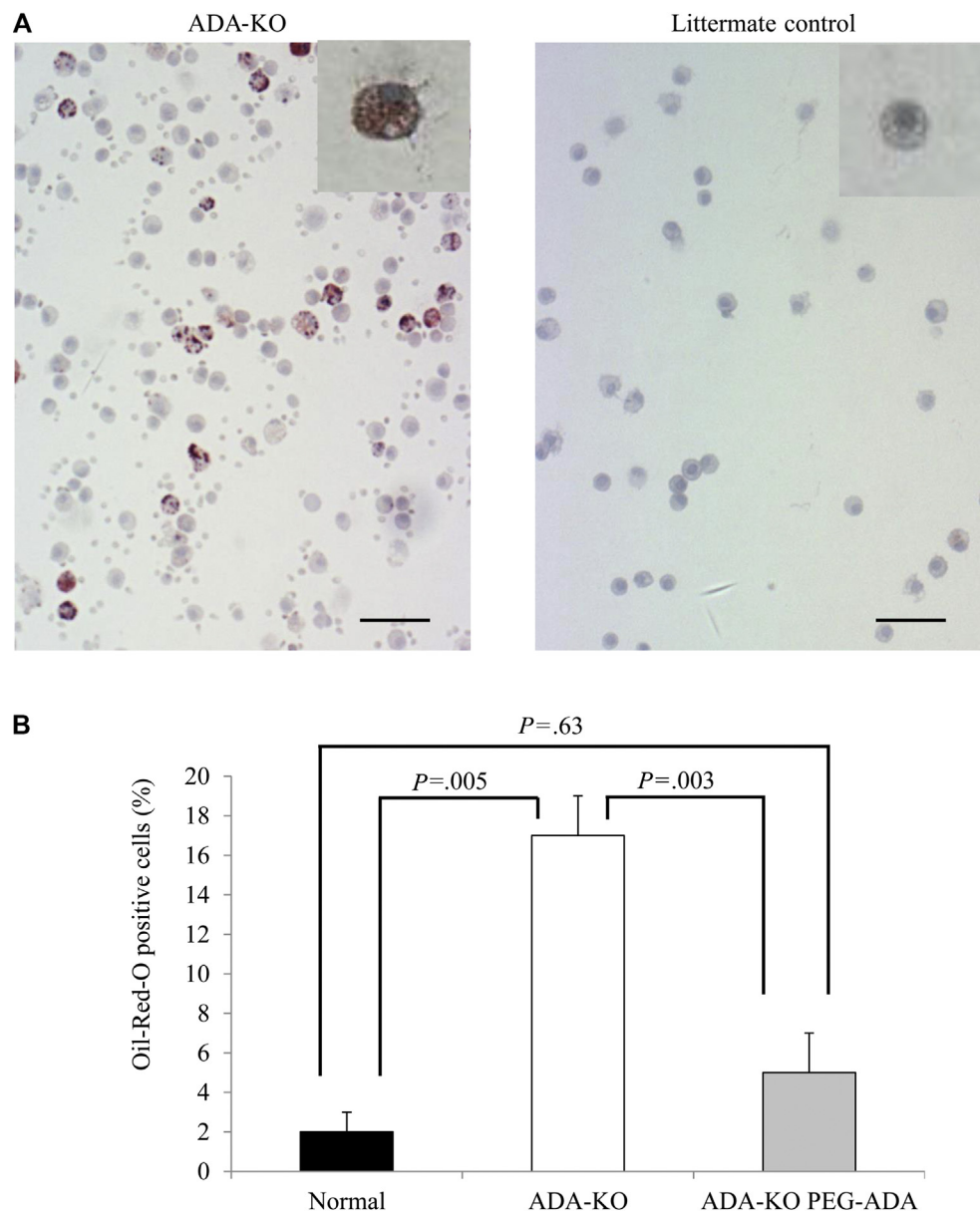


FIG E2. Increased Oil-Red-O (ORO) staining of AMs in the lungs of ADA-KO mice. **A**, Excessive ORO staining of AMs from ADA-KO mice compared with littermate control mice. Figures representative from N = 5 mice of each genotype ($\times 10$ magnification, $\text{bar} = 50 \mu\text{m}$). **B**, Percentage of ORO-stained cells among AMs from ADA-KO mice, ADA-KO mice treated with PEG-ADA, and littermate control mice. Graph bar represents mean $\pm$ SD of 500 cells for each group from 4 independent experiments.

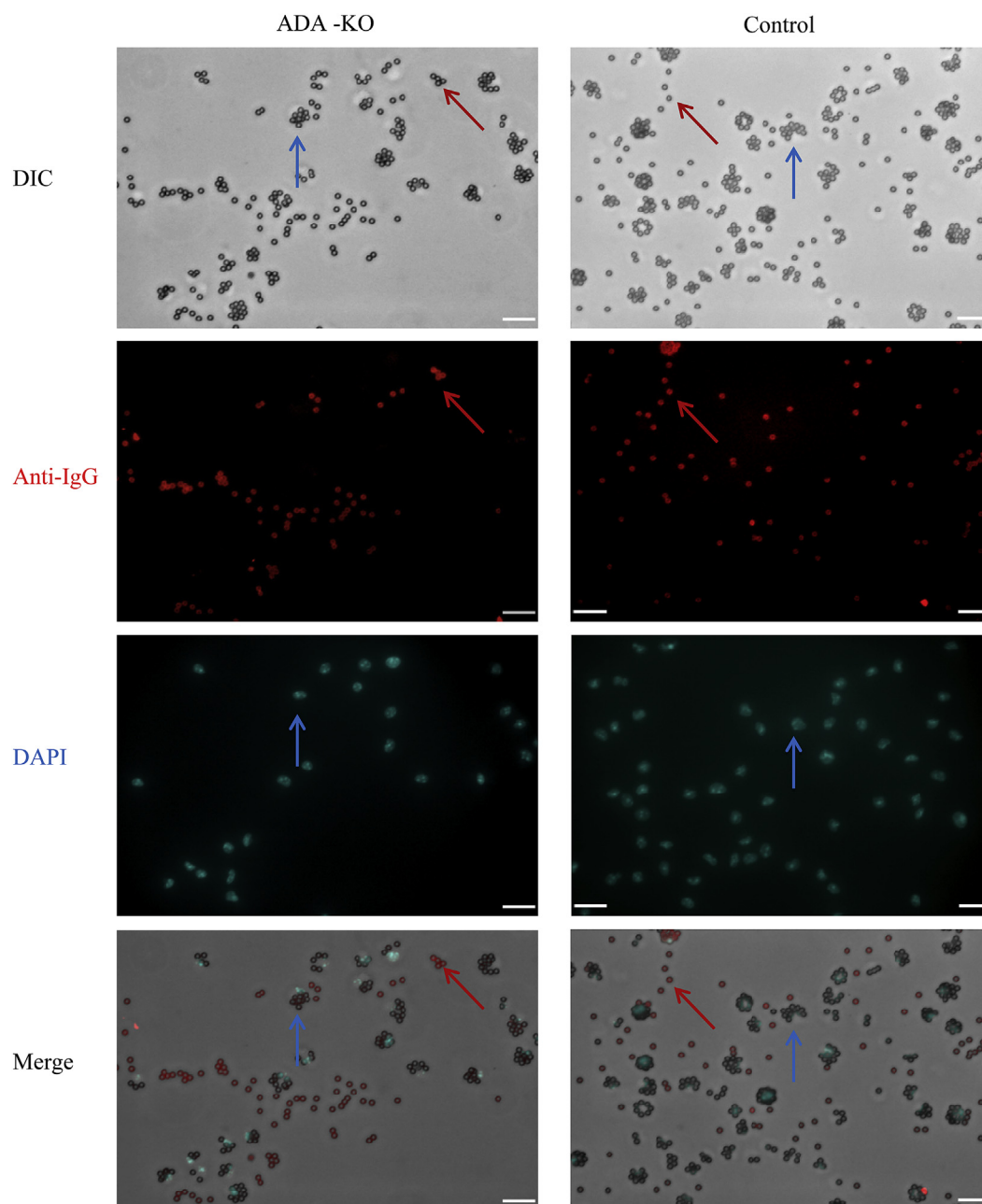


FIG E3. AMs of ADA-KO mice demonstrate normal phagocytosis. Uptake of IgG-coated beads by AMs from 16- to 18-day-old ADA-KO mice (*left*) is similar to that from littermate control mice (*right*). Images in the top row are differential interference contrast (DIC), the second row are Cy3-conjugated anti-IgG antibodies, and the third row are stained with 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI). The bottom row shows merged images. *Blue arrows* denote intracellular beads, and *red arrows* indicate extracellular beads. Figures are representative of more than 35 images from 3 independent experiments for each genotype ($\times 40$ magnification, *bar* = 40 μm).