A disintegrin and metalloproteinase domain-15 Deficiency Leads to Exaggerated Cigarette Smoke-induced Chronic Obstructive Pulmonary Disease (COPD)-like Disease in Mice

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Abstract

A disintegrin **a**nd **m**etalloproteinase domain-15 (ADAM15) is expressed by cells implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD), but its contributions to COPD are unknown. To address this gap, ADAM15 levels were measured in samples from cigarette smoke (CS)-versus air-exposed wild-type (WT) mice. CS-induced COPD-like disease was compared in CS-exposed WT, *Adam15*^{−/−}, and *Adam15* bone marrow chimeric mice. CS exposure increased Adam15 expression in lung macrophages and CD8^{+} T cells and to a lesser extent in airway epithelial cells in WT mice. CS-exposed *Adam15*^{−/−} mice had greater emphysema, small airway fibrosis, and lung inflammation (macrophages and CD8^{+} T cells) than WT mice. *Adam15* bone marrow chimera studies revealed that *Adam15* deficiency in leukocytes led to exaggerated pulmonary inflammation and COPD-like disease in mice. *Adam15* deficiency in CD8^{+} T cells was required for the exaggerated pulmonary inflammation and COPD-like disease in CS-exposed

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Adam15^{−/−} mice (as assessed by genetically deleting CD8^{+} T cells in *Adam15*^{−/−} mice). *Adam15* deficiency increased pulmonary inflammation by rendering CD8^{+} T cells and macrophages resistant to CS-induced activation of the mitochondrial apoptosis pathway by preserving mTOR signaling and intracellular Mcl-1 levels in these cells. These results strongly link ADAM15 deficiency to the pathogenesis of COPD.

Keywords

macrophage; CD8^{+} T cell; mammalian target of rapamycin; myeloid cell leukemia-1 (Mcl-1); mitochondria

Introduction

COPD is the 3rd leading cause of death worldwide[1]. COPD is characterized by airflow limitation that is poorly reversible and associated with an abnormal pulmonary inflammatory response to noxious particles or gases including those contained in cigarette smoke (CS)[2].

Airflow limitation in COPD is caused both by pulmonary emphysema and small airway disease[3]. Inhaling CS induces alveolar septal cell apoptosis[4] and stimulates the recruitment of leukocytes in the lung. Proteinases released by CS-activated leukocytes and epithelial cells degrade extracellular matrix (ECM) proteins within the alveolar walls to promote emphysema development[2].

Prior studies of proteinases in COPD have focused on the activities of matrix metalloproteinases (MMPs) and serine and cysteine proteinases[2,5,6]. Since the 1960s, proteinases were thought to exclusively promote the development of COPD[6,7]. However, this dogma was recently challenged when **a d**isintegrin **a**nd **m**etalloproteinase domain-8 (Adam8) was identified as the first proteinase that protects mice from developing CS-induced COPD-like disease[8]. ADAMs are type I transmembrane proteinases with multiple domains including: 1) a pro-domain which maintains latency of the metalloproteinase (MP) domain; 2) a MP domain which can shed proteins from cell surfaces; 3) disintegrin and cysteine-rich domains which can regulate cell adhesion and migration; 4) a surface-anchoring transmembrane domain; and 5) a cytoplasmic domain which can regulate intracellular signaling[9–11]. Whether other ADAMs regulate CS-induced COPD-like disease in mice is unclear.

ADAM15 has potential to contribute to the pathogenesis of COPD. Human ADAM15 and murine Adam15 are expressed by cells implicated in the pathogenesis of COPD including leukocytes, epithelial cells, and fibroblasts[2]. Although ADAM15 has minimal ECM protein-degrading activity[12], it has activities that could be relevant to the pathogenesis of COPD including shedding growth factor receptors[13], and regulating cellular adhesion and migration[12–14]. However, ADAM15's contributions to COPD have not been explored. We quantified and localized ADAM15 expression in the lungs of CS-versus air-exposed WT mice and compared COPD-like lung disease in CS-exposed WT versus *Adam15*^{−/−} mice and *Adam15* bone marrow (BM) chimeric mice. Our results show that *Adam15* deficiency promotes exaggerated COPD-like disease in CS-exposed mice by inciting an

excessive pulmonary inflammatory response by increasing the survival of CD8^{+} T cells and macrophages by inhibiting CS-induced activation of the mitochondrial apoptosis pathway by abrogating CS-induced inactivation of mTOR-Mcl-1 signaling in these cells.

Results

Adam15 expression is increased in the lungs of CS-exposed WT mice:

Exposing WT mice to CS for 1 month increased lung Adam15 transcript and protein levels (Fig. 1A–1B); levels increased further after 2 months of CS exposure but did not increase further after 6 months of CS exposure. Adam15 immuno-staining was detected in alveolar macrophages (AMs), CD8^{+} T cells, bronchial and alveolar epithelial cells, and α-smooth muscle actin (α-SMA)-positive cells around the small airways in WT mice that were exposed to CS for 6 months (Fig. E1).

Surface Adam15 staining levels were measured on AMs isolated from WT mice that were exposed to air or CS for 1 week. Acute CS exposure increased surface Adam15 levels on AMs ~3-fold (Fig. 1C). CS extract (CSE) induced concentration- and time-dependent increases in surface Adam15 protein levels on AMs isolated from unchallenged WT mice (Fig. 1D–1E). CD8^{+} T cells are increased in the lungs of CS-exposed mice and COPD patients and are required for CS-induced lung inflammation and airspace enlargement in mice[15–17]. We investigated whether CSE regulates surface Adam15 levels on CD8^{+} T cells. CSE induced concentration-dependent increases in surface Adam15 levels on CD8^{+} T cells isolated from unchallenged WT mice (Fig. 1F). Maximal increases in surface Adam15 levels occurred rapidly (within 30 min of adding CSE [(Fig. 1G]) as reported for release of perforin and granzyme B from the lytic granules of antigen-activated CD8^{+} T cells[15]. CSE also increased ADAM15 expression in human cells including *ADAM15* transcript levels in human bronchial epithelial cells (HBECs), and surface ADAM levels on monocytes and CD8^{+} T cells *in vitro* (Fig. 1H–1J)

Adam15^{−/−} mice develop exaggerated CS-induced COPD-like disease and pulmonary inflammation:

Adam15^{−/−} mice have normal lung development and a normal phenotype in the unchallenged state[18]. When exposed to CS for 6 months, *Adam15^{−/−}* mice had greater emphysema development (Fig. 2A–2B) and small airway fibrosis (Fig. 2C–2D), and more α-SMA-positive cells in their small airways (Fig. 2E–2F) than WT mice. WT and *Adam15^{−/−}* mice had similar CS-induced airway mucus metaplasia (Fig. E2).

Inhaling CS induces a chronic pulmonary inflammatory response in mice and humans which promotes airspace enlargement[2,15,19]. Thus, we evaluated whether the exaggerated airspace enlargement observed in CS-exposed *Adam15^{−/−}* mice is associated with increased pulmonary inflammation. CS-exposed *Adam15^{−/−}* mice had greater increases in bronchoalveolar lavage (BAL) total leukocyte, macrophage, and lymphocyte counts than CS-exposed WT mice (Fig. 3A–3C). *Adam15^{−/−}* mice had lower increases in BAL PMN counts than WT mice after 6 months CS exposure (Fig. E3A). Mean total blood leukocyte

and leukocyte subset counts were similar in air- and CS-exposed $Adam15^{-/-}$ and WT mice (Table E1).

When exposed to CS for 1 and/or 3 months, $Adam15^{-/-}$ mice had a higher percentage of CD4$^+$ lymphocytes (Fig. 3D), CD8$^+$ lymphocytes (Fig. 3E), and CD3-positive NKT cells (Fig. 3F) but a lower percentage of lung CD3$^+$CD4$^+$Foxp3$^+$ T regulatory cells (Tregs; Fig. 3G) in enzymatic lung digest samples than WT mice. The percentage of B cells in lung digest samples was lower in $Adam15^{-/-}$ mice than WT mice after 1 month of CS exposure, but similar after 3 months of CS exposure (Fig. 3H). $Adam15^{-/-}$ mice had a lower percentage of CD3-negative natural killer (NK) cells in enzymatic lung digest samples when exposed to air or CS for up to 3 months than WT mice (Fig. E3B). Flow cytometry dot plots are shown in Fig. E4.

Lung Ccl-2, Cxcl-10, Gm-csf, and Mmp-12 levels were higher in CS-exposed $Adam15^{-/-}$ versus WT mice (Fig. 4A–4D). Lung Il-10, Il-6, and Il-1β levels were lower in CS-exposed $Adam15^{-/-}$ versus WT mice (Fig. 4E–4G). Lung Tgf-β, Tnf-α, Ccl-5, M-csf, and Ccl-3 levels were similar in CS-exposed WT and $Adam15^{-/-}$ mice (Fig. E5). CS exposure increased lung levels of granzyme A (a CD8$^+$ T cell product which injures pulmonary epithelial cells[16]) in $Adam15$ but not WT mice (Fig. 4H).

Adam15 deficiency in leukocytes leads to exaggerated pulmonary inflammation and COPD-like disease:

Adam15 is expressed by leukocytes and lung parenchymal cells. Thus, $Adam15$ bone marrow (BM) chimeras were generated and exposed to air or CS for 6 months to identify the $Adam15$-deficient cells that drive the greater CS-induced COPD-like disease. $Adam15$ deficiency in leukocytes (not lung parenchymal cells) increased CS-induced emphysema development (Fig. 5A), small airway fibrosis (Fig. 5B) and lung inflammation as: 1) WT recipients transplanted with $Adam15^{-/-}$ BM had greater CS-induced emphysema, small airway fibrosis, and lung macrophage counts (Fig. 5C) than WT recipients transplanted with WT BM; and 2) $Adam15^{-/-}$ recipients transplanted with WT BM had reduced CS-induced emphysema development, small airway fibrosis, and macrophage counts than $Adam15^{-/-}$ recipients transplanted with $Adam15^{-/-}$ BM (Fig. 5A–5C).

As $Cd8^{-/-}$ mice are protected from CS-induced accumulation of macrophages in the lung and emphysema development[15], $Adam15^{-/-}xCd8^{-/-}$ compound mutant mice were generated and exposed to air or CS for up to 6 months. $Adam15$ deficiency in CD8$^+$ T cells was crucial for driving the enhanced pulmonary inflammation and emphysema development in $Adam15^{-/-}$ mice as CS-exposed $Adam15^{-/-}xCd8^{-/-}$ mice had lower BAL total leukocyte, macrophage, and lymphocyte counts and reduced emphysema development than CS-exposed $Adam15^{-/-}$ or WT mice (Fig. 6).

Adam15 is not required for CD8$^+$ T cell or macrophage adhesion or migration:

ADAM15 increases glomerular mesangial cell migration and cell-cell interactions of a fibroblast cell line[12,14]. Whether Adam15 regulates the adhesion of migration of leukocytes is unknown. WT and $Adam15^{-/-}$ macrophages and CD8$^+$ T cells did not differ in their capacity to adhere to an ECM protein (Fig. E6) or migrate towards chemoattractants (Fig.

E7) *in vitro*. Thus, it is unlikely that *Adam15* deficiency increases CS-induced pulmonary inflammation by increasing the trans-endothelial migration of monocytes or CD8^{+} T cells into the lung.

Adam15 deficiency in CD8^{+} T cells and macrophages abrogates CS-induced activation of the mitochondrial apoptosis pathway to increase cell survival by reducing CS-induced inactivation of mTOR signaling:

CS-induced apoptosis of leukocytes regulates leukocyte accumulation in the lung[2]. Compared with CS-exposed WT mice, CS-exposed *Adam15*^{–/–} mice had reduced rates of apoptosis in their alveolar macrophages (AMs) and CD8^{+} T cells (assessed as lower intracellular active caspase-3 levels; Fig. 7A and 7B, respectively) and reduced CS-induced activation of the mitochondrial apoptosis pathway in their AMs (as assessed by a lower percentage of cells that did not stain with the mitochondrial dye, JC-1 indicative of loss of mitochondrial membrane potential; Fig. 7C). CSE-treated macrophages and CD8^{+} T cells isolated from naive *Adam15*^{–/–} mice exhibited reduced activation of the mitochondrial apoptosis pathway *in vitro* (Fig. 7D–7E).

The PI3 kinase-Akt-mTOR pathway inhibits activation of the mitochondrial apoptosis pathway by increasing the expression of anti-apoptotic Bcl-2 family members, especially intracellular myeloid cell leukemia sequence-1 (Mcl-1) in macrophages and T cells[20,21]. Translocation of Bcl-2 proteins to the mitochondrial membrane prevents mitochondrial membrane depolarization and cytochrome C release[22]. The mTOR-Mcl-1 pathway pro-survival pathway was measured in CD8^{+} T cells isolated from the lungs mice that had been exposed to CS for 3 months, or in macrophages isolated from unchallenged mice and exposed to CSE *in vitro*. CD8^{+} T cells isolated from the lungs of *Adam15*^{–/–} mice had greater intracellular levels of phosphorylated ribosomal protein S6 (P-S6; a marker of mTOR signaling; Fig. 8A–8B) and higher intracellular levels of Mcl-1 (Fig. 8C–8D). CSE exposure substantially reduced intracellular levels of P-S6 (Fig. 8E) and Mcl-1 in WT but not *Adam15*^{–/–} macrophages *in vitro* (Fig. 8F–8G). Thus, *Adam15* deficiency promotes the survival of CD8^{+} T cells and macrophages in the CS-exposed lungs by rendering these cells resistant to CS-triggered activation of the mitochondrial apoptosis pathway by preserving their mTOR-Mcl-1 signaling when they are exposed to CS.

ADAM15 over-expression reduces CSE-induced activation of the mitochondrial apoptosis pathway in monocyte/macrophage-like cells:

ADAM15 was over-expressed in human differentiated THP-1 (monocyte-macrophage-like) cells using a vector containing an ADAM15 complementary DNA (cDNA) and electroporation. Robust *ADAM15* expression was detected using real-time quantitative PCR in THP-1 cells 24 h after electroporation but not in cells treated with the control vector (Fig. 9A). THP-1 cells over-expressing ADAM15 had increased CSE-induced activation of the mitochondrial apoptosis pathway (as assessed by a higher percentage of cells that did not stain with JC-1; Fig. 9B) and reduced intracellular MCL-1 protein levels compared with cells treated with the control vector (Fig. 9C–9D). Thus, ADAM15 overexpression promotes CSE-induced inactivation of mTOR-Mcl-1 signaling to increase the mitochondrial apoptosis pathway in monocyte/macrophage-like cells.

Discussion

This study links Adam15 to the pathogenesis of COPD-like disease, leukocyte function, and the mTOR pathway in CS-exposed mice for the first time. Acute CS exposure increased lung Adam15 transcript and protein levels in WT mice, and Adam15 was localized to pulmonary macrophages, CD8$^+$ T cells, epithelial cells, and airway α-ASM-positive cells in CS-exposed WT mice. When exposed to CS for 6 months, *Adam15$^{-/-}$* mice developed exaggerated pulmonary inflammation, emphysema development, and small airway fibrosis compared with WT mice. *Adam15* deficiency in BM-derived leukocytes (especially CD8$^+$ T cells) was crucial for driving the exaggerated COPD-like disease in CS-exposed *Adam15$^{-/-}$* mice. *Adam15* deficiency increased CD8$^+$ T cell and macrophage counts in the CS-exposed murine lung by increasing the resistance of these cells to CS-induced activation of the mitochondrial apoptosis pathway thereby increasing the survival of these cells in the lung. *Adam15* deficiency reduced activation of the mitochondrial apoptosis pathway in pulmonary CD8$^+$ T cells and macrophages by abrogating CS-induced inactivation of mTOR signaling in CD8$^+$ T cells and macrophages. The greater mTOR signaling in CS-exposed CD8$^+$ T cells and macrophages leads to higher intracellular levels of Mcl-1 (a Bcl-2 pro-survival protein) which translocates to the mitochondrial membrane to reduce loss of mitochondrial membrane potential, downstream caspase-3 activation, and apoptosis [Fig. 10]. Conversely, *ADAM15* over-expression in THP-1 cells increases CSE-induced inactivation of mTOR-MCL-1 signaling and promotes CSE-induced activation of the mitochondrial apoptosis pathway.

ADAM15 has not been studied previously in COPD. *ADAM15* gene expression is upregulated macrophages and fibroblasts when they are activated[9]. Although ADAM15 was reported not to be expressed by CD8$^+$ T cells in healthy subjects[23], we report increased Adam15 staining in CD8$^+$ T cells in CS-exposed WT lungs and on the surface of CSE-activated murine CD8$^+$ T cells *in vitro*.

The increased *Adam15* gene expression in the lungs of WT mice that were exposed acutely to CS could be mediated by the increased lung levels of pro-inflammatory mediators present in their lungs. (Epi)genetic processes could also increase *Adam15* expression in CS-exposed murine lungs. The murine Adam15 promoter contains three Sp1-binding sites[24]. CS increases Sp1 binding to the promoter regions of other genes to activate their transcription[25]. Whether Sp1 binding to the *Adam15* promoter contributes to the increased *Adam15* expression observed in CS-exposed WT murine lungs mice is unclear. Single-nucleotide polymorphisms in the *ADAM15* locus are linked to rheumatoid arthritis[26], but to our knowledge have not linked to human COPD. MicroRNAs (miRNAs) regulate gene transcription in human and murine COPD[27]. MiR-147b binds the 3′ untranslated region of the *ADAM15* mRNA in human endothelial cells to reduce *ADAM15* expression in these cells[28]. Whether other miRNAs increase *Adam15* expression in CS-exposed murine lungs will be investigated in our future studies.

CSE increased ADAM15 surface levels on human monocytes and CD8$^+$ T cells and *ADAM15* transcript levels in HBECs *in vitro* indicating that the expression data obtained from murine cells are translatable to humans. In publicly-available Gene Expression

Omnibus (GEO) microarray and *RNA* sequencing data-sets, *ADAM15* transcript levels were higher in bronchial epithelial cells from COPD patients versus non-smokers and smokers but were not related to GOLD stage (GSE5058 and GSE 63127; data not shown). *ADAM15* transcript levels in AMs were higher in smokers versus non-smokers, but not increased in AMs from COPD patients (GSE130928; data not shown).

CS-exposed *Adam15*^{-/-} mice developed exaggerated COPD-like disease suggesting that Adam15 has protective activities in lungs exposed chronically to CS. However, WT mice exposed to CS for up to 2 months had increased lung levels of Adam15. Heme oxygenase-1 (HO-1) is a cyto-protective protein which is upregulated during various acute insults in humans and mice[29,30] including hypoxia- and ischemia-reperfusion (IR)-induced tissue injury. However, *HO-1*^{-/-} mice have exaggerated disease compared with WT mice in these injury models[29,30]. While 2 months of CS exposure increased lung Adam15 protein levels in WT mice 7-fold, 6 month CS exposures did not further increase Adam15 lung levels. Thus, increases in Adam15 lung levels induced by acute CS exposures may represent an acute cyto-protective stress response similar to that observed in *HO-1*^{-/-} mice with acute hypoxia- or ischemia-reperfusion (IR)-induced tissue injury[31,32]. We hypothesize that the failure to upregulate Adam15 lung levels further during more chronic CS exposures contributes to inadequate protection of the lungs of WT mice from the deleterious effects of CS. The absence of Adam15 in the lungs of *Adam15*^{-/-} mice likely sensitizes them to the development of CS-induced COPD-like disease.

Studies of *Adam15* BM chimeras and *Adam15*^{-/-}x*Cd8*^{-/-} mice revealed that *Adam15* deficiency in leukocytes (especially CD8^{+} T cells) was responsible for the exaggerated pulmonary inflammation and COPD-like disease in CS-exposed *Adam15*^{-/-} mice. Macrophages are required for CS-induced emphysema development in mice[19,33] as their products (e.g. Mmp-12 and oxidants) injure the alveolar walls[34]. However, CD8^{+} T cells are required for macrophage accumulation and activation in the CS-exposed lung[15] (Fig. 10). CD8^{+} T cells release ligands (e.g. Cxcl-10) for Cxcr3 expressed on macrophages which induce macrophage activation and Mmp-12 release[15,16]. CD8^{+} T cells also induce alveolar epithelial cell apoptosis by releasing perforin and granzymes[15]. It is noteworthy that CS-exposed *Adam15*^{-/-} mice had elevated lung levels of Cxcl-10, granzyme A, and Mmp-12. CS-exposed *Adam15*^{-/-} mice also had reduced Treg counts in their lungs which may have contributed to their increased pulmonary inflammatory response to CS as Treg release anti-inflammatory molecules such as Il-10[35], and COPD patients have a blunted Treg response to CS[36].

Adam15 deficiency promotes pulmonary inflammation and emphysema development, in part, by reducing CS-induced activation of the mitochondrial apoptosis pathway in CD8^{+} T cells and macrophages thereby increasing their survival in the lung by abrogating CSE-induced reductions in mTOR signaling and downstream synthesis of Mcl-1 protein. Mcl-1 binds to and stabilizes the mitochondrial membrane thereby preventing CS-induced mitochondrial depolarization, cytochrome c release, and cellular apoptosis. *Adam15* deficiency in macrophages rendered these cells resistant to CSE-induced apoptosis *in vitro*. However, studies of CS-exposed *Adam15*^{-/-}x*Cd8*^{-/-} mice demonstrated that *Adam15* deficiency in CD8^{+} T cells was required for the increased lung inflammation (including

the increased lung macrophage counts) in CS-exposed $Adam15^{-/-}$ mice. CD8$^+$ T cells release Cxcl10 which activates mTOR signaling in other leukocytes by binding to Cxcr3 expressed on leukocytes and lung Cxcl-10 levels were increased in CS-exposed $Adam15^{-/-}$ mice[37]. We hypothesize that the increased numbers of apoptosis-resistant CD8$^+$ T cells in the lungs of CS-exposed $Adam15^{-/-}$ mice release increased quantities of ligands for Cxcr3 expressed by macrophages which, in turn, drives increased mTOR signaling (and survival) of macrophages in the lungs of $Adam15^{-/-}$ mice (Fig. 10). The mechanism by which $Adam15$ deficiency maintains mTOR signaling in CS-exposed CD8$^+$ T cells and macrophages is unclear, but Adam15 has a cytoplasmic tail which binds to intracellular proteins including Src kinases which regulate the PI3 kinase-Akt pathway upstream of mTOR signaling[38].

$Adam15$ deficiency may also increase macrophage counts in CS-exposed lungs by increasing lung levels of mediators that promote macrophage recruitment (Ccl-2) or survival (Gm-csf), or by decreasing lung levels of anti-inflammatory mediators including Il-10. The reduced lung Il-10 levels in CS-exposed $Adam15^{-/-}$ mice may be due to their reduced pulmonary Treg counts as Il-10 is a Treg product[35]. To our knowledge this is the first report linking deficiency of an Adam family member to Treg counts in any organ.

$Adam15$ deficiency increased CS-induced small airway fibrosis and the numbers of α-SMA-positive cells (which could be myofibroblasts or smooth muscle cells) in the small airways of mice. $Adam15$ expression was increased in small airway α-SMA-positive cells in CS-exposed WT mice, and human ADAM15 increases human fibroblast migration and survival[14,39]. Thus, $Adam15$ deficiency in myofibroblasts may promote exaggerated small airway fibrosis in CS-exposed $Adam15^{-/-}$ mice by increasing myofibroblast activation, survival, and/or ECM protein deposition. Although Tgf-β promotes small airway fibrosis in mice[34], CS-exposed WT and $Adam15^{-/-}$ mice had similar lung Tgf-β levels. The greater CS-induced small airway fibrosis in $Adam15$ mice was linked to their greater lung inflammation by BM transplant experiments. It is noteworthy that macrophage products promote small airway remodeling in CS-exposed rodents[40].

A limitation of this study is that we employed only a loss-of-function approach in mice. Some of our findings could be due to compensatory changes in lung levels of other proteinases in $Adam15^{-/-}$ mice. Small molecules inhibitors which are selective for Adam15 do not exist. However, our $ADAM15$ over-expression results in THP-1 cells support our hypothesis that ADAM15 has protective activities in CS-exposed lungs by limiting the survival of leukocytes in the lung. Future studies will employ gain-of-function approaches in CS-exposed mice to test the hypothesis that Adam15 has protective activities in the CS-exposed lung. We did not fully elucidate the mechanisms by which Adam15 regulates small airway fibrosis or the contributions of PMN-derived Adam15 to CS-induced COPD-like disease which will be explored in future studies. Our *in vitro* studies evaluated the effects of CSE on leukocytes and epithelial cells using CSE concentrations that were optimized for each cell type and readout. These studies only provide information on the effects of aqueous-soluble CS components, and it is not possible to relate the CSE concentrations that were tested to concentrations of gaseous-phase CS which contains other noxious components.

Our results suggest that strategies that increase lung levels of ADAM15 have therapeutic potential in COPD. However, increased expression of ADAM15 has been detected in various tumors including non-small cell lung cancer and is linked to tumor cell migration and metastasis by increasing MMP-9 expression[41,42]. Additional studies are needed to evaluate ADAM15 as a potential therapeutic target for COPD.

In conclusion, our results strongly link *Adam15* deficiency to the pathogenesis of COPD-like disease in mice and mTOR signaling in pulmonary CD8$^+$ T cells and macrophages in CS-exposed mice for the first time. In CS-exposed mice, *Adam15* deficiency leads to exaggerated COPD, in part, by increasing the pulmonary inflammatory response to CS by protecting CD8$^+$ T cells and macrophages from CS-induced activation of the mitochondrial apoptosis pathway by preserving mTOR-Mcl-1 signaling in these leukocytes.

Methods

Ethics

Studies conducted on mice were approved by the Institutional Care and Use Committee at Brigham and Women's Hospital (BWH). Studies conducted on human subjects were approved by the BWH Institutional Review Board.

Mice: *Adam15* mice were obtained from Dr. CP Blobel (Cornell University, New York, NY) and backcrossed into a pure C57BL/6 strain (to the F8 generation). *Adam15*$^{-/-}$ mice have normal lung development and no abnormal phenotype in the unchallenged state[18]. Pure strain C57BL/6 WT mice and *Cd8*$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Adam15*$^{-/-}$x*Cd8*$^{-/-}$ mice were generated by breeding *Adam15*$^{-/-}$ mice and *Cd8*$^{-/-}$ mice. Genotypes were confirmed using PCR methods performed on DNA extracted from tail biopsies. Mice were housed under specific pathogen-free conditions until they were 8-12 weeks of age before experiments were conducted. Male and female were studied and randomized to the experimental groups by an observer not involved in the conduct of the experiments.

Adam15 bone marrow (BM) chimeric mice: Unchallenged C57BL/6 WT and *Adam15*$^{-/-}$ recipient mice aged 10-12 weeks were lethally-irradiated (using 450 centigray dose from a ^{137}Cesium source[8,43,44] twice 4 h apart) and then received complete BM (erythrocytes and leukocytes) containing two million leukocytes from non-irradiated donor mice *via* a tail vein injection. Four groups of *Adam15* BM chimeras were generated by transplanting: 1) WT BM into WT recipients; 2) WT BM into *Adam15*$^{-/-}$ recipients; 3) *Adam15*$^{-/-}$ BM into WT recipients; and 4) *Adam15*$^{-/-}$ BM into *Adam15*$^{-/-}$ recipients. Mice were house for 10 weeks to permit engraftment of donor BM and then 6 month air and CS exposures were initiated and lungs were removed (at ~46 weeks of age)[8].

Cigarette smoke (CS) exposures: Mice were exposed to air or mixed mainstream and side-stream CS from 3R4F Kentucky Research cigarettes for 2 h/day 6 days-a-week in Teague TE 10z chambers for 1-6 months[45].

CS extract (CSE): Smoke from two 3R4F cigarettes was bubbled through 20 ml of DMEM medium yielding a 100% CSE solution which was filter sterilized (0.2 μm syringe filter), diluted, and used within 5 min of preparation[7,8]. Pilot experiments optimized the CSE concentrations needed to induce Adam15 expression or apoptosis for each cell type studied.

Adam15 protein and mRNA levels: Adam15 protein levels were measured in homogenates of WT lungs using an ELISA (CUSABIO, College Park, MD) and normalized to total protein levels measured in the same samples using a kit (Thermo-Fisher Scientific, Rockford, IL). *Adam15* mRNA levels were measured using a SYBR green-based real-time PCR assay with *Ppia* as the housekeeping gene (Table E2) and the comparative threshold method[7].

Immunostaining of alveolar macrophages (AMs) and CD8+ T cells for surface Adam15: AMs were isolated from murine lungs using BAL. Macrophages were isolated from the peritoneal cavities of mice 4 days after intraperitoneal injection of 40 mg of thioglycollate in 1 ml of DMEM medium using peritoneal lavage. Macrophages were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h in 8 well Lab-Tek® Chamber slides™ to render them quiescent. Macrophages were then incubated at 37°C for 8-48 h with 0-5% CSE.

CD8+ lymphocytes were isolated from the spleens of unchallenged mice using EasySep™ Mouse CD8+ T Cell Isolation Kits (STEMCELL Technologies, Cambridge, MA). CD8+ T cells were incubated at 37°C for up to 2 h with 0-2.5% CSE. Cells were fixed for 3 min at 4°C in 3% paraformaldehyde and 0.25% glutaraldehyde in PBS, and immunostained for surface Adam15 using rat anti-Adam15 IgG or non-immune rat IgG followed by Alexa-488-conjugated goat anti-rat F(ab)$_2$. Cell surface immunofluorescence was quantified on images of the cells using MetaMorph software (Molecular Devices LLC, Sunnyvale, CA)[7,46].

ADAM15 mRNA levels in HBECs: Normal HBECs (Lonza, Allendale, NJ) were incubated for 4-24 h with or without 2.5% CSE. *Adam15* mRNA levels were measured using quantitative real-time-PCR using *PPIA* as the housekeeping gene (Table E2).

Immunostaining of human leukocytes for surface ADAM15: Monocytes and CD8+ T cells were isolated from the blood of healthy donors by positive section for CD14 or CD8α using immunomagnetic beads (STEMCELL Technologies). Monocytes and CD8+ T cells were incubated with and without CSE and then immunostained for surface ADAM15, as described in the online supplement.

COPD-like disease: Mice were exposed to air or CS for 6 months, and serum samples were obtained post-euthanasia *via* right ventricular puncture. Lungs were inflated to 25 cm H$_2$O pressure and fixed in a 10% buffered formalin. Distal airspace size or fibrosis around small airways having a mean diameter of 300-699 microns was quantified on Gill's-stained or Masson's Trichrome-stained lung sections, respectively, using morphometric methods[7,8]. Lung sections were immunostained for airway epithelial α-SMA (a myofibroblast or smooth muscle cell marker)[7].

BAL leukocyte counts: Total and differential leukocyte counts were performed on BAL samples[47].

Lymphocyte subsets in enzymatic lung digests: Mice were exposed to air or CS for 1-3 months. Lung tissue was digested with 1 mg/ml collagenase D (Sigma Aldrich, St. Louis, MO) in medium at 37°C for 45 min. The digests were passed through a 70 μm strainer and washed with cold PBS containing 0.5% bovine serum albumin. The cells were fixed and permeabilized using the Foxp3 Staining Buffer Set (eBioscience, San Diego, CA). Cells were incubated with Fc block solution (Biolegend, San Diego, CA), and incubated with antibodies (Table E3) or isotype-matched control antibodies (rat IgG1κ, IgG2aκ, or IgG2bκ) for 45 min at 4°C (Table E3) and washed. After dead cell and doublet exclusion, lymphocyte subsets were identified as follows: a) $CD4^+$ T cells ($CD45^+CD3^+CD4^+$), b) T regulatory cells (Tregs; $CD45^+CD3^+CD4^+$ Foxp3$^+$), c) $CD8^+$ ($CD45^+CD3^+CD8\alpha^+$), c) B cells ($CD45^+B220^+$), f) CD3-negative natural killer cells ($CD45^+CD3^-Nk1.1^+$), or g) CD3-positive NKT cells ($CD45^+CD3^+NK1.1^+$). Cells were identified and quantified using flow cytometry with a BD LSR Fortessa Cell Analyzer (San Jose, CA) and counting beads (ThermoFisher, Waltham, MA). Data were analyzed with FlowJo software (Tree Star Inc., Palo Alto, CA).

Lung mediator levels: Mediators were measured in lung homogenates using ELISA kits and normalized to lung total protein levels measured on the aliquots of the same samples[7].

Leukocyte apoptosis: AMs and splenic $CD8^+$ T cells were isolated from unchallenged mice, as described above, and induced to undergo apoptosis by incubating them at 37°C with up to 10% CSE for up to 24 h at 37°C. Cells were fixed (as described above), permeabilized with 100% methanol at 4°C for 20 min, and immunostained for intracellular active caspase-3 using Alexa-488-conjugated rabbit anti-caspase-3 IgG or Alexa-488-conjugated rabbit IgG as a control (Cell Signaling, Beverly, MA). The mean integrated fluorescence of 200 cells/ group was quantified using MetaMorph software. Data were corrected for non-specific staining in the presence of the non-immune antibody[48]. Other aliquots of cells were immediately stained for 30 min at 4°C with JC-1 dye using a Mitoprobe® kit (Thermo-Fisher Scientific, Rockford, IL). JC-1 staining in >500 cells/group was measured using MetaMorph software.

Ribosomal protein S6 phosphorylation in bone marrow-derived macrophage (BMDMs): BMDMs were isolated from naïve mice[7,49], cultured in 8-well chamber-slides in L929-conditioned medium for 7 days. The media were changed to complete DMEM medium (DMEM [Corning, Manassas, VA] supplemented with 10% heat-inactivated FBS [Sciencell Research Laboratories, Carlsbad, CA], 100 units/ml penicillin, and 100 μg/mL streptomycin [ThermoFisher Scientific, Waltham, MA]). Cells were incubated with or without 10% CSE and, at intervals, were fixed and incubated with rabbit antimurine phospho-S6 (Ser$^{235/236}$) IgG (Cell Signaling Technology, Beverly, MA), or non-immune rabbit IgG (Dako, Santa Clara, CA) followed by Alexa-488-conjugated goat anti-rabbit F(ab)$_2$ (Invitrogen, Charlestown, MA). MetaMorph software was used to measure intracellular staining for phospho-S6.

Myeloid cell leukemia sequence-1 (Mcl-1) protein levels in CSE-treated BMDMs: BMDMs were isolated from naïve mice, and then cultured to render them quiescent[7]. Cells were incubated with or without 10% CSE and lyzed at intervals in radioimmunoprecipitation assay (RIPA) buffer containing proteinase inhibitors. Western Blot analysis for Mcl-1 and heat shock protein 90 (Hsp90; as a loading control) was performed and Mcl-1 signals were normalized to Hsp90 signals[7].

Ribosomal protein S6 phosphorylation and Mcl-1 protein levels in pulmonary CD8$^+$ T cells: Mice were exposed to air or to CS for 3 months. CD8$^+$ T cells were isolated from enzymatic digests of lungs, as outlined above. Cells were immediately lyzed in RIPA buffer containing proteinase inhibitors and PhosSTOP (Sigma, St. Louis, MO). Western Blot analysis for Mcl-1, phospho-S6 (Ser$^{235/236}$), total S6, and heat shock protein 90 (Hsp90; as a loading control) was performed. Mcl-1 signals were normalized to Hsp90 signals, phospho-S6 (Ser$^{235/236}$) signals were normalized to total S6 signals[7].

Overexpression ADAM15 in human THP-1 cells: Human ADAM15 cDNA (isoform 6 pre-proprotein as the longest transcript variant) amplified by RT-PCR was subcloned into the BamHI and XhoI sites of pcDNA3.1 vector to obtain pcDNA-ADAM15 (the primer sequences used for cloning were: forward: 5′ -CGCGGTACCATGCGGCTGGCGCTGCTCTGGG-3'; reverse: 5′ -CCGCTCGAGTCAGAGGTAGAGCGAGGACACT-3'). Human THP-1 monocytes (from ATCC) were maintained in RPMI-1640 containing 10% FBS and differentiated into macrophage-like cells by incubating them with 150 nM phorbol myristate acetate for 24 h. Cells were electroporated (Neon Transfection System; Thermo Fisher Scientific, Waltham, MA) with the ADAM15 or control (pcDNA3.1) vector[50]. *ADAM15* expression was measured using qRT-PCR and the THP-1 cells were treated with 10% CSE for up to 24 h. JC-1 staining and intracellular levels of MCL-1 were measured as described above.

Statistics: Data are presented as box-plots showing medians and 25th and 75th percentiles and whiskers showing 10th and 90th percentiles (for data that were not normally distributed) or mean ± SD (or SEM; for data that were normally distributed). Data were analyzed with Kruskal-Wallis One-Way ANOVA followed by pair-wise comparisons using Mann-Whitney U-tests (non-parametric data), or One-Way ANOVAs followed by pair-wise comparisons using two-tailed Student's t-tests (parametric data). *P* values ≤ 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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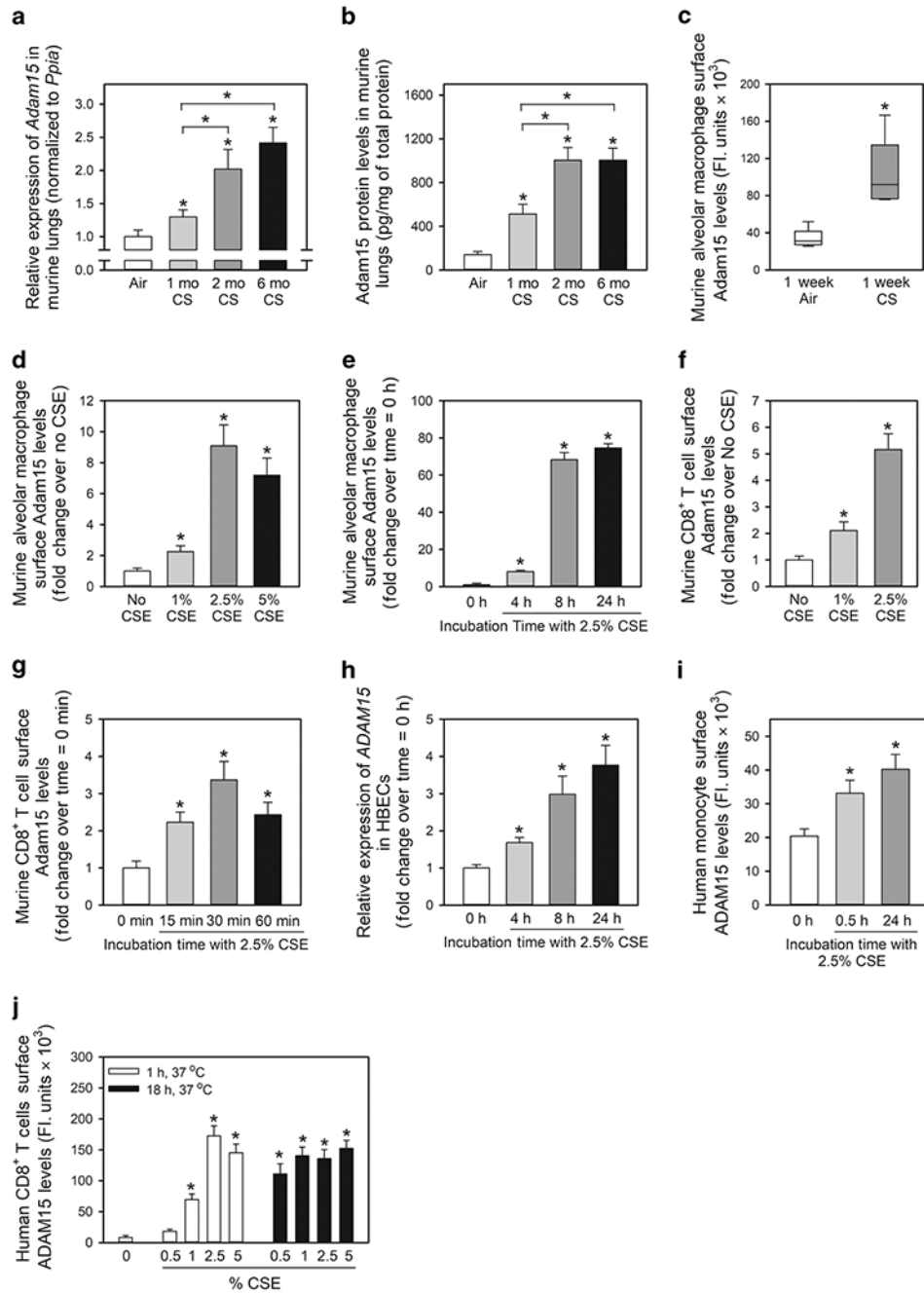
Figure 1: A Disintegrin and A Metalloproteinase Domain 15 (Adam15) expression is induced in the lungs of wild-type (WT) mice exposed to cigarette smoke (CS) and is upregulated on the surface of macrophages, monocytes and CD8+ T cells exposed to CS extract (CSE) in vitro.
In **A** and **B**, Adam15 steady state mRNA and protein levels were measured in the lungs of C57BL/6 WT mice that were exposed to air or CS for up to 6 months using quantitative real-time PCR and an ELISA, respectively (n = 9-10 mice/group). The bar graphs show means ± SD. Data were analyzed using one-way ANOVAs followed by pair-wise testing with two-tailed Student's t-tests. *, P 0.004 versus air-exposed mice belonging to the same genotype control or the group indicated. In **C**, C57BL/6 WT mice were exposed to

air or CS for 1 week, and alveolar macrophages (AMs) were isolated using bronchoalveolar lavage. Surface Adam15 protein levels were quantified on AMs using immunostaining techniques, as described in Methods. The boxes in the box-plots show the medians and 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles; n = 5 mice/group with 200-300 cells analyzed/mouse. Data were analyzed using a Kruskal-Wallis One-Way ANOVA followed by pair-wise testing with Mann-Whitney U tests. *, $P = 0.008$ versus air group. In **D-E**, AMs were isolated from naïve WT mice and incubated for 24 h at 37°C with varying concentrations of CS extract (CSE; **D**) or for varying times with 2.5% CSE (**E**). Cells were then fixed and immunostained for surface Adam15, as described in Methods. Data are mean ± SEM; n = 150-200 cells/group. The experiments shown are representative of at least three independent experiments yielding similar results. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.001$ compared to unstimulated cells. In **F-G**, CD8+ lymphocytes were isolated from the spleens of naïve WT mice and incubated at 37°C for 2 h with varying concentrations of CSE (**F**) or with or without 2.5% CSE for varying times up to 60 min (**G**). Cells were fixed and immunostained for surface Adam15. Data are mean ± SEM; n = 150-200 cells/group. The experiments shown are representative of at least three independent experiments yielding similar results. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.001$ compared to unstimulated cells. In **H**, steady state *ADAM15* mRNA levels were measured in normal human bronchial epithelial cells (HBECs) incubated at 37°C for varying times with or without 2.5% CSE using quantitative realtime PCR. Data are mean ± SD; n = 3 experiments. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.03$ versus cells incubated without CSE. In **I** and **J**, human monocytes and CD8+ T cells were isolated from the blood of healthy donors by positive section for CD14 or CD8α using immunomagnetic beads. Monocytes and CD8+ T cells were incubated with or without CSE for varying times and/or with concentrations of CSE. Cells were fixed and non-permeabilized cells were immunostained for surface ADAM15 as described in Methods. Data are mean ± SEM; n = 150-200 cells/group. The experiments shown are representative of at least three independent experiments yielding similar results. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.001$ compared to unstimulated cells.
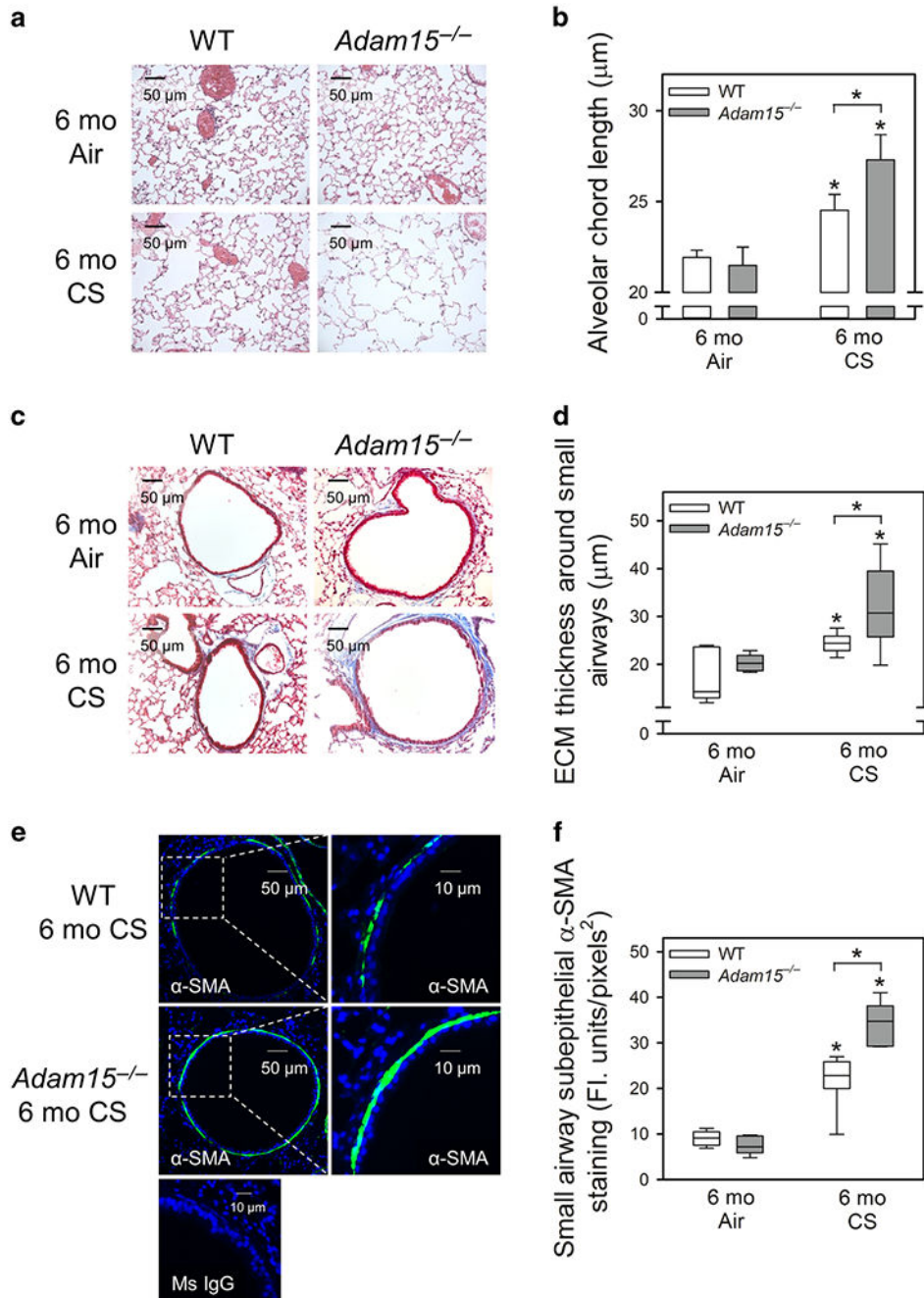
Figure 2: Adam15 deficiency in mice increases CS-induced emphysema development and small airway fibrosis.

Adult *Adam15*^{−/−} mice and WT mice were exposed to air or CS 6 days-a-week for 6 months (Mo). **A**: Images of hematoxylin and eosin-stained inflated lung sections from mice. **B**: Airspace size was quantified in Gills-stained lung sections using Scion image analysis software, as described in the Methods. The mean chord length is the average of the horizontal and vertical distances between the alveolar walls. Data are mean ± SD; n = 8-11 mice exposed to air and 11-14 mice exposed to CS. Data were analyzed using a One-Way

ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, P 0.035 versus the same genotype exposed to air or the group indicated. **C** shows representative images of extracellular matrix (ECM) deposition around small airways (300-699 μm in diameter) in Masson Trichrome-stained lung sections from WT and *Adam15*$^{-/-}$ mice exposed to air or CS for 6 months. **D**: The thickness of the ECM protein layer deposited around small airways was quantified using morphometry methods, as described in the Methods. The boxes in the box-plots show the medians and 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles; n = 7-10 mice/group. Data were analyzed using a Kruskal-Wallis One-Way ANOVA followed by pair-wise testing with Mann-Whitney U tests. *, P 0.038 versus the same genotype exposed to air or the group indicated. In **E**, murine lung sections were immunostained for α-smooth muscle actin (α-SMA, a marker of myofibroblasts or smooth muscle cells) around small airways of WT and *Adam15*$^{-/-}$ mice that were exposed to air or CS for 6 months. Nuclei were counterstained blue with 4′,6-diamidino-2-phenylindole. As a control, lung sections were stained with a non-immune murine (Ms) IgG (bottom panel). The merged images shown in **E** are representative of 5-7 mice/group. **F**: Box-plots of α-SMA staining that was quantified per unit area of the walls of small airways having a luminal diameter of 300-699 μm using MetaMorph software (5 air-exposed mice/group and 6-7 CS-exposed mice/group). The boxes in the box-plots show the medians and 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles. Data were analyzed using a Kruskal-Wallis One-Way ANOVA followed by pair-wise testing with Mann-Whitney U tests. *, P 0.005 versus air-exposed mice belonging to the same genotype or the group indicated.
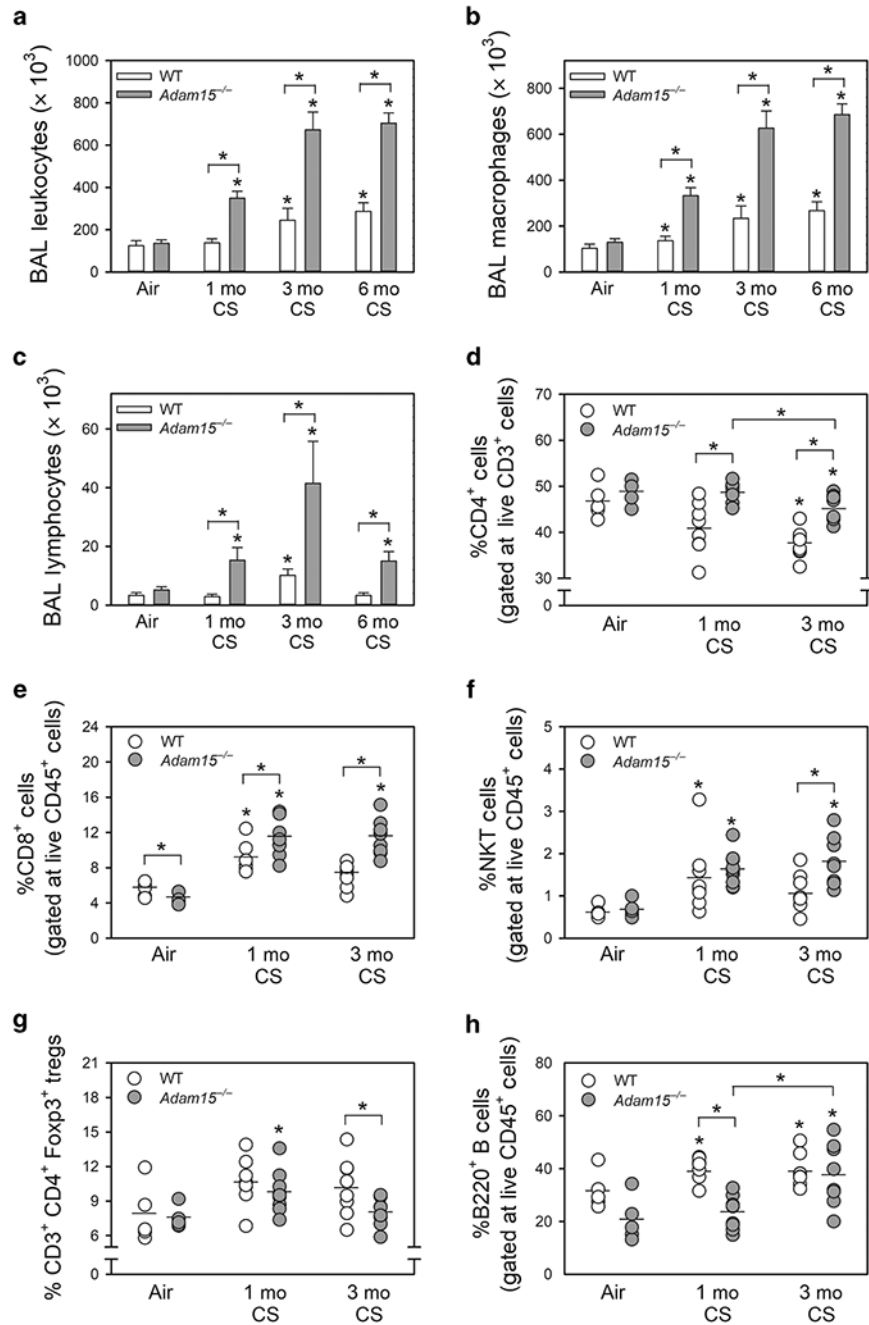
Figure 3: Deficiency of Adam15 in mice increases CS-induced lung inflammation.
Wild-type (WT) and *Adam15*^{−/−} mice were exposed to air or CS for 1 month (Mo),
3 months or 6 months. Absolute numbers of all leukocytes (**A**), macrophages (**B**), and
lymphocytes (**C**) were counted in bronchoalveolar lavage (BAL) samples. Data are mean ±
SEM; n = 12-14 mice exposed to air and 6-11 mice exposed to CS. Data were analyzed
using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *,
P 0.005 versus the same genotype exposed to air or the group indicated. In **D-H**, WT and
Adam15^{−/−} mice were exposed to air or CS for 1 or 3 months, lungs were enzymatically

digested, and leukocytes were immunostained for markers of lymphocyte subsets including CD4$^+$ lymphocytes (**D**), CD8$^+$ lymphocytes (**E**), CD3-positive NKT cells (**F**), Tregs (**G**), and B cells (**H**). Lymphocyte subsets were quantified using flow cytometry. Data are mean ± SD; n = 5 mice exposed to air and 8-9 mice exposed to CS. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P <$ 0.05 air-exposed mice belonging to the same genotype or the group indicated.
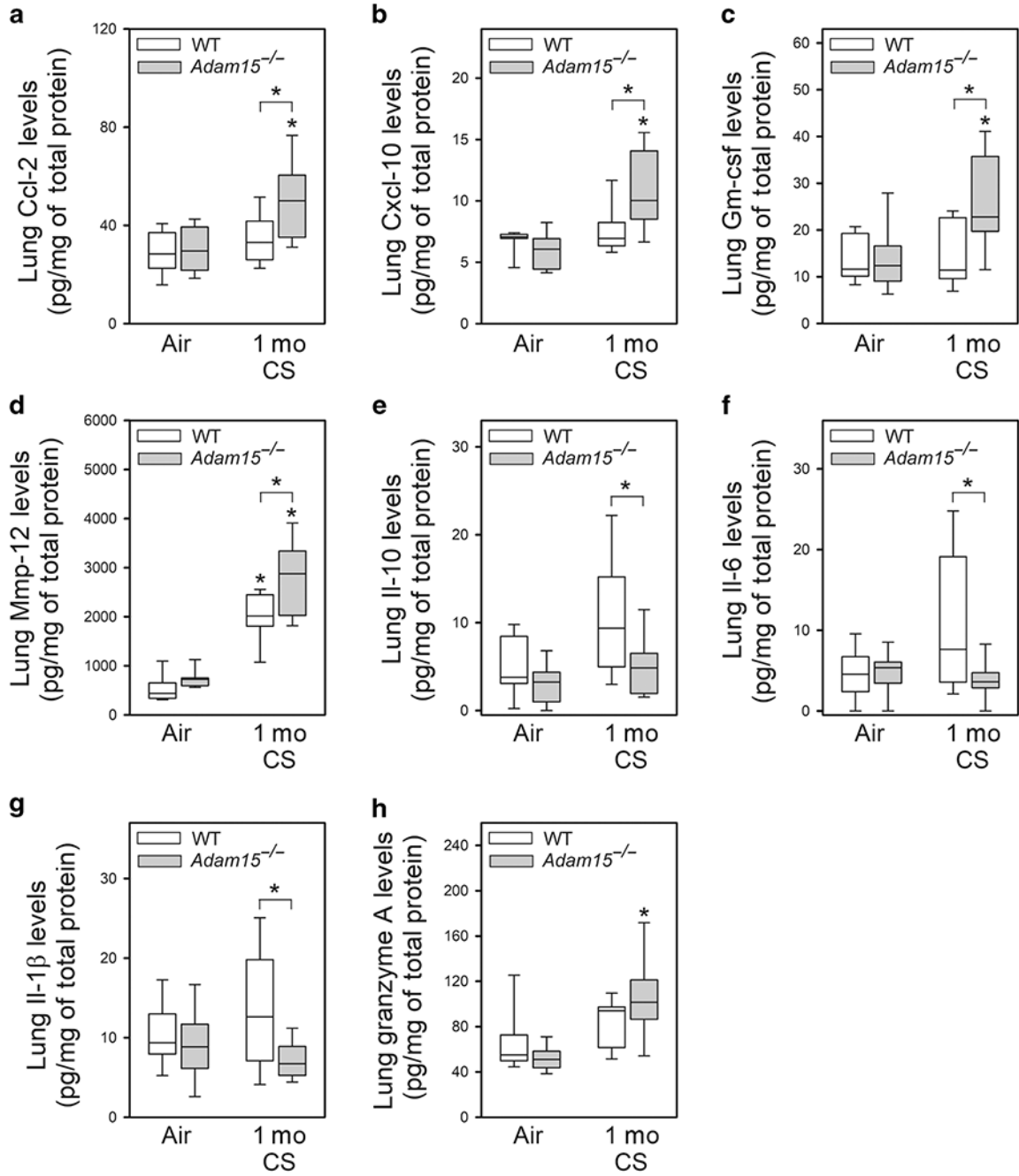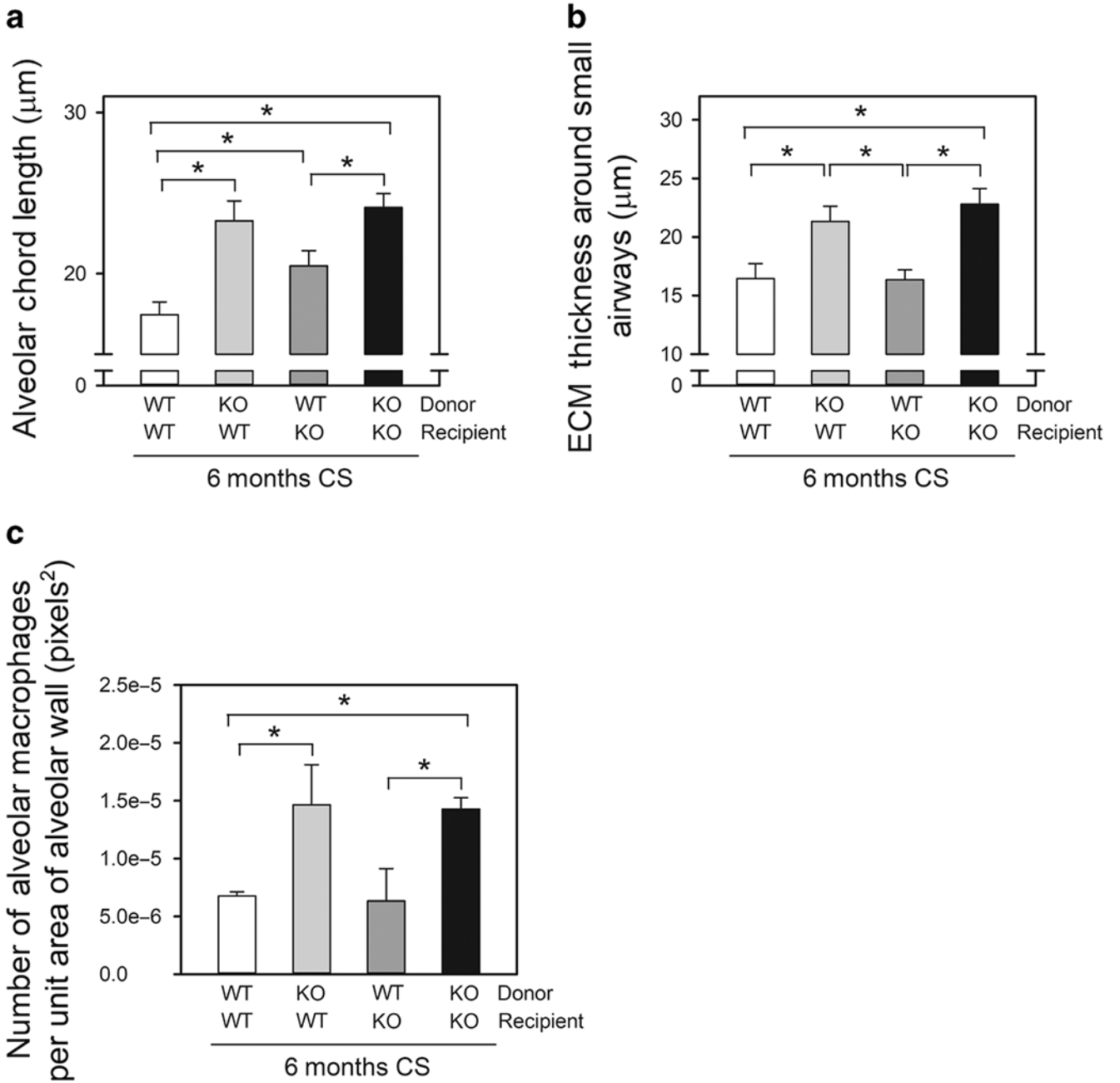
Figure 4: Deficiency of Adam15 in mice increases lung levels of mediators that promote CS-induced lung inflammation and destruction.

WT and *Adam15*^{−/−} mice were exposed to air or CS for 1 month (Mo), lung levels of Ccl-2 (**A**; n = 10-12 mice/group), Cxcl-10 (**B**; n = 7 mice/group), Gm-csf (**C**; n = 10-12 mice/group), Mmp-12 (**D**; n = 8 mice/group), Il-10 (**E**; n = 10-12 mice/group), Il-6 (**F**; n = 14-22 mice/group), Il-1β (**G**; n = 13-22 mice/group) and granzyme A (**H**; n = 8 mice/group) were measured in homogenates of lung samples using commercial ELISA kits. Data were normalized to total protein levels measured in the same samples. The boxes in the

box-plots show the medians and 25th and 75th percentiles, and the whiskers show the 10th and 90th percentiles. Data were analyzed using a Kruskal-Wallis One-Way ANOVA followed by pair-wise testing with Mann-Whitney U tests. *, P ≤ 0.025 versus air-exposed mice belonging to the same genotype or the group indicated.

Figure 5: Deficiency of Adam15 in bone marrow (BM)-derived leukocytes drives the exaggerated COPD-like disease detected in CS-exposed Adam15⁻/⁻ mice.

In **A-C**, 4 groups of *Adam15* BM chimeric mice (WT BM into WT recipients [WT WT], *Adam15⁻/⁻* BM into WT recipients [KO WT], WT BM into *Adam15⁻/⁻* recipients [WT KO], and *Adam15⁻/⁻* BM into *Adam15⁻/⁻* recipients [KO KO]) were exposed to air or CS for 6 months. Panel **A** shows the mean alveolar chord lengths measured in the CS-exposed mice. The mean chord length in air-exposed mice was 14 to 19 μm in all groups (data not shown). Data are mean ± SEM; n = 4-6 mice/group. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.05$ versus

the group indicated. In **B**, the thickness of the ECM protein layer deposited around small airways was quantified in microns, as described in Methods. Data are mean ± SEM; n = 5-8 mice/group. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, P 0.022 versus the group indicated. In **C**, the number of alveolar macrophages in lung sections normalized to alveolar wall area was measured, as described in the Methods. Data are mean ± SEM; n = 5-6 mice/group. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.05$ versus the group indicated.
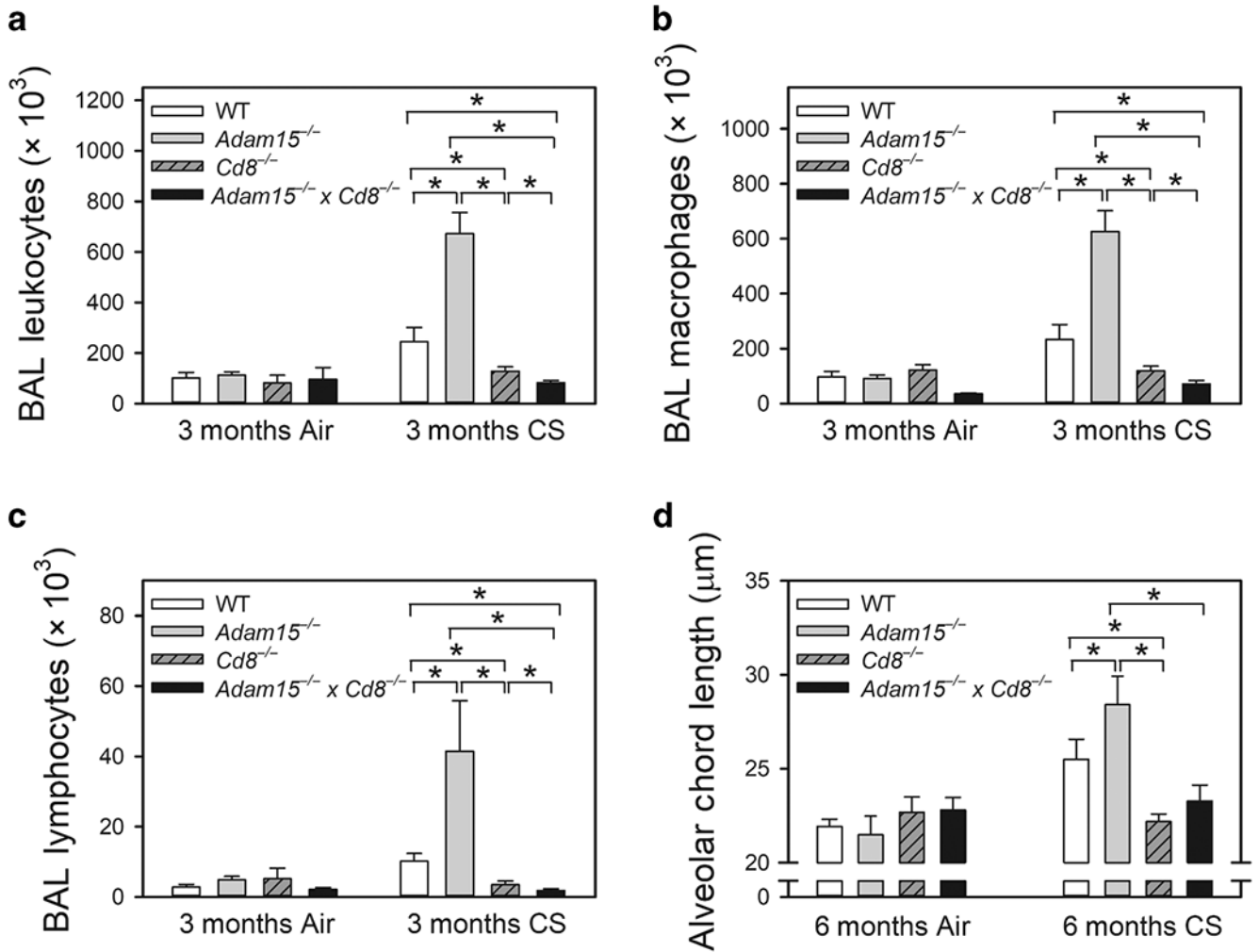
Figure 6: Deficiency of Adam15 in CD8$^+$ T cells is crucial for driving the exaggerated lung inflammation and destruction detected in CS-exposed Adam15$^{-/-}$ mice.
Adult WT mice, *Adam15* mice, *Cd8*$^{-/-}$ mice and *Adam15*$^{-/-}$ x *Cd8*$^{-/-}$ compound mutant mice were exposed to air or CS 6 days-a-week for 3 months or 6 months. Absolute numbers of all leukocytes (**A**), macrophages (**B**), and lymphocytes (**C**) were counted in bronchoalveolar lavage (BAL) samples. Data are mean ± SEM; n = 7-17 mice exposed to air for 3 months air and 6-11 mice exposed to CS for 3 months. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P <$ 0.05 versus the group indicated. **D**: alveolar chord length was measured on lung sections as a measure of airspace enlargement. Data are mean ± SEM; n = 5-11 mice exposed to air for 6 months and 10-16 mice exposed to CS for 6 months. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P <$ 0.05 versus the group indicated.
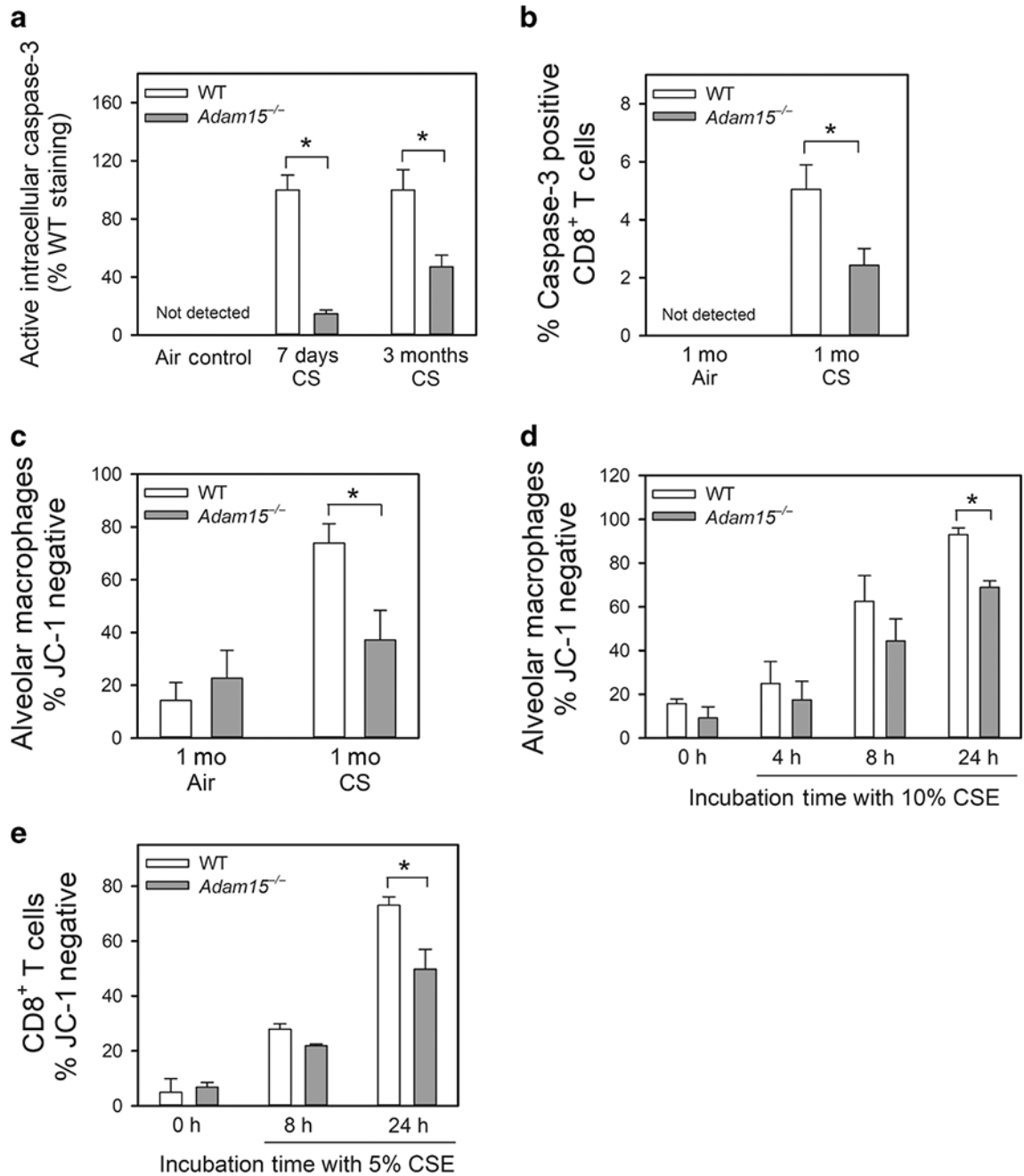
Figure 7: Deficiency of Adam15 in CD8+ T cells and macrophages protects the cells from CS-induced activation of the mitochondrial apoptosis pathway in vivo and in vitro.

A: Alveolar macrophages (AMs) were isolated from the lungs of WT and *Adam15*$^{-/-}$ mice that had been exposed to air or CS for 1 week or 3 months (Mo) using BAL. **B**: CD8+ T cells were isolated from the lungs of WT and *Adam15*$^{-/-}$ mice exposed to air or CS for 1 month as described in Methods. In **A** and **B**, immediately after the AMs and CD8+ T cells were isolated they were fixed and then immunostained for intracellular active caspase-3. Staining was quantified, as described in the Methods. Active caspase-3 was not

detected in macrophages or CD8$^+$ T cells isolated from air-exposed WT or *Adam15*$^{-/-}$ mice. Data are mean ± SEM; n = 5 mice/group in **A**, and n = 4-5 mice/group in **B**. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.05$ versus the group indicated. **C**: AMs were isolated from the lungs of WT and *Adam15*$^{-/-}$ mice exposed to air or CS for 1 month using BAL. Activation of the mitochondrial apoptosis pathway was measured as loss of mitochondrial membrane potential (lack of staining of the cells with the mitochondrial dye, JC-1 which stains only viable mitochondria). Data are mean ± SEM; n = 5 mice/group. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.05$ versus the group indicated. **D**: AMs isolated from naïve WT and *Adam15*$^{-/-}$ mice were incubated at 37°C for up to 24 h with or without 10% CS extract (CSE). **E**: CD8$^+$ T cells isolated from spleen of naïve WT *Adam15*$^{-/-}$ mice were incubated at 37°C for up to 24 h with or without 5% CSE. In **D** and **E**, activation of the mitochondrial apoptosis pathway was measured as loss of mitochondrial membrane potential (lack of staining of the cells with JC-1). Data are mean ± SEM; n = 3 separate experiments in **D** and **E**. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.05$ versus the group indicated.
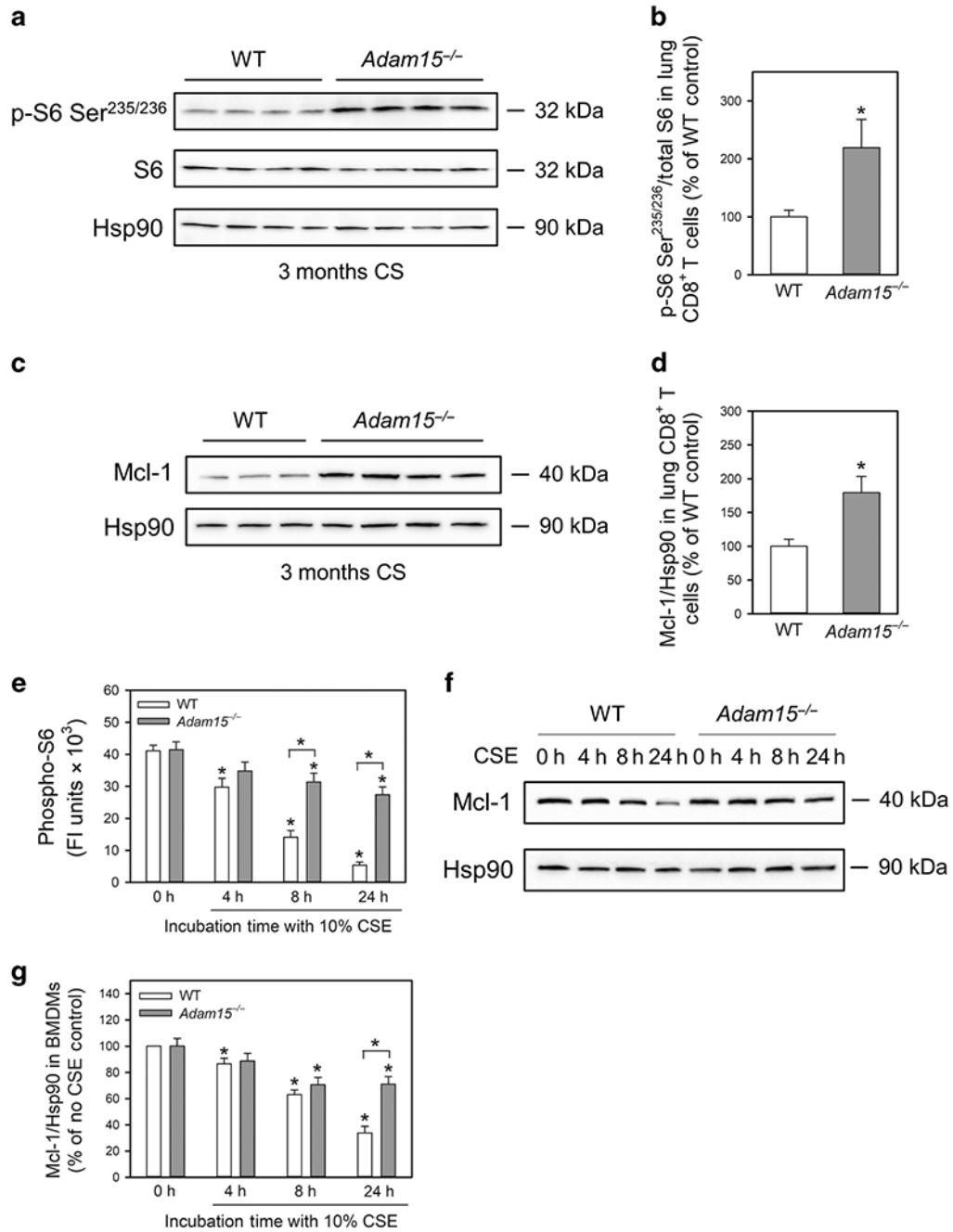
Figure 8: Deficiency of Adam15 in macrophages and CD8^{+} T cells presences activation of the mTOR-Mcl-1 pro-survival pathway when cells are exposed to CS.

In **A-D**, CD8^{+} T cells were isolated from the lungs of WT and *Adam15^{−/−}* mice that had been exposed to CS for 3 months. In **A**, phosphorylated S6 (p-S6-Ser[235/236]), total S6 (S6), and a housekeeping control (heat shock protein-90; Hsp90) were quantified in extracts of the pulmonary CD8^{+} T cells using Western blotting and densitometry. The p-S6-Ser[235/236] signals were normalized to total S6 levels and expressed as a % of the values for WT mice. The images shown in **A** are representative of 4 mice per group. **B**: The bars show

means ± SD (n = 4 mice/group). Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Students t-tests. *, P 0.01 versus the WT control. In **C-D**, intracellular protein levels of Mcl-1 and Hsp90 were quantified in pulmonary CD8$^+$ T cells from CS-exposed mice using Western blotting and densitometry. The Mcl-1 signals were normalized to Hsp90 levels and expressed as a % of the values for WT mice. The images shown in **C** are representative of 3-4 mice per group. **D**: The bars show means ± SD (n = 3-4 mice/group). Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Students t-tests. *, P 0.004 versus the WT control. In **E-G**, bone marrow-derived macrophages (BMDMs) were isolated from unchallenged WT and *Adam15*$^{-/-}$ mice and exposed to 10% CSE for up to 24 h. In **E**, BMDMs were fixed, permeabilized, and immunostained with a green fluorophore for intracellular phosphorylated ribosomal protein S6 (p-S6), and the nuclei were counterstained blue with 4′,6-diamidino-2-phenylindole. Control cells were stained with a primary non-immune rabbit (Rb) IgG (right panels). P-S6 staining in BMDMs was quantified, as described in the Methods. Data are mean ± SD and data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Students t-tests. *, P 0.01 versus the control. In **F-G**, intracellular protein levels of Mcl-1 and Hsp90 were quantified in CSE-treated BMDMs using Western blotting and densitometry. The images shown in **G** are representative of 7 mice/group. In **G**, the Mcl-1 signals were normalized to Hsp90 levels and expressed as a % of the values for controls cells incubated without CSE. Data are mean ± SD; n = 7 mice/group. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, P 0.004 versus no CSE control belonging to the same genotype or the group indicated.
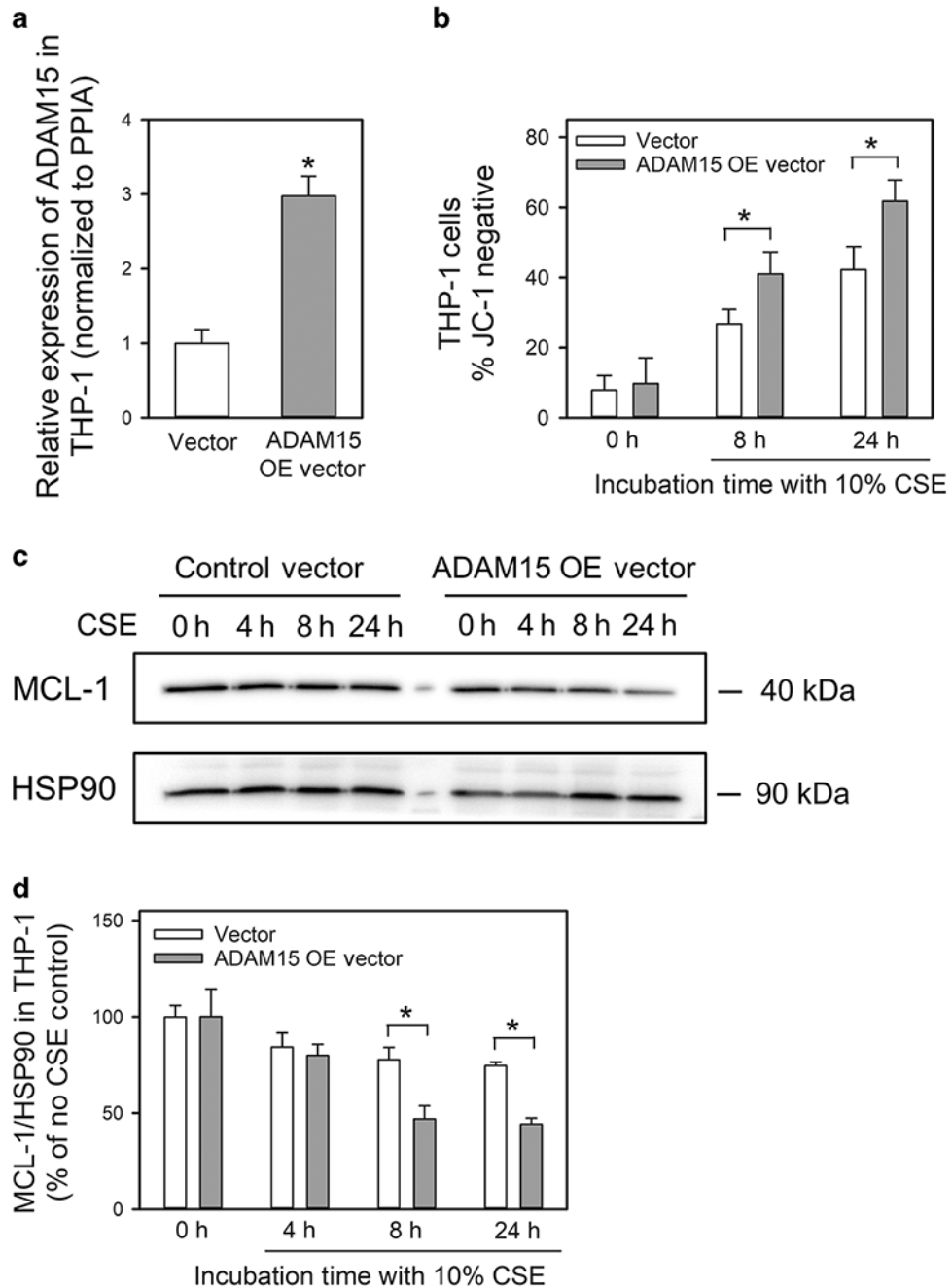
Figure 9: Overexpression of ADAM15 in human THP-1 cells promotes CSE-induced apoptosis and reductions in intracellular Mcl-1 protein levels.

In **A**, *ADAM15* steady state mRNA levels were measured in total cellular RNA samples from differentiated THP-1 24 h after electroporation of an ADAM15 over-expression [OE] vector (pcDNA3.1-ADAM15, a plasmid containing an ADAM15 complementary DNA) or a control vector (Vector) using quantitative real-time PCR. In **B-D**, differentiated THP-1 cells were electroporated with either the ADAM15 OE vector or the control vector and then incubated with 10% CSE for the time indicated. In **B**, loss of mitochondrial membrane

potential was measured by staining the cells with JC-1 dye and quantifying the staining using MetaMorph software. In **C-D**, intracellular protein levels of myeloid cell leukemia-1 (MCL-1) and heat shock protein-90 (HSP90; a housekeeping control) were quantified in CSE-treated THP-1 cells using Western blotting and densitometry. The MCL-1 signals were normalized to HSP90 levels and expressed as a % of the values for controls cells incubated without CSE. Data are mean ± SD. Data were analyzed using a One-Way ANOVA followed by pair-wise testing with two-tailed Student's t-tests. *, $P < 0.03$ versus the group indicated.
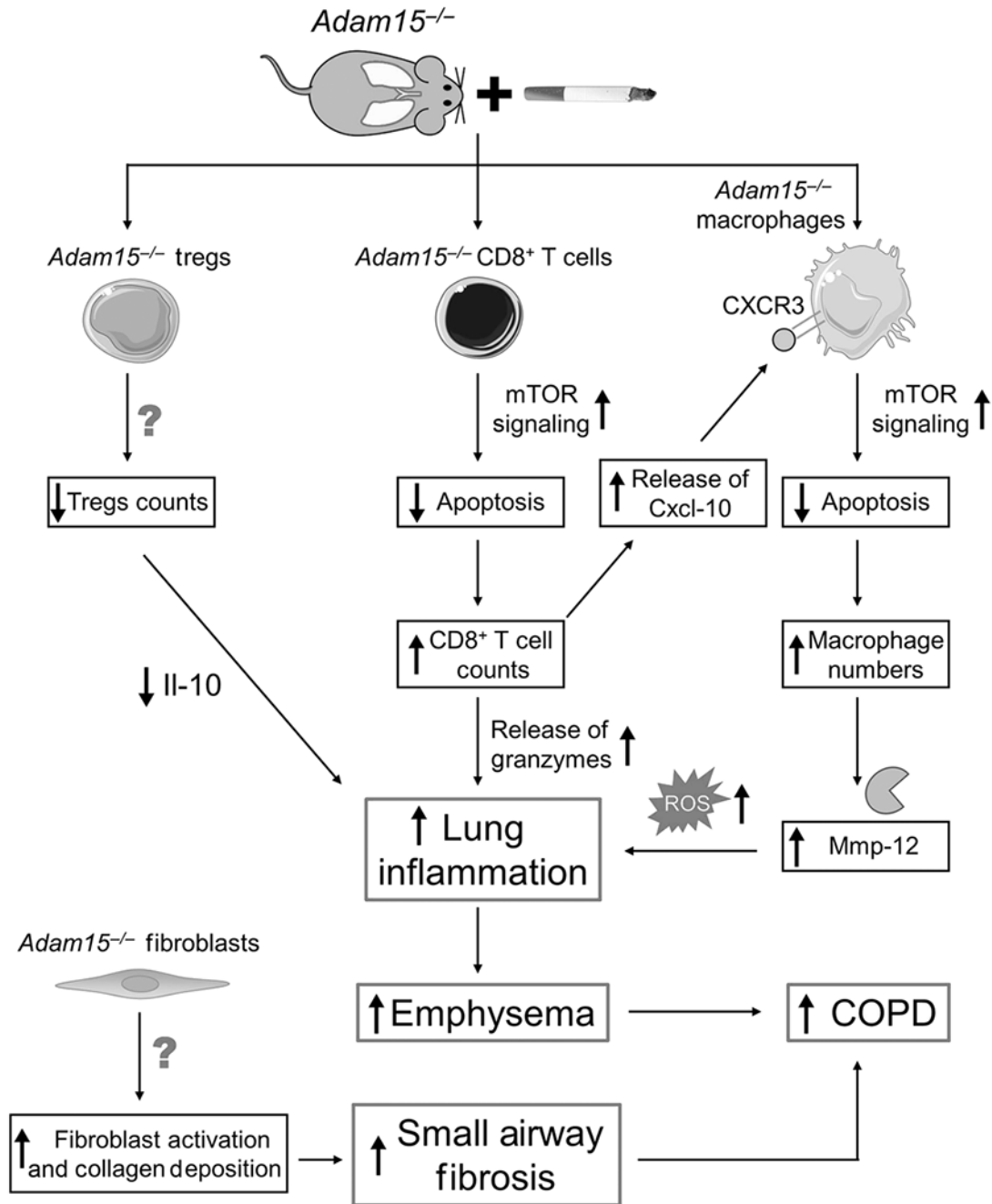
Figure 10: cartoon illustrating the mechanisms by which deficiency of Adam15 leads to exaggerated COPD in mice.

Exposing $Adam15^{-/-}$ mice to CS leads to a phlogistic pulmonary response characterized by increases in the number of innate and adaptive immune cells (alveolar macrophages [AMs], CD4+ lymphocytes, CD8+ lymphocytes and CD3-positive NKT cells) and reductions in leukocytes having anti-inflammatory activities (Foxp3+ T regulatory cells) in the lung, along with exaggerated emphysema development and small airway fibrosis. The results of studies of $Adam15$ bone marrow chimeras and $Adam15^{-/-}$ X $Cd8^{-/-}$ mice indicate that deficiency of $Adam15$ in leukocytes (especially CD8+ T cells) drives the increased

emphysema development and small airway fibrosis observed in CS-exposed $Adam15^{-/-}$ mice. Likely, increased release of Mmp-12 and oxidants from the increased numbers of macrophages and the increased release of granzymes from the increased numbers of CD8^{+} T cells detected in the lungs of CS-exposed $Adam15^{-/-}$ mice contributed to their increased emphysema development. The exaggerated small airway fibrosis in CS-exposed $Adam15^{-/-}$ mice may be due to: 1) increased release of mediators from leukocytes and other cells that activate (myo)fibroblasts; and 2) $Adam15$ deficiency in (myo)fibroblasts increasing the survival and/or profibrotic activities of these cells in the small airways. $Adam15$ deficiency increases CS-induced pulmonary inflammation by several mechanisms. First, $Adam15^{-/-}$ CD8^{+} T cells (and macrophages) are protected from CS-induced activation of the mitochondrial apoptosis pathway and consequently accumulate in, and injure, the lung. $Adam15$ deficiency preserves mammalian target of rapamycin (mTOR) signaling in CD8^{+} T cells (and macrophages), leading to preserved expression of myeloid cell leukemia sequence-1 (Mcl-1) protein (an anti-apoptotic protein which binds to the mitochondrial membrane to prevent loss of mitochondrial membrane potential, mitochondrial release of cytochrome c and downstream caspase activation). The increased number of CD8^{+} T cells in lungs of $Adam15^{-/-}$ mice also promotes the survival of macrophages in the lung via $Adam15^{-/-}$ CD8^{+} T cells releasing increased quantities of Cxcl-10 (and possibly other ligands for Cxcr3) which bind to Cxcr3 on $Adam15^{-/-}$ macrophages to increase mTOR signaling in macrophages leading to increased macrophage survival and activation and release of Mmp-12, oxidants and other mediators that promote lung inflammation and destruction. Second, the accumulation of leukocytes leads to increased release of pro-inflammatory mediators that amplify pulmonary inflammation and destruction in $Adam15^{-/-}$ lungs. Third, lung levels of anti-inflammatory mediators, such as interleukin-10 (Il-10) are reduced in CS-exposed $Adam15^{-/-}$ lung, which may be due to the reduced numbers of Foxp3^{+} T regulatory cells (which are a source of Il-10) in their lungs. A reduced anti-inflammatory immune response likely contributes to the phlogistic environment that increases emphysema development and small airway fibrosis in CS-exposed $Adam15^{-/-}$ lungs.