Interleukin-15 Regulates Retinoic Acid Receptor Beta in the Lung During Cigarette Smoking and Influenza Virus Infection

Jianmiao Wang\textsuperscript{1,2}, Wei Liu\textsuperscript{1}, Chad Marion\textsuperscript{1}, Rajvir Singh\textsuperscript{1}, Nathaniel Andrews\textsuperscript{1}, Chun Geun Lee\textsuperscript{3}, Jack A. Elias\textsuperscript{3} and Charles S. Dela Cruz\textsuperscript{1*}

\textsuperscript{1}Section of Pulmonary, Critical Care and Sleep Medicine, Yale University School of Medicine, New Haven, Connecticut, USA
\textsuperscript{2}Department of Respiratory and Critical Care Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
\textsuperscript{3}Medicine and Biologic Sciences, Brown University, Warren Alpert Medical School, Providence, Rhode Island, USA

\textsuperscript{*}Corresponding author: Charles S. Dela Cruz, M.D., Ph.D., Section of Pulmonary, Critical Care and Sleep Medicine, Yale University School of Medicine, 333 Cedar Street, PO Box 208057, New Haven, CT 06520-8057. Office Phone: (203) 785-3627. E-mail: charles.delacruz@yale.edu

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Abstract

Virus-induced exacerbations often lead to further impairment of lung function in chronic obstructive pulmonary disease. Interleukin (IL)-15 is critical in antiviral immune responses. Retinoic acid (RA) signaling plays an important role in tissue maintenance and repair, particularly in the lung. We studied RA signaling and its relation to IL-15 in the lung during cigarette smoke (CS) exposure and influenza virus infection. In vivo studies show that RA signaling is diminished by long-term CS exposure or influenza virus infection alone, which is further attenuated during infection following CS exposure. RA receptor β (RARβ) is specifically decreased in the lung of IL-15 transgenic (overexpression; IL-15Tg) mice, and a greater reduction in RARβ is found in these mice compared with wild type (WT) after infection. RARβ is increased in IL-15 knockout (IL-15KO) mice compared with WT after infection and the additive effect of CS and virus on RARβ downregulation is diminished in IL-15KO mice. IL-15 receptor α (IL-15Rα) is increased and RARβ is significantly decreased in lung interstitial macrophages from IL-15Tg mice compared with WT. In vitro studies show that IL-15 downregulates RARβ in macrophages via IL-15Rα signaling during influenza virus infection. These studies suggest that RA signaling is significantly diminished in the lung by CS exposure and influenza virus infection. IL-15 specifically downregulates RARβ expression and RARβ may play a protective role in lung injury caused by CS exposure and viral infections.

Keywords: interleukin-15; chronic obstructive pulmonary disease; retinoic acid receptor; cigarette smoke; influenza
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality worldwide and is projected to become the third leading cause of death by 2020 (1). It is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the lung to noxious particles or gases (2). Cigarette smoke (CS) exposure is the main cause of lung inflammation that induces parenchymal tissue destruction (resulting in emphysema) and disrupts normal repair and defense mechanisms (resulting in small airway fibrosis) leading to progressive airflow limitation (2, 3).

Respiratory virus infections are associated with up to 40-60% of COPD exacerbations (4, 5), and these exacerbations accelerate the decline in lung function, contribute to disease progression and increase the risk of death (6, 7). As compared with non-viral exacerbations, virus-induced COPD exacerbations are associated with more severe symptoms, more frequent hospitalizations and longer recovery period (8, 9). A range of respiratory viruses has been shown to cause COPD exacerbations, among which the most common viruses are rhinoviruses, but in more severe exacerbations requiring hospitalization, influenza is more common (10). However the mechanisms that mediate these viral exacerbations and their effects on the lung in CS exposure and COPD have not been adequately defined.

Previous studies from our laboratory and others have explored the effects of viral infections following CS exposure on the lung in mouse models (11-15). These studies demonstrated that CS and viruses interact in a manner to induce exaggerated pulmonary inflammation and accelerated emphysema and airway fibrosis (11). However almost all of these studies focused on the innate immune mechanisms (11-14), the possibility that other signaling pathways could also contribute to these effects has not been fully addressed. Interleukin (IL)-15 is a pro-inflammatory cytokine that is expressed by antigen-presenting cells (APCs) including macrophages and dendritic cells and epithelial cells. It is important for the activation and proliferation of natural killer (NK)
and CD8 T cells (16, 17). IL-15 is also critical for the activation and function of APCs (18, 19). IL-15 is induced in respiratory virus infections and plays an important role in antiviral immune responses (20, 21); however, excessive IL-15 expression within tissue is associated with lung injury (20).

Retinoic acid (RA) signaling is critical in biological processes such as lung development (22, 23) and immune homeostasis (24, 25). It also plays an important role in tissue maintenance and repair, particularly in the lung (26, 27). RA is the main active metabolite of vitamin A and many clinical studies have demonstrated a positive relationship between vitamin A status and lung function (28, 29). It was reported that CS exposure causes vitamin A depletion and the deficiency of vitamin A induces the development of emphysema in rats (30, 31). Previous studies have also shown that RA treatment can promote the repair and or re-alveolarization of parenchymal lesions in the animal models of emphysema (32, 33, 34). However, the RA signaling and its relation to cytokines such as IL-15 in the lung during influenza virus infection following CS exposure have not been studied.

Here we show that RA signaling is diminished in the lung by long-term CS exposure, or by influenza virus infection, which is further attenuated during influenza virus infection following CS exposure. IL-15 specifically inhibits RA receptor β (RARβ) expression both in vivo and in vitro in a dose-dependent manner, and RARβ may play a role in the lung inflammatory process caused by CS exposure and influenza virus infection.
MATERIALS AND METHODS

Mice
C57BL/6 mice and IL-15 knockout (IL-15KO) mice were purchased from The Jackson Laboratory and Taconic, respectively. IL-15 transgenic (IL-15Tg) mice that use the Clara cell 10-kD protein promoter and reverse tetracycline transactivator to target IL-15 to the lung on a C57BL/6 background have been previously generated using approaches described by our laboratory (35). All animal studies were approved and according to the guidelines of the Yale Institutional Animal Care and Use Committee (IACUC).

CS Exposure
C57BL/6 mice and IL-15KO mice were exposed to room air/no smoking (NS) or the smoke from non-filtered 3R4F research cigarettes (University of Kentucky, Lexington, KY) using the smoking apparatus and protocol previously described (11, 15, 36). During the first week, mice received a half cigarette twice a day to allow for acclimation, and thereafter they received 1 cigarette twice a day.

In Vivo Administration of Influenza Virus
After 1 month of NS/CS exposure or 2-4 weeks of oral doxycycline water treatment, the mice were lightly anesthetized and $5.0 \times 10^{3.375}$ TCID$_{50}$ (50% tissue culture infective doses) of A/PR8/34 (H1N1) influenza virus (equivalent to 0.05 LD$_{50}$ in C57BL/6 mice) was administered via nasal aspiration in 70 µl PBS, using techniques previously described by our laboratory (37).

Flow Cytometry
Lung single cell suspensions were prepared using the Lung Dissociation Kit (Miltenyi Biotec, Auburn, CA) according to the standard protocol previously described (38). Cells were incubated with anti-mouse CD16/CD32 (eBioscience, San Diego, CA) to reduce nonspecific binding. Staining reactions were performed at 4°C with anti-mouse F4/80 PE and anti-mouse CD11c APC (eBioscience). Alveolar macrophages (AMs)
and interstitial macrophages (IMs) were sorted by flow cytometry (BD FACSARia) based on their differential F4/80 and CD11c expression as previously described (39). For the analysis of IL-15 receptor α (IL-15Rα) expression, cells were stained with anti-mouse F4/80 PE, anti-mouse CD11c eFluor 450 and anti-mouse IL-15Rα APC (eBioscience), and acquired on a BD FACS LSRII.

**Cell Culture**

RAW264.7 cells were purchased from American Type Culture Collection. Cells were cultured and treated with influenza virus, recombinant mouse IL-15 (rmIL-15, R&D Systems, Minneapolis, MN) or anti-IL-15Rα antibody (Abcam, Cambridge, MA). Details are provided in the online supplement.

**Quantitative PCR**

Total RNA was isolated from the lung tissue, sorted cells including IMs and AMs, and RAW264.7 cells. Quantitative PCR was carried out using the specific primers for the retinaldehyde dehydrogenases (RALDHs), RA receptors (RARs), cytochrome P450 family 26 subfamily B polypeptide 1 (Cyp26b1), IL-15 and IL-15Rα. For microRNA-29b (miR-29b) analysis, specific TaqMan probe was used. Details are provided in the online supplement.

**Western Blotting**

Whole lung lysates and RAW264.7 cell lysates were prepared and the total protein concentration was determined. Equal amounts of sample proteins were used for Western blot analysis. Details are provided in the online supplement.

**Statistical Analysis**

Results are reported as mean (± SEM) values, unless otherwise specified. The student unpaired two-tailed t test was performed for all statistical analyses using GraphPad Prism 6. Differences between groups were considered significant when $P < 0.05$. 
RESULTS

CS Exposure Regulates RA Signaling Components in the Lung

First, to test whether CS exposure would attenuate RA expression and signaling, we exposed C57BL/6 mice to NS or CS for 1 month, 3 months or 6 months. The mRNA expression of the RA signaling components in the lung was measured using quantitative PCR including the key RA-synthesizing enzymes (RALDH1 and RALDH2), RARs (RARα, RARβ and RARγ) and the major enzyme for RA metabolism (Cyp26b1). The protein level of the various RARs in the lung of mice exposed to NS or CS for 6 months was also assessed using Western blot. We found that 1-month and 3-month exposure to CS did not significantly alter expression of the RA signaling components in the lung (Figure 1). However, the mRNA expression of RARβ and Cyp26b1 was significantly decreased after 6-month exposure of CS compared with NS (Figure 1B and 1I). There were no significant changes in the expression of RARα, RARγ, RALDH1 and RALDH2 after CS exposure (Figure 1A, 1C, 1G and 1H). Western blot analyses showed similar results in RAR expression patterns at the protein level in the lung of mice after 6-month exposure to CS (Figure 1D-F).

Influenza Virus Infection in the Setting of CS Exposure Downregulates RARβ

Given the importance of virus-induced exacerbations in COPD and that CS can enhance the inflammatory and remodeling effects of influenza virus (11-15), C57BL/6 mice were exposed to NS or CS for 1 month and then infected with influenza virus or vehicle control. On day 7 after infection, total leukocyte counts in bronchoalveolar lavage fluid (BALF) and IL-15 protein levels by ELISA in lung tissue were determined. We found that the total leukocyte counts in BALF and IL-15 protein levels in lung tissue were increased after CS exposure or influenza virus infection; there was additional increase in the levels of inflammation and IL-15 protein levels after dual exposure of CS and virus (Figure S1). The mRNA expression of the RA signaling components in the lung was measured using quantitative PCR, and the protein level of RARs in the lung was assessed using Western blot (Figure 2). We found that the mRNA expression of RARβ, RARγ and RALDH1 in the lung was significantly decreased on day 7 after infection.
with influenza virus (Figure 2B, 2C and 2G). Importantly, dual exposure of CS and influenza virus resulted in further decrease in RARβ and RALDH1 expression (Figure 2B and 2G). There was no significant effect with dual exposure of CS and virus on RARγ expression in the lung (Figure 2C). There were also no significant changes in the expression of RARα, RALDH2 and Cyp26b1 in the lung of mice exposed to CS and/or influenza virus (Figure 2A, 2H and 2I). Western blot analyses showed similar results in RARs expression at the protein level (Figure 2D-F).

**Overexpression of IL-15 in the Lung Results in Significant Repression of RARβ during Influenza Virus Infection**

To study the role of IL-15 in the lung in our models, we utilized mice that overexpress IL-15 in the lung epithelium for the purpose of examining the effect of this cytokine during pulmonary viral infections. Wild type (WT) and IL-15Tg mice were infected with influenza virus or vehicle control. On day 7 after infection, total leukocyte counts in BALF and IL-15 levels in lung tissue were determined. We found that increased leukocyte counts in BALF of IL-15Tg mice compared to control WT mice (Figure S2). Moreover, IL-15 levels in lung tissue were further increased in IL-15Tg mice compared with WT mice after influenza virus infection (Figure S2). The mRNA expression of the RA signaling components in the lung was measured using quantitative PCR, and the protein level of RARs in the lung was assessed using Western blot (Figure 3). We found that the mRNA expression of RARβ and RARγ was significantly decreased on day 7 after infection with influenza virus (Figure 3B and 3C). RALDH1 and Cyp26b1 expressions were also decreased after infection (Figure 3G and 3I). Importantly, RARβ expression was specifically decreased in IL-15Tg lungs compared to WT controls, and there was a greater significant reduction in RARβ expression found in IL-15Tg mice compared with WT mice after influenza virus infection (Figure 3B). There were no significant changes in the expression of RARα and RALDH2 after infection in this modeling system (Figure 3A and 3H). Western blot analyses showed similar results in RARs at the protein level (Figure 3D-F).
**IL-15 is Required for RARβ Downregulation in the Lung during CS Exposure and Influenza Virus Infection**

To determine the requirement of IL-15 in the RARβ downregulation in the lung during CS exposure and influenza virus infection, WT and IL-15KO mice were exposed to NS or CS for 1 month and then infected with influenza virus. On day 7 after infection, total leukocyte counts in BALF were determined. We found that the total leukocyte counts were significantly decreased in IL-15KO mice during influenza virus infection following NS or CS exposure compared with control WT mice (Figure S3). The mRNA expression of the RA signaling components in the lung was measured using quantitative PCR, and the protein level of RARs in the lung was assessed using Western blot (Figure 4). We found that the mRNA expression of RARβ was significantly increased in the lung of IL-15KO mice compared with WT mice on day 7 after infection with influenza virus (Figure 4B). Importantly, the effect of dual exposure to CS and virus on RARβ downregulation was significantly diminished in IL-15KO mice (Figure 4B). There were no significant changes in the expression of RARα, RARγ, RALDH1, RALDH2 and Cyp26b1 in the lung of IL-15KO mice compared with WT mice after infection or after dual exposure to CS and virus (Figure 4A, 4C and 4G-I). Western blot analyses showed similar results in RARs expression at the protein level (Figure 4D-F).

**Increased IL-15 and IL-15Rα in Lung Interstitial Macrophages Promotes Downregulation of RARβ**

Given the predominant cell population after viral infections is macrophages, we focused on studying the role of IL-15 and IL-15Rα and their effects on RARs in subpopulations of lung macrophages. WT and IL-15Tg mice were infected with influenza virus or vehicle control. On day 7 after infection, lung IMs (F4/80+ CD11c+) and AMs (F4/80+ CD11c+) were isolated using flow cytometry and the mRNA expression of IL-15, IL-15Rα and RARs in these two cell populations was measured using quantitative PCR (Figure 5). Despite the fact there was a net increase in the absolute numbers of total lung macrophages (AMs and IMs) in infected IL-15Tg compared to infected WT mice (data not shown), only the proportion of AMs was increased after influenza virus
infection in the IL-15Tg lungs, whereas there were no significant changes in the proportion of IMs in the lung (Figure 5A). The proportion of AMs in the lung of IL-15Tg mice was higher compared with WT after infection (18.5±1.0% versus 15.0±0.7%; P<0.05). IL-15 and IL-15Rα expression was significantly increased in the lung IMs from WT or IL-15Tg mice after influenza virus infection, and IL-15Rα expression in IMs from IL-15Tg mice was higher compared with WT after infection (Figure 5B and 5C). On the contrary, the expression of RARβ and RARγ was significantly decreased after infection, and specifically RARβ expression in IMs from IL-15Tg mice was lower compared with WT after infection (Figure 5E and 5F). There were no significant changes in the expression of RARα in the lung IMs after influenza virus infection (Figure 5D). In the AMs, IL-15 and IL-15Rα expression was also significantly increased after infection; however, there was no significant difference in IL-15Rα expression between IL-15Tg and WT mice after infection (Figure 5B and 5C). The expression of RARα was significantly decreased in the lung AMs after infection, while there were no significant changes in RARβ and RARγ expression (Figure 5D-F). In addition, IL-15Rα expression on IMs and AMs in the lung was also analyzed by flow cytometry. We found similar results as the mRNA expression (Figure 6A-D).

**IL-15Rα is Required for RARβ Downregulation in Macrophages by Influenza Virus or Recombinant IL-15**

To determine the dose response effects of influenza virus on RARs, RAW264.7 macrophages were treated with influenza virus at three different dosages for 24 hours. The mRNA expression of IL-15, IL-15Rα and RARs was measured using quantitative PCR and the protein levels of RARβ were also assessed using Western blot. We found that the expression of IL-15 and IL-15Rα was significantly increased with the stimulation of influenza virus in a dose-dependent manner (Figure 7A). The RARβ and RARγ expression was significantly decreased after virus treatment, while no changes were observed in RARα expression (Figure 7B). Importantly, influenza virus inhibited the expression of RARβ in a dose-dependent manner (Figure 7B and 7D).

To determine the dose response effects of IL-15 on RARs, RAW264.7
macrophages were treated with recombinant mouse IL-15 (mIL-15) at three different dosages for 24 hours. The expression of RARs in these cells was measured using quantitative PCR. We found that the expression of RARβ was inhibited by rmIL-15 in a dose-dependent manner, while there were no significant changes in RARα and RARγ expression after treatment with rmIL-15 (Figure 7C). IL-15 and IL-15Rα signaling is critical for the activation of APCs including macrophages and dendritic cells upon microbial infection (18, 19). Blocking IL-15Rα by using anti-IL-15Rα antibody decreased IL-15-mediated RANTES (chemokine ligand 5) and cytokines production in these cells (19). We found that RANTES and tumor necrosis factor α (TNFα) levels in the culture supernatant were also detected via ELISA. Both of these cytokines were increased by the stimulation of rmIL-15 in a dose-dependent manner (Figure S4A and S4B).

To determine the requirement of IL-15Rα in the downregulation of RARβ by IL-15 or influenza virus, RAW264.7 macrophages were preincubated with IL-15Rα blocking antibody and then treated with influenza virus or rmIL-15. The protein levels of RARβ were assessed using Western blot. We found that IL-15Rα blocking resulted in increased RARβ expression in these cells treated with influenza virus or rmIL-15 (Figure 7E and 7F).

Previous studies have suggested that RARβ reduction is mainly caused by DNA hypermethylation (40) and IL-15 can regulate expression of DNA methyltransferase 3b (Dnmt3b) via the repression of miR-29b (41). Thus, we also measured the miR-29b expression using quantitative PCR in RAW264.7 macrophages treated with rmIL-15. We found that miR-29b expression was also inhibited by rmIL-15 in a dose-dependent manner (Figure S4C). In addition, IL-15Rα blocking also resulted in increased miR-29b expression in these cells treated with rmIL-15 (Figure S4D).

**DISCUSSION**

COPD is characterized by an imbalance between tissue inflammation, injury and repair that ultimately results in the progressive destruction of pulmonary parenchyma (42).
The disruption of normal repair mechanisms also causes airway fibrosis in COPD (2). Previous studies have suggested that RA signaling plays an important role in tissue maintenance and repair processes in the lung (26). In this study, we show that RARβ expression is significantly reduced in the lung after long-term CS exposure, which is consistent with the findings from previous related studies (43, 44). These results support the hypothesis that the inhibition of alveolar repair by CS is one of the mechanisms for the development of emphysema (42).

Enhanced morbidity in virus-infected and second-hand smoke-exposed children, and enhanced disease severity in virus-infected normal smokers have been described clinically (45, 46). These clinical findings suggest that the interactions between CS exposure and viral infections play important roles in clinical scenarios that include virus-induced COPD exacerbations, which have become important clinical parameters in understanding the pathogenesis of COPD. Studies from our laboratory and others have demonstrated that CS and viruses interact in a manner to induce exaggerated inflammatory, emphysema-like and airway fibrotic changes in animal CS-exposure and infection models (11-15). Studying the mechanisms of how CS exposure and viral infections interact in the lung and affect these pulmonary tissue changes will provide potentially important therapeutic target for diseases such as COPD. Our work described here show that RARβ and RALDH1 expression are diminished in the lung after influenza virus infection and are further attenuated after dual exposure to CS and influenza virus, a relevant and common scenario in COPD. This pattern of downregulation was not observed in the other subtypes of RARs. Our results additionally suggest that the RA signaling mediated repair pathway is further inhibited by the combination of CS exposure and influenza virus infection, which could worsen the imbalance between tissue injury and repair in the COPD lung, and ultimately lead to the exaggerated emphysema and airway fibrosis during virus-induced COPD exacerbations.

Here we show that IL-15 is induced in the lung by CS exposure or influenza virus infection alone and further increased by the combination of CS and influenza virus in the mouse models and in vitro cell exposure systems, and that the increase in IL-15
level that is observed with CS exposure and virus infection is associated with increased lung inflammation. Moreover, similar exaggerated lung inflammation can be observed in IL-15Tg mice after influenza virus infection. In the present study, by using IL-15Tg mouse model of influenza virus infection, we also show that RARβ expression is specifically and significantly decreased in the lung of IL-15Tg mice compared with WT mice. A greater reduction in RARβ expression is found in IL-15Tg mice compared with WT mice after influenza virus infection. In addition, by using IL-15KO mice, we show that RARβ is significantly increased in the lung of IL-15KO mice compared with WT after infection and the effect of dual exposure to CS and virus on RARβ downregulation is significantly diminished in IL-15KO mice. These results suggest that IL-15 regulates RA signaling in the lung as shown by its ability to significantly inhibit RARβ expression during CS exposure and influenza virus infection. The impairment of this repair signal is associated with the exaggerated inflammatory lung injury.

Macrophages are highly heterogeneous based on their anatomical location, specialized function and activation state (47). Lung macrophages play a central role in the development and disease progression of COPD (48). AMs and IMs represent the two main lung macrophage subsets, which are localized in distinct anatomical compartments in the lung, the air spaces and lung connective tissue, respectively (49, 50). AMs have been described in detail (47), while IMs have not yet been fully characterized and their in vivo function remains unknown. A number of studies have suggested that IMs are actually an intermediary stage in the maturation of AMs. There is also evidence that AMs and IMs are distinct cell populations with differing functions and that each population contributes to different inflammatory and immune responses in the lung (47). However, because IMs are in direct contact with the lung matrix and other pulmonary connective tissue components, the release of mediators or enzymes by these cells may have greater biological and/or pathological effects than those released by macrophages in the alveolar compartment.

Previous studies have demonstrated that RA signaling decreases the matrix metalloproteinases (MMPs) and increases the tissue inhibitors of metalloproteinases (TIMPs) in macrophages and peripheral blood mononuclear cells (51, 52). In the
present study, IL-15 and IL-15Rα expression is significantly induced in lung IMs from WT and IL-15Tg mice after influenza virus infection. IL-15Rα expression is further increased in the lung IMs from IL-15Tg mice compared with WT mice after infection. Expression of IL-15 in AMs is not as robust, but IL-15Rα can be induced with influenza virus infection in both WT and IL-15Tg mice. Interestingly, the expression of RARβ is downregulated in lung IMs after infection, and a greater reduction in RARβ expression is found in the lung IMs from IL-15Tg mice compared with WT mice after infection. These results suggest that the RARβ-mediated RA signal is further diminished in the lung IMs from IL-15Tg mice after influenza virus infection. Given the important role of RARβ in tissue repair, decrease in the RARβ-mediated RA signal with IL-15 and IL-15Rα expression and influenza virus infection could lead to the further release of MMPs from macrophages and contribution to lung tissue destruction as often seen in COPD.

In the in vitro studies, we demonstrate that influenza virus infection increases the IL-15 and IL-15Rα expression and inhibits the RARβ expression in macrophages in a dose-dependent manner. Previous studies have shown that IL-15 and IL-15Rα signaling is critical for the activation of APCs including macrophages and dendritic cells upon microbial infection (18, 19). IL-15Rα knockdown or blocking with antibody decreases IL-15-mediated RANTES production in these cells (19). Here we show that the RANTES and TNFα are induced in macrophages treated with IL-15 in a dose-dependent manner, and IL-15 treatment specifically reduces the RARβ expression in a dose-dependent manner. We also demonstrate that blocking IL-15Rα results in increased RARβ expression in macrophages treated with influenza virus or IL-15. These results suggest that IL-15 downregulates RARβ expression in macrophages via IL-15Rα during influenza virus infection.

RARβ is unique among its family members since its gene expression is lost during early development in a variety of tumors. A number of studies have demonstrated its unique physiological role among the RAR subtypes as a tumor repressor protein (53). The aberrant methylation of CpG islands is an epigenetic change that induces the transcriptional silencing of tumor suppressor genes such as RARβ gene. It has been reported that RARβ expression is lost or reduced in a large percentage of patients with
lung cancer and in a population at high risk of lung cancer (54, 55). The hypermethylation of RARβ gene is considered a major cause of the loss of RARβ expression (40) and Dnmt3b has been reported to be a direct, negatively regulated target of miR-29b (56). Previous studies have also demonstrated that IL-15 represses miR-29b via induction of Myc/NF-κBp65/ Hdac-1 resulting in Dnmt3b overexpression and DNA hypermethylation (41). Here we show that miR-29b is inhibited in a dose-dependent manner by IL-15 in macrophages that are dependent on IL-15Ra, suggesting that IL-15 may downregulate RARβ expression in this fashion.

Given the beneficial effects of RA in animal models of emphysema, the therapeutic potential of RA and RARγ agonist were evaluated in human patients with emphysema that were mostly focused on patients with α1-antitrypsin deficiencies (57, 58). Unfortunately both of these trials failed to show significance in the benefit on the primary outcomes of lung function and density. Our results indicate that the potentially protective RARβ is significantly downregulated during CS exposure and further more with influenza virus infection. Given the importance of RARβ in mediating the repair signaling during lung injury, we report that RARβ is significantly inhibited during CS exposure, influenza virus infection and or IL-15 expression, and may play an important protective role in the virus-induced COPD exacerbations. Since RARβ is suppressed in COPD, particularly during viral exacerbations, agents that could reverse the silencing of RARβ could prove to be important at restoring this protective lung response.
Acknowledgments

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Figure Legends

**Figure 1.** CS exposure regulates RA signaling components in the lung. C57BL/6 mice were exposed to room air/no smoking (NS) or cigarette smoke (CS) for 1 month (1M), 3 months (3M) or 6 months (6M). The mRNA expression of the RA signaling components in the lung was measured using quantitative PCR. The relative mRNA levels of RARα (A), RARβ (B), RARγ (C), RALDH1 (G), RALDH2 (H) and Cyp26b1 (I) are shown (n = 5 mice/group). The protein levels of RARα (D), RARβ (E) and RARγ (F) in the lung of mice exposed to NS or CS for 6M were assessed using Western blotting. Data are representative of three experiments. * P < 0.05.

**Figure 2.** Influenza virus infection in the setting of CS exposure downregulates RARβ. C57BL/6 mice were exposed to cigarette smoke (CS) for 1 month and then infected with influenza virus (Flu). On day 7 after infection, the mRNA expression of the RA signaling components in the lung was measured using quantitative PCR. The relative mRNA levels of RARα (A), RARβ (B), RARγ (C), RALDH1 (G), RALDH2 (H) and Cyp26b1 (I) are shown (n = 5 mice/group). The protein levels of RARα (D), RARβ (E) and RARγ (F) in the lung were assessed using Western blotting. Data are representative of three experiments. * P < 0.05; ** P < 0.01.

**Figure 3.** Overexpression of IL-15 in the lung results in significant repression of RARβ during influenza virus infection. WT and IL-15Tg mice were infected with influenza virus (Flu). On day 7 after infection, the mRNA expression of the RA signaling components in the lung was measured using quantitative PCR. The relative mRNA levels of RARα (A), RARβ (B), RARγ (C), RALDH1 (G), RALDH2 (H) and Cyp26b1 (I) are shown (n = 3-5 mice/group). The protein levels of RARα (D), RARβ (E) and RARγ (F) in the lung were assessed using Western blotting. Data are representative of three experiments. * P < 0.05; ** P < 0.01.

**Figure 4.** IL-15 is required for RARβ downregulation in the lung during CS exposure.
and influenza virus infection. WT and IL-15KO mice were exposed to room air/no smoking (NS) or cigarette smoke (CS) for 1 month and then infected with influenza virus. On day 7 after infection, the mRNA expression of the RA signaling components in the lung was measured using quantitative PCR. The relative mRNA levels of RARα (A), RARβ (B), RARγ (C), RALDH1 (G), RALDH2 (H) and Cyp26b1 (I) are shown (n = 5 mice/group). The protein levels of RARα (D), RARβ (E) and RARγ (F) in the lung were assessed using Western blotting. Data are representative of three experiments. * P < 0.05; ** P < 0.01.

Figure 5. Increased IL-15 and IL-15Rα in lung interstitial macrophages promotes downregulation of RARβ. WT and IL-15Tg mice were infected with influenza virus (Flu). On day 7 after infection, lung interstitial macrophages (IMs) and alveolar macrophages (AMs) were isolated using flow cytometry (A). The mRNA expression of IL-15 (B), IL-15Rα (C), RARα (D), RARβ (E) and RARγ (F) in the lung IMs and AMs were measured using quantitative PCR. Data are representative of three experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 6. IL-15Rα expression on lung macrophages is increased during influenza virus infection. WT and IL-15Tg mice were infected with influenza virus (Flu). On day 7 after infection, lung single cell suspensions were prepared. Cells were incubated with anti-mouse F4/80, anti-mouse CD11c and anti-mouse IL-15Rα and analyzed by flow cytometry. Representative flow cytometry histograms and mean fluorescence intensity (MFI) of IL-15Rα expression on lung interstitial macrophages (A, B) and alveolar macrophages (C, D) are shown. Data are representative of three experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 7. IL-15Rα is required for RARβ downregulation in macrophages by influenza virus or recombinant IL-15. RAW264.7 macrophages were cultured without influenza virus (CTRL) or with virus at low multiplicity of infection (MOI) (Flu(L)), medium
MOI (Flu(M)) and high MOI (Flu(H)) for 24 hours. The mRNA expression of IL-15 and IL-15Rα (A) and RARs (B) was measured using quantitative PCR and the protein levels of RARβ (D) were assessed using Western blot. Cells were cultured with rmIL-15 at 0, 5, 30 and 120 ng/ml for 24 hours. The mRNA expression of RARs (C) was measured. Cells were preincubated with 20µg/ml anti-IL-15Rα antibody or isotype control for 30 minutes and then treated with influenza virus at high MOI or rmIL-15 at 120 ng/ml for 24 hours. RARβ (E, F) was assessed using Western blot. Data are representative of three experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls.
Figure 6

A

Cell counts

IL-15Ra

B

IL-15Ra expression (MFI)

WT
IL-15Tg
WT+Flu
IL-15Tg+Flu

C

Cell counts

IL-15Ra

D

IL-15Ra expression (MFI)

WT
IL-15Tg
WT+Flu
IL-15Tg+Flu
A

B

C

D

E

F

RARβ

Actin

CTRL  Flu(L)  Flu(M)  Flu(H)

RARβ

Actin

Isotype  Anti-IL-15Rα  Flu

Isotype  Anti-IL-15Rα  rmIL-15

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Interleukin-15 Regulates Retinoic Acid Receptor Beta in the Lung During Cigarette Smoking and Influenza Virus Infection

Jianmiao Wang, Wei Liu, Chad Marion, Rajvir Singh, Nathaniel Andrews, and Charles S. Dela Cruz

ONLINE DATA SUPPLEMENT
Online Materials and Methods

Bronchoalveolar Lavage
Mice were sacrificed using intraperitoneal ketamine/xylazine injection. The trachea was cannulated and perfused twice with 0.7 ml PBS. The bronchoalveolar lavage fluid (BALF) was centrifuged, and the cell pellet was resuspended with PBS. Total leukocyte counts in BALF were determined using a hemocytometer.

Cell Culture
RAW264.7 cells (ATCC) were cultured in 12-well plates at a density of 2×10^5 cells/well in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator under 5% CO₂ at 37°C. After washing with PBS, fresh medium was added. Cells were then treated with influenza virus at a multiplicity of infection (MOI) of 0.1, 0.5 or 2.5. In the experiments of IL-15 stimulation, cells were treated with recombinant mouse IL-15 (rmIL-15, R&D Systems) at 0, 5, 30 or 120 ng/ml. Cells were harvested for mRNA or microRNA-29b (miR-29b) and Western blot analysis after incubation with virus or rmIL-15 for 24 hours, and the culture supernatants were collected for RANTES and TNFα detection. In the experiments of IL-15Rα blocking, cells were preincubated with 20 µg/ml anti-IL-15Rα monoclonal antibody (Abcam) or isotype control for 30 minutes and then treated with influenza virus at an MOI of 2.5 or rmIL-15 at 120 ng/ml. Cells were harvested for miR-29b and Western blot analysis after incubation with virus or rmIL-15 for 24 hours.

ELISA
IL-15 levels in the mouse lung homogenates and RANTES and TNFα levels in the cell culture supernatants were quantified using commercial ELISA kits (R&D Systems) following the manufacturer’s instructions.

Quantitative PCR
Total RNA was isolated from the lung tissue, sorted cells including IMs and AMs, and
RAW264.7 cells using RNeasy Plus Mini Kit (Qiagen, Valencia, CA). After synthesis of cDNA, quantitative PCR was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and the specific primers for the retinaldehyde dehydrogenases (RALDHs), RA receptors (RARs), cytochrome P450 family 26 subfamily B polypeptide 1 (Cyp26b1), IL-15 and IL-15 receptor α (IL-15Rα). The primer sequences were as follows: RARα (5’-GAGGCTGTAAGGGCTTCTTCTTCTT-3’ and 5’-CTTGGGTGCTCCTTTCTTCTT-3’), RARβ (5’-GTGTGGAAGCTTCTTCTGC-3’ and 5’-CTGGCAGGTTTCAGCGATACA-3’), RARγ (5’-CCACCAAAATGCATCATCAAG-3’ and 5’-ATCCGCAGCATTAGATGC-3’), RALDH1 (5’-ATGGTTTAGCAGCAGGACTCTTC-3’ and 5’-CCAGACATCTTGAAATCCACCGAA-3’), RALDH2 (5’-GTGTGGAAGCTTCTTCTGC-3’ and 5’-CTGGCAGGTTTCAGCGATACA-3’), Cyp26b1 (5’-CAGCGACTTACCCCTCCGAAT-3’ and 5’-GGTCCACTGGCGAGAGGAG-3’), IL-15 (5’-CATTTTGGGCTGTGTGAGTGC-3’ and 5’-ACTGGGATGAAAGTCACTGTTGCACTG-3’) and IL-15Rα (5’-GCCTCAAGTGCTCAGAGACC-3’ and 5’-ACCTTTGGGTGCTACTGTTGGC-3’). The relative mRNA expression was determined using the 2^{A∆Ct} methods with the 18S rRNA as endogenous control. For microRNA quantitative PCR, total RNA was isolated from RAW264.7 cells using miRNeasy Mini Kit (Qiagen), 10 ng of total RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit with microRNA-specific RT primers (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed using microRNA-specific TaqMan probe and TaqMan Universal Master Mix (Applied Biosystems). The relative miR-29b expression was determined using the 2^{A∆Ct} methods with the snoRNA55 as endogenous control.

**Western Blotting**

Whole lung lysates and RAW264.7 cell lysates were prepared and the total protein
concentration was determined using BCA assay (Thermo Scientific, Rockford, IL). Equal amounts of sample proteins were separated on 4-20% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After incubation with blocking buffer (5% w/v nonfat dry milk in TBS/0.1% Tween) for 1 hour at room temperature, membranes were incubated with primary antibodies (anti-RARα polyclonal antibody and anti-β-actin monoclonal antibody (Cell Signaling Technology, Beverly, MA), anti-RARβ polyclonal antibody and anti-RARγ polyclonal antibody (Abcam, Cambridge, MA)) overnight at 4°C, washed 3 times with TBS/0.1% Tween, and incubated for 1 hour with secondary antibody (HRP linked anti-Rabbit IgG antibody (Cell Signaling Technology)) at room temperature. Immunoreactive signal was detected using a chemiluminescent procedure (SuperSignal West Femto substrate for RARβ and Pierce ECL substrate for the other proteins, Thermo Scientific) according to the manufacturer’s instructions.
Supplementary Figure Legends

**Figure S1.** Total leukocyte counts in bronchoalveolar lavage fluid and IL-15 levels in lung tissue during CS exposure and influenza virus infection. C57BL/6 mice were exposed to cigarette smoke (CS) for 1 month and then infected with influenza virus (Flu). On day 7 after infection, total leukocyte counts (A) in bronchoalveolar lavage fluid were determined (n=5 mice/group). IL-15 levels (B) in the mouse lung homogenates were quantified by ELISA (normalized to lung homogenate total protein) (n=3 mice/group). * P < 0.05.

**Figure S2.** Total leukocyte counts in bronchoalveolar lavage fluid and IL-15 levels in lung tissue of IL-15Tg mice after influenza virus infection. WT and IL-15Tg mice were infected with influenza virus (Flu). On day 7 after infection, total leukocyte counts (A) in bronchoalveolar lavage fluid were determined (n=3-5 mice/group). IL-15 levels (B) in the mouse lung homogenates were quantified by ELISA (normalized to lung homogenate total protein) (n=3 mice/group). * P < 0.05; ** P < 0.01.

**Figure S3.** Total leukocyte counts in bronchoalveolar lavage fluid of IL-15KO mice during influenza virus infection following CS exposure. WT and IL-15KO mice were exposed to room air/no smoking (NS) or cigarette smoke (CS) for 1 month and then infected with influenza virus. On day 7 after infection, total leukocyte counts in bronchoalveolar lavage fluid were determined (n=5 mice/group). * P < 0.05; ** P < 0.01.

**Figure S4.** Induction of RANTES and TNFα and downregulation of miR-29b expression in macrophages treated with rmIL-15. RAW264.7 macrophages were cultured with rmIL-15 at 0, 5, 30 and 120 ng/ml for 24 hours. The RANTES (A) and TNFα (B) levels in the culture supernatant were detected via ELISA. The miR-29b (C) expression was measured using quantitative PCR. Cells were preincubated with IL-15Rα blocking antibody and then treated with rmIL-15 at 120 ng/ml for 24 hours. The
miR-29b (D) expression was measured. Data are representative of three experiments. *

\[ P < 0.05; ** P < 0.01; *** P < 0.001 \] compared with controls.