Deletion of vitamin D receptor leads to premature emphysema/COPD by increased matrix metalloproteinases and lymphoid aggregates formation

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ABSTRACT

Deficiency of vitamin D is associated with accelerated decline in lung function. Vitamin D is a ligand for nuclear hormone vitamin D receptor (VDR), and upon binding it modulates various cellular functions. The level of VDR is reduced in lungs of patients with chronic obstructive pulmonary disease (COPD) which led us to hypothesize that deficiency of VDR leads to significant alterations in lung phenotype that are characteristics of COPD/emphysema associated with increased inflammatory response. We found that VDR knock-out (VDR$^{-/-}$) mice had increased influx of inflammatory cells, phospho-acetylation of nuclear factor-kappaB (NF-κB) associated with increased proinflammatory mediators, and up-regulation of matrix metalloproteinases (MMPs) MMP-2, MMP-9, and MMP-12 in the lung. This was associated with emphysema and decline in lung function associated with lymphoid aggregates formation compared to WT mice. These findings suggest that deficiency of VDR in mouse lung can lead to an early onset of emphysema/COPD because of chronic inflammation, immune dysregulation, and lung destruction.

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1. Introduction

Vitamin D deficiency has become an important global public-health problem with an estimate of more than 1 billion people worldwide having low/insufficient serum levels of vitamin D [1–4]. Vitamin D deficiency is linked to decline in lung function, reduced immunity, and increased inflammation [4–9]. Hence, studies to understand the role of vitamin D in pathogenesis of lung diseases with respect to vitamin D, vitamin D receptor (VDR) and vitamin D-binding protein are considerable interests [6].

Vitamin D belongs to a steroid hormone superfamily of nuclear receptors that has pleotropic protective effects on several diseases and disorders including asthma and chronic obstructive pulmonary disease (COPD) [4,7,8]. 1,25(OH)$_2$D$_3$ (1,25-dihydroxyvitamin D$_3$) an active metabolite of vitamin D (which binds to nuclear receptor VDR and interacts with other steroid hormone receptors), is a potent regulator of the immune response in Th1 cell-directed diseases [10,11]. Ligand binding activates VDR, which forms a heterodimer with its partner, the retinoid X receptor (RXR), and this complex VDR/RXR further binds to specific genomic sequences in the promoter region of target genes (vitamin D response elements) and thus recruit transcription factors and co-regulatory molecules to activate or suppress gene transcription [12,13].

Vitamin D and VDR are important regulators of inflammation in the lungs. Recent epidemiological studies showed a significant correlation between low serum concentrations of 25-hydroxy vitamin D and chronic lung diseases, such as asthma [14] and COPD [4], and accelerated decline in lung function [2,7]. However, the molecular mechanisms underlying these phenomena are not known. It may be possible that vitamin D or VDR deficiency would invoke lung inflammation and alteration in lung function by proteinase/antiproteinase imbalance. Lung levels of VDR protein is decreased in patients with COPD which led us to hypothesize that VDR deficiency in mouse (VDR$^{-/-}$ mice) can lead to abnormal lung phenotype, due to differential modulation of signaling mediators, and immune dysfunction further leading to spontaneous airspace enlargement and altered lung function. We tested the hypothesis by studying the lung inflammatory cellular influx, posttranslational modifications (phosphorylation and acetylation) of NF-κB RelA/p65, levels of proinflammatory mediators, levels/activities of extracellular matrix proteins, mean airspace enlargement, lymphoid aggregates formation and pulmonary function in lungs of VDR$^{-/-}$ mice.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma Chemicals (St. Louis, MO). Anti-

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bodies used to detect proteins include VDR, MMP-2, MMP-9, MMP-12, TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Santa Cruz Biotechnology, Santa Cruz, CA) for immunoblot analysis.

2.2. Animals

Wild-type (WT) mice of genetic background C57BL/6 J (Jackson Laboratory, Bar Harbor, ME) and VDR-deficient (VDR$^{-/-}$) mice [13,15–18] were housed in the vivarium at the University of Rochester. Experiments were performed on 4–6 months old, average body weight (24 ± 1.5 gm) and sex-matched (male and female) WT and VDR$^{-/-}$ mice which were provided with water *ad libitum*, and maintained in a 12 h dark/light cycle. All experimental protocols described in this study were approved by the animal research committee of the University of Rochester.

2.3. Bronchoalveolar lavage and tissue harvest

The mice were intraperitoneally injected with 100 mg/kg body weight of pentobarbiturate (Abbott laboratories, Abbott Park, IL) and killed by exsanguination. The lungs were lavaged three times with 0.6 ml of 0.9% sodium chloride and removed *en bloc*. The bronchoalveolar lavage (BAL) fluid cell pellet was resuspended in saline, and the total cell numbers were determined by counting on a hemocytometer. Differential cell count (500 cells/slide) was performed on cytospin-prepared slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Bering, Newark, DE).

2.4. Cytokine analysis

The levels of proinflammatory mediators, such as monocyte chemoattractant protein-1 (MCP-1) and chemokine keratinocyte chemoattractant (KC) in the lung homogenates, were measured by ELISA using respective duo-antibody kits (R&D Systems, Minneapolis, MN).

2.5. Immunohistochemistry and trichrome staining

Immunohistochemical staining for macrophages in mouse lung sections was performed as described previously [19]. Gomori's trichrome staining was performed according to the manufacturer's instructions (Richard-Allan Scientific, Kalamazoo, MI). The nuclei stains black, cytoplasm and muscle fibers in red and the collagen stains blue. Pictures were captured using Advance SPOT software version 4.6 (Diagnostic Instruments Inc. Sterling Heights, MI).

2.6. Hematoxylin and Eosin (H&E) staining and morphometric analysis

Mouse lungs (which had not been lavaged) were inflated by 1% low-melting agarose at a pressure of 25 cm H_2O, and then fixed with neutral buffered formalin. Tissues were embedded in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin (H&E). The alveolar size was estimated from the mean linear intercept (Lm) of the airspace which is a measure of airspace enlargement/emphysema [19,20].

2.7. Measurement of lung mechanical properties

Lung mechanical properties were determined using the Scireq Flexivent apparatus (Scireq, Montreal, Canada) as described earlier [21].

2.8. Protein extraction from lung tissues

One lobe of the lung tissue (~50 mg) was homogenized in 0.5 ml of ice-cold RIPA buffer containing complete protease inhibitor cocktail (Sigma) as described previously [19,21].

2.9. Immunoblot analysis

Protein level in lung samples were measured by bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL), and Western blotting was performed as previously described [19,21]. The intensity of bands were quantified using image J software (Version 1.41, NIH, Bethesda, MD, USA).

2.10. MMPs activity assay

Zymography was performed to determine the activity of MMP-2 and MMP-9 in mouse lung as described previously [21].

2.11. Statistical analysis

Data were presented as means ± SEM. Statistical analysis of significance was calculated using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test for multigroup comparisons using the Statview software. $P < 0.05$ was considered as significant.

3. Results

3.1. Inflammatory cellular influx in lung of VDR$^{-/-}$ mice

VDR$^{-/-}$ mice showed increased neutrophil influx ($P < 0.01$) into the BAL fluid compared to WT (Fig. 1A). The number of macrophages and total cells in BAL was not significantly altered among VDR$^{-/-}$ and WT mice (Fig. 1B and C). Interestingly, we observed a significant increase in infiltration of macrophages into the lung interstitium of VDR$^{-/-}$ mice, but not in WT (Fig. 1D and E). Wild-type mice did not show any significant changes in the inflammatory cellular influx into the lungs.

3.2. Genetic ablation of VDR led to increased activation of RelA/p65 and increased release of NF-κB responsive proinflammatory cytokines in the lung

The levels of total RelA/p65 were significantly increased in the lung of VDR$^{-/-}$ compared to WT mice. VDR$^{-/-}$ mice also showed increased phosphorylation at Ser276 and acetylation at Lys310 of RelA/p65 compared to WT mice (Fig. 2A and B). These results suggest that increased phosphorylation and acetylation of RelA/p65 are among the important contributing factors that induced NF-κB-dependent lung inflammation in VDR$^{-/-}$ mice.

The basal levels of MCP-1 ($P < 0.05$) and KC ($P < 0.01$) were significantly increased in VDR$^{-/-}$ mice when compared to WT (Fig. 2C and D). These results corroborate that increased levels of MCP-1 and KC may contribute to enhanced macrophage and neutrophil influx in the lung of VDR$^{-/-}$ mice.

3.3. Increased levels of extracellular matrix proteins in VDR$^{-/-}$ mice

The protein levels of MMP-2, MMP-9 and MMP-12, and enzyme activity of MMP-2 and MMP-9 were significantly increased in the lungs of VDR$^{-/-}$ mice compared to WT (Fig. 3A, B, C and D) as determined by immunoblot analysis and zymography assay, respectively. The expressions of tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 and TIMP-2 levels were significantly increased
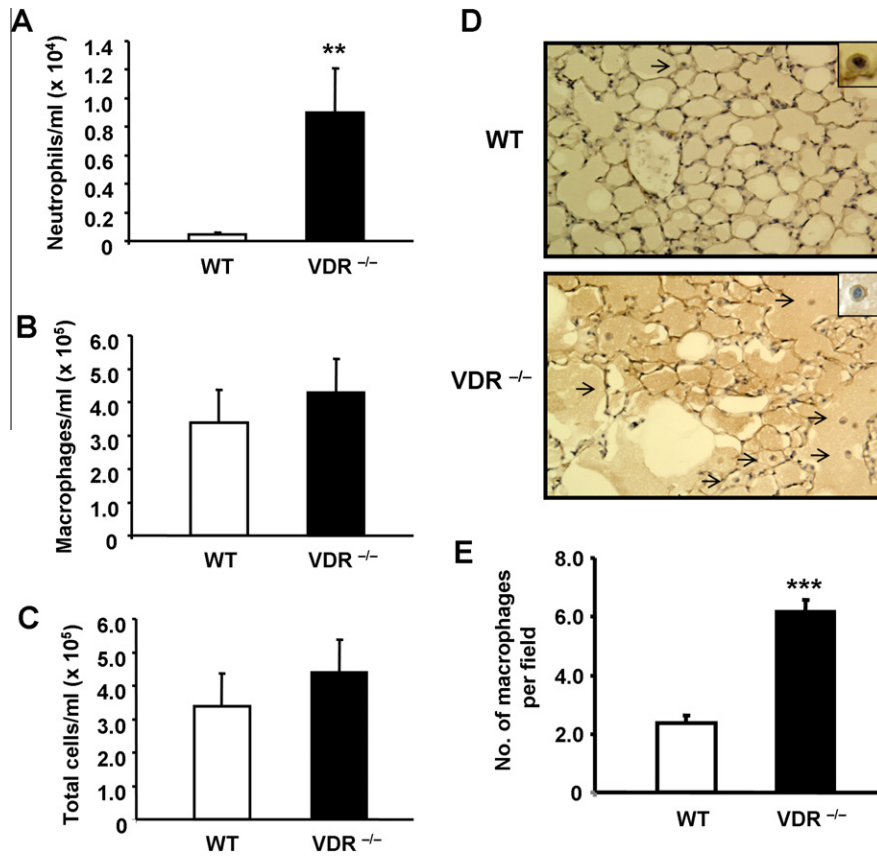
Fig. 1. Neutrophil and macrophage influx into BAL fluid of VDR$^{-/-}$ and WT mice. The number of neutrophils (A), macrophages (B) and total cells (C) on cytospin slides was determined by Diff-Quik staining. Lung sections of VDR$^{-/-}$ and WT mice were stained with anti-mouse Mac-3 antibody (D). Representative figure of Mac-3 positive cells identified by immunohistochemical staining (dark brown stained macrophages were indicated by arrows), Original magnification: 200×. Histogram (E) Values are mean ± SEM (n = 3–5 mice per group). $^{**}P < 0.01$, $^{***}P < 0.001$, significant compared to WT mice.
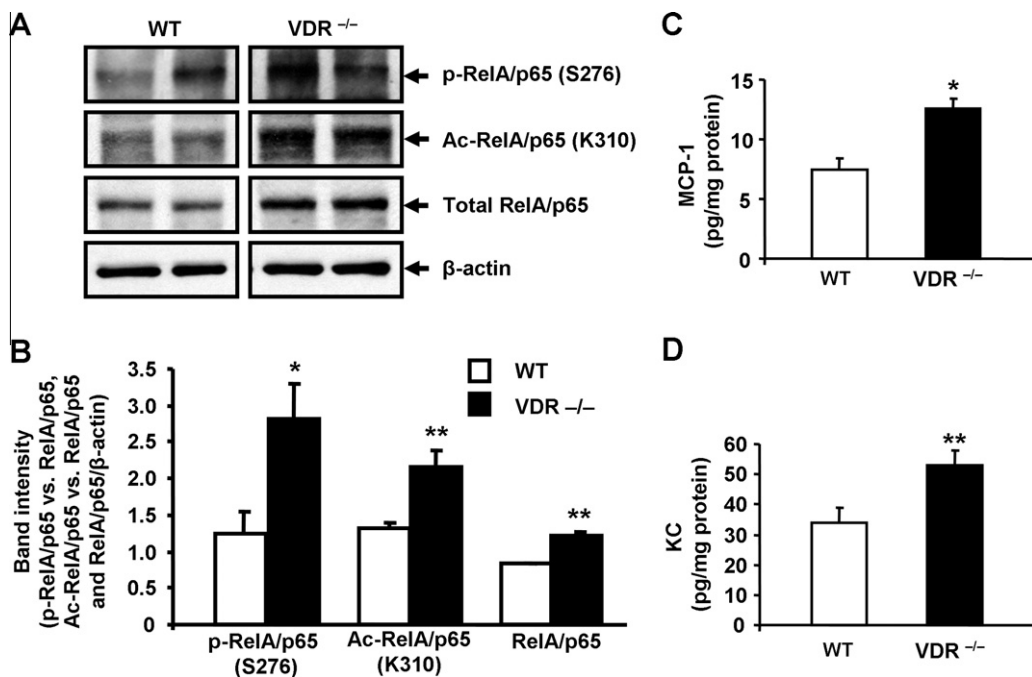

Fig. 2. Phospho/acetylation of NF-κB subunit RelA/p65 and NF-κB responsive proinflammatory cytokines in the lung of VDR$^{-/-}$ and WT mice. Phosphorylation (S276) and acetylation (K310) of NF-κB RelA/p65 was assessed in the whole lung homogenates of VDR$^{-/-}$ and WT mice by immunoblot analysis (A), and bands were measured by densitometry (relative band intensity versus total RelA/p65 or β-actin) (B). β-actin was used as a loading control. After densitometric analysis, values were normalized against loading controls. The levels of proinflammatory cytokines, MCP-1 (C) and KC (D) were measured by ELISA using the lung homogenates from VDR$^{-/-}$ and WT mice. Values are mean ± SEM (n = 3–4 mice per group). $^*P < 0.05$, $^{**}P < 0.01$, significant compared to WT mice.
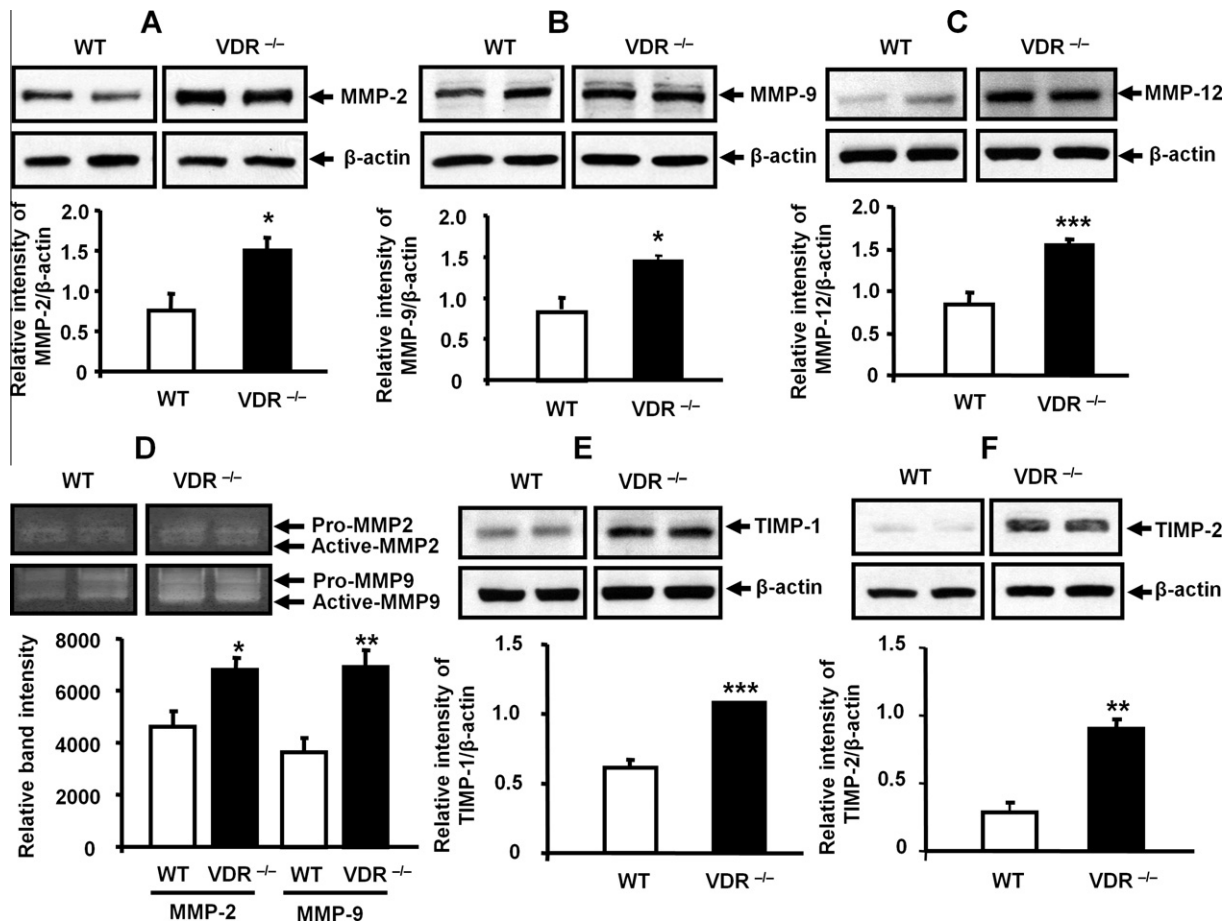
Fig. 3. Expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in the lungs of VDR$^{-/-}$ and WT mice. The protein abundance of MMP-2 (A), MMP-9 (B), MMP-12 (C) MMP-2 and MMP-9 enzyme activity (D), levels of TIMP-1 (E), and TIMP-2 (F) were assessed in the whole lung homogenates of VDR$^{-/-}$ and WT mice by immunoblot analysis and zymography assay. β-actin was used as a loading control. After densitometric analysis, values were normalized against loading controls. Values are mean ± SEM (n = 3–4 mice per group). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significant compared to WT mice.

in VDR$^{-/-}$ mice as compared to WT mice ($P < 0.01$) (Fig. 3E and F). There was no significant alteration in levels of TIMP-3 and TIMP-4 in the lungs among VDR$^{-/-}$ and WT mice (data not shown).

3.4. VDR$^{-/-}$ mice show emphysema/COPD phenotype and alterations in respiratory mechanics

Mean linear intercept (Lm) is commonly used as an index for characterizing airspace enlargement (emphysema) and the severity associated with structural destruction in the lungs [22]. To assess the airway enlargement, Lm was determined in age-matched VDR$^{-/-}$and WT mice. Lungs from the VDR$^{-/-}$ mice had greater Lm than the WT mice, in general the normal alveolar size being significantly increased ($P < 0.001$) compared to WT mice (Fig. 4A). Trichrome staining showed increased collagen deposition (blue stain) in the peribronchial area with lymphoid aggregates in VDR$^{-/-}$ mice as compared with WT (Fig. 4B). These lymphoid aggregates contain population of CD4$^+$ and CD8$^+$ T- and B-cells that are involved in obstruction of the small airways and thickening of the airway wall in patients with COPD and in mouse lung showing airspace enlargement [23,24]. These observations suggest that vitamin D/VDR deficiency is linked with proliferation of B-cells. Which contribute to the immune-inflammatory process in the aggregates of lymphoid follicles and/or development and perpetuation of emphysema.

The respiratory mechanics were assessed by the Flexivent using parameters, such as dynamic resistance (R), elastance (E), compli-

ance (C), Newtonian resistance (Rn), tissue damping (G) and tissue elastance (H). The dynamic resistance of the lung reflects the sum of both the airway resistance and tissue resistance. Dynamic resistance indicates the level of pulmonary constriction. The dynamic resistance was significantly increased in VDR$^{-/-}$ mice compared to WT mice ($P < 0.05$) (Fig. 4C). Dynamic elastance is the elastic rigidity of the lungs and compliance is the reciprocal of elastance. The lung compliance in VDR$^{-/-}$ mice was significantly decreased compared to WT mice ($P < 0.05$) and the lung elastance in VDR$^{-/-}$ mice was significantly increased compared to WT mice ($P < 0.05$) (Fig. 4D and E). Other parameters, such as Rn and G remained, unaltered among WT and VDR$^{-/-}$ mice (data not shown), whereas tissue elastance was significantly increased in VDR$^{-/-}$ mice compared to WT mice (data not shown). Based on the respiratory mechanics, it is evident that VDR$^{-/-}$ mice show fibrosis phenotype, which lack correlation between the emphysema phenotype measured by lung morphometry (Lm). Overall, these results suggest that VDR$^{-/-}$ mice had several lung phenotypic alterations, which make it an appropriate *in vivo* model to study the pathogenesis of chronic lung diseases.

4. Discussion

In the current study, we reported that VDR deletion led to increased influx of inflammatory cells, elevated phospho-acetylation of NF-κB RelA/p65, and up-regulation of MMP-2, MMP-9, and MMP-12 in the lung. These changes were associated with emphy-
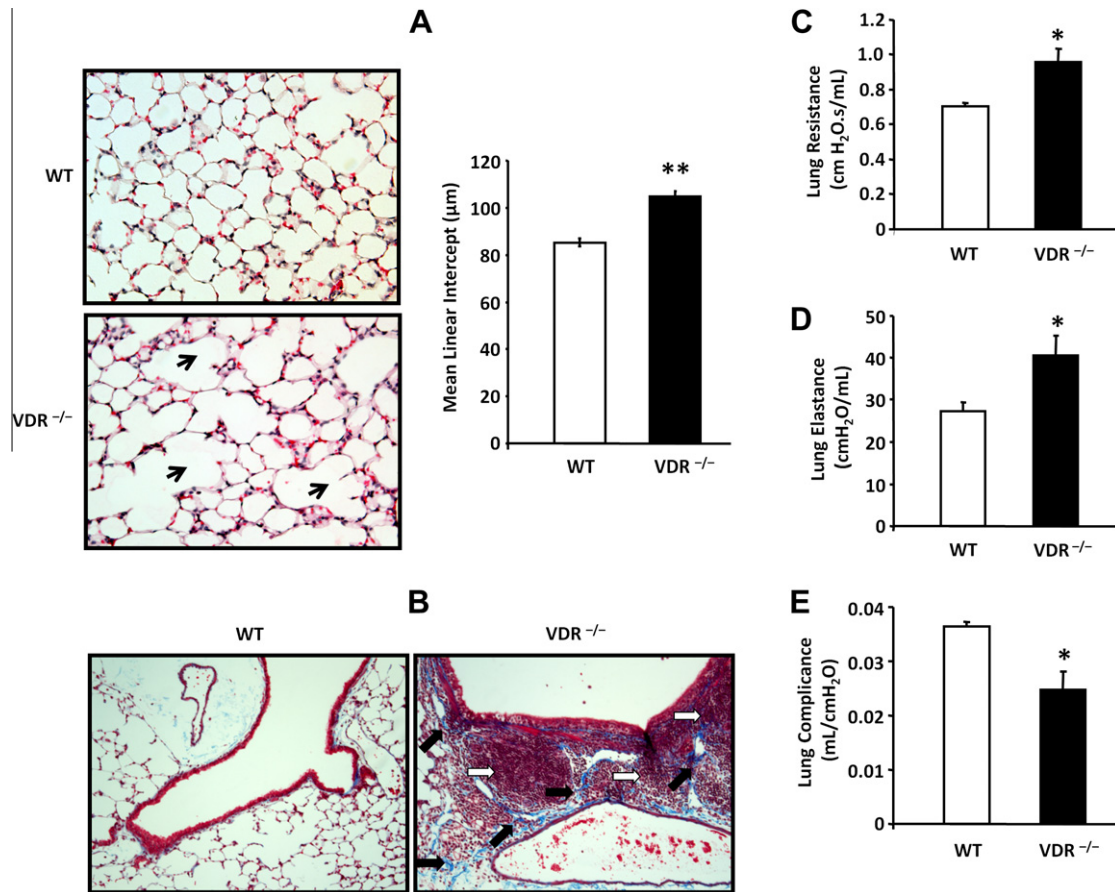
Fig. 4. Morphometric analysis, trichrome staining and respiratory mechanics in lungs of VDR$^{-/-}$ and WT mice. Representative figure of hematoxylin and eosin (H&E) stained lung sections from VDR$^{-/-}$ and WT mice. Original magnification: 200×. Mean linear intercept (Lm) was calculated using H&E stained slides (A). Representative figure of trichrome stained lung sections from WT and VDR$^{-/-}$ mice showing peribronchial collagen deposition stained dark blue (dark arrows), and lymphoid aggregates (light arrows). Original magnification: 100× (B). Significant increase in Lm between the VDR$^{-/-}$ and WT mice was observed. Dark arrow indicates alveolar airspace enlargement in (A). Dynamic lung resistance (C), lung elastance (D) and lung compliance (E) as a measure of lung mechanics were determined in WT and VDR$^{-/-}$ mice using Flexivent. Values are mean ± SEM (n = 3–5 mice per group). $*P < 0.05$, $***P < 0.001$, significant compared to WT mice.

sema and decline in lung function in the VDR$^{-/-}$ mice. Our findings indicate that deficiency of VDR in mouse lung can lead to an early onset of emphysema/COPD possibly due to inflammation, immune dysregulation, and lung destruction.

Vitamin D regulates genes that are involved in inflammation, cellular proliferation, differentiation, and apoptosis [9,10,12,13]. It also plays an important role in steroid resistance and airway remodeling which are the hallmarks of severe asthmatics and patients with COPD, and their exacerbations [5,7]. The levels of VDR were significantly decreased in lungs of patients with COPD compared with smokers (unpublished observation). Therefore, we studied the lung phenotypic, physiological characteristics, associated key signaling molecules and respiratory mechanics in the lungs of VDR$^{-/-}$ mice. VDR$^{-/-}$ mice showed significant increase in neutrophil influx in BAL fluid and increased macrophage influx into lung interstitium compared to WT mice. These data suggest that deficiency of VDR can recruit inflammatory cells in the lungs possibly due to increased release of NF-κB-dependent chemoattractants/mediators. Activation of NF-κB (RelA/p65), measured as phosphorylation of (Ser276) and acetylation (Lys310), is increased in lungs of VDR$^{-/-}$ as compared to that of WT mice. This was associated with increased levels of NF-κB-dependent proinflammatory mediators, MCP-1 and KC in lungs of VDR$^{-/-}$ mice. The mechanism for activation of NF-κB pathway in VDR$^{-/-}$ mice has been studied earlier [15,16,25]. VDR interacts with components of NF-κB pathway molecules directly or indirectly, and upon deficiency of VDR then leads to activation of NF-κB. We have recently demonstrated

the inhibitory role of VDR in NF-κB activation by regulating IκBα (decreased) levels, and interaction of VDR-RelA/p65 using the VDR$^{-/-}$ mouse embryonic fibroblasts [15,16,25]. However, the exact role of VDR in relation to NF-κB signaling in lung inflammation remains elusive.

Inflammatory cells, which are recruited in the lung, are known to release numerous mediators that can cause airway constriction and remodeling as well as produce proteases (elastases, cathepsins, granzymes and MMPs) that could destroy the lung parenchyma. Therefore, we determined the levels and activities of MMPs and tissue inhibitors of metalloproteinases (TIMPs) that are potentially involved in alveolar destruction (emphysema) and extracellular matrix remodeling. VDR$^{-/-}$ mice show activation of MMP-2, MMP-9 and MMP-12 in the lungs as compared to WT. Activity analysis correlates with protein expression showing increased enzyme activity of MMP-2 and MMP-9 in VDR$^{-/-}$ mice compared to WT. This implies that increased activation of NF-κB along with MMPs may further lead to progression of increased alveolar destruction and decline in lung function. Earlier studies in the heart of VDR$^{-/-}$ mice have demonstrated a significant increase in MMP-2 and MMP-9 mRNA levels, as well as enzyme activity along with collagen deposition, and fibrosis, which contribute to progression of cellular hypertrophy [26]. This finding is consistent with the gene profile study that vitamin D modulates expression and metabolism of extracellular matrix genes in the heart of VDR$^{-/-}$ mice [26]. The increased levels of MMPs may also be due to direct NF-κB activation or activation of mediators which can activate

MMPs. For example, 1,25-$(OH)_2$D directly or indirectly modulates extracellular matrix homeostasis in tissues particularly in lung and skin tissues by regulation of transforming growth factor-β (TGF-β) and MMPs [27,28]. The mRNA levels of anti-aging related genes, such as fibroblast growth factor-23 (Fgf-23), p53 and insulin-like growth factor 1 receptor (IGF1R) were significantly decreased in older VDR$^{-/-}$ mice suggesting that ablation of VDR promotes premature aging and vitamin D_3 homeostasis regulates physiological aging [13,17].

Since the VDR$^{-/-}$ mice had increased lung inflammatory response without any stimuli associated with activation of NF-κB and MMPs/TIMPs, we further determined the airspace enlargement as mean linear intercept in the lungs of VDR$^{-/-}$ mice. VDR$^{-/-}$ mice showed significant increase in alveolar destruction and airspace enlargement (emphysema phenotype) with altered lung function (lung resistance and compliance) compared to the WT. The results from respiratory mechanics show that VDR$^{-/-}$ mice had fibrosis phenotype and lacked correlation between the airspace enlargement confirmed by lung morphometry. Our observations corroborate with earlier report that structural emphysema does not correlate with lung compliance [20]. This might be due to a lack of correlation involved in anatomic emphysema, which is distinct from that which causes the loss of elastic recoil [20]. The exact reason for increased airspace enlargement or reduced alveologenesis/alveolar septation remains unclear, and requires further studies in VDR$^{-/-}$ mouse lung over a developmental time course from postpartum day 1 (PP1) through PP14.

Several studies that links vitamin D and COPD have highlighted the impact of low vitamin D levels and associated polymorphisms of vitamin D-binding protein on progression and severity of chronic respiratory infections, innate immune response, susceptibility to microbial infections, osteoporosis, muscle wasting, and steroid resistance associated with COPD is modulated both by innate and adaptive immunity [4–6,9,10,29–34]. Inflammatory immune cells form an aggregate as large volume together with the pool of inflammatory cells (polymorphonuclear leukocytes, macrophages, lymphocyte subtypes CD4$^+$ and CD8$^+$ T-cells, and B lymphocytes) as lymphoid follicles in severe cases of COPD [23,24]. Trichrome staining clearly shows increased collagen deposition in the lung peribronchial and perivascular areas along with lymphoid aggregates, which may include immune regulatory T-cells and or B-cells in VDR$^{-/-}$ mice as compared to WT mice.

In summary, vitamin D dyshomeostasis is implicated in chronic lung disease with altered lung function. Vitamin D deficiency is common in patients with asthma and COPD which further correlates with decreased VDR levels compared to smokers. Ablation of VDR resulted in increased inflammatory cellular influx into the lung, which was associated with increased site-specific posttranslational modifications, such as phosphorylation and acetylation of NF-κB RelA/p65 subunit, release of NF-κB-dependent proinflammatory cytokines MCP-1 and KC, and lymphoid aggregates formation. Activation of NF-κB and MMPs in the lung of VDR$^{-/-}$ mice further led to increased airspace enlargement and decline in lung function. These data suggest that VDR plays a vital role in lung development, function and extracellular matrix remodeling. These findings conclude the importance of VDR$^{-/-}$ as a model to understand molecular mechanisms underlying various chronic lung disease including COPD/emphysema, asthma and their exacerbations which are associated with vitamin D deficiency.

Acknowledgments

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