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Mycobacterium tuberculosis Infection is Exacerbated in Mice Lacking Lecithin:Retinol Acyltransferase.

Steven E. Trasino[1,2], **Xiao-Han Tang**[1], **Carolina Trujillo**[3], **Jaclynn Andres**[3], **Sabine Ehrt**[3], **Lorraine J. Gudas**[1]

[1]Department of Pharmacology, Weill Cornell Medical College of Cornell University, New York, NY.

[2]School of Urban Public Health, Nutrition Program, Hunter College, City University of New York, New York, NY.

[3]Department of Microbiology and Immunology, Weill Cornell Medical College of Cornell University, New York, NY.

(1.2) INTRODUCTION

Vitamin A [(VA), retinol, (ROL)] and its family of metabolites, including retinaldehyde, retinyl-esters (RE), and all-trans retinoic acid (RA), are referred to as retinoids. RA signals by binding retinoic acid receptors (RAR α, β and γ) and regulates the transcription of genes vital to human health (1,2), including genes involved in innate and adaptive immune responses [Reviewed in (3, 4)]. In humans VA deficiency (VAD) is associated with increased severity of *Mycobacterium tuberculosis (Mtb)* infection (5–8), but VAD is often found in the setting of wasting and other micronutrient deficiencies (6), making it challenging to determine a causal relationship between VA and *Mtb*. Using wild-type (WT) mice and a genetic VAD model, LRAT−/− mice, that are unable to synthesize and store RE in lung,

*CORRESPONDING AUTHOR: To whom correspondence should be addressed: Lorraine J. Gudas, Ph.D., Department of Pharmacology, Weill Cornell Medical College of Cornell University, 1300 York Avenue, New York, NY, 10065; telephone: +1-212-746-6250; ljgudas@med.cornell.edu.

(1.8) AUTHORSHIP CONTRIBUTIONS

SET, XHT, CT, SE, JA & LJG designed and conducted research, and data collection.

SET, XHT, CT, SE & LJG conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review.

CRediT author statement

Steven E. Trasino: designed and conducted research, data collection, conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review, **Xiao-Han Tang**: designed and conducted research, data collection, conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review, **Carolina Trujillo:** designed and conducted research, data collection, conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review, **Jaclynn Andres:** designed and conducted research, and data collection, **Sabine Ehrt:** designed and conducted research, data collection, conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review, **Lorraine J. Gudas:** designed and conducted research, data collection, conducted molecular and statistical analysis, provided data interpretation, manuscript writing and critical review.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

liver, and other tissues (9), here we explored the interrelationship among VAD, *Mtb* infection, and lung pathology using an experimental model of *Mtb* infection.

(1.3) ABRIDGED MATERIALS AND METHODS.

(see expanded MATERIALS AND METHODS in supplemental file)

(1.3.1) Animals.

Generation of LRAT $^{-/-}$ mice was previously described (9). We performed all procedures involving wild-type (WT) C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME) and LRAT −/− mice according to NIH guidelines for housing and care of laboratory animals, that were reviewed and approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

(1.3.2) Vitamin A Deprivation.

We conducted the dietary vitamin A deprivation using vitamin A sufficient (VAS) and VAD diets in WT and LRAT −/− mice as previously described (10).

(1.3.3) Aerosol Infections.

We performed aerosol infections of WT and LRAT −/− C57BL/6 mice as previously described (11).

(1.3.4) Immunohistochemistry (IHC).

We performed IHC as previously described (12).

(1.3.5) High Performance Liquid Chromatography (HPLC) of Retinoids.

We analyzed tissue retinoids extracted from lung, liver, and serum by HPLC as described (13).

(1.3.6) RNA Isolation and Quantitative RT-PCR (q-PCR).

We isolated total RNA from whole lung homogenates and performed quantitative RT-PCR (qRT-PCR) with gene specific primers (Table S1) as described (13).

(1.4) RESULTS AND DISCUSSION

(1.4.1) Mice lacking LRAT have lower lung retinol levels than WT mice, even on a VAS (vitamin A sufficient) diet.

We first sought to determine the VA status of WT and LRAT −/− mice after being fed either a VAS or VAD diet for 5 weeks (extended supplemental methods) prior to aerosol infection with *Mtb*. At day 1 (day of infection), as expected, we did not detect RE in lungs from VAS and VAD LRAT$^{-/-}$ mice (Fig. 1A). Also, VAD LRAT−/− mice exhibited >3-fold and 2-fold reductions in lung and serum ROL levels, respectively, compared to VAS WT mice (Fig. 1A), supporting the onset of a VAD state. In contrast, in VAS LRAT−/− mice day 1 serum and lung ROL levels were the same as, and ~1.4 fold lower, respectively, than those in VAS WT mice (Fig 1A); these results are consistent with our and others' previous findings that

LRAT −/− mice in the presence of dietary VA have lower retinol, but no RE, in their lungs compared to VAS WT mice (9, 14).

(1.4.2) Mice lacking LRAT have higher pulmonary *Mtb* burden than WT mice.

We next measured *Mtb* colony forming units (CFUs) at various time points post-infection and found that compared to VAS WT mice, *Mtb* CFUs were not different between any of the groups at day 14 or 28 post-infection (Fig. 1B, C). However, at day 28, compared to VAS WT mice, both VAS and VAD LRAT−/− mice had more severe lung pathology, marked by greater numbers and areas of inflammatory regions (Fig. 1E, G, H, and I). Similarly, at day 56 post-infection, in both VAS and VAD LRAT−/− mice lesion numbers and areas (Fig. 1F, J, K, and L), as well as CFUs (Fig. 1B, D), were higher than in VAS WT mice. Interestingly, *Mtb* CFUs did not differ between VAS and VAD LRAT −/− mice at any time point through the infection course (Fig. 1B, C, D), despite our hypothesis, based on the risk of *Mtb* in humans with VAD (5–8), that LRAT −/− mice could be more susceptible to *Mtb* infection regardless of their diet.

(1.4.3) Lung and liver retinoid levels are altered in response *Mtb*.

We measured tissue retinoid levels at numerous time points to determine the temporal changes in lung, serum, and liver retinoids in response to *Mtb* infection. Compared to day 1, lung total RE, but not ROL (Fig. S1A), increased by day 56 in VAS WT in response to *Mtb* infection (Fig. S1B), suggesting the decreased mobilization of lung RE pools, and/or increased uptake and esterification of diet and liver-derived ROL. Consistent with this idea, we found a different pattern in the liver. VAS WT showed an increase in liver total RE and ROL levels from day 1 to day 28, but then a steady decline in both from day 28 to day 56 (Fig. S1C, D). These data suggest that in response to lung and systemic *Mtb* infection, VAS WT mice initially increase lung and liver retinoid pools, potentially reflecting enhanced uptake of dietary VA, followed by hepatic mobilization of RE and export of ROL for uptake by lung or other tissues as *Mtb* infection becomes chronic. This idea is supported by a steady and concomitant increase in serum ROL from day 1 to day 56 in VAS WT mice (Fig. S1D). Thus, in the future it will be critical to measure the levels of SAA1, 2, and 3 (serum amyloid A proteins 1,2, and 3) in the liver and lung after Mtb infection, since SAAs, rather than RBP1, carry retinol throughout the body after bacterial infections(15, 16). Figure S1A shows at day 1,28, and 56 lower lung ROL levels in LRAT−/− mice regardless of their diet than that in VAS WT mice, indicating that low lung retinol level is a potential cause for more severe lung pathology observed in LRAT−/− mice regardless of their diet.

(1.4.4) *Mtb* Infection Reduces Lung Retinoid Signaling and LRAT Expression.

Using qPCR we then analyzed mRNA levels of a panel of retinoid relevant genes to determine lung retinoid pathway and signaling changes in response to *Mtb* infection. *RARβ2, CRBP1*, and *Cyp26A1* are RA-responsive genes (1), and thus provide a reliable indicator of tissue retinoid signaling. Our analysis showed that transcripts of *RARα, β2, γ, CRBP1*, and *Cyp26A1* steadily decreased from day 1 to day 56 (Fig. S1F–J) and, with the exception of *RARα*, the decreases in these mRNAs were greater in VAS and VAD LRAT−/− compared to VAS WT mice (Fig. S1F–J). We also measured lung LRAT mRNA in VAS WT, and similarly found that both lung LRAT mRNA (Fig. S1K) and protein levels (Fig. S1L–

O), decreased by ~5 and 2.5 fold, respectively, in VAS WT mice from day 1 to day 56. These results collectively demonstrate that, despite the increased levels of lung total RE (Fig. S1B), pulmonary retinoid signaling is greatly reduced with chronic *Mtb* infection, in line with reduced lung retinol levels (Fig. S1A). It remains unclear if the reduced LRAT and lung retinoid signaling are related, and whether these reductions in response to *Mtb* infection reflect an immune-evasion program. The latter may be more likely, given the anti-*Mtb* properties of retinoids (17), and their vital role in immune responses involved in *Mtb* control (3, 4).

(1.4.5) Mice Lacking LRAT Have Increased *Mtb* Pulmonary Immune Cell Infiltrates.

Using IHC, we next measured lung parenchyma protein expression of markers of macrophages (F4/80), T-cells (CD3e), and neutrophils (Ly6G), key inflammatory mediators involved in the innate and adaptive immune response to *Mtb* [Reviewed in (18)]. Consistent with the lung CFU levels and histology data (Fig. 1D, J–L), at day 56, lung inflammatory regions in VAS and VA LRAT−/− mice showed higher immunoreactivity and levels of F4/80 (Fig. S2A–C, J), CD3e (Fig. S2D–F, K) and Ly6G (Fig. S2G–I, L) compared to VAS WT. Also, with the exception of CD3e, lung parenchymal expression of F4/80 and Ly6G was also higher in both LRAT−/− cohorts groups compared to VAS WT (Fig S2J, L). Consequently, we then measured lung mRNA levels of *TNFα, IL1β, IL6, IL12p40* and *IFNγ*, pro-inflammatory cytokines that have a central role in *Mtb* infection control [Reviewed in (19)]. We found that *TNFα* and *IL1β* mRNAs were higher in VAD LRAT −/− at days 28 and 56, and *TNFα* in VAS LRAT −/− at day 56 compared to VAS WT mice (Fig S2M, N). Lung *IL6* and *IL12p4* mRNA levels were also higher in VAS and VAD LRAT −/− on day 56 compared to VAS WT (Fig. S2O, P). There were no differences in lung *IFNγ* mRNA levels among any of the experimental groups (Fig. S2Q). The increased lung immune-infiltrate and expression of pro-inflammatory cytokines in both VAS and VAD LRAT −/− cohorts is consistent with their higher *Mtb* burden at day 56 (Fig. 1B, D), and with human and murine models of *Mtb* showing that excessive lung inflammation leads to excessive lung immunopathology and worse clinical outcomes (20, 21). Retinoids possess anti-inflammatory properties through modulation and inhibition of Th1 pro-inflammatory immune responses (3, 4). Conversely, VA deficient states are associated with increased inflammation and risk for infections, including *Mtb*(4, 5). Thus the reductions in pulmonary ROL and RA signaling in *Mtb* infected LRAT −/− mice suggest a causal relationship between pulmonary retinoid status and increased levels of pulmonary cell infiltrate and mRNA transcripts of Th1 proinflammatory cytokines.

In conclusion, data from this initial study demonstrate that mice lacking LRAT are potentially at increased risk for an aggravated response to *Mtb* infection because of their lower lung ROL levels. Although serum levels of ROL were unchanged in VAS LRAT −/− mice throughout the study, which generally indicates a normal VA status, (Figure S1E), their lung levels of ROL were lower than those in VAS WT on day 1 (preinfection) (Fig. 1A), which likely contributed to their higher *Mtb* CFUs on day 56 (Fig 1B, D). We also recognize that the differences in CFUs between LRAT −/− and WT VAS mice were modest (Fig. 1B), but do suggest, in light of the more severe lung immunopathology in LRAT −/− mice, that

with longer, more chronic infection the differences in *Mtb* burden between VAS WT and LRAT −/− mice might be more pronounced.

Data from this pilot study reveal for the first time potentially complex bi-directional relationships among pulmonary retinoid status, signaling, and *Mtb* infection, and provide the first genetic and molecular support for a role of lung retinoid status in *Mtb* infection. Since these data are from a single experiment, future and more expansive studies should further investigate the impact of VAD on *Mtb* infection and examine the subtypes of immune cells and circulating cytokine profiles in *Mtb* infected LRAT −/− mice. Further examination of the role of pulmonary VA and LRAT in host-response to *Mtb* is also warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mice lacking LRAT show increased risk for an aggravated response to *Mycobacterium tuberculosis (Mtb)* infection.
- *Mtb* infection in wild-type mice is associated with increased levels of lung retinoid levels, but reductions in lung retinoid-related transcriptional signaling.

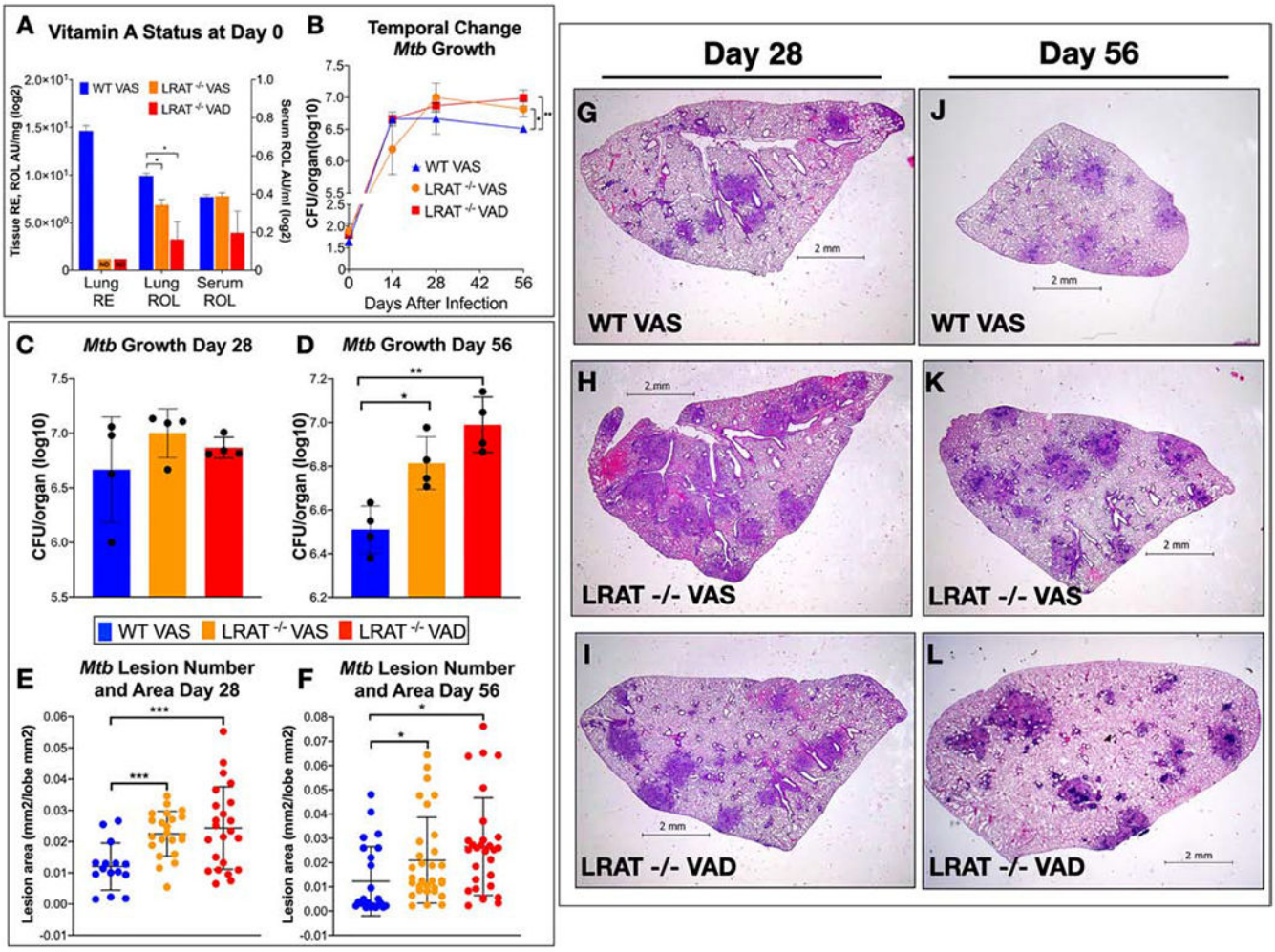

Figure 1. Mice lacking LRAT have higher TB burden than WT mice.

A) Lung and serum levels of total retinyl-esters (RE) and serum retinol (ROL) in Wt C57BL/6 (Wt) and LRAT −/− mice after 5 -weeks of being fed either vitamin A sufficient diet (VAS), [Wt VAS (n=4), LRAT -−/− VAS (n=4)] or a vitamin A deprivation diet (VAD), [LRAT −/− (VAD) (n=4)]. **B)** Colony-forming units (CFU) in lungs from mice described in **A)** infected by aerosol with *Mtb* strain H37Rv from day 1 to day 56 post-infection. Data are means ± SD of 4 mice per group. **C-D)** CFU in lungs from mice described in **A)** on day 28 and day 56 post-infection. **E-F)** Mean inflammatory lesion area (mm2/lobe mm2) in lungs from mice described in **A)** on day 28 and day 56 post-infection. **G-L)** Representative hematoxylin and eosin-stained whole lungs demonstrating inflammatory lesions on post-infection day 28 **(G-I)** and post-infection day 56 **(J-L)** of *Mtb* infected mice described in A). Errors bars represent ± SD, with *p<0.05, ***p*<0.01, ****p*<0.001 significant values representing differences in LRAT −/− VAS and VAD compared to VAS WT derived by with ANOVA and Dunnet's multiple comparison test.