RETINOIC ACID SYNTHESIS BY LUNG ANTIGEN PRESENTING CELLS AND INDUCTION OF ITS SYNTHESIS BY *MYCOBACTERIUM TUBERCULOSIS*.

By

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(date) 12/15/13

*We also certify that written approval has been obtained for any proprietary material contained therein.
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<td>Autofluorescence</td>
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<td>AG</td>
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<td>AM</td>
<td>Alveolar macrophages</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>atRA</td>
<td>All trans retinoic acid</td>
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<td>BAL</td>
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<td>BCG</td>
<td><em>Mycobacterium bovis</em> bacillus Calmette-Guerin</td>
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<td>CMI</td>
<td>Cell-mediated immunity</td>
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<td>Cyto D</td>
<td>Cytochalasin D</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IFN-γ</td>
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<td>MHC</td>
<td>Major histocompatibility concept</td>
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<tr>
<td>MLN</td>
<td>Mediastinal lymph node</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
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<td>Retinal dehydrogenases</td>
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<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TLR</td>
<td>Toll like receptors</td>
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<td>Treg</td>
<td>Regulatory CD4+ T cells</td>
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<td>WT</td>
<td>Wild type</td>
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VII. Abstract

Retinoic Acid synthesis by lung antigen presenting cells and induction of its synthesis by *Mycobacterium tuberculosis*.

By

Yeritza I. Hernandez-Collazo

Retinoic acid (RA), the biologically active form of vitamin A (retinol) plays a pivotal role in immunity. RA is initially produced by the oxidation of retinol to retinal and subsequently of retinal to RA. The three isoforms of RALDH enzyme, RALDH-1, RALDH-2, and RALDH-3, catalyze the oxidation of retinal to RA. Expression of the RALDH enzymes is restricted to certain cell types. We hypothesized that lung antigen presenting cells (APCs) express RALDH and are capable of synthesizing RA. We also suggested that infection with *Mycobacterium tuberculosis* (MTB) induces RALDH expression in lung APC contributing to the lung’s immune suppressive environment. Thus different lung cell subsets were subjected to RT-PCR analysis to detect RALDH expression. Whole Lung expressed RALDH-1 and RALDH-2 isoforms. Brochoalveolar lavage (BAL) cells and magnetically sorted lung CD11c⁺ cells expressed RALDH-2. MTB induces RALDH-2 expression in spleen and lung CD11c⁺ cells. Induction of RALDH-2 is dose dependent and peaks after 24 hours. Phagocytosis of MTB bacillus was required for the induction of RALDH-2 mRNA expression. GM-CSF, a strong inducer of RALDH-2 expression, was secreted by MTB infected APCs and mediated the MTB-induced RALDH-2 expression by APC.
1a. *Mycobacterium tuberculosis*: The Causative Agent of Tuberculosis

*Mycobacterium tuberculosis* (MTB) is the causative agent of tuberculosis (TB). In 2011, 8.7 million people were diagnosed with TB and 1.4 million died from the disease. Although the estimated number of people who will be develop disease each year declined, TB is still the second greatest single infectious agent killer worldwide (1). The immune system is generally successful in controlling the primary infection and only 5-10% of the infected population is estimated to develop primary disease. However MTB bacilli are rarely eradicated, and individuals can retain a residual dormant bacterial within granulomas in the lungs (2-5). These mycobacteria can reactivate later when the immune system becomes compromised and lead to re-activation disease. The currently licensed MTB vaccine, live *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), was discovered nearly 100 years ago and confers variable to incomplete protection against pulmonary TB. Development of a new vaccine has been unsuccessful due to limited understanding of the immune response to *M. tuberculosis* infection and disease in the lung (6).
1b. Immunity to MTB

MTB is spread from person to person by inhalation of aerosolized bacilli. Transmission usually involves direct inhalation of the airborne pathogen. After inhalation, MTB bacilli enter the alveolar space and invade phagocytic cells, mostly alveolar macrophages (AM) to establish infection (7-8). The host, in turns, initiates an innate followed by an adaptive immune response to the mycobacteria (9). Most responses during the early stages of MTB infection represent a balance between the ability of the mycobacteria to replicate and the ability of infected macrophages to rally mechanisms to destroy the invading pathogen. MTB survives inside the macrophages by inhibiting the normal maturation of the phagosome (10-11). If alveolar and other lung macrophages do not destroy or inhibit MTB, the bacilli will multiply logarithmically within the lung.

The logarithmic phase of growth can be arrested with the development of cell-mediated immunity (CMI) (9,12-14). During the first two to three weeks after infection, T cell immunity develops and antigen-specific T cells migrate to the lungs including the alveolar space. CD4+ T cells proliferate and differentiate at the site of infection orchestrating a Th-1 response. Interactions between T cells and infected macrophages initiate the formation of the granuloma, hallmark of MTB infection (5, 13-14). The CMI response to MTB infection is so potent that on average, it is estimated that 90% of infected individuals are able to contain MTB infection and do not develop disease during their lifetime.
The involvement of adaptive immunity and Th-1 mediated response in controlling MTB has been well established. In contrast, how antigen presenting cells (APCs) and innate immunity mediate host defense against mycobacteria is less well understood.

1c. Lung Antigen Presenting Cells (APC) and MTB

The lungs are divided into two major compartments: the alveolar space and the parenchyma. In a steady state, the majority of cells in the alveolar space are alveolar macrophages (15-16). AM are the primary cell type involved in the initial uptake of MTB. Resident AM modulate T cell responses in the lung, but they also may have immunosuppressive functions (17-21). On the other hand, lung parenchymal tissue that includes airway epithelium comprises a variety of APC types including dendritic cells (DCs), macrophages and B cells (16). Using CD11c/CD11b surface marker expression to differentiate lung APCs, there are three major APC populations in the lung: 1) CD11c\textsuperscript{high}/CD11b\textsuperscript{low} (AM), 2) CD11c\textsuperscript{high}/CD11b\textsuperscript{high} (DCs), and 3) CD11c\textsuperscript{low}/CD11b\textsuperscript{high} (interstitial macrophages or monocytes). In MTB, Depletion of mouse AM with dichloromethylene diphosphonate containing liposomes enhances protective immunity against MTB and augments DC function (20, 21).

DCs are present along the entire respiratory tract. They are abundant in the upper airway, but few are present in the distal airways and alveolar spaces. In mice, after aerosol infection with MTB, myeloid DCs become infected in the lungs and transport the bacilli to the mediastinal lymph nodes (MLN) (22). However, where
MTB and DC encounter one another is still not clear since MTB infects the alveolar space. DCs transport of MTB to the local lymph node is a critical determinant for the initiation of adaptive immune responses to MTB.

**1d. APCs: Pro-Inflammatory vs anti-inflammatory responses**

Recognition of MTB by phagocytic cells leads to cell activation and cytokine production. These cytokines will induce further activation and cytokine synthesis in other cell types resulting in a cascade of cross-regulation. MTB bacilli induce cytokines with both pro-inflammatory and anti-inflammatory responses. The main cytokines involved in MTB clearance, TNF-α and IFN-γ are consider pro-inflammatory cytokines. Monocytes and T cells are the main producer of these cytokines, respectively. TNF-α plays a key role in granuloma formation and macrophage activation (23). INF-γ production in response to MTB antigens can be used as a surrogate marker for MTB infection. It is secreted after T cell activation and in return activates infected macrophages to kill intracellular MTB. In addition, IFN-γ enhances the processing and presentation of MTB antigens to T cells. While IFN-γ is an essential cytokine it is not sufficient to control MTB infection (24-25). MTB infection also induces the production of IL-1β, IL-6, and IL-12 (crucial for the induction of INF-γ), IL-18 and IL-15. With exception of IFN-γ, these pro-inflammatory cytokines are mainly produced by APCs.

Anti-inflammatory cytokines are also a key component of responses to MTB infection and generation of pathology. IL-4, IL-10 and TGF-β mainly inhibit the
production or the effect of pro-inflammatory cytokines. Macrophages and some reactive T cells produce IL-10 and it is known to down-regulate the production of IFN-γ, TNF-α and IL-12 (26-28). TGF-β is synthetized by monocytes and DCs and produced in excess at sites of MTB infection. In T cells, TGF-b inhibits T cell proliferation and INF-γ production. In macrophages it inhibits activation and antagonizes APC function (29).

Regulatory T (Treg) cells are characterized by expression of the transcription factor Foxp3 and play a key role in immune homeostasis. Treg cells can down-regulate the immune system and are essential for the prevention of autoimmunity. Tregs also can suppress antimicrobial immune responses, especially against pathogens that establish persistent chronic infections (30-31). These cells have been found during MTB infection and in a mouse model require TGF-β for their generation.

**1e. Retinoic Acid as immune regulator**

Retinoic acid (RA) is a metabolite of vitamin A. There are two different RA isoforms, all-trans-RA (at-RA) and 13-cis-RA. RA has a number of regulatory functions in the immune system. It is required for the differentiation of promyelocytes into neutrophils (32) and contributes to the differentiation and induction of homing receptors on T and B cells (33-34).

RA is obtained through foods containing vitamin A precursors (i.e.,
carotenoids) or vitamin A itself in the form of retinyl esters (35). After absorption, retinol is oxidized to retinaldehyde, by alcohol dehydrogenases (ADHs). The final step for RA synthesis, oxidation of retinaldehyde to RA, is catalyzed by retinal dehydrogenases (RALDH). There are three isoforms of retinaldehyde dehydrogenase: RALDH1, RALDH2, and RALDH3 (36-38). These enzymes have tissue-specific patterns of expression.

Vitamin A and RA are considered immune regulators of both humoral and cellular immune responses (39-42). However, the cellular and molecular mechanisms used by retinoids in the immune system remain poorly understood. RA can effectively induce and enhance regulatory CD4+ T cell (Treg cell) frequencies and function (43-44). In conjunction with TGF-β, it induces the differentiation of CD4+ naive T cells into Treg cells (43, 45-46). RA also enhances the expression of α4β7 and CCR9 on T cells upon activation after antigenic stimulation with dendritic cells from the gut-associated lymphoid organs (33) and contributes to B cell homing and isotype switching (34). It has also been shown that RA can induce the secretion of anti-inflammatory cytokines by APCs. RA can enhance the production of IL-10 and reduce the synthesis of IL-12 and TNF-α from DCs and macrophages (47-48). In turn, vitamin A deficiency leads to increments in pro-inflammatory cytokines such as IL-12 and IFN-y whereas supplementation with retinoids leads to anti-inflammatory responses and secretion of IL-10 and IL-4 (49-52).

During this study we focus on the analysis of RA synthesis by APC and the MTB mediated induction of RALDH-2. We dissected the mechanism used by the
mycobacteria to induce RALDH-2 expression and discuss the importance of RALDH-2 expression by APC.
2. Methods

Mice

Eight- to twelve-week-old female, C57BL/6 mice were purchased from Jackson Laboratories (Wilmington, MA). Mycobacterial Ag 85B specific- TCR transgenic (P25 TCR-Tg) mice were provided by Kiyoshi Takatsu (53). Tlr-2 gene knockout mice in the C57BL/6J background (Tlr2−/−) were generously provided by O. Takeuchi and S. Akira (Osaka University, Osaka, Japan). Mice were housed under specific-pathogen-free conditions in ventilated micro-isolator cages (Lab Products, Inc., Maywood, NJ) and fed a standard rodent diet and water ad libitum. All studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Bacterial stocks and peptides

*M. tuberculosis* H37Ra was cultured in Middlebrook 7H9 medium with ADC enrichment, and frozen stocks were prepared as described previously (54). Bacterial counts and viability were performed by counting CFU on 7H10 medium. *M. tuberculosis* H37Ra stocks were tested periodically for viability.

The 15-mer sequence of Ag85B, i.e. peptide 25 (aa 240-254) with the sequence FQDAYNAAGGHNAVF was synthesized by Invitrogen. MTB cell lysate was obtained
from Colorado State University and original stock was diluted in PBS to a concentration of 1mg/ml, stored at -20°C and used at concentrations of 1-100μg/ml.

**Antibodies and cytokines**

The following mouse antibodies were purchased conjugated to either Alexa Fluor® 647, PE-CF594, PerCP-Cy5.5, APC or Alexa Fluor 488. From BD Bioscience: rat anti-mouse CD8 (557682) and rat anti-mouse FoxP3 (562466). Anti-mouse CD3e (45-0031), anti-mouse CD4 (17-0042), anti-mouse CD25 (53-0251) and Anti-mouse CD16/32 used for Fc receptor blocking, were purchased from eBioscience.

Exogenous cytokines used were IL-2 (200u/ml), TGF-β1 (5 ng/ml), (R&D Systems, USA) and GMCSF (40ng/ml) (R&D).

*All-trans* retinoic acid (RA) was purchased from Sigma (USA), dissolved in DMSO (10 mM) and stored at -20°C and protected from light and used at a 100nM concentration. Cytochalasin D was purchased from Sigma (USA C8273) and dissolved in DMSO.

**Purification of Antigen presenting cells (APCs)**

In brief, spleen, lungs and mesenteric lymph nodes from C57BL/6 mice were harvested and processed using gentlyMACSDissociator© (Miltenyi Biotec, USA). Lungs were digested with collagenase type 4 in complete DMEM for 30 min at 37°C.
Processed and digested tissues were passed through a 70μm cell strainer (Fisher Scientific, USA) and red blood cells (RBC) were lysed in hypotonic lysis buffer (10 mM Tris-HCl and 0.83% ammonium chloride). Subsequently, CD11c+ cells were enriched by positive selection using anti-CD11c micro beads according to the manufacturer’s protocol (Miltenyi Biotec: 130-052-001). The resulting purity of CD11c+ cells was approximately 85-90%.

To obtain BAL, lungs of 8-12 weeks old mice were lavaged with 1ml PBS using a Angiocath syringe (BD: 381144). After lavage of the lungs, cells were washed with PBS and centrifuged to remove excesses. Cell pellets were resuspended in DMEM and kept in cold.

**CD4+ T cell isolation**

Purified CD4+ T cells were isolated from spleens using immune-magnetic cell sorting (IMACS). The CD4+ T cell negative selection kit (Miltenyi; 130-095-248) was used to obtain total resting CD4+ T cells by following the manufacturer’s instructions. The purity of CD4+ T cells was confirmed by flow cytometry and ranged between 88 and 95%. Additionally, contaminating CD8+ T cells were depleted with CD8+ T cell positive selection microbeads (Miltenyi; 130-049-401).
Cell culture

All experiments were performed with purified CD11c+ cells cultured in IMDM (BioWhittaker, Walkersville, Md.) supplemented with fetal calf serum (10% v/v, Gemini-Bioproducts, West Sacramento, CA; 100-106), HEPES (100mM), nonessential amino acids (100mM), L-glutamine (2 mM), and 2-mercaptoethanol (0.05 mM) at 37°C in 5% CO2. Experiments without M. tuberculosis H37Ra were supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml).

MTB infection of APCs

RALDH-2 induction in CD11c+ cells was evaluated as follows: CD11c+ cells from spleen and lungs were seeded in a 48 well plate (1x10^6 cells/well). Following plating of the cells, M. tuberculosis H37Ra was used to infect CD11c+ at MOIs of 0.3, 1, 3, 5 or 10 depending on the experiment. Experiments were done in triplicate. Cells were incubated at 37°C in a 5%CO2 atmosphere and collected 3, 6, 12 or 24 hours post infection for RNA extraction. Additionally cells were stimulated with GM-CSF (40ng/ml) (R&D) and MTB cell lysate (Colorado State University) in a concentration range of 1-100μg/ml.

Treg cell induction

To generate Treg cells from lung and splenic CD11c+ cells, we preceded as follows: CD11c+ cells from spleen, lungs and BALs of C57BL/6 mice were isolated
and stimulated with 4 ng/ml of murine recombinant IFN-γ (Peprotech, Rocky Hill, NJ; 315.05) for 48 hours prior to use to upregulate MHC-II expression, then plated as 50,000 - 250,000 cells/well in a 96-well plate and cultured overnight in supplemented IMDM with or without Ag85B. APCs were then co-cultured with CD4+ T cells isolated from P25 TCR Tg mice with or without TGF-β (5ng ml⁻¹) (R&D, 100-B) and RA (100nM) (Sigma, R2625). Medium was changed after 3 days of culture and cells were harvested after 5 days for flow cytometric analysis.

**Flow cytometry**

Prior to staining, cells were washed and re-suspended in staining buffer containing 1x PBS and 5% BSA. To block non-specific staining, the 2.4G2 anti-CD16/32 antibody was added. For surface receptor expression on CD4+ T cells, antibodies were added and cells were incubated 25 min. on ice. For intracellular cytokine staining cells were re-suspended in Pharmigen Transcription Factor Buffer Set (BD Bioscience: 562574) and intracellular cytokine staining was performed according to the manufacturer’s protocol.

**RALDH measurement and RT-PCR**

Total RNA was isolated from bronchioalveolar lavages (BAL) of the lungs, lungs, spleens, mesenteric lymph nodes and purified CD11c+ cells of C57BL/6 mice using the RNeasy Plus Mini Kit (Qiagen, Germantown, MD; 74134), according to the
manufacturer’s protocol. cDNA was generated using Superscript III First-Strand Synthesis System for RT-PCR and oligoDTs (Invitrogen; 18080051) or the QuantiTect® Reverse Transcription kit (Qiagen; 205310), according to the manufacturer’s protocol. Inventoried TaqMan gene expression Assays (Applied Biosystems; 4331182), were used to perform Real Time-PCR with primers for RALDH-1 (Invitrogen, Mm00657317_m1), RALDH-2 (Invitrogen, Mm00501306_m1), RALDH-3 (Invitrogen, Mm00657317_m1) and house keeping gene β-actin ((Invitrogen, Mm00657317_m1) on cDNA samples. PCR and analysis was done using the StepOne Real-Time PCR Systems and Software (Applied Biosystems; 4376374). The comparative $C_T$ method ($\Delta \Delta C_T$) as described in the manufacturer’s guide was used to assess relative quantities of mRNA.

### Western Blotting

In order to assay for RALDH-2 protein in different lung samples we harvested BAL, whole lungs, spleens, mesenteric lymph nodes and CD11c$^+$ cells from C57BL6 mice. After removing dead cells, $2 \times 10^6$ cells were re-suspended in cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 1% Triton), 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 μg of leupeptin/ml) and lysed on ice. Cell lysate proteins (30 μg/lane) were resolved on a 4-12% gradient SDS-PAGE gel and transferred to Trans-Blot nitrocellulose membrane (Bio-Rad). Blots were incubated with ALDH1/2 Antibody (Santa Cruz Biotechnologies: sc-166362) followed by horseradish peroxidase-conjugated
donkey anti-goat IgG(H+L) mAb (Jackson, West Grove, PA; 705-035-003) and bands were detected by chemiluminescence using SuperSignal West Pico substrate (Fisher; PI-34078). Blots were analyzed on a Versa Doc Imaging Systems (Bio-Rad) with the Quality One-4.4.0 Software.

**Cytokine quantification**

GM-CSF was measured by sandwich ELISA (R&D Systems; DY415) following the manufacturer's protocol.
3. Current Progress

3a. Lung APCs express RALDH-1 and RALDH-2

RA biosynthesis is dependent on the intracellular oxidation of retinal, which is catalyzed by the enzyme RALDH (36). We investigated the expression of RALDH isoforms in the lung by RT-PCR. RALDH-1 and RALDH-2 were the two main isoforms found in lung homogenates (Fig. 1a, b). RALDH-1 was highly expressed in lung homogenates when compared to spleen and MLN (Fig. 1a). RALDH-2 is expressed mostly in secondary lymph nodes (55). We found that lung parenchymal cells expressed RALDH-2 (Fig. 1b). RNA obtained from BAL cells expressed mostly RALDH-2 but not RALDH-1. This pattern was also observed in bead sorted lung CD11c+ cells (Fig. 1c, d). RALDH-1 is present in the remaining subsets of the lung (Fig. 1d). Western Blot analysis confirmed expression of RALDH protein in lung parenchymal cells, BAL and lung CD11c+ cells (Fig 1e). Total RALDH-2 expression is higher in BAL cells compared to total lung APC (Fig 1c, e). Western blotting result hardly show any RALDH1 protein present. These results determined the presence of RALDH enzymes in lung APCs. RT-PCR data suggest that lung APC mostly express RALDH-2 while the remaining cell types, i.e. CD11c depleted lung cells express RALDH-1.
3b. MTB induces RALDH-2 expression in splenic and lung APC

To investigate if MTB can modulate RA biosynthesis in APC, we infected bead sorted lung and splenic CD11c+ cells with a non-virulent strain of MTB, *M. tuberculosis* H37Ra and determined whether MTB infection induced RALDH-2 expression. In these experiments we focused exclusively on RALDH-2 since this isoform was the main isoform found in lung CD11c+ cells. RALDH2 is also the main isoform producing *all-trans* retinoic acid. Although splenic CD11c+ cells do not express RALDH-2 at baseline, we found that MTB can induce RALDH-2 expression in splenic CD11c+ cells when infected at MOI of 0.3-10 for 24 hours at 37°C (Fig. 2a). MTB also increased RALDH-2 expression in lung CD11c+ cells at MOI of 3 (Fig. 2b). RALDH-2 mRNA expression was dose dependent as well as time dependent, peaking at 24 hours at MOI of 3:1 (Fig. 2a, c). After 48 hours RALDH-2 expression varied.

3c. MTB bacilli are better inducers of RALDH-2 mRNA in APC than soluble MTB lysate.

To determine whether soluble MTB molecules and/or MTB bacilli induce RALDH-2 expression, we cultured splenic CD11c+ cells with MTB lysate or MTB bacilli and examined RALDH-2 synthesis by RT-PCR. MTB lysate failed to fully up-regulate RALDH-2 expression in spleen APCs (Fig 3a) when compared to whole live MTB bacilli. These results raised the possibility that phagocytosis of bacilli is necessary for optimal expression of RALDH-2 mRNA.
by MTB. RALDH-2 induction was tested using MTB H37Ra at MOI of 0.3, 1, 3 and 10:1. Up-regulation of RALDH-2 mRNA occurred at MOI 0.3 but it was at MOI 10 that the induction exceeded the induction generated by the positive control, GM-CSF (40ng/ml). Even at high concentrations (up to 100μg/ml) of MTB lysate little upregulation of RALDH-2 was detected.

MTB pathogen-associated molecular patterns (PAMPs) are recognized by toll like receptors (TLR) expressed on and in APC. TLR2, TLR9 and, possibly, TLR4 are primarily responsible for recognizing MTB associated PAMPs. We determined the capacity of MTB to induce RALDH-2 expression through TLR2. Spleen APCs from TLR2 knockout animals were infected with MTB bacilli at MOI of 5:1 and 10:1 for a period of 24 hours and RT-PCR for mRNA analysis was performed. The absence of TLR2 receptors on APC did not block RALDH-2 induction by MTB. TLR2 knockout animals were capable of inducing RALDH-2 (Fig. 4).

To determine if the enhanced ability of intact MTB bacilli to upregulate RALDH-2 mRNA was dependent on uptake by APC, we cultured spleen CD11c+ cells with MTB bacilli in the presence or absence of cytochalasin D. Cytochalasin D inhibits phagocytosis. Cytochalasin D treatment reduced RALDH-2 expression in MTB infected splenic APCs (Fig. 3b). Cytochalasin D treatment did not affect the ability of GM-CSF to upregulate RALDH-2 in APC (Fig. 3b). These results demonstrate that MTB bacilli were more effective at
inducing RALDH-2 mRNA expression than MTB lysate and that inhibition of phagocytosis of MTB reduced this expression.

RALDH-1 also synthetizes RA in vivo and in-vitro. Since RALDH-1 was the main enzyme found in whole lung cells, we investigated whether MTB can induce RALDH-1 expression in APC. Live MTB was unable to induce RALDH-1 expression in splenic CD11c⁺ cells (Fig. 3a). MTB lysate (100 μg/ml) induced minimal RALDH-1 expression (Fig. 3a). RALDH-1 and RALDH-2 induction by MTB lysate was minimal but we have not ruled out the possibility that mechanisms mediated by soluble MTB molecules are involved in the induction of RALDH enzymes. Although phagocytosis of MTB appears to be more efficient mechanism for RALDH-2 mRNA expression, some mycobacterial components in MTB lysate may have the ability to induce RALDH-1 and RALDH-2 mRNA expression.

3d. GM-CSF induces RALDH-2 expression

We hypothesized that RALDH-2 induction by MTB was dependent on soluble factors secreted by the bacteria after phagocytosis. To test our hypothesis, we incubated splenic CD11c⁺ cells with MTB H37RA MOI 10:1 for 24 hours. After incubation, supernatants were harvested and passed through a 0.22μm filter. The bacteria-free supernatants were transferred to a new culture of splenic CD11c⁺ cells and incubated for another 24 hours at 37°C. We observed that cells stimulated with supernatants from infected cells had increased RALDH-2 expression (Fig. 4a). We hypothesized that this enhanced
induction by both MTB infected cell and GM-CSF supernatants, was due to a soluble inducible factor.

We hypothesized that GM-CSF was the soluble factor carried over in the supernatants to induce RALDH-2 expression in splenic CD11c+ cells. In order to determine if GM-CSF was responsible for RALDH-2 induction we first examined if GM-CSF was secreted by MTB infected APC. GM-CSF cytokine synthesis was observed 24 hours after infecting APC at MOI of 3 and 10:1 (Fig. 4b). We did not measure GM-CSF production at MOI of 0.3:1. Minimal GM-CSF levels were detected in supernatants from cells infected observed at MOI 1:1.

Next we decided to block soluble GM-CSF in supernatants to determine if RALDH-2 induction was inhibited. GM-CSF neutralizing antibody (1μg/ml) was added to fresh infected splenic CD11c+ cells (MOI 10:1). GM-CSF (100pg/ml) stimulated APCs served as positive control. GM-CSF neutralizing antibody completely inhibited induction of RALDH-2 by splenic APC infected with MTB or exposed to GM-CSF (Fig. 4c). In summary, we determined that MTB induction of RALDH-2 in APC is was mediated by GM-CSF. GM-CSF was secreted into supernatants after APC had phagocytized MTB bacilli and interacted with surrounding APC to induce mRNA RLADH-2 expression.
4. Discussion

Retinaldehyde dehydrogenase (RALDH) is the enzyme involved in the oxidation of retinal to RA. This enzyme in contrast to other ADH has tissue specific patterns and only cells able to express RALDH will synthesize RA. Lung cells have the capacity to synthetize RA. They express two isoforms of RALDH enzyme: RALDH-1 and RALDH-2. Lung CD11c+ cells mainly express the RALDH-2 isoform. This makes RALDH-2 relevant to the adaptive immune response in the lungs. RALDH-1 is known to be expressed in pulmonary epithelial tissues (56) and at very low levels in lung APC subsets compared to whole lung parenchyma (Fig. 1).

MTB induces expression of RALDH-2 in splenic CD11c+ cells in a dose dependent manner. The induction peaks at 24 hours and although lung CD11c+ cells express higher constitutive levels of RALDH-2, this can be further up-regulated by infection with MTB bacilli. Stronger up-regulation of RALDH-2 was observed in splenic CD11c+ cells. Whereas as most splenic CD11c+ APCs are DC, lung CD11c+ cells are heterogeneous and contain subsets of DC, monocytes and macrophages. Our results suggest that the constitutive levels of RALDH-2 observed in the lungs are due to this heterogeneous cell population. We hypothesize that resident macrophages and residual alveolar macrophages are contributing to the constitutive expression of RALDH-2 mRNA. In the intestinal tract, another mucosal system, RALDH-2 is constitutively expressed and induced in CD103+ DC and some macrophages (46). We attribute the up-regulation of RALDH-2 mRNA in lung APC due to a contribution of all the different lung APC subsets. We also examined
RALDH-1 expression after MTB infection. Whole MTB bacilli did not induce RALDH-1 expression. When we compared MTB lysate to whole bacteria, lysate had minimal RALDH-2 induction capability in contrast to whole MTB bacilli. This suggested that phagocytosis of the pathogen might be required in order to induce RALDH-2 expression. Experiments with cytochalasin D suggested that MTB uptake had a significant role in RALDH-2 mRNA expression by MTB in APC. Although inhibition of phagocytosis inhibited RALDH-2 mRNA induction, we have not ruled the possibility that specific MTB molecules are involved in inducing RALDH-1 and RALDH-2 expression and that MTB bacilli just represent a very local and concentrated means to deliver these molecules. It is also possible that the process of phagocytosis itself triggers RALDH2 mRNA expression in APC. The latter could be addressed with different sized latex beads. Phagocytosis of MTB resulted in a more efficient means to induce RALDH-2 but not RALDH1 mRNA expression.

It was important to understand what was the principal factor inducing RALDH-2 expression by MTB. After stimulating splenic CD11c+ cells with supernatants obtained from MTB infected macrophages we noticed that stimulation still occurred (Fig 4a). This indicated that the principal factor involved in RALDH-2 expression was soluble and could be transferred by supernatants of MTB infected cells. Inhibition of GM-CSF’s in supernatants inhibited RALDH-2 expression by uninfected cells upon transfer. Although previously reported that GM-CSF is the main inducer of RA synthesis (57-58), the mechanism by which GM-CSF induces RALDH-2 expression and RA synthesis is undefined. These results together allow us to conclude that MTB induction of RALDH-2 enzyme is dependent on GM-CSF
secretion. GM-CSF is a soluble molecule secreted after MTB infection (Fig. 4b). Although little is known about the role of GM-CSF during MTB infection, it is known to act as an immune-regulatory cytokine and can decrease bacterial burden (59-60).

In this study we demonstrate that lungs contain the machinery necessary for RA synthesis. RALDH-2 is present in lung and splenic APCs and can be induced after MTB infection. The induction of RALDH-2 is dependent on GM-CSF secretion and whole MTB bacilli are necessary for the expression of the enzyme. RA may play a role in regulating the pro and anti-inflammatory balance of APCs during MTB infection.
Figures:

**Figure 1: Lung CD11c⁺ cells express RALDH-2 enzyme.** Lung, splenic, mesenteric lymph node (MLN) and BAL cells where isolated from 2-4 normal C57Bl/6 mice and cells were lysed for mRNA (A-D) or protein extraction (E) and analysis. Lung cells expressed RALDH-1 and RALDH-2 (A, B). BAL and sorted lung CD11c⁺ cells express RALDH-2 (C, D). RT-PCR was normalized for actin-β. Results where confirmed by western blot analysis using a goat anti-mouse polyclonal antibody for RALDH-2 (E). Samples were normalized for protein concentration. Graphs are representative of 4-5 experiments.
Figure 2. RALDH-2 expression is induced in splenic and lung APC after MTB infection. Splenic CD11c+ cells where infected with MTB H37Ra at indicated MOI. GM-CSF (40ng/ml) served as a positive control. (A) RALDH-2 expression increased overnight as the bacterial burden increased from an MOI of 0.3 to MOI of 10:1. (B) MTB also induced RALDH-2 expression in lung CD11c+ cells at MOI 3:1. (C) Time course experiments showed that RALDH-2 mRNA expression peaks at 24 hours after MTB infection (MOI 3:1). RALDH-2 mRNA expression was determined by RT-PCR (TaqMan) and expressed as a relative quantity of β-actin using the 2-ΔΔC\textsubscript{T} method. Shown is a representative experiment of three. Each experiment was performed in triplicate using cells isolated and pooled from five to ten animals.
Figure 3: Inhibition of phagocytosis of MTB by cytochalasin D reduces RALDH-2 mRNA induction. (A) Splenic CD11c+ cells were stimulated with MTB H37Ra at MOI 0.3-10:1 and MTB cell lysate (1-100μg/ml). MTB cell lysate failed to induce RALDH-2 expression in spleen CD11c+ 24 hour incubation. RALDH-1 was not induced after infection of splenic CD11c+ with the whole bacilli. Induction of RALDH-1 was similar to RALDH-2 when stimulated with cell lysate. (B) MTB infection of splenic CD11c+ cells failed to fully induce RALDH-2 expression in presence of cytochalasin D (1μg/ml). GM-CSF stimulated induction of RALDH-2 was not affected by cytochalasin D treatment. mRNA expression was determined by RT-PCR (TaqMan) and expressed as a relative quantity to β-actin using the 2-ΔΔC_T method. Means ± SEM of three independent experiments are shown. Each experiment was conducted with a separate pool of cells isolated from four to six animals. * p < 0.05.
Figure 4. Induction of RALDH-2 expression is not mediated by TLR-2. Splenic CD11c+ cells from wild type (WT) and TLR-2 knockout (TLR2 -/-) animals were stimulated with MTB H37Ra at MOI 5:1 and 10:1 and. Lack of TLR-2 receptor on APCs did not eliminate RALDH-2 expression by APCs. GM-CSF stimulated induction of RALDH-2 was a positive control. mRNA expression was determined by RT-PCR (TaqMan) and expressed as fold change after determining the relative quantity to β-actin using the comparative \(2^{-\Delta\Delta C_T}\) method. Shown are the preliminary results of one experiment with means of triplicates for each sample. The experiment was conducted with pooled cells isolated from four animals.
**Figure 5. GM-CSF induces MTB mediated RALDH-2 expression.** (A) Supernatants of 24 hour uninfected, GM-CSF stimulated and MTB infected splenic CD11c+ cells (solid bars) were harvested, filtered (0.22μm) and transferred to fresh cultures of splenic CD11c+ cells (striped bars). After overnight incubation cells were harvested and RALDH-2 mRNA expression measured. Supernatants from MTB infected and GM-CSF stimulated cells induced RALDH-2 expression; (B) GM-CSF measured by ELISA in supernatants of splenic CD11c+ cells infected with MTB H37Ra (MOI 0.3-10:1 for 24 hours). (C) Anti GM-CSF mAb (1μg/ml) blocked RALDH-2 expression by MTB-infected and GM-CSF stimulated CD11c cells. Means ± SEM of three experiments are shown. Each experiment was conducted with a separate pool of cells isolated from 4-6 animals. * p < 0.005, ** p < 0.0005, *** p < 0.0001.
IX. Future Directions

RA plays a pivotal role in the intestinal mucosal immune response. Also, RA’s capacity to induce Tregs has been well documented (33, 43, 46) and helps understand its role in immunity. Our studies demonstrate the capacity of RA to be synthesized by lung APC and that MTB can control its induction or up-regulation. However, there remain many unanswered questions:

1. Is the induction of RALDH-2 expression in APC part of a general response to phagocytosis or an MTB specific mechanism?

Our studies show that after MTB infection, secreted GM-CSF stimulates APC to induce RALDH-2 expression. Previous studies have suggested that GM-CSF can be secreted after phagocytosis (61). To fully understand RALDH-2 induction and RA synthesis by APC is important to clarify if GM-CSF mediated induction of RALDH-2 is due to a general function of phagocytic cells or specific to MTB infection. For these experiments we could use latex coated beads in order to induce phagocytosis. Latex coated beads can be phagocytized by APC and if GM-CSF is induced after phagocytosis, we would predict to see RALDH-2 mRNA induction after latex bead phagocytosis. This induction might be size dependent and to address that it is important to use different size of latex beads, fluctuating from 0.5μm to 3μm of size. If RALDH-2 induction is not observed then this suggest that the phagocytosis of MTB is associated with unique signaling resulting in GM-CSF production. To prove that
the induction of RALDH-2 is dependent on the phagocytosis of MTB we could utilize latex beads coated with mycobacterial cell lysate. The mixture of beads and cell lysate provide MTB molecules that can be ingested by the cell and we predict that this combination would induce RALDH-2 expression.

2. What subset of lung APC induce RALDH-2 expression?

Our previous experiments focused on constitutive expression of RALDH-2 by lung APC and BAC and the induction of RALDH-2 expression after MTB infection. It is still unclear what lung APC subset induces RALDH-2 expression. To direct this subject we could sort different lung APC. Alveolar macrophages have been traditionally obtained by brochoalveolar lavage of the lung. BAL consists mostly of alveolar macrophages (>80%). To sort DC from monocytes and resident macrophages we would use a flow cytometric approach. Lung resident macrophages can be separated from DCs by using auto-florescence (AF) and the markers CD11c, MHC II, Siglec F, F4/80. Lung macrophages are known to be CD11c+, AF\textsuperscript{HI}, MHC II\textsuperscript{lo}, Siglec F+, and F4/80+. Lung DCs are CD11c+, AF\textsuperscript{LO}, MHC II\textsuperscript{hi}, Siglec F−, and F4/80−. MTB infection of the three different lung APC subsets should indicate us what specific cells are inducing RALDH-2 expression in the lungs.
3. Does induction of RALDH-2 expression correlate with T reg cell generation in the lungs?

RALDH-2 expression in lung resident macrophages has been linked to Treg cell generation \textit{in vitro} (62). Also it is known that MTB infection induces Treg cell differentiation (63-65). In preliminary experiments we have shown the capacity of AM and lung macrophages to induce Treg cells (data not shown). In order to link these results we could infect AM with MTB and pulse with ova peptide. After ON infection and removal of the bacteria by serial washes, CD4+ T cells are added for an antigen specific system where ova specific (DO11 mice or OT II) mice are used and cells are supplemented with TGF-β for an optimal Treg differentiation environment. APCs that were pre-treated with MTB are expected to generate more Treg cells than the control conditions. We also could add GM-CSF to an antigen specific experiment using Tg25 mice and Ag85b. RALDH-2 synthesis is mediated by GM-CSF, in the case that Treg induction is due to RA, we expect to see differentiation in the presence of a RALDH-2 and RA inducer.

4. Does RA synthesis have a role \textit{in vivo}?

Little is known about the role of vitamin A during MTB infection. RA is obtained through foods containing vitamin A precursors and a diet can be modified to be a vitamin A-rich or vitamin A-free diet. In order to address the importance of RA during infection we propose experiment with control groups that are fed with a
vitamin A-rich or free diet. After treatment of the animals will be infected using aerosolized bacteria. Total CFU counts; lung morphology and cell counts would be performed and tested for any difference at day 1, 7, 14, 21 and 3 months after infection. We also suggest the examination of total Treg cell numbers in infected animals. Any difference in total CFUs would suggest a role for vitamin A during MTB infection but can be also a sign of malnutrition. To address this concern we suggest using RAR inhibitors (i.e. LE135). LE135 can be administrated intra-peritoneally to mice at day -5, 0 and 5 after MTB infection and examine any differences in total CFU.
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