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Mycobacterial Strains of Different Virulence Trigger Dissimilar Patterns of Immune System Activation *In Vivo*

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1. Introduction

Tuberculosis (TB), one of the major world health problems, is a chronic infection caused by members of the Mycobacterium tuberculosis complex (MTC). In 2009, tuberculosis (TB) caused 1.7 million deaths and 9.4 million new cases. Although recent efforts to improve TB prevention, diagnosis and treatment have contributed to a 35% decrease in the death rate, the emergence of mycobacterial strains with highly virulent phenotypes combined with pandemic HIV infections has added new challenges to control TB.

Host-pathogen interactions during experimental pulmonary tuberculosis have been studied using laboratory mycobacterial strains of well defined, relatively homogeneous virulence. These studies have contributed to uncover immune evasion mechanisms evolved by mycobacteria, and their role to establishing chronic infections. Despite the successful models of experimental tuberculosis and the high homology among MTC strains, the immune mechanisms and the mycobacterial characteristics that cause the remarkable varying degrees of clinical virulence remain barely studied. Although previous reports partially described differences in immunopathogenesis and bacterial growth (R. Chacon-Salinas et al., 2005; J. Dormans et al., 2004; B. Lopez et al., 2003), the effects of different MTC strains both on airways DC and on T cell activation have not been assessed, especially in vivo.

Broadly, mycobacterium of intermediate virulence (e.g. M. tuberculosis H37Rv) seems to reduce DC migration to the mediastinal lymph nodes (A. J. Wolf et al., 2007) which could be associated with a delayed onset of specific effector T cell responses (G. S. Garcia-Romo et al., 2004; R. J. North & Y. J. Jung, 2004; A. J. Wolf et al., 2008), thus allowing early (during the first 4 weeks of infection) exponential Mtb replication. Around 30 days post-infection,

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mycobacterial replication rate is diminished (B. J. Rogerson et al., 2006) while diverse immune evasion mechanisms avoid bacterial killing by T cell-activated macrophages (J. A. Armstrong & P. D. Hart, 1971) and cytotoxic CD8+ T cells (E. M. Weerdenburg et al., 2010). The analysis of these evasion mechanisms used by MTC strains, however, have barely been comparatively assessed (L. Quintero-Macias et al., 2011).

To evaluate the in vivo differences in host-pathogen interaction across the wide range of virulence among MTC strains we used three mycobacterial strains as representative of low (*Mycobacterium canettii*), intermediate (*Mycobacterium tuberculosis* H37Rv), and high (*Mycobacterium beijing*) virulence degrees. The recently defined *Mycobacterium tuberculosis* Beijing (M. Beijing) strains are associated with high virulence and multidrug resistance (I. Parwati et al., 2010), and cause in mice a quick increase of cellular infiltrate with high numbers of colony forming units in the lungs (J. Dormans et al., 2004). Conversely, smooth-type *Mycobacterium tuberculosis* Canettii (M. canettii) strains rarely cause TB in humans and in the experimental mouse model show low cellular infiltrate with limited chronic infection (M. Fabre et al., 2010). Interestingly, our previous results assessing the mechanisms causing the difference in virulence showed an inverse correlation between strain virulence and in vivo cytotoxic responses, as well as higher bacterial burden in the lungs of M. Beijing infected mice (L. Quintero-Macias et al., 2011).

We decided to assess a profile of dendritic cell maturation and T cell exhaustion in vivo during pulmonary infection with these three mycobacterial strains. Since MTC strains are intracellular pathogens, T cells have an important role in mediating cytolysis of infected cells and to induce activation of other immune cells (Y. He et al., 2001; S. Inoue et al., 2005; E. A. Murphy et al., 2001; S. C. Oliveira et al., 2002). For intracellular pathogens like MTb, one way to subvert T cell responses could well be by altering activation/maturation of DCs. Importantly, DCs play an important role both in inducing effector cytotoxic T cells in vivo as well as in Ag-surveillance of mucosal surfaces and in the uptake and transport of mycobacterial bacilli to the lung draining lymphoid tissue, the mediastinal lymph nodes (MedLN)(G. S. Garcia-Romo et al., 2004; A. Pedroza-Gonzalez et al., 2004; A. J. Wolf et al., 2007).

We aimed to describe our recent findings regarding the differential stimulation of DCs and T cells by MTC strains with different virulence. We consider that increasing the research on the differences among MTC strains pathogen-host interactions in vivo might help to better understand, among other things, the underlying limitations of anti-TB vaccines.

2. Materials and methods

2.1 In vivo effects of different mycobacterial lysates over the subsequent activation of DCs

Separate groups of 3-5 BALB/c female mice were intratracheally primed with each lysate (40μg/mice in 40μL) from the three strains used in our experiments (lyCan: M. canettii lysate; lyH37: Mtb H37Rv lysate; lyBei: M. Beijing lysate). Five hours after the mycobacterial lysate, mice were given an intranasal (i.n.) challenge with LPS to assess in DCs the potential effects of prior mycobacterial lysate treatment. Control groups included a) mice primed-boosted both with LPS, or b) mice treated with endotoxin-free saline solution before the LPS challenge, c) mice treated only with endotoxin-free saline solution without further LPS.

Then, 10 hours after LPS challenge, a time which is around the peak of lung DC activation induced by LPS alone, we obtained cell suspensions from lung, BAL (Bronchio-Alveolar Lavage) and MedLN to assess CD86 expression in (Gr1-, MHC-II hi, CD11c+) DCs. Mycobacterial lysates were prepared by one of us (I. Estrada-Garcia, ENCB-IPN).

2.2 Experimental model of airways-induced pulmonary tuberculosis in mice

M. tuberculosis strains were grown in Middlebrook 7H9 medium (Difco Laboratories) supplemented with OADC (Difco Laboratories). After 1 month of culture, mycobacteria were harvested, adjusted to $2.5x10^5$ bacteria in 100µl sterile endotoxin-free saline solution, aliquoted, and maintained at -70ºC until used. Before use, bacteria were stained with fluorescein diacetate (InvitroGen, F1303) and viable bacteria (Kvach, J. T. and Veras, J. R. 1982) (green fluorescence) were counted with an epifluorescence microscope and adjusted to the infective dose. We used the murine model of intra-tracheal infection as described previously (Hernandez-Pando, R. 1996), with some modifications.

Briefly, 3-5 male BALB/c mice from 6-8 weeks of age were anaesthetized with sevoflurane, and 100 µl isotonic sterile endotoxin-free saline solution with 2.5×10^5 viable bacilli were inoculated intra-tracheally. Control animals were inoculated only with isotonic, sterile endotoxin-free saline solution without bacilli. Animals were then maintained in cages fitted with microisolators in a P-3 biosecurity level facility. The protocol was institutionally approved according to ethical norms for use of animals in experimentation. Following infection, at least three to five mice per group were euthanized at every time point selected for the various analysis.

2.3 Staining of cell suspensions for flow cytometry analysis

Monoclonal antibodies used for phenotypic analysis of DC and T cells were anti-CD3-FITC (BD Pharmingen 553062), anti-CD4-PerCP (BD Pharmingen 553052), anti-CD8a-APC (BD Pharmingen 553035), PD-1-PE (BD Pharmingen 551892), anti-CD11c-APC (BD Pharmingen 550261), anti-CD40-PE (BD Pharmingen 553791), anti-MHCII-FITC (BD Pharmingen 553623), anti-Ly6c-A700 (e-biosciences 56-5981-32), and Streptavidin-conjugated with PerCP fluorochrome (SAV-PerCP, BD Pharmingen 554064), biotinylated anti-CD103 (R&D Systems BAF1990). Cell suspensions were prepared by disgregating the organs using a 70µm cell strainer (BD Falcon 352350) and the piston of a 3mL Syringe (BD 309585). Spleen, Lungs, BAL and Mediastinal lymph nodes cell suspensions were washed, incubated 10 min at 4°C with Power Block reagent (Biogenex, HK085-5K) to block Fc receptors, washed, and stained with fluorochrome-coupled mAbs for 15 min at 4°C. Cells were centrifuged and resuspended in FACS buffer. 10^6 and 10^5 live MHC-II high or CD3+ cell cells were acquired respectively. Data was acquired in a Dako Cyan Flow Cytometer and analyzed with FlowJo Software 7.2 (Tree Star, Inc., San Carlos, CA).

3. Results

3.1 Lysates of highly virulent mycobacteria decrease activation of BAL DC in vivo

To test whether mycobacterial components differentially affected the activation patterns of lung DCs, we intra-tracheally treated separate groups of mice with different mycobacterial

lysates prepared from each mycobacterial strain. After five hours mice were challenged with intranasal LPS to determine the subsequent DC activation. CD86 expression on DCs from bronchio-alveolar lavage (BAL), lung and MedLN was assessed after 10 hours of LPS challenge.

Compared to all control groups, M. tuberculosis H37Rv and M. Beijing lysates reduced the LPS-triggered activation of BAL and lung DCs, whereas M. Canettii lysate showed no difference. Interestingly, in these two groups of mice (figure 1, top and middle panels), the reduced activation is observed regarding both the percentage of CD86+ DCs (left Y axis) and the intensity of expression of CD86 (MFI, right Y axis). In the lungs, slight differences were observed only in mice treated with lyBei. In the MedLN, all lysates increased the percentage of CD86+DCs when compared to mice that received two doses of LPS (figure 1, bottom panel, left Y axis).

Our results suggest that the one factor contributing to the different virulence observed among MTC strains lies in how DCs respond to mycobacterial components. These slight but relevant differences are clearly seen in the activation patterns of BAL and lung DCs. Although it remains uncertain whether the patterns observed with lyH37 or lyBei lysates are product of increased DC migration from the BAL to the lungs to the MedLN (which could be associated with the virulence-dependent exacerbated lung infiltrate characteristic of chronic tuberculosis) or a gradient of DC inhibition (which could be associated with the early inhibition of specific T cell responses).

3.2 The CD103+ dendritic cell subset is preferentially activated during virulent Mtb infection

To assess whether infection with different MTC strains induced divergent patterns of DC activation we evaluated the expression of CD40 on lung, MedLN and spleen DCs. CD40 in DCs is a crucial coestimulatory molecule required for naive T cell activation and –especially- for appropriate induction of cytotoxic T cells (P. Bjorck et al., 1997; G. Grouard et al., 1996; A. M. Moodycliffe et al., 2000), in lung, MedLN and spleen DCs. We compared CD40 expression between uninfected mice and mice infected with M. canettii, M. tuberculosis H37Rv, or M. Beijing at chronic infection (60 days after infection). Also, we analyzed two DC subsets distinguished by the expression of the CD103 molecule. In particular the lung CD103+ DC subset, as this is associated with proinflammatory responses and CD8+ T cell activation during intracellular pathogen infections (G. T. Belz et al., 2004; M. L. del Rio et al., 2010; T. S. Kim & T. J. Braciale, 2009).

We found that the infection with virulent mycobacterial strains (Mtb. H37Rv and M. Beijing) mainly activated lung CD103+ DCs (figure 2). In contrast, lung CD103+ DCs of mice infected with M. canettii had the lowest expression of CD40 (figure 2, top panel white bar). Interestingly, in M. Beijing infected mice, MedLN CD103+ DCs showed reduced expression of CD40 (figure 2, middle panel), even below the expression of the control group (uninfected mice). In the spleen, CD40 expression in both DC subsets from infected mice was below levels observed in uninfected mice (figure 2, bottom panel), but showed a direct correlation with virulence (M. Beijing infected mice had the highest expression of CD40 in spleen DCs).

These results indicate that mycobacteria of different virulence induce each a different activation pattern in the regional DCs. M. Beijing infection induced CD103+ DC activation

in the lungs with moderate inhibition in the MedLN (the regional, draining lymph node) and spleen (systemic response). Mtb H37Rv induced the activation of CD103+ DC in both lungs and MedLN while inhibiting CD40 expression in the spleen; and M. canettii reduced CD40 expression in CD103+ DCs in the lungs and spleen, with a slight increase in the MedLN.

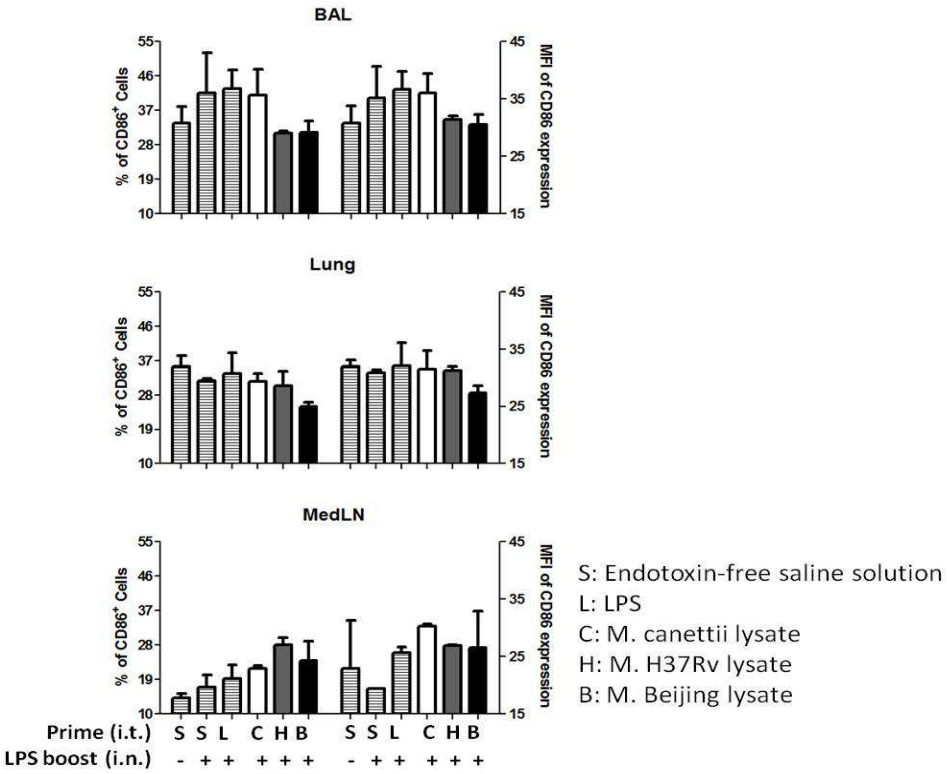

Fig. 1. Prior inoculation of mycobacterial lysate from virulent strains reduces subsequent activation of airways DCs.

Prior inoculation of mycobacterial lysate from virulent strains reduces subsequent activation of airways DCs. Groups of 3-5 BALB/c mice received intra-tracheal (i.t.) lysates prior to intra-nasal (i.n.) LPS stimulation. After 10 hours of LPS inoculation CD86 expression on DCs was determined and compared to control groups (striped bars). Bronchio-alveolar lavage (BAL) and lung DCs from mice that received M. Beijing lysate (black bars) showed a reduction in CD86 expression (mean fluorescence intensity (MFI) and percentage of positive cells). Mtb. H37Rv lysate (gray bars) induced a reduction of CD86 only in BAL DCs while M. canettii lysate (white bars) did not induced a reduction in BAL and lung DCs and neither increased CD86 expression in Spleen DCs.

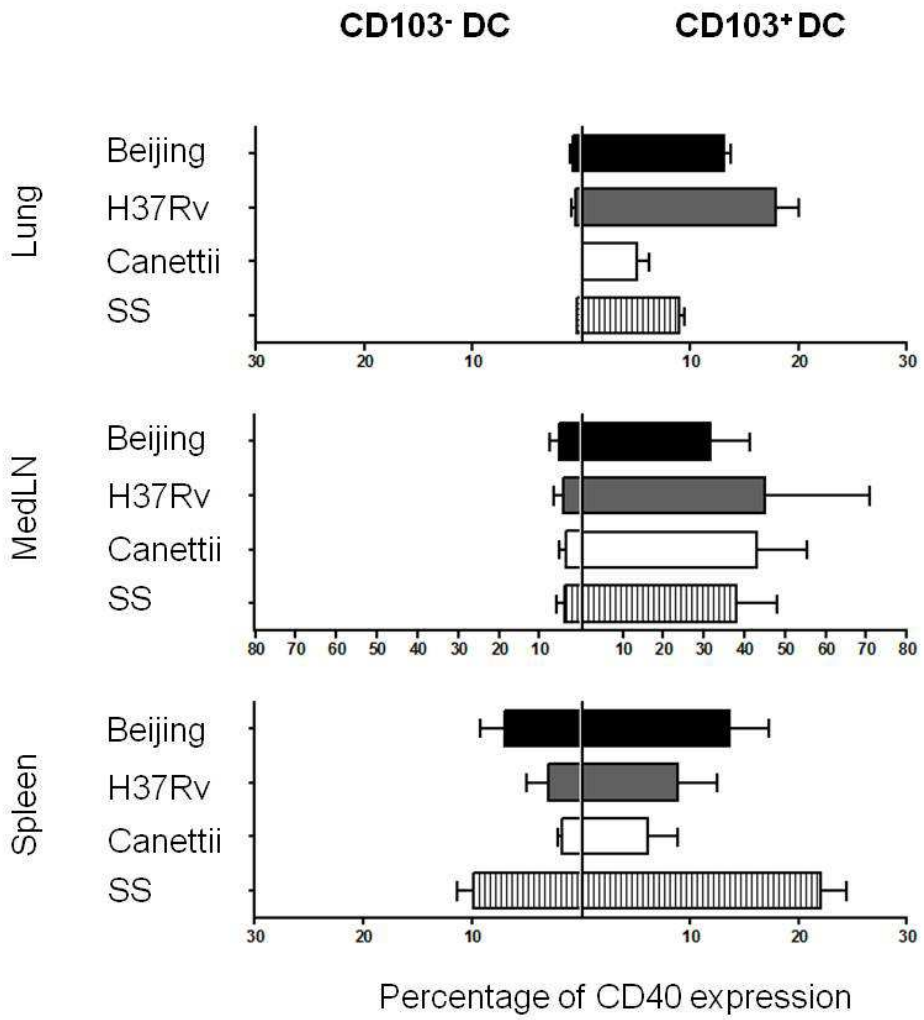

Fig. 2. CD40 expression in CD103+ and CD103- DC subsets during chronic infection with different mycobacterial strains.

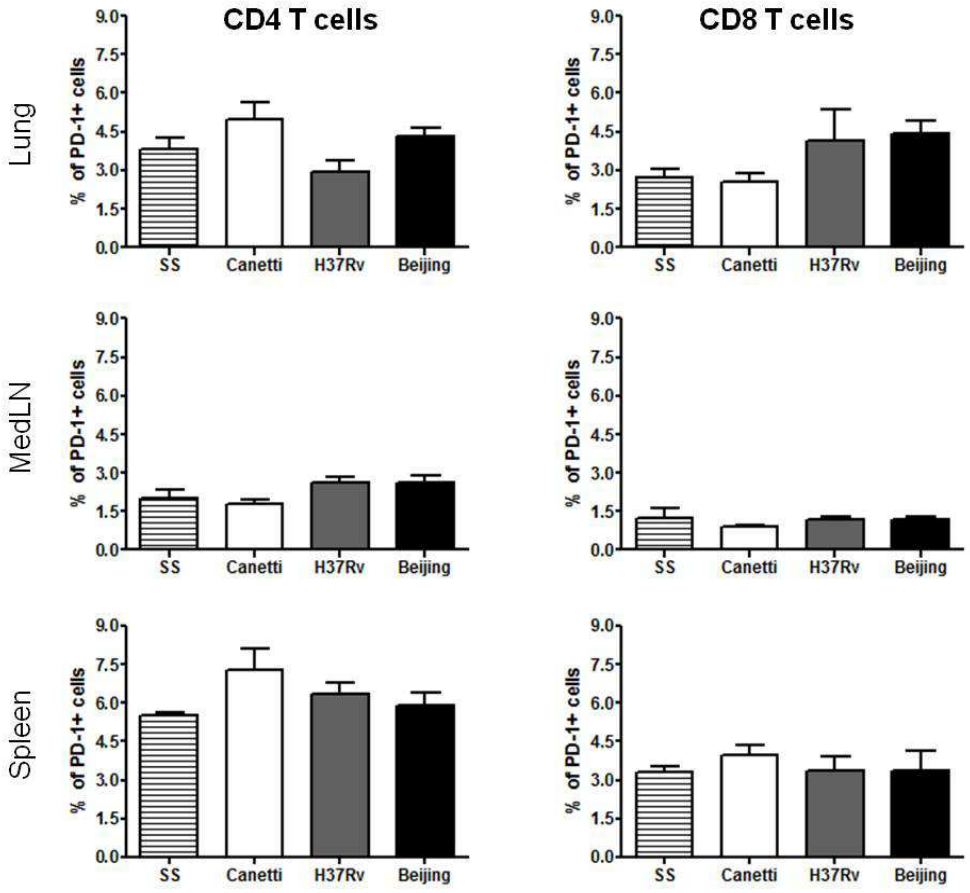

Fig. 3. PD-1 expression in CD4+ and CD8+ T cells during chronic infection with different mycobacterial strains.

3.3 Increased expression of PD-1 on lung CD4+ and CD8+ T cells is observed only during M. Beijing chronic infection

Since T cell expression of the PD-1 molecule has been shown associated in vivo to T cell exhaustion during chronic intracellular infections, we assessed the expression of this molecule during the infection with these three different mycobacteria. T cells showed different patterns of PD-1 expression among groups of infected mice. In M. canettii infected mice only PD-1+ CD4+ T cells are increased in lungs (figure 3, top panel-left plot), in M. tuberculosis H37Rv infection only PD-1+ CD8+ T cells are increased (figure 3, top panel-right plot), and in M. Beijing infected mice both T cell subsets showed increased PD-1 expression (figure 3, top panel). Interestingly, strain virulence and PD-1 expression in both T cell subsets showed an inverse correlation in the spleen, although slight differences in CD8+ PD-1+ percentage were observed among groups (figure 3, bottom panel). Seemingly, none of the strains induced overt changes in PD-1 expression on T cells, although there is a tendency for virulent mycobacteria to increase PD-1 expression on lung CD8+ T cells while M. canettii infection affects CD4+ T cells.

Groups of 3-5 BALB/c mice were intra-tracheally infected with different mycobacterium strains. At 60 days after infection CD40 expression on CD103+ and CD103- DCs was determined. In all groups CD103+ DCs had the highest expression. Mtb. H37Rv (gray bars) and M. Beijing (black bars) increased CD40 expression on lung CD103+ DCs. In MedLN CD103+ DCs, M. Beijing infection reduced CD40 expression while Mtb. H37Rv increased it. In contrast, M. canettii (white bars) infection reduced the percentage of CD103+ CD40+ DC in the lungs with a slight increase in MedLN. In the spleen and compared to uninfected mice (striped bars), all infected mice had reduced expression of CD40 in both CD103+ and CD103- DCs. In infected mice, for both DC subsets in the spleen, CD40 expression followed a direct correlation with virulence (Beijing>H37Rv>Canettii).

T cells expression of the exhaustion-associated marker PD-1 was analyzed at 60 days after infection with different mycobacteria. Overall, slight differences were observed when compared to uninfected mice (stripped bars). Mice infected with M. canettii (white bars) showed a tendency to increase PD-1 expression on lung and spleen CD4+ T cells and on spleen CD8+ T cells. Mice infected with Mtb. H37Rv (gray bars) or M. Beijing (black bars) showed no differences in PD-1 expression on both CD4+ and CD8+ T cells from the spleen or MedLN. In the lungs the two virulent mycobacteria increased PD-1 expression on CD8+ T cells.

4. Discussion

Tuberculosis is a major health problem worldwide, causing around 2 million deaths yearly. Although more than 100 years of research have led to significant improvement in disease control (in 2009, WHO reported a 35% drop in the death rate), it has also revealed a complex landscape of intricate interactions between mycobacteria and host immune system. The relative recent appearance and description of highly virulent strains in combination with the high incidence of tuberculosis in immuno-compromised patients entitles for a deeper understanding of how the immune system reacts to mycobacteria with a broader virulence spectrum.

Most of the current knowledge comes from studying murine models of pulmonary infection with strains of intermediate virulence (e.g. M. tuberculosis H37Rv). In the early phase of infection, mycobacteria are recognized and internalized by resident phagocytic cells like alveolar macrophages and pulmonary dendritic cells. Within these cells, mycobacterium bacilli can escape degradation and start replication. Concomitantly, it appears that lung DC crucial role in migration and activating specific T cells in the MedLN is inhibited. During the chronic phase mycobacteria apparently avoid killing associated with apoptosis of infected cells and remain confined inside granulomas.

Infection with the highly virulent Mycobacterium tuberculosis Beijing (M. Beijing) causes a quick increase of cellular infiltrate with high numbers of colony forming units in the lungs (D. Aguilar et al., 2010; B. Marquina-Castillo et al., 2009). Conversely, infection with smooth-type M. Canettii strains rarely cause TB in humans, and in the experimental mouse model, M. canettii strains show low cellular infiltrate with limited lung bacterial burden during chronic infection (M. Fabre et al., 2010). Importantly, among these three strains, virulence showed a direct correlation with inhibition of in vivo cytotoxicity (L. Quintero-Macias et al., 2011).

In the present study we tried to further define in vivo the virulence differences by assessing the potential effects upon DCs. Regarding DC activation, we observed an apparent differential recognition of M. canettii components by the DCs. When mice were treated with M. canettii lysate, BAL and lung DCs expressed similar levels of CD86 after LPS stimulation in vivo. During infection, lung and spleen CD103+ DCs showed less CD40 expression as compared to the other mycobacterial infection and to uninfected mice. Unlike M. canetti, both Mtb H37Rv and M. Beijing components reduced DC activation in BAL, and during infection, increased CD40 expression in lung DCs. Conceivably, virulent mycobacteria might induce a strong activation of BAL DCs causing the migration towards lung parenchyma and MedLN. The differences observed between Mtb H37Rv and M. Beijing infection suggest a probable scenario where Mtb H37Rv induces DC migration to MedLN whereas M. Beijing prevents MedLN recruitment while increasing systemic distribution.

Homeostatic mechanisms during chronic inflammatory responses on mucosal surfaces tend to increase and bias T cell differentiation to anti-inflammatory and regulatory phenotypes. PD-1 expression on T cells is associated with T cell exhaustion during chronic intracellular infections. Our results showed only slight variations in PD-1 expression during chronic infection. Of note, Mtb H37Rv and M. Beijing infections induced similar increase in the percentage of CD3+CD8+PD1+ lung T cells, while M. canettii infection increased PD-1 expression on lung and spleen CD3+CD4+ T cells. Although PD-1 expression had small variations compared to uninfected mice, a tendency of virulent mycobateria to induce CD8+ PD-1+ T cells was observed and might relate to decreased in vivo cytotoxicity (L. Quintero-Macias et al., 2011).

Several mycobacterial components have been associated to immune system subversion. RD-1-encoded secreted proteins (e.g. ESAT-6) mediate macrophage inhibition by TLR2 recognition and have the potential to form pores in membranes probably facilitating bacterial escape from phagosomes. RD-1 region is associated with virulence since is absent in attenuated M. bovis BCG. However the three strains used in our experiments

carry the RD-1 region (T. A. Halse et al., 2011), suggesting that their differing degrees of virulence might not be directly related to this genomic region. Recent publications have shown the involvement of other RD-1 proteins (EspF, EspG (D. Bottai et al., 2011)) or other genomic regions (RD-2 also lost in attenuated M. bovis BCG (R. A. Kozak et al., 2011)) in virulence.

Mycobacterial cell wall also contributes to virulence. The high content of heterogeneous lipids is a hallmark of mycobacteria. Lipoarabinomanans and lipomanans are two of the major lipidic components that can reduce the activation and cytokine secretion of macrophages. Although limited evidence suggest that cell wall lipids are key in immune response subversion (L. Quintero-Macias et al., 2010; L. M. Rocha-Ramirez et al., 2008), strain-specific lipid characterization during infection represents a challenge yet to be fully engaged. Among the strains used in our experiments, clear differences on the lipid synthesis have only been described for M. Beijing (G. Huet et al., 2009; M. B. Reed et al., 2007).

On the other hand, a decade of intense research on the mucosal immune system provides an outlook of similar complexity with the interaction of an increasing number of cell types within highly specialized microenvironments. In the case of tuberculosis is necessary to consider that the interaction of at least four histological compartments (alveolar space, lung parenchyma, draining lymph nodes, blood) is affected by the balance between mycobacterial virulence and host resistance. Apparently, virulent mycobacteria would preferentially target lung CD103+ DCs and thus avoid activation of the CD103- DCs. Further research is required to determine whether this constitutes an active evasion mechanism and to clarify the role of lung CD103+ DCs in the induction of Mtb-specific T cells or regulatory T cells.

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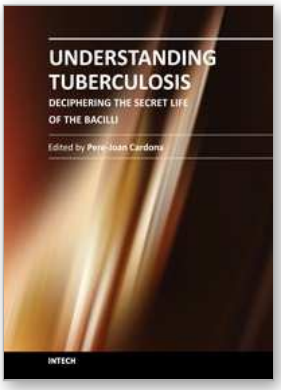

Understanding Tuberculosis - Deciphering the Secret Life of the Bacilli

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Mycobacterium tuberculosis, as recent investigations demonstrate, has a complex signaling expression, which allows its close interaction with the environment and one of its most renowned properties: the ability to persist for long periods of time under a non-replicative status. Although this skill is well characterized in other bacteria, the intrinsically very slow growth rate of Mycobium tuberculosis, together with a very thick and complex cell wall, makes this pathogen specially adapted to the stress that could be generated by the host against them. In this book, different aspects of these properties are displayed by specialists in the field.

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