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Comparing efficacy of BCG/lactoferrin primary vaccination versus booster regimen

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Summary

Lactoferrin is an iron binding glycoprotein possessing multiple immune modulatory activities, including ability to affect macrophage cytokine production, mature T- and B- lymphocytes and immature dendritic cells, and enhance the ability of macrophages and dendritic cells to stimulate antigen-specific T-cells, and. These characteristics of lactoferrin suggested that it could function as an effective adjuvant enhance efficacy of the BCG, the current vaccine for tuberculosis disease. Admix of lactoferrin to the BCG vaccine promoted host protective responses that surpasses activity of the BCG vaccine alone as determined by decreasing pulmonary pathology upon challenge with virulent *Mycobacterium tuberculosis* (MTB). This study builds on previous reports by examining the effectiveness of the lactoferrin adjuvant comparing primary vaccination versus an immunization schedule with a booster administered at 8 weeks. BCG/lactoferrin vaccinating, given once or twice, demonstrated an improvement in pulmonary disease compared to both the BCG vaccinated and non-immunized groups. The splenic recall profiles showed a difference in cytokine production induced by mycobacterial antigen from splenocytes isolated from mice immunized with BCG/lactoferrin once or twice. Production of IL-17 is increased in the BCG/lactoferrin 2x group compared to the primary vaccinated group. Both BCG/lactoferrin vaccinated group exhibited increase production of IFN- γ compared to the non-immunized group and decreased production of IL-10 compared to the group vaccinated with only BCG. This study illustrates that the adjuvant activity of lactoferrin to enhance BCG efficacy occurs whether the vaccination regimen is a single delivery or combined with a booster, leading to enhanced host protection and decreased disease manifestation.

Keywords

Lactoferrin; BCG; Vaccine; Adjuvant; Tuberculosis

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1. Introduction

Lactoferrin is a natural iron binding glycoprotein found primarily in mucosal secretions and secondary granules of neutrophils (1). It possesses a wide spectrum of immune modulatory activities. Lactoferrin is capable of enhancing natural killer T-cell activity (2), increasing macrophage production of inflammatory mediators (3, 4), promoting maturation of T- and B- lymphocytes (5, 6) and dendritic cells (7), and increasing T-cell antigen specific activity (8, 9). Recently, it was discovered that lactoferrin could upregulate and maintain dendritic cell and macrophage expression of antigen presentation and co-stimulatory molecules during mycobacterial infection (10, 11). This ability to enhance both antigen presentation cell function and T-cell activation correlates well with empirical findings showing lactoferrin as an effective adjuvant to augment the BCG vaccine.

The current vaccine for Tuberculosis (TB) is an attenuated strain of *Mycobacterium bovis* bacillus Calmette Guerin (BCG). BCG is well known to be effective against childhood onset disease, but protection is mostly ineffective against adult onset disease (12, 13). Considering that adult TB disease remains a growing global health concern (14), effective vaccination strategy is the only method to limit transmission and spread of the pathogen *Mycobacterium tuberculosis* (MTB) (15). Most developing TB vaccines focus on either novel vaccine candidates or improving the existing BCG (16). The advantage of the latter strategy is that BCG has a well-established safety profile (17), thus admix of the lactoferrin adjuvant with no toxic profiles makes the BCG/lactoferrin vaccine a promising candidate for human use.

Previous studies demonstrated that addition of 100 µg/mouse lactoferrin to BCG enhanced vaccine efficacy, offering host protection against MTB challenge defined by decreased pulmonary pathology. Those results correlated with increased production of IFN-γ in response to mycobacterial antigens (18–20). The lactoferrin adjuvant remained effective at a 10x lesser dose (10 µg/mouse)(21), indicating feasibility for use of a recombinant lactoferrin used as an adjuvant incorporated into the existing BCG vaccine. This study further investigates the utility of the lactoferrin adjuvant to control pathology after infection by comparing a primary vaccination with a booster regimen. The study addresses if a single immunization with BCG/lactoferrin will be effective to promote host pathological protection against MTB challenge, and what correlates are found with developed antigen specific recall responses compared to a booster vaccination schedule.

2. Materials and Methods

2.1 Animals

Female C57BL/6 mice (6 weeks old, Jackson Laboratories, Bar Harbor, ME) 20–25 g initial body weights were used. All *in vivo* experiments were conducted under approved guidelines of the animal ethics committee at the University of Texas, Health Science Center at Houston (HSC-AWC-08-050 and AWC-11-020).

2.2 Lactoferrin, BCG, and MTB

Low endotoxin bovine milk lactoferrin (< 1E.U./mg, < 20% iron saturated, >95% purity) was provided by PharmaReview Corporation (Houston, TX). *Mycobacterium bovis* Calmette Guerin (BCG), Pasteur strain, (TMC 1011, ATCC, Manassas, VA) was grown in Dubos base (without addition of glycerol) with 10% supplement (5% BSA and 7.5% dextrose in saline) on an orbital shaker at 37°C for 2 weeks before use. BCG was diluted with 1x Dulbecco's phosphate-buffered saline (PBS) (Cellgro, Herndon, VA) to 3×10⁸ organisms/mL, estimated using McFarland standards (Sigma) and confirmed by plating dilutions onto 7H11 agar plates (Remel, Lenexa, KS) and colony enumeration after incubation at 37°C with 5% CO₂ for 3–4 weeks. Erdman strain *Mycobacterium tuberculosis* (MTB) (TMC 107,

ATCC) was used for challenge studies. MTB was grown in Dubos base with 5.6% glycerol and 10% supplement for 3–4 weeks. Bacteria taken during log phase growth were resuspended in 1x PBS and sonicated for 10 seconds to dislodge clumping. Dilutions were made to 3×10^8 bacteria/mL, estimated using McFarland standards and confirmed by enumeration after growth on 7H11 agar plates.

2.3 Vaccination and Challenge

C57BL/6 (6 mice/group) were immunized subcutaneous at the tail base with BCG (1×10^6 CFU/mouse), immunized with BCG and lactoferrin (100 μ g) in 1xPBS, or remained non-immunized, as previously described (18–20). Primary vaccinated mice received the first immunization coinciding with the 8 weeks booster vaccination of the matched group. After 12 weeks, mice were aerosol challenged with Erdman strain of *Mycobacterium tuberculosis* (MTB) (TMC 107, ATCC) at 1×10^8 CFU/mL suspended in 5 mL 1x PBS, using an inhalation exposure system (IES) (GLAS-COL Model #A4212 099c Serial #377782). Inoculation dose (50–250 CFU/mouse) was confirmed by sacrificing 4 mice at day 1 post-infection and assessing for lung bacterial load after plating homogenates on 7H11 plates. Groups of mice were sacrificed at 65 days post-challenge; lung, liver, and spleen sectioned for colony forming units (CFU) and histological analysis. Experiments were repeated to confirm results.

2.4 Histological techniques

Mice were sacrificed on day 65 following aerosol infection. Lungs were fixed in 10% formalin and embedded in paraffin using standard techniques. Sections, 5 μ m thick, were stained with hematoxylin and eosin (H&E) and subsequently reviewed histologically; the pathologist viewing and interpreting the slides was blind to the type of experiment and treatment. Lungs from 4 to 6 mice of each group were analyzed. Multiple sections of each lung from groups were digitized analyzed using digital software (NIH Image J). Relative area of occluding lesions was obtained and calculated as percent of total (100%) lung area.

2.5 Splenocyte recall response

Heat-killed BCG (HK-BCG) was achieved by autoclaving BCG suspension in 1x PBS at 121°C for 20min, with killing verified by no growth after plating on 7H11 agar after 3 weeks. Splenocytes were isolated from immunized and non-immunized C57BL/6 mice (6 mice/group) at 6 weeks post primary vaccination or booster, as previously described (19). Briefly, spleens were minced, and red blood cells lysed with ACK lysing buffer (Cambrex Bio Sciences, East Rutherford, NJ). Splenocytes were cultured at 2×10^6 cells/mL in Dulbecco's Modified Eagles Medium (DMEM, Sigma, St. Louis, MO) supplemented with 2.2g/L sodium bicarbonate, 0.05g/L of HEPES (Sigma), 0.05g/L L-arginine (Sigma), 100 μ g/mL penicillin G (Sigma), 50 μ g/mL gentamycin sulfate (Sigma) 0.005% 2-mercaptoethanol (2-Me, Gibco, Carlsbad, CA), and 10% fetal bovine serum (FBS, Sigma). Assay controls were performed to assess potential for cellular activation, including stimulation with HK-BCG at multiplicity of infection (MOI) 1:1 or 10:1 (organisms:cell) ratio for 24 and 72 hrs. Supernatants were collected and stored at -20°C for later ELISA analysis.

2.6 ELISA (Enzyme linked immuno-sorbant assay)

Supernatants were assayed for cytokine production using the DuoSet ELISA kits (R&D Systems, Minneapolis, MN), according to manufacturer's instructions. Supernatants were assayed for production of T-cell cytokines, IFN- γ , IL-17, IL-12p40, IL-10, and TGF- β 1. The lower limits of detection for the assay are between 15–32pg/mL.

2.7 Statistics

In vivo experiments were repeated with 6 mice/group. Splenocyte cultures were repeated at least 6 times and data reported as mean $\pm$ standard deviation. All analytical assays were repeated in triplicate. All ELISA determinations were performed using triplicate wells (average $\pm$ standard deviation). Statistical analyses were performed using OneWay ANOVA followed by the Tukey post-hoc test for group-by-group comparisons, using GraphPad Prism 4.0 (La Jolla, CA); significance was reported at p values ≤ 0.05 .

3. Results

3.1 Reduced bacterial load in vaccinated mice post infectious challenge

Addition or incorporation of adjuvants into BCG based vaccines can successfully decrease early lung bacterial loads and delay dissemination of organisms to other tissues. However, this CFU reduction relatively short lived compared to BCG alone-immunized mice. We previously reported similar findings using lactoferrin with BCG, where CFUs were nearly identical to BCG alone immunized groups at later times (>2 months post infection), even though marked differences were seen in pulmonary pathology (20). These findings were repeated in mice aerosol challenged with a low dose MTB. Lung, liver, and spleen were collected from non-immunized mice or those immunized once or twice with BCG or BCG/lactoferrin. At day 65 post-challenge, non-immunized mice demonstrated relatively high bacterial loads in all organs examined (Fig 1). Immunization with BCG alone as a primary vaccination or with a booster at 8 weeks, significantly decreased bacteria loads in all tissues examined (Fig 1). A similar significant decrease in organ CFU was seen in the BCG/lactoferrin vaccinated group, regardless of whether it was given as a primary vaccination or with a booster immunization. However, at this later time post infection, there was no significant difference maintained between BCG alone or the BCG plus lactoferrin group after aerosol challenge.

3.2 BCG immunization with lactoferrin adjuvant decreases pulmonary pathology resulting from MTB infection

Adult onset TB disease is marked by distinctive pulmonary pathology, the development of granuloma structures that play a role in transmission of disease (22–24). As such, an effective vaccine against TB is required to alleviate development of destructive granuloma pathology. Lung sections were examined at 65 days post infectious challenge from non-immunized mice, mice immunized with BCG alone, or with BCG and lactoferrin. Non-immunized exhibited well defined granulomatous structures that characterized by diffuse activated macrophages with lymphocytic clusters (dark blue) that occupy large areas of tissue surrounded by inflamed and thickened alveolar walls. The tissues from the BCG vaccinated mice, administered either once or twice, showed characteristic changes in granuloma structure, with smaller diameter focal responses and reduction in surrounding inflammation compared to the non-immunized controls. Immunization with BCG and lactoferrin given as a primary vaccination, or with a booster dose, demonstrated a significant change in pulmonary pathology compared to both BCG vaccinated and non-immunized groups. Specifically, there was marked decrease in granuloma size, reduced peripheral inflammation, and an apparent increase in lymphocytic focal clusters within the granuloma (Fig 2a).

Histological sections were further examined to quantitate the level of occlusion between groups. Granuloma structures were evaluated in respect to whole lung area, analyzed in a blinded manner using Image J analytical software (Fig 2b). Mice immunized with BCG and lactoferrin once or twice had a significant decrease in granuloma size as defined by reduced overall percent lung occlusion compared to both the BCG vaccinated and non-immunized

groups ($p<0.05$). In addition, the single immunization with BCG and lactoferrin group had reduced occlusion when directly compared to single immunization with BCG alone; the boosted groups were also significantly different when lactoferrin was included as a vaccine component.

3.3 Splenic recall responses to mycobacterial antigens do not correlate with reduced granulomatous pathology

The specific immune responses generated by vaccination directly affect outcome of MTB infection. Splenic recall responses were examined in vaccinated mice for specific responses generated to heat-killed (HK) BCG, and assessed for production of IFN- γ , IL-2, IL-17, IL-12p40, IL-10, TNF- α , IL-6, and TGF- β 1.

Single immunization with BCG or with BCG and lactoferrin significantly increased IFN- γ production from splenocytes, compared to the non-immunized group, with no differences observed between the BCG or BCG and lactoferrin groups. However, splenocyte responses from mice twice immunized with BCG demonstrated a significant relative decrease in IFN- γ production compared to the single immunized group. That reduction was abrogated when lactoferrin is admixed with BCG, with levels remaining high regardless of single or multiple lactoferrin immunizations (Fig 3a).

Examination of IL-17 production did not correlate with the IFN- γ response. In this case, splenocytes from mice immunized once with BCG and lactoferrin significantly increased production of IL-17 in response to HK-BCG, compared to both the non-immunized and single BCG immunized groups. Levels of IL-17 produced from splenocytes isolated from mice immunized twice with BCG were comparable to the single BCG and lactoferrin group. However, splenocytes from mice twice immunized with BCG and lactoferrin decreased production of IL-17 to nearly levels of the non-immunized controls (Fig 3b).

Production of IL-10 in recall responses were also examined, and found to be increased in all immunized groups. However, generally the mice immunized with BCG alone were superior in IL-10 production compared to mice immunized with the BCG and lactoferrin groups. However, this difference was not significant (Fig 3c). Other cytokine recall responses were examined; there were no differences observed in production of other mediators, including IL-2, IL-12p40, TNF- α , IL-6, or TGF- β 1 (data not shown).

4. Discussion

Lactoferrin has demonstrated to be a potential adjuvant capable of enhancing efficacy of the existing BCG vaccine to promote host protection against MTB infection in mouse models. This study examined the effects of lactoferrin adjuvant activity between a primary vaccination protocol and a previously reported booster regimen. Both protocols demonstrated that addition of lactoferrin led to reduced pulmonary pathology, even when no additional reduction of bacterial load occurred relative to BCG vaccination alone. However, there was a distinct difference in splenocyte recall response between mice immunized with BCG or BCG with lactoferrin, and this varied based on whether the vaccine was administered once or twice.

Vaccination in the presence of lactoferrin demonstrated comparable effects on organ bacterial loads comparable to use of BCG alone, which is consistent with previously reported findings (18–20). While addition of lactoferrin led to organ CFU reduction early in MTB infection (<30 days post-MTB challenge), no differences compared to BCG vaccination were apparent at later time points. The field of TB vaccine study has found few candidates capable of lowering organ bacterial load that surpasses the capabilities of the

current BCG vaccine. Several hypotheses have been proposed, including the lack of effective CD8⁺ cell activity (25–28), restriction of T-cell activation of macrophages at the site of infection once the granuloma structure is established (29), and recruitment of T-regulatory cells to suppress effector T-cell function (30, 31). What is required is a redefinition of vaccine success that would include reduction in disease, rather than relying on bacterial load reduction alone as the primary outcome for success. The reduced inflammation in lungs from MTB challenged mice immunized with BCG and lactoferrin further support that vaccines should be examined for shifts in T cell function, such as examination of contribution of T-regulatory response which may be tied to pathological damage regulating disease states.

Tuberculosis disease results in dramatic modification and restructuring of lung tissue to promote spread of infection. The hallmark of TB disease is the formulation of the granuloma, defined as a localized accumulation of lymphocytes and infected and uninfected macrophages. The granuloma structure has always been considered as a two-edged sword, allowing focused sequestration of organisms from the host while at the same time serving as a nidus for focused inflammatory responses culminating in tissue destruction that facilitates release of infectious organisms (32). Current theories challenge this observation (33, 34). Regardless of the cause, the benefit of successful pathological vaccination (one that reduces destructive pathology) is a major goal (35). Vaccination with BCG and lactoferrin appears to meet those overall goals to reduce MTB induced pulmonary pathology, suggesting adjuvant promotion of mechanisms that maintain normal tissue structures and deter spread of infectious organisms.

BCG and lactoferrin immunization demonstrated similar host protective responses upon MTB challenge, regardless of use of single or double regimen vaccination protocols. However, a noticeable difference in recall cytokine profiles was observed in response to mycobacterial antigens. The most notable difference was the increased production of IL-17 in the single immunized group. The role of IL-17 in protecting against MTB infection is shown to be both beneficial and detrimental (36). IL-17 is associated with neutrophil recruitment and activation (37); it is well established that early neutrophilic responses during MTB infection contributes to initiation of the granuloma (38, 39). Yet sustained elevated neutrophils at the local infection site is associated with increased pulmonary pathology (36, 40, 41), leading to disruption of normal lung function. It should be noted that immunization with BCG resulted in increased IL-17 production but only when the vaccine was twice administered. This is consistent with a study that examined pathological role of IL-17 when the host is repeatedly exposed to mycobacterial antigens (42). These studies indicate that repeated exposure to mycobacterial antigen, as in repeated BCG vaccination, may generate a detrimental IL-17 response that could be controlled by addition of lactoferrin adjuvant in vaccination protocols. Similar overall effects were noted with IFN- γ ; lactoferrin was able to support and maintain elevated IFN- γ recall production from T cells when given either once or twice in the vaccine, whereas the BCG response diminished upon multiple vaccinations. The opposite was true for IL-10, with reduced production in the lactoferrin immunized groups. This is encouraging, as IL-10 has been linked to increased incidence of disease (43); the absence of IL-10 also leads to increases in IFN- γ (44).

In conclusion, the beneficial differences in lung pathological outcome post-MTB challenge was maintained in both lactoferrin adjuvant protocols, even though differential generation cytokine profiles in splenic recall response. This suggests that the efficacy of lactoferrin adjuvant can be achieved with a primary vaccination and that a secondary booster may not be required. These studies also illustrate the need to examine vaccines in a new light, incorporating parameters that include pathological protection in addition to reliance on standard bacterial load enumeration in organs. We feel that lactoferrin has strong potential

as an adjunct component to the current BCG vaccine to boost efficacy through enhancing host protection leading to decreased pulmonary pathology upon subsequent infectious challenge.

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References

1. Actor JK, Hwang SA, Kruzel ML. Lactoferrin as a natural immune modulator. *Curr Pharm Des.* 2009; 15:1956–1973. [PubMed: 19519436]
2. Damiens E, Mazurier J, el Yazidi I, Masson M, Duthille I, Spik G, Boilly-Marier Y. Effects of human lactoferrin on NK cell cytotoxicity against haematopoietic and epithelial tumour cells. *Biochim Biophys Acta.* 1998; 1402:277–287. [PubMed: 9606986]
3. Hwang SA, Wilk KM, Bangale YA, Kruzel ML, Actor JK. Lactoferrin modulation of IL-12 and IL-10 response from activated murine leukocytes. *Med Microbiol Immunol.* 2007; 196:171–180. [PubMed: 17377816]
4. Curran CS, Demick KP, Mansfield JM. Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways. *Cell Immunol.* 2006; 242:23–30. [PubMed: 17034774]
5. Dhennin-Duthille I, Masson M, Damiens E, Fillebeen C, Spik G, Mazurier J. Lactoferrin upregulates the expression of CD4 antigen through the stimulation of the mitogenactivated protein kinase in the human lymphoblastic T Jurkat cell line. *J Cell Biochem.* 2000; 79:583–593. [PubMed: 10996849]
6. Zimecki M, Mazurier J, Spik G, Kapp JA. Human lactoferrin induces phenotypic and functional changes in murine splenic B cells. *Immunology.* 1995; 86:122–127. [PubMed: 7590872]
7. Spadaro M, Caorsi C, Ceruti P, Varadhachary A, Forni G, Pericle F, Giovarelli M. Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells. *Faseb J.* 2008; 22:2747–2757. [PubMed: 18364398]
8. Fischer R, Debbabi H, Dubarry M, Boyaka P, Tome D. Regulation of physiological and pathological Th1 and Th2 responses by lactoferrin. *Biochem Cell Biol.* 2006; 84:303–311. [PubMed: 16936801]
9. Frydecka I, Zimecki M, Bocko D, Kosmaczewska A, Teodorowska R, Ciszak L, Kruzel M, Wlodarska-Polinski J, Kuliczowski K, Kornafel J. Lactoferrin-induced up-regulation of zeta (zeta) chain expression in peripheral blood T lymphocytes from cervical cancer patients. *Anticancer Res.* 2002; 22:1897–1901. [PubMed: 12168890]
10. Hwang SA, Kruzel ML, Actor JK. Influence of bovine lactoferrin on expression of presentation molecules on BCG-infected bone marrow derived macrophages. *Biochimie.* 2009; 91:76–85. [PubMed: 18486627]
11. Hwang SA, Actor JK. Lactoferrin modulation of BCG-infected dendritic cell functions. *Int Immunol.* 2009; 21:1185–1197. [PubMed: 19692539]
12. Andersen P, Doherty TM. The success and failure of BCG - implications for a novel tuberculosis vaccine. *Nat Rev Microbiol.* 2005; 3:656–662. [PubMed: 16012514]
13. Brennan MJ. The tuberculosis vaccine challenge. *Tuberculosis (Edinb).* 2005; 85:7–12. [PubMed: 15687021]
14. WHO. WHO. Global Tuberculosis Control: Epidemiology, Strategy, Financing. 2009.
15. Thaiss CA, Kaufmann SH. Toward novel vaccines against tuberculosis: current hopes and obstacles. *Yale J Biol Med.* 83:209–215. [PubMed: 21165340]
16. Hawkrige T, Mahomed H. Prospects for a new, safer and more effective TB vaccine. *Paediatr Respir Rev.* 12:46–51. [PubMed: 21172675]
17. Ho MM, Southern J, Kang HN, Knezevic I. WHO Informal Consultation on standardization and evaluation of BCG vaccines Geneva, Switzerland 22–23 September 2009. *Vaccine.* 28:6945–6950. [PubMed: 20692219]

18. Hwang SA, Arora R, Kruzel ML, Actor JK. Lactoferrin enhances efficacy of the BCG vaccine: comparison between two inbred mice strains (C57BL/6 and BALB/c). *Tuberculosis (Edinb)*. 2009; 89(Suppl 1):S49–54. [PubMed: 20006305]
19. Hwang SA, Wilk K, Kruzel ML, Actor JK. A novel recombinant human lactoferrin augments the BCG vaccine and protects alveolar integrity upon infection with *Mycobacterium tuberculosis* in mice. *Vaccine*. 2009; 27:3026–3034. [PubMed: 19428915]
20. Hwang SA, Wilk KM, Budnicka M, Olsen M, Bangale YA, Hunter RL, Kruzel ML, Actor JK. Lactoferrin enhanced efficacy of the BCG vaccine to generate host protective responses against challenge with virulent *Mycobacterium tuberculosis*. *Vaccine*. 2007; 25:6730–6743. [PubMed: 17698261]
21. Hwang SA, Welsh KJ, Kruzel M, Actor JK. A Mini-Review: Lactoferrin Augmentation of the BCG Vaccine Leads to Increased Pulmonary Integrity. *Tuberculosis Research and Treatment*. 2011
22. Hope JC, Villarreal-Ramos B. Bovine TB and the development of new vaccines. *Comp Immunol Microbiol Infect Dis*. 2008; 31:77–100. [PubMed: 17764740]
23. Locht C, Rouanet C, Hougardy JM, Mascart F. How a different look at latency can help to develop novel diagnostics and vaccines against tuberculosis. *Expert Opin Biol Ther*. 2007; 7:1665–1677. [PubMed: 17961090]
24. Padilla-Carlin DJ, McMurray DN, Hickey AJ. The guinea pig as a model of infectious diseases. *Comp Med*. 2008; 58:324–340. [PubMed: 18724774]
25. Chen CY, Huang D, Wang RC, Shen L, Zeng G, Yao S, Shen Y, Halliday L, Fortman J, McAllister M, Estep J, Hunt R, Vasconcelos D, Du G, Porcelli SA, Larsen MH, Jacobs WR Jr, Haynes BF, Letvin NL, Chen ZW. A critical role for CD8 T cells in a nonhuman primate model of tuberculosis. *PLoS Pathog*. 2009; 5:e1000392. [PubMed: 19381260]
26. Bruns H, Meinken C, Schauenberg P, Harter G, Kern P, Modlin RL, Antoni C, Stenger S. Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against *Mycobacterium tuberculosis* in humans. *J Clin Invest*. 2009; 119:1167–1177. [PubMed: 19381021]
27. van Pinxteren LA, Cassidy JP, Smedegaard BH, Agger EM, Andersen P. Control of latent *Mycobacterium tuberculosis* infection is dependent on CD8 T cells. *Eur J Immunol*. 2000; 30:3689–3698. [PubMed: 11169412]
28. Wang J, Santosuosso M, Ngai P, Zganiacz A, Xing Z. Activation of CD8 T cells by mycobacterial vaccination protects against pulmonary tuberculosis in the absence of CD4 T cells. *J Immunol*. 2004; 173:4590–4597. [PubMed: 15383593]
29. Bold TD, Ernst JD. Who benefits from granulomas, mycobacteria or host? *Cell*. 2009; 136:17–19. [PubMed: 19135882]
30. Kursar M, Koch M, Mittrucker HW, Nouailles G, Bonhagen K, Kamradt T, Kaufmann SH. Cutting Edge: Regulatory T cells prevent efficient clearance of *Mycobacterium tuberculosis*. *J Immunol*. 2007; 178:2661–2665. [PubMed: 17312107]
31. Quinn KM, Rich FJ, Goldsack LM, de Lisle GW, Buddle BM, Delahunt B, Kirman JR. Accelerating the secondary immune response by inactivating CD4(+)CD25(+) T regulatory cells prior to BCG vaccination does not enhance protection against tuberculosis. *Eur J Immunol*. 2008; 38:695–705. [PubMed: 18266274]
32. Paige C, Bishai WR. Penitentiary or penthouse condo: the tuberculous granuloma from the microbe's point of view. *Cell Microbiol*. 12:301–309. [PubMed: 20039878]
33. Hunter RL, Armitage L, Jagannath C, Actor JK. TB research at UT-Houston--a review of cord factor: new approaches to drugs, vaccines and the pathogenesis of tuberculosis. *Tuberculosis (Edinb)*. 2009; 89(Suppl 1):S18–25. [PubMed: 20006299]
34. Welsh KJ, Risin SA, Actor JK, Hunter RL. Immunopathology of postprimary tuberculosis: increased T-regulatory cells and DEC-205-positive foamy macrophages in cavitory lesions. *Clin Dev Immunol*. 2011:307631. [PubMed: 21197439]
35. Fine PE. Herd immunity: history, theory, practice. *Epidemiol Rev*. 1993; 15:265–302. [PubMed: 8174658]
36. Torrado E, Cooper AM. IL-17 and Th17 cells in tuberculosis. *Cytokine Growth Factor Rev*. 21:455–462. [PubMed: 21075039]

37. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity*. 2008; 28:454–467. [PubMed: 18400188]
38. Seiler P, Aichele P, Bandermann S, Hauser AE, Lu B, Gerard NP, Gerard C, Ehlers S, Mollenkopf HJ, Kaufmann SH. Early granuloma formation after aerosol *Mycobacterium tuberculosis* infection is regulated by neutrophils via CXCR3-signaling chemokines. *Eur J Immunol*. 2003; 33:2676–2686. [PubMed: 14515251]
39. Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, Kawakami K, Suda T, Sudo K, Nakae S, Iwakura Y, Matsuzaki G. IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis* bacille Calmette-Guerin infection. *J Immunol*. 2007; 178:3786–3796. [PubMed: 17339477]
40. Eruslanov EB I, Lyadova V, Kondratieva TK, Majorov KB, Scheglov IV, Orlova MO, Apt AS. Neutrophil responses to *Mycobacterium tuberculosis* infection in genetically susceptible and resistant mice. *Infect Immun*. 2005; 73:1744–1753. [PubMed: 15731075]
41. Keller C, Hoffmann R, Lang R, Brandau S, Hermann C, Ehlers S. Genetically determined susceptibility to tuberculosis in mice causally involves accelerated and enhanced recruitment of granulocytes. *Infect Immun*. 2006; 74:4295–4309. [PubMed: 16790804]
42. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, Pereira DR, Randall TD, Pedrosa J, Cooper AM, Castro AG. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with *Mycobacterium tuberculosis*. *J Exp Med*. 2007; 1609–1616. [PubMed: 20624887]
43. Awomoyi AA, Marchant A, Howson JM, McAdam KP, Blackwell JM, Newport MJ. Interleukin-10, polymorphism in SLC11A1 (formerly NRAMP1), and susceptibility to tuberculosis. *J Infect Dis*. 2002; 186:1808–1814. [PubMed: 12447767]
44. Redford PS, Boonstra A, Read S, Pitt J, Graham C, Stavropoulos E, Bancroft GJ, O'Garra A. Enhanced protection to *Mycobacterium tuberculosis* infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung. *Eur J Immunol*. 40:2200–2210. [PubMed: 20518032]

Figure 1. Organ bacterial load at day 65 post-MTB challenge

Lung (A), liver (B), and spleen (C) were collected from MTB challenged mice at day 65, and homogenates plated on 7H11 plates for CFU analysis. At least 6 mice were used per group; numbers represent means of CFU $\pm$ standard deviation, representative of repeated experimentation. All mice immunized with BCG or BCG/lactoferrin, once or twice, demonstrated a significant decrease in organ CFU compared to the non-immunized controls.

*** = $p < 0.001$

Figure 2. Pulmonary pathological changes from mice immunized with BCG or BCG/lactoferrin in response to MTB challenge

Representative lung sections collected from non-immunized (A) or BCG (B) or BCG/lactoferrin (C) immunized mice immunized once, or twice (D, E), and challenged with MTB were fixed in 10% formalin, paraffin sectioned, and stained with hematoxylin and eosin. Sections were imaged at 20x magnification and area analysis (F) conducted using Image J (NIH), depicted in the lower right panel. BCG/lactoferrin immunized mice, both 1x and 2x, demonstrate a significant decrease in percent lung occlusion by granuloma structures compared to the BCG (1x and 2x) vaccinated and non-immunized groups. Lungs from 4 to 6 mice of each group were analyzed. Relative area of occluding lesions was obtained and calculated as percent of total (100%) lung area, represented as mean values $\pm$ standard deviation. * = $p < 0.05$ ** = $p < 0.01$

Figure 3. Splenocyte production of cytokines in response to mycobacterial antigens

At 6 weeks post-last vaccination, splenocytes were isolated and stimulated with HK-BCG. Supernatants were collected at 72hrs and analyzed for production of IFN- γ (A), IL-17 (B), and IL-10 (C). There is a distinct pattern of immune response observed from splenocytes from mice immunized once with BCG/lactoferrin and twice with BCG/lactoferrin. Values calculated as mean pg.ml $\pm$ standard deviation, using 4 animals per stimulation. Limit of detection is between 15–32pg/mL. * = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$