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Lactoferrin augments BCG vaccine efficacy to generate T helper response and subsequent protection against challenge with virulent *Mycobacterium tuberculosis*

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Abstract

The ability to control intracellular *Mycobacterium tuberculosis* (MTB) infection relies on cellular immunity and generation of a strong T-cell helper 1 (T_H1) response. Lactoferrin, an iron-binding protein with immune regulatory functions, was investigated as an adjuvant to boost *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) efficacy. Lactoferrin was initially shown to augment IL-12(p40) production from macrophages stimulated with LPS. A single immunization of mice with Lactoferrin as an adjunct adjuvant resulted in amplified splenocyte proliferative response to heat-killed BCG, and elevated IL-12(p40) production with increased relative ratios of IL-12/IL-10. Furthermore, splenocyte recall response to HK-BCG was augmented for proinflammatory mediators, TNF-α, IL-1β, and IL-6, approaching responses generated to complete Freund's adjuvant (CFA) immunized controls. Specific responses were identified, with significant elevation of IFN-γ generated during antigenic recall. Subsequent aerosol challenge of Lactoferrin adjuvant immunized mice with virulent *M. tuberculosis* revealed decreased mycobacterial loads in the lung, and limitation of organism dissemination to a peripheral organ (spleen). These studies indicate that Lactoferrin can act as an adjunct adjuvant to augment cellular immunity and boost BCG efficacy for protection against subsequent challenge with virulent MTB.

Keywords: Lactoferrin; BCG vaccine efficacy; *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is the leading cause of morbidity due to an infectious disease and is a serious, unresolved burden upon the world's population despite aggressive vaccine implementation and pro-

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gressive antibiotic treatment. The causative agent is *Mycobacterium tuberculosis* (MTB), an intracellular bacterium whose primary host cell is the macrophage. It is estimated that over a third of the world's population is infected with MTB, with incidence of infection continuously on the rise [1].

Protection against MTB infection requires host generation of a strong cell-mediated immunity (CMI); specifically, a T-cell-mediated delayed-type hypersensitivity (DTH) response, involving the activation of both $CD4^+$ and $CD8^+$ lymphocytes and development of a strong T-cell helper type-1 (T_H1) immunity as indicated by production of interferon gamma (IFN-γ) and macrophage-derived interleukin-12 (IL-12), activating host macrophages and leading to bacterial clearance [2,3]. DTH contributes to the development of protective granulomatous response, limiting organism dissemination. The widely used TB vaccine is a live attenuated strain of *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG). The global efficacy of BCG in generating a protective host response against MTB has fallen, especially in regards to preventing adult onset disease. Despite waning efficacy, BCG vaccine is still the gold standard against which all other developing vaccines are measured.

Employing adjuvants as a strategy to improve BCG vaccine efficacy is a major world-wide research focus. Most adjunct adjuvants, while effective in enhancing humoral immunity, fail to increase T-cell responses considered protective during subsequent MTB challenge. Presently, the model adjuvant capable of promoting generation of CMI and DTH responses is complete Freund's adjuvant (CFA); a water-in-oil emulsion containing 50% mineral oil, emulsifying agent Arlacel A, and heat-killed avirulent MTB strain, H37Ra. CFA is highly toxic; it is not suitable for human use and becoming more undesirable for use in animals. Recent advances in adjuvant development are directed towards generating vaccines that approach the efficacy of CFA while possessing none of its toxic properties [4].

A promising natural adjuvant candidate is Lactoferrin, an 80-kDa iron-binding protein commonly found in secretory fluids and present in secondary granules of neutrophils [5,6]. Lactoferrin is non-toxic, and has been examined in depth for its ability to modulate excessive proinflammatory responses during sepsis [7,8]. Cell surface receptors for Lactoferrin have been identified on multiple leukocyte populations, including macrophages and lymphocytes [9]. Lactoferrin stimulates T cells to proliferate, promotes lymphocyte maturation, increases surface expression of leukocyte functional adhesion molecule LFA, and enhances natural killer cell activity [10].

Lactoferrin exhibited adjuvant properties through generation of lymphocytic responses with suboptimal antigen doses [11]. Preliminary studies also demonstrated efficacy of Lactoferrin to boost DTH responses against multiple antigens, including BCG. Lactoferrin stimulated naïve macrophages to increase production of IL-12 [12], an essential cytokine produced by macrophages and dendritic cells to promote T_H1 immunity, thereby identifying a potential molecular mechanism by which Lactoferrin promotes antigen-specific lymphocytic responses.

These studies investigate Lactoferrin adjuvant activities. Experiments were designed to examine Lactoferrin effects on stimulated macrophages and Lactoferrin ability to elicit in vitro and in vivo responses to BCG. Finally, Lactoferrin was evaluated as an adjuvant to enhance efficacy of the BCG vaccine to generate protective host responses to subsequent challenge with virulent MTB.

2. Materials and methods

2.1. Macrophages

Two murine macrophage cell lines, J774A.1 and RAW 264.7, purchased from American Type Culture Collection (ATTC), were used for stimulation experiments. Cell were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma) and 0.01% HEPES (Sigma) and L-arginine (Sigma).

2.2. Macrophage stimulation

Murine macrophages were plated into 24-well plates at 1×10^6 cells/mL/well using the media as outlined in Section 2.1. Triplicate cells of macrophages were stimulated with 200 ng/mL of lipopolysacrride (*Escherichia coli* O111:B4 LPS; appro

ximately 3×10^6 E.U./mg) (Sigma), with increasing concentrations of bovine Lactoferrin (1 μg, 10 μg, 100 μg, 500 μg, and 1 mg/mL), with Lactoferrin and LPS. Low endotoxin bovine Lactoferrin utilized was a gift from PharmaReview Corporation (Houston, TX), characterized as <1 E.U./mg, less than 25% iron saturated, and >95% purity. Cells were incubated at 37 °C with 5% CO_2. Supernatants were collected after 72 h and frozen at −20 °C until analyzed by ELISA for cytokine production.

2.3. Microorganisms

The 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. The Erdman strain of *M. tuberculosis* (TMC 107, ATCC) used for challenge was grown in Dubos base (with 5.6% glycerol) with 10% supplement for 3 weeks before use. Cultures were taken during log growth period. Organisms were washed with 1× PBS (Dulbecco's phosphate-buffered salts 10×, Cellgro, Herndon, VA) and resuspended in 1× PBS. Suspensions were sonicated for 5 s prior to use. Bacterial concentration was determined using McFarland standards, and confirmed by plating dilutions onto 7H11 agar plates (Remel, Lenesa, KS). Plates were incubated at 37 °C with 5% CO_2 for 3–4 weeks, colonies were enumerated. Heat-killed BCG (HK-BCG) was produced by autoclaving the BCG suspension in 1× PBS at 121 °C for 20 min. Death of BCG was verified by plating of autoclaved BCG on 7H11 plates.

2.4. Immunizations

Immunizations were performed using standard NIH protocols for evaluation of BCG vaccines, modified as follows. Ten to twelve female C57BL/6 mice (4–5 weeks, Jackson Laboratories, Bar Harbor, ME) were immunized per group with 100 μl of the formulation, once, subcutaneously (s.c.) at the base of the tail. All formulations of BCG with or without Lactoferrin utilized BCG at 10^6 CFU/mouse. Lactoferrin was administered at 100 μg/mouse. BCG and Lactoferrin were emulsified with Freund's adjuvant in a 1:1 ratio. IFA and CFA were used as described previously [11].

2.5. Antigenic responses

Seventeen days post-immunization, spleens were harvested from each immunization group, homongenized, and red blood cells lysed by ACK buffer (Cambrex Bio Science, East Rutherford, NJ). Splenocytes were washed 2× with 1× PBS and resuspended in DMEM, supplemented with 10% heat-inactivated FBS, 0.005% 2-mercaptoethanol (2Me, Gibco, Carlsbad, CA), 0.01% penicillin G (Sigma) and gentamycin (Sigma), 0.01% HEPES (Sigma) and L-arginine (Sigma). Cells were plated at 10^6 cells/mL and stimulated with HK-BCG at 5:1 ratio. Four to six mice were used per group. Assay was done in duplicate or triplicate. Supernatants were collected at 24 and 72 h and frozen at −20 °C until evaluation by ELISA. Recall response experiments were repeated a total of three times with similar results.

2.6. Proliferation assay

Splenocytes at 5×10^5 cells/100 μL/well were plated in DMEM without phenol red (Sigma) and stimulated with HK-BCG at 5:1 ratio. After 48 h, proliferation was measured using the MTT assay (Sigma) following manufacturer's protocols. MTT was dissolved in 1× PBS at 5 mg/mL and filtered through a 0.2-μm filter. Ten microliters of MTT solution was added to each well, and plates were incubated at 37 °C for 4 h. Supernatants were removed and 100 μL of 0.1 N of hydrochloric acid (HCl) in anhydrous isopropanol was added. Four to six mice were used per group; assay was performed in triplicate. Absorbance was read at 570 nm subtracting background at 690 nm. Proliferation index was calculated relative to naïve control splenocytes in the absence of HK-BCG stimulation.

2.7. ELISA

Supernatants were assayed in triplicate for cytokine production using enzyme-linked immunosorbent assay (DuoSet ELISA kit, R&D Systems, Minneapolis, MN), according to manufacturer's instructions. Supernatants were analyzed for T-cell cytokine IFN-γ, macrophage mediators of T_H1 response (IL-12(p40),

IL-10), and proinflammatory mediators (IL-1β, IL-6, TNF-α). IL-1β and TNF-α were analyzed at 24 h, all others at 72 h. Values (pg/ml) were by regression analysis of data to standard curves generated.

2.8. Erdman challenge

Fourteen days post-immunization, four mice from each group were aerosol challenged with Erdman MTB [13]. Each mouse was infected via aerosol with <100 CFU/mouse. Aerosol infection was achieved using an inhalation exposure system (IES) (GLAS-COL Model #A4212 099c Serial #377782). Verification of infectious dose was accomplished at 1 day post-infection; four mice were sacrificed, lungs collected and homogenized, and dilutions plated onto 7H11 agar plates for CFU counts. Twenty-eight days after Erdman challenge, mice were sacrificed. Lung and spleen tissues were isolated, homogenized, and plated on 7H11 agar plates for CFU determination. Plates were incubated at 37 °C for 3–4 weeks before enumeration.

2.9. Statistics

One-way ANOVA comparison or students *t*-test was used for all data analysis. Statistical significance was assigned for values of $p<0.05$ or as indicated, comparing average values and standard errors between groups.

3. Results

3.1. Lactoferrin enhances IL-12 production from stimulated macrophages

Lactoferrin was examined for the ability to enhance IL-12 production in vitro from LPS-stimulated macrophage cell lines. J774A.1 or RAW 264.7 cultured macrophages were stimulated with LPS (200 ng/mL) in the presence of increasing Lactoferrin concentrations (Fig. 1). Stimulation of both cell lines with LPS led to production of IL-12(p40), with J774A.1 cells slightly more responsive than the RAW 264.7 line. Addition of Lactoferrin led to significant ($p<0.05$) increase in IL-12 from both cell lines. Increased IL-12 was apparent at 100 μg/ml of Lactoferrin and above. The RAW 264.7 cells were more sensitive to Lactoferrin treatment with increased IL-12 production at all Lactoferrin concentrations tested from 1 through 1000 μg/ml. J774A.1 or RAW 264.7 macrophages did not produce any significant levels of IL-12(p40) without stimulation with LPS or when stimulated with Lactoferrin alone (not shown).

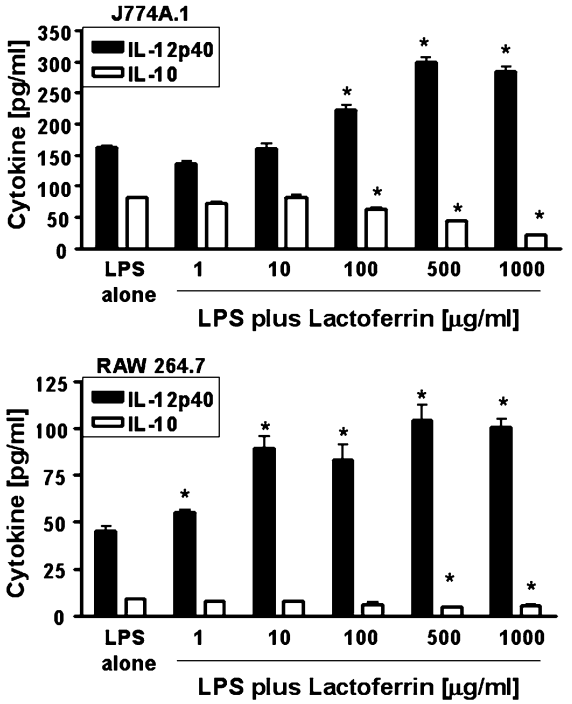

Fig. 1. Lactoferrin amplifies IL-12 production from stimulated macrophages. J774A.1 (top) or RAW 264.7 cells (bottom) were stimulated with LPS (200 ng/ml) and increasing concentrations of Lactoferrin (1–1000 μg/ml). Supernatants were assessed for IL-12 (■) and IL-10 (□). Average values (pg/ml) with standard errors are shown. *$p<0.05$ compared to LPS alone.

In a converse relationship, Lactoferrin reduced the production of IL-10 from LPS-stimulated macrophages (Fig. 1). The J774A.1 cell line produced approximately 81 μg/ml IL-10; addition of 100 μg/ml Lactoferrin and above led to significant ($p<0.05$) decrease. Although the levels of IL-10 produced upon LPS stimulation from the RAW 264.7 cell line were lower, Lactoferrin was able to reduce IL-10 production at higher concentrations. J774A.1 or RAW 264.7 macrophages did not produce any significant levels of IL-10 without stimulation with LPS or when stimulated with Lactoferrin alone (not shown).

Overall, Lactoferrin was able to affect LPS stimulation of macrophage cell lines, resulting in an

Table 1
BCG immunization with adjunct Lactoferrin adjuvant increases IL-12(p40)/IL-10 ratios

Group	IL-12(p40) [pg/ml]	IL-10 [pg/ml]	IL-12(p40) /IL-10 ratio
Non-immunized	45 (4)	61 (4)	0.77 (0.09)
BCG/IFA	50 (11)	69 (9)	0.70 (0.1)
BCG/IFA/Lactoferrin	86 (11)*/**	73 (18)	1.43 (0.22)*/**
BCG/CFA	69 (1.5)*/**	93 (7)*/**	0.78 (0.07)

Splenocytes obtained from mice given a single immunization with BCG as indicated were assessed for IL-12(p40) and IL-10 production to HK-BCG; average pg/ml shown with standard error. Ratio values represent comparisons made on individual mice.

* $p<0.05$ vs. non-immunized.

** $p<0.05$ vs. BCG alone.

increased ratio of IL-12/IL-10 production, leading to increased production of mediators necessary for driving T_H1-mediated functions.

3.2. Lactoferrin enhances in vivo development of T_H1 mediators to BCG antigen

Lactoferrin was examined for ability to effectively increase BCG immunization for promotion of T_H1 immunity. Immunization conditions were stringent to investigate early events in generation of response. Mice were immunized only once s.c. with 10^6 BCG emulsified in IFA, in IFA and Lactoferrin, or in CFA. Splenocytes were harvested 17 days post-immunization and stimulated in vitro with heat-killed BCG (HK-BCG) at a ratio of 5:1. Supernatants were collected after 72 h and analyzed for IL-12(p40) and IL-10 (Table 1).

A single administration of BCG in IFA and Lactoferrin resulted in significantly increased production of IL-12 in the splenic recall assay ($p<0.05$), with 86 pg/ml produced. Likewise, the CFA positive control immunization group also demonstrated significant increase in IL-12 production, relative to non-treated mice. In comparison, with only one immunization and short time to recall, the group receiving BCG emulsified in IFA alone did not generate significantly increased IL-12 production (50 pg/ml). Evaluation of IL-10 was also performed; immunization with Lactoferrin did not significantly increase production of this cytokine. Splenic recall to HK-BCG demonstrated only modest increases in IL-10 production for both the IFA alone and IFA with Lactoferrin groups. In contrast, BCG administered in CFA demonstrated significant increase in IL-10.

A comparison of IL-12 to IL-10 produced revealed strong and significant shift in response generated between groups. Immunization in IFA and Lactoferrin adjuvant demonstrated a significantly higher ratio of IL-12/IL-10 for the IFA and Lactoferrin group (ratio of 1.43) compared to either IFA or CFA alone (ratios of 0.70 and 0.78, respectively).

3.3. Lactoferrin enhances in vivo development of proinflammatory mediators and IFN-γ

Splenocytes from Lactoferrin immunized mice demonstrated strong proliferative response to HK-BCG (Fig. 2). A comparison of stimulation index (SI) revealed specific proliferation to antigen was significantly enhanced for the IFA with Lactoferrin and for the CFA immunized groups (2.81 ± 0.43 and 4.24 ± 0.42) vs. the non-immunized group (1.31 ± 0.23) or BCG emulsified in IFA group (0.58 ± 0.25).

The assay was extended to further examine generation of proinflammatory response (TNF-α, IL-β, and IL-6) in antigenic recall to HK-BCG. Splenocytes from individual mice were stimulated with HK-BCG (5:1). Supernatants were collected and assayed (Fig. 3). In concert with the increased stimulation

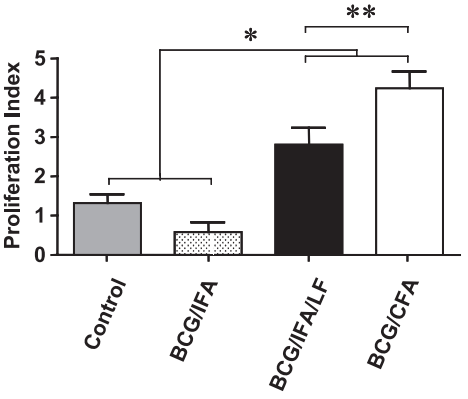

Fig. 2. Stimulation index of splenocytes from mice immunized with BCG. Splenocytes from mice immunized one time with BCG in IFA (▨), BCG in IFA with Lactoferrin (100 μg/mouse) (■), or BCG in CFA (□) were assessed for proliferative response to heat-killed BCG. Average Stimulation Index values with standard errors are shown. Responses are compared to non-immunized control mice (▩). *$p<0.05$; **$p<0.05$ as measured by two-tailed unpaired Student's t-test.

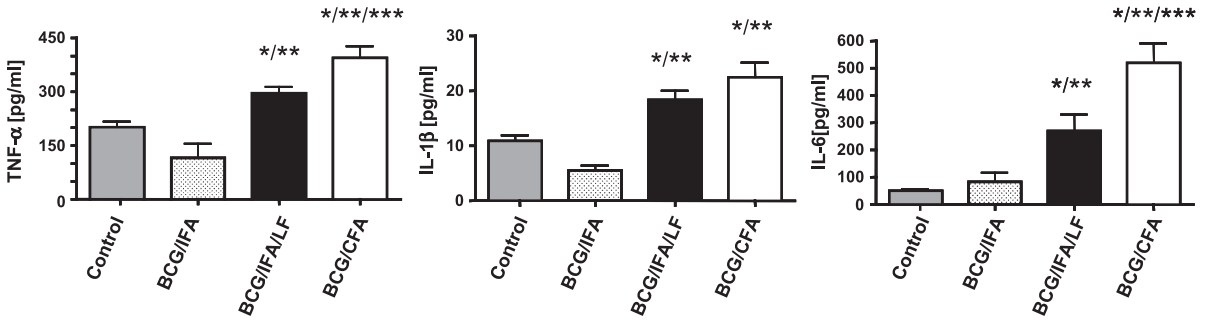

Fig. 3. BCG immunization with adjunct Lactoferrin adjuvant augments proinflammatory mediators from splenocytes. Splenocytes from mice immunized one time with BCG in IFA (▨), BCG in IFA with Lactoferrin (100 μg/mouse) (■), or BCG in CFA (□) were assessed for response to heat-killed BCG. Supernatants were assessed by ELISA for TNF-α, IL-1β, and IL-6. Average values (pg/ml) with standard errors are shown. Responses are compared to non-immunized control mice (▩). *p<0.05 vs. non-immunized; **p<0.05 vs. BCG alone; ***p<0.05 CFA vs. Lactoferrin group.

index, there was significant production of all three proinflammatory mediators in the Lactoferrin immunization group compared to both the non-immunized and IFA immunized groups (p<0.05). Likewise, the CFA immunization group remained statistically higher for TNF-α, IL-β, and IL-6, with TNF-α and IL-6 markedly elevated compared to all other groups.

IFN-γ response was measured as a direct indicator of generated T_H1 response, and as a marker for required function of vaccine efficacy to protect against virulent mycobacterial infection (Fig. 4). Increased IFN-γ was found upon in vitro stimulation with HK-BCG. Specifically, the IFA/Lactoferrin group produced 556.2±63.8 pg/ml IFN-γ compared with 149.5±7.6 pg/ml for the IFA alone group. As expected, the positive control CFA adjuvant group generated the highest level of IFN-γ with 2668.0±136.6 pg/ml.

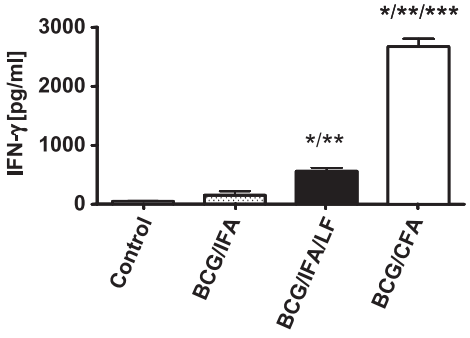

Fig. 4. Adjunct Lactoferrin adjuvant increases T_H1 cytokine IFN-γ from splenocytes. Splenocytes from mice immunized one time with BCG in IFA (▨), BCG in IFA with Lactoferrin (100 μg/mouse) (■), or BCG in CFA (□) were assessed for production of IFN-γ to heat-killed BCG. Average values (pg/ml) with standard errors are shown. Responses are compared to non-immunized control mice (▩). *p<0.05 vs. non-immunized; **p<0.05 vs. BCG alone; ***p<0.05 CFA vs. Lactoferrin group.

3.4. Vaccination with Lactoferrin adjuvant increases protection against challenge with virulent M. tuberculosis

Immunized mice were aerosol challenged with virulent *M. tuberculosis*, strain Erdman (<100 CFU per lung). Tissue was obtained 4 weeks following implantation and CFUs were enumerated. All immunization groups showed significant reduction in lung organism load (p<0.05) compared to the non-immunized control, with nearly 1 log less organisms present indicating local growth control within the tissue of implantation (Fig. 5, left). Of major importance is the ability to restrict organism dissemination to peripheral organs following aerosol challenge. In this regard, CFUs enumerated from spleen tissue detailed differences between immunization groups (Fig. 5, right). In the spleen, the non-immunized mice showed 4.49±0.25 log CFU, indicating spread of organisms to that tissue. In contrast, all BCG immunized groups limited dissemination. The IFA and Lactoferrin adjuvant group demonstrated the largest and most consistent reduction (p<0.065) in bacterial load within the spleen (2.760±0.32 log CFU); three of four mice

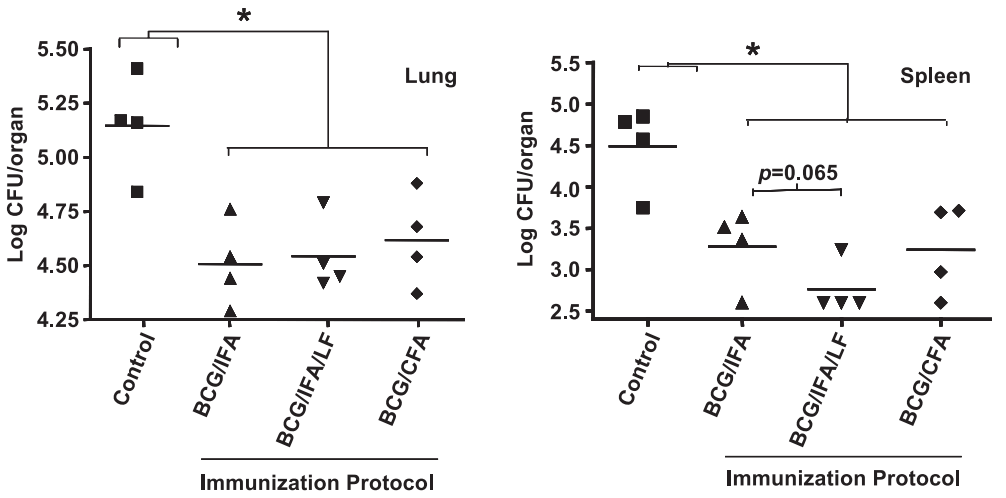

Fig. 5. BCG vaccination with adjunct Lactoferrin adjuvant limits mycobacterial dissemination following aerosol challenge with MTB. Mice immunized one time with BCG in IFA (▲), BCG in IFA with Lactoferrin (100 μg/mouse) (▼), or BCG in CFA (♦) were aerosol challenged with <100 CFU virulent MTB, strain Erdman. Non-immunized controls are indicated (■). Twenty-eight days post infection, lung tissue (left) was assessed for bacterial load and spleen tissue (right) was examined for dissemination of organisms to peripheral tissue. Individual CFU values for four mice per group are shown; bars indicate average for group. Responses are compared between groups. *$p<0.05$ vs. non-immunized. Three of four mice in the Lactoferrin group exhibited sterilizing immunity in the spleen; values were placed at organism limit of detection (Log_{10} of 500 CFU/organ=2.69). Data representative of two experiments with similar results and reduction in CFU.

demonstrated sterilizing immunity in that tissue with CFU levels below the limit of detection (500 CFU per organ). Indeed, the Lactoferrin immunized group further reduced bacterial loads compared to the IFA alone group (3.278±0.23 log CFU) and the CFA group (3.243±0.27 log CFU). At this day 28 time point, no detectable organisms were found in the liver for any group, including the non-vaccinated controls (data not shown).

4. Discussion

These studies were designed to examine utility of Lactoferrin as an adjunct adjuvant to increase efficacy of BCG vaccination to generate IFN-γ and protection against subsequent challenge with virulent MTB. There remains a global challenge to combat tuberculosis; more people die from tuberculosis each year than as a result of any other infectious disease. The 2003 World Health Organization (WHO) report on tuberculosis (TB) incidence rates around the world shows dense epidemic areas where TB vaccine is widely applied [1], clearly illustrating the limitations of the BCG vaccine towards spread of disease [14]. Delining BCG efficacy will continue to be an obstacle in eradicating tuberculosis; a modeling study predicted that a MTB vaccine with only 50% efficacy would save thousands of lives in the next 10 years [15,16]. Accompanying strategies for the eradication of TB have included aggressive managed treatment, such as the directly observed treatment short course program (DOTS); however, dramatic strides in improving TB incidences will only occur with the development of improved vaccines and adjuvants.

It is widely accepted that host protection against MTB requires development of T-cell-mediated, protective delayed-type hypersensitivity (DTH) response. This involves activation of both CD4+ and CD8+ T cells, generation of T-cell helper1 (T_H1) type immunity and proinflammatory cytokine production, including IFN-γ, IL-12, and TNF-α [2,17]. BCG as a live vaccine has historically been more efficacious than killed or subunit vaccines by being able to induce cell-mediated immunity (CMI) required for protection. BCG remains the gold standard by which other vaccines are judged. Advantages of the BCG vaccine lie in its potential ability to persist in vivo for longer periods of time and to induce prolonged immunological memory, thus generating CMI and immune

responses at mucosal surfaces for relatively low cost. However, efficacy of BCG has fallen during the past two decades, especially in protection against adult pulmonary tuberculosis. Clinical studies indicate a range of efficacy from 80% effective in the United Kingdom to almost 0% efficacy in India and Malawi [4,18]. The reasons for this are unclear at this time. The most important problem with the current BCG vaccine is its inability to generate and sustain the necessary protective DTH responses needed for control of MTB infection. What is clear is that there is a great need to develop novel vaccines that can overcome the failure of BCG, offering protection against infection and limiting dissemination of organisms.

Lactoferrin is a naturally occurring glycoprotein that possesses a wide range of immunomodulatory effects, including the ability to enhance DTH response against BCG [11] and augment production of IL-12(p40) (a critical T_H1-generating cytokine) from peritoneal macrophage in naïve mice [12]. Lactoferrin enhances IL-12/IL-10 ratio in LPS-stimulated J774A.1 and RAW 264.7 macrophages, possibly by binding to CD14 receptor as a complex with LPS [19–21]. IL-12 is a critical mediator of protective response during MTB infection [22,23]. This suggested that Lactoferrin would have the potential to generate a local environment to promote development of antigen-specific T_H1 cellular response from activated presenting cells. Indeed, when Lactoferrin was admixed with BCG vaccine, greater antigen-specific proliferative responses were generated. Likewise, recall responses to HK-BCG revealed relative increases of proinflammatory mediators TNF-α, IL-1β, and IL-6. Lactoferrin was able to significantly augment the production of IFN-γ compared to vaccine preparations in IFA alone. Overall, Lactoferrin, acting as an adjunct adjuvant, was able to augment vaccine efficacy.

Stringent conditions were applied to test Lactoferrin's ability to enhance BCG vaccine efficacy for protection against subsequent aerosol challenge. For these studies, mice were administered a single dose of vaccine, thus allowing for identification of differences in response generation. All three vaccines were productive in limiting mycobacterial growth in the organ of implantation, with lung CFUs reduced nearly 1 log after only 28 days. However, the Lactoferrin group was superior in limiting bacterial dissemination, with three of four mice exhibiting sterilizing immunity in the spleen. Although the other two vaccinated groups had reduction in splenic CFUs, neither was able to achieve sterilizing immunity and completely inhibit or control spread of organisms to that tissue.

To date, no research vaccine against tuberculosis has been able to consistently surpass BCG in reduction of lung organisms following challenge with virulent MTB [24]. Multiple alternative vaccines failed to protect against MTB. Indeed, surpassing BCG seemed an unrealistic goal that may never be accomplished. The need for new MTB vaccines is paramount, as evident by the number of new vaccine formulations currently in the process of Phase I clinical testing. These include vaccines utilizing live attenuated MTB, recombinant BCG with MTB Ag85 antigens, inactived *M. vaccae* organisms, DNA vaccines utilizing heat shock protein 65 DNA, dendritic vaccines incorporating Ag85-ESAT6 proteins, and modified (auxotrophic) organisms [25–29]. The data described in this report indicate that Lactoferrin may be a useful adjunct adjuvant to boost efforts to BCG-based vaccines, and perhaps to assist through augmentation of other vaccine strategies as well.

Acknowledgements

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References

[1] World Health Organization. Global tuberculosis control. Surveillance, planning, financing. The world health report 2003. Geneva (Switzerland): World Health Organization; 2003.
[2] Flynn JL. Immunology of tuberculosis and implications in vaccine development. Tuberculosis (Edinb) 2004;84:93.
[3] Bloom BR, Flynn J, McDonough K, Kress Y, Chan J. Experimental approaches to mechanisms of protection and pathogenesis in *M. tuberculosis* infection. Immunobiology 1994;191:526.

[4] Brennan M. A new generation of tuberculosis vaccine. In: de Quadros CA, editor. Vaccines: preventing disease & preventing health. Washington (DC): Pan American Health Organization; 2004. p. 177.
[5] Zimecki M, Artym J, Chodaczek G, Kocieba M, Kruzel ML. Protective effects of lactoferrin in *Escherichia coli*-induced bacteremia in mice: relationship to reduced serum TNF alpha level and increased turnover of neutrophils. Inflamm Res 2004;53:292.
[6] Legrand D, Elass E, Pierce A, Mazurier J. Lactoferrin and host defense: an overview of its immuno-modulating and anti-inflammatory properties. Biometals 2004;17:225.
[7] Kruzel ML, Harari Y, Mailman D, Actor JK, Zimecki M. Differential effects of prophylactic, concurrent and therapeutic lactoferrin treatment on LPS-induced inflammatory responses in mice. Clin Exp Immunol 2002;130:25.
[8] Adamik B, Zimecki M, Wlaszczyk A, Berezowicz P, Kubler A. Lactoferrin effects on the in vitro immune response in critically ill patients. Arch Immunol Ther Exp (Warsz) 1998;46:169.
[9] Suzuki YA, Lonnerdal B. Characterization of mammalian receptors for lactoferrin. Biochem Cell Biol 2002;80:75.
[10] Kruzel ML, Zimecki M. Lactoferrin and immunologic dissonance: clinical implications. Arch Immunol Ther Exp (Warsz) 2002;50:399.
[11] Zimecki M, Kruzel ML. Systemic or local co-administration of lactoferrin with sensitizing dose of antigen enhances delayed type hypersensitivity in mice. Immunol Lett 2000;74:183.
[12] Actor JK, Hwang SA, Olsen M, Zimecki M, Hunter Jr RL, Kruzel ML. Lactoferrin immunomodulation of DTH response in mice. Int Immunopharmacol 2002;2:475.
[13] Actor JK, Breij E, Wetsel RA, Hoffmann H, Hunter Jr RL, Jagannath C. A role for complement C5 in organism containment and granulomatous response during murine tuberculosis. Scand J Immunol 2001;53:464.
[14] Lewinsohn DM, Lewinsohn DA, Grotzke JE. TB vaccines at the turn of the century: insights into immunity to *M. tuberculosis* and modern approaches for prevention of an ancient disease. Semin Respir Infect 2003;18:320.
[15] Lietman T, Blower SM. Potential impact of tuberculosis vaccines as epidemic control agents. Clin Infect Dis 2000; 30(Suppl. 3):S316.
[16] Murray CJ, Salomon JA. Modeling the impact of global tuberculosis control strategies. Proc Natl Acad Sci USA 1998; 95:13881.
[17] Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci U S A 2001;98:5752.
[18] Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 1994;271:698.
[19] Crouch SP, Slater KJ, Fletcher J. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. Blood 1992;80:235.
[20] Elass-Rochard E, Legrand D, Salmon V, Roseanu A, Trif M, Tobias PS, et al. Lactoferrin inhibits the endotoxin interaction with CD14 by competition with the lipopolysaccharide-binding protein. Infect Immun 1998;66:486.
[21] Miyazawa K, Mantel C, Lu L, Morrison DC, Broxmeyer HE. Lactoferrin–lipopolysaccharide interactions. Effect on lactoferrin binding to monocyte/macrophage-differentiated HL-60 cells. J Immunol 1991;146:723.
[22] Cooper AM, Kipnis A, Turner J, Magram J, Ferrante J, Orme IM. Mice lacking bioactive IL-12 can generate protective, antigen-specific cellular responses to mycobacterial infection only if the IL-12 p40 subunit is present. J Immunol 2002;168:1322.
[23] Cooper AM, Magram J, Ferrante J, Orme IM. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with mycobacterium tuberculosis. J Exp Med 1997;186:39.
[24] McMurray DN. Recent progress in the development and testing of vaccines against human tuberculosis. Int J Parasitol 2003;33:547.
[25] Nor NM, Musa M. Approaches towards the development of a vaccine against tuberculosis: recombinant BCG and DNA vaccine. Tuberculosis (Edinb) 2004;84:102.
[26] Wang J, Xing Z. Tuberculosis vaccines: the past, present and future. Expert Rev Vaccines 2002;1:341.
[27] Kumar H, Malhotra D, Goswami S, Bamezai RN. How far have we reached in tuberculosis vaccine development? Crit Rev Microbiol 2003;29:297.
[28] Collins DM. New tuberculosis vaccines based on attenuated strains of the *Mycobacterium tuberculosis* complex. Immunol Cell Biol 2000;78:342.
[29] Collins HL, Kaufmann SH. Prospects for better tuberculosis vaccines. Lancet, Infect Dis 2001;1:21.