Predicting protection against tuberculosis from BCG using the guinea pig model

Honors Thesis

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Abstract

Guinea pigs, while having been used as a model animal for tuberculosis infections for over a century, modern literature about their response to the commonly used BCG vaccination is limited. In this study, we investigated whether immune responses to the BCG vaccine are variable and if these differences could predict protection after exposure to *Mycobacterium tuberculosis*. Using the guinea pig model, the only rodent species that reliably develops delayed-type hypersensitivity responses similar to humans, we evaluated inflammation in response to BCG vaccination, tuberculin skin test (TST) response, and capacity for antigen-specific secretion of IFN-γ by ELISpot in response to M. bovis purified protein derivative (PPD). We hypothesized that the degree of inflammation incited by BCG vaccination would correlate with TST size and frequency of antigen-specific IFN-γ production. Most of the animals developed an inflammatory response to TST within 24-72 hours, indicating a Th1 response was developed post-vaccination. However, 4 out of 18 individuals lacked an inflammatory response that lasted over 24 hours. To confirm this, IFN-γ production was assessed in PBMCs isolated from BCG-vaccinated and unvaccinated guinea pigs, finding little correspondence between IFN-γ production and increased skin inflammation. We hypothesized that IFN-γ secretion would be highest in guinea pigs with the largest TST response. The results of this study demonstrate the development of a delayed-type hypersensitivity response to BCG vaccination, albeit inconsistent in its systemic effects. Collectively, our results a highly differential response to BCG vaccination. If vaccination confers variable protection against *M. tuberculosis* infection, this variable vaccine response may offer insight into correlates of immune protection.

Introduction

The BCG (Bacille-Calmette-Guérin) vaccine was created in 1921 (Setiabudiawan et al., 2022) to protect humans against the development of disease from the *Mycobacterium tuberculosis* (*Mtb*) bacterial infection. Like many early vaccines, the BCG vaccine is live-attenuated (Setiabudiawan et al., 2022), consisting of weakened organisms to be injected intradermally into an individual so that they may develop an adaptive immune response to the pathogen. However, it has been proposed that because *Mtb* is a bacterial organism (Suliman, 2023) and not viral, it possesses a cell wall that hides its extracellular proteins beneath, making it harder for the immune system to recognize the disease and mount a response. Coupled with the fact that *Mtb* can live inside its host's cells for years at a time undetected (Setiabudiawan et al., 2022), it also might be hard for cells to target antigen sequences specific to the species and mount an adaptive response.

Most bacterial vaccinations, such as tetanus, get around this issue by targeting virulence factors (Setiabudiawan et al., 2022) secreted by the desired organism. Yet because the BCG vaccination contains the entire organism (Setiabudiawan et al., 2022), the antigen(s) that the body is exposed to can vary substantially depending on each individual's immune system. While exposure to a variety of antigens can build a more reliable immune response to a pathogen (Martinez et al., 2022), it also leads to a larger variety of reaction strengths and types (Martinez et al., 2022) between individuals.

Historically, the efficacy of BCG vaccination was measured using tuberculin skin tests (TSTs) (Setiabudiawan et al., 2022). However, this method often garnered false positive results (Suliman, 2023), requiring a more accurate test to be developed. An alternative test measures the response to BCG based on the cytokine IFN-γ, a signaling protein used by white blood cells

when in contact with an *Mtb* antigen (Ordway et al., 2007). IFN-γ acts as a pro-inflammatory signal (Setiabudiawan et al., 2022), promoting more leukocytes to migrate to the site and respond to the pathogen that was detected in that area. This response can be replicated *in vitro* (Kuan et al., 2022), allowing systemic adaptive response to *Mtb* to be measured from collected blood samples (Kuan et al., 2022). This has led to the development of IFN-γ ELISpot assays, which use a 96-well ELISpot plate and overlaying stimulated PBMCs to produce IFN-γ for downstream measurement (Setiabudiawan et al., 2022), allowing for easy quantification of an antigen-specific response.

Through the use of IFN-γ assays in humans and other model animal rodents, BCG has not been found to produce consistent systemic inflammatory results when their PBMCs have been re-exposed to an *Mtb* antigen (Kuan et al., 2022). This can contrast with the inflammatory results seen at the vaccination and/or skin test sites, making BCG's efficacy unpredictable and inconsistent (Martinez et al., 2022) across tissues, organs, individuals, and species.

The BCG vaccination has been recorded to have a correlate of protection against *Mtb* disease anywhere between 20% to 90% (Suliman, 2022), depending on the studies referenced. This protection has only been shown to develop in children under 2 years old (Martinez et al., 2022). This protection wanes in adults, leaving them unprotected from *Mtb* disease despite being vaccinated (Martinez et al., 2022). Due to this loss of efficacy, research has been underway for decades to make a more reliable vaccination (Setiabudiawan et al., 2022) that works on all age groups, lasts for a longer time, and can be readily distributed in low-income *Mtb*-endemic areas.

While a new vaccination is being developed, the specific mechanism causing the variability in responses to BCG is still poorly understood. With this, research is currently occurring to delve into the reasoning behind the lack of correlates of protection specific to BCG (Suliman, 2022). By knowing specifically why BCG is unpredictable, new *Mtb* vaccinations can be developed with knowledge of this problem and avoid the complications associated with BCG inefficacy.

Development of new *Mtb* vaccinations (and countless other medical products) has historically begun in rodents like mice and rats (Orme and Ordway, 2016). While these animal models are invaluable for short-term research studies due to their small size and many similarities to human biology, they can only sustain an adaptive immune response against *Mtb* from BCG for a few months (Orme and Ordway, 2016) before protection is lost.

By comparison, guinea pigs' neutrophil-dominant initial response (McMurray, 1994) more closely mirrors that of the human response to *Mtb*. Mice, which primarily rely on macrophages for their innate immune response (Orme and Ordway, 2016), show differences in immunohistochemical signaling between the innate and adaptive immune systems. Because macrophages primarily rely on phagocytosis instead of granule release like neutrophils (Orme and Ordway, 2016), mice ultimately diverge more from humans in their histopathological development of *Mtb*-derived lesions.

While IFN-γ has historically been shown to drive the penultimate immune response to *Mtb* in both guinea pigs and mice (Orme and Ordway, 2016), the cellular signaling route between a neutrophil-dominant innate response and an adaptive immune response is largely conserved (Ordway et al., 2007) between guinea pigs and humans. This means that guinea pigs often show similar necrotic patterns at BCG vaccination and TST inoculation sites to humans, as their neutrophilic granules destroy tissue similarly at both injection sites (Ordway et al., 2007). With this shared signaling and pathologic routes, results from guinea pig-based vaccination studies can

give greater insight into the possible pathologic consequences the BCG vaccine and *Mtb* antigen(s) may have in humans.

In this study, we examined the local and systemic responses to BCG vaccination in guinea pigs. We hypothesized that inflammation at the vaccination site would correlate with inflammation at the skin test site, and a proportional production of *in vitro* IFN-γ when exposed to an *Mtb* antigen. By comparing the responses of these animals, we can assess if any commonly used immunological measure of vaccine response can predict protection from tuberculosis.

Methods

90 outbred adult Dunkin-Hartley guinea pigs were acquired for this study. 72 of them were vaccinated intradermally on their ventral abdomen with BCG at day 0, while the other 17 were mock-vaccinated with saline in the same area. The animals were then left to mount an immune response, having the surface area of their inflammation around the vaccination site measured at 3, 7, and 14 days post-vaccination. Additional characteristics, such as the presence of scarring or necrosis at the vaccination site, were also recorded as they could be indicative of a higher immune response toward the vaccination.

8 weeks after their initial vaccination, a tuberculin skin test (TST) was performed intradermally on their ventral abdomen, parallel to the vaccination site. 2 μg of purified protein derivative (PPD) derived from the *M. bovis* organism was administered to both the BCG and saline vaccinated animals. Over a period of 72 hours post-inoculation, each animals' surface area of inflammation at the TST site was measured daily, with any scarring and necrosis at the site also being recorded if present.

Four weeks after the 72-hour TST monitoring was complete, PBMCs were collected from the cranial vena cava of 12 animals. Three unvaccinated guinea pigs were compared to nine BCG-vaccinated animals with varying responses to TST, as follows: three with no TST response, three with a mid-level TST response, and three with a strong TST response. PBMCs were then isolated from blood samples by density gradient using LymphoLyte solution to be used fresh in an IFN-γ ELISpot assay.

Each animal's PBMCs were transferred into Opti-MEM cell culture media, plated on a 96-well ELISpot plate coated with VE4 capture antibody, stimulated with PMA/Ionomycin (positive control) in one well, *M. bovis* PPD in another, or left unstimulated in a third well (negative control), then left to incubate at 37°C for 72 hours. Additional controls included wells with no addition of cells to fully control for background in the assay.

After the 72-hour incubation period, the cells were lysed, and the plate was rinsed thoroughly with PBS + Tween 20 (0.05%). Once dried, 100 μL/well containing 3μg of NG3 detection antibody was added to all used wells and left to incubate at room temperature for 2 hours. Following the first incubation, the wells were washed with PBS + Tween again before placing 100 μL/well of 1:3000 diluted goat anti-mouse IgG2a HRP conjugate antibody solution into each used well and left to incubate at room temperature for another hour.

Following the completion of the second incubation, the wells were thoroughly washed with PBS + Tween and dried before being washed again with PBS. After being completely rinsed out, 100 μL/well of TMB was added to each used well and left to develop for 30 minutes. Once darkened, the plate was thoroughly rinsed with DI water to remove any excess TMB or cellular particulates. The dried plate was then left in a drawer to develop for 48 hours before being scanned and read.

This ELISpot assay was repeated the following week, this time with 6 BCG vaccinated animals. Three animals were picked due to their high inflammatory response at the vaccination site and/or TST site, while the other three were picked for their low inflammatory response at one or both sites.

Once both plates were complete, the number of spots per well was counted, ensuring that each animal was capable of producing IFN-γ using the positive control while simultaneously comparing the increase in IFN-γ production in response to the PPD with the unstimulated cells. These values were then cross-referenced with each animal's inflammatory responses to see if there were any similarities or trends in the overall data.

Results

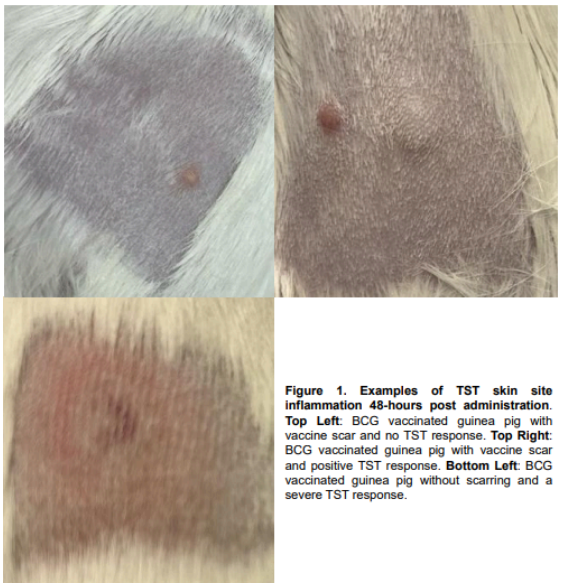

Figure 1. Examples of TST skin site inflammation 48-hours post administration. **Top Left**: BCG vaccinated guinea pig with vaccine scar and no TST response. **Top Right**: BCG vaccinated guinea pig with vaccine scar and positive TST response. **Bottom Left**: BCG vaccinated guinea pig without scarring and a severe TST response.

<u>Local inflammatory response to BCG vaccination</u>

Out of 72 BCG-vaccinated guinea pigs, 93% showed inflammation at the BCG vaccination site at one point (see Fig. 2) during the two weeks of monitoring post-injection. Of those individuals, only 32% showed consistent inflammation (see Fig. 2) over the entire two weeks, ranging from 0.5-6 mm in width (see Fig. 1). Of those individuals, 4% of the overall individuals developed 2-4 mm wide scabs at the injection site at some point during the two weeks. Two more individuals developed a 5 mm raised and red inflammatory reaction at the site during the two weeks instead. Ultimately, 19% of individuals were left with scars (see Fig. 1) at the vaccination site, each ranging from 2-10 mm^2. All 18 individuals mock-vaccinated with saline showed no signs of inflammation or scarring at the injection site at any point during the two weeks.

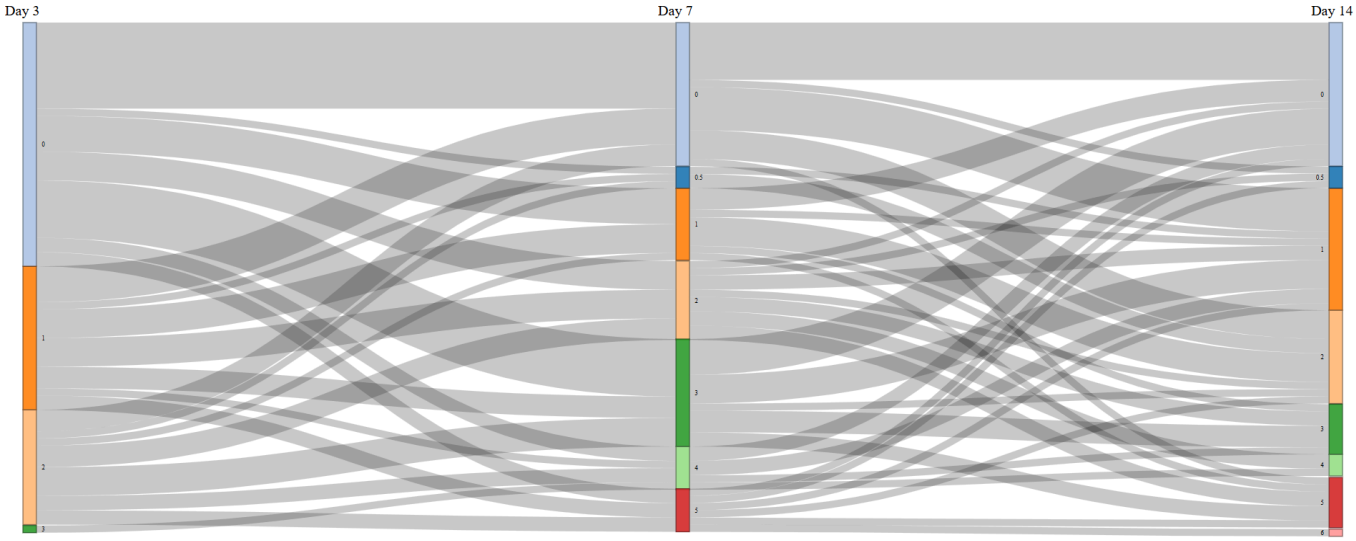

Figure 2. Inflammatory response to BCG vaccine over time. Data collected from 72 guinea pigs. The size of skin site inflammation was recorded at days 3, 7, and 14 post-vaccination. Each colored bar represents the width of the lesion in mm.

Tuberculin Skin Test (TST) Response

Of all 72 BCG-vaccinated animals given *M. bovis* PPD intradermally, 93% of individuals showed inflammation at the TST site (see Fig. 3) at some point during the 72-hour monitoring period post-inoculation. Lesions ranged anywhere from 6 to 460 mm^2. No lesions were seen in the individuals mock-vaccinated with saline.

46% of all lesions appeared firm for at least 24 hours. 8% of all lesions stayed firm for 48 hours. Firm lesions ranged from 16-208 mm^2 (see Fig. 3). 33% of lesions appeared soft for 24 hours. No lesions remained soft for longer than 24 hours. Soft lesions ranged from 4-90 mm^2 (see Fig. 3). No lesions appeared firm or soft during the first 24 hours.

14% of all individuals showed necrosis at the TST site for at least 24 hours, with 10% of all individuals showing necrosis at the site for 48 hours or more. Only one individual showed necrosis for all 72 hours of monitoring. Necrotic lesions ranged in size from anywhere between 24 to 460 mm^2.

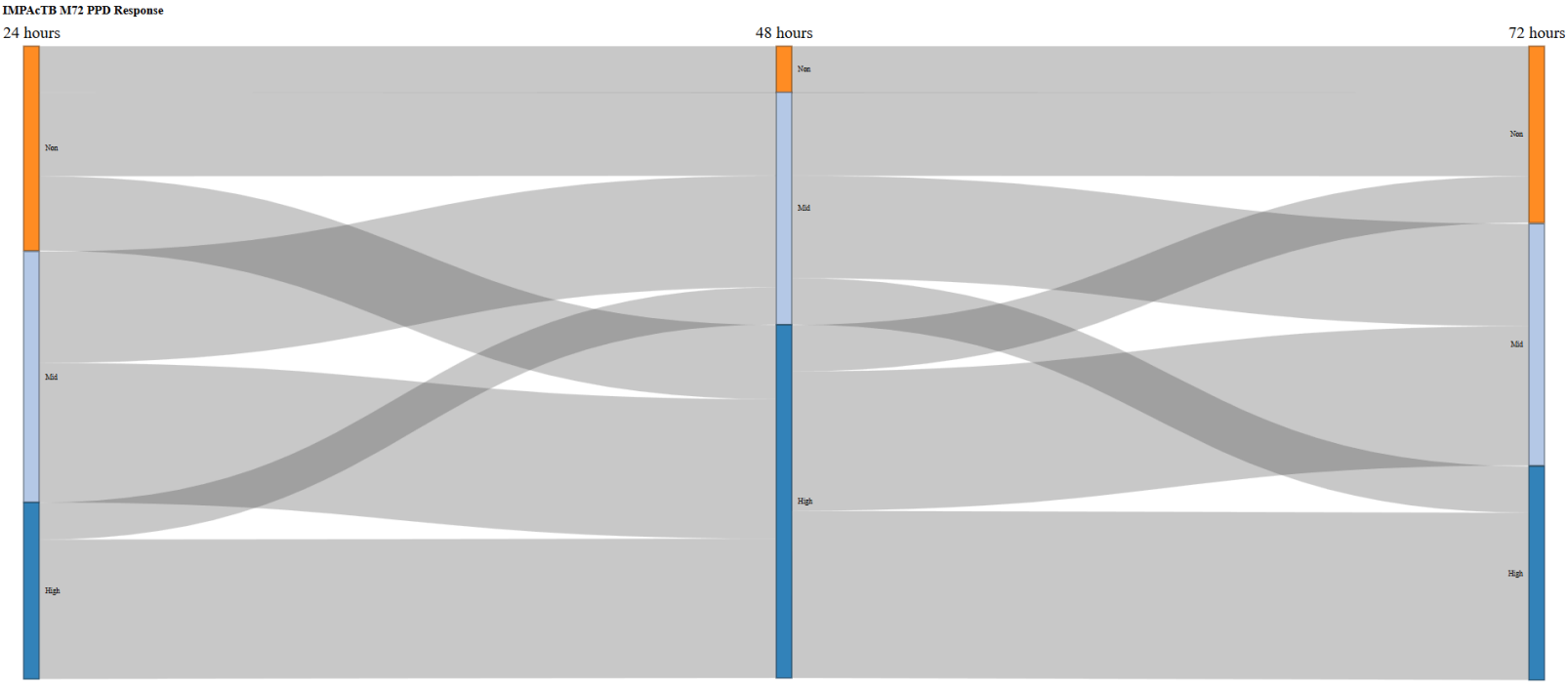

Figure 3. Size of skin induration in response to tuberculin skin test (TST). Data collected from 72 guinea pigs. An intradermal injection of 2µg PPD was performed and the skin site induration measured at 24-, 48-, and 72-hours after placement.

Antigen-Simultated IFN-γ Secretion

All positive control wells showed too many spots (i.e. the number of cells producing IFN-γ out of 200,000 cells per well) to count (see Fig. 4). All no-cell negative control wells showed no spots (see Fig. 4). Cell-only wells showed a baseline of 9-150 spots per well (see Fig. 5). *M. bovis* PPD-stimulated wells showed 12 to 1500 spots per well (see Fig. 5).

Animals mock-vaccinated with saline ranged from 12-22 spots per well (see Fig. 4), and overall averaged 17 spots per well when stimulated with *M. bovis* PPD. This same group ranged from 9-21 spots per unstimulated well, averaging 13 spots per well overall. BCG-vaccinated

animals ranged from 23-1500 spots per *M. bovis* PPD-simulated well (see Fig. 4), averaging 434 spots per well overall. These animals also ranged from 14-150 spots per unstimulated well (see Fig. 4), with an average of 73 spots per well overall.

Strong responders at the vaccine site ranged from 23-1000 spots per *M. bovis* PPD-stimulated well (see Fig. 4), averaging 355 spots per well altogether. Comparatively, these animals' unstimulated wells ranged from 34-150 spots per well (see Fig. 4), averaging 232 spots per well. Weak responders at the vaccine site ranged from 36-150 spots per *M. bovis* PPD-stimulated well (see Fig. 4), averaging 96 spots per well. These animals' unstimulated wells ranged from 73-150 spots per well (see Fig. 4), averaging 348 spots per well.

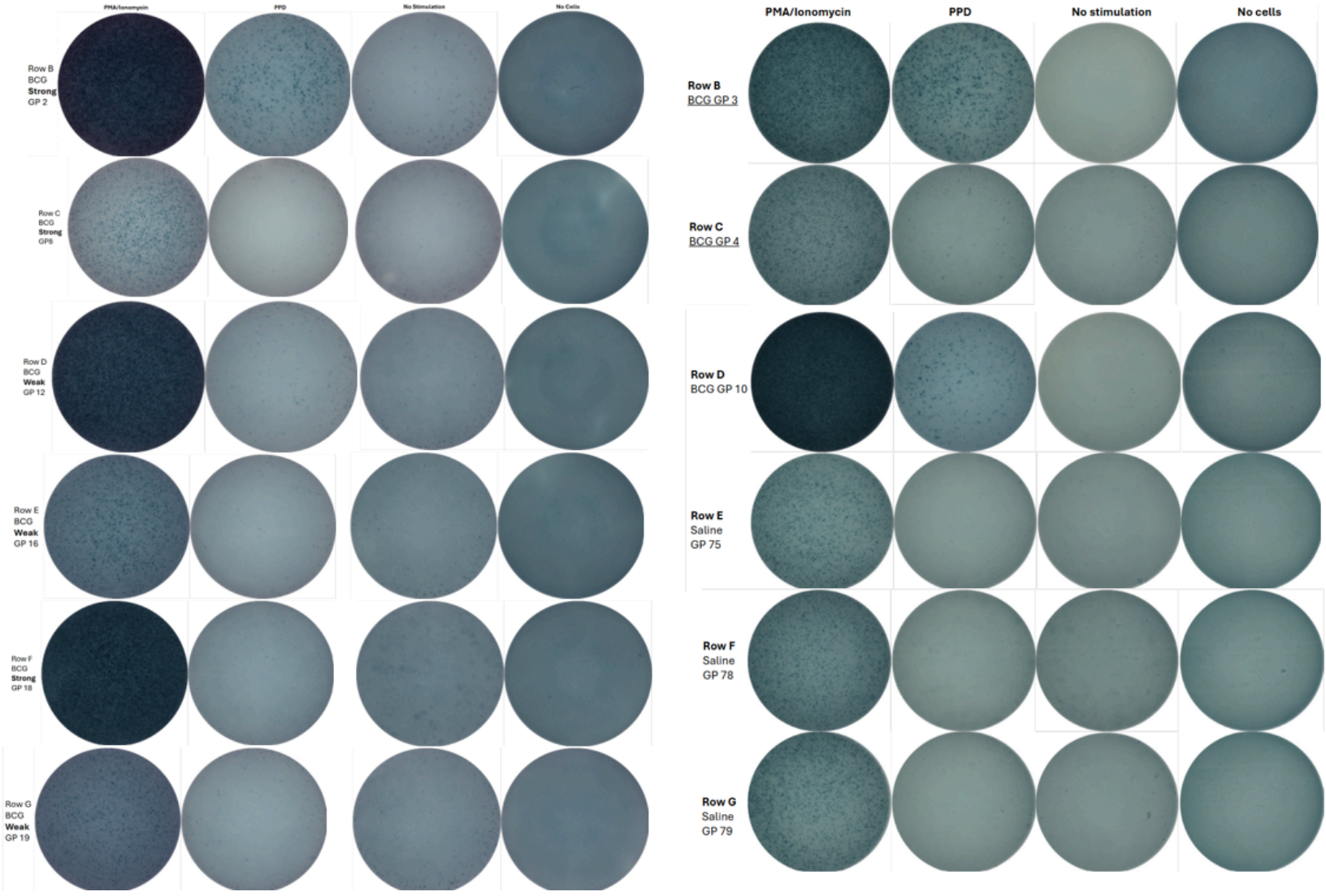

Figure 4. Images of antigen-stimulated IFNγ ELISpot. Data are shown from guinea pigs with a high response at the vaccine site, compared to an unvaccinated guinea pig. Positive control stimulation with PMA and ionomycin generates numerous spots in all guinea pigs. The unvaccinated guinea pig has no response to the PPD antigen, while the response to the PPD antigen is variable among BCG-vaccinated guinea pigs despite a high vaccine site response.

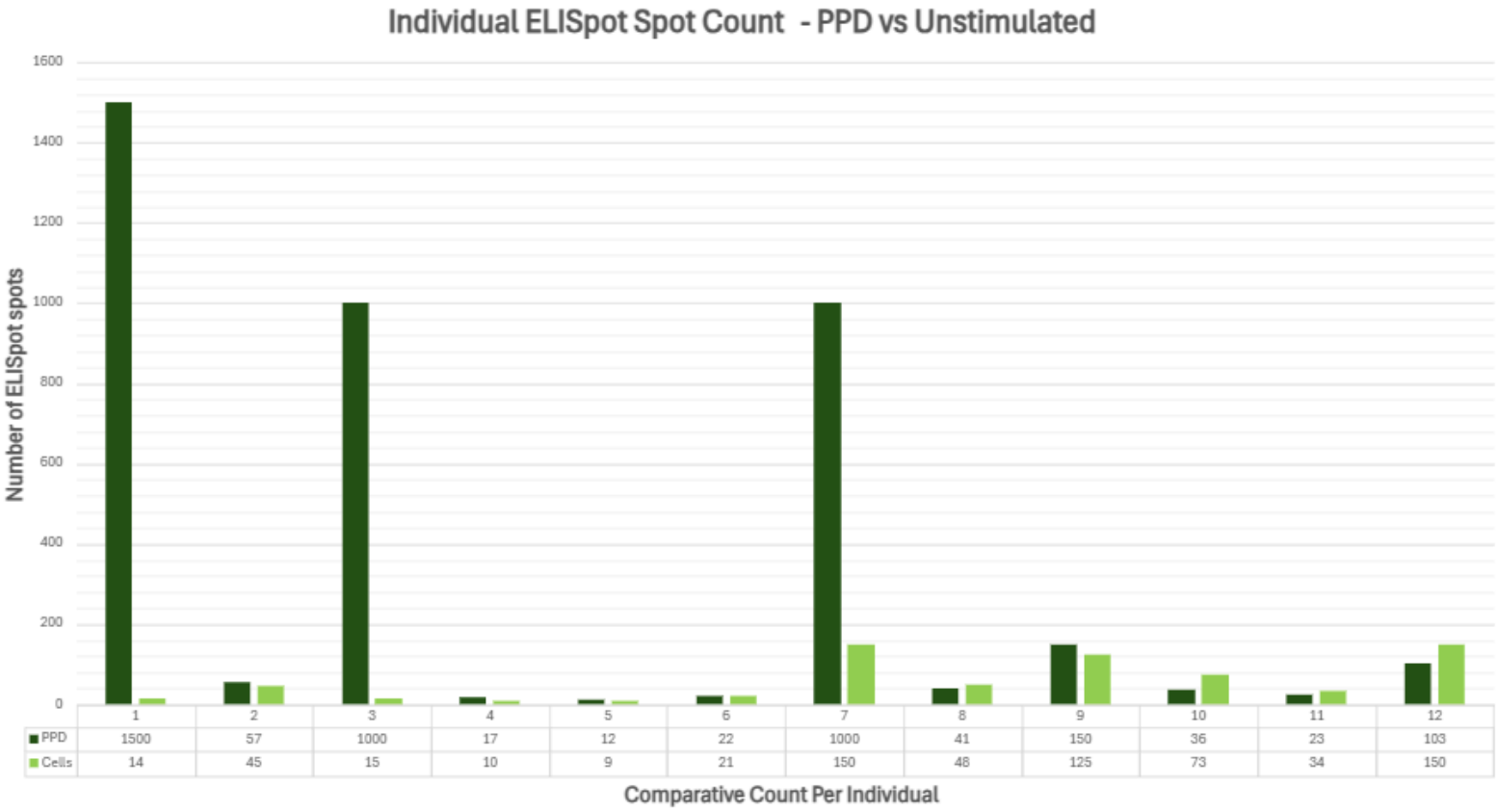

Figure 5. PPD-stimulated vs unstimulated PBMC ELISpot Spot Count. Spot counts represent the frequency of IFN-γ producing cells in response to TB antigen or levels naturally present as background among the cells isolated.

Conclusion

In short, quantifiable inflammatory responses were identified in 93% of individuals at the BCG vaccination site or the TST skin test site. While a majority of individuals showed inflammation at both sites, some individuals showed a response at only one site. A large portion of the individuals that showed inflammation at both sites showed a much stronger inflammatory reaction at one site compared to another, neither of which correlated with how much IFN-γ they produced when stimulated with *M. bovis* PPD on ELISpot.

Out of the 9 BCG-vaccinated individuals' whose IFN-γ levels were measured on ELISpot, there was no correlation between the strength of response at the vaccine site and the amount of cells per experimentally stimulated well producing IFN-γ. While the BCG-vaccinated animals generally showed higher IFN-γ production when experimentally stimulated, the lowest responders produced similar levels of IFN-γ to those mock-vaccinated with saline. These lower performing or unvaccinated individuals also showed very little spot count difference between unstimulated IFN-γ production and stimulated IFN-γ production, indicating these values are not significantly statistically different enough to conclude BCG vaccination reliably confers a higher inflammatory response rate than innate immunity alone.

Following this study, the BCG-vaccinated animals will be exposed to aersolized *Mtb* and monitored to see if disease develops. From this, conclusions can be drawn about the correlates of protection against TB disease from BCG vaccination can be drawn in guinea pigs. By comparing

the inconsistent rate of inflammation from BCG to the disease protection correlates from the vaccine, more knowledge about the variable performance of the vaccine can be found.

Discussion

Throughout these data, inconsistencies such as this make themselves apparent when comparing different inflammatory testing modalities against each other. For example, a lot of individuals that showed highly inflammatory reactions at the TST site showed little IFN-γ produced on ELISpot. One particular individual, who had the largest TST response site inflammation at 460 mm^2, produced just 150 IFN-γ spots per experimentally stimulated well. This number dwarfs in comparison to the 1000 -1500 spots per well produced by the highest recorded individuals, each whose TST response area was less than half of 460 mm^2. All of these individuals mentioned showed a larger width and more consistent inflammation throughout the three weeks of tracking at the vaccination site than the large TST site responder.

Overall, trends showed that while 93% of individuals had an inflammatory response at the vaccination site and the skin test site, there are some individuals that expressed reactionary responses at just one site and not the other. Similarly, a portion of individuals that showed necrosis at the TST site did not show exacerbated inflammatory response (i.e. scarring or scabbing) at the vaccination site.

When examining IFN-γ ELISpot spot counts, we see that the individual that displayed the lowest spot count (out of all the vaccinated individuals measured) when exposed the *M. bovis* antigen PPD was a strong responder at the vaccination site, showing just 23 spots per experimentally stimulated well for a vaccine-site scarring area of 12 mm^2. It is worth noting that this individual's experimentally stimulated well showed less spot forming units compared to the unstimulated control from the same animal, highlighting a lack of systemic antigen-specific response to the antigen despite its notable local inflammatory response.

When comparing animals mock-vaccinated with saline and weak responders at the vaccination site, IFN-γ spot counts appear extremely similar. For both groups' experimentally stimulated wells and unstimulated cell wells, ELISpot counts typically differed by 10-20 spots between both groups. For each individual, the difference in spots between *M. bovis* PPD stimulated and unstimulated wells showed a difference of around 10 spots or less. Multiple individuals also showed the higher IFN-γ production in their unstimulated wells, indicating that these counts are not statistically significant enough to show a cause-and-effect relationship on IFN-γ production from the BCG vaccination.

Because an overall trend between receiving the BCG vaccine and higher rates of inflammation cannot be drawn, we cannot conclude that BCG vaccination produces a uniform response in guinea pigs. Even though certain individuals did show outlying displays of inflammatory markers, the type of response and where it appears is variable on an individual basis.

While the lack of correlation between BCG vaccination and antigen-specific systemic inflammatory responses disproves our hypothesis, this trend (or lack thereof) indicates a similar response in guinea pigs to that of mice and human to that of BCG. As the variable correlate of protection from BCG has historically been noted in these groups, we can conclude that the failure of BCG to reliably produce inflammatory responses in all organs and systems is a fault of the vaccination and not of the model species. Knowing that these guinea pigs are showing a similar response to BCG as humans and other well-studied model species, the value of these animals as a model for future *Mtb* and BCG vaccination-based studies is affirmed.

Citations

Birkhaug, Konrad E. “Protection against Tuberculosis with BCG in Guinea Pigs.” *American Review of Tuberculosis*, vol. 27, no. 1, 1933, pp. 6–31.

Martin, C, et al. “The live mycobacterium tuberculosis phop mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs.” *Vaccine*, vol. 24, no. 17, 24 Apr. 2006, pp. 3408–3419, https://doi.org/10.1016/j.vaccine.2006.03.017.

Martinez, Leonardo, et al. “Infant BCG vaccination and risk of pulmonary and extrapulmonary tuberculosis throughout the life course: A systematic review and individual participant data meta-analysis.” *The Lancet Global Health*, vol. 10, no. 9, Sept. 2022, https://doi.org/10.1016/s2214-109x(22)00283-2.

McMurray, David N. “Guinea pig model of tuberculosis.” *Tuberculosis*, 16 May. 1994, pp. 135–147, https://doi.org/10.1128/9781555818357.ch9.

Ordway, Diane, et al. “The cellular immune response to *mycobacterium tuberculosis* infection in the guinea pig.” *The Journal of Immunology*, vol. 179, no. 4, 15 Aug. 2007, pp. 2532–2541, https://doi.org/10.4049/jimmunol.179.4.2532.

Orme, Ian M., and Diane J. Ordway. “Mouse and Guinea pig models of tuberculosis.” *Microbiology Spectrum*, vol. 4, no. 4, 12 Aug. 2016, https://doi.org/10.1128/microbiolspec.tbtb2-0002-2015.

Suliman, Sara. “BCG: From veins to correlates.” *Cell Host &amp*; Microbe, vol. 31, no. 6, 14 June 2023, pp. 921–923, https://doi.org/10.1016/j.chom.2023.05.021.

Setiabudiawan, Todia P., et al. “Protection against tuberculosis by Bacillus Calmette-Guérin (BCG) vaccination: A historical perspective.” *Med*, vol. 3, no. 1, 14 Jan. 2022, pp. 6–24, https://doi.org/10.1016/j.medj.2021.11.006.

van der Meijden, A.P.M., de Jong, W.H., Steerenberg, P.A. *et al.* Intravesical BCG administration in the guinea pig. *Virchows Archiv B Cell Pathol* 55, 207–215 (1988). https://doi.org/10.1007/BF02896577

Williams, A., et al. “Boosting with poxviruses enhances *Mycobacterium bovis* BCG efficacy against tuberculosis in Guinea pigs.” *Infection and Immunity*, vol. 73, no. 6, 1 June 2005, pp. 3814–3816, https://doi.org/10.1128/iai.73.6.3814-3816.2005.