**Nanoparticle-Fusion protein complexes protect against *Mycobacterium tuberculosis* infection**

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**Short title:** nanoparticle-based TB vaccine

**Abstract**

Tuberculosis (TB) is the leading cause of death from infectious disease and the current vaccine, *Bacillus Calmette-Guerin* (BCG), is inadequate. Nanoparticles (NPs) are an emerging vaccine technology with recent successes in oncology and infectious diseases. NPs have been exploited as antigen delivery systems and also for their adjuvantic properties. However, the mechanisms underlying their immunological activity remain obscure. Here, we developed a novel mucosal TB vaccine (Nano-FP1) based upon yellow carnauba wax NP (YC-NPs), coated with a fusion protein consisting of three *Mycobacterium tuberculosis* (*Mtb*)antigens: Acr, Ag85B and HBHA. Mucosal immunisation of BCG-primed mice with Nano-FP1 significantly enhanced protection in animals challenged with low-dose, aerosolised *Mtb*. Bacterial control by Nano-FP1 was associated with dramatically enhanced cellular immunity compared to BCG, including superior CD4+ and CD8+ T-cell proliferation, tissue resident memory T-cell (Trm) seeding in the lungs, and cytokine polyfunctionality. Alongside these effects, we also observed potent humoral responses such as the generation of Ag85B-specific serum IgG and respiratory IgA. Finally, we found that YC-NPs were able to activate antigen-presenting cells via an unconventional IRF-3-associated activation signature, without the production of potentially harmful inflammatory mediators, providing a mechanistic framework for vaccine efficacy and future development.

**Introduction**

As of 2017, tuberculosis (TB) is the deadliest infectious disease worldwide [1](#_ENREF_1), killing 1.8 million people annually with an associated economic impact in the region of $12 billion [2](#_ENREF_2). The outlook for the control and management of TB is mixed. For the first time in recent years, two new drugs; bedaquiline and delamanid have been licensed [3](#_ENREF_3) and newer, more effective, treatment regimens are coming to the fore [4](#_ENREF_4), [5](#_ENREF_5). Despite these innovations, deficits in TB control programs and the challenge of antibiotic resistance means that there has been little change in the overall incidence of TB in the last decade [6](#_ENREF_6). Indeed, the 2025 WHO global TB targets are unlikely to be met, particularly in heavily affected countries such as China and India. [7](#_ENREF_7).

The current vaccine for TB, BCG, is nearly a century old. While moderately effective in infants and adolescents, the protection it offers is highly variable and it is poorly effective in adults [8](#_ENREF_8). The failure of MVA85A, one of only two vaccines to reach phase 2b clinical trials in recent years, illustrates the challenges remaining in TB vaccine development [9](#_ENREF_9). Generation of an effective TB vaccine represents the only route to achieving long-term control or eradication of the disease.

Nanoparticles (NPs) are a nascent and attractive platform for vaccine development due to their low toxicity, ease of manufacturing and low cost. Many NPs are able to act simultaneously as adjuvant and antigen delivery system [10](#_ENREF_10), thereby conferring benefits absent in traditional antigen-adjuvant approaches. NPs are biochemically heterogeneous and may be composed of various synthetic (e.g. polystyrene) or natural (e.g. chitosan) compounds, with important variations in charge, size and structure. The unique physiochemical composition of each type of NP dictates their immunological properties from affecting antigen uptake and diversion to lymphoid organs, to the induction of autophagy and activation of the inflammasome. Importantly for vaccines against intracellular pathogens, some NPs can facilitate the cross-presentation of antigen thereby eliciting cytotoxic immune responses [11](#_ENREF_11), [12](#_ENREF_12). Given these benefits, NPs have played a significant role in advances within infectious disease vaccine development. Testament to their potential is the recent licensing of a landmark vaccine, Mosquirix, containing nanoparticulate-scale liposomes, which is the first ever licensed malaria vaccine [13](#_ENREF_13), [14](#_ENREF_14).

In the current study, we use NPs produced via the emulsification of yellow carnauba (YC) palm wax with sodium myristate (NaMA). YC wax and its derivatives are classified by the European Food Standards Agency as non-toxic and their use is licensed in a wide range of edible and cosmetic products [15](#_ENREF_15). These NPs have an average diameter of ~400 nm (Ranging from 200 – 800 nm) and are anionic, with a zeta potential of approximately -75mV thus imparting a high colloidal stability on YC-NaMA in suspension [16](#_ENREF_16), [17](#_ENREF_17). YC-NaMA nanoparticles have shown considerable promise as a vaccine antigen delivery system, stimulating potent immune responses when being used to deliver the HIV-gp140 and tuberculosis Ag85B antigens in murine vaccine studies [16](#_ENREF_16), [17](#_ENREF_17). However, the mechanism(s) responsible for these effects remain undefined.

Here, we tested a novel tuberculosis vaccine (‘Nano-FP1’) composed of YC-NaMA coated with a fusion protein (FP1) consisting of the *Mtb* antigens Ag85B (early expression), Acr (latent expression) and HBHA (epithelium-targeting). The latter antigen comprised only the heparin-binding domain and was therefore included not as an immunogen but a vaccine ‘guide’ to epithelial tissue. We show that mucosal immunization with Nano-FP1 significantly enhances protection in BCG-primed, and also BCG-naïve, mice, making it an appealing vaccine for immunocompromised individuals. This protection was associated with a breadth of antigen-specific cellular and humoral immune responses at both systemic and mucosal sites. Importantly, we demonstrate for the first time that YC-NPs induce a specific IRF3-biased activation signature in APCs that results in a phenotypically mature, yet hypo-inflammatory phenotype, corresponding with muted NF-κB activity and prolonged type I interferon (IFN) production. These data show that NPs derived from an abundant natural product can effectively mimic the function of expensive and highly-developed synthetic adjuvants, and that the mucosal vaccine Nano-FP1 can play a major role in preventing TB.

**Results**

**Adsorption of FP1 onto YC-NPs**

Effective carriage of antigen by YC-NaMA is integral to the success of this delivery system. Interaction of protein antigens with YC-NaMA is likely to occur due to both electrostatic and hydrophobic interactions [18](#_ENREF_18), [19](#_ENREF_19) with the availability of hydrophobic or charged moieties on the surface of the nanoparticle and the osmolarity of the adsorption solution both playing a role in the efficiency of adsorption. First, a fusion protein (FP1, Fig.1A) was constructed that expressed three *Mtb* antigens with unique properties: Ag85B, a protective Th1-inducing and early-expressed antigen; Acr, an antigen expressed during latency; and a portion of HBHA, responsible for binding to host epithelium. FP1 was cloned and expressed in *E. coli*, followed by purification to over 97% (Fig. 1B,C). Adsorption of FP1 onto YC-NaMA was required prior to immunisation. Therefore, we tested whether FP1 bound to NPs under experimental conditions. To assess protein loading the size of the NPs was measured pre- and post-adsorption. Moreover, after ultracentrifugation we measured the amount of free FP1 in the supernatant, allowing quantification of the amount of bound protein. After adsorption, there was a significant increase in the mean size of YC-NaMA NPs from 324.4+/-2.4 nm for naked NPs to 353.4+/-3.5 nm after protein loading (paired t-test, *P=*0.03)Additionally, there was a significant increase in the Zeta Potential of the nanoparticles from -85.3+/-0.95 mv to -79.1+/-1.6 mv (*P=*0.003) after FP-1 loading. Although size and Zeta Potential increased after protein loading, the polydispersity index remained low (PDI = 0.26) and the Zeta Potential remained strongly negative indicating that FP-1 loaded NPs retain high colloidial stability. After adsorption, NPs were separated from unbound FP1. Of the starting amount of 100 µg, ~35 µg free FP1 was detected indicating 65+/-4.6% binding of FP1 to YC-NaMA NPs under experimental conditions.

**Nano-FP1 Protects against TB in BCG-primed and BCG-naïve Animals**

Next, FP1-loaded NPs (Nano-FP1) were tested for protective efficacy against two *Mtb* strains: *Mtb* H37Rv in London, and *Mtb* Harlingen in Stockholm (Fig 2). Low-dose infection models are physiologically representative of natural infection, and so we utilized aerosolisation technology to challenge mice with *Mtb*. Mice were immunized via the intranasal or intratracheal (Stockholm only) routes (± subcutaneous BCG priming). After two vaccine boosts, animals were challenged via aerosolized *Mtb* (100 - 250 CFUs).

Immunisation with BCG alone led to a significantly reduced bacterial burden in the lungs in all experiments by an average of 0.7 log (*P*ADJ ≤ 0.005 for all experiments) compared with animals mock-immunised with PBS. BCG immunisation also significantly reduced the spleen burden in 3/4 experiments by 1.2 log on average (*P*ADJ < 0.03for all experiments). Strikingly, in all BCG-primed experiments, immunisation with Nano-FP1 conferred significantly better protection than BCG alone in (Fig 2A and C), reducing lung CFUs by a further 0.6 log on average compared with BCG (*P*ADJ < 0.01 for all experiments). Moreover, protection in the lungs afforded by Nano-FP1 was significantly better than PBS on a BCG-naive background (Fig 2B, *P*ADJ = 0.04), giving comparable levels of protection as BCG. Importantly, we found that Nano-FP was also superior at preventing extra-pulmonary *Mtb* dissemination, as evidenced by significantly reduced *Mtb* CFUs in the spleens compared to BCG alone (*P*ADJ = <0.05). We therefore concluded that our vaccine could enhance protection by parenteral BCG, but also offer similar levels of protection when administered in BCG-naïve mice. Nano-FP1 efficacy was observed in two independent laboratories in two separate geographical locations (London and Stockholm).

**Nano-FP1 Induces Systemic and Mucosal Antibodies against *Mtb* Antigens**

Given the ability of Nano-FP1 to enhance bacterial control, we hypothesized that the vaccine was broadly enhancing immunological function, and first assessed whether humoral responses were being bolstered. An elegant study by Lu *et al.* (2016) has recently underscored the importance of antibody in controlling TB [20](#_ENREF_20). We therefore measured antibody titres to the FP1 constituent antigens (Ag85B and Acr) in the blood and airway surface liquid of immunised animals as a measure of recognition of the Nano-FP1 vaccine by the immune system. Levels of specific IgG in the sera and IgA in bronchoalveolar lavage (BAL) were assessed by ELISA and compared between the Nano-FP1, BCG and PBS mock-immunised animal groups. Immunisation with Nano-FP1 generated robust IgG and IgA responses to Ag85B in the sera and BAL (Fig. 3) respectively, whereas ACR-specific responses were relatively lower, particularly in the BAL. The variable humoral responses indicate differential recognition of these FP1 component antigens, but an overall boost to antigen-specific humoral immunity by Nano-FP1 when taken together. Interestingly, BCG alone induced minimal Ag85B/Acr-specific IgG or IgA responses.

**Enhanced Memory T-cell Proliferation induced by Nano-FP1**

Antibody class switching in naïve B cells depends on T-dependent effector cytokines such as IFN-γ and IL-4. Since high IgG/IgA titres were observed after Nano-FP1 immunisation, we speculated that there would be a co-occurring memory T-cell response of similar magnitude. To measure memory T-cell proliferation, splenocytes were pulsed with recall antigen and the cell cycle marker Ki67 was measured (Fig. 4). As expected, no T-cell proliferation was observed in the PBS control group upon exposure to recall antigen, with the exception of a small percentage of CD8+ T cells (~7.5%) responding to Acr (possibly due to intrinsic immunomodulatory effects of Acr on APCs, as previously documented [21](#_ENREF_21)). In the BCG vaccine group, there were modest responses to Acr in both the CD4 and CD8 compartments (<10% and <5% Ki67+, respectively) and no detectable responses to Ag85B, in keeping with studies showing that BCG induces sub-optimal antigen presentation *in vivo* [22](#_ENREF_22). By contrast, large percentages of Ki67+ CD4+ (>15%) and Ki67+ CD8+ (>30%) T cells were detected in splenocytes from the Nano-FP1 vaccine group after stimulation with Ag85B or FP1, with values being markedly higher than those from BCG-vaccinated animals. Consistent with the levels of Acr-specific antibodies, there were muted but notable proliferative responses to Acr in the Nano-FP1 group. These data indicate that mucosal booster immunisation with Nano-FP1 may lead to increased frequency of antigen-specific memory T cells compared to BCG alone.

**Enhanced T-cell Cytokine Production by Nano-FP1**

The enhanced T-cell proliferative responses induced by Nano-FP1 led us to question what specific T-cell phenotype was being induced by immunisation. To address this, splenocytes from immunised animals were pulsed with recall antigens (Ag85B, Acr or FP1), and levels of IFN-γ (Th1), IL-4 (Th2), IL-10 (Treg) and IL-17 (Th17) were quantified by multiplex flow cytometry. There were muted levels of all four cytokines in the BCG group in response to all recall antigens (Fig. 5), which was consistent with the low levels of T-cell proliferation previously detected. However, high levels of IFN-γ (>7000 pg/mL), IL-10 (>2000 pg/mL) and IL-17A (>3000 pg/mL) were noted in the Nano-FP1 group in response to Ag85B and FP1, with Acr inducing more modest levels of these cytokines, although still more than BCG. These data suggested that Nano-FP1 was inducing a mixed Th1-Th17-Treg response *in vivo*. Both Th1 and Th17 cells have pivotal roles in protection against TB and IL-10 is crucial for the survival of CD8+ memory T cells, suggesting a causal link between elevated IL-10 (Fig. 5) and abundant CD8+ T-cell proliferation in response to Ag85B.

**Nano-FP1 Boosts Ag85B-specific CD4+ T-cell Polyfunctionality**

Polyfunctional T cells are able to produce several effector cytokines concomitantly, and their presence during natural infection and immunisation has been linked to protection against TB [23](#_ENREF_23), [24](#_ENREF_24). We hypothesized that Nano-FP1 was inducing a greater degree of T-cell polyfunctionality that could potentiate control of *Mtb* compared with BCG alone. Splenocytes from immunized mice were therefore stimulated with the recall antigens Ag85B and Acr, and CD4+ and CD8+ T cells were interrogated for the presence of four effector cytokines: IFN-γ, IL-2, IL-17A and TNF-α. As can be seen in Fig. 6A, mice immunized with Nano-FP1 had 0.47% polyfunctional (i.e. 3+ cytokine) CD4+ T cells in response to Ag85B compared to only 0.03% in the BCG group (*P* = <0.05). In the CD8 compartment, there was a similar trend, with the Nano-FP1 group containing 0.08% polyfunctional T cells responsive to Ag85B, whereas there were only 0.03% in the BCG group. The responses to Acr, in line with previous observations, were more muted across all groups. Next, we probed specific Ag85B-dependent polyfunctionality in order to determine if there were any distinct cytokine patterns between vaccine groups (Fig. 6B). It was noted that Nano-FP1 strongly induced two subsets in CD4+ cells: IFN-γ+IL-2+TNF-α+ (*P* = <0.05, Nano-FP1 vs BCG) and IFN-γ+TNF-α+, the former possibly representing actively proliferating (IL-2+) cells belonging to the latter subset. In CD8+ cells, there was a similar pattern, however none of the groups reached statistical significance. Evidence suggests that IFN-γ+ IL-2+ TNF-α+ CD4+ T cells are a particularly protective subset in TB, exhibiting high proliferative capacity and resistance to exhaustion [25](#_ENREF_25).

**Pulmonary Seeding of Tissue Resident Memory T-Cells after Nano-FP1 Immunisation**

Tissue resident memory T cells (Trm) are lymphoid cells that do not circulate in the blood or lymphatic system. It has been reported that pulmonary Trm are efficiently induced by mucosal immunization with BCG or viral vectors expressing *Mtb* antigens, and that these Trm offer rapid protection against *Mtb* infection [26](#_ENREF_26), [27](#_ENREF_27). Thus we hypothesized that Nano-FP1, as a mucosal vaccine, was inducing this cell type. To test this, lungs from immunized mice were harvested and assessed for memory T cells (CD44highCD62Llow) expressing the Trm markers CD69 and CD103. To exclude for the possibility of these T cells being directed against the NP itself, YC-NaMA alone was also administered as a control. As shown in Fig. 7, parenteral BCG alone was unable to induce an appreciable level of either CD4+ or CD8+ Trm, in line with other studies. Similarly, YC-NaMA had no effect on levels of CD69+CD103+ cells. FP1 alone was able to increase CD4+ Trm to 5.58% and CD8+ Trm to 5.63%, likely reflecting the high avidity of Ag85B for cognate T-cell receptors (TCRs) even in the absence of adjuvant. Most strikingly, the complete Nano-FP1 vaccine was able to induce 12.6% CD4+ Trm and 17.2% CD8+ Trm. Although the TCR specificity of these cells remains to be elucidated, these are likely to be directed to FP1, given the fact that YC-NaMA is non-proteinaceous and lacks intrinsic ability to induce Trm. Taken together, these data firmly suggest that Nano-FP1 can induce mucosal, tissue resident memory T cells alongside lymphoid organ counterparts.

**YC-NaMA Nanoparticles Activate Antigen-Presenting Cells via IRF-3**

The evidence thus far firmly established the ability of Nano-FP1 to enhance the bacterial control and immunogenicity afforded by the BCG vaccine, bolstering immunological parameters classically associated with protection against TB. However, despite the use of YC-NaMA in several experimental vaccines, its mode of action has hitherto remained unknown. Professional APCs such as macrophages, dendritic cells (DCs) and B cells are known to orchestrate the adaptive immune system, and we theorised that the NP component of Nano-FP could be acting as an adjuvant and inducing APC maturation, thus providing a mechanism for enhanced B- and T-cell immunogenicity, and ultimately, bacterial control. To test this, APCs were pulsed with YC-NaMA and a comprehensive panel of activation markers was measured. We found that YC-NaMA was able to induce up-regulation of numerous surface markers associated with APC activation (Fig. 8A), including CCR7 (*P* = <0.05) and PD-L2 (*P* = <0.05). There was also a strong trend for up-regulation of MHC Class I, MHC Class II, and modest up-regulation of CD86 and PD-L1. Intriguingly, under the same conditions, YC-NaMA was unable to induce any detectable IL-1β or IL-6, with only weak TNF-α production compared to the positive control LPS (Fig. 8B).

Since TLR-dependent inflammatory cytokine production is typically driven by MyD88-dependent NF-κB activation, whereas maturation markers are more dependent on TRIF-dependent IRF-3 activation causing an autocrine type I IFN loop [28](#_ENREF_28), [29](#_ENREF_29), we inferred that YC-NaMA was inducing an IRF-3-biased activation profile in APCs. Using a macrophage NF-κB reporter cell line, we found that while LPS induced strong NF-κB-dependent gene transcription (*P* = <0.0001, 24 hours & 48 hours, LPS vs unstimulated cells), there was no significant induction of this pathway by YC-NaMA (Fig. 8C), explaining the lack of inflammatory cytokines. However when IRF-3 was tested for activation by phosphorylation on serine 396, YC-NaMA strikingly induced almost double the level of phosphorylation observed in the positive control (LPS MFI: 2821, YC-NaMA MFI: 5533) (Fig. 8D). Type I IFN production explicitly requires IRF-3/IRF-7 activation, and accordingly we observed sustained and potent *Ifna* and *Ifnb* gene transcription (~100-fold above *Actb*) due to YC-NaMA stimulation at 24 hours post-stimulation, at which point even LPS was not inducing either of these genes. To confirm our model of APC activation by YC-NaMA, we measured transcriptional levels of the chemokines RANTES (*Ccl5*) and IP-10 (*Cxcl10*) (Fig 8F). RANTES requires dual IRF-3/NF-κB cooperation within its promoter [30](#_ENREF_30) , whereas IP-10 is a classic interferon-stimulated gene induced by autocrine IFN-β [31](#_ENREF_31). In accordance with our hypothesis, YC-NaMA induced significantly more IP-10 transcripts than unstimulated cells (*p* < 0.01), but diminished RANTES transcripts compared to LPS (*p* < 0.001). Collectively, these findings strongly support a novel mechanism of activity in which YC-NaMA is not immunologically inert, but activates IRF-3 to drive maturation marker expression without the accompanying inflammation associated with NF-κB signaling, leading to enhanced cellular and humoral immunity.

**Discussion**

The use of BCG as a worldwide TB vaccine is unlikely to be discontinued. While it offers only limited protection against *Mtb*, it has the significant advantage of providing cross-protection against non-mycobacterial pathogens. BCG can enhance responses to pathogens such as *C. albicans, S. aureus* and *S. pneumoniae* via so-called ‘trained immunity’ involving epigenetic priming of myeloid cells [32](#_ENREF_32); accordingly, clinical trials have highlighted the role of BCG in lowering infant mortality independently of effects on TB [33](#_ENREF_33). Given this situation, a novel TB vaccine would likely be used as a ‘booster’ to pre-existing immunity offered by BCG.

In this study, we developed a novel fusion protein (FP1) and complexed this with YC-NaMA NPs: a cheap, abundant and safe source of NPs. The resultant vaccine, Nano-FP1, was tested *in vivo* via mucosal immunisation and showed enhanced protection against the H37Rv and Harlingen *Mtb* strains in mice that were BCG-primed. This increase in bacterial control was found in both the lungs and the spleen. Protection was associated with multi-system immunological activity. Remarkably, however, we also observed protection in BCG-naïve mice. TB is the biggest killer of HIV-infected individuals globally, yet the use of BCG in this population or in otherwise-immunocompromised individuals carries the risk of vaccine-induced disease, and hence there are safety concerns associated with this practice [34](#_ENREF_34). Since Nano-FP1 is a subunit vaccine containing no live organisms, it has potential applications within this, and other immunocompromised, at-risk groups.

T cells are indispensable for protection against *Mtb*. Mice lacking CD4+ T cells succumb to *Mtb*-mediated lethality [35](#_ENREF_35), and CD8+ T cells may provide a rapid response to pulmonary infection by directing cytotoxicity against infected macrophages [36](#_ENREF_36). Our experiments showed that in the Nano-FP1 booster group, exposure to recall antigen triggered a higher proliferative response (Ki67+) in both CD4+ and CD8+ T cells compared to mice receiving BCG alone. This implies a higher number of central antigen-specific T cells in these animals, or T cells with overall higher proliferative capacity. Since failure of the host to eradicate *Mtb* is linked with T-cell exhaustion and terminal differentiation [37](#_ENREF_37), [38](#_ENREF_38), these data hold promise for Nano-FP1 as an effective vaccine.

Th1 and Th17 cells in particular have been implicated in protection against TB, and we observed elevated levels of both signature cytokines (IFN-γ and IL-17) in the spleens of Nano-FP1 immunised mice, compared to BCG immunisation alone. Besides its conventional role in macrophage priming, IFN-γ can be advantageous during TB disease by acting in a regulatory role in antagonizing tissue-destructive neutrophils [39](#_ENREF_39). Interestingly, we also noted elevated levels of IL-10, a cytokine classically associated with Treg and Tr1 subsets. While typically regarded as detrimental in TB, recent evidence from cellular analysis of granulomas in *Mtb*-infected cynomolgus macaques has shown that granulomas containing a mixture of both Th1/Th17 effector cells and IL-10+ regulatory T cells are strongly associated with sterilization[40](#_ENREF_40), and Th1 cells that co-produce IL-10 are in fact more effective at licensing macrophages to kill intracellular pathogens [41](#_ENREF_41). Levels of the cardinal Th2 cytokine, IL-4, were also elevated in recall groups, however these levels are so negligible as to be physiologically irrelevant. By inducing cytokines associated with diverse helper T-cell subsets, Nano-FP1 could be inducing a desirable and protective cellular profile while limiting collateral immune-mediated tissue destruction.

Alongside enhanced proliferative responses, we also found that mucosal delivery of Nano-FP1 led to the accumulation of lung parenchymal CD69+CD103+ memory T cells. Trm cells are an intriguing effect for any TB vaccine, since they offer the possibility of swift and specific control of *Mtb* before it can establish a microenvironment favourable to its own survival. The delay in T-cell-mediated immunity during the early stages of infection has been described as a ‘bottleneck’ [42](#_ENREF_42) in which T cells must egress from the lymph node to the lung tissue [43](#_ENREF_43), and Trm cells are a potential solution to this problem. Investigating the TCR repertoire of Nano-FP1-induced Trm cells could further elucidate mechanisms of vaccine efficacy.

The role of T-cell polyfunctionality in TB is controversial. While some studies have shown a role for these cells in protection, other studies have shown that T cells expressing multiple cytokines correlate with disease as opposed to protection. However, there is evidence that T-cells expressing multiple cytokines are adequately primed, since detrimental conditions such as myeloid PD-L1 expression [44](#_ENREF_44), inadequate antigen dose [45](#_ENREF_45) and metabolic impairment [46](#_ENREF_46) can inhibit their generation. Therefore, in the context of vaccination at least, polyfunctional T cells are likely to be an indicator of optimal T-cell priming. It has been suggested that the classical Th1 cytokines IFN-γ and TNF-α have distinct roles in the control of pulmonary *Mtb* and its extra-pulmonary dissemination, respectively [47](#_ENREF_47). Since we observed enhanced levels of both cytokines co-expressed in polyfunctional CD4+ T cells (IFN-γ+IL-2+TNF-α+) in the Nano-FP1 group, this provides a possible explanation for enhanced protection in both lungs and spleen.

Nano-FP1 was able to enhance anti-Ag85B and anti-Acr IgG in the serum and IgA in the mucosa. The latter is indicative of engagement with the mucosal immune system, and therefore confirms the success of our immunization route. Whether these humoral responses are protective, or merely indicative of strong immune engagement, remains unclear. Within a polyclonal antibody response there is likely to be heterogeneity with regards to protective capability. Though considered an intracellular pathogen, *Mtb* can be found extracellularly during infection, thus raising the possibility of antibody involvement in immunity. Further work is necessary to elucidate the role of antibody, and particularly glycosylation of the Fc region, in protection against disease.

Interestingly, YC-NaMA was found to be a robust activator of the IRF-3-IFN signaling axis. The autocrine IFN loop in DCs has been noted to underpin the activity of chitosan as a particulate adjuvant [48](#_ENREF_48). In this sense, the biological activity of YC-NaMA also strongly resembles that of the adjuvant MPLA. MPLA activates TLR-4 with a TRIF-IRF-3 bias [49](#_ENREF_49), meaning that it can induce T-cell responses without potentially pathological inflammation. MPLA required three decades of research and development to progress to use in humans [50](#_ENREF_50), and we show here that similar effects can be achieved with a natural product from yellow carnauba palm trees. We propose that the unconventional activation of APCs by YC-NaMA leads to vigorous T- and B-cell responses, while maintaining the safety profile of the particle. In uncovering the biological activity of YC-NaMA, there is case of ‘immunological irony’, in that *Mtb* exploits the IRF-3-IFN cascade to suppress cytokine production during natural infection [51](#_ENREF_51), and here we have used this as a tool to improve protection against the pathogen. Further work is needed to clarify the cellular targets of YC-NaMA NPs and investigate how its immunomodulatory functions can be modified to further enhance protection against TB.

The TB vaccine pipeline is relatively well-stocked at the pre-clinical and early developmental stages but suffers from a high vaccine failure rate leaving limited progression of vaccines to phase 3 clinical trials. Traditional vaccine approaches such as live attenuated or protein-adjuvant vaccines are still in development. However, recent years have seen a rise in the development of vaccines utilizing new technologies such as ‘improved BCG’ vaccines, double attenuated MTB vaccines and virally vectored vaccine delivery platforms. The former two aim to target a ‘sweet spot’ between immunogenicity and virulence. The feasibility of this approach is debatable and raises certain questions, particularly with regards to safety and preventing the return of virulence. These vaccines, unlike Nano-FP1, would not be safe to use in immunocompromised patients nor would they be safe to use via the mucosal route thus highlighting a benefit of our approach. Likewise, immunity generated by virally vectored vaccines can be often be inhibited by antiviral response. The pairing of the traditional (protein + adjuvant) with new technology (nanoparticles) enabled us to design a vaccine which avoids the limitations found in some other approaches to TB vaccine development.

In summary, we show that Nano-FP1 is an effective mucosal TB vaccine that can induce a wide array of immunological benefits, but most importantly, can offer improved protection against *Mtb*, when compared with the current vaccine BCG. The data herein illustrate the advantages of using a YC-NaMA based nanoparticulate antigen delivery system and highlight the potential for repurposing these NPs towards other vaccines and therapeutics. Future work will involve exploiting the immunological mechanism(s) responsible for the activity of Nano-FP1 and optimising the route of delivery to augment Nano-FP1 vaccine efficacy.

**Materials and Methods**

*Ethics*

All mice used in this study were used according to the UK national legislation (Animals in Scientific Procedures Act, 1986) and the Swedish Board of Agriculture and Swedish Animal Protection agency requirements - djurskyddslagen 1988:534; djurskyddsfo¨rordningen 1988:539; djurskyddsmyndigheten DFS 2004:4), as well as the local guidelines (St George’s University, London and Karolinska Institute, Stockholm).

*Vaccines*

YC-NaMA nanoparticles from Particle Sciences (Pensylvania, USA) were supplied as a 10% w/v stock solution in water. Fusion protein 1 (FP1) was provided by Lionex GmbH (Braunschweig, Germany) and was composed of an N-terminal histidine tag, Acr (Rv2031c), Ag85B (Rv1886c) and the heparin binding site of HBHA (Rv0475). FP1 was expressed in *E. coli* and purified via nickel ion chromatography. Purified FP1 was characterised by SDS-PAGE and probed for presence of constituent antigens using Western Blotting and anti-Ag85B/Acr antibody. Endotoxin content of purified FP1 was <7 I.U./mg. Vaccines were formulated for 1 hour at room temperature prior to addition of poly I:C (Sigma) immediately before immunisation. Experimental animals received 10 μg FP1 in 0.1% w/v YC-NaMA and 20 μg poly(I:C) (Sigma) per immunisation. Poly(I:C) was chosen as an adjuvant for its ability to stimulate Tc1 and Th1 responses [52](#_ENREF_52).

*Bacteria*

*M. bovis* BCG (strain Pasteur) and *Mtb* strains H37Rv and Harlingen were grown in 7H11 broth (Becton Dickinson) supplemented with OADC (Becton Dickinson) and enumerated on Mycobacteria Selectatab™ (Kirchner) selective 7H11 agar plates according to standard microbiological techniques. Animals were challenged via the aerosol route with ~100 CFU *Mtb* H37Rv per animal using a 3-jet Collison nebulizer (BGI) controlled by an AeroMP (Biaera Technologies), at St George’s, London, or with ~250 CFU using the In-Tox Products LLC device (small Animal Exposure System), at Karolinska Institute, Stockholm.

*Nanoparticles*

YC-NaMA (Particle Sciences, USA) are lipidic nanoparticles produced as previously described [17](#_ENREF_17) by emulsifying the wax of the yellow carnauba palm with sodium myristate. Particle size and charge was determined using a NanoZS Zetasizer (Malvern, UK). A minimum of three readings were taken and average NP size and Zeta Potential pre-, and post-protein adsorption was then calculated for quantification of protein adsorption. NPs and FP1 were incubated for 1 hour at room temperature and the mixture was then centrifuged at 100,000 x g for 30 mins after which free FP1 in supernatant was quantified by ELISA using a known concentration of FP-1 as a standard.

*Mice, Immunisations and Sample Collection*

Eight week-old C57BL/6J females were purchased from Charles River (Cambridge, UK). BCG-primed animals were immunized with 100 µL 2 x 105 CFU of BCG ten weeks prior to vaccine boosting. For immunisations, mice were anesthetised with isoflurane followed by delivery of either 40 µL PBS or 40 µL Nano-FP1 vaccine via the intranasal or intratracheal routes. Animals received two vaccine boosts three weeks apart and were then challenged via the aerosol route with *Mtb* three weeks after the final boost. Three animals from each experimental group were culled immediately prior to challenge and blood, spleens and bronchoalveolar lavage collected for immunogenicity testing. Remaining animals were sacrificed 3 weeks after challenge for organ CFU enumeration.

*APC Activation*

Macrophages (J774.1 cell line) were cultured in complete DMEM media (10% FCS, 10 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol – all from Sigma). For surface maturation, cells were stimulated for 48 hours with either lipopolysaccharide (LPS; 100 ng/mL, Sigma) or YC-NaMA (0.1, 1 or 10 μg/mL) and stained with Fixable Viability Dye eFluor 780 (eBioscience) and Fc receptors were blocked with TruStain fcX anti-mouse CD16/32 (Biolegend). Cells were then stained in flow cytometry buffer (PBS with 0.5% BSA and 0.1% sodium azide) with the following antibodies for 30 mins at 4°C: CCR7-PerCP/Cy5.5, CD80-APC, CD86-PE/Cy7, H-2-FITC, I-A/I-E-Brilliant Violet 510, PD-L1-Brilliant Violet 421 and PD-L2-PE (All BioLegend), followed by acquisition on a BD FACSCanto II and analysis on FlowJo v10 software. Compensation was performed by UltraComp Compensation Beads (eBioscience). Culture supernatants were tested for cytokines by ELISA in accordance with the manufacturer’s instructions (eBioscience Ready-Set-Go ELISA). Phosphorylation of IRF-3 was tested on cells fixed with 90% methanol as previously described [53](#_ENREF_53). For reporter assays, an NF-κB alkaline phosphatase reporter cell line (Invivogen) was used according to the manufacturer’s instructions.

*ELISAs*

Serum IgG and mucosal IgA against Ag85B and Acr were measured by ELISA. Purified Ag85B and Acr from Lionex GmbH (Braunschweig, Germany) were used for ELISA plate coating (2 μg/mL). After blocking (PBS 1% BSA), mouse sera or lavage were diluted in PBS 1% BSA, 0.05% Tween20 and added in ten-fold dilution series to a final dilution of 1:10,000 (Sera) or five-fold dilution series to 1:2500 (BAL). Specific antibody was detected (1 hour at 37°C) with peroxidase-conjugated anti-mouse IgG/A (Sigma) and OPD substrate (Sigma). Samples in triplicate were read at 482 nm on a plate reader (Tecan200). Relative antibody titers were defined as the reciprocal of the dilution at the cutoff value, with cutoff values determined using the Frey method [54](#_ENREF_54). Levels of FP1 were assessed as above, where FP1 was used to coat plates (varying concentrations) and peroxidase-conjugated anti-His tag antibody (Sigma) was used to detect FP1 binding to the plate. The amount of FP1 present was calculated using a standard curve consisting of known concentrations of FP1.

*T-cell Proliferation and Cytokines*

Splenocytes were isolated from spleens homogenised by mechanical disruption through a 70 μm strainer (BD). Erythrocytes were lysed by brief incubation with ACK Lysis Buffer (Invitrogen). Cells were seeded in technical duplicates in complete RPMI (10% FCS, 10 U/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol) at 1.5 x 106 cells/well and stimulated with 5 µg/mL recall antigen or 1 µg/mL α-CD3 antibody (Biolegend) for 6 days before supernatant recovery for cytokine quantification. For Ki67 staining, cells were stained with viability dye under Fc receptor blockade. Cells were stained with the following antibodies at optimised dilutions: CD4-PerCP/Cy5.5, CD8a-Brilliant Violet 510 and CD90.2-Brilliant Violet 421 — all from Biolegend) for 30 mins at 4°C. Cells were then fixed and permeabilised for 30 min at 4°C using the Foxp3 / Transcription Factor Staining Buffer Set (eBioscience), followed by intracellular staining (45 min, 4°C) using Ki67-APC (Biolegend). Cells were washed extensively and then acquired immediately on a BD FACSCanto II instrument. Compensation was done by beads. Analysis was performed using FlowJo V10. Fluorescence-minus-one (FMO) controls were used to determine gating boundaries, and duplicate values were averaged within experiments, followed by background subtraction from an unstimulated control sample. Cells were gated by size (FSC/SSC), singularity (area/width), viability (eFluor 780low), CD90.2, CD4, CD8 and Ki67 expression. Levels of IFN-γ, IL-4, IL-10 and IL-17 in culture supernatants were measured using the mouse LegendPlexTM kit (Biolegend) according to the manufacturer’s instructions. Samples were acquired on a BD FACSCalibur, and data analysed using the proprietary data analysis software (Biolegend)

*T-cell Polyfunctionality*

T-cell polyfunctionality was assessed as previously reported [55](#_ENREF_55), with minor modifications. Briefly, 1.5 x 106 splenocytes were seeded in complete RPMI in duplicate and stimulated with 5 μg/mL recall antigen in the presence of 10 μg/mL brefeldin A (Sigma). Some cells were additionally stimulated with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL, Sigma) and ionomycin sulfate (20 μg/mL, Sigma). After 4 hours, cells were stained with viability dye under Fc receptor blockade as described above. Cells were fixed with BD Cytofix for 30 min at 4°C, followed by permeablisation in flow cytometry buffer containing 0.5% saponin (Sigma). Cells were incubated with an antibody cocktail (CD3-FITC, CD4-PerCP/Cy5.5, CD8α-Brilliant Violet 510, IFN-γ-Brilliant Violet 421, IL-2-PE, IL-17A-PE/Cy7, and TNF-α-APC — all from Biolegend) for 45 min at 4°C. Cells were extensively washed and then immediately acquired on a BD FACSCanto II. Compensation was performed as described above.

For analysis, FlowJo Software v10 was used and cells were gated by size (FSC/SSC), singularity (area/width), viability (eFluor 780low) and then T-cell lineage (CD3+). Boolean gating was then used to determine individual cytokine combinations, using FMO and biological (PMA/ionomycin) staining controls to determine gating boundaries. Data were analysed in Microsoft Excel by averaging technical duplicates, subtracting background cytokine production in unstimulated cells, and then applying a positivity threshold of 0.01% as recommended by National Institutes of Health (NIH) guidelines [56](#_ENREF_56). A gating strategy and representative data are shown in Supplementary Figure 1.

*RNA Isolation and RT-qPCR*

Macrophages (1 x 106 per condition) were stimulated for 24 hours and then lysed in RNeasy RLT lysis buffer (Qiagen). mRNA was extracted by using the RNeasy Mini RNA Isolation kit (Qiagen) according to the manufacturer’s instructions. The mRNA concentration was then measured by NanoDrop (ThermoFisher). cDNA was synthesised using Superscript II (Thermofisher) and dNTPs (New England Bioscience) according to the manufacturer’s instructions. qPCR was performed by a SYBRGreen (Qiagen) ΔΔ-CT method [57](#_ENREF_57) relative to the housekeeping gene Actb. The following primers were used: Ifna (F): 5’-ATGGCTAGGCTCTGTGCTTTCCT-3’; Ifna (R): 5’-AGGGCTCTCCAGACTTCTGCTCTG-3’; Ifnb (F): 5’-ATGGTGGTCCGAGCAGAGAT-3’; Ifnb (R): 5’-CCACCACTCATTCTGAGGCA-3’; Ccl5 (F) 5’-AGATCTCTGCAGCTGCCCTCA-3’ (R) 5’-GGAGCACTTGCTGCTGGTGTAG-3’;

Cxcl10 (F) 5’-GCCGTCATTTTCTGCCTCA-3’ (R) 5’-CGTCCTTGCGAGAGGGATC-3’; Actb (F): 5’- CCCTAAGGCCAACCGTGAAA-3’; Actb (R): 5’-GTCTCCGGAGTCCATCACAA-3’.

*Lung T-cell Analysis*

Lavaged lungs were perfused by extensively flushing the right cardiac ventricle with PBS. Excised tissue was then cut into ~1 mm pieces, followed by incubation for 45 min in RPMI containing 1 mg/mL collagenase and 0.5 mg/mL DNAse I (Roche). Cells were passed through a 40 μm strainer (BD) and stained with viability dye under Fc receptor blockade, followed by staining with CD3-APC, CD4-PerCP/Cy5.5, CD8α-Brilliant Violet 510, CD44-FITC, CD62L-PE, CD69-PE/Cy7 and CD103-Brilliant Violet 421. Cells were gated as for proliferation, but also on the CD44highCD62Llow population.

*Statistical Analysis*

All statistical tests used are stated in figure legends. Graphpad Prism 7 software was used for analysis of results.

**Figure Legends**

**Figure 1. FP1 composition and loading onto nanoparticles.** The Nano-FP1 vaccine consists of a fusion protein containing the *Mtb* antigens Acr, Ag85B and HBHA adsorbed onto the surface of YC-NaMA nanoparticles (A). Purified FP1 was ~ 55 kDa as measured by SDS-PAGE and Coomassie staining (B). Western blotting with specific antibody demonstrated the presence of the constituent antigens, Acr and Ag85B, within FP1 (C).

**Figure 2. Immunisation with Nano-FP1 equals or enhances BCG-derived protection in naïve or primed animals respectively.** Protective efficacy of Nano-FP1 was measured in the low-dose aerosol Mtb challenge model. BCG priming (when done) was 10 weeks with all other experimental intervals 3 weeks apart. Three experiments in total were performed in London (2 x BCG primed background (A), 1 x unprimed (B)) and a further experiment on a BCG-primed background in Stockholm. Immunisation with BCG significantly reduced CFUs in lungs of challenged animals when compared with PBS group in all experiments (A – C, PADJ <0.005). Immunisation with Nano-FP1 afforded greater protection against *Mtb* H37Rv or Harlingen compared to immunisation with BCG alone in BCG-primed (A & C, PADJ <0.01) or unprimed (B, PADJ 0.04) animals. Each data point is representative of 1 animal. Lines represent mean +/- SEM. Tukey’s multiple comparison test was used for statistical analysis.

**Figure 3. Nano-FP1 generates antigen specific antibody responses in blood and mucus of immunised mice.** ELISA analysis of anti-Ag85B and –AcR IgG responses in sera or IgA in BAL of unimmunised animals, or those immunised with BCG or Nano-FP1. Immunisation with Nano-FP1 resulted in the production of high titres of fusion-protein antigen-specific IgG in the Sera or of anti-Ag85B IgA in the lung mucosa. Negligible antibody responses were observed in naïve mice or those immunised with BCG alone. Data represent means + SEM of three animals per group and are representative of three separate experiments.

**Figure 4. Splenocytes from mice immunised with Nano-FP1 are highly proliferative.** Spleens from immunised or control mice were stimulated with recall antigen for 6 days and T-cell proliferation was quantified by expression of Ki67, depending on expression of CD4 (left) or CD8 (right). Greater CD4 and CD8 T cell proliferation was observed in cells from the Nano-FP1 vaccine group in response to Ag85B of FP1 stimulation. Data represent mean + SEM of pooled values from 3 animals and are representative of three separate experiments.

**Figure 5. Splenocytes from mice immunised with Nano-FP1 exhibit strong recall cytokine responses.** Multiplex quantification of cytokine release by murine splenocytes in response to recall antigens. Splenocytes from animals immunised with Nano-FP1 showed strong cytokine responses after stimulation with Ag85B or FP1, but not to Acr. Cytokine production in the PBS or BCG groups was low-to-minimal. Production in media alone was used as a negative control and data represent cytokine production by splenocytes pooled from 3 animals per experimental group. Data are representative of 3 separate experiments Tukey’s multiple comparison test was used to test for statistical significance.

**Figure 6. Nano-FP1 induces high T-cell polyfunctionality.** Spleens from (n = 1-3) immunised or control mice were stimulated with recall antigen for 4 hours under Golgi blockade and then assessed for the production of four effector cytokines: IFN-γ, IL-2, IL-17A and TNF-α. (A) CD4+ and CD8+ T cells expressing 3 or more cytokines. (B) CD4+ and CD8+ T cells expressing specific combinations of cytokines in response to Ag85B re-stimulation. Data are derived from three independent experiments. Bars depict means ± SEM. Significance was tested between groups by two-way ANOVA with Dunnet’s post-test*\** p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\* p < 0.0001.

**Figure 7. Nano-FP1 immunisation leads to tissue resident memory T cells in the lungs**. Lungs from immunised mice were perfused and then memory T cells (CD44hiCD62Llo) were assessed for co-expression of CD69 and CD103 in tandem. Percentages of Trm are indicated in the relevant plots. Plots are representative and derived from n = 3 pooled mice per group.

**Figure 8. YC-NaMA induces an IRF-3-associated APC activation profile.** Macrophages were assessed for various parameters of activation. (A) Macrophages were stimulated for 48 hours with 0.1, 1 or 10 g/mL YC-NaMA or LPS and assessed for surface phenotype by flow cytometry. (B) Cytokine levels in the supernatants were determined by ELISA. (C) Macrophages expressing an alkaline phosphatase NF-κB reporter were pulsed with YC-NaMA (10 g/mL) or LPS for indicated times, and then assessed for reporter activity by reading optical density. (D) Phosflow detection of IRF-3 phosphorylation at 4 hours post-stimulation. (E) RT-qPCR of *Ifna* and *Ifnb* 24 hours post-stimulation, relative to housekeeping gene *Actb*. (F) RT-qPCR of *Ccl5 and Cxcl10* at 24 hours post-stimulation, relative to housekeeping gene *Actb*. Data are derived from one (D), three (A-C) or four (E-F) independent experiments. Bars depict means ± SEM. Significance was tested against the unstimulated control (A-C) or against other groups (F) by one one-way ANOVA with Tukey’s post-test (A,B, F) or two-way ANOVA with Dunnet’s post-test (C) *\** p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\* p < 0.0001.

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**H:\Papers & Lit. Review\My papers\NanoFP1\Nano-FP1 figures\Slide2.TIFFigure 1**

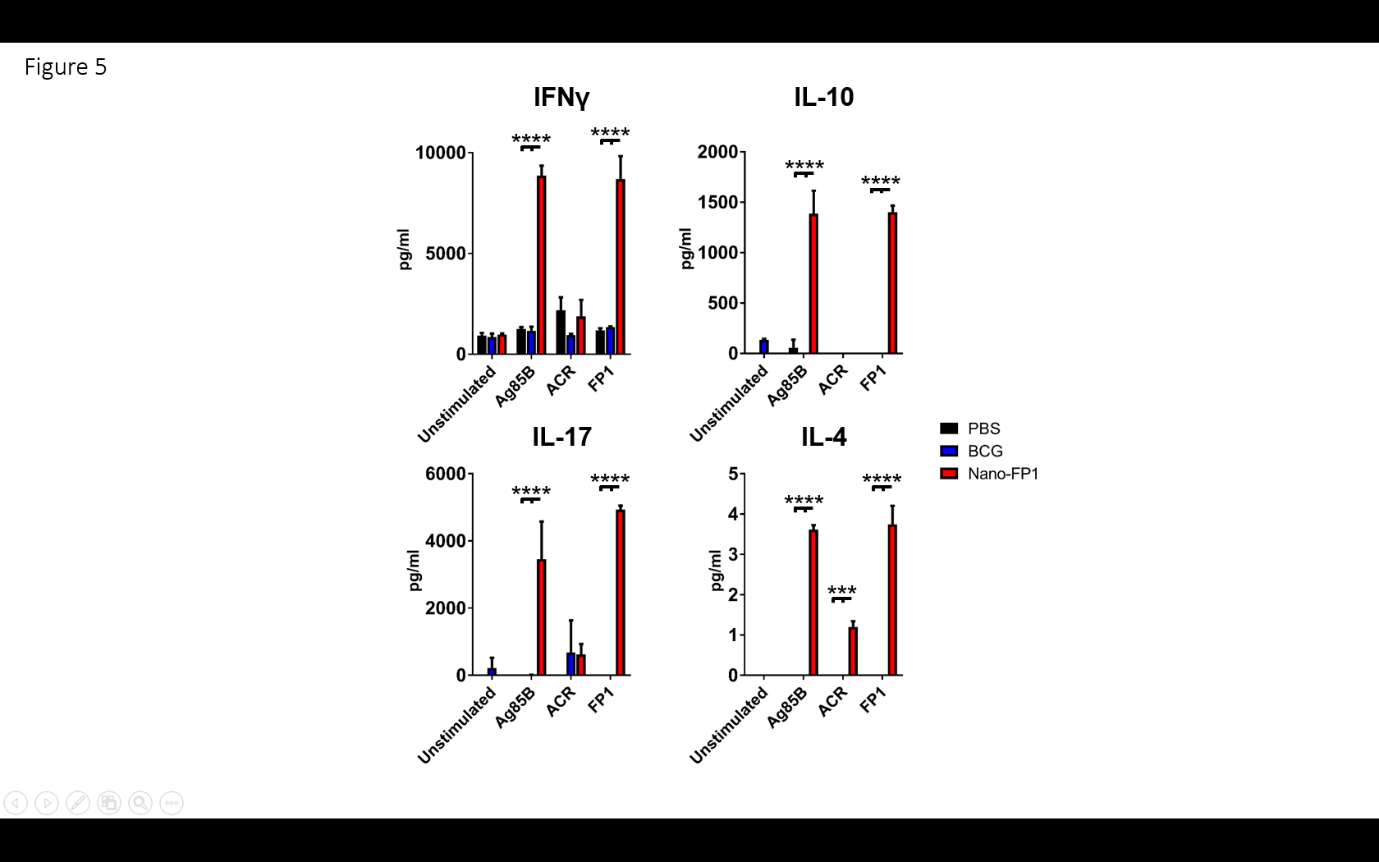
**Figure 2**

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**Figure 5**



**Figure 6**

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