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Selected β-glucans act as immune-training agents by improving anti-mycobacterial activity in human macrophages - a pilot study.

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Short title: β-glucans and *M. tuberculosis* infection

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Abstract

Epigenetic reprogramming of innate immune cells by β-glucan in a process called trained immunity, leads to an enhanced host response to a secondary infection. β-glucans are structural components of plants, algae, fungi and bacteria and thus recognized as non-self by human macrophages. We selected the β-glucans curdlan from *Alcaligenes faecalis*, WGP dispersible from *Saccharomyces cerevisiae,* and β-glucan-rich culture supernatant of *Alternaria* and investigated whether they could produce trained immunity effects leading to an increased control of virulent *Mycobacterium tuberculosis*. We observed a significant *M. tuberculosis* growth-reduction in macrophages trained with curdlan and *Alternar*ia, which also correlated with increased IL-6 and IL-1β release. WGP dispersible-trained macrophages were stratified into 'non responders' and 'responders', according to their ability to control *M. tuberculosis*, with 'responders' producing higher IL-6 levels. The addition of neutrophils to infected macrophage cultures further enhanced macrophage control of virulent *M. tuberculosis*, but not in a stimuli-dependent manner. Pathway enrichment analysis of DNA methylome data also highlighted hypomethylation of genes in pathways associated with signaling and cellular reorganization and motility, and 'responders' to WGP-training were enriched in the interferon-gamma signaling pathway. This study adds evidence that certain β-glucans show promise as immune training agents.

Introduction

Tuberculosis (TB) remains a major global health problem inflicting mainly resource-poor countries [1]. The worldwide yearly incidence rate is 10 million, and 1.5 million deaths are caused by TB every year. TB is caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*) which has co-evolved with humans, thus becoming specialized at immune evading mechanisms [2]. Despite the last decades' many advances in the understanding of the complexity of an *M. tuberculosis*-infection, we are far from the WHO goal of eliminating TB by 2035 [3]. Thus, efforts resulting in improved treatment regimens and more effective vaccines are greatly needed.

Recent studies have highlighted the fact that we possess an innate immune memory and that myeloid cells can resist an infection to the same or an unrelated pathogen via a phenomenon termed trained immunity [4]. Trained immunity has been shown to be driven by epigenetic remodeling [5, 6] and differential gene expression [7], resulting in a rewiring of intracellular metabolic pathways [8, 9] and a long-term proinflammatory phenotype with increased cytokine responses upon a second stimulus or infection. Trained immunity is induced after exposure to some live vaccines, such as BCG, as well as to some microbial components of pathogens, with fungal β-glucan being the most widely studied [6, 10, 11].

Macrophages, one of the primary cell types infected with *M. tuberculosis*, are central mediators of the immune response to TB. Since fungal β-glucan from *Candida albicans* can induce a trained immunity phenotype in human macrophages, we wanted to investigate what other microbial or fungal glucans could mediate similar effects. β-glucans are polysaccharides that serve as energy stores and structural components in cell walls of plants, algae, fungi, and bacteria [12]. In this study we selected β-glucans based on previous reports of immune modulatory effects [13-16]. β-glucans are not produced by mammalian cells and are recognized as non-self, microbe associated molecular patterns (MAMPs) by human innate immune cells.

Curdlan is a high molecular weight, mostly linear, β-(1,3)-glucan from the non-pathogenic bacterium *Alcaligenes faecalis* [17]. Curdlan stimulation can increase macrophage control of *M. tuberculosis*-infection in mice and human THP-1 cells [16]. Curdlan was also able to induce a trained immunity phenotype in M-CSF stimulated human macrophages, which correlated with the enhanced secretion of IL-6 and TNF-α [18]. WGP (whole glucan particles) is a branched β-(1,3)-, (1,6)-glucan from the yeast *S. cerevisiae* [19]. This variety of β-glucan has a non-pathogenic source and has extensively been used as an immunomodulator and sold under numerous commercial names [20, 21]. However, while immune-activating effects have been described when using WGP dispersible, the insoluble, particulate form of this β-glucan, the soluble form also binds to the β-glucan receptor Dectin-1 but without activating it [22]. *Alternaria spp* is a multicellular fungi and plant pathogen, which also can cause allergies in humans. The effects of β-

glucan from *Alternaria* have to our knowledge not been studied regarding immune response in mammals. However, there is structural information available [23], and preliminary results on beneficial trained immunity effects in mice (Dr. Marta Romano, personal communication).

We designed a pilot study with selected β-glucans that could potentially act as immune training agents and tested if they could increase the anti-mycobacterial activity of macrophages. We found that curdlan and the β-glucan-rich *Alternaria* supernatant could act as immune training agents and boost macrophage restriction of mycobacterial growth. In a subset of donors, WGP dispersible also facilitated the control of mycobacteria. The increase in anti-mycobacterial activity was accompanied by an increased IL-6 secretion and altered DNA methylation status of genes involved in pathways associated with metabolic processes, signaling and cellular reorganization and motility.

Materials and methods

Human monocyte isolation and trained immunity stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte rich fractions of blood obtained from healthy volunteers (Linköping University Hospital blood bank, Linköping). Healthy volunteers gave their written informed consent for the use of their blood for scientific purposes. Isolation was performed by the method of density gradient centrifugation using Lymphoprep (Axis-Shield) and Sepmate-50 tubes (Stemcell Technologies) according to the manufacturer's protocol. The mononuclear cells were seeded in 75cm^2 culture flasks (BD Falcon) in Dulbecco's Modified Eagle Medium (DMEM) and allowed to adhere for 1-2 h before the non-adherent lymphocytes were washed away using warm Krebs-Ringer Glucose buffer (made in house). Complete DMEM supplemented with 10% pooled human serum, 2mM GlutaMAX, 1mM Sodium Pyruvate, 100U/ml penicillin and 100µg/ml streptomycin (all from Gibco) was then added to the cells with immune training agents, or medium only as negative control for 24h. The immune training agents used in this study were: β-glucan *Saccharomyces cerevisiae* 10µg/ml (Whole Glucan Particles, WGP dispersible, Invivogen), β-glucan *Alcaligenes faecalis* 1µg/ml (Curdlan, Sigma-Aldrich), and culture supernatant from *Alternaria* (kind gift from Dr. Marta Romano). *Alternaria alternata* (strain 18586) was obtained from the BCCMTM/IHEM (Sciensano, Brussels, Belgium) and cultured as described by Strandberg [24]. The mould was first cultured at 27° C on potato dextrose agar (BD Difco, New Jersey, USA) plates for two weeks before gently harvesting the spores with a cell scraper. Spores were diluted in phosphate buffer saline (PBS) and counted with a hemocytometer. Then, Roux flasks containing 250 ml of liquid Czapek medium were seeded with 107 spores and cultured at 27°C for five weeks. At the end of the culture, the solid fungal mats were removed, and the culture filtrates were retrieved. Filtrates were cleared by centrifugation, diluted 10x in PBS, filtered through a 0.2-µm filter, aliquoted and finally kept at -20°C until used. The β-glucan's source and concentration range are presented in Table 1. After the one-day incubation with B-glucans or *Alternaria* supernatant, medium was replaced with complete DMEM only, and further incubated for 5 days, with medium change after 2 days. 6 days after monocyte isolation the differentiated macrophages all had similar morphology (suppl. Fig. 1). The macrophages were trypsinized (0.05% Trypsin-EDTA from Gibco), counted and reseeded into 96-well plates in antibiotic-free DMEM (ABF medium) at a density of 50 000 cells/well or 10 000 cells/well for neutrophil co-culture experiments.

Neutrophil isolation

Human neutrophils were isolated from whole blood with EDTA obtained from healthy volunteers (Linköping University Hospital blood bank, Linköping), by the method of density gradient centrifugation using Lymphoprep and Polymorphprep (both Axis-Shield). Red blood cells were lysed by hypotonic shock and neutrophils were washed multiple times in Kreb's Ringer Glucose Buffer without calcium. Cells were stained using 2µM PKH26 red dye (Sigma) for 3 minutes followed by multiple wash steps with FBS (Gibco) or complete ABF medium according to the manufacturer's instructions.

M. tuberculosis culture and infection of macrophages

The laboratory *M. tuberculosis* strain, H37Rv, carrying the green fluorescent protein (GFP)-encoding pFPV2 plasmid (*M. tuberculosis*-GFP) was grown in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase (ADC, Becton-Dickinson), 0.05% Tween-80, and the selection antibiotic Kanamycin at 20µg/ml for two to three weeks at 37°C. The bacteria were then re-seeded in fresh broth for an additional 7 days to reach early log phase for use in experiments. For infection, the harvested bacteria were washed, resuspended in antibiotic-free complete DMEM and filtered through a Milliex-®SV 5.0µm syringe filter (Merck) to remove bacterial clumps. Macrophages were infected with *M. tuberculosis* at a multiplicity of infection (MOI) 10 for 2h, followed by medium change to remove extracellular bacteria. For selected experiments, at 1 day post infection, freshly isolated neutrophils were counted and added to infected macrophage cultures at a ratio of 2:1.

Secondary stimulation of macrophages and cytokine measurements

Macrophages were trained with β-glucan (as described earlier) and on day 6 reseeded in 96 well plates with 50 000 cells per well. The following day cells were stimulated with either MOI-20 of γ-irradiated H37Rv (γ-irradiated whole cells, NR 14819, LOT 59585333, BEI Resources), or a cocktail of TLR antagonists: 10ng/ml LPS (Escherichia coli serotype O26:B6, Sigma-Aldrich) and 20ng/ml Pam3CSK4 (Invivogen). Production of TNF-α, IL-6, IL-33, IL-8, IL-10, IL-1β, IFNγ, CXCL9, CXCL10, CXCL11, IL-12p40 and IL-18 was determined in 24-hour supernatants by using a magnetic Luminex® Assay kit from R&D Systems. Reagents and samples were prepared according to the manufacturer's protocol and measured on a Luminex FLEXMAP 3D Instrument. IL-33, IL-12p40 and IL-18 were below detection limit and not presented in the results.

Metabolite measurements

Cell culture supernatants were collected on day 6 after addition of β-glucans and stored in -80°C until measured. Quantitative measurement of lactate and glucose was performed on an ISCUSflex Microdialysis Analyzer using a L-P-G kit (both from M Dialysis).

Live-cell microscopy of M. tuberculosis infected macrophages and neutrophil co-cultures: M. tuberculosis growth and macrophage cell death

Macrophages infected with H37Rv-GFP were studied using an Incucyte S3 (IncuCyte Live-Cell 120 Analysis System, Sartorius). The relative fluorescence signal (RFU) of bacteria was measured every 4 hours (20x, 4 images/well) until day 5 post-infection, analyzed using Incucyte S3 software and expressed as total integrated intensity of green objects (GCUxµm^2/image). For experiments where neutrophils were added, green fluorescence values were normalized to time-point 0h and results shown as fold change of total GCUxµm^2/image. Macrophage cell death, in the same infected cultures, was quantified using DRAQ7™ (Deep Red Anthraquinone 7, BD Pharmingen), far-red fluorescent DNA dye that enters the cell when the cell membrane becomes permeable upon death. Cell death was expressed as DRAQ7 mean count/image from images taken every 4 hours (20x, 4 images/well) until day 5 post-infection. Macrophage cell numbers were calculated using the Incucyte S3 Cell-by-Cell module and data extracted as mean count per image taken every 4 hours (20x, 4 images/well) until day 5 post-infection. Accumulated cell death was calculated by adding up the DRAQ7 positive cells for each time point measured.

DNA extraction and DNA methylation data pre-processing

DNA was isolated from macrophages using AllPrep DNA/RNA Mini Kit (Qiagen). DNA concentration was measured by Qubit fluorometer using dsDNA HS Assay Kit (Invitrogen). Genome-wide methylation profiling was done using the

Infinium methylation EPIC 850K platform. DNA methylation IDAT files were processed in R (v3.6.3) using *ChAMP* package [25] from bioconductor. CpG sites with less detection p-value (≥0.01) were removed and the filter was also applied to reduce the biasness of sex chromosomes, cross-hybridising probes, probes with SNPs at the CpG site using the default filtering criteria of ChAMP package. Within the sample, normalization was performed using the BMIQ method and the methylation β value was calculated for each sample. After quality control filtering, a total of 710,895 CpG sites in 7 samples (with 3 different stimulants) were available for the downstream analysis. An unsupervised hierarchical clustering dendrogram was generated using the *hclust* and *ape* packages in R based on the Euclidean distance matrix calculation from the methylation β value.

DNA methylation data post-processing and enrichment analysis

To evaluate the effect of each stimulant, the fold change was calculated with respect to the control sample for each CpG site (Curdlan vs Control = CC, WGP disp vs Control = WC, *Alternaria* vs Control = AC). Only those CpG sites which changed/altered in the same direction in all samples (positive: hypermethylation and negative: hypomethylation), were selected for further analysis. Density plot was used to determine the optimal cut-off score (β > |0.6|) for differentially methylated CpG-sites. In WebGestalt [26], a Gene Set Enrichment Analysis (GSEA) was performed using the panther database with the default parameters.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software. Wilcoxon matched-pairs signed rank test with two-tailed *P*-value was used to compare samples. Statistical significance values were presented as exact *P*-values with $P < 0.05$ considered as significant.

Results

Selected immune training agents enhance macrophage mycobacterial control

We adapted a scheme for induction of trained immunity published by Bekkering et al. [27], and started by testing the hypothesis that selected β-glucans could enhance macrophage control of *M. tuberculosis* (Fig. 1a). Monocytes were trained during 24 h with curdlan, WGP dispersible or *Alternaria* supernatant (Table 1). The concentrations used were optimized by testing a range of different concentrations and measuring cell viability with and without infection. Macrophages were then infected with H37Rv-GFP at a multiplicity of infection (MOI) of 10 after one week of differentiation. As antimycobacterial efficacy of macrophages typically varies between donors, experiments in which the bacterial load increased by five-fold in non-trained cells were selected for further analysis (suppl. Fig. 2). We could observe a significantly reduced bacterial load at 5 days post infection in curdlan- ($p = 0.03$) and *Alternaria*-trained cells ($p = 0.03$) (Fig. 1b-c). The training procedure did not influence the phagocytic capacity (Fig. 1d), allowing us to rule out this factor as an explanation for the observed differences in bacterial load. Neither did cell death, expressed as cumulative DRAQ7 counts, differ significantly between the conditions (at day 5 post infection, control vs curd $p = 0.8$, control vs WGP $p = 0.2$, control vs *Alternaria* $p = 0.7$, suppl. Fig. 3a-b). A metabolic shift from oxidative phosphorylation to increased glycolysis in cells trained with β-glucan from *C. albicans* has previously been shown to be an essential component of the trained immunity phenotype of the cells [8]. However, in our experiments the enhanced anti-mycobacterial capacity of the trained macrophages that we observed was not accompanied by increased amounts of lactate (suppl. Fig. 3c) or decreased amounts of glucose (suppl. Fig. 3d) in culture supernatants.

Neutrophils enhance macrophage mycobacterial growth restriction

Neutrophils are important players in the early immune response to *M. tuberculosis* [28] and we hypothesized that the addition of neutrophils might synergize with the anti-mycobacterial capacity of immune-trained macrophages. Freshly isolated neutrophils stained with the membrane dye PKH26 were added to the infected macrophage cultures at 1 day post-infection. Inspection of the images revealed that bacteria were all located intracellularly in the macrophages indicating that the neutrophils did not directly assist in the clearance of the bacteria (Fig. 2a). However, macrophages readily phagocytosed neutrophils which could be detected as red spots in the macrophage's cytoplasm (Fig. 2a). At 5 days post-infection a significant reduction in *M. tuberculosis* load was observed in all conditions co-cultured with neutrophils except the *Alternaria*-trained macrophages (Fig. 2b). The mycobacterial load in *Alternaria*-trained macrophages co-cultured with PMN was significantly lower than in mock-trained macrophages co-cultured with PMN ($p = 0.0273$, suppl. Fig. 4). However no significant synergy with the immune-training agents could be detected for WGP- and curdlan-trained co-cultures ($p = 0.9201$ and $p = 0.1641$, respectively).

Increased proinflammatory cytokine production in β-glucan trained human macrophages after secondary stimulation

β-glucan training of monocytes *in vitro* has previously been demonstrated to increase cytokine production upon secondary stimulation with LPS and Pam3CSK4 (Pam3) [27], but also with various bacteria [29]. Therefore, macrophages trained with β-glucans were given a secondary stimulation on day 7 after immune training. Either a TLR-agonist cocktail consisting of LPS and Pam3, to study the heterologous trained immunity response to microbial stimuli, or γ-irradiated *M. tuberculosis* (γRv), for study of the *M. tuberculosis*-specific secondary response. Cytokines (IL-6, TNF-α, IL-1β, IL-10, IL-8, CXCL10, CXCL9, CXCL11) were measured using a multiplex Luminex assay in 24-hour supernatants with the different β-glucan trained samples compared to mock-trained cells from the same donor (Fig.3).

In response to LPS and Pam3, WGP dispersible-trained cells released significantly decreased amounts of IL-10 (Fig. 3d), while *Alternaria* supernatant-training was associated with significantly increased IL-6, TNF-α, IL-10, IL-8, CXCL10 and CXCL9 production (Fig. 3a-b, d-g). The mycobacterial secondary stimulation with γRv resulted in significantly increased amounts of IL-6 and IL-1β from curdlan trained cells (Fig. 3a, c), while cells trained with *Alternaria* supernatant released increased amounts of IL-6, TNF-α, IL-1β and IL-10 (Fig. 3a-d).

Since we observed that WGP dispersible enhanced bacterial control in just four out of six donors (Fig. 1c), we divided these donors into 'responders' (>2.5 fold enhanced anti-mycobacterial capacity over control) and 'non-responders' (Fig. 4a). The 'responders' to WGP dispersible training also released increased amounts of IL-6 (Fig. 4b). However, the other cytokines measured did not display as consistent results (suppl. Fig. 5). Macrophage cell death patterns were similar for 'responders' and 'non-responders' (not shown).

Pathway enrichment analysis reveals enrichment of immune-related pathways in β-glucan trained macrophages

Since innate immune memory has been shown to be epigenetically regulated [6], we wanted to study the changes in DNA methylation in our cells induced by β-glucan training. Genome-wide DNA methylation data from the Illumina 850k methylation EPIC platform was analyzed for 7 macrophage donors and the 4 different training conditions (mock, curdlan, WGP dispersible and *Alternaria* supernatant). First, we performed an unsupervised hierarchical clustering analysis to see whether we could discover any specific grouping of samples induced by the immune training (suppl. Fig. 6). The samples mainly clustered according to macrophage donor, which shows that the epigenetic changes to DNA induced by immune training is subtle compared to the overall differences between the individual donors. We identified the differentially methylated genes (DMGs) by comparing the β-glucan-trained (curdlan, WGP dispersible and *Alternaria*) cells with mock-trained controls. Using the DMGs that had Δβ-values going in the same direction, i.e. that showed a consistent hypo- and hypermethylation after β-glucan training we identified the pathways that were enriched upon immune training (suppl. Table 1-3) . A graph was generated to show the pathways in which at least 5 of the donors had enrichment, the size of the circles describes the number of genes present in the leading edge of the pathway which were differentially methylated, and the color demonstrates the normalized enrichment score (Fig. 5).

Generally, the epigenetically modulated pathways were more conform among donors with the purified glucan stimuli, including curdlan and WGP dispersible (Fig. 5 a-b). *Alternaria*, on the other hand, which is a crude extract, caused variable responses among donors (Fig 5c). The different stimuli did not generate coherent epigenetic modulation of pathways, with overlaps only between WGP dispersible and *Alternaria* (EGFR signaling pathway and angiogenesis, Fig. 5b-c). Among responders to WGP dispersible, a negative enrichment in the interferon gamma signaling pathway can be noted (absent in non-responders, Fig. 5b). A negative enrichment (hypomethylation) indicates enhanced accessibility for transcription of the interferon-gamma signaling pathway. The interferon-gamma signaling pathway was not epigenetically modulated in the curdlan- or *Alternaria*-trained cells. However, other signaling pathways like cytokine signaling and interleukin signaling were found to be modulated in curdlan- and *Alternaria*-trained cells, respectively.

Discussion

It has previously been shown that β-glucan can induce trained immunity, characterized by functional changes in monocytes and macrophages, which can result in increased protection against various microbial infections [6]. While β-glucan from *C. albicans* has been most widely studied, little is known whether β-glucans from other species can mediate similar effects. In this study we show that β-glucan from a bacterial source (curdlan from *A. faecalis*), from yeast (WGP dispersible from *S. cerevisiae*) and from the supernatant of a multicellular fungi (*Alternaria*) are to different degrees able to induce functional changes and epigenetic reprogramming in human macrophages that resemble the trained immunity phenotype described by others. While the β-glucans curdlan and WGP dispersible used in this study are purified β-glucans, the *Alternaria* supernatant is a crude stimulus. Although it is known to be rich in β-glucans, other components may also be responsible for the effects seen in the experiments, which is a limitation to this study.

Since β-glucans are not produced by members of the kingdom *Animalia* [12], it is likely that the innate immune system has developed mechanisms to recognize them as foreign molecules, but also that the encounter subsequently leads to a functional reprogramming in order to protect from future microbial infections. The different β-glucans that can mediate a trained immunity reprogramming that can boost human macrophages' anti-mycobacteria control, are still very unexplored. However, curdlan and WGP have been found to induce a functional immune memory phenotype resulting in increased proinflammatory cytokines in response to LPS [18]. Size, branching and solubility of the glucan have been found to be important for the immunomodulatory action [12], but also more specifically for the different binding affinity to the β-glucan receptor, Dectin-1 [30, 18]. Consequently, further studies are required to better understand the immunomodulatory role of β-glucans and how they can reprogram macrophages to better control *M. tuberculosis*.

β-glucan induced trained immunity has been shown to be epigenetically regulated at the level of histones [6]. In this study, we chose to analyze DNA methylation to see whether specific gene associated pathways were enriched after immune training. A diverse range of epigenetically modulated pathways were enriched after the cells were exposed to the training agents, and the diversity of methylation changes complicated interpretation In curdlan-trained macrophages, chemokine- and cytokine related pathways were found modulated. In WGP dispersible-trained macrophages, interferon-gamma signaling pathway, angiogenesis components as well as the EGF receptor pathway were modulated. In *Alternaria*-trained cells, the pattern was less concise but overlapped with WGP dispersible in that both modulated the angiogenesis and EGF receptor pathway. How these effects link to enhanced mycobacterial control in macrophages remains obscure, however one of the hypomethylated pathways (indicating enhanced accessibility for transcription) was the interferon-gamma signaling pathway. It is well established that IFN-γ is an important factor in the control of a *M. tuberculosis* infection [31], and we have previously shown that IFN-γ gene hypomethylation correlates with increased mycobacterial control in BCG-vaccinated subjects [32]. Other cytokines [33], including interleukins [34], have also been demonstrated to play a role in macrophages' ability to restrict mycobacterial growth and we found that pathways for cytokine and chemokine signaling were modulated in response to curdlan and *Alternaria*-training.

An active macrophage defense can lead to control of *M. tuberculosis* at lower multiplicities of infection [35, 33], which has been shown to be dependent on functional IFN-γ signaling, but also IL-β signaling is important for macrophage restriction of *M. tuberculosis* growth [34]. Based on that observation, we attempted to boost the macrophage ability to control *M. tuberculosis* using β-glucans as immune training agents when using a higher multiplicity of infection. When conducting experiments, we encountered large donor variability when it came to the control of mycobacteria, but also variability in the enhanced control from training with β-glucan, and the cytokine response when given the secondary stimulation with LPS/Pam3 cocktail or the mycobacterial stimulus γ-irradiated *M. tuberculosis* (γRv). Still, training human monocytes with the β-glucans curdlan and *Alternaria* resulted in macrophages that could restrict the growth of virulent *M. tuberculosis* to a higher extent than mock-trained control macrophages in the mono-culture experiments. Moreover, curdlan- and *Alternaria*-training resulted in increased release of IL-1β and IL-6 when given a secondary stimulus with γRv. Previous studies have found curdlan to be able to enhance the anti-mycobacterial activity in mouse macrophages and THP-1 cells [16]. We show similar results with regard to anti-mycobacterial boosting effects. However, we also found an increased cytokine-release in response to mycobacterial stimuli, along with epigenetic reprogramming of the cells where genes in pathways associated with inflammation and receptor signaling are differentially methylated.

Interestingly, when studying WGP dispersible trained macrophages we observed a donor variability where some donors, but not all, responded with an increased mycobacterial control after training. When stratifying the donors into 'responders' and 'non-responders', we found that the 'responders' also released more IL-6 upon secondary stimulation with either LPS/Pam3 or γRv. Bimodal responses are common in biological systems and in this case the observed difference in cytokine release was also associated with enhanced mycobacterial control. The small group size is a limitation in this study and further validation is needed. However, the fact that IL-6 that has been found to be pivotal for innate immune memory in response to both β-glucan [6, 36, 37] and BCG [37], separated the 'responders' from the 'non-responders' highlight the fact that this finding may hold biological relevance. This functional separation between 'responders' and 'non-responders' was also evident in the epigenome where an enrichment in genes in the interferon-gamma-signaling pathway was only found in the 'responders'. The fact that cells from different human donors are at outset differently apt to controlling mycobacteria, but also show divergent responses to the training stimuli, probably reflects the human donors' heterogenous genetical background and environmental exposure to microbial stimuli. This concept that some human subjects have an inherent ability to control mycobacteria and to respond to a training stimulus such as the BCG vaccine has been described earlier [32]. More recently a study on BCG immune training of neutrophils also identified high and low responders when looking at immune priming lncRNAs and the pro-inflammatory cytokine release [38].

During a tuberculosis infection in vivo, multiple innate immune cells contribute to limiting bacterial growth and the spread of infection. A previous study found that efferocytosis of apoptotic PMN can enhance the control of *M. tuberculosis* in human macrophages [39-41]. We added viable PMN in selected experiments, however the PMN started showing signs of cell death after a few hours. By adding PMN to β-glucan-trained macrophages, we investigated if this resulted in additional restriction of *M. tuberculosis*-growth in the co-cultures. Indeed, the addition of PMN resulted in a significant enhanced anti-mycobacterial control in the macrophages. However, this was also the fact for mock-trained controls. In the *Alternaria*-trained macrophages, the bacterial growth appeared to already be maximally reduced, so no further reduction could be observed after the addition of PMN, when using our experimental setup. Considering the recent findings where BCG vaccination led to immune training of neutrophils [38], it would be interesting to add β-glucan-trained neutrophils to the infected macrophages. But due to the short lifespan of PMN in vitro this would require a different immune training scheme than what has been used when training macrophages in vitro, and hence not something we attempted in the current study.

Trained immunity, in a wider perspective, has been shown to confer some of the beneficial effects of the tuberculosis vaccine BCG [5, 42], and our group has earlier identified "responders" to BCG vaccination that were characterized by exhibiting an enhanced anti-mycobacterial response at the level of peripheral macrophages [43]. One of the key findings was that the responder's macrophages exhibited increased phagocytosis of *M. tuberculosis*. Hence, we wanted to investigate whether the enhanced anti-mycobacterial response in macrophages trained with β-glucan likewise could be related to increased phagocytosis. However, when studying the number of phagocytosed bacteria

there was no difference between control macrophages and trained macrophages, nor between the different β-glucan-trained macrophages. This can also be compared to the study performed by [16], where a short pre-treatment with curdlan did increase the phagocytic ability of mice macrophages.

Cell death dynamics during infection are interesting as cell death can be beneficial to the host cell when threatened by an intracellular bacterium, but it can also be a means for the bacterium to escape and spread [44]. Even though cell death mechanisms are not within the scope of this study, we wanted to examine whether there was a difference between differently trained macrophages and the rate of cell death during infection. Infection with virulent *M. tuberculosis* led to increased cell death in all macrophages. Yet, there was no clear correlation between cell death rates and mycobacterial control in our experiments.

The limitations of the current prevention and treatment regimens for tuberculosis along with continued high world-wide mortality rates, emphasize the need for new prevention and treatment strategies. Host-directed therapies have emerged as a promising approach and stimulating a trained immune response either to prevent or treat tuberculosis could be a game-changer in fighting the disease. With this study we explored β-glucans as possible immune training agents and show promising results as to how they can strengthen the macrophage control of an *M. tuberculosis* infection. We found that β-glucans can upregulate key pathways that are important for cellular signaling and motility as well as being able to modulate functional characteristics like increased mycobacterial control and cytokine release. However more experiments are needed to better characterize the effects of β-glucan observed in this study.

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Statement of Ethics

Ethical approval is not required for this study in accordance with local and national guidelines. Informed, written consent was obtained from healthy human volunteers for the use of their blood for scientific purposes.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Clara Braian and Maria Lerm designed the study. Clara Braian performed the laboratory work, the related analyses, interpreted the data and created the figures. Jyotirmoy Das, Lovisa Elisabet Karlsson designed and performed the bioinformatic analyses of the data, wrote the scripts for analysis, interpreted the data, and created the figures. Clara Braian and Maria Lerm wrote the manuscript. Clara Braian, Maria Lerm, Jyotirmoy Das and Lovisa Elisabet Karlsson revised the manuscript and approved the final version.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

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Figure legends:

Fig. 1. Immune training with selected β-glucans increases macrophage control of *M. tuberculosis*. **a** Schematic overview of experimental setup. Human monocytes were trained with curdlan 1μg/ml, WGP dispersible 10μg/ml or *Alternaria* supernatant 0.01% for 1 day and then infected with H37Rv-GFP, MOI-10 on day 7. **b** Growth of H37Rv-GFP over time presented as fold change of total integrated green fluorescence. Mean values with SEM from 6 experiments are shown in graph. **c** Endpoint comparison of bacterial growth at 5 days post-infection. As has been indicated with closed symbols, two donors did not show improved bacterial control after WGP dispersible training. * = $P < 0.05$, ns = not significant. **d** Phagocytosis as measured by internalized GFP-expressing bacteria (total integrated green fluorescence) at 4h post-infection. * = $P < 0.05$, ns = not significant.

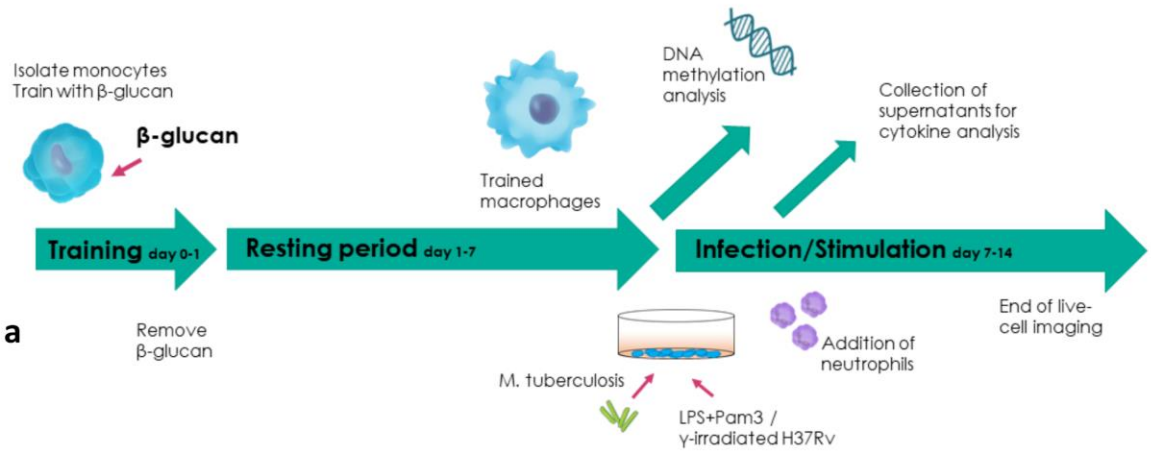
Fig. 2. Co-incubation of neutrophils with trained macrophages infected with H37Rv-GFP. **a** Representative microscopy images of cells from the same donor, 1.5 days post addition of neutrophils (PMN). Neutrophils were stained with the red membrane dye PKH26 and added to macrophages 1 day post-infection. PMN phagocytosed by macrophages can be detected as red spots in the macrophage's cytoplasm (arrows). **b** Bacterial load in trained macrophages co-cultured with neutrophils. Endpoint comparison of bacterial growth at 5 days post-infection in trained macrophages or medium control macrophages with or without neutrophils (+PMN) added at 1 day hours post-infection. Data presented as fold change (compared to 1 day post-infection when PMN where added) of total integrated green fluorescence. ns = not significant. * = $P < 0.05$. n=9

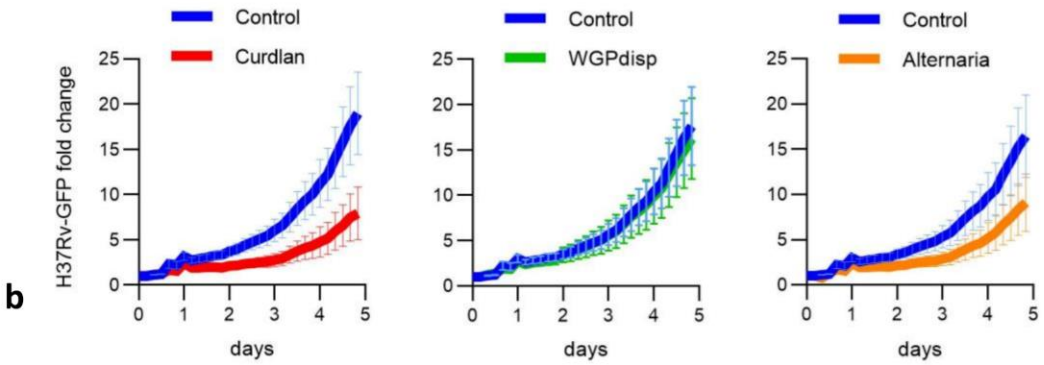
Fig. 3. Cytokine production in β-glucan trained human macrophages after secondary stimulation. Human monocytes were trained with curdlan 1μg/ml (red), WGP dispersible 10μg/ml (green) or *Alternaria* supernatant 0.01% (orange) for 1 day. After one week of washout and differentiation the cells were either unstimulated or stimulated with a cocktail of LPS 10ng/ml and Pam3Cys 20ng/ml (LPS/Pam3), or γ-irradiated H37Rv MOI-10 (γRv). After 1 day supernatants were collected and analyzed for cytokines. β-glucan trained samples were compared to their mock-trained controls (blue) from the same blood donor. IL-6. **b** TNF-α. **c** IL-1β. **d** IL-10. **e** IL-8. **f** CXCL10. **g** CXCL9. **h** CXCL11. ns = not significant. * = $P < 0.05$. n=6

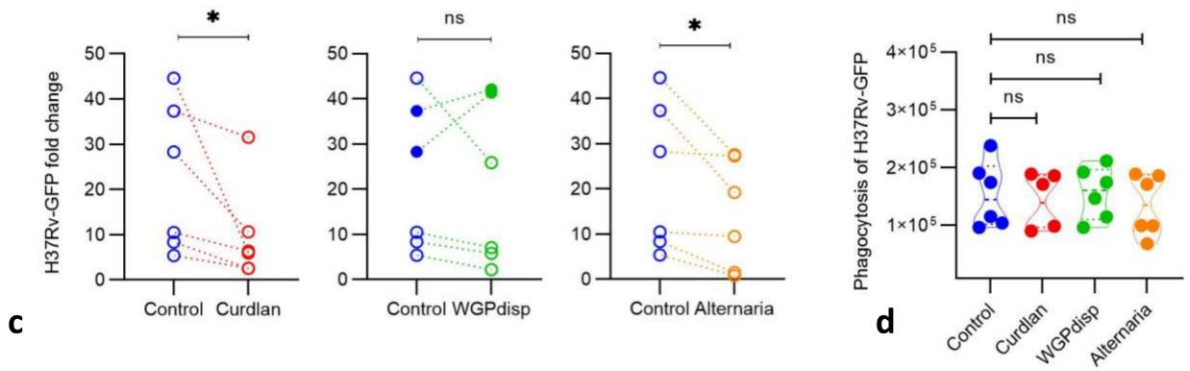
Fig. 4. IL-6 production in WGP dispersible-trained macrophages correlates to mycobacterial growth reduction. **a** Growth of H37Rv-GFP in WGP dispersible-trained macrophages at 120h presented as fold change over 4h post infection. **b** IL-6 concentration in 24-hour supernatants after secondary stimulation with either a cocktail of LPS 10ng/ml and Pam3Cys 20ng/ml (LPS/Pam3), or γ-irradiated H37Rv MOI-10 (γRv). β-glucan trained samples (green) were compared to their mock-trained controls (blue) from the same blood donor. Donors were divided into 'responders' (open circles) and 'non-responders' (closed circles) according to their ability to control growth of H37Rv, as seen in panel a. ns = not significant. * = $P < 0.05$. n=6

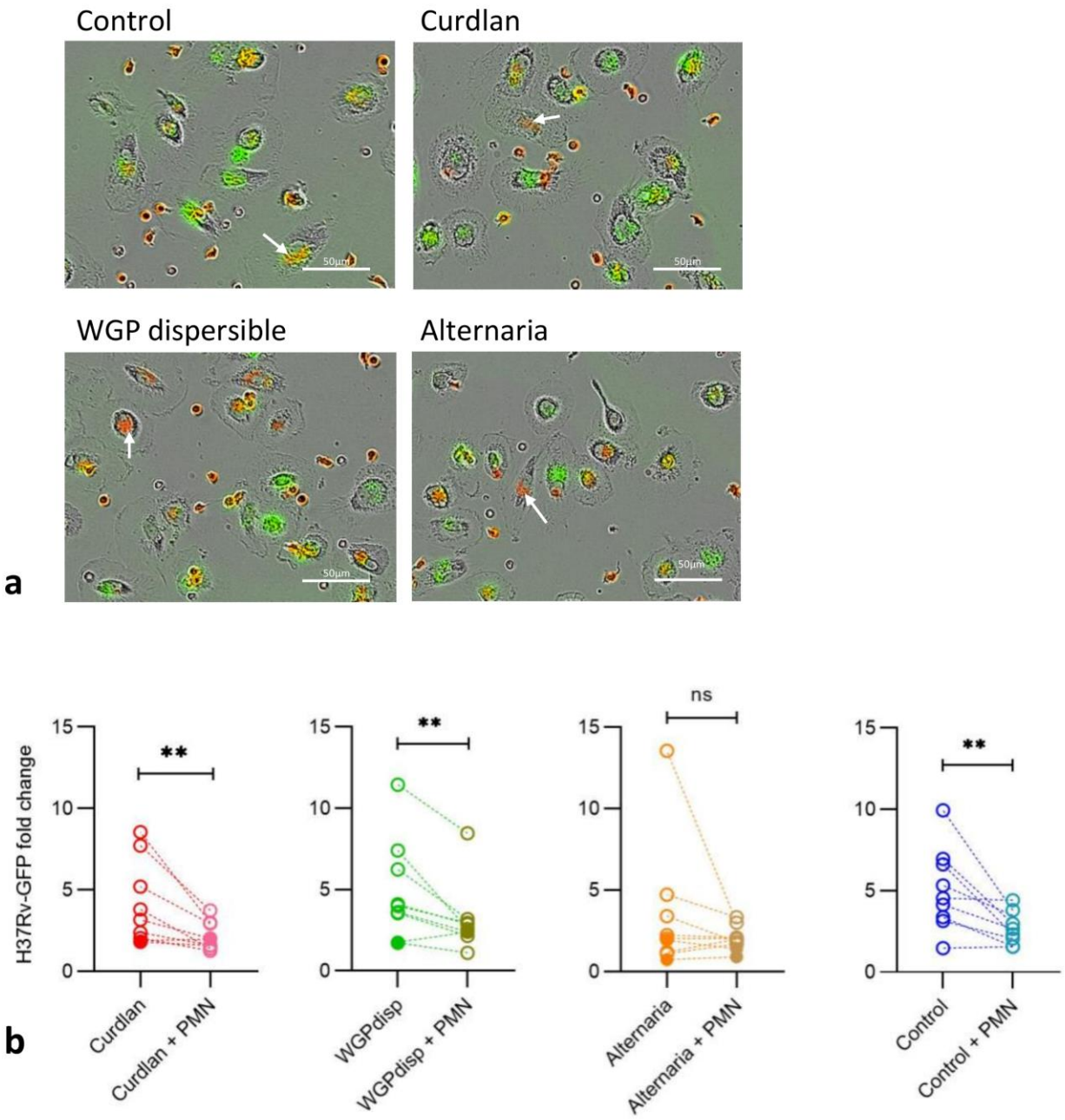
Fig 5. DNA methylation pathway analysis of macrophages trained with β glucan.

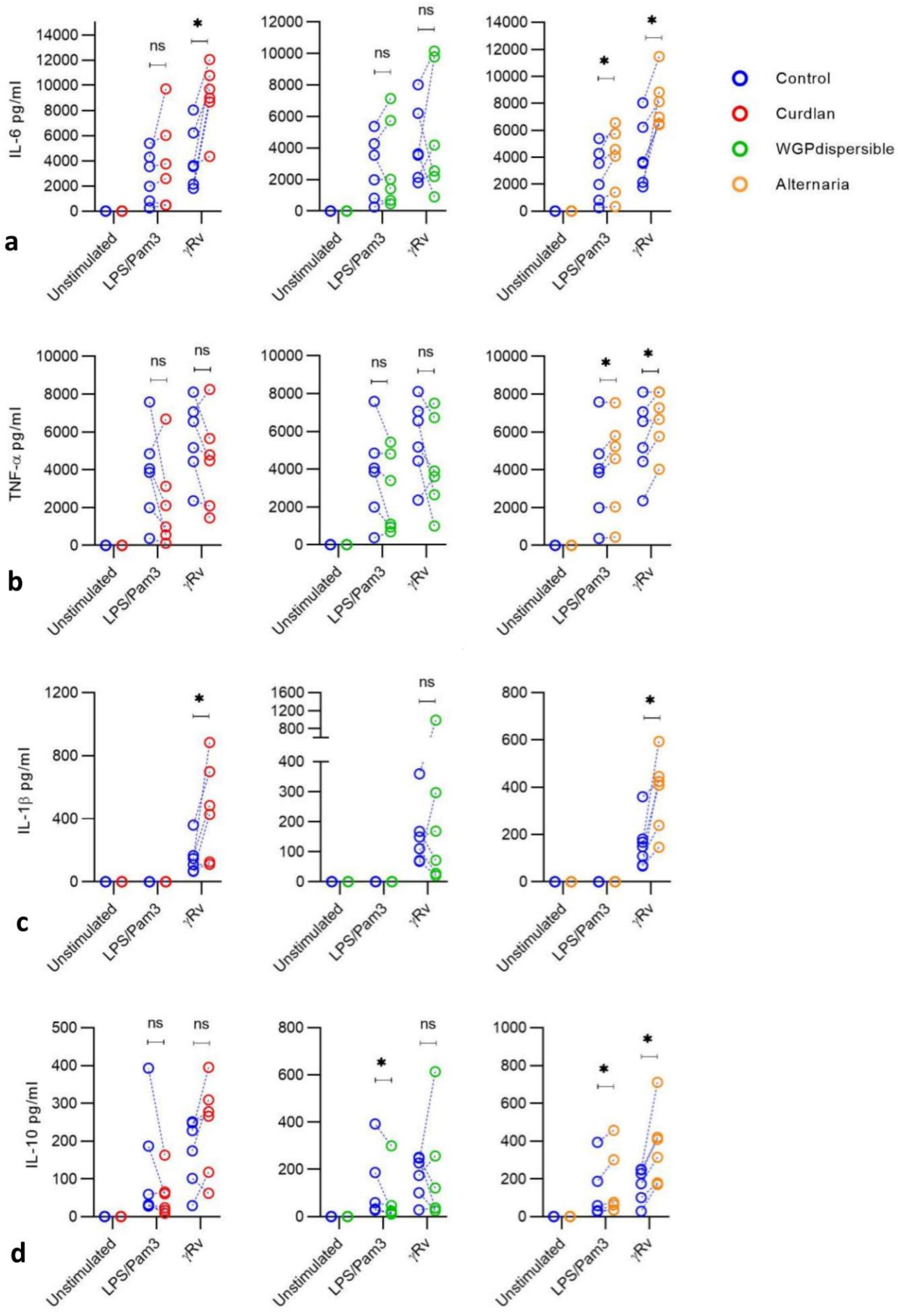
Macrophages from 7 donors, trained with **a** curdlan, **b** WGP dispersible or **c** Alternaria for 24 hours, followed by 5 days wash out/resting period. Δβ values of differentially methylated genes were analyzed using the PANTHER Database. Pathways with enrichment in over 5 donors are shown in figure for each β glucan training agent. The size of the circle describes the number of genes from the input list present in the leading edge of the pathways genes, the color of the circle demonstrates the normalized enrichment score from the PANTHER Database, the circles' location on the x-axis describes the total number of genes involved in the pathway that was identified in each donor. For WGP dispersible trained cells donors are divided into 'responders' (green) and 'non responders' (orange) according to their ability to control growth of H37Rv.

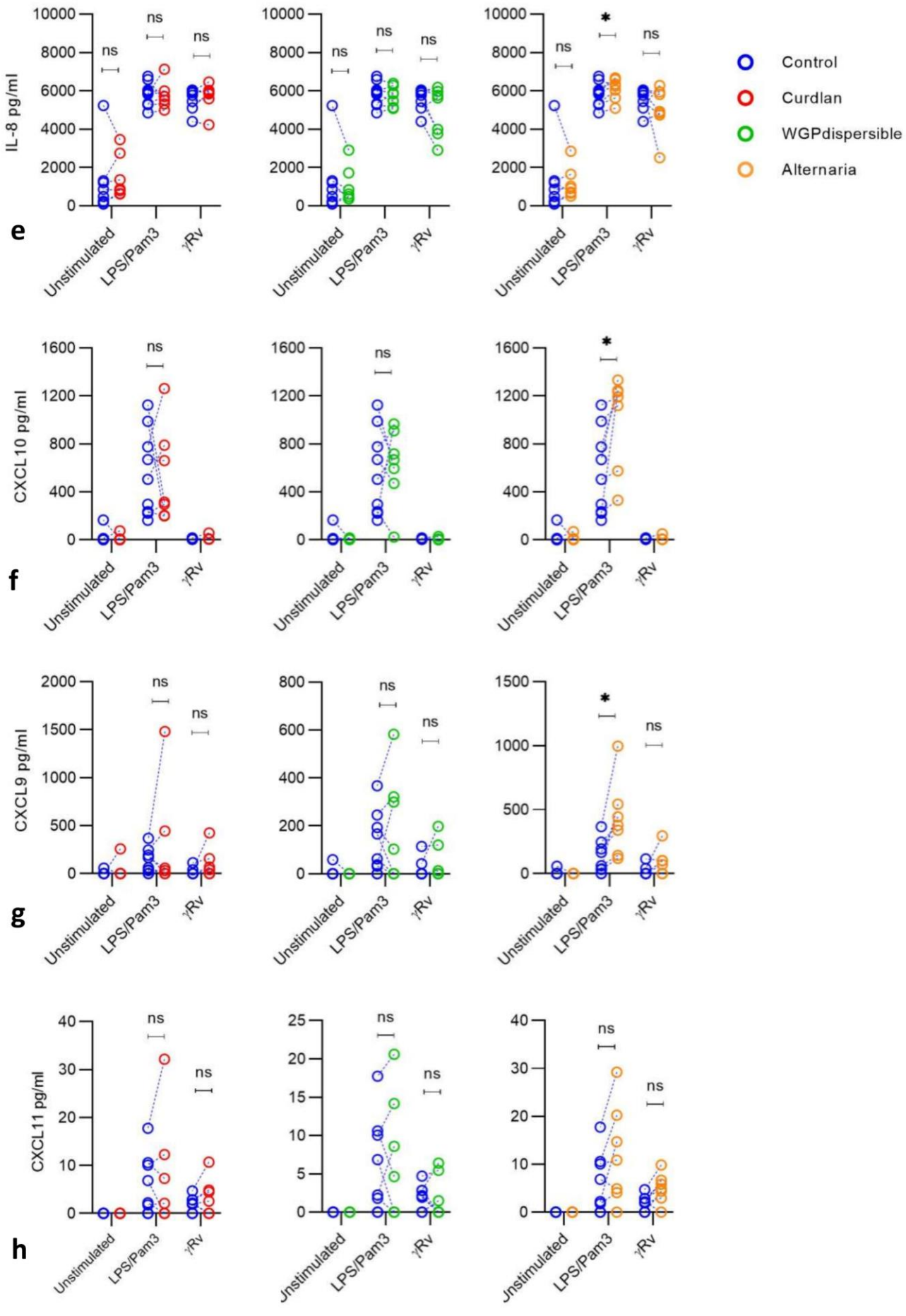


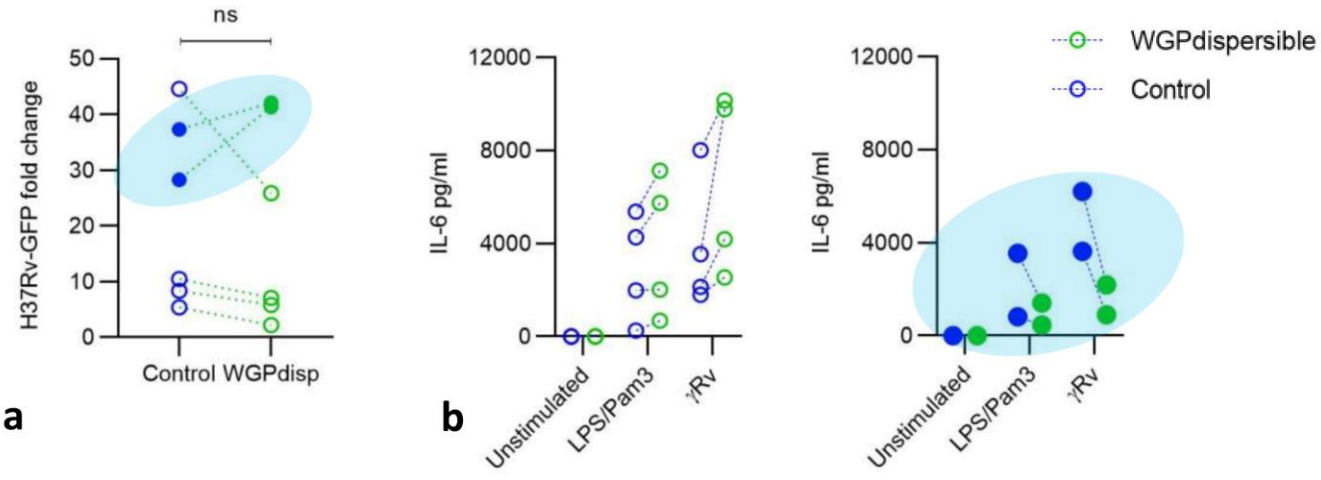












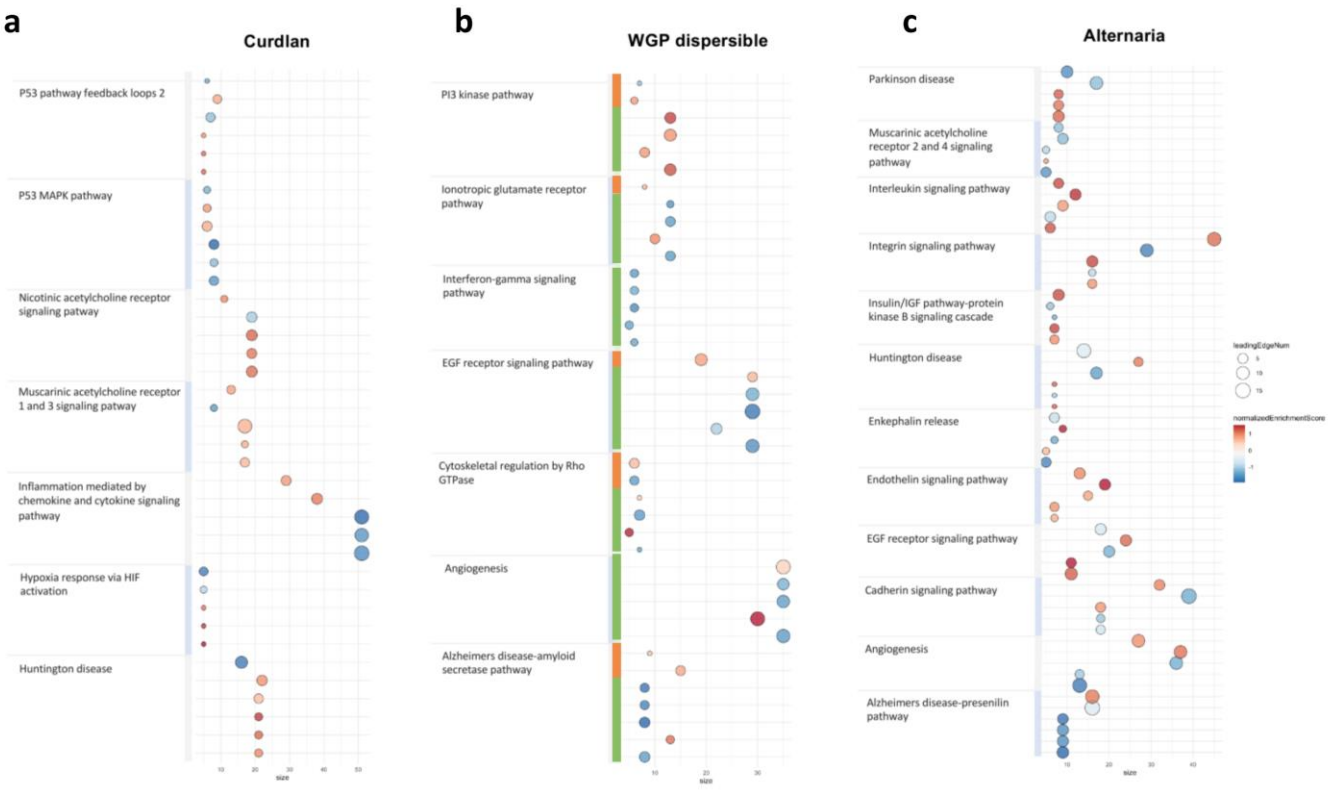


Table 1. β-glucan immune training agents

	Taxon	Species	Reference	Tested range	Selected conc.
Curdlan	Bacteria	*Alcaligenes faecalis*	[16]	0.1-10μg/ml	1μg/ml
WGP dispersible	Yeast	*Saccharomyces cerevisiae*	[14]	0.1-10μg/ml	10μg/ml
Alternaria	Fungi	*Alternaria*	[22]	1%-0.01%	0.01%