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The lack of defined correlates of protection hampers development of vaccines against tuberculosis (TB). In vitro mycobacterial outgrowth assays are thought to better capture the complexity of the human host/*Mycobacterium tuberculosis* (Mtb) interaction. We used a PBMC-based "mycobacterial-growth-inhibition-assay" (MGIA) to investigate the capacity to control outgrowth of Bacille Calmette-Guérin (BCG). Interestingly, strong control of BCG outgrowth was observed almost exclusively in individuals with recent exposure to Mtb, but not in (long-term) latent TB infection, and only modestly in BCG vaccinees. Mechanistically, control of mycobacterial outgrowth strongly correlated with the presence of a $CD14^{dim}$ monocyte population, but also required the presence of T cells. The nonclassical monocytes produced CXCL10, and CXCR3-receptor blockade inhibited the capacity to control BCG outgrowth. Expression of CXCR3 splice variants was altered in recently Mtb exposed individuals. Cytokines previously associated with trained immunity were detected in MGIA supernatants, and CXCL9, CXCL10, and CXCL11 represent new markers of trained immunity. These data indicate that CXCR3-ligands are associated with trained immunity and critical factors in controlling mycobacterial outgrowth.In conclusion, control of mycobacterial outgrowth early after exposure to Mtb is the result of trained immunity mediated by a CXCL10-producing non-classical $CD14^{dim}$ monocyte subset.

Mycobacterial Growth Inhibition is associated with trained innate immunity

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Abstract:

The lack of defined correlates of protection hampers development of vaccines against tuberculosis (TB). *In vitro* mycobacterial outgrowth assays are thought to better capture the complexity of the human host/ *Mycobacterium tuberculosis* (Mtb) interaction..

Here, we used a PBMC-based "mycobacterial-growth-inhibition-assay" (MGIA) to investigate the capacity to control outgrowth of Bacille Calmette-Guérin (BCG). Interestingly, strong control of BCG outgrowth was observed almost exclusively in individuals with recent exposure to Mtb, but not in (long-term) latent TB infection, and only modestly in BCG vaccinees. Mechanistically, control of mycobacterial outgrowth strongly correlated with the presence of a $CD14^{dim}$ monocyte population, but also required the presence of T-cells. The non-classical monocytes produced CXCL10, and CXCR3-receptor blockade inhibited the capacity to control BCG outgrowth. Expression of CXCR3 splice variants was altered in recently Mtb exposed individuals. Cytokines previously associated with trained immunity were detected in MGIA supernatants, and CXCL9, CXCL10 and CXCL11 represent new markers of trained immunity. These data indicate that CXCR3-ligands are associated with trained immunity and critical factors in controlling mycobacterial outgrowth.

In conclusion, control of mycobacterial outgrowth early after exposure to Mtb is the result of trained immunity mediated by a CXCL10-producing non-classical $CD14^{dim}$ monocyte subset.

(195 words)

Introduction:

Tuberculosis (TB) remains a major threat to human global health, with an estimated one fourth of the world population latently infected and lifetime reactivation rates between 3-10% (1). Annually approximately 2 million people die of TB disease, including those suffering from concomitant HIV infection (2). WHO aims to reduce the burden of TB disease by 2035 (2) and mathematical modelling predicts that successful novel vaccination strategies would be essential to reach that goal (3, 4). However, clinical evaluation of new TB vaccines is hampered by the long latency period following infection with *Mycobacterium tuberculosis* (Mtb) and the low proportion of infected individuals that eventually develop TB disease, thus necessitating large and long term follow-up studies to determine efficacy against clinical endpoints such as prevention of (or progression to) disease. Production of IFN-γ in response to Mtb antigen stimulation has been the golden standard of anti-Mtb immunity. Although IFN-γ is required, it is not sufficient for protection against TB disease development (5, 6), and preclinical studies even show that high IFN-γ can be detrimental such that "more can be worse" (7). Despite intense research efforts, correlates and mechanisms of (vaccine-induced) protection in TB have remained elusive, hampering rationalized correlate-based down-selection of the expanding list of novel vaccine candidates.

Mycobacterial growth inhibition assays (MGIA) measure the capacity of whole blood (8) or isolated peripheral blood mononuclear cells (PBMCs) to inhibit the outgrowth of mycobacteria, including Bacillus Calmette Guérin (BCG) (9-13) and Mtb (14). There is a long-standing interest in measuring and understanding the capacity of the immune system to control mycobacterial growth. Several biological assays to quantitate mycobacterial growth have been employed over the years, including the use of reporter (luciferase) expressing mycobacteria (9, 15, 16). In addition, Hoft et al employed primary as well as secondary lymphocyte inhibition assays followed by CFU counting (17-19). Wallis et al adapted mycobacterial cultures in the BACTEC MGIT system to investigate not only drug sensitivity but also effector immunity (20, 21). Over time, several aspects of these different

approaches have been integrated into the MGIA assay that is currently employed by several groups and includes infection of PBMCs with mycobacteria for several days, followed by incubation in BACTEC MGIT tubes to assess mycobacterial growth capacity (9, 10, 12, 22). Development and optimization of the MGIA assay as it is currently used have been recently reviewed *in extenso* (10, 23). MGIA assays have been used already for cells of human (9, 12, 24-26), non-human primate (NHP) (26) and mouse (11, 13, 14) origin.

Although these functional assays may reflect the capacity of individuals to control mycobacterial outgrowth, the underlying mechanism remains unknown. BCG vaccinated mice have reduced Mtb loads following infection compared to non-vaccinated animals, and this is paralleled by their splenocytes' capacity to reduce mycobacterial outgrowth in MGIA (11, 13), supporting the assay's biological validity. BCG vaccination has mostly been used in such studies, because BCG affords at least partial protection against severe manifestations of TB disease, and efficiently induces IFN-γ producing T-cells (9, 11, 13, 14, 25, 26). Although many researchers have attempted to identify the cellular subset(s) responsible for mycobacterial growth control, their identity remains inconclusive, and to some extent even elusive. Associations have been found with monocyte-lymphocyte ratios (24), multifunctional $CD4^+$ T-cells (25), and iron metabolism (26). Increased iron concentrations, in particular in whole blood based MGIA assays but also in PBMC assays, significantly promoted the *in vitro* growth capacity of mycobacteria, such that some caution should be applied to interpreting data from whole-blood based MGIA assays (26).

Mtb infection results in progression to disease in only a small proportion (3-10%) of those infected, while the majority of individuals apparently is able to control mycobacterial outgrowth (2). Recent meta analyses have shown that latent infection with Mtb (LTBI) results in a striking 79% protection against re-infection with Mtb, suggesting strong Mtb infection induced protective host responses (27). Although protection against infection is often thought to be associated with the induction of

adaptive immune responses, a novel recent concept suggests that epigenetic modification of monocytes may also contribute to protection (28). This phenomenon has been termed 'trained immunity', and hallmark cytokines produced by these trained monocytes are IL-1β, IL-6 and TNF-α (28, 29). To explore this biological process and to uncover new biology and corresponding correlates of mycobacterial inhibition, we decided to investigate the capacity of PBMCs from cohorts of BCG vaccinees, as well as individuals with LTBI, patients with active TB, and persons from contact investigations following recent TB exposure. PBMC samples with unexpectedly strong control of mycobacterial outgrowth were observed. These were subsequently used to unravel the mechanism of mycobacterial growth control.

Results:

MGIA in BCG cohorts: limited and transient bacterial growth control.

Mycobacterial growth inhibition assays provide an important and hypothesis free read out of functional (bacterial) growth inhibition by the human immune system. However, in order to apply such functional assays in further research and eventually for rationalised TB vaccine selection, it is essential to optimise the assay towards high reproducibility and robustness. Utilizing a frozen stock of BCG and an extensive thawing procedure, we were able to obtain solidly reproducible standard curves across experiments (Supplementary Figure 1A). Serial dilutions were generated as standard curves for each independent experiment and each standard was plated in a 10-fold dilution series (Supplementary Figure 1D) to obtain CFU counts. Cumulative CFU counts over 9 individual experiments were highly reproducible (Supplementary Figure 1B), as were the time-to-positivity (TTP) read-outs from the BACTEC over the same dilutions (Supplementary Figure 1C).

BCG vaccination has been widely used for almost a century, but the protection it affords in human populations has been highly variable, likely depending on population geography and previous exposure to environmental factors. We analysed a cohort of young, mycobacterially naïve (PPD negative) Dutch adults before and at several time points after BCG vaccination (30). We observed considerable variation in mycobacterial growth inhibition between these individuals during follow up time points, and there appeared no single common time point at which mycobacterial outgrowth across all participants was significantly improved following vaccination (Figure 1A). However, we noticed that several individuals had considerable growth control at 4 weeks post vaccination (left panel), whereas others controlled BCG outgrowth only at 8 (middle) or 12 weeks (right panel) post vaccination, suggesting different kinetics in the response (30). Therefore, we compared the pre-vaccination MGIA result with the results at the peak response for each individual (4, 8 or 12 weeks post vaccination), and then observed a significant inhibition of mycobacterial outgrowth (Figure 1B). It was remarkable that samples with significant control at one time point (e.g. week 4 or 8) had lost

that capacity at the next sampling time point, which was only 4 weeks later (Figure 1A), suggesting a limited role of imprinted adaptive immunity. Thus, although there was significant BCG growth reduction following BCG vaccination, the magnitude of BCG growth control was rather modest and transient.

LTBI and TB infection: recent exposure associated with strong bacterial growth control.

Given the observation that LTBI protects significantly against TB (see above) (27), we decided to assess the capacity of PBMCs from several Mtb infected cohorts to control BCG outgrowth. We also included TB patients with active disease as well as a control group comprised of healthy, mycobacterially naïve Dutch donors. Altogether, patients with active TB did not control BCG outgrowth in the MGIA assay (Figure 1C), although we could discriminate 2 'subgroups' with a different functional response. We have critically analysed the available clinical parameters of these groups, including disease localization (pulmonary vs. extrapulmonary localization), ethnicity, and suggestion of long-term existing disease, but we were not able to identify any parameter associated with good or poor inhibition of BCG outgrowth.

As only a proportion of latently infected individuals progresses to disease, the capacity to inhibit mycobacterial outgrowth may be expected to characterize the majority of latently infected donors, especially those that have been latently infected for a long period without developing disease. Contrary to our expectation, however, a cohort of latently infected individuals from the Netherlands (31) as well as a group of elderly Norwegian individuals (32, 33) known to be latently infected with Mtb for about 3 decades (based on TST conversion and IGRA positivity) failed to inhibit BCG outgrowth in the MGIA assay (Figure 1D & E). The LTBI cohort from the Netherlands was comprised of individuals with a positive tuberculin skin test (TST), and most individuals also had a positive Quantiferon TB Gold (QFN) response., For most individuals in our LTBI cohort it is hard to estimate the time since Mtb infection, and since our BCG vaccination studies also revealed a temporal

inhibition in BCG growth following vaccination, we hypothesized that mycobacterial growth control might be strongest recently after mycobacterial (TB) exposure.

Next, in order to study this directly, we included samples from 3 fully independent TB contact investigations in the Netherlands: these involved a professional soccer club (34), a supermarket outbreak (35-37), and a group of immigrants with recent (re-)exposure to Mtb (38, 39). Interestingly, all 3 cohorts considerably controlled BCG outgrowth in the MGIA assay (Figure 1F), with delta median values around 1 log. To the best of our knowledge, this is the highest level of mycobacterial control reported for human PBMCs so far. Combination of all independent cohorts, previously shown above, according to infectious status, showed that recently exposed individuals inhibited BCG outgrowth best (Figure 1G). Good control, defined as more than 1 log reduction compared to the control population, was almost only observed in donors that were recently exposed. Intermediate control (between the lower confidence interval (CI) value and 1 log reduction of the median of the controls) was observed most frequently in recently exposed individuals but also in BCG vaccinated individuals as well as a subset of TB patients. Taken together, most individuals in the recently exposed group had intermediate or good control, whereas only a small proportion of individuals in the other groups showed such BCG growth inhibition.

The capacity to control BCG outgrowth was compared to clinical measures of Mtb infection status, the TST and QFN, both measures of induced adaptive immunity. Surprisingly, PPD induced TST induration correlated inversely with the capacity to control BCG outgrowth in the MGIA (Supplementary Figure 2A). However, this was mostly due to the large proportion of individuals that had negative skin test results and relatively good MGIA control. If these individuals were excluded from the analysis the negative correlation was lost. There also was no positive correlation between the absolute QFN result and BCG growth control (Supplementary Figure 2B). However, also here a

considerably sized group of individuals lacked cellular immunity towards Mtb antigens while having good control of BCG outgrowth in the MGIA.

Immune correlate analysis: $CD14^{dim}$ monocytes correlate with MGIA control.

To decipher the mechanism of mycobacterial growth control mediated mostly by the samples from recently exposed individuals, we analysed associations with several immune cell subsets and functions. The percentage of $CD19^+$ B-cells correlated with the capacity to control BCG in the MGIA, as higher proportions of B-cells were associated with decreased CFU (Figure 2A). In contrast, the percentage of $CD3^+$ T-cells was inversely related to control in the MGIA (Figure 2B). Since it was previously reported that multifunctional T-cells were associated with MGIA results (25), we also enumerated multifunctional T-cells in our samples (gating strategies are given in supplementary Figure 7A,B). However, the frequency of $CD4^+$ T-cells producing IFN-γ, TNF-α and IL-2 was very low in our cohorts, although significant differences were identified (Figure 2C). The same was true for $CD8^+$ multifunctional T-cells (supplementary figure 3A). There was no correlation between the frequency of $CD4^+$ multifunctional T-cells and the MGIA results (Figure 2D), nor was there for $CD8^+$ multifunctional T-cells (supplementary figure 3B). In addition, none of the single cytokine producing $CD4^+$ or $CD8^+$ T-cells (supplementary figure 3C and 3D) was associated with MGIA activity (supplementary figure 3E and F).

In contrast, however, analysis of different memory T-cell populations revealed a strong association ($P<0.0001$) of $CD4^+$ central memory (CM) T-cells with the capacity to control BCG outgrowth (Figure 2E) (gating strategies are given in supplementary Figure 7C). Both $CD4^+$ and $CD8^+$ effector cell frequencies were inversely related with MGIA control (Figure 2F) (all data in supplementary Figures 4A and 4B).

In addition to cells involved in adaptive immunity we also assessed monocytes, being the primary targets of BCG infection. The percentage of $CD14^+$ monocytes correlated significantly with the

capacity to control BCG outgrowth in the MGIA (p=0.0006) (Figure 3A). An even stronger correlation with MGIA activity was seen when expressing monocyte/lymphocyte (ML) ratios, which were obtained by dividing the number of $CD14^{+}$ cells by that of $CD3^{+}$ cells (p<0.0001)(Figure 3B). When analysing these CD14 data we noticed that particular samples had a very abundant $CD14^{dim}$ population (Figure 3C). Therefore, we analysed $CD14^{dim}$ and $CD14^{bright}$ populations separately and found that the $CD14^{dim}$ population was most abundantly present in individuals recently exposed to Mtb (Figure 3D). Interestingly, not the frequency of the $CD14^{bright}$ (Figure 3E), but that of $CD14^{dim}$ cells (p<0.0001)(Figure 3F) correlated with the capacity to control BCG outgrowth in the MGIA.

Thus we have identified a $CD14^{dim}$ monocyte subset that is increased upon recent exposure to Mtb, and correlates with the capacity to control BCG outgrowth.

Non-classical monocytes: associated with MGIA control and production of CXCL10.

$CD14^{dim}$ monocytes are non-classical monocytes that are further characterized by the expression of particular chemokine receptors. Indeed, $CD14^{bright}$ cells expressed increased levels of CD163, CCR2 and CX3CR1, but $CD14^{dim}$ cells expressed higher levels of CCR5, consistent with a non-classical monocyte subset phenotype (Figure 4A). Further work revealed that a large proportion of the $CD14^{dim}$ population produced the chemokine CXCL10. In contrast, TNF-α was generally produced by $CD14^{bright}$ cells (Figure 4B). Most importantly, the proportion of CXCL10 producing monocytes was associated with the capacity to control BCG outgrowth (Figure 4C). The capacity to control BCG outgrowth was not associated with CXCL10 producing $CD14^{bright}$ cells (Figure 4D), but to CXCL10 producing $CD14^{dim}$ cells (Figure 4E). The proportion of total $CD14^{+}$, $CD14^{bright}$ or $CD14^{dim}$ cells producing TNF-α did not correlate with the capacity to reduce BCG outgrowth (supplementary Figure 5 A,B,C).

Interestingly, follow-up of individuals exposed in the supermarket outbreak allowed longitudinal assessment of the capacity to control BCG outgrowth. The capacity to control BCG outgrowth persisted during the first 2 years post exposure, and time points with the strongest reduction in BCG

outgrowth contained the highest frequency of $CD14^{dim}$ monocytes (Figure 4F). Longitudinal follow-up was available only for the supermarket study and for the Dutch cohort of LTBI individuals. In the supermarket contact investigation with recent Mtb exposure, control in the MGIA was maintained throughout the 2-year follow up. Although strong control was achieved, this did not result in sterile eradication of the BCG in the MGIA assay (Supplementary Figure 6A). In the more remote LTBI cohort, however, no control was observed during follow up (Supplementary Figure 6B). In agreement with the above results, the supermarket group had increased ML ratios, increased frequencies of $CD14^{dim}$ cells, and increased frequencies of CXCL10-producing $CD14^{dim}$ cells, whereas none of these were observed during follow up of the LTBI cohort (Supplementary Figure 6C-H).

Thus, our functional and immunological analyses identify strong correlations between the capacity to control BCG outgrowth in the MGIA and the presence of a non-classical monocyte subset characterized by decreased expression of CD14, that produced CXCL10

Mechanistic involvement of CXCL10/CXCR3 signalling requires the presence of T-cells.

Since the frequency of CXCL10 producing $CD14^{dim}$ monocytes was strongly associated with MGIA control, we next investigated the concentration of CXCL10 and its closely related family member CXCL9 in the supernatants of the 4 day MGIA assay. CXCL9 and CXCL10 were present at highest levels in donors recently exposed to TB, although concentrations varied between individuals (Figure 4G). The third CXCR3 binding-family member, CXCL11, could not be detected in the same MGIA supernatants. CXCL9 and CXCL10 bind to CXCR3 as their cognate receptor. NBI-74330, a chemical CXCR3 receptor antagonist was added to the MGIA assay during the 4 days incubation.. Controls with the CXCR3 antagonist and BCG bacteria only, in the absence of PBMCs, revealed no difference in BCG outgrowth kinetics, excluding direct effects of the inhibitor on bacterial growth. Addition of the inhibitor to PBMCs from samples that controlled BCG outgrowth abrogated the reduction in BCG CFU, indicating that the CXCR3/CXCL10 axis is functionally involved in controlling BCG outgrowth (Figure 5A, raw data given in supplementary Table 1). Interestingly, PBMC samples that lacked BCG

outgrowth control showed increased capacity to control BCG outgrowth upon addition of the CXCR3 antagonist NBI-74330, to a small but significant extent (Figure 5B). Analysis of the supernatants of these co-cultures revealed a similar pattern in the production of CXCL10 at day of 4 of the cultures, suggesting a feedback loop between chemokine production and receptor function (Figure 5C).

These opposing effects of the CXCR3 antagonist in donors that controlled versus those that did not control BCG outgrowth prompted us to assess expression of CXCR3 receptor alternative splice variants. Interestingly, although these splice variants differ only in the N-terminal part of this 7-transmembrane G-protein coupled receptor, their intracellular effector coupling is different (40, 41): CXCR3A is an immune activating receptor and has the highest binding affinity for CXCL9, 10 and 11, whereas CXCR3B exerts a more inhibitory role and can also bind CXCL4 (42). The regulation of expression of each of the splice variants has not been resolved yet, but epigenetic modifications may play a role.

RNA samples were isolated from most PBMC samples (depending on cell yields) directly after thawing, without any stimulation and stored. Taqman quantitative RT-PCR was performed for both splice variants on all samples that were available. Recently exposed individuals had a decreased ratio of CXCR3A over CXCR3B, as a result of both increased CXCR3B and reduced CXCR3A expression (Figure 5D). LTBI donors showed a similar trend towards a decreased ratio, but this did not reach statistical significance.

The CXCR3 receptor is most abundantly expressed on T-cells, although it can also be expressed on monocytes, NK and B-cells. Since all MGIA data were obtained from total PBMCs we wondered if monocytes alone would be sufficient or whether the presence of T-cells was required. First, data were compared for the percentage of Tcm cells, since these cells typically express CXCR3 and may be responsible for the effector function (43). Since the presence of CXCL10 producing CD14dim monocytes seems a first prerequisite for BCG control, groups were subdivided based on the percentage of CXCL10 positive cells as determined in Figure 4E, using a cut-off of 3%. Indeed, donors

with good control of BCG outgrowth had higher frequencies of Tcm cells, suggesting that T-cells and/ or T-cell expressed CXCR3 is involved in control of BCG outgrowth (Figure 5E, F). Secondly, we performed an additional series of MGIA experiments, in which we included not only total PBMCs, but also isolated monocytes and monocytes supplemented with purified autologous T-cells. PBMCs from BCG controllers again inhibited outgrowth of BCG, which could be reversed with the CXCR3 antagonist as shown above (Figure 5G). However, isolated monocytes did not reduce the bacterial burden unless T-cells were added (Figure 5G). Thus, both monocytes and T-cells are required to control BCG outgrowth.

Trained immunity.

Since we observed control of BCG outgrowth mostly in donors that were recently exposed to Mtb and since control of BCG outgrowth was associated with a particular subset of monocytes, we evaluated whether trained innate immunity was responsible for the observed bacterial control. Trained immunity has previously been described in studies following BCG vaccination and *in vitro* restimulation with unrelated pathogen derived pattern recognition receptor (PRR)agonists (28, 29, 44). Cytokines that thus far have been reported as primary indicators of trained immunity are IL-1β, IL-6 and TNF-α. Samples from the MGIA assays of the above tested clinical groups were therefore analysed for the production of these cytokines. Confirming our hypothesis, all three were found at increased levels in recently Mtb exposed individuals (Figure 6A, B, C).

Next, in order to confirm that our above defined CXCR3-binding chemokines are indeed associated with trained immunity, we assessed their concentrations in supernatants from an independent cohort of recently BCG-vaccinated individuals in which 'classic' trained immunity human samples were obtained 4 weeks after vaccination. PBMC isolated from volunteers vaccinated with placebo or BCG were restimulated for 24 hours *in vitro* with heat-killed *Candida albicans, Staphylococcus aureus,* Mtb or lipopolysaccharide *(*LPS). Although CXCL9 was detected in these BCG-trained samples (Figure 6D), CXCL10 was much stronger upregulated (Figure 6E). Also CXCL11 was found to be

increased in the classical trained immunity samples (Figure 6F). These data show that CXCL9, CXCL10 and CXCL11 may be newly identified markers of trained immunity.

Additional cytokine and chemokine data were collected using a 40-plex bead array. We also analysed these cytokines and chemokines for their induction in both classically trained samples and in our recently exposed individuals. Intriguingly, next to CXCL9, CXCL10 and CXCL11 also IL-4, CCL8 (MCP2), CCL11 (eotaxin), CCL17 (TARC), CCL19 (MIP3β), CCL23 (MIP3), CCL25 (TECK), CCL26 (MIP4α), CCL27 (C-TACK) and CXCL13 (BLC) were identified as potential novel markers of trained immunity, deserving further research (Figure 6G).

Hence, we identified new markers of trained immunity and a series of candidate markers that deserve further investigation for their possible contribution to trained immunity effector mechanisms. The CXCR3 axis, involving both CXCL9 and CXCL10 as ligands, has a functional role in mediating mycobacterial outgrowth control in the context of trained immunity. Monocyte frequency, and in particular the $CD14^{dim}$ non-classical monocyte population that produced CXCL10 may serve as surrogate marker for BCG outgrowth control. However, it remains important to validate these findings in additional, independent cohorts, preferable comprised of recently exposed individuals. In addition, validation of these findings in human of NHP studies with novel vaccine candidates that induce protection when available would be extremely valuable. Detailed analysis of samples with strong control of bacterial outgrowth may contribute to the identification of novel correlates of protection.

Discussion:

The present study reports that mycobacterial growth inhibition can be measured in humans using a refined and robust MGIA assay. Contrary to our expectation, improved mycobacterial growth control was not associated with (Th1) cytokine producing (or polyfunctional) T-cells, but rather with the presence and activity of a non-classical $CD14^{dim}$ monocyte subset, but Tcm-cells were required for growth control. Individuals with recent, but not remote, exposure to Mtb had the strongest capacity to control BCG outgrowth, suggesting that a temporary change in effector immunity occurred that mediated growth control. Indeed, non-classical monocytes producing CXCL10 were found to be involved in BCG growth control through the CXCR3 receptor. Expression of CXCR3 splice variants, with immune activating or inhibiting functions, was altered upon recent Mtb exposure. Moreover, both CXCR3 ligands, CXCL9 and CXCL10, were also detected in samples of recently BCG-vaccinated individuals in which trained immunity was induced, suggesting that trained innate immunity was the mechanism behind control of BCG outgrowth in the MGIA following recent exposure to Mtb.

Mycobacterial growth control has been considered an important new readout of the capacity of the immune system to control BCG or Mtb, particularly because these assays are functional and do not require a specific *a priori* hypothesis regarding possible mechanisms involved. Since TB vaccines are designed to induce protective T-cell immunity, the responsible immune effector cells in the MGIA assay have also been presumed to be T-cell subsets. Recently, Smith *et al.* suggested an association between MGIA control capacity and the frequency of multifunctional $CD4^+$ T-cells, studying a small cohort of BCG vaccinated infants (25). However, these data could not be replicated in our much larger cohort of individuals from different settings. In our view, the frequencies of multifunctional T-cells observed in both Smith's and our study were extremely low; moreover, the negative control populations that lack both multifunctional T-cells and MGIA control skewed the trend lines strongly. Therefore, we conclude that multifunctional T-cells are not associated with the capacity of human PBMCs to control BCG growth, at least in our larger cohorts, but likely also in others. Previous

studies in mouse models of MGIA identified a role for IFN-γ in the control of BCG outgrowth (11). IFN-γ deficient mice had reduced growth control, but these effects were most clear in condition where low numbers of splenocytes and relatively high BCG inocula were used (11).

Also in line with this observation is our finding that a considerable proportion of individuals that lack cellular immune responses against Mtb, in TST and/or by IFN-γ production in QFN, controlled BCG outgrowth quite well. Similarly, two-third of recently exposed individuals from the soccer club contact investigation did not convert their TST. All TST negative individuals were tested by TST a second time 6 weeks after initial TST reading, but many remained negative. When comparing TST positive (≥10mm) and negative recently exposed individuals for their capacity to control BCG outgrowth in the MGIA, no differences were observed, both subgroups controlled BCG outgrowth equally well. We therefore hypothesized that these individuals may efficiently have controlled bacterial growth through innate immunity, which could imply that induction of adaptive immunity was not needed and the TST therefore remained negative.

The ML-ratio was very strongly associated with the capacity to control BCG outgrowth. Previous studies have also identified ML ratio as an important factor associated with the capacity to control in MGIA, however, a whole blood version of the MGIA was used in that study precluding a direct comparison with our data (24). Intriguingly, transcriptomic analysis of blood monocytes from those individuals identified IFN-γ amongst the top 5 upstream regulators, together with type I IFNs associated with early responses against intracellular infections (24). Interferons can have direct effects, but also augment effector responses through induction of several downstream inflammatory mediators such as interferon-inducible protein 10 (IP-10) or CXCL10. Thus, early activation of these transcriptomic pathways in monocytes may also result in activation of CXCL10 and some of its family members. Gene expression studies in BCG vaccinated mice already had identified CXCL9 as one of the most differentially expressed genes (12). Stimulation with BCG resulted in strong transcriptional

upregulation of CXCL9 as well as CXCL10, next to TNF-α, IL-1β and IL-6 (12), fitting perfectly with our trained immunity hypothesis. Also in studies with the novel vaccine candidate MVA-Ag85A, CXCL10 was the top differentially transcribed gene at day 1 post vaccination both in South African infants as well as in UK adults (45). Vaccinated infants at day 28 also expressed more CXCL9 and CXCL10 compared to their pre-vaccination sample (45). Monocyte signatures were most abundantly expressed and correlated with increased T-cell responses to vaccination (45). These indicate that early activation of innate immune cells, with production of their respective cytokines, is critical for guiding the adaptive immune system.

Unexpectedly, non-classical $CD14^{dim}$ monocytes were found to correlate strongly with the capacity to control BCG outgrowth. This cell population was abundantly present in individuals with recent TB exposure and strong MGIA growth reduction. Non-classical monocytes have been associated with a predominantly scavenging and sensing function within the immune system, in particular in case of intracellular viral infections (46). These cells may also be involved in local tissue surveillance and have been linked to autoimmune diseases (46). Non-classical monocytes are efficient recognizers of extracellular TLR ligands, but also of immune complexes. TLR triggering did not induce ROS or cytokine production by $CD14^{dim}$ cells, but triggered specific production of TNF-α, IL-1β and CCL3, *e.g.* by virus induced TLR7/8 triggering (47). TLR7/8 triggering also induced CXCL10 production by these cells, perhaps via a slightly different signalling pathway, since MYD88 was not essential for CXCL10 production, whereas it is required for TNF-α and IL-1β production (47). In addition, next to their ability to release multiple cytokines, $CD14^{dim}$ cells are also highly motile, in agreement with their patrolling function, but are relatively weak phagocytes (47).

Intracellular analysis of several cytokines and chemokines revealed that the $CD14^{dim}$ monocyte population produced the CXCR3 ligand CXCL10, and CXCL10 production correlated strongly with BCG growth reduction. Indeed, CXCR3 receptor blockade using a chemical antagonist confirmed mechanistic involvement of the CXCR3 signalling pathway in BCG growth reduction. CXCR3 can bind

not only CXCL10, but also CXCL9 and CXCL11. These ligands were not present in our intracellular staining panel, but CXCL9, not CXCL11, was abundantly detected in supernatants. It remains to be seen whether a single or rather a combination of CXCR3 ligands is responsible for mediating BCG growth reduction. The effect of these chemokines may further depend on the balance in expression of splice variants of CXCR3, since in recently Mtb exposed individuals a decreased ratio of CXCR3A over CXCR3B was observed. Although monocyte alterations were strongest associated with changes in control of BCG outgrowth, the presence of T-cells was found to be indispensable to mediate ultimate BCG growth control by monocytes. In any event, we think this is the first molecular identification of a host effector molecule responsible for mycobacterial growth inhibition, next to granulysin (48, 49).

Since our data suggested that the cellular ability to control mycobacterial growth was associated with recent exposure to Mtb, was transient, and was linked to the (non-classical) monocyte phenotype and (CXCR3 signalling related) function, we hypothesized that this ability may have been acquired as a result of trained innate immunity by epigenetic changes in the myeloid cell lineage (28, 50). Trained immunity refers to the altered functional state of innate immune cells that persists after elimination of the initial stimulus, and allows an increased response to restimulation of the cells through both the same and different pattern recognition receptors (50, 51). In humans, we (MN) have shown that BCG vaccination induces trained immunity (28, 50). Indeed, cytokines previously associated with trained immunity such as TNF-α, IL-1β and IL-6 were also increased in the current study in individuals with recent exposure to Mtb, suggesting trained immunity in these samples. Importantly, samples from previous trained immunity studies were shown in the current study to contain large amounts of CXCL10, and also CXCL9 and CXCL11, thus bridging these previous trained immunity studies now with our TB cohort observations reported in the current study, and newly identifying CXCL9 and CXCL10 as markers of trained immunity. Future studies into the epigenetic

alterations of these genes will be required to fully establish these chemokines as novel trained immunity markers.

Trained immunity associated cytokines were detected in the majority of individuals with recent exposure to Mtb, but only in a limited number of individuals with more remote LTBI or TB disease. This suggests that Mtb infection induced training of monocytic cell lineages is a temporary phenomenon during the first period after infection. This is in line with our earlier observation that BCG vaccination exerts strong trained immunity effects for several months, but these effects diminish one year after vaccination (29). It is difficult to estimate the duration of persisting trained immunity after recent exposure, in particular in Mtb infection since in many cases the exact timing of infection is unknown. However, the contact investigation in the supermarket allowed identification of the presumed infectious period and included longitudinal follow up (37). Somewhat to our surprise, most contacts from that outbreak maintained the capacity to control BCG in the MGIA during the 2 years of follow up from initial exposure. The lifespan of circulating monocytes is estimated to be in the magnitude of days, thus implying that training must occur at the monocytic progenitor level to allow longer lasting training effects. Mycobacteria have been found in human and mouse stem cells (52) in the bone marrow, and it is possible that this niche could be involved in training innate immune cells. Future studies in animals would be needed to demonstrate a causal link between functional reprogramming of myeloid cell progenitors and trained immunity in TB.

BCG-induced trained innate immunity may underlie some well-known observations such as that infants vaccinated with BCG are protected to secondary infections against unrelated pathogens (53). Epidemiological studies suggested that these effects can last for several months until up to possibly multiple years (54). Circulating monocytes with trained immunity functional phenotypes have been detected at 3 and 12 months following BCG vaccination (29). Epidemiological evidence has furthermore indicated that prior latent TB infection strongly protects against development of active

TB upon re-infection (27). Moreover, BCG vaccination has the highest efficacy in the absence of prior sensitization or pre-existing infection (55). In analogy with these epidemiological findings, experimental vaccination studies revealed that MGIA growth reduction is induced by priming BCG vaccination but not upon secondary BCG vaccination (9). The mechanisms behind this protective efficacy in primary infection/ vaccination has not been resolved, but may be explained by continuous innate immune training, which is capable of limiting unrelated as well as related bacterial growth upon reinfection. Obviously, while this new hypothesis will need to be corroborated further in suitable animal models such a NHP, collectively these observations point to an important role for innate and trained immunity in protective immunity against mycobacterial infections. These initial events are likely also important in guiding and steering the adaptive response, which is key to optimal protection as well. How exactly these different phenomena are causally, mechanistically and temporally related remain topics for further study.

Recently, a mouse MGIA study was published that showed control of Mtb Erdman outgrowth in vaccinated mice (14). However, also mice vaccinated with the CAF01 adjuvant only showed such control, which was interpreted as an artefact. However, this observation could also fit with the induction of trained immunity. Moreover, the authors were unable to associate growth control with the number of cytokine-producing vaccine-specific T-cells and found that there was no persistence of growth reduction at 29 weeks post vaccination (14). These data in fact fit well with the actual induction of temporary trained innate immunity as we report here.

Trained immunity has been described for monocytes. However, also other circulating cells may be instructed as a result of pathogen induced training. MGIA control was not only associated with monocytic cell populations but also with B-cells and $CD4^+$ central memory cells. In contrast, numbers of total $CD3^+$ T-cells as well as $CD4^+$ and $CD8^+$ effector cells inversely correlated with mycobacterial growth control.CXCR3 expression is most abundant on CD4 memory T-cells and T-cells were required

to mediate BCG growth control as isolated monocytes were unable to control BCG outgrowth. These data suggest an interplay between T-cells and monocytes in the control of BCG outgrowth through the CXCL10-CXCR3 axis. It has previously been reported that the $CXCR3^{+}CCR6^{+}$ memory T-cell population contained the vast majority of anti-Mtb reactive Th1-cells, and these rather unusual Th1 cells have been coined Th1* (43, 56). Thus not only innate cells but also CXCR3 expressing Mtb specific T-cells may be directed by CXCL10 produced by trained monocytes to mediate Mtb specific immunity. An additional cell population in which trained immunity has been shown to be induced by BCG vaccination are NK-cells (57). The importance of trained NK cells for protection has also been suggested by a recent clinical trial in which correlates of protection after BCG vaccination have been reported to be not $CD8^{+}$ T-cells, but IFN-γ-producing NK-cells (58).

Although MGIA has been developed in principle as *in vitro* measure of vaccine induced protection, our data now show that growth control in the MGIA was the result of trained innate immunity, rather than of effector T-cell induction. However, T-cells appear to be involved in execution of growth control. To date, BCG is the only vaccine that induced partial protection against TB in humans and is well known to induce trained innate immunity. However, multiple new vaccine candidates are currently in clinical testing and it is hard to speculate if these vaccines induce protection in humans and whether these utilise (different) effector mechanisms. Therefore, we recommend future studies that analyse mechanisms of protection against mycobacteria to take into account not only effector T-cell induction but also trained innate immunity.

Originally, trained immunity was demonstrated measuring the production of IL-6, TNF-α and IL-1β upon stimulation. Here, we identify CXCL9 and CXCL10, and potentially CXCL11, as new markers of trained immunity. Moreover, our measurements of cytokines and chemokines in supernatants of recently exposed individuals as well as classical trained immunity samples revealed a number of additional candidate molecules induced during trained immunity: IL-4, CCL8 (MCP2), CCL11

(eotaxin), CCL17 (TARC), CCL19 (MIP3β), CCL23 (MIP3), CCL25 (TECK), CCL26 (MIP4α), CCL27 (C-TACK) and CXCL13 (BLC). A considerable number of these chemokines are associated with the macrophage inflammatory response, supporting an association with trained immunity. However additional studies are required to demonstrate their functional role in trained immunity.

In conclusion, we have shown that MGIA control in human PBMCs is associated with recent Mtb infection. Control of BCG outgrowth was strongly linked to the presence of a non-classical, $CD14^{dim}$ monocyte subset. The combination of the association with recent exposure, the temporary nature of growth reduction and the presence of a particular monocyte subset led to the identification of trained innate immunity as the mechanism behind mycobacterial growth control. Indeed we were able to identify classical markers of trained innate immunity induced in mycobacterial control samples. In addition, we mechanistically link the CXCL10-CXCR3 axis as critical to BCG growth control as well as to trained immunity. Involvement of the CXCR3 axis may reflect the link between innate and adaptive cells involved in mycobacterial growth control. These studies have opened novel avenues to assess protective immunity against a major human pathogen, and may redirect our thinking on vaccine induced immunity and correlates of protection in TB.

Materials and Methods

An extended version of the materials and methods is available as supplementary information.

Ethics and study samples

Human participation in this research was according to the U.S. Department of Health and Human Services and Good Clinical Practice guidelines. This included protocol approval by the Leiden University Medical Center Ethics Committee and written informed consent by all donors. Anonymous buffy coats from healthy blood bank donors were only used if donors had consented scientific use of blood products. Specifically, healthy control donor samples were *in vitro* PPD negative healthy buffy coats obtained from Sanquin blood transfusion services, The Netherlands. The group of healthy donors was supplemented with healthy Dutch donors prior to BCG vaccination, after extensive testing of their mycobacterially naïve status. These donors were subsequently BCG vaccinated and analysed at 4, 8, 12 weeks and 1 year post BCG vaccination; the BCG vaccination study was approved with protocol number P12.087 (30). A longitudinal follow up study of individuals with latent TB infection (LTBI) was also run at LUMC and approved under protocol number P07.048 (31). In addition, a cohort with known long-term TB latency, and age-matched uninfected controls was recruited in Norway after approval by the Regional Committees for Medical and Health Research Ethics in Norway (protocol number P027/99) (32, 33).

In addition, we obtained specific permission (protocol number B16.002) from the ethical review board at Leiden University Medical Center to use archived samples remaining after completion of previous studies, in particular contact investigations at a soccer club (original protocol number 136/97)(34), in a supermarket (original protocol number P05.053)(35-37) and amongst immigrants with a recent TB contact (original protocol number P04.114)(38, 39).

Finally, BCG vaccination studies in healthy male, volunteers were executed in Nijmegen, approved by the Arnhem-Nijmegen Medical Ethical Committee with protocol number NL50160.092.24.

Experimental design

Archived PBMCs were thawed, rested and counted. Cells were split for BCG-based MGIA as well as direct stimulation with live BCG for flowcytometry including intracellular cytokine staining. Remaining cells were dispensed in Tri-zol reagents. Supernatants were collected after 4 days of co-culture in the MGIA system. Upon analysis of MGIA data as well as flowcytometry data, cytokine and chemokine measurements were initiated and functional experiments were designed.

Statistical analysis

All data were analysed assuming a non-Gaussian distribution and therefore non-parametric testing was applied. All legends contain detailed information on the statistical tests applied and the number of individuals included for analysis. Generally, when comparing groups Kruskal-Wallis or Wilcoxon-paired rank tests were applied depending on the number of groups in the equation. For the analysis of longitudinal data an ANOVA with Friedman test was applied. Correlations were based on linear regression modelling. All analyses were performed using Graph Pad prism version 7.00, Graph Pad Software Inc.

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Author contributions:

SAJ, KEvM, THMO designed the study, analysed data and wrote the manuscript. KEvM performed the experiments. SMA provided important clinical advice to the study and collected samples from various clinical cohorts. CP was responsible for sample collection in many of the clinical cohorts utilized in the study. MGN, RJWA, RvC provided samples and contributed to study design. FO, GEK and SVK provided clinical samples utilized in the study.

The authors have no conflict of interest to report.

Data will be available upon request to the corresponding author.

Figure 1

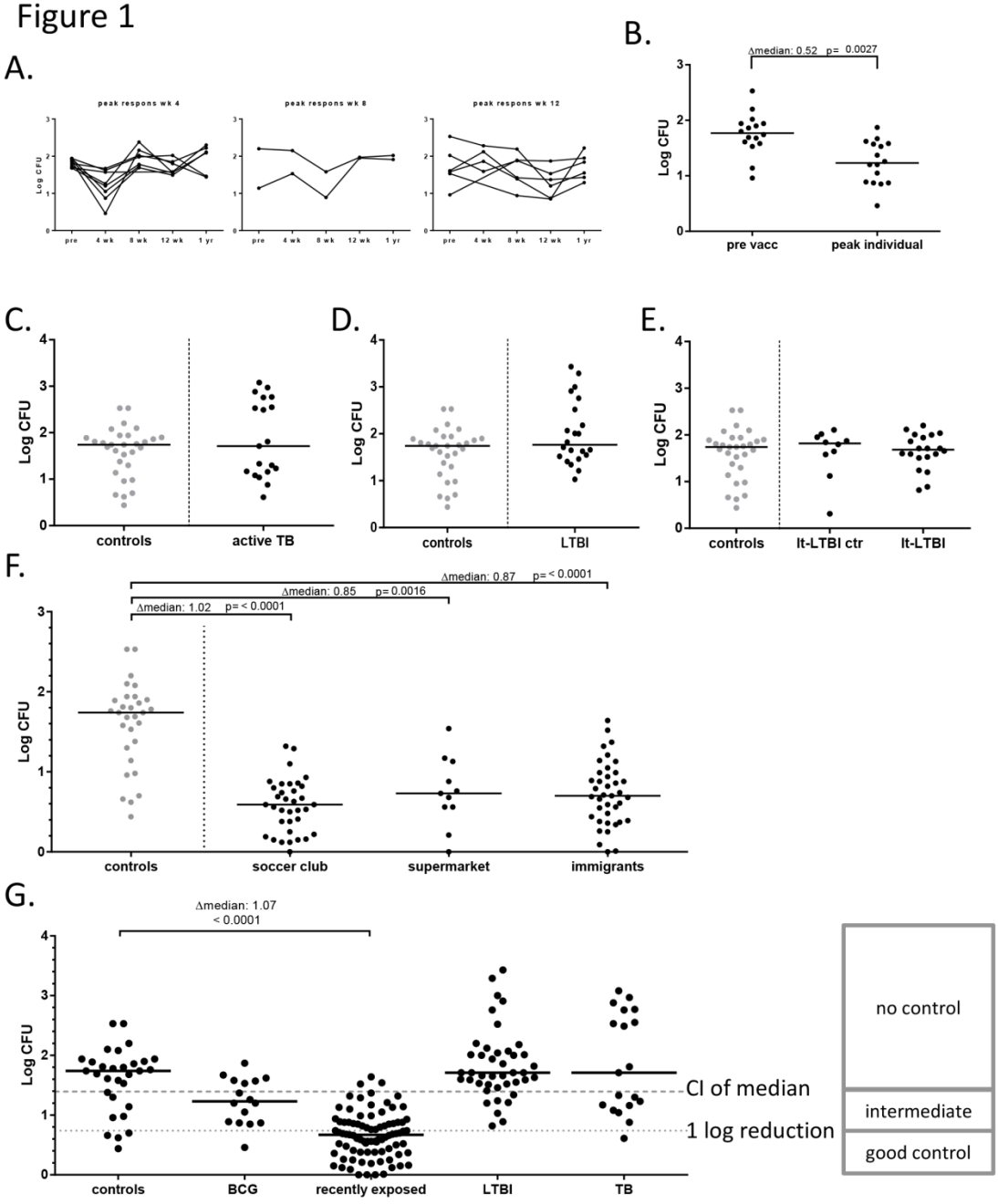


Figure 1: **Mycobacterial growth control is associated with recent TB exposure**

PBMCs were infected with live BCG, rotated 4 days and incubated in MGIT tubes in the BACTEC machine. Horizontal lines indicate medians. Δ median indicates the difference in medians between groups.

A. PBMCs collected prior to BCG vaccination, 4, 8 and 12 weeks post vaccination and 1 year post-vaccination. Left plot indicates donors with the strongest reduction in BCG growth at week 4 (n=8), middle at week 8 (n=2) and right at week 12 (n=6).

B. For each individual from Fig1A the time point with maximal MGIA control was compared to the pre-vaccination sample and compared using the Wilcoxon paired rank test.

C. All groups in Figures 1C-G were compared to a common group of 30 controls: 16 individuals prior to BCG vaccination and 14 bloodbank donors using the Kruskal-Wallis test. Patients with active TB disease (n=19) .

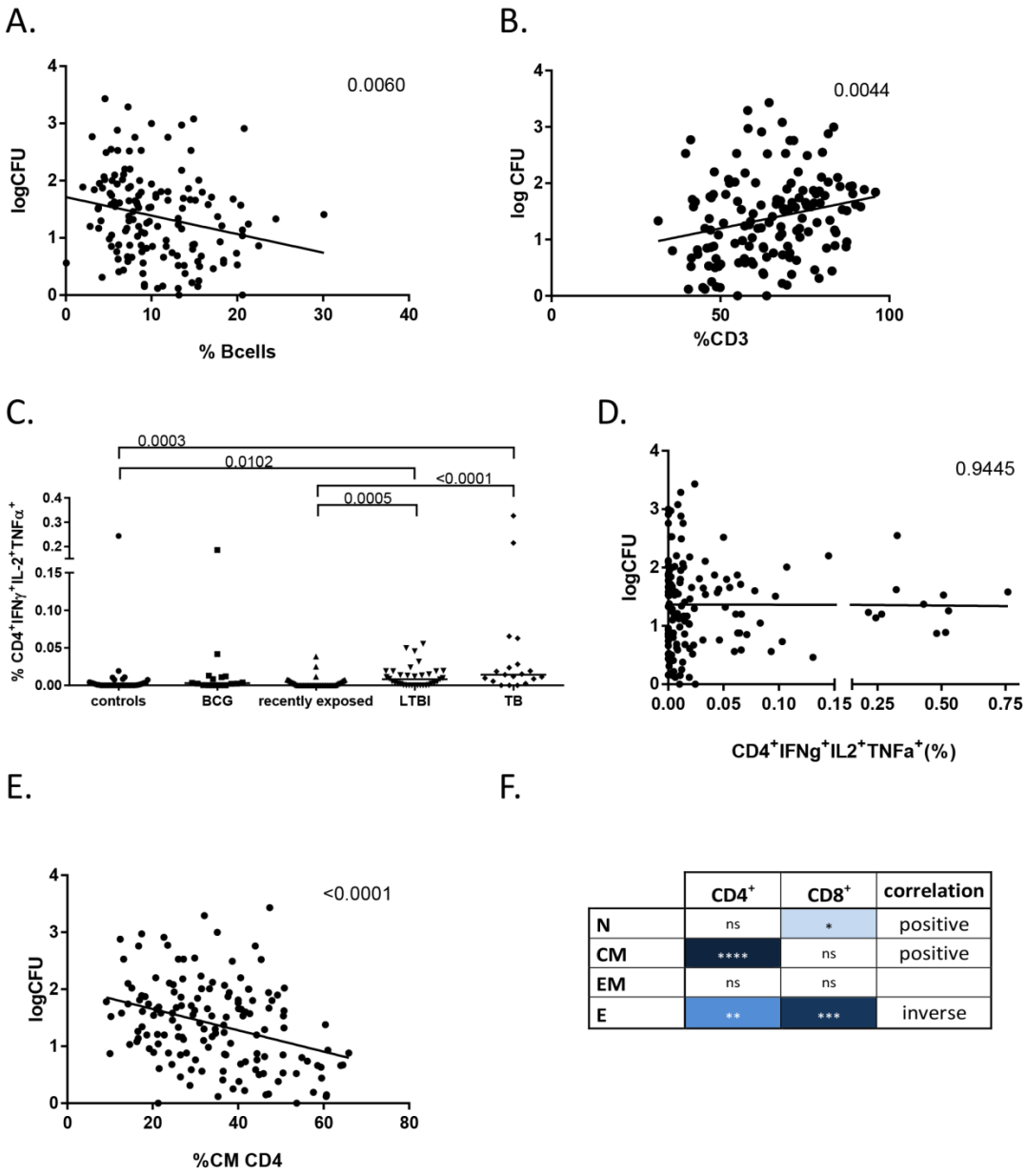
D. Individuals with latent TB infection (n=22) .

E. Individuals with long-term latent TB infection (lt-LTBI; n=20). An age-matched control group (lt-LTBI ctr, n=10) was included

F. Three independent cohorts of recently TB exposed individuals collected during contact investigations, comprised of 35 individuals at a Soccer Club, 11 individuals in a supermarket, 39 immigrants with recent exposure to active TB.

G. Combined analysis of all groups tested: BCG (n=16 at peak response); recently exposed (from 1F: n=85); LTBI (from 1D + E: n=42), active TB (from 1C) . Data were compared using the Kruskal-Wallis test. Dashed lines indicate the lower limit of the confidence interval of the median of the controls, respectively the 1 log reduction compared to this median. Samples above the CI of median lacked control of mycobacterial outgrowth, samples between this lower limit and 1 log reduction featured intermediate control and samples with more than 1 log reduction had good control.

Figure 2



F.

	CD4+	CD8+	correlation
N	ns	*	positive
CM	****	ns	positive
EM	ns	ns	
E	**	***	inverse

Figure 2: **Mycobacterial growth control is not associated with multifunctional T-cells**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine. Antibody marker staining for FACS analysis was performed after 16 hours of negative control or live BCG stimulation. Data from all independent cohorts were combined. Associations were determined using linear regression modelling (n=144).

A. The percentage of $CD19^+$ B-cells was positively correlated with mycobacterial growth control.

B. The percentage of $CD3^+$ T-cells was correlated inversely with mycobacterial growth control.

C. The percentage of $CD4^+$ T-cells producing IFN-γ, IL-2 and TNF-α (multifunctional T-cells) following 16 hours of BCG stimulation over the different clinical groups. Groups were compared using the Kruskal-Wallis test. Higher numbers were observed in LTBI and TB groups. Controls n=38, BCG n=16, exposed n=50, LTBI n=35, TB n=19.

D. The percentage of $CD4^+$ T-cells producing IFN-γ, IL-2 and TNF-α did not correlate with the capacity to control mycobacterial outgrowth (n=144).

E. The percentage of $CD4^+$ central memory (CM, $CD45RA^-CCR7^+$) T-cells correlated with the capacity to control mycobacterial outgrowth (n=148).

F. Summary of correlation data between $CD4^+$ or $CD8^+$ T-cell subsets based on the expression of CD45RA and CCR7, with the capacity to control mycobacterial outgrowth. Positive correlations indicate increased expression of the population is associated with better growth control, inverse correlations indicate increase in the population is associated with decreased growth control.

Figure 3

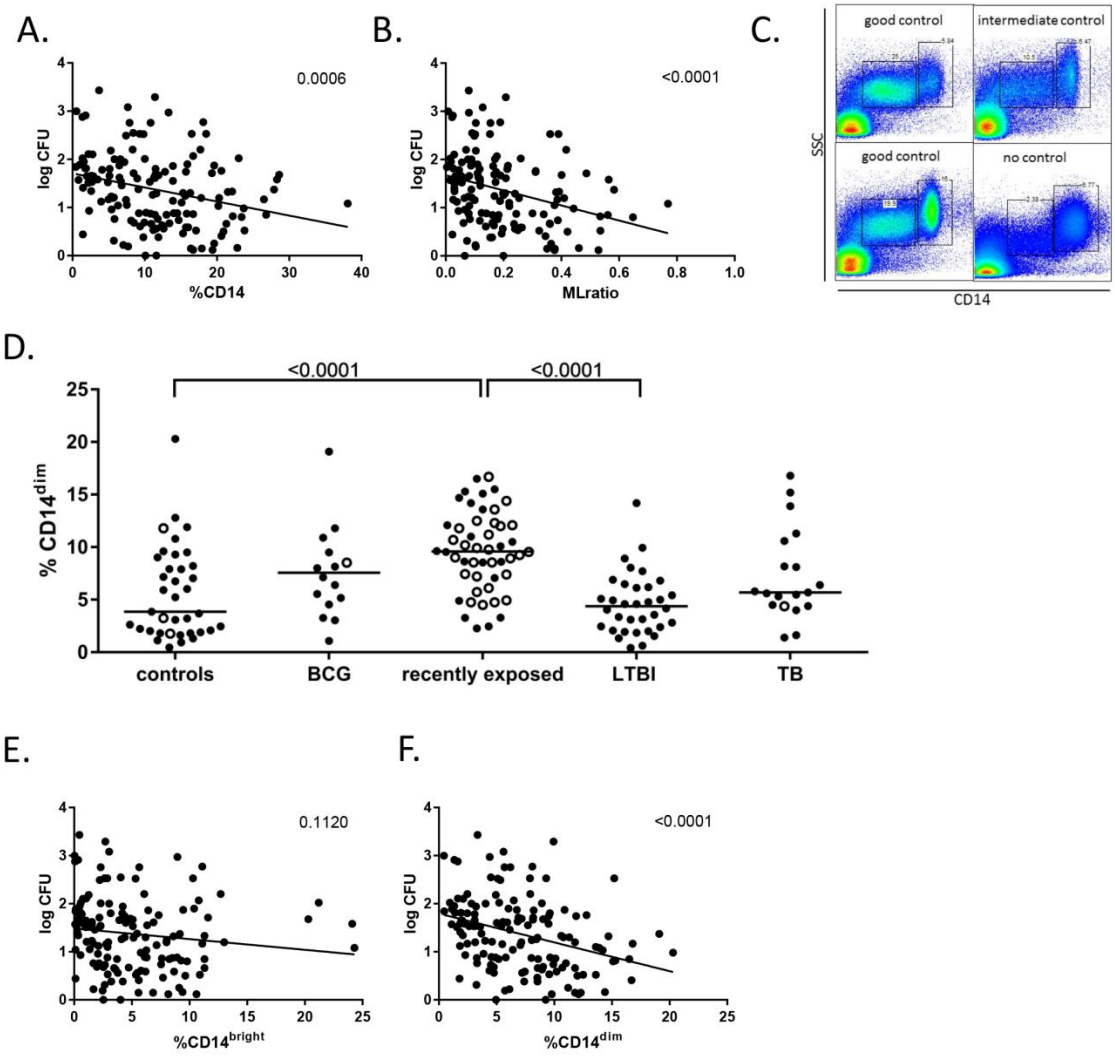


Figure 3: **Capacity to control mycobacterial growth is associated with a CD14^{dim} myeloid population**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine Antibody marker staining for FACS analysis was performed after 16 hours of negative control stimulation. Associations were determined using linear regression modelling (n=144).

A. The percentage CD14^{+} monocytes was positively correlated with mycobacterial growth control.

B. The ML ratio (monocyte/lymphocyte ratio, %CD14/CD3) correlated with mycobacterial growth control.

C. Representative dot-plots of monocyte profiles in individuals with good mycobacterial growth control (more than 1 log reduction of BCG outgrowth compared to the median of the control population), intermediate control (between 1 log reduction and lower limit of the CI of median of control population, see Fig 1G) or no control (above lower limit of the CI of median of control population). There was a strong $CD14^{dim}$ population in the individuals with mycobacterial growth control.

D. Recently exposed individuals had the highest proportion of $CD14^{dim}$ cells, open symbols indicate donors with good control in the MGIA. Controls n=37, BCG n=16, exposed n=50, LTBI n=34, TB n=19.

E. The percentage of $CD14^{bright}$ monocytes was not correlated with mycobacterial growth control (n=144).

F. The percentage of $CD14^{dim}$ monocytes was positively correlated with mycobacterial growth control (n=144).

Figure 4

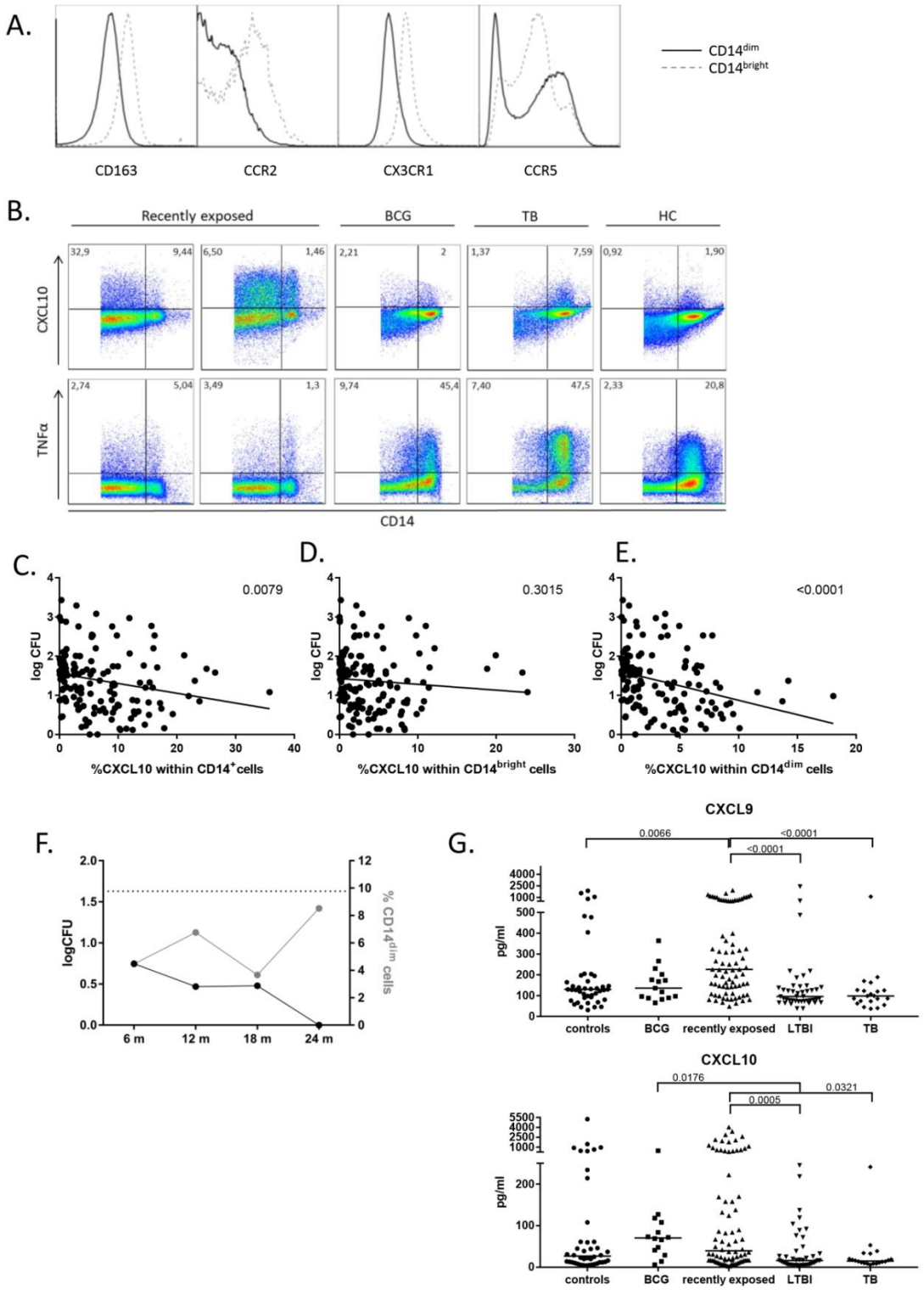


Figure 4: **Non-classical monocytes produce CXCL10 and are involved in mycobacterial growth control**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine. Antibody marker staining for FACS analysis was performed after 16 hours in the absence of specific stimulation in the presence of BFA. Associations were determined using linear regression modelling and a $p<0.05$ was considered significant.

A. Concatenate gate of $CD14^{dim}$ and $CD14^{bright}$ cells for monocyte subset markers revealed a non-classical monocyte expression pattern of the $CD14^{dim}$ population consisting of low CD163, low CCR2, high CX3CR1 and high CCR5 expression.
B. CXCL10 (upper row) and TNFα (bottom row) production by monocytes in recently exposed individuals, a BCG vaccinated donor, a TB patient and a healthy control donor.
C. The percentage of total monocytes-derived CXCL10 was positively correlated with the capacity to control mycobacterial outgrowth (n=144).
D. The percentage of $CD14^{bright}$ cells producing CXCL10 did not correlate with mycobacterial growth control (n=144).
E. The percentage of $CD14^{dim}$ cells producing CXCL10 correlated strongly with mycobacterial growth control (n=144).
F. Longitudinal follow up of individuals from the supermarket contact investigation revealed increasing levels of growth control which coincided with an increased percentage of CXCL10 producing $CD14^{dim}$ cells (line indicates mean of 8 samples).
A. Supernatants of 4 days MGIA co-cultures were tested for cytokine and chemokine production using a multiplex bead array. Shown is CXCL9 and CXCL10 production and CXCL9 production, expressed as pg/ml, over the clinical groups. Groups were compared using the Kruskal-Wallis test. Controls n=43, BCG n=15, exposed n=79, LTBI n=44, TB n=21.

Figure 5

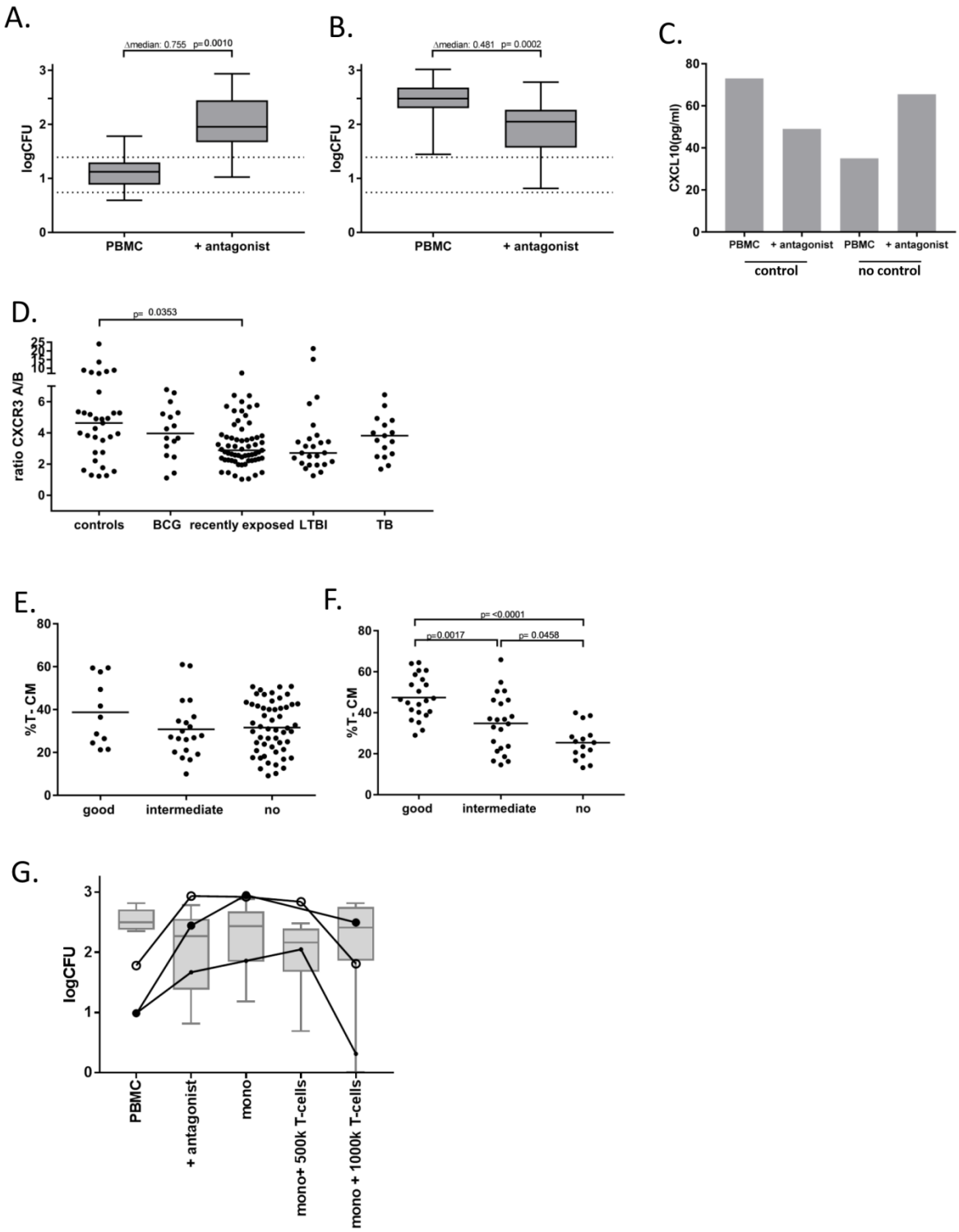


Figure 5: **CXCR3 is key in determining mycobacterial outgrowth control**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine.

A. A CXCR3 receptor antagonist (NBI-74430) was added daily during the MGIA incubation period to inhibit activity of CXCL10, this reverted the capacity to control mycobacterial outgrowth. Data represent 11 donors with MGIA control run in 2 independent experiments, differences were assessed using the Wilcoxon matched-pairs signed rank test.

B. Similar to A, addition of the CXCR3 receptor antagonist during the MGIA assay. Data represent 27 donors without MGIA control run in 3 independent experiments, differences were assessed using the Wilcoxon matched-pairs signed rank test.

C. CXCL10 production was measured in supernatants of the MGIA assay at day 4 of co-culture in samples with MGIA control or no MGIA control, in the absence or presence of NBI-74430, the CXCR3 antagonist. Data are expressed as median.

D. CXCR3 splice variants were determined by real-time quantitative RT-PCR. A ΔCt was calculated for both CXCR3A and CXCR3B using the in-well GAPDH control. Subsequently, the ratio of CXCR3A over CXCR3B was calculated and plotted. Data were compared using the Kruskal-Wallis test. Controls n=33, BCG n=16, exposed n=63, LTBI n=26, TB n=16.

E. The percentage of Tcm cells was plotted for donors with a low frequency of $CD14^{dim}$ monocytes producing CXCL10 (<3%). Good (n=11), Intermediate (n=20), no (n=54).

F. The percentage of Tcm cells plotted for donors with a high frequency of $CD14^{dim}$ monocytes producing CXCL10 (>3%), groups were compared using the Kruskal-Wallis test. Good (n=22), Intermediate (n=22), no (n=15).

G. MGIA assay with PBMCs and isolated fractions of monocytes with or without T-cells. Cells were separate and combined in various ratios before infection with live BCG and 4 day co-culture. Boxes and whiskers indicate donors that lack MGIA control (n=8), lines indicate 3 individual donors with MGIA control.

Figure 6

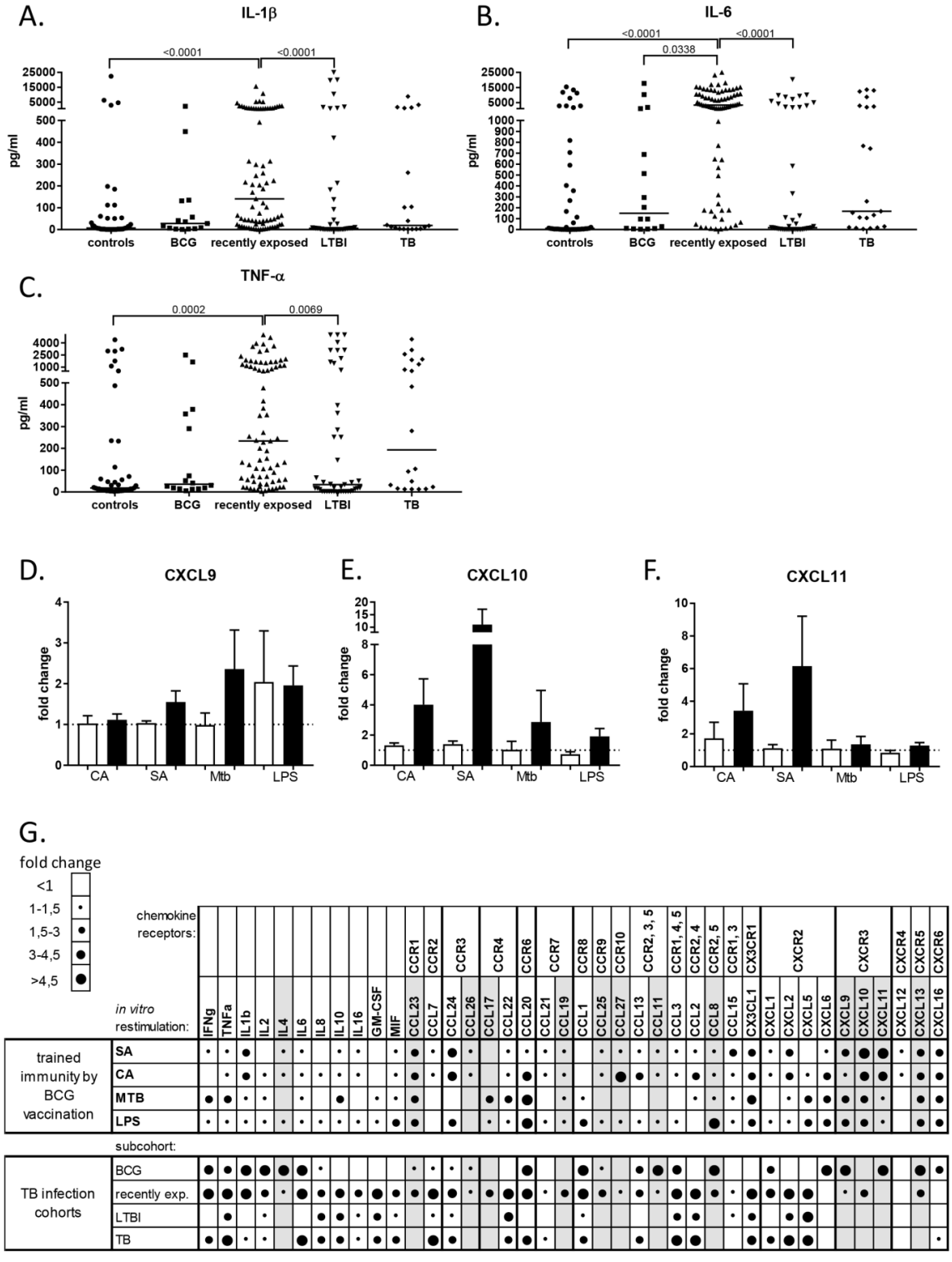


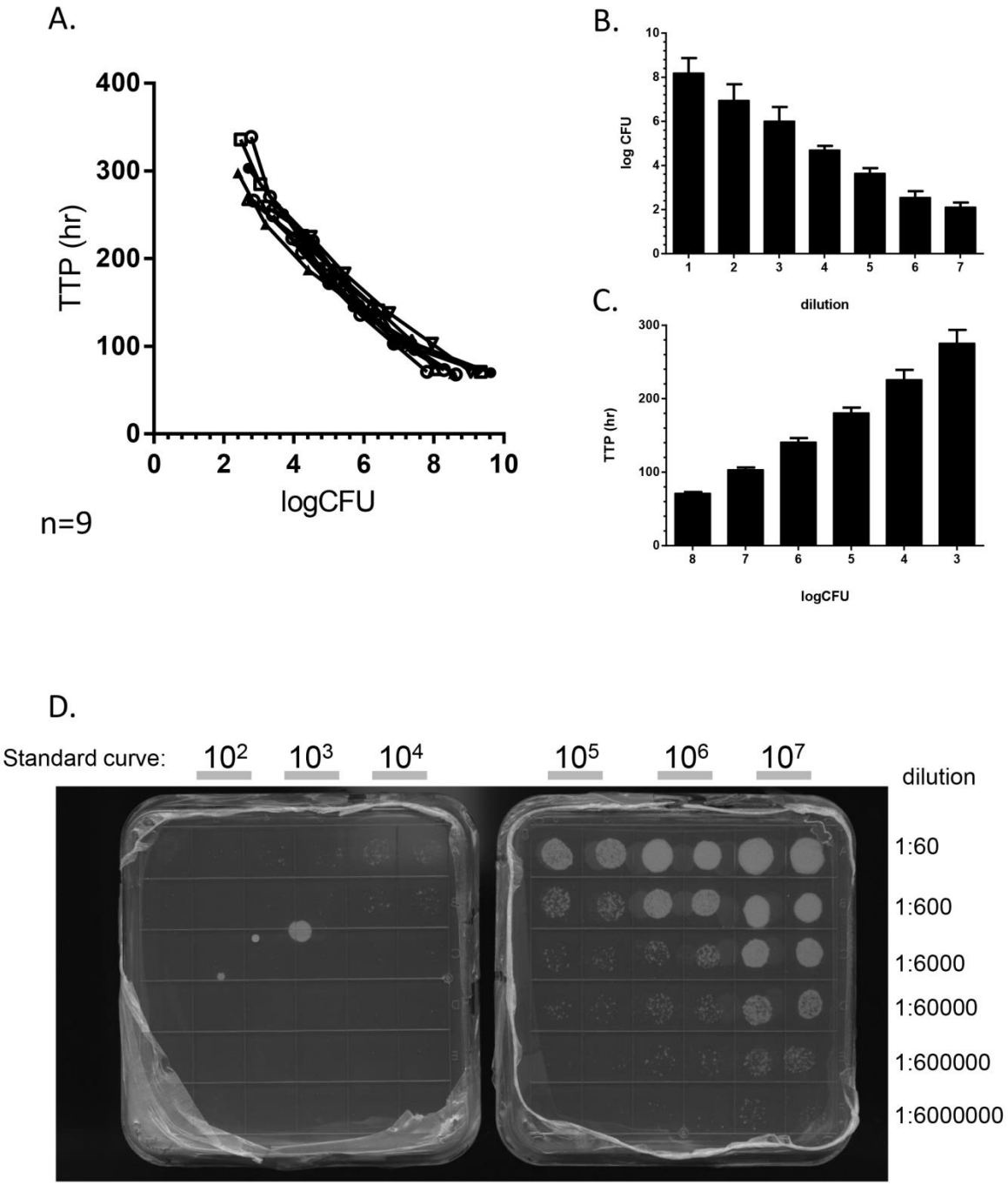
Figure 6: **CXCL10 production is a novel marker of trained immunity**

Supernatants from the 4 day co-culture with BCG were collected and cytokine/ chemokine production determined using a multiplex bead array. In addition, samples were collected from *in*

vivo, BCG-vaccinated individuals and stimulated *ex vivo* (restimulation with microbiological agents). Groups were compared using the Kruskal-Wallis test (A-C).

A. IL-1β (as classical Trained Immunity marker) production was assessed in 4 day culture supernatant. Controls n=43, BCG n=15, exposed n=79, LTBI n=40, TB n=20.
B. IL-6 (as classical Trained Immunity marker) production was assessed in 4 day culture supernatant. Controls n=43, BCG n=16, exposed n=79, LTBI n=44, TB n=21.
C. TNF-α production (as classical Trained Immunity marker) was assessed in 4 day culture supernatant. Controls n=42, BCG n=16, exposed n=78, LTBI n=45, TB n=20.
D. CXCL9 (as new Trained Immunity marker) was measured in supernatants of *ex vivo* stimulated PBMCs, black bars indicate BCG vaccinated donors (n=10), open bars indicate placebo vaccinated donors (n=5). Results are expressed as fold change of 4 weeks post-vaccination over the pre-vaccination time point.
E. CXCL10 (as new Trained Immunity marker) was measured and expressed similarly as in D.
F. CXCL11 (as new Trained Immunity marker) was measured and expressed similarly as in D. .
G. Summary of all cytokine and chemokine data collected in the multiplex bead array analysis using supernatants from individuals with trained immunity following BCG vaccination (top part; vaccinated donors only) and samples from individuals from TB infection cohorts from the current study (bottom parts). Dot sizes indicate the fold change compared to prevaccination for the trained cohort and fold change vs. the control population in our clinical TB infection groups. Grey filled cytokines and chemokines are potentially associated with trained immunity.

Supplementary Figure 1



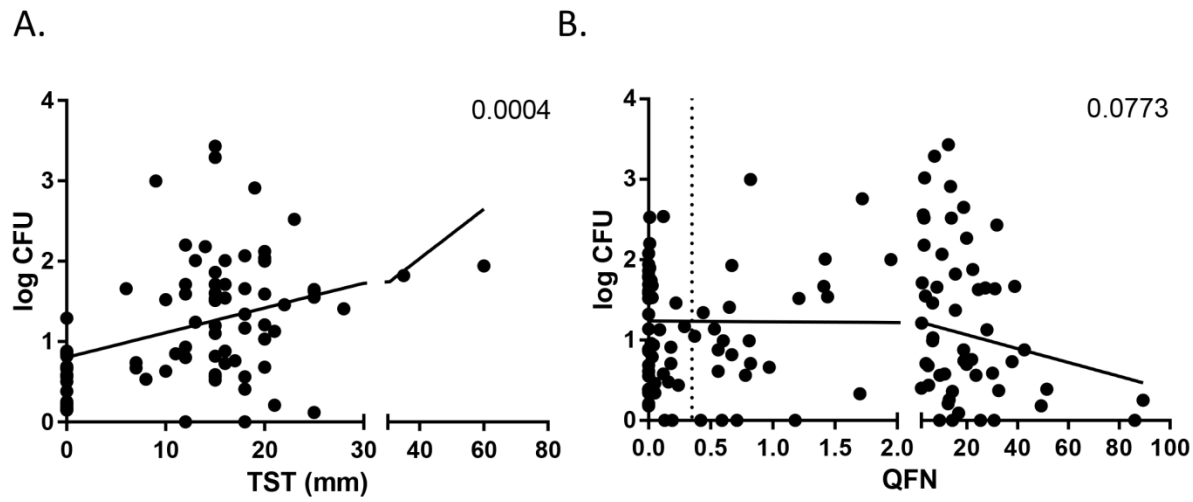
Supplementary Figure 1: **Reproducibility of standard curves in MGIT system using frozen standardized batch of BCG**

In all MGIA assays BCG was used from a single frozen stock. In each individual assay one vial was thawed using serial washes with Tween80 and diluted to generate standard curves. Dilutions were added to BACTEC MGIT tubes and incubated in the BACTEC machine until positive (TTP).

Simultaneously, all dilutions were plated on 7H10 and colony forming units were counted after 14-21 days.

A. Relation between CFU and TTP for the 9 standard curves used in this study.
B. CFU of the different dilutions of the standard curve; plotted are the mean + SD CFU for each condition out of 9 experiments.
C. Mean + SD CFU out of 9 experiments vs. TTP for all dilutions of the standard curve.
D. Scan of CFU plate of MGIA standard curve, diluted 10-fold. All samples are spotted in duplicates. CFU are determined by counting spots in highest countable dilution using high-resolution scanned images and ImageJ software.

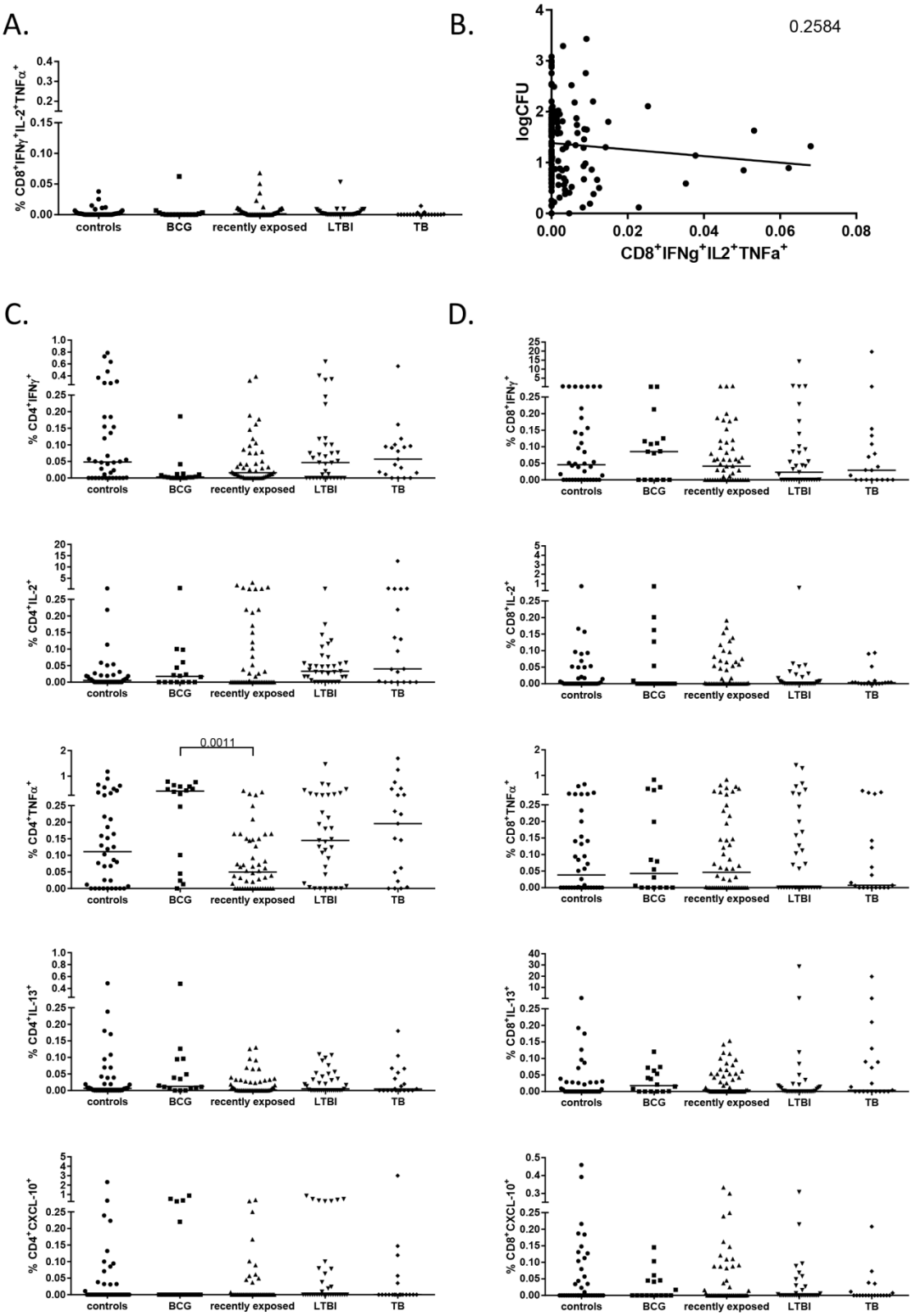
Supplementary Figure 2

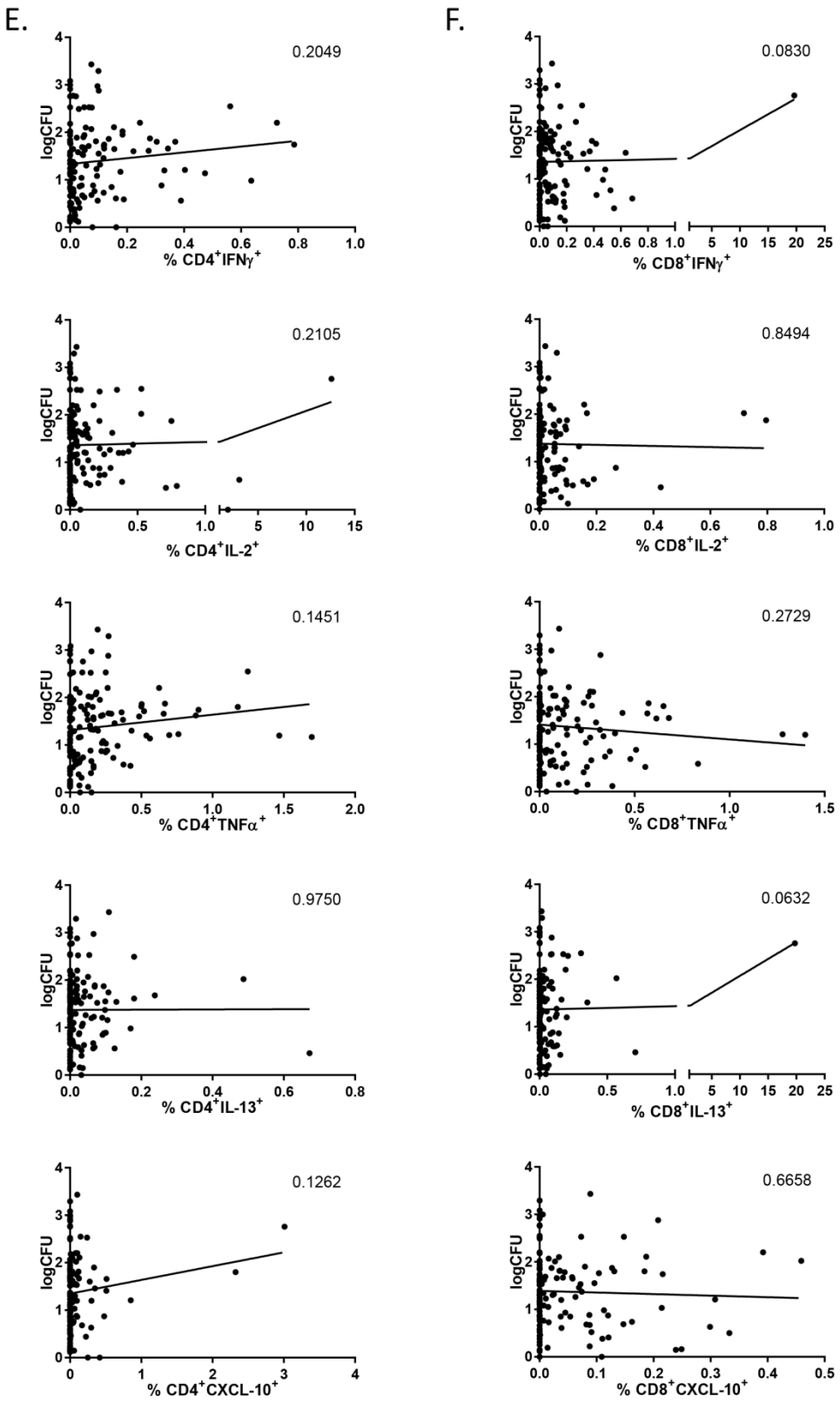


Supplementary Figure 2: **Capacity to control BCG outgrowth correlates with TST but not QFN test results**

Combining all results from all cohorts, mycobacterial growth is indicated in log CFU on the Y-axis and TST in mm (panel A, n=81) or QFN IFN-γ production in IU/ml (panel B, n=125) on the X-axis. Associations were determined using linear regression modelling.

Supplementary Figure 3



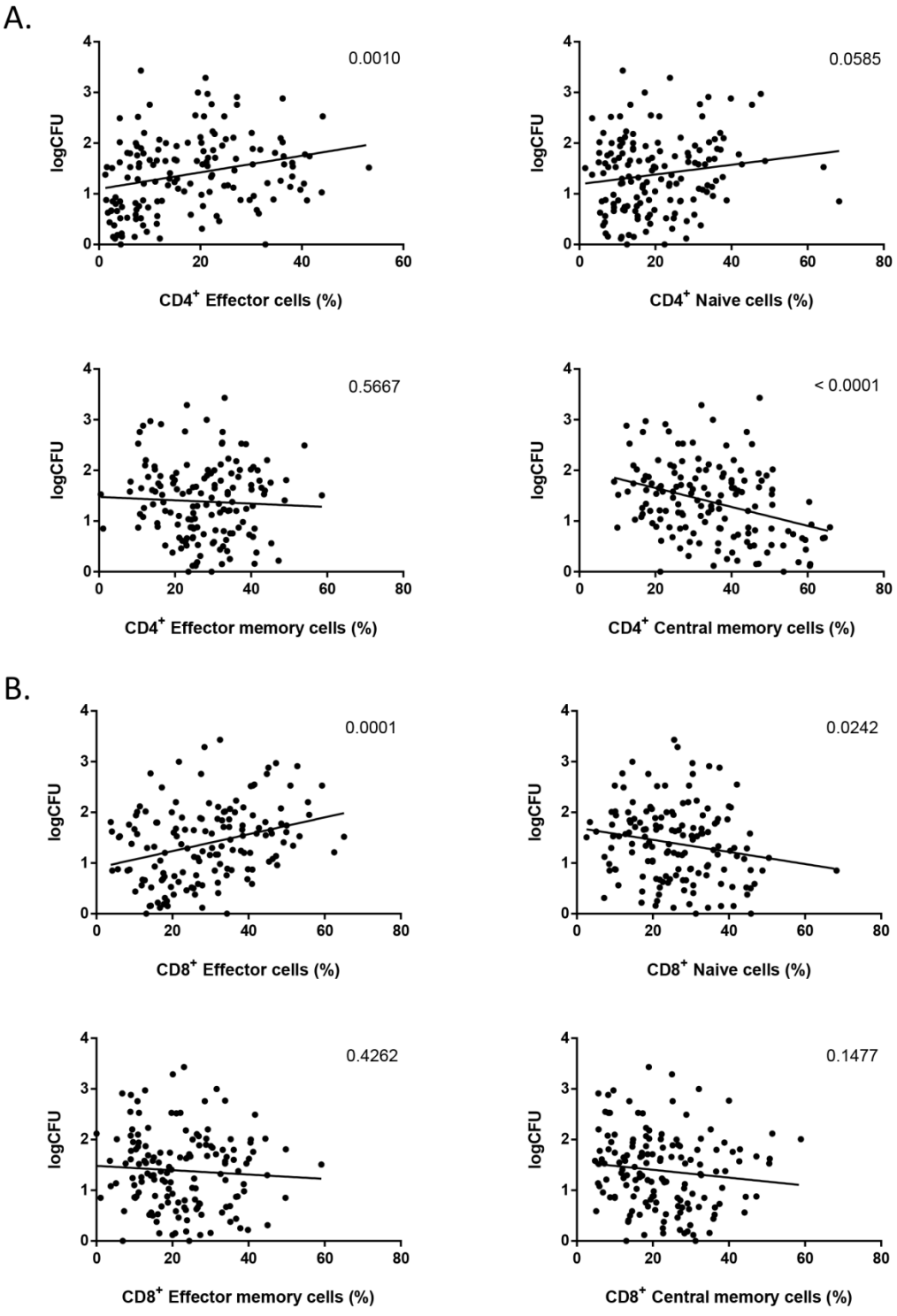


Supplementary Figure 3: **CD4 or CD8 T-cell cytokine production do not correlate with capacity to control mycobacterial outgrowth**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine. Antibody marker staining for FACS analysis was performed after 16 hours of BCG stimulation in the presence of BFA. Associations were determined using linear regression modelling. Controls n=38, BCG n=16, exposed n=50, LTBI n=35, TB n=19.

A. $CD8^+$ multifunctional T-cells (IFN-γ^+ TNF-α^+ IL-2^+) are hardly detectable in clinical groups.
B. The frequency of $CD8^+$ multifunctional T-cells does not correlate with the capacity to control mycobacterial outgrowth (n=142).
C. $CD4^+$ T-cells producing single cytokines (IFN-γ, IL-2, TNF-α, IL-13 or IL-10) are not different across the clinical groups, only $CD4^+$ T-cell TNF-α production is significantly higher in BCG vaccinated donors compared to recently exposed individuals.
D. $CD8^+$ T-cells producing single cytokines (IFN-γ, IL-2, TNF-α, IL-13 or IL-10) are not different across the clinical groups.
E. $CD4^+$ T-cells producing single cytokines (IFN-γ, IL-2, TNF-α, IL-13 or IL-10) do not correlate with the capacity to control mycobacterial outgrowth (n=144).
F. $CD8^+$ T-cells producing single cytokines (IFN-γ, IL-2, TNF-α, IL-13 or IL-10) do not correlate with the capacity to control mycobacterial outgrowth (n=144).

Supplementary Figure 4

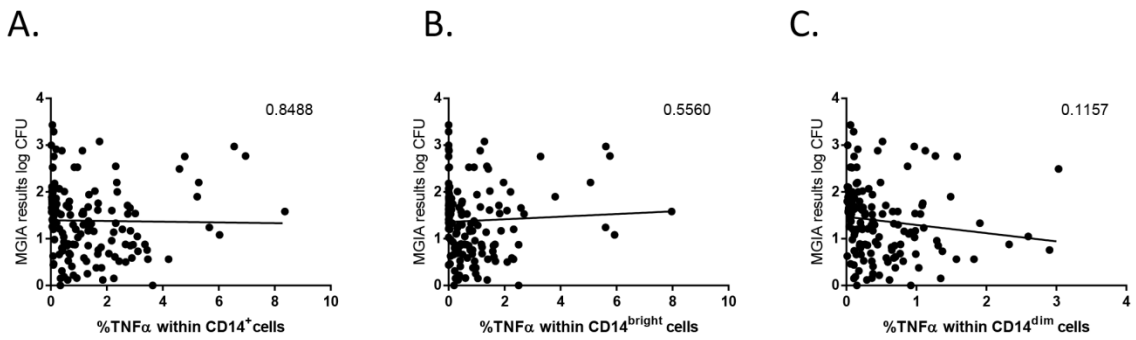


Supplementary Figure 4: **Central memory and effector T-cell populations correlate with capacity to control mycobacterial outgrowth**

PBMCs were infected with live BCG, rotated for 4 days and incubated in MGIT tubes. FACS staining was performed after 16 hours of BCG stimulation. Associations were determined using linear regression modelling (n=148).

A. Correlation between $CD4^+$ subsets based on the expression of CD45RA and CCR7 with the capacity to control mycobacterial outgrowth. $CD45RA^+CCR7^+$: naïve cells, $CD45RA^-CCR7^+$: central memory cells, $CD45RA^-CCR7^-$: effector memory cells, $CD45RA^+CCR7^-$: effector cells.
B. Correlation between $CD8^+$ subsets based on the expression of CD45RA and CCR7 with the capacity to control mycobacterial outgrowth. $CD45RA^+CCR7^+$ cells are naïve cells, $CD45RA^-CCR7^+$ cells are central memory cells, $CD45RA^-CCR7^-$ cells are effector memory cells and $CD45RA^+CCR7^-$ cells are effector cells.

Supplementary Figure 5

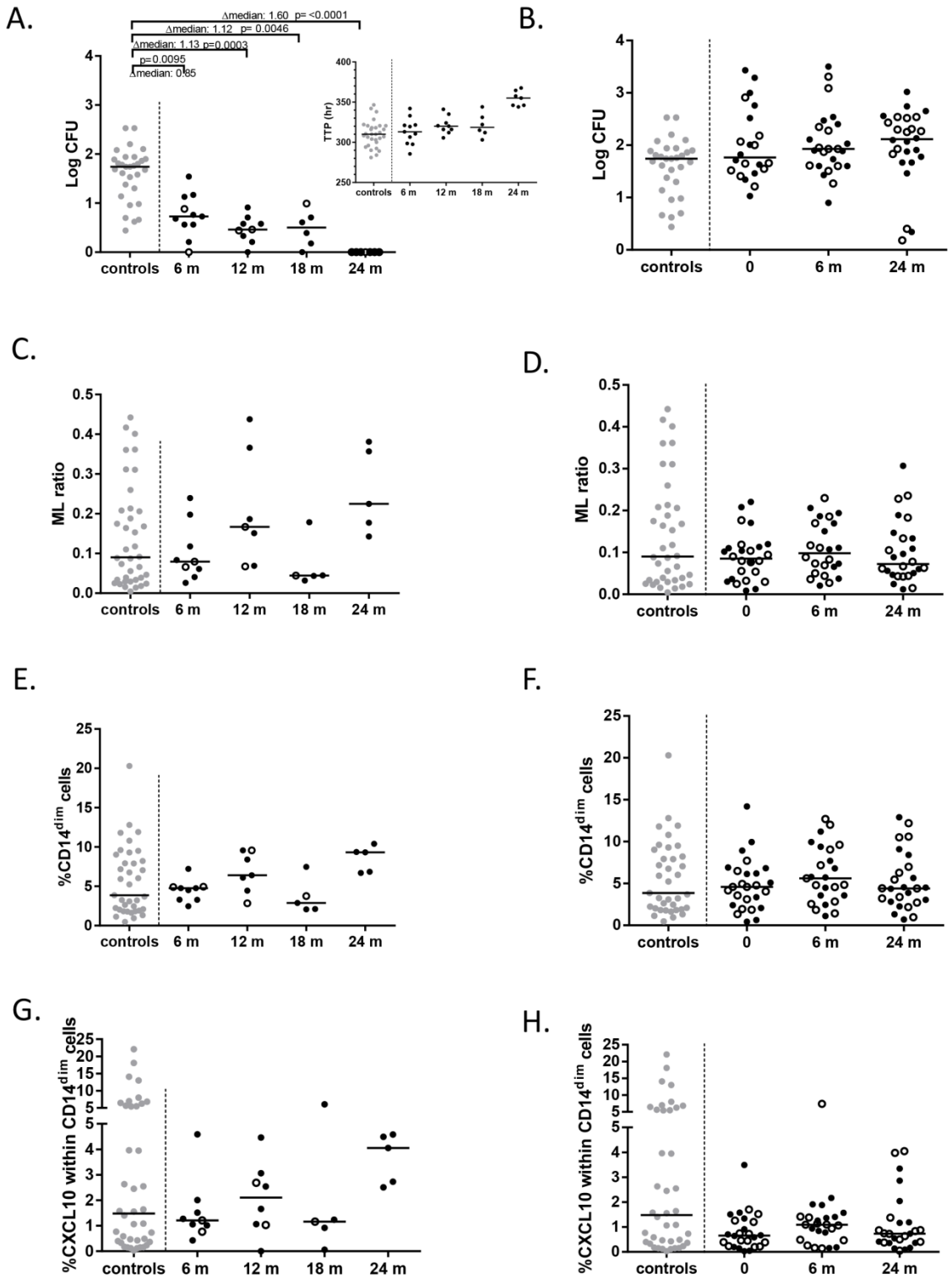


Supplementary Figure 5: **Monocyte TNF-α production does not associate with the capacity to inhibit mycobacterial control**

PBMCs were infected with live BCG, rotated for 4 days and samples incubated in MGIT tubes in the BACTEC machine. FACS staining was performed after 16 hours of negative control stimulation in the presence of BFA. Associations were determined using linear regression modelling (n=144).

A. TNF-α production in the total CD14 population does not correlate with mycobacterial outgrowth control.
B. TNF-α production in the $CD14^{bright}$ population does not correlate with mycobacterial outgrowth control.
C. TNF-α production in the $CD14^{dim}$ population does not correlate with mycobacterial outgrowth control.

Supplementary Figure 6

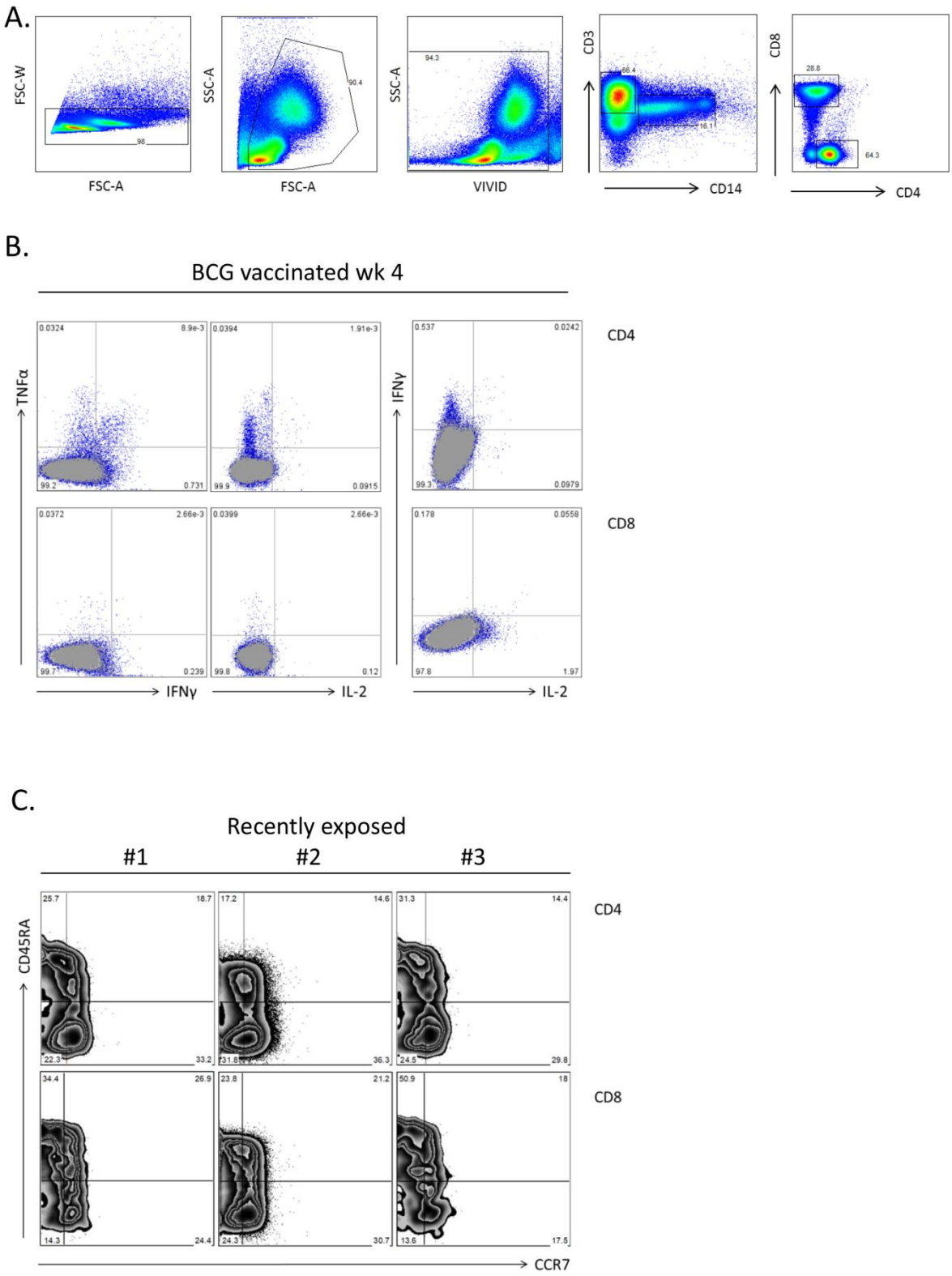


Supplementary Figure 6: **Mycobacterial growth control may increase in early stages following infection**

The Dutch LTBI cohort as well as the contact investigation in the Dutch supermarket both included longitudinal follow up. All samples from the same individual were tested in the same MGIA assay. The control population shown in this graph is the same as used in figure 1C-G and was comprised of healthy Dutch individuals.

A. The contact investigation in the supermarket also included longitudinal follow up at 3-monthly intervals. Only 2 individuals were treated prophylactically (open symbols). All groups had increased BCG outgrowth control compared to the healthy control population. (Kruskal Wallis). The insert shows the actual time to positivity (TTP) in the BACTEC culture to illustrate that although values at 12 months are very low they do not indicate sterile elimination of BCG. Controls n=30, m6 n=11, m12 n=9, m18 n=6, m24 n=7.
B. The LTBI cohort from the Netherlands had samples collected at inclusion (month 0), 6 and 24 months later. Individuals with prophylactic treatment are depicted with open symbols. Controls n=30, m0 n=22, m6 n=25, m24 n=26.
C. Monocyte lymphocyte ratios over different time points of follow up in the supermarket contact investigation. Controls n=30, m6 n=11, m12 n=9, m18 n=6, m24 n=7.
D. Monocyte lymphocyte ratios over different time points of follow up in the LTBI cohort. Controls n=30, m0 n=22, m6 n=25, m24 n=26.
E. The proportion of $CD14^{dim}$ cells at different time points of follow up in the supermarket contact investigation. Controls n=30, m6 n=11, m12 n=9, m18 n=6, m24 n=7.
F. The proportion of CXCL10 producing $CD14^{dim}$ cells at time points of follow up in the LTBI cohort. Controls n=30, m0 n=22, m6 n=25, m24 n=26.
G. The proportion of CXCL10 producing $CD14^{dim}$ cells at different time points of follow up in the supermarket contact investigation. Controls n=30, m6 n=11, m12 n=9, m18 n=6, m24 n=7.
H. The proportion of $CD14^{dim}$ cells at time points of follow up in the LTBI cohort. Controls n=30, m0 n=22, m6 n=25, m24 n=26.

Supplementary Figure 7



Supplementary Figure 7: **gating strategies**

A. Flow cytometry data were analysed using Flow Jo software Initial gating included gating on singlets using FSC-A and W gates, followed by gating on lymphocytes and live cells ($VIVID^-$).

T-cells were gated as $CD3^{+}CD14^{-}$ cells, followed by CD4 and CD8 gates and myeloid cells were $CD14^{+}CD3^{-}$.

B. Cytokine production upon BCG stimulation (blue) compared to unstimulated samples (grey), for CD4 in top row and CD8 in bottom row. Boolean gating was used to analyse T-cell cytokine production capacities according to MIATA guidelines (59).

C. Example of gates used to identify memory T-cell populations based on CD45RA and CCR7 for $CD4^{+}$ and $CD8^{+}$ T-cells.

	MGIA: donors with BCG growth control							
	log CFU				log CFU + Antagonist			
	replicates			mean	replicates			mean
1	1,7	0,28		0,99	1,64	1,7		1,67
2	1,7	1,86		1,78	3,06	2,81		2,94
3	1,92	0,03		0,98	2,81	2,08		2,45
4	0,01	0,37	1,41	0,60	1,95	1,95	2,32	2,07
5	0,48	0,77		0,63	0	2,05		1,03
6	0,71	1,06		0,89	1,76	3,17		2,47
7	1,04	1,07	1,26	1,12	1,62	1,65	2,29	1,85
8	0,71	1,56		1,14	2,29	1,62		1,96
9	1,83	0,92	1,04	1,26	0,86	1,47	1,59	1,31
10	0	2,59		1,30	2,56	1,54		2,05
11	1,92	0,87		1,40	2,5	1,41		1,96

	MGIA: donors without BCG growth control							
	log CFU				log CFU + Antagonist			
	replicates			mean	replicates			mean
1	2,81	2,62		2,715	1,92	2,59		2,26
2	2,56	2,52		2,54	2,27			2,27
3	2,46	2,40		2,43	1,10			1,10
4		2,68		2,68	1,51	0,12		0,82
5	3,00	3,03		3,02	2,37	1,42		1,90
6	2,46	2,14		2,30	1,16	1,39		1,28
7	2,24	2,46		2,35	2,62	2,94		2,78
8	2,27	2,43		2,35	2,30	2,46		2,38
9	2,49	2,40		2,45	2,75	2,46		2,61
10	2,84	2,78		2,81	2,43	1,96		2,20
11	1,74	2,56		2,15	2,17			2,17
12	2,35	2,35		2,35	2,05	1,89		1,97
13	2,11	2,74	2,50	2,45		2,23		2,23
14	2,90	2,05		2,48	2,17	2,44		2,31
15	2,78	2,26		2,52	2,05	1,89	2,29	2,08
16	2,59	2,59		2,59	2,17	1,71		1,94
17	2,47	2,84	2,68	2,66	2,14	2,35		2,25
18	2,65	2,78		2,72	2,68	2,29		2,49
19	2,84	2,71		2,78	2,02	2,08		2,05
20	2,40	2,72		2,56	1,70	1,86		1,78
21	2,08	3,10		2,59		2,62		2,62
22	3,23	0,71		1,97	0,48	1,63		1,06
23	2,69	3,45		3,07	1,57	1,57		1,57
24	1,03	2,21		1,62	1,41	0,71		1,06
25	1,51	1,38		1,45	1,35	1,06		1,21

26	1,54	1,95		1,75		2,27	1,95		2,11
27	2,72	0,96		1,84			1,98		1,98

Supplementary table 1: raw data of MGIA experiment with CXCR3 antagonist.

Supplementary Materials and Methods

Ethics and study samples

Human participation in this research was according to the U.S. Department of Health and Human Services and Good Clinical Practice guidelines. This included protocol approval by the Leiden University Medical Center Ethics Committee and written informed consent by all donors. Anonymous buffy coats from healthy blood bank donors were only used if donors had consented scientific use of blood products. Specifically, healthy control donor samples were *in vitro* PPD negative healthy buffy coats obtained from Sanquin blood transfusion services, The Netherlands. The group of healthy donors was supplemented with healthy Dutch donors prior to BCG vaccination, after extensive testing of their mycobacterially naïve status. These donors were subsequently BCG vaccinated and analysed at 4, 8, 12 weeks and 1 year post BCG vaccination; the BCG vaccination study was approved with protocol number P12.087 (30). A longitudinal follow up study of individuals with latent TB infection (LTBI) was also run at LUMC and approved under protocol number P07.048 (31). In addition, a cohort with known long-term TB latency, and age-matched uninfected controls was recruited in Norway after approval by the Regional Committees for Medical and Health Research Ethics in Norway (protocol number P027/99) (32, 33).

In addition, we obtained specific permission (protocol number B16.002) from the ethical review board at Leiden University Medical Center to use archived samples remaining after completion of previous studies, in particular contact investigations at a soccer club (original protocol number 136/97)(34), in a supermarket (original protocol number P05.053)(35-37) and amongst immigrants with a recent TB contact (original protocol number P04.114)(38, 39).

Finally, BCG vaccination studies in healthy male, volunteers were executed in Nijmegen, approved by the Arnhem-Nijmegen Medical Ethical Committee with protocol number NL50160.092.24.

Experimental design

Archived PBMCs were thawed, rested and counted. Cells were split for BCG-based MGIA as well as direct stimulation with live BCG for flowcytometry including intracellular cytokine staining. Remaining PBMCs were dispensed in Tri-zol reagens for later RNA isolation. Supernatants were collected after 4 days of co-culture in the MGIA system. Upon analysis of MGIA data as well as flowcytometry data, cytokine and chemokine measurements were initiated and functional experiments were designed.

BCG

BCG (Pasteur strain) was grown in Middlebrook 7H9 medium supplemented with 10% ADC (BD Biosciences), log phase bacteria were frozen in aliquots in glycerol (Sigma-Aldrich). The number of viable bacilli per ml was determined by plating serial dilutions of bacteria on Middlebrook 7H10 agar plates supplemented with 10% OADC (BD Biosciences) and counting of visible colonies after 3 weeks. When aliquots were thawed for infection of PBMCs they were first washed three times with PBS/Tween80 (0.05%) (Sigma-Aldrich), and were left to set for a minute to remove big clumps from the suspension.

MGIA

The EC Fp7 project Euripred optimized the protocol for human MGIA assays (22), we have utilized the optimized 'in tube' MGIA Euripred protocol in our studies.

Cryopreserved PBMCs were thawed and rested in RPMI (Gibco life sciences, ThermoFisher Scientific Inc., Bleiswijk, the Netherlands) supplemented with glutamax (Gibco) and 10% FBS (Hyclone, ThermoFisher Scientific Inc.) (=R10 medium) at a concentration of $2x10^6$ cells/ml for 2 hours in the presence of benzonase (10U/ml, Merck, Amsterdam, the Netherlands). After resting, cells were washed with R10 medium and counted with a Casy Cellcounter (Roche, Woerden, the Netherlands). $1x10^6$ PBMC's were co-cultured for 4 days in RPMI supplemented with glutamax and 10% inactivated pooled human serum with 2.6 logCFU (+/- 0.36 SD) BCG P3 on a rotator in a 37 ^{0}C humidified CO_2

incubator in a final volume of 600 µl. All samples were run in duplicates. After 4 days, 100 µl supernatant was harvested and stored for future analysis and the remaining 500 µl per sample were transferred to a PANTA/Enrichment supplemented MGIT tube (Becton Dickinson, Erembodegem, Belgium) and placed in a Bactec 900 system (BD) until time to positivity (TTP) was reached. All tubes included in the analysis were checked visually for mycobacterial growth. Samples reaching positivity within 100 hours were considered contaminated and thus deleted as false positive, since the inoculum of 2.6 logCFU BCG is expected to reach TTP after more than 250 hours.

As a control for the BCG inoculum all experiments included a serial dilution (10^7 to 10^2) of the BCG stock for time to positivity in PANTA/Enrichment supplemented MGIT tubes and plating on Middlebrook 7H10 agar plates, supplemented with 10% OADC (BD) for CFU determination. When all dilutions showed colonies on the 7H10 plates, plates were scanned on a Canon Scanner 9000F and colonies were counted using the ImageJ software. CFU's were converted to logCFU and plotted against the TTP. Linear regression analysis was applied (GraphpadPrism software v7) and all samples were transposed and data are plotted as logCFU.

For the inhibition of mycobacterial outgrowth the same experimental setup as described above was used with the daily addition of the CXCR3 antagonist (NBI-74330, Tocris Bioscience, Bristol, United Kingdom) at a final concentration of 1 µM. After 4 days of incubation, samples were transferred to a supplemented MGIT tube and placed into the Bactec900 system.

To identify subsets responsible for the observed BCG outgrowth control, cells were separated and combined before initiation of the BCG infection experiment. Briefly, PBMCs were thawed and $CD14^+$ monocytes were enriched by magnetic bead separation using CD14 beads (Miltenyi Biotec, Leiden, The Netherlands), followed by positive selection of $CD3^+$ T-cells using CD3 beads (Milteny Biotec) on the CD14negative fraction. For each donor, PBMCs (1x10e6) were compared to isolated monocytes (150.000), and monocytes (150.000) + 0,5x10e6 T-cells or monocytes + 1x10e6 T-cells. Cells were

combined into sarstedt tubes before addition of BCG, the remainder of the procedure was identical as described above.

Flowcytometric analysis

$1x10^6$ rested PBMC's were incubated with medium only or with $1x10^6$ CFU BCG for 6 hours after which Brefeldin A (3 µg/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added and samples were incubated for another 16 hours. After incubation cells were washed with PBS/0.1% BSA (Pharmacy LUMC, The Netherlands)/ (Sigma-Aldrich) and stained with Vivid live-dead stain (Invitrogen, ThermoFisher Scientific Inc.) for 10 minutes at 4 ^{0}C followed by staining with surface markers CD4-APC-H7 (clone RPA-T4), CD8-AlexaFluor700 (clone RPA-T8), CD3-Brilliant Violet510 (clone UCHT-1), CD45RA-PE-Cy5 (clone MEM-56) and CCR7-Brilliant Violet605 (Clone 3D12) (all BD Biosciences), CD19 PE-TexasRed (clone HIB19) and CD14-Qdot655 (clone 61D3) (both Ebiosciences, ThermoFisher Scientific Inc.) for 30 minutes at 4 ^{0}C. Cells were washed, fixed with fixation buffer A (fix/perm reagents Nordic MuBio, ITK, Uithoorn, The Netherlands) for 15 minutes at RT. After fixation cells were washed again and stained for the cytokines and chemokines IFN-γ-PerCP-Cy5.5 (clone 4S.B3) (Biolegend), IL-2-FITC (clone 5.344.111), TNF-α-PE-Cy7 (clone Mab11), IL-13-APC (clone JES10-5A2) and CXCL10-PE (clone 6D4/D6/G2) (all BD Biosciences) in permeabilization buffer B. After incubation for 30 minutes at room temperature (RT), cells were washed and resuspended in 1% paraformaldehyde and measured within 24 hours after staining on a LSRFortessa (BD Biosciences) using Diva software v6.2. Analysis was done using Flowjo v9.7.3 (Treestar Inc., Ashland, OR, USA) and a minimum of $7.5x10^5$ PBMC's were analysed.

For further analysis of the monocyte population also CD163-PerCP-C5.5 (clone GHI/61),CCR2-APC (clone 48607), CCR5-APC-H7 (clone 2D7/CCR5) and CX3CR1-Pacific Blue (clone 2A9-1) (all BD Biosciences) were used as surface markers but staining was performed at 37°C to prevent temperature based changes in, and thereby low detection of, the chemokine receptors.

Real-time Quantitative RT-PCR

For all PBMC samples that had cells left after the initial MGIA and flowcytometry experiments were initiated, remaining cells were directly transferred to Tri-zol reagent (Qiagen, Venlo, the Netherlands). Total RNA was extracted using direct-zol kits, including an on-column DNA digestion, according to instructions of the manufacturer (Zymo-research, Baseclear, Leiden, The Netherlands). Total RNA was reverse transcribed using oligo(dT) 12-18 primers (Thermofisher Scientific, Bleiswijk, The Netherlands) and superscript IV enzymes (Thermofisher).

Real-time quantitative RT-PCR was performed with Taqman technology and TaqMan universal PCR master mix (Thermofisher) using the following primers and probes were used: CXCR3-A: FAM probe, 5-TGAGTGACCACCAAGTGCTAAATGACGC-3; forward 5-ACCCAGCAGCCAGAGCACC-3; reverse 5-TCATAGGAAGAGCTGAAGTTCTCCA-3. CXCR3-B: FAM probe, 5-CCCGTTCCCGCCCTCACAGG-3; forward 5-TGCCAGGCCTTTACACAGC-3; reverse 5-TCGGCGTCATTTAGCACTTG-3 based on (42). GAPDH was used for normalization using VIC labelled probes in both PCR reactions (Thermofisher). Taqman PCR was run on a Quantstudio 6 Flex (Applied Biosystems) with QuantStudio Real Time PCR v1.1 software and data expressed as the difference in Ct values between GAPDH and CXCR3A/B. Undetectable signals for CXCR3A were set at a Ct of 40 to allow calculation of the ΔCt value. Ratios were determined by dividing the ΔCt of CXCR3A by that of CXCR3B.

BCG-induction of trained immunity

To assess induction of trained immunity by BCG vaccination, 30 healthy male subjects (age: 19-37 years) were randomly assigned to receive either BCG (SSI, Denmark) or placebo (the diluent used to dissolve BCG) in a double-blind fashion. Blood was drawn before BCG/placebo and 4 weeks later. PBMCs were isolated by density centrifugation of Ficoll-Paque (GE healthcare, UK) . Cells were washed twice in PBS and resuspended in RPMI culture medium (Roswell Park Memorial Institute medium; Invitrogen, CA, USA) supplemented with 50 μ g/mL gentamicin, 2mM Glutamax (Gibco), and 1mM pyruvate (Gibco). 5x10^6/mL cells in 100 μ L/well were added to 96 well plates, and cells

were incubated for 24h with RPMI, sonicated *Mycobacterium tuberculosis* H37Rv (5 μ g/mL), heat-killed *Candida albicans* (1x10^6/mL, strain UC820) or *Staphylococcus aureus* (1x10^6/mL clinical isolate). Supernatants were collected after 24h and stored at -20^{o}C until further analysis. The study was approved by the Arnhem-Nijmegen Medical Ethical Committee, NL50160.092.24.

Luminex®

A 40-plex chemo-cytokine Luminex® assay was performed according to manufacturer's instructions (Bio-Rad, Veenendaal, The Netherlands) on BCG culture supernatants and on supernatants of the trained immunity experiment.

Statistical analysis

All data were analysed assuming a non-Gaussian distribution and therefore non-parametric testing was applied. All legends contain detailed information on the statistical tests applied and the number of individuals included for analysis. Generally, when comparing groups Kruskal-Wallis or Wilcoxon-paired rank tests were applied depending on the number of groups in the equation. For the analysis of longitudinal data an ANOVA with Friedman test was applied. Correlations were based on linear regression modelling. All analyses were performed using Graph Pad prism version 7.00, Graph Pad Software Inc.