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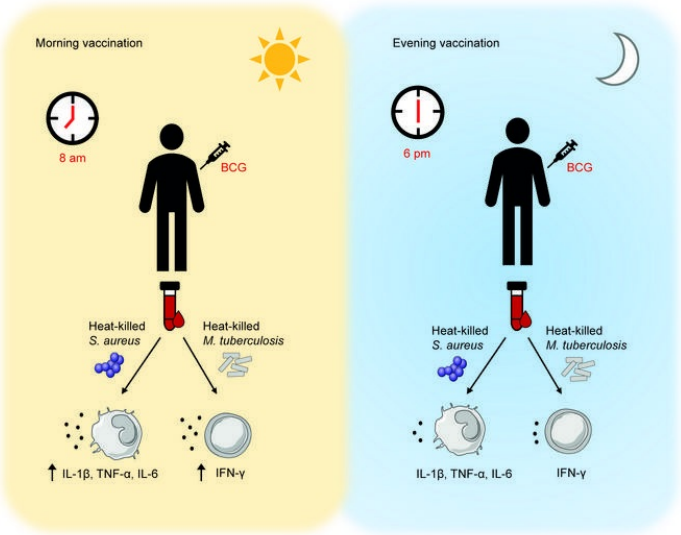
Graphical abstract



Circadian rhythm influences induction of trained immunity by BCG vaccination

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Declaration of Interests

MGN and LABJ are scientific founders of Trained Therapeutics Discovery. All other authors declare no financial interest.

ABSTRACT

BACKGROUND. The anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) reduces overall infant mortality. Induction of innate immune memory, also termed trained immunity, contributes towards protection against heterologous infections. Since immune cells display oscillations in numbers and function throughout the day, we investigated the effect of BCG administration time on the induction of trained immunity.

METHODS. 18 volunteers were vaccinated with BCG at 6pm and compared with 36 age- and sex-matched volunteers vaccinated between 8-9 am. Peripheral blood mononuclear cells were stimulated with *Staphylococcus aureus* and *Mycobacterium tuberculosis* before, as well as two weeks and three months after BCG vaccination. Cytokine production was measured to assess the induction of trained immunity and adaptive responses, respectively. Additionally, the influence of vaccination time on induction of trained immunity was studied in an independent cohort of 302 individuals vaccinated between 8am-12pm with BCG.

RESULTS. Compared to evening vaccination, morning vaccination elicited both a stronger trained immunity and adaptive immune phenotype. In a large cohort of 302 volunteers, early morning vaccination resulted in a superior cytokine production capacity compared with later morning. A cellular, rather than soluble, substrate of the circadian effect of BCG vaccination was demonstrated by the enhanced capacity to induce trained immunity in vitro in morning compared to evening isolated monocytes.

CONCLUSIONS. BCG vaccination in the morning induces stronger trained immunity and adaptive responses compared to evening vaccination. Future studies should take vaccine administration time into account when studying specific and non-specific effects of vaccines: early morning should be the preferred moment of BCG administration**.**

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INTRODUCTION

The anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) has the highest vaccine coverage worldwide (1). BCG protects primarily against disseminated tuberculosis (TB) in children (2), whereas protection against pulmonary TB in adults is modest (3, 4). While the world is striving for a more effective anti-TB vaccine (5), there is mounting evidence that BCG possesses general immune modulatory properties (6). Epidemiological data and randomized trials have shown that BCG vaccination reduces all cause morbidity and mortality in neonates and children (7-13). In these trials, a reduced incidence of respiratory infections and sepsis, as well as in-hospital mortality, has been found.

Previously we have demonstrated that BCG vaccination induces non-specific innate immune memory responses. This phenomenon called trained immunity (14) has been postulated to explain, at least in part, the non-specific beneficial effects of BCG vaccination on child morbidity and mortality. Epigenetic modification and metabolic reprogramming of monocytes are shown to be responsible for upregulated cytokine responses upon ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) with BCG-unrelated stimuli such as *Staphylococcus aureus*, *Candida albicans*, and Toll-like receptor 4 (TLR4) ligand lipopolysaccharide (LPS) (15-17). These longer lasting non-specific immunological effects, up to 1 year after BCG vaccination, are explained by transcriptional changes associated with myeloid cell development and function in hematopoietic stem and progenitor cells, which are epigenetically conveyed into peripheral blood $CD14^+$ monocytes (18, 19). Induction of trained innate immunity has primarily been studied in monocytes (16, 20-22), but BCG exerts non-specific effects on other innate immune cells such as NK cells and γδ-T cell as well (15, 23). In human challenge models, BCG vaccination reduced yellow fever vaccine viremia upon subsequent vaccination (20), and in a controlled human malaria infection model, a subset of BCG vaccinated volunteers responded with reduced *Plasmodium falciparum* parasitemia, which correlated with early monocyte, NK, and γδ-T cell activation during bloodstream infection (23).

In multiple hematopoietic cell lineages, the importance of intrinsic circadian molecular clocks has been identified (24-26). Circadian rhythm reflects any biological process with a 24-hour rhythm that adapts to environmental changes due to the Earth's rotation. A central clock situated in the central suprachiasmatic nucleus of the hypothalamus coordinates peripheral molecular clocks present within cells located throughout the body, including immune cells (the intrinsic circadian molecular clocks). Circadian clock genes have been shown to oscillate in human PBMCs (24). Approximately 8% of the macrophage transcriptome oscillates in a circadian fashion, including many important regulators for pathogen recognition and cytokine secretion (26). A growing body of literature has acknowledged the importance of circadian rhythms in immune function (27). Relative and absolute numbers of hematopoietic stem cells and most mature leukocytes in the circulation fluctuate throughout the day (28). In addition, cellular functions such as phagocytosis capacity, migration, and proliferation display circadian oscillations (29).

Although the attention for chronobiology and pharmacology is rising, little is known about the influence of circadian immune rhythms on vaccine immunogenicity. To our knowledge, previous studies have exclusively focused on timing of vaccine administration on induction of vaccine specific antibody responses. While results of studies focusing on influenza and hepatitis A immunogenicity hinted towards increased induction of specific immune responses when individuals were vaccinated in the morning (30, 31), another study found the effect to be dependent on the moment of sample collection rather than the timing of influenza vaccine administration (32).

The influence of timing of vaccine administration on non-specific immunological effects of vaccines has not been investigated so far. Knowledge about possible oscillations in induction of BCG-induced trained immunity in vivo would be of great importance for assessing (non-specific) vaccine efficacy, as well as for potential implications of BCG vaccination as immune modulator (12, 33, 34). Therefore, we investigated the effect of timing of BCG administration on the induction of trained immunity, in order to assess whether time of the day should be taken into account to maximize health benefits after BCG vaccination.

RESULTS

BCG-vaccinated healthy individuals

Eighteen healthy volunteers (eleven females, seven males) of Western-European ancestry participating in the 300BCG cohort study were vaccinated in the evening and each individual was retrospectively age- and sex-matched with two individuals from the 300BCG cohort, who were vaccinated between 8am and 9am, resulting in a subgroup of 36 age- and sex-matched morning vaccinated controls (**figure 1** and **figure 2A**). The median age of both groups was 26 years. All volunteers developed a scar three months after BCG vaccination and the average scar size did not differ significantly between groups. An overview of participant characteristics is presented in **table 1**. Complete blood counts were measured on EDTA blood samples. Whole blood leukocyte counts, monocyte, lymphocyte or neutrophil counts did not differ significantly between the morning and evening vaccinated subgroups at baseline, two weeks, or three months after BCG vaccination (**figure 2B**).

BCG vaccination in the morning elicits a stronger trained immunity phenotype compared to evening vaccination

BCG vaccination in the morning induced trained immunity, resulting in significantly enhanced *S. aureus*-induced interleukin (IL) 1β and tumor necrosis factor α (TNF-α) production two weeks and/or three months after BCG vaccination (**figure 3A-C**). Overall, no induction of trained immunity was observed in the evening vaccinated subgroup (**figure 3D-F**). IL-1β and IL-6 production after 24h *M. tuberculosis* stimulation was induced in both groups (**supplementary figure 1**). However, a significant upregulation of IL-1β production upon *M. tuberculosis* stimulation two weeks after BCG vaccination was only apparent in the evening vaccinated group, whereas three months after BCG vaccination this was only apparent in the morning vaccinated group. *M. tuberculosis*-induced IL-6 production was upregulated in both groups after two weeks (**supplementary figure 1**). Specific *M. tuberculosis* IFN-γ

responses were significantly higher in morning vaccinated individuals three months after BCG vaccination (**figure 3G**). In contrast to BCG administration in the morning, BCG vaccination in the evening did not elicit an increased IFN-γ response upon *M. tuberculosis* restimulation (**figure 3H**). The significant changes in cytokine production upon restimulation after BCG vaccination due to timing, as shown in figure 3A-H, could explain between 7 to 12 percent of the overall variation in cytokine production within this cohort.

Changes in cytokine production after BCG vaccination were not explained by altered percentages of monocytes or lymphocytes in the PBMC fraction after Ficoll isolation, since those remained stable between time points and did not differ between groups (**figure 3I**).

Trained immunity responses induced by BCG vaccination are most profound in the early morning

Subsequently we assessed whether BCG administration at different time points during the morning (between 8am and 12pm (noon)) within the entire 300BCG cohort (**figure 4A**), comprising of 302 (171 female and 131 male) volunteers, would affect the induction of trained immunity in terms of cytokine production. Participant characteristics of the entire 300BCG cohort can be found in **supplementary table S1**. Interestingly, significant differences in fold changes were found between the early morning vaccinated group (8am-9am, n=68) and late morning vaccinated group (11am-12pm, n=66) for *S. aureus*-induced IL-1β and TNF-α, and between 8am-9am and 10am-11am (n=8) subgroups for *S. aureus*-induced IL-6 two weeks after vaccination in favour of the early morning vaccinated group (**figure 4B-D**). After three months, *S. aureus*-induced IL-1β and TNF-α remained significantly higher in the early morning vaccinated group (**figure 4B-D**). No significant differences were observed between different morning vaccinated subgroups in *M. tuberculosis*-induced IFN-γ responses two weeks and three months after vaccination (**figure 4E**), which is also seen for IL-1β and IL-6 production (**supplementary figure 2**).

In contrast to baseline *S. aureus*-induced IL-6 and TNF-α responses, which did not differ between groups, baseline *S. aureus*-induced IL-1β responses were significantly higher in the late morning (11am-

12pm) vaccinated individuals, compared to those vaccinated between 8am and 11am (**supplementary figure 3A-C**). *M. tuberculosis* induced IFN-γ responses did not differ between morning subgroups (**supplementary figure 3D**). When *S. aureus*-induced IL-1β responses are corrected for time of blood drawing, the differences in fold changes between early morning and late morning vaccination are no longer significant (**supplementary figure 3E**). We found a modest but significant inverse correlation between percentages of lymphocytes in the isolated PMBC fraction at baseline and time of BCG administration, and a positive correlation between percentages of monocytes in isolated PBMCs at baseline and time of BCG administration (**figure 5A-B**). Thus, on average, the earlier the moment of blood collection during the morning, the higher the lymphocyte percentages and lower monocyte percentages. Nevertheless, fold changes of lymphocytes and monocytes percentages did not significantly differ between the different morning subgroups (**figure 5C-D**). After correction for monocyte percentages within the PBMC fraction, differences between morning vaccinated subgroups remained apparent on the level of IL-1β, IL-6 and TNF-α production to *S. aureus* stimulation (**supplementary figure 4**).

Cell intrinsic effect of circadian rhythm on induction of trained immunity

There are two main mechanisms that could explain the differential induction of trained immunity by morning or evening BCG vaccination: i. changes in circulating modulators of immune responses, and ii. cell-intrinsic circadian changes. The most obvious candidate for circulating modulators affected by circadian rhythm are corticosteroid hormones (35). As expected, circulating cortisol levels were significantly higher in the early morning compared to later during the morning (**supplementary figure 5**). We did not find differences in cortisol concentrations between the morning- and evening-vaccinated groups, also no significant correlations were observed between circulating cortisol concentrations and ex vivo PBMC-derived *S. aureus* induced IL-1β, IL-6 or TNF-α responses, or *M. tuberculosis*-induced IFN-γ responses (data not shown). Moreover, addition of morning-derived serum to freshly isolated Percoll monocytes during 24 hours priming with BCG had an inhibitory effect on in

vitro BCG-induced trained immunity compared to serum collected from volunteers in the evening (**figure 6A-C**), which thus cannot explain the better induction of trained immunity in the morning.

The differential response to the BCG vaccine could also be explained by cell-intrinsic circadian changes. We therefore isolated monocytes from a separate group of healthy volunteers in the morning and in the evening. Indeed, when monocytes were collected and isolated in the morning, they displayed an increased capacity to mount a trained immunity response (higher proinflammatory cytokine production after LPS restimulation) in an experimental in vitro model, compared to cells isolated in the evening (**figure 6A, D-E**). No significant differences were found in the production of the anti-inflammatory cytokine IL-10 (**figure 6F**).

Circadian transcriptional and epigenetic changes in BCG-vaccinated individuals

Furthermore, we studied whether the expression of key clock genes *CLOCK* and *ARNTL* (BMAL1) are affected in morning BCG-vaccinated individuals from an independent cohort (20). As seen in **figure 7**, *CLOCK* and *ARNTL* mRNA expression in monocytes were increased one month after BCG vaccination in individuals which were protected to subsequent yellow fever viremia (responders). Additionally, histone 3 lysine 27 acetylation (H3K27ac), an epigenetic modification associated with gene activation, was increased at the promoter of *CLOCK* and *ARNTL* one month after BCG vaccination. Since cells were available only from a limited number of individuals, caution in the interpretation of the results is required.

Since epigenetic rewiring is one of the hallmarks of trained immunity, we assessed the accessibility of chromatin in evening- versus morning-vaccinated individuals by assay for transposase-accessible chromatin using sequencing (ATAC-seq) (see **supplementary figure 6** and **supplementary table 2** for quality statistics). **Figure 8A** shows differences in chromatin accessibility three months after BCG vaccination for evening- compared to morning-vaccinated individuals (see also **supplementary table 3**). Less differences are seen two weeks after vaccination (**supplementary figure 7**), which suggests that the epigenetic changes induced by BCG need a relatively long time to become apparent.

Individuals vaccinated in the morning had more accessible chromatin in genes of PBMCs important for the mTOR pathway, which is crucial for the induction of trained immunity. The evening-vaccinated individuals showed increased chromatin accessibility in PBMCs for TNF signalling and NOD-like receptor (NLR) signalling (**figure 8B** and **supplementary table 4**), which do not explain the higher TNF production capacity in the morning. This suggests that more molecular mechanisms should be activated and involved in BCG-induced trained immunity. Analysis of transcription factor (TF) binding profiles from JASPAR also showed enrichment of TFs involved in mTOR signalling for morning vaccinated individuals (**figure 8C** and **supplementary table 5**). TF binding profiles from CODEX, which focuses on haematopoietic cell lines, shows in addition enrichment of TFs associated with active histone marks and with removal of repressive histone marks in morning vaccinated individuals (**figure 8D** and **supplementary table 5**).

DISCUSSION

An increasing amount of evidence shows that BCG vaccination elicits a non-specific innate immune memory phenotype (15-17, 20, 23), likely contributing towards decreased overall morbidity and mortality after neonatal BCG vaccination (7-10). However, immunological effects after BCG vaccination are highly variable in both children and adults (7, 20, 23, 36), and understanding sources of this variation would be of great importance for attempts to improve vaccination strategies. Circadian rhythms play an important role in modulation of immune function (27, 37, 38), and we hypothesized that the time of BCG administration might be an important factor to explain the variability of the immunological effects and protective efficacy of the vaccine. In line with this hypothesis, the present study demonstrates that timing of BCG administration is an important factor influencing both *M. tuberculosis* specific, but especially the induction of trained immunity after vaccination. Early morning vaccination resulted in superior cytokine production capacity upon ex vivo restimulation with related (*M. tuberculosis*) and non-related pathogens (*S. aureus*) compared to evening vaccination. Strikingly, both the non-specific trained innate immunity as well as *M. tuberculosis*-specific adaptive responses were practically absent in the volunteers vaccinated in the evening. Furthermore, the earlier volunteers were vaccinated in the morning, the stronger the trained immunity phenotype in terms of cytokine production.

Our findings are in line with previous studies, showing enhanced immunogenicity after influenza and hepatitis A vaccination in the morning compared to the evening (30, 31). The findings from our study confirm previous observations in mice showing an effect of timing of BCG administration on immune function. For example, neutrophil migration in mice implemented with BCG-impregnated cell traps displayed a circadian rhythm (39, 40). In an experimental BCG inflammation mouse model, a diurnal inflammatory response was noticed at the site of inoculation thirty days after BCG administration (41). In another study, circadian oscillations have been observed in non-specific protection of BCG treated mice at different times during the day and subsequent Ehrlich ascites carcinoma challenge (42). Interestingly, the magnitude of the protozoan *Leishmania* infection is shown to be modulated by a

circadian clock in immune cells (43), and one of its causative species *Leishmania braziliensis* is a potent inducer of in vitro trained immunity (44). Trained immunity responses induced by *S. aureus* and specific memory responses to *M. tuberculosis* upon BCG vaccination are differently affected by the circadian rhythm. Previous studies showed that different types of viruses display distinct interactions with core clock proteins (45). Similar differences might explain the different contribution of the circadian rhythm observed in this study for cytokine production upon *S. aureus* versus *M. tuberculosis* stimulation, but this needs to be investigated in future studies.

Chromatin accessibility showed differences between evening- and morning-vaccinated volunteers three months after vaccination. Importantly, these differences were not present after two weeks, indicating a relative long period needed by BCG vaccination to induce trained immunity. Chromatin that was more accessible for morning vaccinated volunteers was enriched for genes of the mTOR pathway, which is crucial for trained immunity (17). This is in line with the observed more pronounced cytokine production in the morning vaccinated individuals. Unexpectedly, several genes of the TNF and NLR signaling pathways has decreased accessibility in the PBMCs of morning vaccinated individuals, which argues that more molecular mechanisms are at play. In addition, enrichment was observed of TFs associated with mTOR signaling for the individuals with BCG administration in the morning (Specificity protein 1 (SP1) (46, 47), TF AP-2 alpha (TFAP2A) (47), and Krüppel-like factor 4 (KLF4) (48, 49)).

Recently we have shown that the longevity of BCG-induced trained immunity in humans is explained by an imprint on hematopoietic stem and progenitor cells (HSPCs), as demonstrated by a bias towards myelopoiesis and upregulated myeloid function on transcriptional level of HSPCs (19). The results of the current study suggest that the impact of BCG on bone marrow progenitors depends on the time of day when the vaccine was administered. There are several hypotheses how the priming of bone marrow precursors could be under influence of a circadian rhythm. Firstly, a molecular intrinsic clock within monocytes and their progenitors could contribute towards the observed effects. This hypothesis is supported by the experiments performed here showing that monocytes isolated from

healthy volunteers in the morning mount a stronger trained immunity phenotype if exposed ex vivo to BCG. Similarly, we demonstrated that monocyte production of IL-1β was partially influenced by the time of the day. These data could explain our findings that BCG administration time influences trained immunity in vivo, as well as that the time of blood drawing determines the efficacy in induction of in vitro trained immunity. Regulators for cytokine secretion and pathogen recognition receptors are examples of genes which have been shown to oscillate on transcriptional level in a circadian manner in macrophages (26). Here we show that the key clock genes, *CLOCK* and *ARNTL,* are more strongly expressed in monocytes one month after BCG vaccination, accompanied by increased H3K27ac at the gene promoters (when blood was taken at the same timepoint in the morning). Multiple studies have shown that the time of day of the infection (bacterial, viral and parasitic) affects the outcome of the infection (50). Previously, it has been shown in mice that low expression of BMAL1 led to increased herpes, influenza (51), and respiratory syncytial virus infection (52). One could hypothesize that increased CLOCK and BMAL1 expression upon BCG vaccination contributes to the observed effect: better protection against heterologous infections upon BCG vaccination.

Secondly, an intrinsic neutrophil timer might be involved in the time-dependent induction of trained immunity by BCG in vivo. Interestingly, mouse studies have shown neutrophils, and not dendritic cells or macrophages, transfer live BCG bacilli to draining lymph nodes (53). In an intradermal Modified Vaccinia Ankara (MVA) vaccination mouse model, CD8 memory responses were surprisingly elicited in the bone marrow compartment, mediated by neutrophils trafficking MVA from the dermis to the bone marrow (54). Recently, a neutrophil timer has been discovered which coordinates immune defense (25). One of the core clock proteins BMAL1, as well as the chemokine CXCR2, has been shown to regulate tissue migration and neutrophil clearance (25). However, it remains to be proven by future studies that neutrophils play a role in the circadian effects of BCG vaccination.

Thirdly, it has been shown in mice that hematopoietic stem cell release is regulated by circadian oscillations, and that HSPCs and leukocytes circulate between bone marrow and peripheral blood following circadian oscillations (55, 56). These circadian oscillations may influence the trained

immunity phenotype of the myeloid progeny. Of note, HSPCs are known to express pathogen recognition receptors as well, which are known to oscillate on transcriptional level in monocytes in a circadian manner (57, 58). Another hypothesis is that the circadian rhythm induction of trained immunity is mediated by IL-1β, one of the key cytokines in BCG-induced trained immunity in vivo (20), of which the production has lately been shown to be under control of the circadian clock protein BMAL1 (59). Finally, a soluble rather than cellular substrate of circadian effects may be hypothesized, since multiple hormones and lipids display diurnal variations in concentration in the circulation (35). Among them, one of the prime candidates for a modulatory role during the circadian rhythm is cortisol. Cortisol has long been known to display a circadian rhythm in concentrations in the blood (35), and its immunomodulatory effects are also well documented (60). However, a role for cortisol is unlikely as it is a known immunosuppressive factor, yet its highest circulating concentration is in the morning, when induction of trained immunity is most effective. Moreover, incubation of monocytes with serum collected in the morning did not result in an increased induction of trained immunity in an in vitro model, arguing that a soluble factor is unlikely to be responsible for the circadian effects of BCG vaccination. Nevertheless, we do not exclude possible involvement of circulating factors, such as insulin, hormones, or dietary components, which could dampen BCG-induced trained immunity in the morning. However, this effect might be masked by cell-intrinsic circadian oscillations.

In conclusion, in the present study we demonstrate the importance of timing of BCG vaccine administration, with a preference for vaccination in the early morning to induce both trained immunity and *M. tuberculosis* specific adaptive responses. This effect was most likely mediated by an intrinsic circadian clock of innate immune cells, rather than soluble factors in the circulation that display circadian rhythms. Future studies should test whether optimized timing of BCG administration results in increased protection after challenge with non-related pathogens in vivo. Moreover, deciphering the mechanism behind the influence of time of vaccination on induction of trained immunity and *M. tuberculosis*-specific responses could lead to possible targets to increase BCG efficacy. Since this study

focused exclusively on individuals of Western-European ancestry, our findings should be validated in cohorts with a different environmental setting and genetic background. At last, time of administration of novel and already existing vaccines should be taken into account when testing specific and non-specific vaccine efficacy. These findings suggest that administering partially effective vaccines like BCG in the morning, in contrast to after school (showing 50% efficacy against sustained infection after adolescent revaccination (61)), might increase immune training and thus vaccine efficacy. This possible effect remains speculative but within the realm of possibility, and it should be investigated in future studies.

MATERIAL AND METHODS

Experimental design

The effect of timing of BCG administration on the induction of trained immunity in vivo was studied as a nested sub study within the 300BCG cohort. In the 300BCG study, 321 healthy (male and female) adult volunteers of Western European ancestry were included from April 2017 until June 2018. Exclusion criteria were: use of systemic medication other than oral contraceptives and acetaminophen, use of antibiotics three months before inclusion, previous BCG vaccination, history of TB, any febrile illness four weeks before participation, vaccination three months before participation, and a medical history of immunodeficiency. Healthy volunteers were recruited using flyers and advertisement in Nijmegen, and received compensation. At the Radboudumc, blood was collected, followed by administration of a standard dose of 0.1 mL BCG Bulgaria (InterVax) intradermally in the left upper arm by a medical doctor. Additionally, blood was collected in the morning two weeks as well as three months after vaccination.

Volunteers participating in the 300BCG trial were vaccinated in the morning (between 8am and 12pm). In order to be able to study differences between morning and evening vaccinated individuals, 18 (seven male and eleven female) volunteers were asked to be vaccinated between 6pm and 6.30pm. All participants, including the volunteers that received their BCG vaccination in the evening, donated blood for immunological assessments in the morning between 8-12 am, and no significant changes in time of blood drawing before versus post-vaccination were seen within the evening- and morning-vaccinated group. For the analysis, each volunteer vaccinated in the evening was age- and sex-matched with two participants vaccinated in the morning between 8am and 9am, resulting in 36 matched controls. A schematic overview of the nested morning-evening study can be found in **figure 2A.** The 18 evening vaccinated volunteers were included in morning-evening sub study analyses but excluded from the main cohort. One volunteer belonging to the main cohort was excluded from analysis because

the vaccination time was not registered, resulting in a cohort with 302 morning vaccinated volunteers (see **figure 1**).

RNA expression of clock genes was measured in healthy individuals vaccinated with BCG from the BCG-Yellow Fever study, and was performed as described previously (GSE104149 (20)). The study was approved by the Arnhem-Nijmegen Medical Ethical Committee (NL50160.092.24).

PBMC isolation and stimulation

PBMCs were isolated from EDTA whole blood with Ficoll-Paque (GE healthcare, UK) density gradient separation. PBMCs were washed twice with phosphate buffered saline (PBS) and counted with a Sysmex hematology analyzer (XN-450). Cells were suspended in Dutch modified RPMI 1640 medium (Roswell Park Memorial Institute, Invitrogen, CA, USA), supplemented with 50 μg/mL gentamicin, 2 mM Glutamax (GIBCO) and 1 mM pyruvate (GIBCO). 5 x 10^5 PBMCs were cultured in a final volume of 200 μL/well in round bottom 96-well plates (Greiner) and stimulated with RPMI (medium control), heat-killed *Mycobacterium tuberculosis* (*M. tuberculosis*) HR37v (5 μg/mL), or heat-killed *Staphylococcus aureus* (*S. aureus*) (10^6CFU/mL, clinical isolate) as a non-specific stimulus and incubated on 37˚C. After 24 hours and 7 days, supernatants were collected and stored at -20˚C until analysis. Cytokines were determined with ELISA (IL-1β, IL-6, and TNF-α (R&D Systems)) in 24 hours supernatants and IFN-γ in 7 days supernatants with Luminex (ProcartaPlex ThermoFischer), according to the manufacturers' protocol. To minimize batch effects during measurements, samples were sorted per stimulus. All time points belonging to one volunteer were measured on the same plate.

In vitro training experiments

In vitro training experiments were performed according to the previously described experimental in vitro trained immunity model (22). Healthy volunteers donated blood at 8am (fasting) and 6pm (fasting from 1pm) on the same day. PBMCs were isolated from EDTA whole blood with Ficoll-Paque density gradient separation. Percoll (Sigma-Aldrich, St 120 Louis, MO, USA) isolation of monocytes was

performed according to the previously described protocol (22). Cells were suspended in Dutch modified RPMI medium, supplemented with 50 µg/mL gentamicin, 2 mM Glutamax and 1 mM pyruvate and counted with a Sysmex hematology analyzer (XN-450). As an additional purification step, 1 x 10^5 isolated Percoll monocytes were plated on polystyrene flat bottom plates (Corning, NY, USA) and incubated for 1 hour at 37˚C, after which non-adherent cells were washed away with warm PBS. Adherent monocytes were primed with either RPMI (negative control) or BCG Bulgaria (5 µg/mL) for 24 hours in the presence of 10% human pooled serum. Cells were washed after 24 hours, and fresh medium (RPMI supplemented with 10% human pooled serum) was added. Medium was refreshed once after three days. After 6 days, cells were restimulated with RPMI (negative control) or LPS (10 ng/mL). IL-6, TNF-α and IL-10 concentrations were determined with ELISA in harvested culture supernatants according to the manufacturer's instructions. All conditions belonging to one volunteer were measured on the same plate.

Chromatin accessibility mapping by ATAC-seq

ATAC-seq was performed as previously described (62, 63), with minor adaptations. In each experiment, 50,000 PBMCs were collected at 300 g for 5 min at 4 °C. After centrifugation, the pellet was carefully resuspended in the transposase reaction mix (12.5 µl 2xTD buffer, 2 µl TDE1 (Illumina), 10.25 µl nuclease-free water, and 0.25 µl 1% digitonin (Promega)) for 30 min at 37 °C. Following DNA purification with the MinElute kit eluting in 11 µl, 1 µl of eluted DNA was used in a qPCR reaction to estimate the optimum number of amplification cycles. The remaining 10 µl of each library were amplified for the number of cycles corresponding to the Cq value (the cycle number at which fluorescence has in-creased above background levels) from the qPCR. Library amplification was followed by SPRI (Beckman Coulter) size selection to exclude fragments larger than 1,200 bp. DNA concentration was measured with a Qubit fluorometer (Life Technologies). Library amplification was performed using custom Nextera primers15. Libraries were sequenced by the Biomedical Sequencing Facility at CeMM using the Illumina HiSeq 3000/4000 platform and the 50-bp single-end configuration.

Chromatin accessibility mapping by ATAC-seq was done in two biologically independent experiments. Sequencing statistics are provided in **supplementary table S2**.

Chromatin accessibility data processing

ATAC-seq reads were trimmed using Skewer (64) and aligned to the GRCh38 assembly of the human genome using Bowtie 2 (65) with the "-very-sensitive" parameter. Duplicate reads were removed using the sambamba (66) "markdup" command and reads with mapping quality >=30 and alignment to the genome were kept. Peak calling was performed with MACS2 (67) using the "-nomodel" and "-extsize 147" parameters, and peaks overlapping blacklisted features as defined by the ENCODE project (68) were discarded. Transposase cutting loci were generated from the filtered BAM files taking into account the transposase bias in a strand-specific way.

We calculated the following sample quality statistics. The fraction of reads in peaks (*FRIP*) was calculated as the fraction of transposase cutting events overlapping identified peaks. Similarly, we quantified the fraction of transposase cutting events overlapping all regions defined in the Ensembl Regulatory Build (*Oracle_FRIP*) (69) and the fraction of peaks overlapping the promoters (*Promoter_FRIP*). We calculated the transcription start site (TSS) enrichment plot as the histogram of transposase cutting events in the peaks around all TSSs (±1,000 bp), normalized by the average tail value (calculated as the average coverage on the last 100 bp of both the left and right tails of the histogram). The *TSS_enrichment* value was defined as the maximum value of the enrichment plot.

Upon examining the sample quality statistics, we selected samples with at least 100,000 sequenced reads and 5,000 called peaks for further analysis. Next, peak lists were aggregated to a consensus peak list by merging called peak summits extended on both sides by 250 bp across all samples which passed the quality control. We quantified the accessibility of each consensus peak in each sample by counting the number of transposition events that overlapped the peak.

The peaks were annotated using UROPA (70) with features defined based on the GENCODE v31 reference annotation (69) as follows: *TSS* if the peak was within ±100 bp from the TSS, *TSS_proximal* if

the peak was within 1000 bp upstream or 500 bp downstream from the TSS, *gene_body* if the peak overlapped a gene, *distal* if the peak was within 100,000 bp from the TSS, *intergenic* otherwise. For each peak only the closest GENCODE feature was considered and the annotations took precedence in the following order: *TSS*, *TSS_proximal*, *gene_body*, *distal*, and *intergenic*.

Differential chromatin accessibility analysis

To identify open chromatin regions that respond differently to training induced by BCG conditioned on the time of vaccine administration, we used the following linear model with interaction: *accessibility ~ TSS_enrichment + batch + sex + age + monocytes + T_cells + B_cells + NK_cells + NKT_cells + visit + time + visit:time*, where TSS*_enrichment* is a sample quality statistic defined in the previous section, *batch* refers to the experimental batch of the ATAC-seq library, *sex* and *age* refer to respective donor annotations, *monocytes*, *T_cells*, *B_cells*, *NK_cells*, and *NKT_cells* refer to cell proportions of the respective cell types estimated with Coulter counter (from PBMCs) and flow cytometry (from whole blood samples), *visit* is a categorical term encoded as 0, 1, and 2 for the first (baseline), second (two weeks post BCG), and third (three months post BCG) visit, respectively, and *time* is a categorical term encoded as 0 for morning (8:00-9:00) and 1 for evening (18:00-18:30) vaccination. We fit this model using R package LIMMA-voom (71) with TMM normalization (72) and duplicate correlation function with blocking on donor identity followed by empirical Bayes function for shrinking the variance across all tested peaks. We used false discovery rate (FDR) to correct for multiple testing across all tested peaks with Benjamini-Hochberg method. We were interested in the regression coefficients and P-values of the *visit:time* interaction term. Given the aforementioned encoding of *visit* and *time* terms, positive coefficients identify regions where BCG-induced chromatin remodeling (second or third visit compared to first visit) results in a relative increase in accessibility if vaccinated in the evening compared to morning. We used R package LOLA (73) to identify enrichment of transcription factor binding sites (TFBSs) in the open chromatin regions. To this end, we tested all peaks passing the FDR of 0.1, separately for peaks with positive and negative coefficients, against CODEX (74), a database of

TFBSs determined with ChIP-seq in hematopoietic and embryonic cell lines, and JASPAR (75), a database of manually-curated experimentally-defined TF binding profiles. We used the BED files from *LOLACore* v180412 and *LOLAExt* v170206 for CODEX and JASPAR, respectively.

To identify enrichment of known molecular pathways, we mapped each peak to the nearest feature based on GENCODE (see previous section for details). Next, we retained only peaks that mapped to protein-coding features and were annotated as *TSS*, *TSS_proximal*, *gene_body*, or *distal* but within a distance of 10,000 bp from the TSS. Finally, we tested peaks passing the FDR of 0.1, separately for peaks with positive and negative coefficients, using the online platform Enrichr (76), which performs the Fisher's exact test, for enrichment of *KEGG_2019_Human* pathways (77).

Complete blood count

Complete blood counts were performed on EDTA whole blood and PBMC fractions after Ficoll isolation on a Sysmex XN-450 haematology analyzer.

Serum cortisol measurements

Serum cortisol was analyzed by LCMSMS after protein precipitation and solid-phase extraction as described previously (78), with the following additional compound specific configurations and characteristics. Internal standard [$^{13}C_3$]-cortisol (Isoscience, King of Prussia, PA) was used. Retention time was 1.46 min. A 9-point calibration curve was used (Sigma). Two transitions (qualitative and quantitative) were monitored. Transitions (Q1>Q3) were m/z 363.4 > 121.1 (25kEV) and m/z 363.4 > 97.1 (34 kEV) for cortisol; m/z 366.4 > 124.1 (25 kEV) and m/z 366.4 > 100.1 (35 kEV) for $^{13}C_3$-cortisol. Dwell time was 100 ms. The method was linear assessed by CLSI EP6 protocol. Recovery was within 96.5 – 102%. Total CV for cortisol is 3.6% at 301 nmol/L and 3.1% at 1092 nmol/L. LOQ was 1.91 nmol/L (13.4% CV).

Statistical analysis

Raw cytokine values were first log-transformed, and then corrected for batch effects using a linear regression model. These data conversions were performed using the statistical programming language R. R-squared represents the explained variance. Corrected cytokine values were converted to fold changes from baseline. Cytokine values are non-normally distributed, as previously demonstrated in detail (79). Mann-Whitney U test was used to compare fold changes between morning and evening vaccinated groups. Kruskal-Wallis test with Dunn's multiple comparison test was used to test for differences in the four different vaccinated subgroups divided by time of vaccination in the 300BCG cohort. Wilcoxon matched-pairs signed rank test was used to compare fold changes of in vitro trained samples belonging to the same volunteer. Friedman test with Dunn's multiple comparison test was used to compare multiple groups of samples belonging to the same volunteer. Complete blood count values were converted to fold changes from baseline. A two-sided p-value of < 0.05 was considered statistically significant.

Study approval

The 300BCG study is approved by the Arnhem-Nijmegen medical ethical committee (NL58553.091.16). The study was performed in accordance with the declaration of Helsinki. Written informed consent was obtained of the healthy volunteers as first during the first visit.

Author contributions

MN and LCJdB designed the study. VPM, LCJdB, VACMK, SJCFMM, HL and HD conducted the cohort study and performed the experiments. LCJdB and RJ performed the in vitro experiments. LCJdB, VPM, and VACMK analyzed the data. TK and VFG performed the ATAC-seq library preparation, DB processed the sequenced ATAC-seq samples, LF performed the differential chromatin accessibility analysis. RA performed experiments and BN analyzed RNA-seq data from the previous study. MN, RvC, LABJ and CB supervised the analysis and interpretation of results. LCJdB and VPM wrote the manuscript which was critically reviewed and approved by all authors. The order of co-first authorship of LCJdB and VPM is defined by the primary responsibility.

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REFERENCES

1. WHO. BCG Immunization coverage estimates by WHO region. *Global Health Observatory data repository.* 2018.
2. Trunz BB, Fine P, and Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. *Lancet (London, England).* 2006;367(9517):1173-80.
3. Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *Jama.* 1994;271(9):698-702.
4. Brewer TF. Preventing tuberculosis with bacillus Calmette-Guerin vaccine: a meta-analysis of the literature. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America.* 2000;31 Suppl 3:S64-7.
5. Voss G, Casimiro D, Neyrolles O, Williams A, Kaufmann SHE, McShane H, et al. Progress and challenges in TB vaccine development. *F1000Research.* 2018;7:199.
6. Kleinnijenhuis J, van Crevel R, and Netea MG. Trained immunity: consequences for the heterologous effects of BCG vaccination. *Trans R Soc Trop Med Hyg.* 2015;109(1):29-35.
7. Aaby P, Roth A, Ravn H, Napirna BM, Rodrigues A, Lisse IM, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis.* 2011;204(2):245-52.
8. Biering-Sorensen S, Aaby P, Napirna BM, Roth A, Ravn H, Rodrigues A, et al. Small randomized trial among low-birth-weight children receiving bacillus Calmette-Guerin vaccination at first health center contact. *The Pediatric infectious disease journal.* 2012;31(3):306-8.
9. Biering-Sorensen K, Aaby P, Lund N, Monteiro I, Jensen KJ, Benn CS, et al. Early BCG-Denmark and Neonatal Mortality Among Infants Weighing <2500 g: A Randomized Controlled Trial. *Clinical Infectious Diseases.* 2017;65(7):1183-90.
10. Biering-Sorensen S, Jensen KJ, Monterio I, Ravn H, Aaby P, and Benn CS. Rapid Protective Effects of Early BCG on Neonatal Mortality Among Low Birth Weight Boys: Observations From Randomized Trials. *J Infect Dis.* 2018;217(5):759-66.
11. Benn CS, Netea MG, Selin LK, and Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. *Trends Immunol.* 2013;34(9):431-9.
12. de Bree LCJ, Koeken V, Joosten LAB, Aaby P, Benn CS, van Crevel R, et al. Non-specific effects of vaccines: Current evidence and potential implications. *Semin Immunol.* 2018;39:35-43.
13. Schaltz-Buchholzer F, Biering-Sorensen S, Lund N, Monteiro I, Umbasse P, Fisker AB, et al. Early BCG Vaccination, Hospitalizations, and Hospital Deaths: Analysis of a Secondary Outcome in 3 Randomized Trials from Guinea-Bissau. *J Infect Dis.* 2019;219(4):624-32.
14. Netea MG, Latz E, Mills KH, and O'Neill LA. Innate immune memory: a paradigm shift in understanding host defense. *Nat Immunol.* 2015;16(7):675-9.
15. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Jacobs C, Xavier RJ, et al. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clin Immunol.* 2014;155(2):213-9.
16. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci U S A.* 2012;109(43):17537-42.
17. Arts RJ, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. *Cell Rep.* 2016;17(10):2562-71.
18. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonca LE, Pacis A, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell.* 2018;172(1-2):176-90 e19.

19. Cirovic B, de Bree LCJ, Groh L, Blok BA, Chan J, Van der Velden JFM, et al. Human Bacille Calmette-Guérin vaccination elicits trained immunity via the hematopoietic progenitor compartment. *Cell Host Microbe.* 2020; S1931-3128(20)30296-1.
20. Arts RJW, Moorlag S, Novakovic B, Li Y, Wang SY, Oosting M, et al. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host Microbe.* 2018;23(1):89-100 e5.
21. Cheng SC, Quintin J, Cramer RA, Shepardson KM, Saeed S, Kumar V, et al. mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science.* 2014;345(6204):1250684.
22. Bekkering S, Blok BA, Joosten LA, Riksen NP, van Crevel R, and Netea MG. In-vitro experimental model of trained innate immunity in human primary monocytes. *Clin Vaccine Immunol.* 2016;23(12):926-33.
23. Walk J, de Bree LCJ, Graumans W, Stoter R, van Gemert GJ, van de Vegte-Bolmer M, et al. Outcomes of controlled human malaria infection after BCG vaccination. *Nat Commun.* 2019;10(1):874.
24. Boivin DB, James FO, Wu A, Cho-Park PF, Xiong H, and Sun ZS. Circadian clock genes oscillate in human peripheral blood mononuclear cells. *Blood.* 2003;102(12):4143-5.
25. Adrover JM, Del Fresno C, Crainiciuc G, Cuartero MI, Casanova-Acebes M, Weiss LA, et al. A Neutrophil Timer Coordinates Immune Defense and Vascular Protection. *Immunity.* 2019;50(2):390-402.
26. Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, et al. A circadian clock in macrophages controls inflammatory immune responses. *Proc Natl Acad Sci U S A.* 2009;106(50):21407-12.
27. Curtis AM, Bellet MM, Sassone-Corsi P, and O'Neill LA. Circadian clock proteins and immunity. *Immunity.* 2014;40(2):178-86.
28. Scheiermann C, Kunisaki Y, and Frenette PS. Circadian control of the immune system. *Nat Rev Immunol.* 2013;13(3):190-8.
29. Labrecque N, and Cermakian N. Circadian Clocks in the Immune System. *Journal of biological rhythms.* 2015;30(4):277-90.
30. Long JE, Drayson MT, Taylor AE, Toellner KM, Lord JM, and Phillips AC. Morning vaccination enhances antibody response over afternoon vaccination: A cluster-randomised trial. *Vaccine.* 2016;34(24):2679-85.
31. Phillips AC, Gallagher S, Carroll D, and Drayson M. Preliminary evidence that morning vaccination is associated with an enhanced antibody response in men. *Psychophysiology.* 2008;45(4):663-6.
32. Kurupati RK, Kossenkoff A, Kannan S, Haut LH, Doyle S, Yin X, et al. The effect of timing of influenza vaccination and sample collection on antibody titers and responses in the aged. *Vaccine.* 2017;35(30):3700-8.
33. Stevens WB, Netea MG, Kater AP, and van der Velden WJ. 'Trained immunity': consequences for lymphoid malignancies. *Haematologica.* 2016;101(12):1460-8.
34. Mulder WJM, Ochando J, Joosten LAB, Fayad ZA, and Netea MG. Therapeutic targeting of trained immunity. *Nat Rev Drug Discov.* 2019;18(7):553-66.
35. van Kerkhof LW, Van Dycke KC, Jansen EH, Beekhof PK, van Oostrom CT, Ruskovska T, et al. Diurnal Variation of Hormonal and Lipid Biomarkers in a Molecular Epidemiology-Like Setting. *PLoS One.* 2015;10(8):e0135652.
36. Nissen TN, Birk NM, Blok BA, Arts RJW, Andersen A, Kjaergaard J, et al. Bacillus Calmette-Guerin vaccination at birth and in vitro cytokine responses to non-specific stimulation. A randomized clinical trial. *Eur J Clin Microbiol Infect Dis.* 2018;37(1):29-41.
37. Early JO, and Curtis AM. Immunometabolism: Is it under the eye of the clock? *Semin Immunol.* 2016;28(5):478-90.
38. Man K, Loudon A, and Chawla A. Immunity around the clock. *Science.* 2016;354(6315):999-1003.

39. Bureau JP, Garrelly L, and Vago P. Nycthemeral variations on LPS- and BCG-induced PMN migration in normal mice. *International journal of tissue reactions.* 1991;13(4):203-6.
40. Bureau JP, Labrecque G, Coupe M, and Garrelly L. Influence of BCG administration time on the in-vivo migration of leukocytes. *Chronobiology international.* 1986;3(1):23-8.
41. Lopes C, Mariano M, and Markus RP. Interaction between the adrenal and the pineal gland in chronic experimental inflammation induced by BCG in mice. *Inflammation research : official journal of the European Histamine Research Society [et al].* 2001;50(1):6-11.
42. Tsai TH, Burns RE, and Scheving LE. Circadian influence on the immunization of mice with live Bacillus Calmette-Guerin (BCG) and subsequent challenge with Ehrlich ascites carcinoma. *Chronobiologia.* 1979;6(3):187-201.
43. Kiessling S, Dubeau-Laramee G, Ohm H, Labrecque N, Olivier M, and Cermakian N. The circadian clock in immune cells controls the magnitude of Leishmania parasite infection. *Sci Rep.* 2017;7(1):10892.
44. Dos Santos JC, Barroso de Figueiredo AM, Teodoro Silva MV, Cirovic B, de Bree LCJ, Damen M, et al. beta-Glucan-Induced Trained Immunity Protects against Leishmania braziliensis Infection: a Crucial Role for IL-32. *Cell Rep.* 2019;28(10):2659-72 e6.
45. Tognini P, Thaiss CA, Elinav E, and Sassone-Corsi P. Circadian Coordination of Antimicrobial Responses. *Cell Host Microbe.* 2017;22(2):185-92.
46. Astrinidis A, Kim J, Kelly CM, Olofsson BA, Torabi B, Sorokina EM, et al. The transcription factor SP1 regulates centriole function and chromosomal stability through a functional interaction with the mammalian target of rapamycin/raptor complex. *Genes Chromosomes Cancer.* 2010;49(3):282-97.
47. Jablonska A, and Polouliakh N. In silico discovery of novel transcription factors regulated by mTOR-pathway activities. *Front Cell Dev Biol.* 2014;2:23.
48. Yamada T, Gierach K, Lee PH, Wang X, and Lacorazza HD. Cutting edge: Expression of the transcription factor E74-like factor 4 is regulated by the mammalian target of rapamycin pathway in CD8+ T cells. *J Immunol.* 2010;185(7):3824-8.
49. Wang Y, Zhao B, Zhang Y, Tang Z, Shen Q, Zhang Y, et al. Kruppel-like factor 4 is induced by rapamycin and mediates the anti-proliferative effect of rapamycin in rat carotid arteries after balloon injury. *Br J Pharmacol.* 2012;165(7):2378-88.
50. Rijo-Ferreira F, and Takahashi JS. Genomics of circadian rhythms in health and disease. *Genome Med.* 2019;11(1):82.
51. Edgar RS, Stangherlin A, Nagy AD, Nicoll MP, Efstathiou S, O'Neill JS, et al. Cell autonomous regulation of herpes and influenza virus infection by the circadian clock. *Proc Natl Acad Sci U S A.* 2016;113(36):10085-90.
52. Majumdar T, Dhar J, Patel S, Kondratov R, and Barik S. Circadian transcription factor BMAL1 regulates innate immunity against select RNA viruses. *Innate Immun.* 2017;23(2):147-54.
53. Abadie V, Badell E, Douillard P, Ensergueix D, Leenen PJ, Tanguy M, et al. Neutrophils rapidly migrate via lymphatics after Mycobacterium bovis BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. *Blood.* 2005;106(5):1843-50.
54. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, et al. Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. *Immunity.* 2012;37(5):917-29.
55. Mendez-Ferrer S, Lucas D, Battista M, and Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature.* 2008;452(7186):442-7.
56. Garcia-Garcia A, Korn C, Garcia-Fernandez M, Domingues O, Villadiego J, Martin-Perez D, et al. Dual cholinergic signals regulate daily migration of hematopoietic stem cells and leukocytes. *Blood.* 2019;133(3):224-36.
57. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, et al. Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity.* 2006;24(6):801-12.

58. Takizawa H, Boettcher S, and Manz MG. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood.* 2012;119(13):2991-3002.
59. Early JO, Menon D, Wyse CA, Cervantes-Silva MP, Zaslona Z, Carroll RG, et al. Circadian clock protein BMAL1 regulates IL-1beta in macrophages via NRF2. *Proc Natl Acad Sci U S A.* 2018;115(36):E8460-e8.
60. Coutinho AE, and Chapman KE. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Molecular and cellular endocrinology.* 2011;335(1):2-13.
61. Nemes E, Geldenhuys H, Rozot V, Rutkowski KT, Ratangee F, Bilek N, et al. Prevention of M. tuberculosis Infection with H4:IC31 Vaccine or BCG Revaccination. *N Engl J Med.* 2018;379(2):138-49.
62. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, and Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods.* 2013;10(12):1213-8.
63. Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. *Nat Genet.* 2016;48(10):1193-203.
64. Jiang H, Lei R, Ding SW, and Zhu S. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. *BMC Bioinformatics.* 2014;15:182.
65. Langmead B, and Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9(4):357-9.
66. Tarasov A, Vilella AJ, Cuppen E, Nijman IJ, and Prins P. Sambamba: fast processing of NGS alignment formats. *Bioinformatics.* 2015;31(12):2032-4.
67. Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, et al. Model-based analysis of ChIP-Seq (MACS). *Genome Biol.* 2008;9(9):R137.
68. Consortium EP. An integrated encyclopedia of DNA elements in the human genome. *Nature.* 2012;489(7414):57-74.
69. Frankish A, Diekhans M, Ferreira AM, Johnson R, Jungreis I, Loveland J, et al. GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res.* 2019;47(D1):D766-D73.
70. Kondili M, Fust A, Preussner J, Kuenne C, Braun T, and Looso M. UROPA: a tool for Universal RObust Peak Annotation. *Sci Rep.* 2017;7(1):2593.
71. Law CW, Chen Y, Shi W, and Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.* 2014;15(2):R29.
72. Robinson MD, and Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* 2010;11(3):R25.
73. Sheffield NC, and Bock C. LOLA: enrichment analysis for genomic region sets and regulatory elements in R and Bioconductor. *Bioinformatics.* 2016;32(4):587-9.
74. Sanchez-Castillo M, Ruau D, Wilkinson AC, Ng FS, Hannah R, Diamanti E, et al. CODEX: a next-generation sequencing experiment database for the haematopoietic and embryonic stem cell communities. *Nucleic Acids Res.* 2015;43(Database issue):D1117-23.
75. Fornes O, Castro-Mondragon JA, Khan A, van der Lee R, Zhang X, Richmond PA, et al. JASPAR 2020: update of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* 2020;48(D1):D87-D92.
76. Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Res.* 2016;44(W1):W90-7.
77. Kanehisa M, Furumichi M, Tanabe M, Sato Y, and Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res.* 2017;45(D1):D353-D61.
78. Ter Horst R, Jaeger M, Smeekens SP, Oosting M, Swertz MA, Li Y, et al. Host and Environmental Factors Influencing Individual Human Cytokine Responses. *Cell.* 2016;167(4):1111-24 e13.

79. Li Y, Oosting M, Deelen P, Ricano-Ponce I, Smeekens S, Jaeger M, et al. Inter-individual variability and genetic influences on cytokine responses to bacteria and fungi. *Nat Med.* 2016;22(8):952-60.

Figure 1

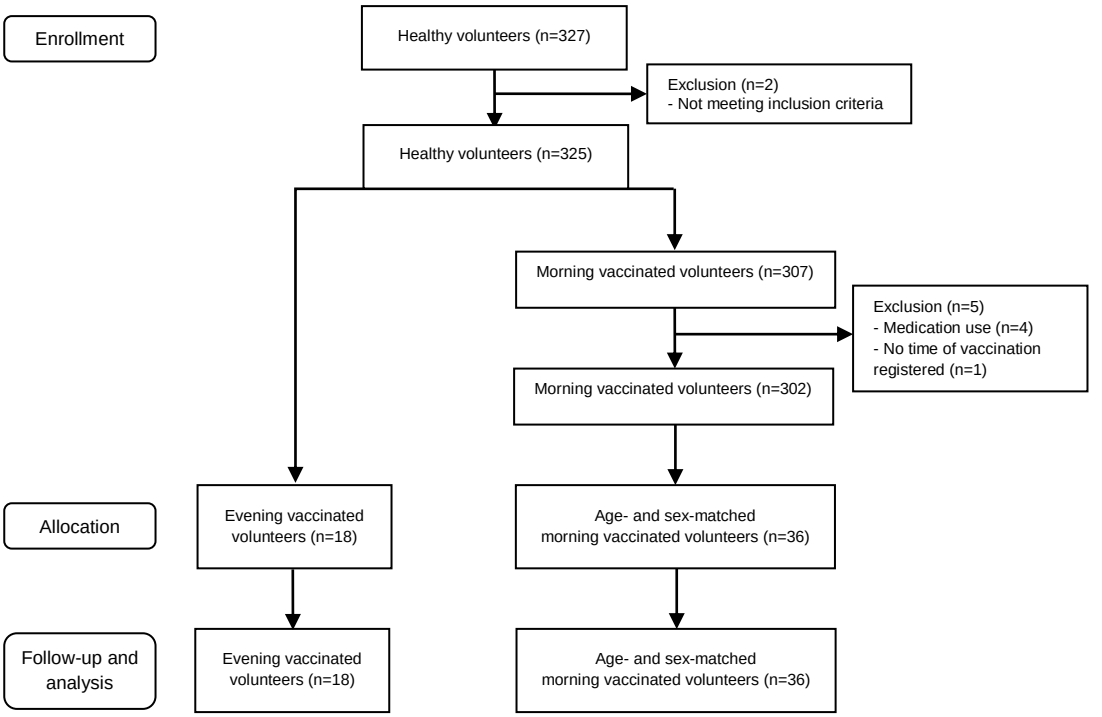

Figure 1. Flow diagram of individuals included in this study. 327 healthy volunteers were included, of which 2 did not meet inclusion criteria. Of the 325 individuals vaccinated, 18 participants were vaccinated in the evening and 307 were vaccinated in the morning. Of the 307 morning-vaccinated individuals, 5 were excluded due to medication use or lack of information. Of the 302 morning-vaccinated individuals, 36 sex- and age-matched controls were selected for further analysis with the evening-vaccinated individuals.

Figure 2

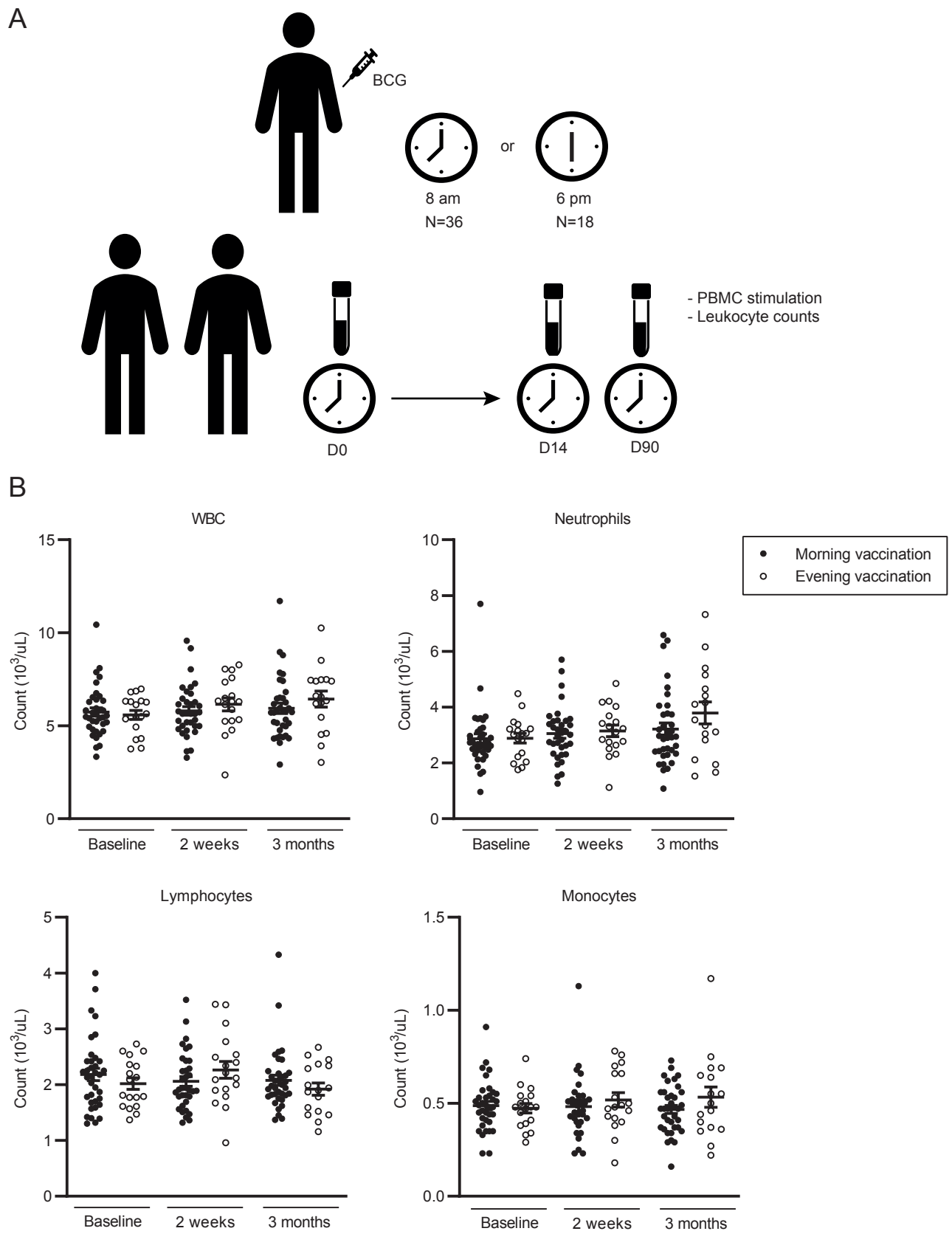

Figure 2. Two groups of healthy volunteers were vaccinated with BCG at two time points: 18 volunteers between 18.00 and 18.30pm, while 36 (1:2 ratio) sex and age-matched controls were vaccinated between 8am and 9am. Blood was collected in the morning at baseline, two weeks, and three months after BCG vaccination (A) Whole blood complete blood counts and leukocyte differential (neutrophil, lymphocyte and monocyte counts) of morning vaccinated individuals and evening-vaccinated individuals (B). (Mean ± SEM, morning n = 36, evening n = 18, Kruskal-Wallis test, Dunn multiple comparison test).

Figure 3

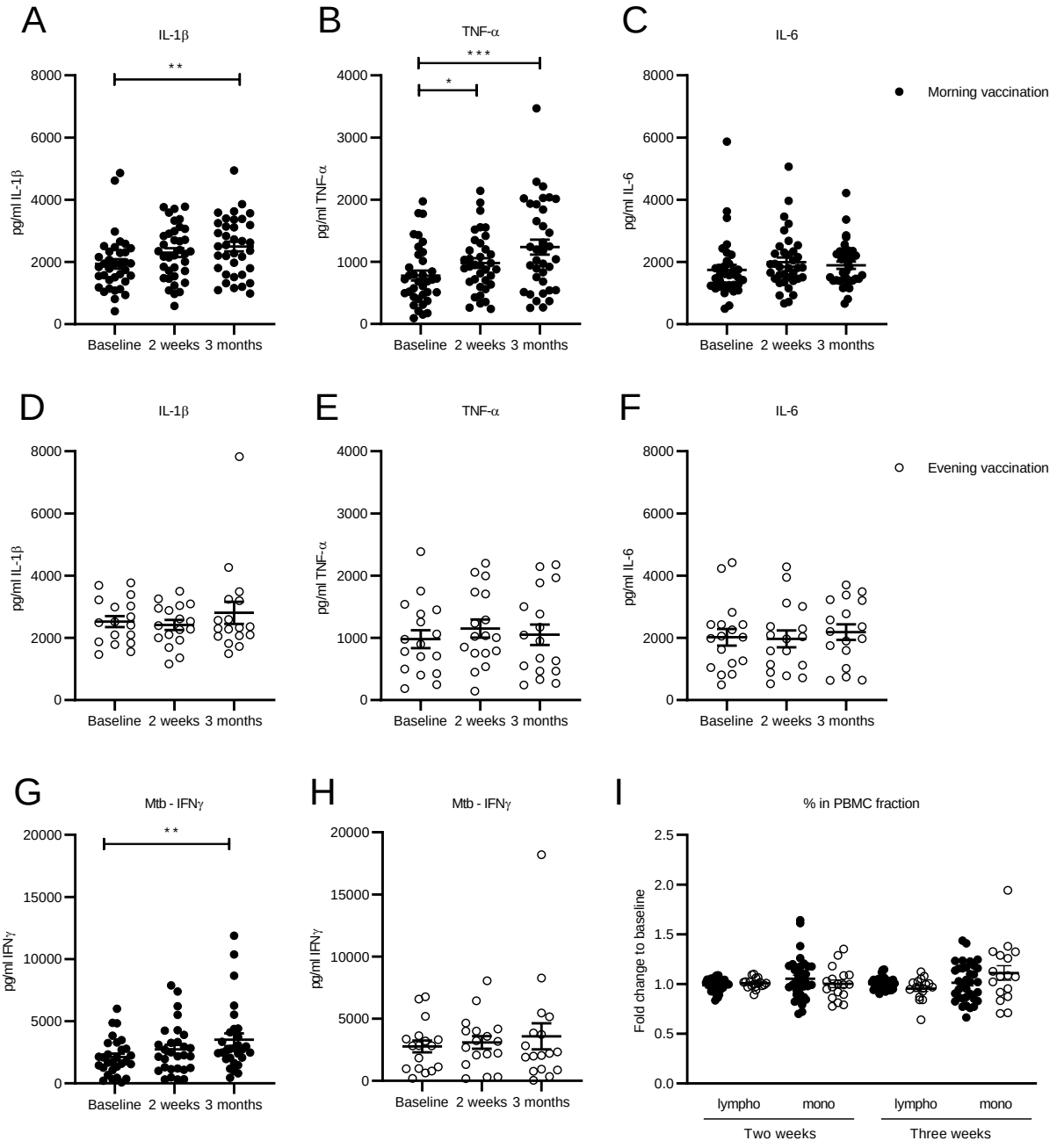

Figure 3. Cytokine values of IL-1β, IL-6 and TNF-α, production to S. aureus stimulation two weeks and three months after BCG vaccination, and production of IFN-γ to M. tuberculosis stimulation of morning (A-C, G) and evening (D-F, H) vaccinated individuals. (Mean ±SEM, n=36 morning vaccinated, n=18 evening vaccinated, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Friedman Test, Dunn multiple comparison test). Fold changes (compared to baseline) of monocyte and lymphocyte percentages within PBMC fraction (I). (Mean ± SEM, morning n = 36, evening n = 18, Kruskal-Wallis test, Dunn multiple comparison test).

Figure 4

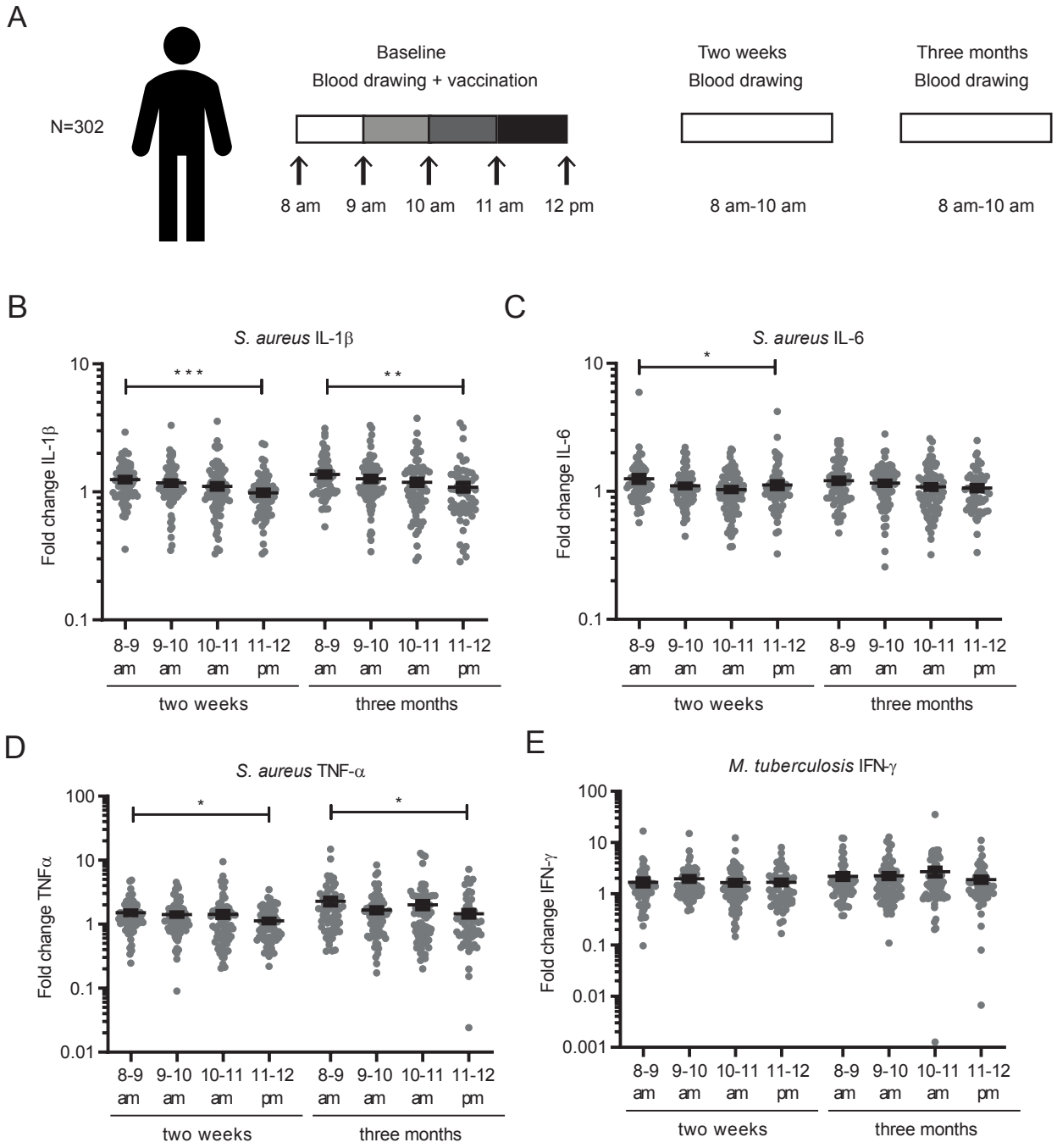

Figure 4. 302 healthy volunteers were BCG vaccinated between 8am and 12pm and blood was collected before, two weeks after and three months after BCG vaccination (A). Fold changes (compared to baseline) two weeks and three months after BCG vaccination of PBMC derived IL-1β (B), IL-6 (C) and TNF-α (D) production to S. aureus stimulation, and IFN-γ production to M. tuberculosis stimulation (E). (Mean ± SEM, n=68 vaccinated between 8am-9am, n=80 vaccinated between 9am-10am, n=84 vaccinated between 10am-11am, n=66 vaccinated between 11am-12pm, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Kruskal-Wallis test, Dunn multiple comparison test).

Figure 5

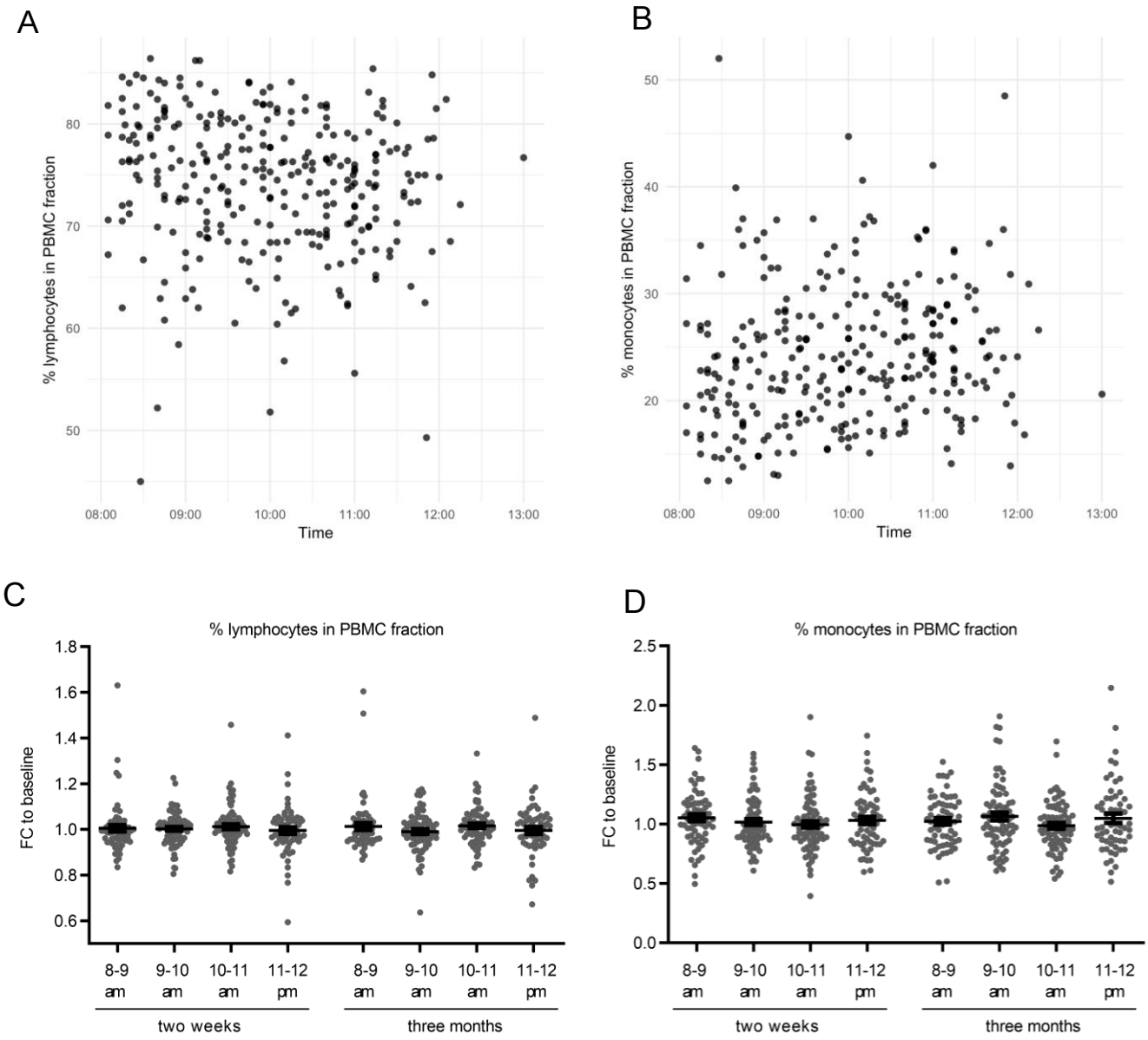

Figure 5. Spearman correlation plot of lymphocyte percentages (p=0.004) (A) and monocyte percentages (p=0.002) (B) within the PBMC fraction against time of blood collection at baseline visit. Comparisons of fold changes in lymphocyte (C) and monocyte percentages (D) between morning vaccinated subgroups. (Mean ± SEM, n=68 vaccinated between 8am-9am, n=80 vaccinated between 9am-10am, n=84 vaccinated between 10am-11am, n=66 vaccinated between 11am-12pm, Kruskal-Wallis test, Dunn multiple comparison test).

Figure 6

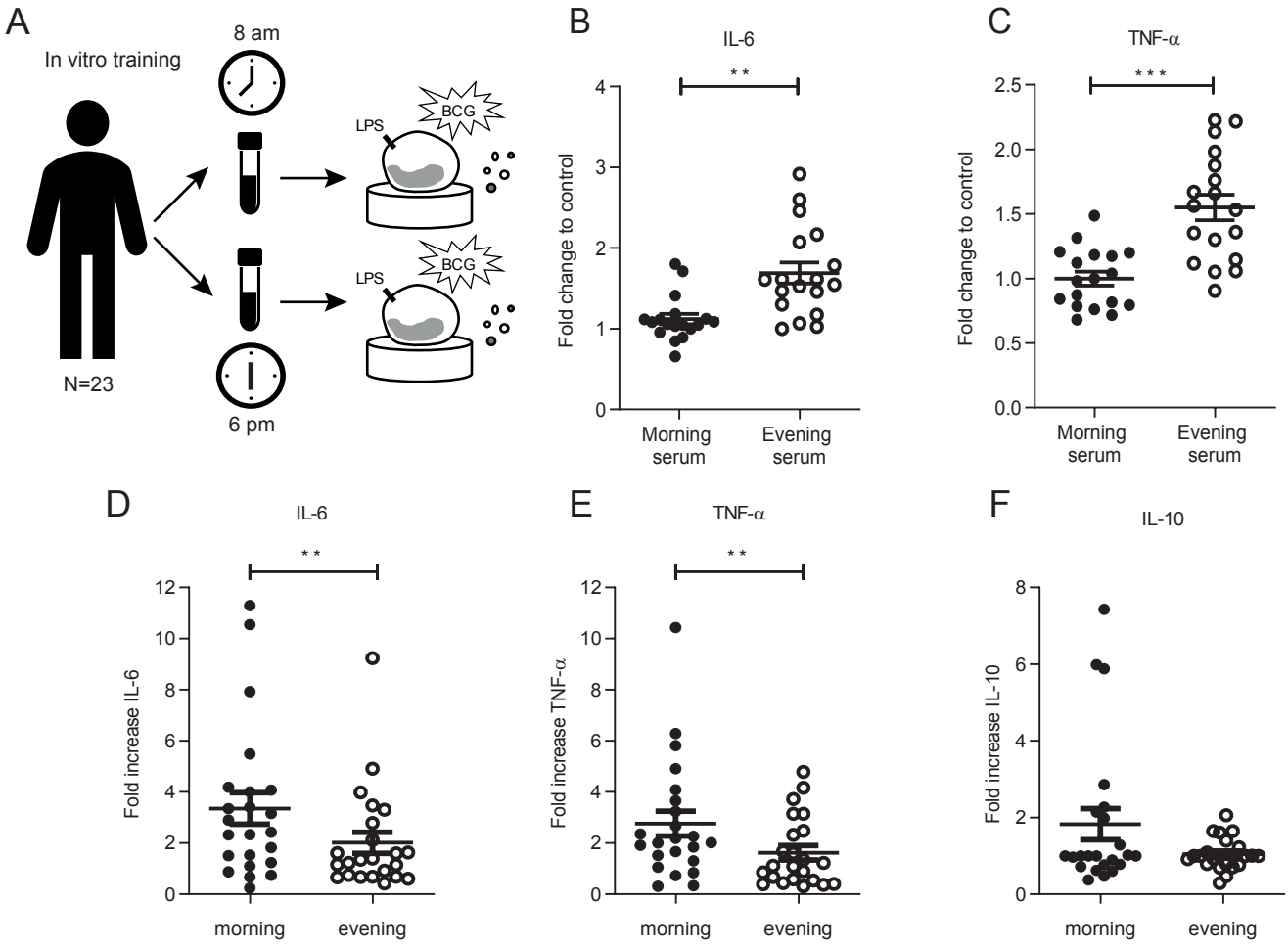

Figure 6. Blood was collected from healthy volunteers for isolation of serum and Percoll monocytes during the morning (8am) and evening (6pm) on the same day (A). Fold changes (compared to medium primed – LPS restimulated conditions) of IL-6 (B) and TNF-α (C) production of BCG primed monocytes supplemented with morning-derived serum versus evening-derived serum. Fold changes (to medium primed – LPS restimulated conditions) of IL-6 (D), TNF-α (E), and IL-10 (F) production after LPS restimulation of BCG trained monocytes derived after morning blood donation versus evening blood donation. (Mean ± SEM, n = 18 morning evening serum, n = 23 morning evening monocytes, *** $p < 0.001$, ** $p < 0.01$, Wilcoxon matched-pairs signed rank test).

Figure 7

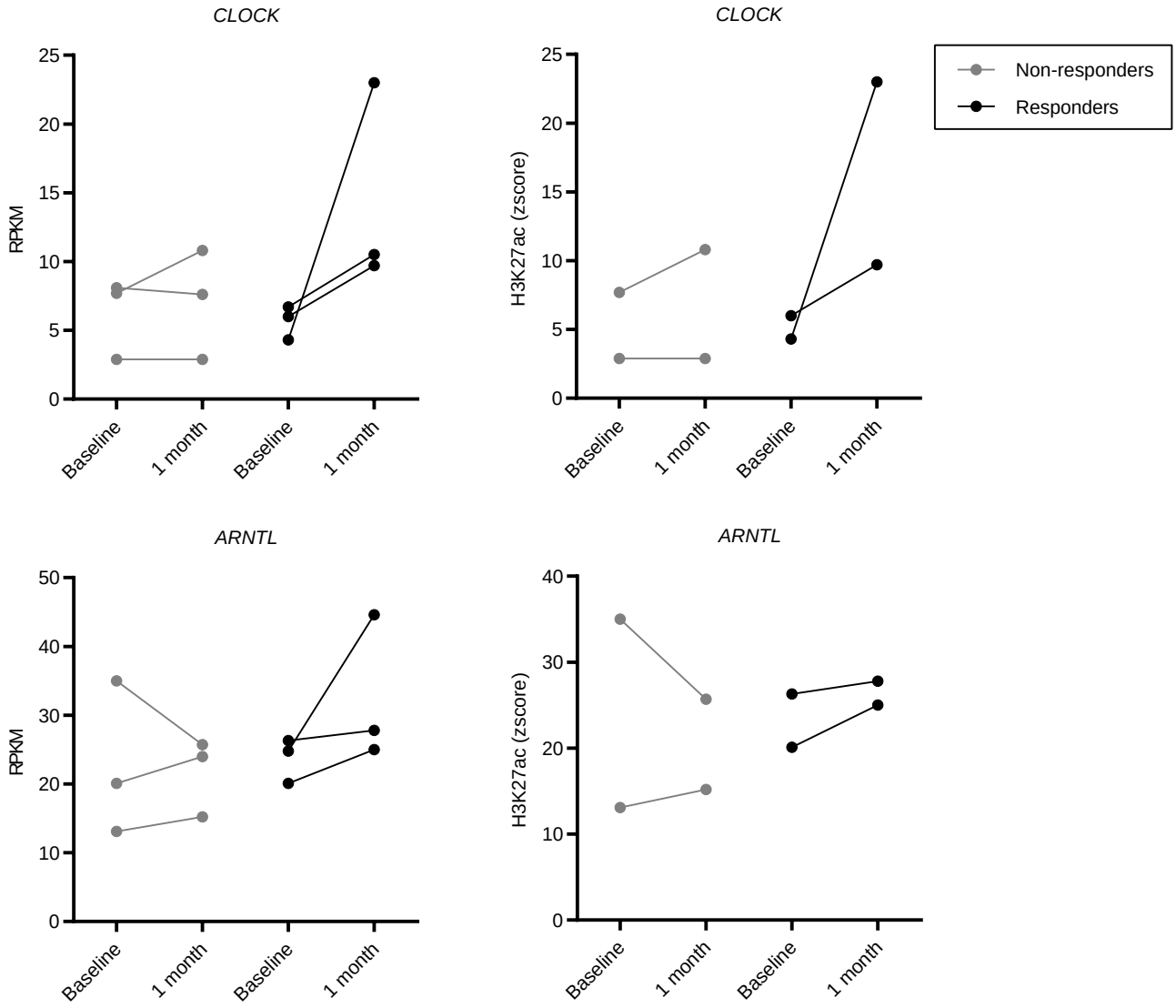

Figure 7. mRNA expression of CLOCK and ARNTL in monocytes from individuals before and 1 month after BCG vaccination. Individuals were divided into responders, which were protected from subsequent yellow fever viremia (maximum yellow fever viremia CT > 36 (n=3)), and non-responders (CT < 36) (n=3). H3K27ac levels at gene promoters (Z-score) of CLOCK and ARNTL before and 1 month after BCG vaccination in monocytes (n=2 both groups).

Figure 8

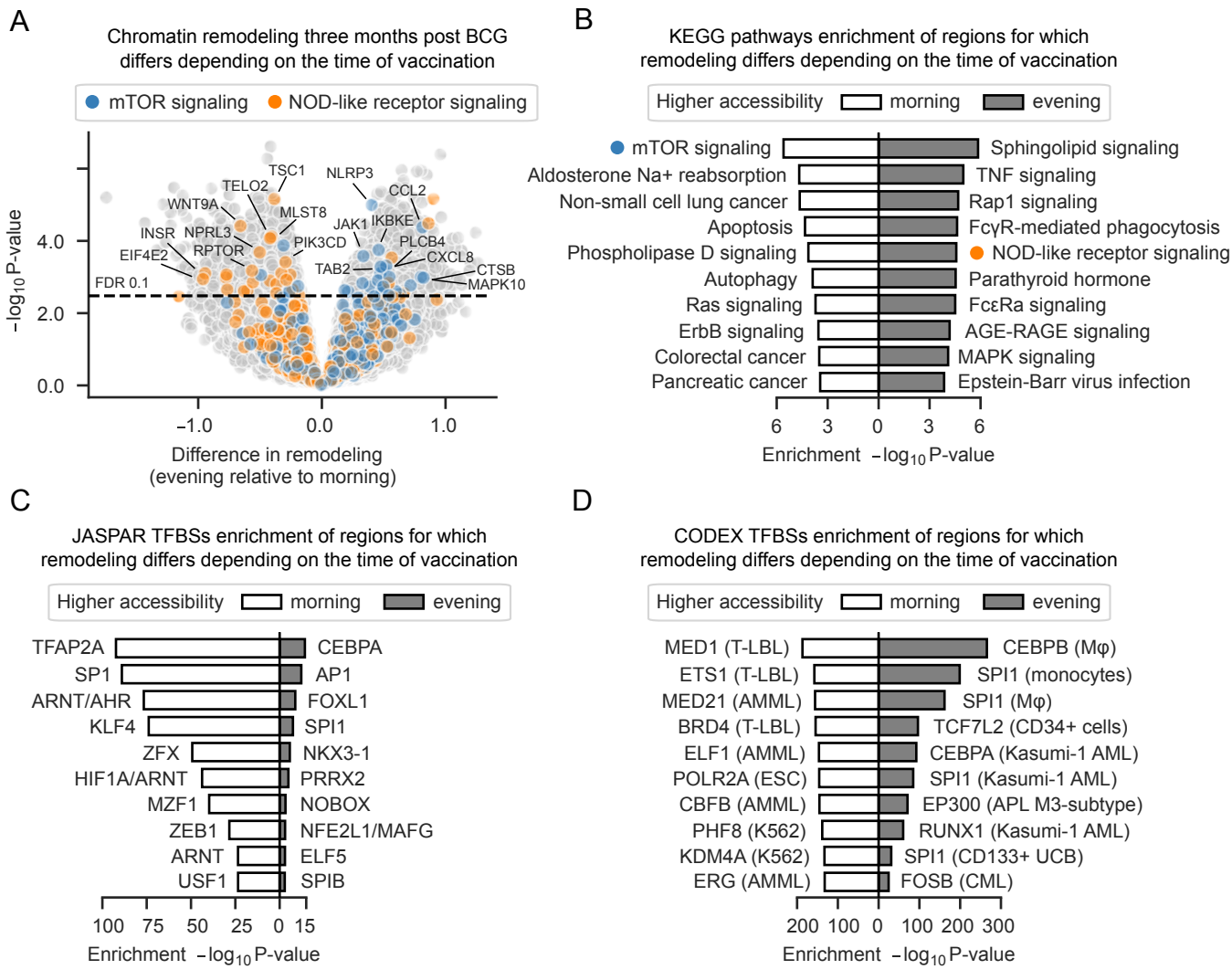

Figure 8. The effect of BCG vaccination on epigenome remodeling in PBMCs differs based on the time of vaccination. Differential chromatin accessibility (DA) analysis of an interaction effect between BCG training (three months post BCG compared to baseline) and time of vaccination (evening compared to morning). Open chromatin regions were assigned to genes based on proximity (A). KEGG pathways enrichment of regions for which remodeling three months post BCG differs based on the time of vaccination (showing top ten pathways for each direction, all displayed pathways passed FDR of 0.02) (B). JASPAR and CODEX transcription factor binding sites (TFBSs) enrichment of regions for which remodeling three months post BCG differs based on the time of vaccination (showing top ten TFs for each direction, all displayed TFs passed FDR of 0.005) (C-D). (n=36 morning vaccinated, n=18 evening vaccinated; DA was performed with LIMMA which computes P-values with a moderated T-test; enrichment analysis was performed with a Fisher's exact test; Benjamini-Hochberg procedure was used to control the FDR).

Table 1

	Morning vaccinated (n=36)	Evening vaccinated (n=18)
Age (years)	26.2 (SD 9.8)	25.8 (SD 10.7)
Sex (% female)	61%	61%
BMI	22.0 (SD 2.0)	21.9 (SD 2.0)
BCG scar size (cm)	0.44 (SD 0.14)	0.41 (SD 0.12)
Smoking (n)	1	1

Table 1. Characteristics of morning and evening vaccinated volunteers (mean ± SD, morning vaccinated n=36, evening vaccinated n=18).