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Alendronate modulates cytokine responses in healthy young individuals after BCG vaccination

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ABSTRACT

Bacillus Calmette–Guérin (BCG) vaccination induces memory characteristics in innate immune cells and their progenitors, a process called *trained immunity* mediated by epigenetic and metabolic reprogramming. Cholesterol synthesis plays an amplifying role in trained immunity through mevalonate release. Nitrogen-containing bisphosphonates (N-BPs), such as alendronate, can inhibit cholesterol synthesis. We explored their effects on trained immunity induced by BCG in a placebo-controlled clinical study (NL74082.091.20) in young, healthy individuals. Participants receiving single-dose oral alendronate on the day of BCG vaccination had more neutrophils and plasma cells one month after treatment. Alendronate led to reduced proinflammatory cytokine production by PBMCs stimulated with heterologous bacterial and viral stimuli one month later. Furthermore, the addition of alendronate transcriptionally suppressed multiple immune response pathways in PBMCs upon stimulation. Our findings indicate that N-BPs modulate the long-lasting effects of BCG vaccination on the cytokine production capacity of innate immune cells.

1. Introduction

The innate immune system responds early and rapidly after an infection. Although able to distinguish self from non-self, responses generated by innate immunity are antigen-agnostic [1]. Traditionally, only the adaptive immune system was thought capable of developing immunological memory. However, more recent research revealed that the innate immune system also mounts a memory-like response through epigenetic and metabolic programming of innate immune cells, which subsequently exhibit a more robust response to secondary infections. This trait, also termed trained immunity, can be induced by several live-attenuated vaccines, including Bacillus Calmette–Guérin (BCG), measles, and oral polio, that were shown to induce protection against heterologous infections [2].

Initially developed against tuberculosis, BCG also reduces all-cause childhood mortality through protection against a wide range of infections [3]. This non-specific protection is partly mediated by the changes in the epigenetic regulation of the function of innate immune cells and metabolic reprogramming marked by an increase in glycolysis and oxidative phosphorylation [4]. Moreover, BCG vaccination leads to persistent transcriptomic changes in human hematopoietic stem and progenitor cells and a myeloid differentiation bias in the bone marrow [5]. However, the efficacy of inducing trained immunity differs between individuals [6], and strategies to improve trained immune responses are needed.

Bisphosphonates (BPs) are a class of drugs mainly used to treat and prevent bone resorption [7]. They have an affinity for the bone and work as a calcium-chelating agent. So far, there are around ten BPs for human

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use in different conditions, varying from osteoporosis to Paget's disease to cancer. Some BPs, including alendronate, contain nitrogen in their side chains and have a different mechanism of action compared to simple BPs. Aside from targeting bone-resorbing osteoclasts, nitrogen-containing BPs (N-BPs) are internalized by monocytes and macrophages [8–10], and they inhibit cholesterol synthesis by blocking the activity of farnesyl pyrophosphate (FPP) synthase [11]. This inhibition leads to the accumulation of isopentenyl diphosphate and dimethylallyl diphosphate in peripheral blood mononuclear cells (PBMCs), with immunomodulatory activities such as activation of γδ T cells [8, 12].

Blockade of FPP synthase by alendronate can also lead to the accumulation of metabolites in the mevalonate pathway. Mevalonate accumulation in monocytes was previously linked to a stronger trained immunity phenotype [13]. Furthermore, another N-BP zoledronate drove both peritoneal and tumor-associated macrophages toward the pro-inflammatory and tumoricidal M1 phenotype in a mouse model of breast cancer [14]. A few observational studies found a lower risk of infections and cancer in people with chronic use of N-BPs [15–17]. Due to its low adverse event profile and potential immune-stimulating properties, we hypothesized that N-BPs could be a good candidate to improve BCG vaccine efficacy.

To test the potential of N-BPs as an adjuvant to improve trained immunity, we designed a clinical study in which the participants received either a placebo vaccination, the BCG vaccine, or the BCG vaccine together with oral alendronate tablets. We collected blood samples from participants before and one month after the intervention and measured cytokine responses following *ex vivo* stimulation of PBMCs with various stimuli. Furthermore, we performed flow cytometry from the whole blood to identify immune cell subsets and RNA sequencing (RNAseq) from PBMCs to assess the transcriptional responses.

2. Materials and methods

2.1. Study design and subjects

Healthy adults were recruited between June and August 2020 at the Radboud University Medical Center. Study subjects did not use any chronic medication except for oral contraceptives, did not have comorbidities, and were BCG-naïve by the time of inclusion. No power calculation was possible due to the absence of information on alendronate's potential effect size; therefore, this clinical trial was designed as an exploratory study. Participants were randomized as 1:1:1 to each group using the Castor electronic data capture platform to receive 9 mg/ml intradermal sodium chloride (Centrafarm, Netherlands) in 0.1 ml as placebo for BCG vaccination, 0.75 mg/ml intradermal BCG vaccine (AJ Vaccines, Denmark) in 0.1 ml, or intradermal BCG vaccine right after orally ingesting a 70 mg alendronate tablet (Aurobindo Pharma, India). 18, 21 and 18 participants completed the study in each arm, respectively. Age, sex, and BMI distributions in each group are provided in Table 1. Blood was collected from participants before and 1 month after vaccination. Ethical approval for the study (NL74082.091.20) was granted by the local ethics committee CMO region Arnhem-Nijmegen.

Table 1
Demographics of the study participants.

	Placebo (n = 18)	BCG (n = 21)	BCG+Alendronate (n = 18)	p-value
Age (years, mean ±sd)	26.9 ± 7.4	26.9 ± 8.1	30.5 ± 9.5	0.296
Sex (F/M)	7/11	16/5	8/10	0.039
BMI (kg/m2, mean±sd)	23.6 ± 1.8	22.9 ± 3.1	23.8 ± 3.7	0.604

2.2. Flow cytometry from whole blood

Before flow cytometry staining, the number of immune cells in the whole blood was determined using a hematology analyzer (Sysmex, Japan). The hematology analyzer relies on flow cytometry principles to identify different cell populations in the blood: the forward scatter light determines cell volume, the side scatter light gives information about cell nuclei and granules, while the side fluorescence indicates nucleic acids and organelles. As a result, cells having similar properties appear in a cluster.

For flow cytometry staining, erythrocytes were first lysed in isotonic NH4CL buffer and washed twice with PBS. White blood cell counts were determined by Coulter Ac-T Diff® cell counter (Beckman Coulter, CA, USA) and used to calculate the absolute numbers of CD45+ leukocytes identified by flow cytometry. 500.000 total leukocytes were used per staining panel. Cells were transferred to a V-bottom 96-well plate, washed twice with PBS + 0.2 % bovine serum albumin (BSA) (Sigma-Aldrich, MO, USA), and stained in the dark for 20 min at room temperature. Afterward, cells were washed twice more with PBS + 0.2 % BSA and measured with the Navios™ flow cytometer (Beckman Coulter). Details of the panels and antibodies are provided in Supplementary Table 1. Kaluza 2.1® software (Beckman Coulter) was used for data analysis.

2.3. PBMC isolation and ex-vivo stimulation

Whole blood was diluted with PBS, and PBMCs were isolated using density gradient centrifugation with Ficoll-Paque (GE Healthcare, IL, USA). The middle PBMC fraction was collected and washed three times with cold PBS. The cells were resuspended and stimulated in RPMI 1640 Medium (Dutch modification) (Thermo Fisher Scientific, MA, USA) supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific), 2 mM GlutaMAX supplement (Thermo Fisher Scientific), and 50 μg/mL gentamicin (Centrafarm, Netherlands). The PBMCs were isolated and used for experiments within 4 h after blood collection.

500.000 PBMCs per well were stimulated with 10 ng/ml *E. coli*-derived LPS, 10^6 /ml *S. aureus*, 10 μg/ml poly(I:C) (Invivogen, CA, USA), 3 μg/ml R848 (Invivogen), 3.3×10^5 K/mL TCID50 heat-inactivated influenza A H1N1 and 1.4×10^3 K/mL TCID50 heat-inactivated SARS-CoV-2 Wuhan strain for 24 h or 7 days in the presence of 10 % pooled human serum at 37 °C with 5 % CO_2. Virus inactivation was performed at 60°C for 30 min. After stimulation, cytokine levels in supernatants were measured using DuoSet® ELISA kits (R&D Systems, MN, USA) following the manufacturer's protocols. Only for IFNα, Human IFN-Alpha ELISA Kit from PBL Assay Science (NJ, USA) was used.

2.4. RNA isolation and sequencing

10 subjects from each intervention group were selected for RNA sequencing. The demographics of the subjects were given in Supplementary Table 2. Cryopreserved PBMCs were thawed and cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (Corning, NY, USA) and 2 mM l-glutamine (Thermo Fisher) for 2 h. After incubation, samples were washed with PBS, filtered, and counted. For samples which were also stimulated with Poly(I:C) (limited to 5 individuals per group with sufficient remaining cells, details in Supplementary Table 3), an additional 1 million cells were plated into one well of a 12-well plate and stimulated with 20 ug/mL poly(I:C) (Invivogen) for 4 h. For each sample, 1 million cells were lysed in RLT buffer (Qiagen, MD, USA) and stored at −80 °C for RNA extraction and sequencing. RNA extractions were performed using the miRNeasy mini or miRNeasy micro kits (Qiagen). RNA quality was evaluated with the 2100 Bioanalyzer (Agilent Technologies).

RNA library preparations were carried out on 100–500 ng of RNA

using the Illumina TruSeq Stranded Total RNA Sample preparation kit, according to the manufacturer's instructions. The libraries were size-selected using Ampure XP Beads (Beckman Coulter) and quantified using the KAPA Library Quantification kit – Universal (KAPA Biosystems). The RNA-Seq libraries were sequenced on the Illumina NovaSeq 6000 system using 100-bp single-end sequencing.

The quality of raw sequencing reads was assessed using FastQC v0.11.5 [18]. Reads were mapped to GENCODE human genome model (GRCh38 V34) using STAR 2.7.9a [19]. Gene transcripts were assembled and quantified on their corresponding human genome using the count-based method featureCounts [20] available in R from package Subread 2.0.3.

2.5. Differential gene expression and gene set enrichment analyses

Gene expression levels across all samples were first normalized using the calcNormFactors function implemented in the edgeR R package (version 3.34.0), which utilizes the TMM algorithm (weighted trimmed mean of M-values) to compute normalization factors. Then, the voom function implemented in the limma package (version 3.38.3) was used to log-transform the data and to calculate precision weights. A weighted fit using the voom-calculated weights was performed with the lmFit function from limma.

To investigate the impact of vaccination on baseline (before intervention) PBMC gene expression, normalized, log-transformed gene expression levels of unstimulated samples were fit to the linear model Expression ~ 1 + individual + timepoint:vaccination, which corrects for natural differences in baseline gene expression between individuals and therefore captures the independent effect of each vaccination condition on gene expression after 1 month.

To investigate the impact of vaccination on the PBMC response to poly(I:C) stimulation, we fit normalized, log-transformed gene expression levels to the linear model Expression ~ 1 + individual + timepoint + stimulus:timepoint and used the makeContrasts and contrasts.fit functions implemented in limma to compare the gene expression response to poly(I:C) before vaccination, with the response to poly(I:C) one month post-vaccination.

Gene set enrichment analyses (GSEA) were performed using the fgsea R package (version 1.18.0) with parameters: minSize = 15. To investigate biological pathway enrichments among genes responsive to vaccination (placebo, BCG, or BCG+Alendronate) or with altered responses to poly(I:C) stimulation before compared to after vaccination (placebo, BCG, or BCG+Alendronate), genes were ordered by the rank statistic: -log10(pvalue)*logFC and compared with the Hallmark gene sets from the MSigDB collections.

2.6. IL-6 concentration measurements in plasma

Whole blood in EDTA tubes was centrifuged at 3800 RPM for 10 min to obtain plasma. The plasma samples were then stored at −80 until testing. IL-6 levels in the plasma samples were measured using the Human IL-6 Quantikine HS ELISA (R&D Systems) according to the manufacturer's instructions.

2.7. Statistical analyses

Statistical analyses apart from RNA sequencing were performed using GraphPad Prism 8 (GraphPad Software Inc., CA, USA) or R 3.6.1 (www.R-project.org). Comparisons between the two time points were performed with the Wilcoxon matched-pairs signed rank test. Mann–Whitney U test was used for comparisons between treatment groups. p values below 0.05 were considered statistically significant.

3. Results

3.1. Study design and demographics

Eighteen, twenty-one, and eighteen participants who completed the study were randomized to receive a placebo vaccine, BCG vaccine, and BCG vaccine with an oral alendronate tablet, respectively. Venous blood was collected before the intervention and during the follow-up visit one month later. Flow cytometry was performed to analyze the changes in the immune cell populations in the blood. PBMCs isolated from blood were incubated with different bacterial and viral stimuli for 24 h and 7 days, and cytokine levels were measured. The study design is visually presented in Fig. 1.

The participant demographics are given in Table 1. There were no significant age and BMI differences between the intervention groups; however, there were more females in the BCG group compared to the other study groups.

3.2. Alendronate used together with BCG vaccination led to increased plasma cell and neutrophil numbers in the circulation

Using different cell surface markers, we identified circulating immune cell populations in the blood by flow cytometry. Principal component analysis (PCA) showed that the baseline immune cell counts were similar between the groups (Fig. 2A, left). One month after treatment, there was no significant difference in immune cell populations between different treatment groups (Fig. 2A, right). Although the abundance of immune cells at the baseline and after treatment remained similar between the groups, the size of plasma and naïve B cell populations exhibited differences after treatment compared to baseline (Fig. 2B). The number of naïve B cells increased after the intervention in all groups, including the placebo, although the increase was higher in the BCG and BCG+alendronate groups. Furthermore, the number of plasma cells in the blood became significantly higher in the BCG+alendronate group after the treatment compared to the other groups. We found no additional differences in immune cell numbers after the interventions, except for more intermediate monocytes and regulatory T cells (Tregs) one month after treatment in all groups (Supplementary Figure 1A). The reason for this is unclear, but the fact that it was observed in all groups argues for either a seasonality effect or batch-effect between time points.

Since neutrophils were not included in the flow cytometry panel, we also used the whole blood counts obtained with a hematology analyzer. Interestingly, we found that the total numbers of white blood cells and neutrophils were significantly increased only in the BCG+alendronate group one month after treatment compared to baseline (Fig. 2C). Monocyte and lymphocyte numbers did not change after the treatments (Supplementary Figure 1B). These data show increased peripheral neutrophil and plasma cell counts one month after receiving the BCG vaccine and oral alendronate treatment.

3.3. Combining alendronate with BCG vaccination reduced TNFα production against bacterial and viral stimuli

BCG vaccine can improve cytokine response to unrelated pathogens starting from 2 weeks up to a year after vaccination [21,22]. However, the effects of BCG vaccination in the present study were lower, and IL-6, TNFα, and IL-1RA production did not significantly increase one month after BCG vaccination (Fig. 3). Without any secondary stimulation, BCG vaccination (with or without alendronate) led to a trend of higher basal IL-1RA production, but no statistical significance was reached (Supplementary Figure 2). Of note, when sexes were analyzed separately, BCG vaccination's impact was more apparent in female participants, although statistical significance was reached only for the TNFα response against poly(I:C) (Supplementary Figure 3). However, this might simply be due to the low number of BCG-vaccinated male participants in the

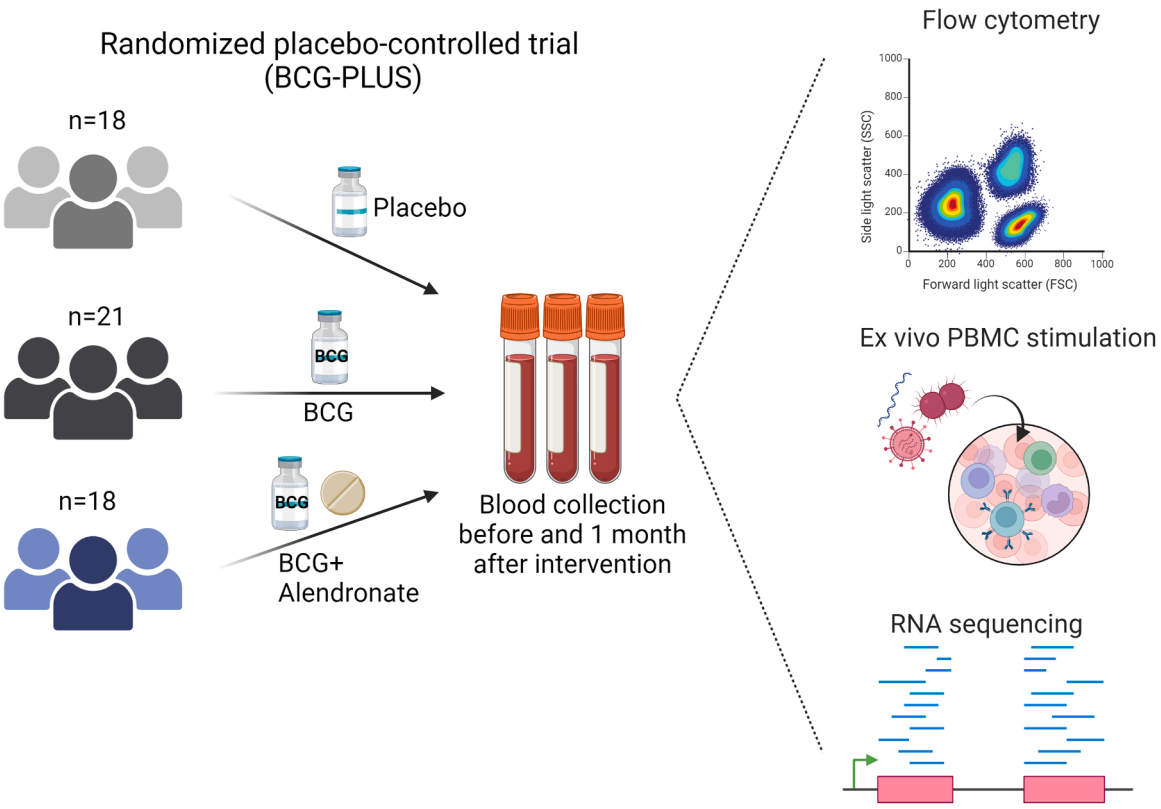

Fig. 1. Study Design. Blood was collected from the study participants before and 1 month after the intervention. Flow cytometry was performed using whole blood. Following PBMC isolation from the blood, *ex vivo* stimulations were performed to measure cytokine production, and cells were stored for RNA sequencing.

study.

The simultaneous administration of alendronate and BCG did not significantly impact IL-6 production (Fig. 3A). Upon LPS or *S. aureus* stimulation, TNFα production was lower in the group that received alendronate combined with BCG compared to the group receiving BCG alone (Fig. 3B). Alendronate also decreased the TNFα response against the Influenza A virus in the combination group compared to the BCG-alone group. IL-1RA production was similar between groups in all stimulation conditions (Fig. 3C).

Next, we assessed the impact of BCG vaccination and alendronate on the modulation of interferon responses. Generally, BCG vaccination led to a trend of enhanced IFNα and IFNγ production at baseline and after viral stimulation, although statistical significance was not reached (Supplementary Figure 2 and Fig. 4). IFNα and IFNγ production was overall not significantly modulated by alendronate (Fig. 4). However, sex-specific analyses revealed a significant reduction of IFNα production against poly(I:C) in females of the combination group compared to the placebo (Supplementary Figure 4).

Lastly, we compared the cytokine productions at baseline and one month later for each group with a paired analysis to complement the fold change analysis (Supplementary Figures 5 and 6). Although most data were consistent, we have observed a few differences between these two representations: IL-6 production was significantly lower one month later after SARS-CoV-2 stimulation only in the combination group (Supplementary Figure 5A), while the fold changes of the treatment groups were not statistically different (Fig. 3A). Fig. 3B showed a lower TNFα production capacity in the combination group than in the BCG-only group after LPS stimulation. Supplementary Figure 5B showed no statistical significance on TNFα production against LPS. Finally, the placebo group produced more IFNα after poly I:C, and less IFNα after influenza and SARS-CoV-2 stimulation 1 month after treatment. However, there was a stronger decline in IFNα after influenza in the BCG+alendronate group (Supplementary Figure 6).

3.4. Single alendronate treatment suppressed transcriptional priming by BCG vaccination

RNA sequencing was performed with PBMCs from 10 individuals per group before and one month after the interventions to investigate if any transcriptional differences were present. When baseline gene expression patterns were compared, BCG and BCG+alendronate treatments led to similar upregulation of pathways, including glycolysis, inflammatory response, and IFNγ response (Fig. 5A). However, the addition of alendronate together with BCG vaccination led to higher TNFα signaling and IFNα response in the unstimulated condition.

We also wanted to compare the transcriptomic changes in a stimulated condition. Only 5 participants per group with adequate number of cells for the stimulation were included in this analysis. Upon poly(I:C) stimulation, alendronate treatment led to the striking downregulation of the pathways upregulated by BCG (Fig. 5B). These pathways included cholesterol homeostasis, glycolysis, IL-2/STAT5 signaling, IL-6/JAK/STAT3, inflammatory response, IFNα and IFNγ responses, and reactive oxygen species pathway. When the TNFA and IL1B gene expressions were individually analyzed, priming by BCG compared to placebo and its reversal by alendronate was evident, although not statistically significant due to the low sample size (Fig. 5C-D). Overall, the RNA sequencing data support the functional observations suggesting that alendronate suppresses the induction of trained immunity by BCG.

4. Discussion

In this study, we explored the effect of alendronate on the induction of trained immunity responses by BCG vaccination in healthy individuals. We observed that oral administration of alendronate simultaneous with BCG vaccination reduced the cytokine production capacity of PBMCs upon immunological challenge with various stimuli. At the transcriptional level, PBMCs of the individuals who received BCG with

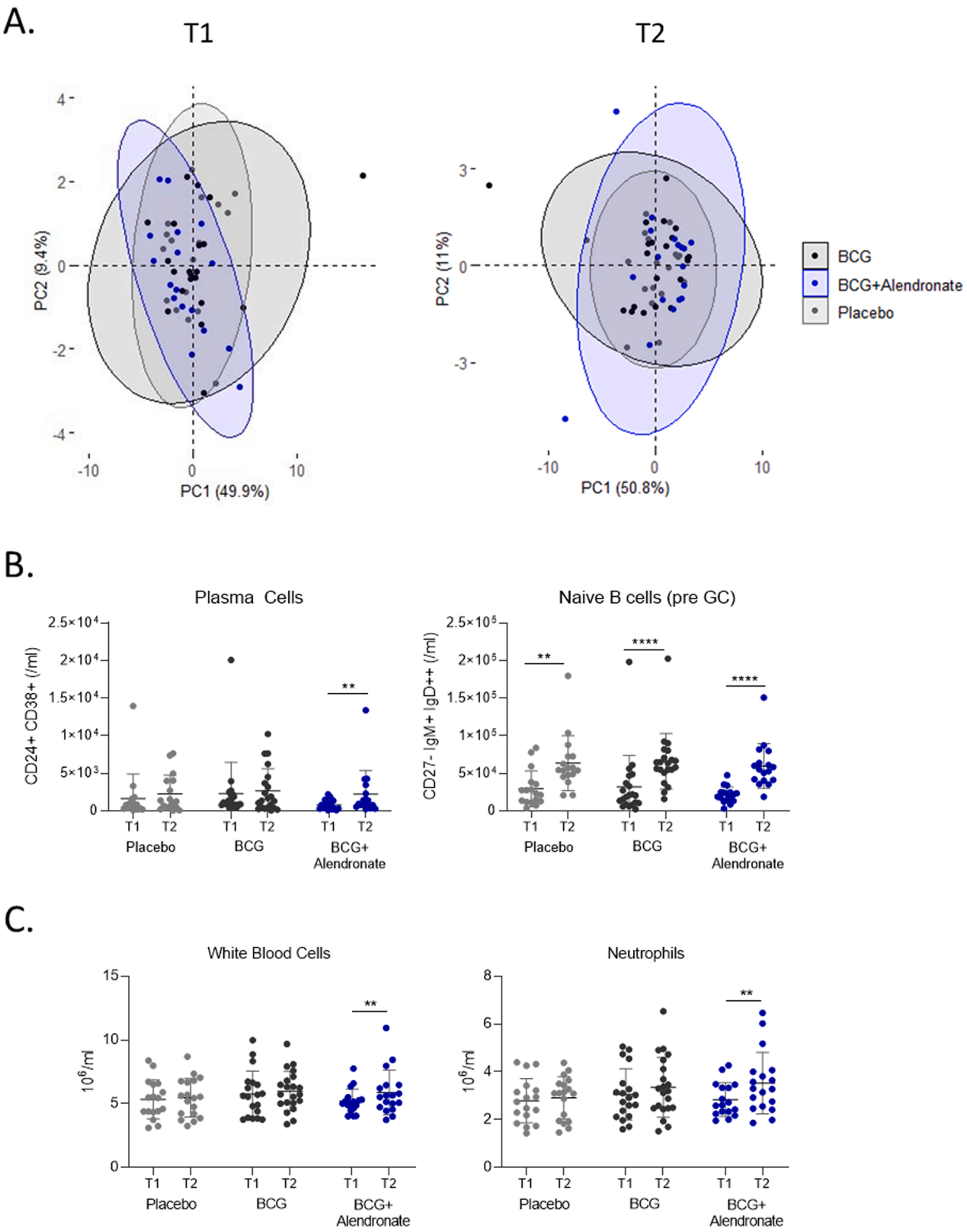

Fig. 2. Immune cell counts measured by flow cytometry and a hematology analyzer before (T1) and after (T2) treatment. A) Principal component analyses of the immune cell populations before (left) and one month after (right) treatment. B) Plasma and naïve B cell counts measured using flow cytometry. C) Total white blood cell and neutrophil counts measured by a hematology analyzer. Cell numbers before and after treatment were compared using the Wilcoxon matched-pairs signed-rank test. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$.

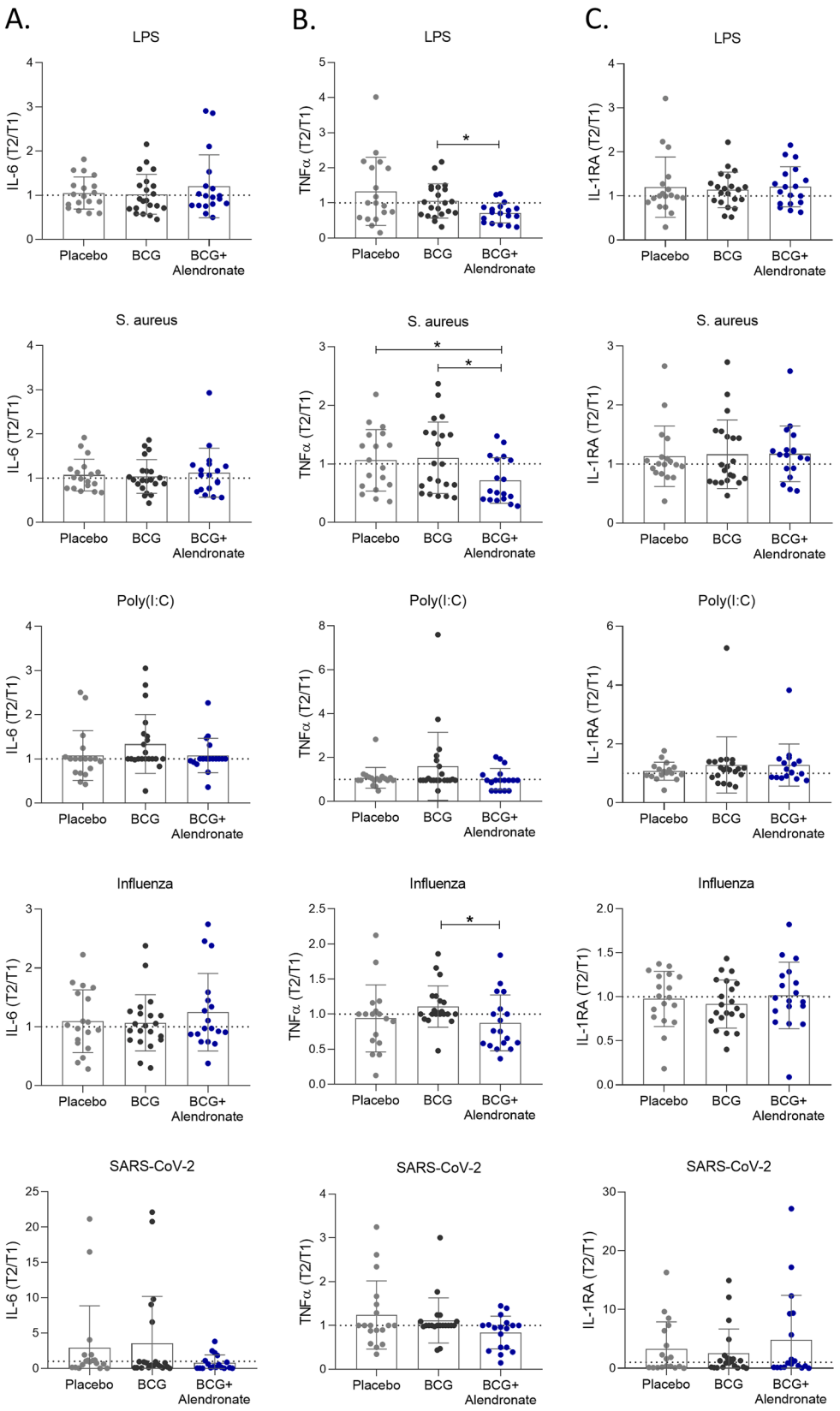

Fig. 3. Fold changes (T2/T1) of A) IL-6, B) TNFα, and C) IL-1RA production by PBMCs upon bacterial and viral stimulation. Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention. *$p \leq 0.05$.

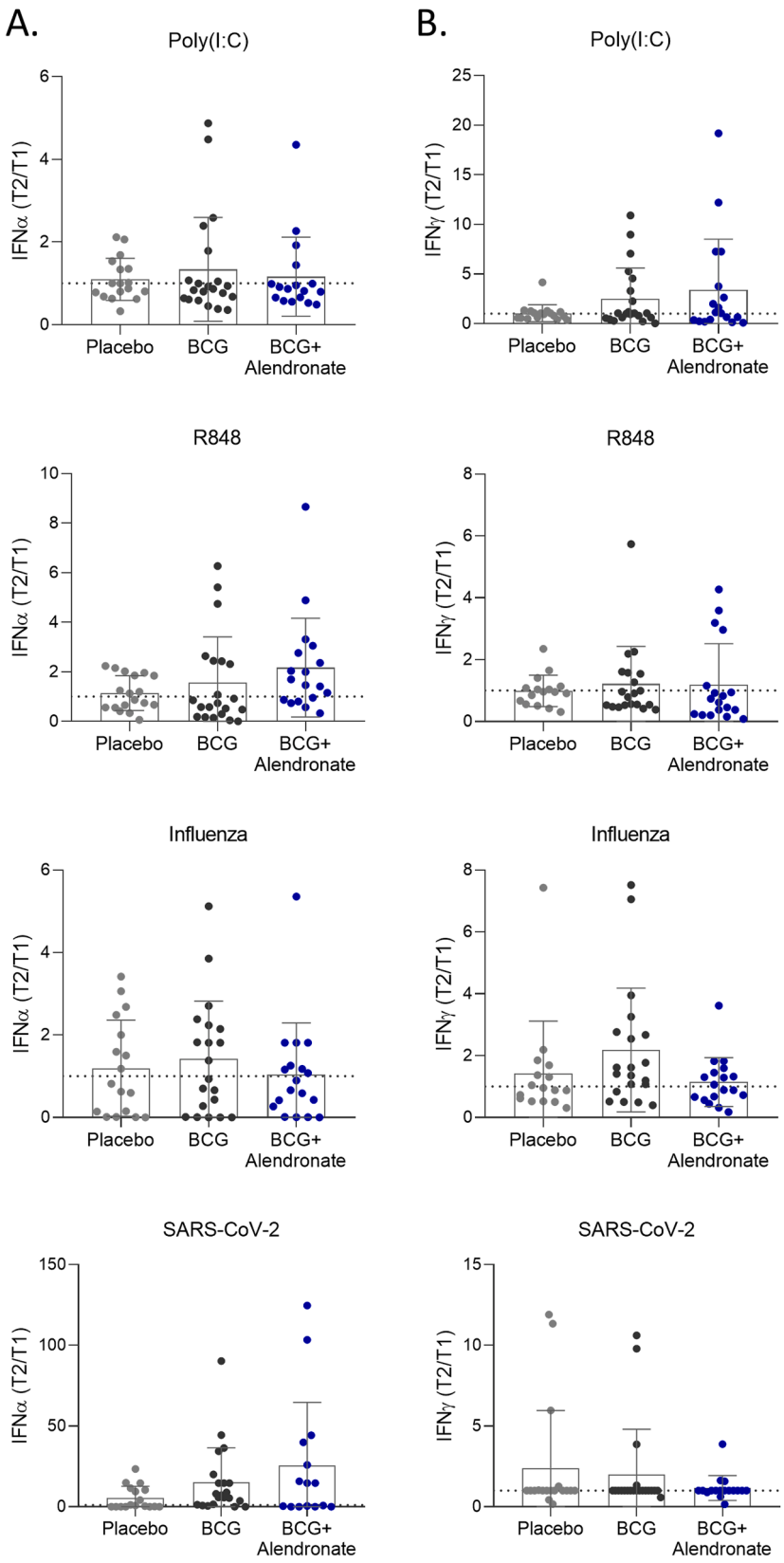

Fig. 4. Fold changes (T2/T1) of A) IFNα and B) IFNγ production by PBMCs upon viral stimulation. Dotted lines depict the fold change of 1. Groups were compared with the Mann-Whitney U test. T1: before the intervention, T2: one month after the intervention.

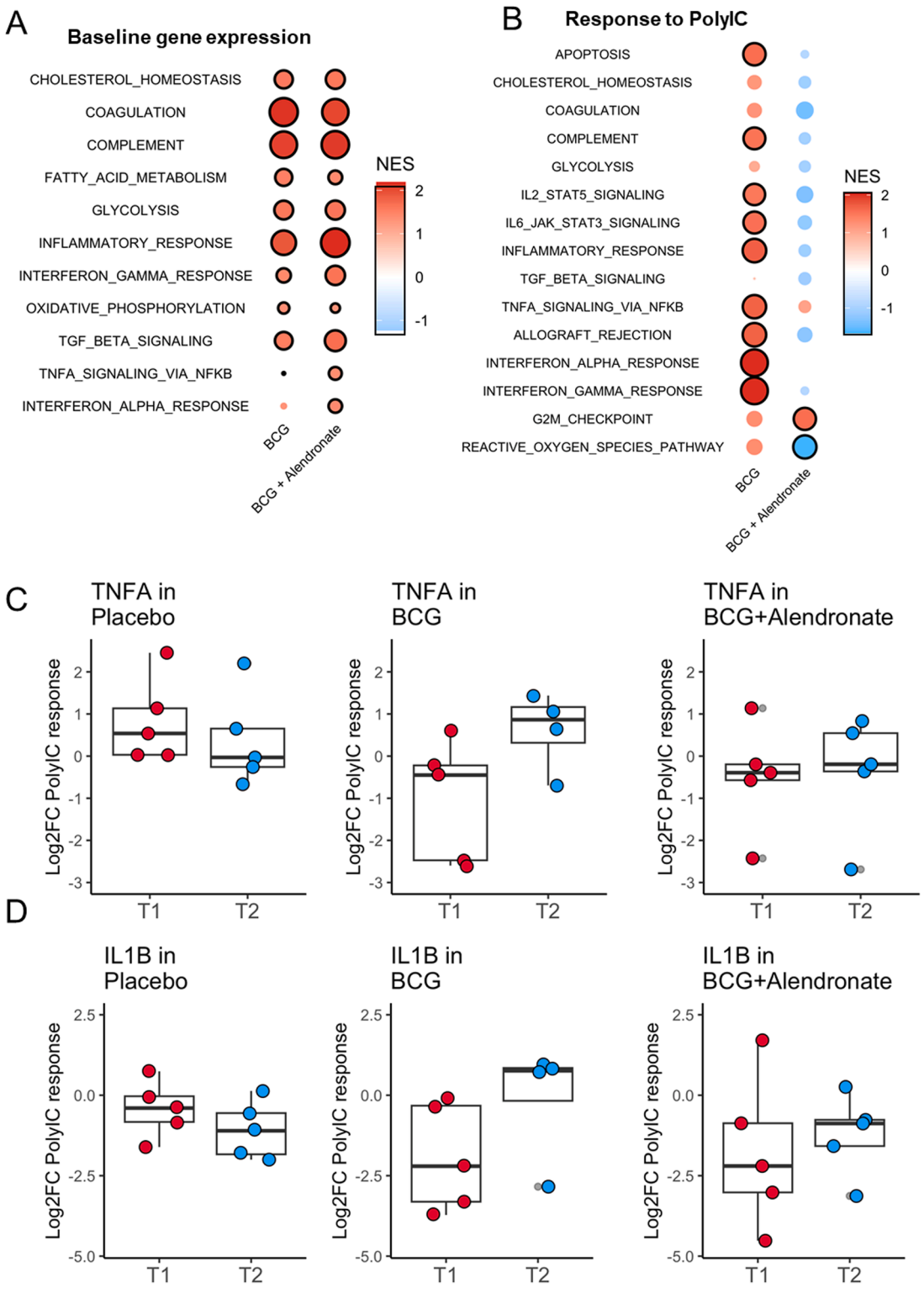

Fig. 5. Gene set enrichment analysis (GSEA) from A) unstimulated and B) poly(I:C)-stimulated PBMCs of treatment groups compared to placebo, and example fold change plots of C) TNFA and D) IL1B expression upon poly(I:C) stimulation. Genes were ordered by the rank statistic –log10(pval)*logFC for the effect of priming on baseline gene expression and for response to poly(I:C) in the indicated primed condition and compared against Hallmark gene sets. Circle size and shading are scaled to a normalized enrichment score (NES). All circles with black border have padj $\leq$ 0.05. On the boxplots showing log2FC change in gene expression to poly(I:C), padj $>$ 0.05 (non-significant) for all panels. T1: before the intervention, T2: one month after the intervention.

alendronate showed downregulated immune-related pathways, such as IFNα and IFNγ response and TNFα, IL-2, and IL-6 signaling upon activation with poly(I:C), compared to the individuals who received only BCG.

Furthermore, BCG vaccination combined with oral alendronate treatment increased white blood cell, neutrophil, and plasma cell numbers in circulation. In a study of patients with chronic idiopathic neutropenia-associated osteopenia or osteoporosis taking 10 mg of

alendronate daily for one year, neutrophil counts were elevated at one month and continued to rise throughout the year [23]. Together with our observations, this suggests a stimulatory effect of alendronate on granulopoiesis in the bone marrow. A recent murine study showed that bisphosphonates promote B cell proliferation and antibody production after antigen encounter [24]. Although the study was primarily on clodronate, alendronate was also shown to improve antibody responses. This corroborates the findings that subjects receiving alendronate had significantly higher numbers of plasma cells one month later compared to baseline.

Bisphosphonates are heavily negatively charged molecules that cannot easily permeate the cell membrane. Fluid-phase endocytosis is required for their intracellular uptake [25]. Immune cells such as neutrophils, monocytes, and macrophages are capable of fluid-phase endocytosis and, therefore, could be susceptible to alendronate's immunomodulatory actions. Alendronate's effects on innate immune cells have not been thoroughly investigated, and the existing literature is inconsistent. A few studies have explored alendronate uptake by macrophages and its functional consequences. However, these were mostly performed with macrophage-like J774 or RAW 264 cell lines and not primary human cells [11,26,27]. Simple BPs such as clodronate, etidronate, and nitrogen-containing BP pamidronate suppressed LPS-induced IL-1β, IL-6, and TNFα production from RAW 264 cells [26]. Of note, pamidronate was cytotoxic in high concentrations. In another study with RAW 264 cells, alendronate also proved to be cytotoxic, but it enhanced LPS-induced IL-1β, IL-6, and TNFα production [28]. In J774.1 cells, alendronate increased lipid A-induced IL-1β production and caused cell death dependent on the activation of the Smad3/NLRP3/ASC axis [29]. A study using PBMCs and monocytes from healthy humans showed that a wide dose range of alendronate inhibited PBMC proliferation in response to lectins or tetanus toxoid (TT) and suppressed IL-1β production from monocytes after LPS or TT stimulation [30].

Our study is the first to explore the effect of alendronate on trained immunity induced *in vivo* by BCG vaccination. Surprisingly, we have not observed a significant trained immunity response one month after BCG vaccination in this study. A reason for this could be the time of vaccination: the participants were vaccinated with BCG during the summer. It was recently shown that the training capacity of BCG is the highest in the winter, while it is lower in the summer [31]. Despite weak BCG-induced training, the results suggest an overall inhibitory effect of alendronate on proinflammatory cytokine production by innate immune cells, particularly TNFα. The observed effects could be considered relatively small, as they generally amounted to 20–25 % differences in the group that received alendronate and BCG compared to the group receiving BCG alone. However, while this may be a relatively small difference at the individual level, inhibition of BCG effects in this range in patients with bladder cancer treated with BCG immunotherapy [32] could have significant deleterious effects at an epidemiological level in large cohorts.

The transcriptome of PBMCs after poly(I:C) stimulation also corroborates our findings on decreased cytokine production capacity. In the unstimulated condition, we did not observe significant differences between the gene expression in PBMCs of the BCG *vs*. BCG+alendronate group. However, several pathways related to innate and adaptive immune response and signaling were transcriptionally downregulated in the BCG+alendronate group after incubation of PBMCs with poly(I:C). This suggests that specific genes might be epigenetically modulated by alendronate treatment, but future studies are needed to characterize the context and duration of these effects.

The observed decrease in the cytokine production capacity of immune cells when volunteers were vaccinated with BCG in the presence of alendronate administration is somewhat surprising: inhibition of FPP synthase by alendronate leads to the accumulation of mevalonate, and this was reported to enhance trained immunity responses [13]. Further studies are needed to study the possible molecular mechanisms of these effects of alendronate on BCG-induced immune modulation.

Alendronate is used in both men and women with osteoporosis, although it is more common in women. The tolerability profile and side effects were similar between the two sexes [33], as well as the drug's effectiveness [34]. However, it is not known if bisphosphonates have sex-specific effects on the molecular level. Considering that bisphosphonates, including alendronate, have immunomodulatory effects and considerable differences exist between men's and women's immune responses [35], it would be plausible that alendronate would differentially influence the male and female immune responses. Although this study was not designed to answer how alendronate influences the immune system of young or older men and women, future studies must address this question. Within the limited sample size of our study, we observed a greater suppressive effect of alendronate on LPS-induced TNFα and poly(I:C)-induced TNFα and IFNα in women. Since a significantly improved cytokine response upon BCG vaccination was also only observed in women, the impact of alendronate might be sex-specific. However, these findings need to be confirmed in larger studies.

An increasing body of research suggests a lower risk of infections and cancer associated with bisphosphonate use. Injection of N-BPs 3 days after infection with influenza resulted in protection against influenza in mice through the expansion of γδ T cells [16]. N-BP use was also linked to a reduced rate of epithelial ovarian cancer compared to no use in women over 50 [17]. An observational study in people with hip fractures reported a lower risk of pneumonia and pneumonia mortality in people using N-BPs than those using non-NB-P anti-osteoporosis medications [15]. On the other hand, a study assessing the incidence of COVID-19 hospitalization and mortality in people using N-BPs found no effect of bisphosphonate use on the risk of getting severe COVID-19 [36]. As these studies are only observational and cannot prove causality, randomized controlled trials are needed to determine whether bisphosphonate use has beneficial or detrimental effects on immunity. Our results indicate a lower *ex-vivo* cytokine production against viral and bacterial stimuli in individuals receiving alendronate with BCG, but whether this translates to clinical outcomes such as infection incidence and severity remains to be investigated.

Though this study investigated alendronate's immunological effects on healthy young individuals, alendronate is primarily used to prevent and treat osteoporosis, a disease of old age [37]. Immune cell numbers and functions are compromised in advanced age, accompanied by sustained low-grade systemic inflammation, which leads to increased susceptibility to infections, higher morbidity, and mortality [38]. Induction of trained immunity is a promising approach to overcoming immune dysregulation in the elderly and relieving the healthcare burden due to infections. BCG vaccination improves cytokine responses of innate immune cells while decreasing systemic inflammation in healthy elderly [39]. Our observations show that even a single use of oral alendronate tablets together with BCG vaccination can have long-term modulatory effects on cytokine and interferon responses against bacterial, viral, and fungal pathogens. Whether this effect would be beneficial or deleterious remains to be investigated in larger studies. As a systemic inflammation marker, we also measured IL-6 concentrations in the plasma before and after placebo, BCG, or BCG+alendronate treatment. However, no differences existed between time points in any group (Supplementary Figure 7). Whether chronic use of alendronate in older individuals would impact systemic inflammation, prevent the induction of trained immunity, or further suppress the host response to pathogens are important open questions to be considered.

One limitation of this study was the sample size. Although decreasing cytokine production patterns were observed after the simultaneous use of BCG and alendronate compared to the BCG-only group, these changes sometimes failed to reach statistical significance. A study with a larger sample size is necessary to further validate the effects of alendronate on trained immunity induction.

In conclusion, we show that simultaneous administration of BCG with oral alendronate reduces the cytokine production capacity of PBMCs against heterologous stimuli one month later in young, healthy

individuals. These findings align with the transcriptome of poly(I:C)-stimulated PBMCs where BCG+Alendronate treatment results in downregulated inflammatory pathways. These observations could have implications for BCG vaccination in the elderly with chronic use of N-BPs, and larger studies investigating the effect of long-term alendronate use on BCG-induced trained immunity are required.

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CRediT authorship contribution statement

Ozlem Bulut: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gizem Kilic:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Priya A. Debisarun:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software. **Rutger Jan Röring:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Software. **Sarah Sun:** Data curation, Formal analysis, Investigation, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. **Manon Kolkman:** Data curation, Formal analysis, Investigation, Resources. **Esther van Rijssen:** Data curation, Formal analysis, Investigation, Resources. **Jaap ten Oever:** Conceptualization, Project administration, Supervision. **Hans Koenen:** Resources, Supervision. **Luis Barreiro:** Resources, Supervision, Writing – review & editing. **Jorge Domínguez-Andrés:** Resources, Supervision. **Mihai G. Netea:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mihai G. Netea reports financial support was provided by European Research Council. Mihai G. Netea reports financial support was provided by Dutch Research Council. Jorge Dominguez-Andres reports financial support was provided by Dutch Research Council. Mihai G. Netea reports a relationship with Trained Therapeutix Discovery (TTxD) that includes: board membership and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2024.106851.

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