Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes

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Adaptive features of innate immunity, recently described as “trained immunity,” have been documented in plants, invertebrates, and mice, but not yet in humans. Here we show that bacille Calmette-Guérin (BCG) vaccination in healthy volunteers led not only to a four- to sevenfold increase in the production of IFN-γ, but also to a twofold enhanced release of monocyte-derived cytokines, such as TNF and IL-1β, in response to unrelated bacterial and fungal pathogens. The enhanced function of circulating monocytes persisted for at least 3 mo after vaccination and was accompanied by increased expression of activation markers such as CD11b and Toll-like receptor 4. These training effects were induced through the NOD2 receptor and mediated by increased histone 3 lysine 4 trimethylation. In experimental studies, BCG vaccination induced T- and B-lymphocyte–independent protection of severe combined immunodeficiency (SCID) mice from disseminated candidiasis (100% survival in BCG-vaccinated mice vs. 30% in control mice). In conclusion, BCG induces trained immunity and nonspecific protection from infections through epigenetic reprogramming of innate immune cells.

**The general perception in immunology is that innate immunity, as opposed to adaptive immunity, is static and does not adapt to an enhanced functional state. However, there is an increasing body of scientific literature indicating enhanced nonspecific protection against infections after previous exposure to certain microbial components in plants (1), invertebrates (2, 3), and mice (4). In our recent review of the relevant literature, we proposed the term “trained immunity” for this effect (5). Prominent microbial components that can induce this enhanced effector function are mycobacterial components such as bacille Calmette-Guérin (BCG), complete Freund adjuvant, and muramyl dipeptide (MDP) (6–9).

BCG, the live attenuated vaccine against tuberculosis (TB), is one of the world’s most widely used vaccines (10). It usually is given to newborns, protecting them especially against severe forms of TB (e.g., TB meningitis, disseminated TB) (11). Soon after its introduction in the 1930s, epidemiological studies surprisingly demonstrated that BCG also protects against childhood mortality independent of its effect on TB (12–15). Recent studies corroborated these findings and suggested a reduction in the burden of infections other than TB (16–20). For example, in a case-control study in Brazil, BCG reduced the risk of death from pneumonia by 50% (17). Although little is known about the mechanisms responsible for these protective effects of BCG, macrophages from BCG-vaccinated mice displayed a greater release of oxygen radicals and intracellular fungal killing (8), suggesting an important role of innate immune mechanisms.

In this paper, we explore the mechanisms of the enhanced immune function induced by BCG both in vitro and in vivo.

**Results**

Monocyte Phenotype Is Modified on BCG Vaccination. In the first series of experiments, blood was collected from 20 naïve (nonexposed) volunteers, before and after (2 wk and 3 mo) vaccination with BCG (Fig. 1A).

As expected, 2 wk after BCG vaccination, IFN-γ production induced by *Mycobacterium tuberculosis* (MTB) was sevenfold higher than it was before vaccination, and this effect remained for at least 3 mo (Fig. 1B). Surprisingly, however, IFN-γ production also increased significantly when cells were exposed to unrelated pathogens, such as yeasts (7.7-fold increase, Fig. 1B) or hyphae (5.2-fold increase, Fig. S1A) of *Candida albicans* and *Staphylococcus aureus* (3.5-fold induction, Fig. 1B). Exposure of peripheral blood mononuclear cells (PBMCs) to LPS induced production of IFN-γ in very low amounts that did not differ before and after BCG vaccination (Fig. S1B).

Interestingly, in the same series of experiments, production of the proinflammatory cytokines TNF-α and IL-1β was also found to be enhanced when cells isolated from the volunteers after BCG vaccination were exposed to mycobacterial and nonmycobacterial stimuli (Fig. 1 C and D). Of note, the increase in cytokine production persisted 3 mo after BCG vaccination (Fig. 1 B–D).

These data demonstrate that BCG alters the functional state of circulating mononuclear cells. To further explore this finding, we investigated the expression of known cell surface activation markers of monocytes. We observed a slight increase in the population of CD14+ monocytes after BCG vaccination (Fig. 2A), all of which expressed the differentiation markers CX3CR1 and CD11b (Fig. S2 C–E). Interestingly, the proportion of CD14+ monocytes expressing Toll-like receptor 4 (TLR4) changed significantly after vaccination (Fig. S2B). In addition, the expression


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BCG-Induced Protection Is T- and B-Cell Independent. To establish whether BCG-induced trained immunity is indeed mediated by innate immune cells (e.g., monocytes) and independent of T and B lymphocytes, we injected either BCG or saline in two groups of severe combined immunodeficiency (SCID) mice that lack both T and B cells. Two weeks later, both groups of mice were infected with a lethal inoculum of *C. albicans*. The BCG-vaccinated mice had a significantly better survival rate than the saline-injected animals (Fig. 3A), and this was accompanied by a decreased fungal burden in the kidneys, the target organ of disseminated candidiasis in mice (Fig. 3B). These data strongly support the notion that BCG can induce nonspecific protection against nonmycobacterial infections through functional reprogramming of innate immune cells, such as monocytes. To demonstrate that the trained immunity function of monocytes also may be found after BCG vaccination of SCID mice in vivo, we performed an additional experiment in vaccinated mice. As previously, SCID mice lacking functional T and B cells and vaccinated with BCG (or with control PBS) were infected with *C. albicans*, and 1 wk later spleen monocytes were restimulated in vitro with LPS, a classic inducer of innate immune responses that is not related to mycobacteria. Innate proinflammatory cytokine production induced by LPS was significantly higher in the BCG-vaccinated mice (Fig. 3C).

Training Effects of BCG Vaccination Are NOD2 and Rip2 Dependent. The mechanisms responsible for training by BCG were explored further in vitro using human cells. Freshly isolated human monocytes were preincubated with either culture medium or BCG for 24 h, followed by washing of the cells. After a washout period of 7 d, during which the cells were incubated solely with culture medium, various secondary stimuli were added to the cell culture (Fig. 4A). During the first week, the monocytes had a viability rate >95% when trained with either culture medium or BCG; however, the experiments could not be extended beyond 10 d because of the death of primary monocytes in longer-term cultures (based on morphology and trypan blue exclusion). Preincubation of cells with BCG markedly increased production of the proinflammatory cytokine TNF-α after the secondary stimulus (Fig. 4B), reproducing the effects observed during BCG vaccination. Remarkably, BCG induced more cytokines than the TLR2 ligand Pam3Cys, which could mimic the effects of BCG preincubation on cytokine production by monocytes (Fig. 4G). In line with this effect of MDP, an inhibitor of the Rip2 kinase also impaired BCG-induced training (Fig. 4H). These data are supported further by a recent study demonstrating priming effects of NOD2 engagement for stimulation of human macrophages with mycobacteria (24).

Finally, as H3K4me3 was increased after BCG vaccination in vivo, we investigated whether blocking histone methylation might reverse monocyte training in vitro. Indeed, inhibition of histone methylation by the methyltransferase inhibitor MTA (5'-deoxy-5'-methylthio-adenosine) almost completely reversed the
training induced by BCG (Fig. 4f). In contrast, the histone demethylase inhibitor pargyline did not influence the BCG-induced training effects.

**Discussion**

Until now, the nonspecific adaptive features of innate immunity (25) that have been demonstrated in plants, invertebrates, and mice and that we termed trained immunity (5) have not been demonstrated to be active in vivo in humans, and the molecular substrate of trained immunity has not yet been identified. In the present study, we demonstrate that monocytes can be functionally reprogrammed, or "trained," to exhibit an enhanced and lasting phenotype after vaccination with BCG. These data point to the mononuclear phagocyte as the cell that mediates the nonspecific protection against reinfection after BCG vaccination. Our data also imply that vaccination with BCG induces two types of immune response: on one hand it induces a classic specific immune response involving antigen-specific T cells and memory

**Fig. 2.** BCG alters the phenotype of circulating monocytes in healthy volunteers. (A) Flow cytometry analysis of CD45+ cells. (B) Histogram showing the expression level of CD14+ (% of total cells) in the cells isolated from 20 volunteers before and after BCG vaccination. (C) Flow cytometry analysis of TLR4 and CD14 in cells isolated from one volunteer before and 3 mo after BCG vaccination. (D and F) Overlays of the surface expression level of TLR4 (D) and CD11b (F) within CD14+ cells isolated from one volunteer before and 3 mo after BCG vaccination. (E and G) Average surface expression level of TLR4 (E) and CD11b (G) within the CD14+ monocyte population isolated from 20 volunteers before and after BCG vaccination. *P < 0.05, **P < 0.01, ***P < 0.005. (H) IL-1β and TNFα mRNA expression after sonicated *M. tuberculosis*, heat-killed *S. aureus* and *C. albicans* hyphae in vitro stimulation of the PBMCs isolated from volunteers before and 3 mo after BCG vaccination (n ≥ 3). (I) ChIP analysis of the enrichment of H3K4me3 at the promoter of *tnfα*, *il6* and *tlr4* in human monocytes isolated from three subjects before and 3 mo after BCG vaccination. *P < 0.05, paired t test.

**Fig. 3.** BCG vaccination protects mice against lethal *C. albicans* infection through a T-/B-lymphocyte–independent mechanism. (A) Survival rate of SCID mice infected with live *C. albicans* (2 × 10^8 cfu/mouse) injected i.v. The mice were vaccinated i.v. either with PBS (control) or BCG 14 d before inoculation with a lethal *C. albicans* dose (n ≥ 15 per group, two independent experiments). (B) Fungal burden of kidneys from control- and BCG-vaccinated SCID mice 3 and 14 d after the lethal *C. albicans* infection (n = 5). (C) TNFα production of spleen monocytes after restimulation in vitro with LPS from control- and BCG-vaccinated SCID mice 3 mo after the lethal *C. albicans* infection (n = 5). *P < 0.05, **P < 0.01, and ***P < 0.005 vs. control (PBS) animals.
leading to protection against TB, whereas on the other hand it induces adaptive trained immunity based on functional reprogramming of mononuclear phagocytes that induces protective effects not only against tuberculosis, but also against other infections. In a combination of in vivo and in vitro experiments, we demonstrate that a NOD2-mediated epigenetic change at the level of histone methylation (H3K4me3) is the mechanism through which BCG enhances innate immune responses.

The mechanisms activated by BCG vaccination are almost certainly multiple and complex: classical adaptive immune memory, epigenetic reprogramming (“training”) of innate immunity as described here, and possibly also secondary nonspecific effects of adaptive immunity on innate immune responses (e.g., increased cytokines, as shown by Strutt et al. (26)). Conceptually, it is important to discern classical adaptive immunity mediated by T and B lymphocytes from the trained immunity mediated by innate immune cells. In this respect, trained immunity reflects an increase in nonspecific antimicrobial capacity through epigenetic reprogramming, similar to the concept of adaptive characteristics of innate immunity, as described by Mantovani et al. (25, 27). Moreover, whereas adaptive immune cell–mediated responses take some time to become robust, innate (trained) immunity becomes apparent much sooner. This was also demonstrated recently in epidemiological studies in which the beneficial effects of BCG in newborn children already were apparent within days of vaccination (28). The rapidity of the nonspecific adaptive characteristics of innate immunity underlines that heterologous adaptive responses cannot fully explain trained immunity.
The impact of the training effects on innate immune responses has become apparent in our experiments in which SCID mice were vaccinated with BCG: these mice were protected from mortality, and kidney burdens were reduced to 20–40% of the levels in the unvaccinated mice after a secondary lethal infection with C. albicans. Earlier studies with BCG in experimental schistosomiasis demonstrated similar protection, which was at least partially T-cell independent (29). There appears to be an obvious link between these experimental results and the non-specific protection against nonmycobacterial disease conferred by BCG (8, 9, 12–20). In the present study, we provide unique mechanistic insights into the processes mediating the adaptive features of innate immunity, or trained immunity, in humans. The modified methylation status of cytokine promoters after BCG vaccination in human monocytes, as well as the blockade of the in vitro training effects with methyltransferase inhibitors, suggests that the innate immune response in humans can be reprogrammed epigenetically. Similar mechanisms have been demonstrated before in plants, during so-called systemic acquired resistance (1), and in invertebrates (30); however, both classes of organisms are devoid of adaptive immune responses. The present study teaches us that in the presence of adaptive immunity—as is the case in mammals—training of innate immunity also is operational and serves to enhance resistance to infection.

Another important point for discussion is whether the training effects of BCG vaccination are the result of epigenetic reprogramming of innate immune cells exclusively, or whether long-term BCG infection also may play a role. Little is known about how long BCG stays viable in the human body. Fortunately, however, a very recent study by Minassian et al. (31) reports that 4 wk after vaccination, only 50% of individuals still displayed viable BCG at the vaccination site. Although we cannot fully exclude a low BCG persistence in a minority of the volunteers, it is to be expected that in most of them, all the microorganisms were cleared after 3 mo. Moreover, in our in vitro experimental setting of trained immunity, monocytes were trained with live BCG as well as with inert MDP, highlighting that live BCG persistence is not mandatory for the training.

It should be stressed that trained immunity in mammals is not induced solely by BCG. Indeed, murine CMV infection can induce protection from reinfection in a T/B-cell–independent and natural killer cell–dependent fashion (4, 32), and systemic infection with C. albicans induced T/B-cell–independent nonspecific protection in mice (33). The role of epigenetic reprogramming was not investigated in these studies.

In conclusion, in this study we provide firm evidence that innate immunity in humans has adaptive features and that it has the capacity to display an enhanced response upon reinfection. This process of trained immunity likely represents a paradigm shift in immunity, as it demonstrates the existence of (non-specific) immunological memory in the absence of adaptive immune responses. Better insights into the relative role of adaptive immune memory and epigenetic reprogramming of innate immunity (trained immunity) may have important consequences for vaccine design, more specifically with regard to the selection of antigens and development of adjuvants. In this respect, answers remain to be provided regarding the strength and duration of epigenetically induced trained immunity. In this context, it is important to note that BCG and the NOD2-ligand MDP have long been known to induce nonspecific protective effects against infections (7, 15) and neoplasms (34), providing the hope that trained immunity can indeed be harnessed for preventive and therapeutic purposes.

**Materials and Methods**

**Subjects.** Subjects (age range, 20–36 y) who were scheduled to receive a BCG vaccination at the public health service, because of travel or work in TB-endemic countries, were asked to participate in this trial. Twenty healthy individuals were included between August and November 2010. Informed consent was obtained from the human subjects included. Blood was drawn before, 2 wk after, and 3 mo after the BCG vaccination. The study was approved by the Arnhem-Nijmegen Ethical Committee.

In vitro cytokine stimulation experiments were performed with PBMCs isolated from buffy coats obtained from healthy volunteers (Sanquin Bloodbank, Nijmegen, The Netherlands).

**PBMC Stimulation Assays.** The mononuclear cell fraction was isolated from the blood by density centrifugation and diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and resuspended in culture medium (Roswell Park Memorial Institute (RPMI) medium; Invitrogen) supplemented with 10 μg/mL gentamicin, 10 mM L-glutamine, and 10 mM pyruvate. Cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5 × 10^6 cells/mL. A total of 5 × 10^6 mononuclear cells in a 100-μL volume was added to round-bottom 96-well plates (Greiner) with RPMI or sonicated MTB H37Rv (1 μg/mL, end concentration), heat-killed C. albicans, heat-killed C. albicans hypheae (1 × 10^6 microorganisms/mL, strain UC820), S. aureus (1 × 10^6 microorganisms/mL), or Escherichia coli LPS (Sigma-Aldrich, 1 ng/mL). After 24 h or 48 h, the supernatants were stored at −20 °C. Cytokine concentrations were assessed in the supernatants using ELISA.

For ChIP analysis, adherent monocytes from the subjects were obtained in a six-well plate by incubating 15 × 10^6 PBMCs for 1 h. The adherent monocytes then were collected before further treatment for chromatin immunoprecipitation.

Buffyoaks of blood from anonymous blood donors were used for the in vitro “training” experiments. To isolate PBMCs, the previously described procedure was used. Monocytes were used after adherence for 1 h in flat-bottom 96-well plates. For monocyte training, RPMI, BCG (1 μg/mL BCG vaccine SSI from the Netherlands Vaccine Institute) or MDP (1 μg/mL Pam3Cys (10 μg/mL), or LPS (1 ng/mL) was added for 24-h incubation at 37 °C. Thereafter, the supernatant was discarded and replaced with fresh RPMI with 10% serum. After 7 d at 37 °C, the supernatant was discarded and the cells were stimulated with E. coli LPS (10 ng/mL), sonicated MTB H37Rv (1 μg/mL), S. aureus (1 × 10^6 microorganisms/mL), or RPMI as a control for an additional 18 h. Subsequently, the supernatant was collected. ELISA was performed. In some representative experiments, the final cell number after initial training with control medium, BCG, or MDP was assessed using Hoechst 33342 fluorescent stain and showed little variation. The normalized cytokine levels based on the number of cells actually present in the well showed the same trend as the nonnormalized cytokine levels.

In the “inhibition” experiments, before priming with BCG, the adherent monocytes were preincubated for 1 h with Bartonella LPS (1 μg/mL), anti-TLR2 antibody and anti-ILg (10 μg/mL; ebioscience), Rip2/p38 inhibitor (1 μM; Sigma-RBI), pargeline (3 μM; Sigma-Aldrich), or MTA (1 mM; Sigma-Aldrich).

**Cytokine Measurements.** Cytokine measurements of TNF-α, IL-1β, and IFN-γ were performed in the supernatants using commercial ELISA kits from R&D Systems (TNF-α and IL-1β) and eBioscience (IFN-γ). In a small proportion of baseline samples in which cytokine concentrations were beyond the detection limit, these outliers were excluded from the analysis.

**FACS Analysis.** Cells were phenotypically analyzed by 10- and 5-color flow cytometry using a Coulter Navios and Coulter Cytomics FC 500 cytomter (Beckman Coulter), respectively, and evaluated using Kaluza 1.1 software (Beckman Coulter). The cells were washed with PBS with 1% BSA before being labeled with fluorochrome-conjugated mAbs. After incubation for 30 min at 4 °C in the dark, the cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAbs were used: CD11b (IM25814), CD14-EC (IM2707U), and CD45-PC7 (IM3548) (all from Beckman Coulter); CD284-PE (TLR 4) (312806), CD206-PE (321106), and CXCR1-FTTC (314606) (all from Biolegend); CD229-APC (S58319) (from BD Biosciences), and dectin-1-APC (FAB1859A) (from R&D Systems).

**Quantitative RT-PCR.** For quantitative RT-PCR (qRT-PCR), adherent monocytes were primed with either culture medium or β-glucan and subjected to 7 d to a second stimulation with 10 ng/mL LPS, 10 μg/mL Pam3Cys, or 1 × 10^6 heat-killed C. albicans for 4 h. Samples were treated with TRizol Reagent (Invitrogen), and total RNA purification was performed. Isolated RNA was reverse transcribed into cDNA using the Script cDNA Synthesis Kit (Bio-Rad). Quantitative PCR (qPCR) was performed using the SYBR Green method. The following primers were used in the reaction (5′–3′): TNF-α forward: TGGCCCAAGGACCAGTCAGA, and TNF-α reverse: GGTGTTGCTACAAATCAGGTGCATTCA;
were approved by the Ethics Committee on Animal Experiments of Radboud University, Nijmegen. The mice first were injected with BCG vaccine SSI (750 μg/mouse) in a 100-μL volume of sterile pyrogen-free PBS (PBS) or with PBS alone. Fourteen days later, the mice were infected i.v. with a lethal dose of C. albicans blastococnidia (1 × 10⁴ cfu/mouse). Survival then was monitored and kidney fungal burden was assessed 3 and 14 d after the C. albicans infection. To assess cytokine production, splenocytes from BCG or vehicle-vaccinated mice were retrieved 7 d after the i.v. infection with C. albicans and stimulated in vitro with LPS (10 ng/mL). Cytokine concentrations were measured using a specific RIA, as described previously (25) in supernatants collected after 48 h of incubation at 37 °C in 5% CO₂ in a 48-well plate.

Statistical Analysis. Differences were analyzed using the Wilcoxon signed rank test or Friedman test for paired samples. P < 0.05 was considered statistically significant. Unless otherwise stated, data are shown as the cumulative results of levels obtained in all volunteers (mean ± SEM).

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