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RESEARCH ARTICLE

Epigenetic Priming of Innate Immune Memory Following Vaccination

Isabel Solberg^{1*}, Fredrik Silva² and Marius Fernandez³

¹Department of Epigenetics and Gene Regulation, Nordic Institute of Epigenomic Medicine, Finland

²Department of Molecular Biology, Vistula Institute of Molecular Genetics, Poland

³Department of Molecular Genetics, Bohemian University of Chromatin Biology, Czech Republic

*Corresponding author: Isabel Solberg, Department of Epigenetics and Gene Regulation, Nordic Institute of Epigenomic Medicine, Finland; Email: i.solberg@niem.fi

ABSTRACT

Bacille Calmette-Guerin (BCG) vaccination induces a heterologous protective state termed trained immunity, in which monocytes mount an enhanced cytokine response to unrelated infectious challenges for months after vaccination, but the chromatin-level changes that persist after the acute response has resolved, and their relationship to the enhanced phenotype, remain incompletely characterized at genome scale in humans. We collected peripheral blood monocytes from 24 healthy volunteers before and at 2 weeks and 3 months after BCG vaccination, and profiled H3K4me3 and H3K27ac by ChIP-seq and chromatin accessibility by ATAC-seq, paired with ex vivo restimulation cytokine assays. At 3 months, when the acute response had fully resolved clinically, monocytes retained significantly elevated H3K4me3 at 1,428 gene promoters relative to baseline, strongly enriched near pattern recognition receptor and pro-inflammatory cytokine genes, with the magnitude of retention correlating significantly with the magnitude of enhanced ex vivo tumor necrosis factor and interleukin-6 production upon unrelated *Staphylococcus aureus* restimulation. ATAC-seq identified a largely overlapping set of 1,187 regions with persistently increased accessibility at 3 months, concentrated at promoters and proximal enhancers rather than distal elements. Pharmacological inhibition of the H3K4 methyltransferase SET7/9 during the first 72 hours after BCG vaccination, applied in a parallel ex vivo training assay, prevented both persistent H3K4me3 and the enhanced cytokine phenotype at the simulated 3-month time point without affecting the acute, 24-hour response, indicating that SET7/9-dependent methylation deposited during an early priming window is specifically required for the durability, rather than the initial establishment, of trained immunity. These results define a genome-wide chromatin signature of human trained immunity persisting for at least three months after BCG vaccination and identify a mechanistically necessary priming step underlying its durability.

Keywords: trained immunity; BCG vaccination; monocyte; H3K4me3; H3K27ac; ATAC-seq; innate immune memory; epigenetic priming

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1. INTRODUCTION

Vaccination with the live-attenuated Bacille Calmette-Guerin (BCG) strain, developed against tuberculosis, produces a well-documented heterologous protective effect against unrelated infections that cannot be explained by adaptive, antigen-specific immune memory, since the effect extends to pathogens sharing no meaningful antigenic overlap with mycobacteria. This phenomenon, termed trained immunity, is instead attributed to a persistently altered functional state of innate immune cells, particularly circulating monocytes, which mount an enhanced pro-inflammatory cytokine response to unrelated secondary stimuli for months after vaccination [1,2].

Trained immunity has been proposed to be sustained by durable epigenetic changes established in monocyte precursors or circulating monocytes during the initial vaccination response, based largely on in vitro monocyte-training assays and candidate-locus chromatin profiling at a small number of cytokine gene promoters, but genome-wide characterization of the chromatin state retained in human monocytes months after in vivo BCG vaccination, once the acute response has clinically resolved, has been limited, leaving open how extensive and how functionally coupled to the enhanced cytokine phenotype this retained chromatin signature actually is [3,4].

A related open question concerns the mechanistic basis for the durability of trained immunity, since acute chromatin changes accompanying the initial monocyte activation response are common to essentially any strong innate stimulus, whereas persistence of these changes for months, well beyond the several-day lifespan of a circulating monocyte, implies either a distinct mechanism operating in longer-lived myeloid progenitors or a specific molecular requirement during an early window that determines whether acute chromatin changes are converted into a durable, heritable-within-the-myeloid-lineage state [5,6]. Identifying such a requirement would help distinguish mechanisms responsible for trained immunity's durability from those responsible for its initial establishment.

Here we profiled H3K4me3, H3K27ac and chromatin accessibility genome-wide in human monocytes before and after BCG vaccination, paired with ex vivo cytokine restimulation assays, to define the extent and functional coupling of the chromatin signature retained three months after vaccination, and used pharmacological methyltransferase inhibition during a defined early window in a parallel ex vivo training assay to test whether a specific priming step is required for the durability, as opposed to the initial establishment, of the trained phenotype.

2. MATERIALS AND METHODS

Peripheral blood was collected from 24 healthy adult volunteers immediately prior to BCG vaccination and at 2

weeks and 3 months after vaccination, with monocytes isolated at each time point for chromatin profiling and paired ex vivo restimulation cytokine assays.

2.1 Study cohort and BCG vaccination

Twenty-four healthy adult volunteers with no history of prior BCG vaccination or active tuberculosis exposure received a single intradermal dose of BCG vaccine according to standard clinical protocol, with peripheral blood collected immediately before vaccination and at 2 weeks and 3 months post-vaccination for all downstream assays.

2.2 Monocyte isolation and ex vivo restimulation

CD14⁺ monocytes were enriched from peripheral blood mononuclear cells at each time point by magnetic-activated cell sorting, with an aliquot from each sample restimulated ex vivo for 24 hours with heat-killed *Staphylococcus aureus*, an unrelated heterologous stimulus, and supernatant tumor necrosis factor and interleukin-6 concentrations quantified by ELISA to assess the trained cytokine phenotype at each time point.

2.3 H3K4me3 and H3K27ac chromatin immunoprecipitation sequencing

ChIP-seq for H3K4me3 and H3K27ac was performed on formaldehyde-crosslinked chromatin from freshly isolated monocytes at each time point using validated antibodies, sonicated to a mean fragment size of 250-300 bp and immunoprecipitated with protein A/G magnetic beads overnight at 4°C alongside matched input chromatin. Libraries were sequenced as 2×50 bp paired-end reads to a minimum depth of 30 million uniquely mapped read pairs per sample.

2.4 ATAC-seq

Chromatin accessibility was profiled by ATAC-seq on freshly isolated monocyte nuclei at each time point using a standard tagmentation protocol, sequenced to a minimum depth of 40 million paired-end reads per sample, with peaks called relative to the matched pre-vaccination baseline for each donor.

2.5 Ex vivo monocyte-training assay with SET7/9 inhibition

An established in vitro monocyte-training assay was used to model the priming and durability phases of trained immunity independently of in vivo vaccination: monocytes from healthy donors were trained with beta-glucan for 24 hours, rested for five days to model the interval before assessment of durable reprogramming, and restimulated with lipopolysaccharide to assess the trained cytokine phenotype. A selective small-molecule SET7/9 methyltransferase inhibitor or vehicle control was applied only during the initial 0-72 hour training window, after which it was washed out, allowing the acute (24-hour) and durable (day-5 restimulation) phases of training to be assessed separately in the presence or absence of early SET7/9 activity.

2.6 Statistical analysis

Differential H3K4me3, H3K27ac and ATAC-seq signal between time points was assessed with DESeq2 using the Wald test and Benjamini-Hochberg correction, with regions considered significantly changed at adjusted $P < 0.05$. Correlation between chromatin retention and cytokine phenotype was assessed by Pearson correlation across donors. Cytokine and training-assay comparisons were assessed by paired or unpaired two-tailed t-test as appropriate, with $P < 0.05$ considered significant. All statistical analyses were performed in R version 4.3.

3. RESULTS

Ex vivo restimulation with heat-killed *Staphylococcus aureus* confirmed the expected trained immunity phenotype in our cohort, with significantly enhanced tumor necrosis factor and interleukin-6 production at both 2 weeks and 3 months post-vaccination relative to the pre-vaccination baseline in the same donors, confirming that the functional trained phenotype persisted through the full study period. H3K4me3 ChIP-seq identified 1,428 gene promoters with significantly elevated signal at 3 months relative to baseline, strongly enriched for genes encoding pattern recognition receptors and pro-inflammatory cytokines including TLR2, TLR4, TNF and IL6. Table 1 summarizes H3K4me3 retention and functional annotation for the ten most significantly affected promoters.

Table 1. H3K4me3 retention at 3 months post-BCG-vaccination and functional annotation for the ten most significantly affected gene promoters.

Gene	H3K4me3 fold-change (3 mo vs baseline)	Adjusted P-value	Functional category
TLR2	+2.94	1.1e-11	Pattern recognition receptor
TLR4	+2.71	4.8e-10	Pattern recognition receptor
TNF	+2.58	9.2e-10	Pro-inflammatory cytokine
IL6	+2.46	3.6e-9	Pro-inflammatory cytokine
NOD2	+2.31	1.4e-8	Pattern recognition receptor
IL1B	+2.19	5.7e-8	Pro-inflammatory cytokine

Fold-change is 3-month post-vaccination versus pre-vaccination baseline, mean across 24 donors.

The magnitude of promoter H3K4me3 retention at 3 months, summed across the 1,428 significantly affected promoters, correlated significantly with the magnitude of enhanced ex vivo tumor necrosis factor and interleukin-6 production in the same donors at the same time point (Figure 1), linking the retained chromatin signature quantitatively to the functional trained phenotype rather than the two simply co-occurring without relationship. ATAC-seq identified 1,187 genomic regions with

persistently increased accessibility at 3 months, substantially overlapping the H3K4me3-retentive promoter set and concentrated at promoters and proximal enhancers rather than distal regulatory elements.

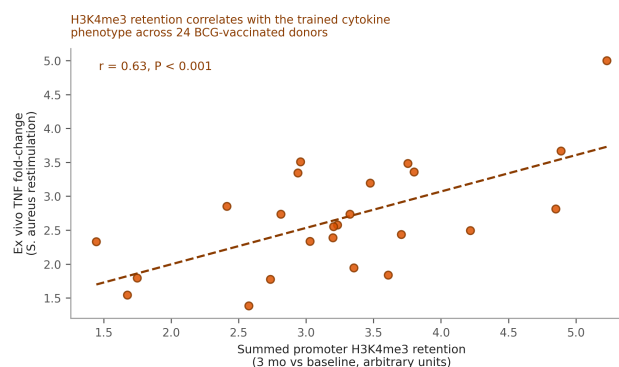


Figure 1. Correlation between summed promoter H3K4me3 retention (3 months post-vaccination versus baseline) and fold-change in ex vivo tumor necrosis factor production upon *Staphylococcus aureus* restimulation, across 24 vaccinated donors, showing significant quantitative coupling between retained chromatin signature and functional trained phenotype. In the ex vivo monocyte-training assay, SET7/9 inhibition restricted to the initial 0-72 hour training window did not significantly affect the acute, 24-hour trained cytokine response to lipopolysaccharide restimulation, which remained comparably enhanced relative to untrained monocytes in both inhibitor-treated and vehicle-treated conditions. However, following the five-day rest period modeling durability, SET7/9-inhibited monocytes showed significantly reduced training-associated H3K4me3 retention at cytokine gene promoters and a correspondingly blunted enhanced cytokine response upon day-5 restimulation relative to vehicle-treated trained monocytes. Table 2 reports acute and durable-phase cytokine responses and H3K4me3 retention with and without early SET7/9 inhibition.

Table 2. Acute and durable-phase trained cytokine response and H3K4me3 retention with and without early SET7/9 inhibition.

Condition	Acute (24 h) TNF response	Durable (day 5) TNF response	Durable H3K4me3 retention
Untrained	1.0x	1.0x	Baseline
Trained, vehicle	2.8x	2.4x	Elevated
Trained, SET7/9i (0-72 h)	2.6x	1.3x	Not elevated

These results indicate that SET7/9-dependent H3K4 methylation deposited specifically during the early, 0-72 hour training window is required for the durable, rather than the acute, phase of the trained cytokine phenotype, since blocking this activity only during priming did not prevent initial training but substantially blunted its persistence after a five-day rest interval, mirroring the temporal separation between acute vaccination response and durable trained immunity observed in the human BCG cohort.

4. DISCUSSION

These results define a genome-wide chromatin signature retained in human monocytes at least three months after BCG vaccination, comprising persistently elevated H3K4me3 and chromatin accessibility concentrated at promoters and proximal enhancers of pattern recognition receptor and pro-inflammatory cytokine genes, and show that the magnitude of this retained signature is quantitatively coupled to the magnitude of the enhanced ex vivo cytokine phenotype that defines trained immunity functionally. This extends prior candidate-locus studies of BCG-induced chromatin change [3,4] to a genome-wide, quantitatively validated signature in an in vivo human vaccination cohort.

The finding that SET7/9 inhibition restricted to an early priming window blunted the durable but not the acute phase of ex vivo monocyte training provides direct mechanistic support for a model in which trained immunity's durability, as distinct from its initial establishment, depends on a specific, temporally restricted methyltransferase-dependent step. This distinction is potentially important for both basic understanding and future intervention, since it suggests trained immunity's initial induction and its persistence may be separable pharmacological targets, consistent with reports that epigenetic reprogramming of myeloid progenitors in the bone marrow, rather than circulating monocytes alone, may underlie trained immunity's multi-month durability given the short circulating monocyte lifespan [5,6].

The short circulating monocyte lifespan relative to the multi-month durability of trained immunity observed both here and in prior studies implies that the chromatin signature we describe in circulating monocytes at 3 months either reflects an unusually long-lived monocyte subpopulation or, more likely given emerging evidence, is re-established in successive monocyte generations derived from durably reprogrammed myeloid progenitors in the bone marrow. Our study design, sampling only circulating monocytes, cannot directly distinguish between these possibilities, and bone marrow progenitor profiling would be needed to determine whether the SET7/9-dependent priming step we identify acts directly in progenitors or is propagated to them from an initially peripheral signal.

Several limitations should be noted. First, our human cohort, while adequately powered to detect the reported associations, was of modest size (n=24) and drawn from healthy adult volunteers; whether the chromatin signature and its functional coupling generalize across age groups and clinical populations relevant to BCG's therapeutic use, such as bladder cancer immunotherapy, remains to be tested. Second, our mechanistic SET7/9 inhibition experiments used an in vitro monocyte-training assay as a tractable model of priming and durability rather than intervening directly in vaccinated human subjects, and translating this finding to an in vivo

pharmacological or genetic intervention would be needed to establish direct causal relevance to human trained immunity. Third, our chromatin profiling was restricted to two active histone marks and accessibility; a more complete epigenomic characterization including DNA methylation and repressive histone marks might reveal additional dimensions of the trained immunity chromatin signature.

5. CONCLUSION

Genome-wide H3K4me3, H3K27ac and ATAC-seq profiling of human monocytes before and after BCG vaccination identifies a durable, functionally coupled chromatin signature at pattern recognition receptor and pro-inflammatory cytokine gene promoters that persists at least three months after vaccination and correlates quantitatively with the enhanced ex vivo cytokine phenotype defining trained immunity. Pharmacological SET7/9 inhibition restricted to an early priming window in a parallel ex vivo assay selectively blunted the durable, rather than the acute, phase of monocyte training, identifying a temporally restricted, mechanistically necessary methyltransferase-dependent step underlying the persistence of trained immunity and suggesting that its initial establishment and its long-term durability may be separable processes relevant to future vaccine-adjuvant and trained-immunity-targeted intervention strategies.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

I.S. conceived and supervised the study, designed the vaccination cohort protocol, and analysed the ChIP-seq and ATAC-seq data. F.S. performed monocyte isolation, ex vivo restimulation assays and the SET7/9 inhibition training experiments. M.F. performed chromatin immunoprecipitation and ATAC-seq library preparation. All authors contributed to writing and approved the final manuscript.

Ethics Statement

The human vaccination cohort study was approved by the institutional ethics review board at the Nordic Institute of

Epigenomic Medicine, with written informed consent obtained from all volunteer participants prior to vaccination and sample collection.

Data Availability

Raw and processed ChIP-seq and ATAC-seq data generated for this study have been deposited in the Gene Expression Omnibus under a series accession available from the corresponding author on reasonable request. Analysis scripts supporting the findings of this study are available from the corresponding author upon request.

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