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Infant RSV immunoprophylaxis changes nasal epithelial DNA methylation at 6 years of age

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Abstract

Background: Respiratory syncytial virus (RSV) infection has been associated with childhood wheeze and asthma, and potential mechanisms include persistent epigenetic effects.

Methods: In the randomized, placebo-controlled MAKI trial, 429 preterm infants randomly received RSV immunoprophylaxis with palivizumab or placebo during their first RSV season. Children were followed until age 6 for asthma evaluation. DNA methylation in cells obtained by nasal brushes at age 6 was measured by Illumina MethylationEPIC array.

Results: RSV immunoprophylaxis in infancy had a significant impact on global methylation patterns in nasal cells at age 6. The first principal component (PC) related to

Abbreviations: DMR, differential methylation region; EWAS, epigenome-wide association studies; FEV, forced expiratory volume; GAMP, global analysis of methylation profiles; IgE, immunoglobulin E; NMDS, nonmetric multidimensional scaling; PCA, principal component analysis; RSV, respiratory syncytial virus.

Louis J. Bont and Gerard H. Koppelman contributed equally to this study as joint senior authors.

the immunoprophylaxis intervention was enriched for the pathway "detection of chemical stimulus involved in sensory perception of smell" and "T cell differentiation." Subsequent analysis of these PCs indicated an effect of RSV immunoprophylaxis on cell type composition of nasal brushed cells. Three CpG sites, cg18040241, cg08243963, and cg19555973 which are annotated to genes *GLB1L2*, *SC5D*, and *BPIFB1*, were differentially methylated at genome-wide significance, but were not associated with asthma.

Conclusion: The study provides the first proof of concept that RSV immunoprophylaxis during infancy has long-term effects on nasal epigenetic signatures at age 6, relating to host sensory perception, epidermal growth factor receptor signaling, and adaptive immune responses.

KEYWORDS

asthma, DNA methylation, epigenetics, RSV immunoprophylaxis, RSV infection

1 | BACKGROUND

Human respiratory syncytial virus (RSV)-related acute lower respiratory tract infection is a leading cause of severe respiratory morbidity and mortality in children.[1] RSV infection in early life is associated with an increased risk of early childhood wheezing.[2–6] Recurrent wheezing and asthma are thought to result from alterations in early life immune development following RSV infection, when the immune system is immature. Premature born children are at higher risk for the adverse effects of early life RSV lower respiratory tract infection.[7,8] However, how RSV infection would have a long-term effect on the host immune system and how this would relate to the development of asthma remains unknown.

Epigenetics refers to (heritable) changes in gene expression that are not encoded by genetic variation. Recent epigenome-wide association studies (EWAS) of allergic diseases have reported encouraging results,[9–14] and epigenetics might explain the high degree of plasticity of the immune response throughout life.[15] For example, in a recent EWAS, we identified 14 CpG methylation sites associated with childhood asthma. Those CpG sites are related to distinct gene expression signatures reflecting early life activation of eosinophils and effector/memory CD8 T cells and NK cells at the expense of naive T cell subsets, and such findings provide support for a role of early life antiviral immunity in asthma inception.[9] Indeed, RSV infection may have epigenetic consequences. RSV infection of Dendritic Cells leads to alterations in histone methylation by the H3K4 demethylase KDM5B, resulting in decreased pro-inflammatory cytokine production and a subsequent increase of Th2 cytokines from T cells.[16] Moreover, RSV has been related to a nasal airway microRNA profile which is predicted to have a downregulated NF-kB signaling pathway.[17] Moreover, environmental exposure can alter cellular epigenetic states.[18,19] Among these epigenetic markers, DNA methylation provides a stable marker that is suitable for investigating mechanisms for long-term regulation of gene expression upon environmental stimulation.[20] Many studies relate early life exposures to DNA methylation signatures later in life, yet these observational studies may be subject to confounding and causation remains to be verified by future studies. A possible solution to this would be to nest epigenetic analyses in a randomized controlled clinical trial, where the exposure can be controlled by the randomized intervention.

We hypothesized that RSV infection in early life causes changes in DNA methylation in the nasal airway epithelium in childhood. To test this hypothesis, we studied the randomized clinical trial MAKI[2] (ISRCTN registry, number ISRCTN73641710) to investigate the direct link between RSV prophylaxis during infancy and DNA methylation changes in the nasal epithelium at age 6. In this trial, we observed significant protective effects of early life RSV prophylaxis on having RSV infection and asthma symptoms in the first years of life.[2,3] We performed an EWAS to identify differentially methylated patterns in relation to palivizumab prevention at the global, individual, and regional CpG site level.

2 | METHODS

2.1 | Study design and participants

In the MAKI trial, 429 otherwise healthy late preterm infants were enrolled between 2008 and 2010, and they were born at 33–35 weeks of gestation. Infants were randomly assigned to receive palivizumab ($n = 214$) or placebo ($n = 215$) during their first RSV season. Baseline characteristics at randomization are available at NEJM.org.[2] Details about the design, definitions, protocol of the primary study, follow-up study, and clinical assessment have been previously described.[2,3] Clinical assessment at age 6 years included questionnaires for respiratory symptoms (parent-reported current asthma) as well as a parent report of a physician's diagnosis of asthma, lung function tests (forced expiratory volume $[FEV]_{0.5,}$ forced vital capacity), and

assessment of specific immunoglobulin E (IgE) to a panel of aeroallergens as well as total serum IgE. Positive specific IgE was defined as an allergen-specific IgE concentration of at least 0.35 kU/L for house dust mite, birch, mugwort, timothy, *Aspergillus*, fumigatus, dog, and cat.[3] After quality control, 274 samples were included in the analyses (Figure 1A).

2.2 | Nasal DNA methylation measurements

Nasal epithelial cells were collected by nasal brushing around age 6 years of age at a home visit by a trained investigator. Briefly, the right nostril of the subjects was examined and the inferior turbinate was located using a speculum and penlight. The lateral area underneath the inferior turbinate was then brushed for 3 s with two brushes (Copan, 56380CS01 FLOQswabs) and these were placed in a 2 ml screw-cap Eppendorf tube and put into a freezer at −80°C until further processing.

DNA was extracted from nasal brushes using the DNA Investigator Kit (Qiagen, Benelux BV). This was followed by precipitation-based purification and concentration using GlycoBlue (Ambion). 500 ng of DNA was bisulfite-converted using the EZ 96-DNA Methylation Kit (Zymo Research), following the manufacturer's standard protocol. After verification of the bisulfite conversion step using Sanger Sequencing, DNA concentration was normalized and the samples were randomized to avoid batch effects. One standard DNA sample per chip was included in this step for quality control. In total, 296 nasal epithelium samples with sufficient DNA quality and quantity were hybridized to the Infinium HumanMethylationEPIC BeadChip array (Illumina).

2.3 | Data pre-processing and statistical analysis

DNA methylation data were pre-processed in R with the Bioconductor package Minfi,[21] using the original IDAT files extracted from the HiScanSQ scanner. We implemented sample filtering to remove 6 bad quality samples (call rate < 99%) and 16 samples with gender mismatch. During processing, bad quality probes which failed more than 10% of the samples (4698 probes), the probes on sex chromosomes (19,681 probes), the probes that mapped to multiple loci (41,167 probes), and the probes containing SNPs at the target CpG sites with a MAF > 5% in European populations were excluded (9907 probes).[22] 790,437 probes were kept for downstream analysis. We subsequently implemented stratified quantile normalization.[23] Methylation β-values were calculated as a percentage: $\beta = M/(M + U + 100)$, where M and U represent methylated and unmethylated signal intensities, respectively, and β-values were then transformed to M-values as $\log2(\beta/(1-\beta))$, and M-values were used in all downstream analyses.

To remove the effect of extreme outliers in data, we trimmed the methylation set using: (25th percentile − 3*IQR) and (75th percentile + 3*IQR), where IQR = interquartile range.

In our predefined analysis plan, we followed the trial design by relating the DNA methylation signatures at age 6 years to the palivizumab intervention. The association of DNA methylation with palivizumab intervention was conducted using robust linear regression

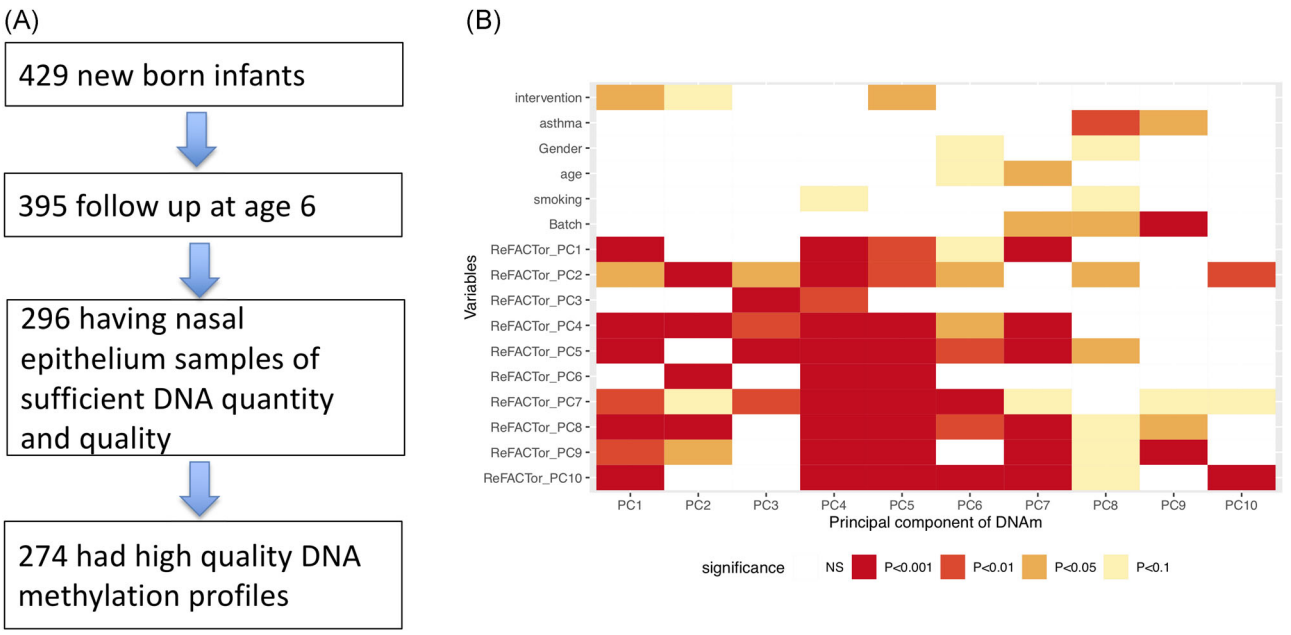

FIGURE 1 Flow chart of enrollment and overview of methylation data. (A) Flow chart of enrollment at age 0, follow-up at age 6, and quality control of DNA methylation data in MAKI study. (B) Association with Global nasal methylation (DNAm) variability. Principal component (PC) regression analysis showing univariable association p value between all covariables and the top 10 PCs of the variance of the nasal DNA methylation data [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Sample characteristics from MAKI methylation study

	Palivizumab	Placebo	*p*
Sample size	132	142	
Age of phenotyping (years)[a]	5.79 (0.20)	5.84 (0.28)	.098
Age of nasal brush sampling (years)	6.61 (0.39)	6.65 (0.41)	.397
Male sex, *n* (%)	84 (63.6)	69 (48.6)	.017
Asthma, *n* (%)	19 (14.4)	34 (23.9)	.065
FEV_{05} (mean (*SD*))	89.51 (9.61)	90.06 (11.49)	.68
Total IgE (mean (*SD*))	148.70 (290.87)	134.23 (177.47)	.692
Total RSV infection (%)	7 (5.3)	22 (15.8)	.01
Medically attended RSV infection (%)	2 (1.5)	13 (9.4)	.011
RSV infection without medical attention (%)	5 (3.8)	9 (6.6)	.461

Note: Missing values were not included. *P* values for categorical variables were calculated using the $\chi 2$ test comparing the palivizumab group and placebo group, and continuous variables by *t*-test.

Abbreviations: FeV_{05}, forced expiratory volume in 0.5 s; IgE, immunoglobulin E; RSV, respiratory syncytial virus.

[a]The age of phenotyping of asthma, FEV05, and Total IgE.

corrected for sex, age at nasal brush sampling, maternal smoking, batch, and surrogate variable analysis (SVA).[24] Surrogate variables were constructed on the residuals of the regression models that accounted for known confounders. Next, we modeled cell type composition by ReFACTor[25] and chose the first 10 PCs of ReFACTor as proxy for nasal cellular heterogeneity. The association of DNA methylation with allergic asthma was conducted using logistic regression corrected for sex, age at nasal brush sampling, maternal smoking, batch, and SVA. The number of surrogate variables was determined by the "leek" method in the SVA R package.[24] Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS)[26] were utilized to check the global effects of palivizumab prophylaxis. R package vegan was used for NMDS analysis. PCs associated with the intervention were analyzed, and the top 20, 50, and 100 CpG sites contributing to the first two PCs were tested. For single CpG site analysis, adjustment for multiple testing was done using Benjamini & Hochberg method. Significant differentially methylated CpG sites were considered when the P-value after false discovery rate (FDR) correction was less than 0.05. For the detection of regions of interest, differentially methylated regions (DMRs) analyses were performed using two methods, comb-p[27] and DMRcate.[28] For comb-p, we set the seed *p* value as 0.05, the adjusted region-level *p* value cutoff to determine DMR as 0.01, and the maximum distance to search for adjacent peaks as 1000 bp. For DMRcate, we set the width of estimation as 1000 bp, and *p* value cutoff to determine DMR as 0.01. Comb-p and DMRcate use different algorithms to identify significantly DMRs. Comb-p uses a one-step Šidák correction method for multiple comparisons,[29] while DMRcate uses the FDR method. To reduce false positives, we only considered a DMR to be significant if it was identified as statistically significant by both methods, according to the definition used in each method.

Global methylation analyses were run using R GAMP package with two models (M.cdf and M.densites) have been tested.[30] All statistical analyses were performed using the computing environment R (version 3.32).

2.4 | Functional annotation analysis

The CpG sites were annotated using GREAT 4.04 (Genomic Regions of Annotations Tool).[31] GREAT assigns a *cis* region of each gene consisting of a basal domain that extends 5 kb upstream and 1 kb downstream from its transcription start site, if nothing is found within this basal domain it extends to the basal regulatory domain of the nearest upstream and downstream genes within 1 Mb. We used GeneNetwork (https://www.genenetwork.nl)[32] for pathway analysis of genes annotated by the GREAT annotation tool of the differentially methylated CpG sites and CpG sites assonated with PCs.

3 | RESULTS

3.1 | Sample characteristics

Figure 1A illustrated the flow chart of initial enrollment, follow-up, and quality control of DNA methylation data in the MAKI study. After quality control, 274 (63.8%) of the collected samples and 790,437 probes remained for further analyses. The baseline characteristics of 274 participating children (132 palivizumab; 142 placebo), representing 63.9% of the original study population, are presented in Table 1. The median age at the time of nasal brush collection was 6.6 years. Current asthma was defined as parent-reported wheeze or asthma medication in the past 12 months[3]; current allergic asthma was defined as having asthma and specific IgE to aeroallergens at age 6 years. Asthma prevalence was somewhat lower in the palivizumab group compared to the placebo group (14% vs. 24%, $p = .065$; Table 1), and no differences were observed in lung function ($FEV_{0.5}$) and total serum IgE levels, as reported previously in the full study population.[3]

3.2 | Differential methylation profiling analysis

After QC, 790,437 CpG sites were available for downstream analyses. Figure 1B illustrates the global DNA methylation variability from nasal methylation data. The first 10 PCs explained 49.8% of the variance of the nasal methylome. Interestingly, the reference-free cell type method ReFACTor[25] showed the strongest association with the first 10 PCs. The association between intervention and the first PCs suggesting treatment effects by RSV immunoprophylaxis.

The PCA plot (Figure 2A) and NMDS plot (Figure 2B–D) illustrate an overall methylation difference at age 6 years between the RSV immunoprophylaxis group and the placebo group. The first two PCs (p value$_{pc1} = 8.1 \times 10^{-3}$; p value$_{pc2} = 1.0 \times 10^{-2}$) were significantly associated with RSV immunoprophylaxis (Table S1). NMDS confirmed this overall methylation difference between the two groups (adonis, $R^2 = 0.107$, $p = .0055$, with 1999 permutations; Figure 2B–D). To test if these global methylation changes are driven by confounders, we applied global analysis of methylation profiles (GAMP)[30] by correcting age, sex, maternal smoking, and SVA. The results show a global association of the intervention with DNA methylation, and that age, sex, and maternal smoking do not influence the global methylation differences between the RSV immunoprophylaxis group and the placebo group (Table S2). However, when surrogate variables were included in the GAMP analysis, there is no significant methylation global difference between the two

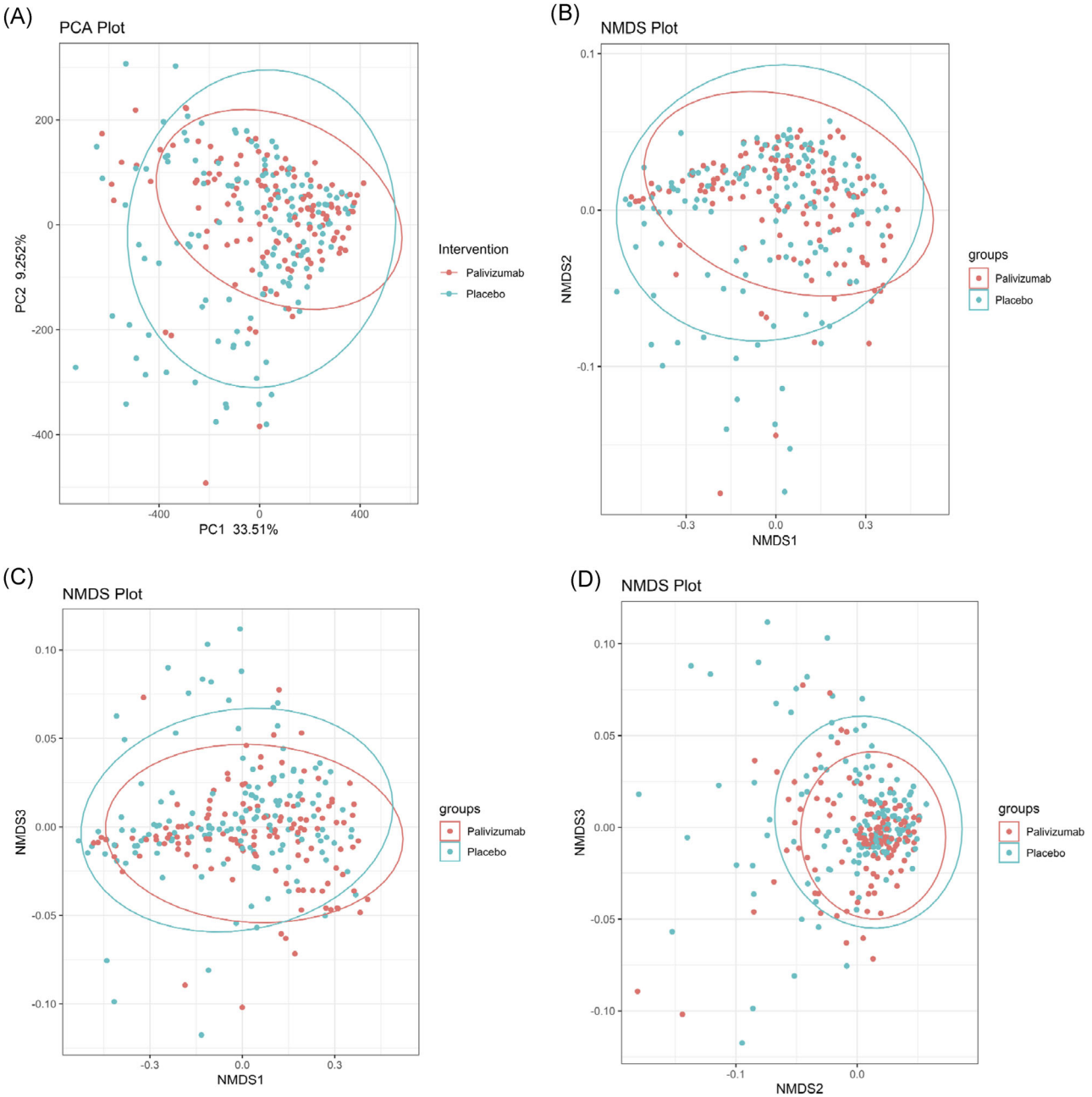

FIGURE 2 Principal component analysis (PCA) and nonmetric multidimensional scaling (NMDS) of global methylation patterns in DNA obtained nasal epithelial brushing in relation to palivizumab intervention. Both biplots depicting the individual overall methylation levels (data points, $n = 274$), colored by treatment group: placebo (blue, $n = 132$) and palivizumab (red, $n = 142$). In PCA analysis, two PCs were significantly associated with the intervention: PC1 ($p = 8.1 \times 10^{-3}$) and PC2 ($p = 1.0 \times 10^{-2}$). In NMDS analysis, the difference in overall methylation levels is significant (adonis, $R^2 = 0.0195$, $p = .0115$, with 1999 permutation. (A) PC1–PC2, (B) NMDS1–NMDS2, (C) NMDS1–NMDS3, and (D) NMDS2–NMDS3 [Color figure can be viewed at wileyonlinelibrary.com]

groups ($p > .05$), which suggests that a significant methylation difference is explained by cell-type composition reflected by the surrogate variables (Figure S1).

We next performed an EWAS on each CpG site, and three CpG sites (cg18040241, cg08243963, and cg19555973) which are localized in Galactosidase *Beta 1 Like 2* (*GLB1L2*), encoding lathosterol oxidase involved in cholesterol biosynthesis (*SC5D*) and BPI Fold Containing Family B Member 1 (*BPIFB1*) were genome-wide significantly associated with RSV immunoprophylaxis versus placebo (Figure 3a and Table S3; $p < .05$ after FDR correction). Among these, cg18040241 which is annotated to gene *GLB1L2* also passed the Bonferroni correction ($p < 6.62 \times 10^{-9}$). These genes have not been previously implicated in viral immune responses or asthma development. The lambda value was 1.123, which indicated no strong inflation in the model after SVA correction (Figure 3B). A sensitivity analysis including asthma status shows very good agreement between the result of analyses with and without asthma correction (epigenome-wide correlation of ß-coefficients = .967, see Table S4). In differential methylation region (DMR) analysis, we identified 8 overlapping DMRs in relation to RSV immunoprophylaxis (Table S5).

3.3 | Pathway analysis

To identify pathways explaining these overall methylation differences after RSV immunoprophylaxis, we selected the top 20 CpG sites contributing most to the first two PCs (Tables S6 and S7). The pathway analysis by gene network of PC1 of top 20CpG indicated enrichment of Go biological pathways involved in "detection of chemical stimulus involved in sensory perception of smell," epidermal growth factor receptor signaling," and "T cell differentiation" (Figure 4A). Those pathways were consistently showing up when the top 50 CpG and 100 CpG sites have been tested (Tables S8 and S9). Based on gene network clustering, Figure 4B depicts two clusters in this gene network: "T cell costimulation" and "detection of chemical stimulus involved in sensory perception of smell" (Tables S10 and S11). The pathway analysis on genes linked to PC2 showed relation to innate immune response genes (Table S12).

3.4 | Link of RSV prophylaxis epigenetic markers to asthma and allergy

We next related PC1 of RSV immunoprophylaxis-associated methylation to patient-reported asthma at age 6 years, but did not observe an association (point-biserial correlation = −0.077, $p = .20$). The three CpG sites which were associated with RSV immunoprophylaxis were also not associated with patient-reported asthma (Table S13). We further tested 30 CpG sites which were reported to be associated with allergic asthma from recent nasal epigenomics studies,[33] yet none of these were associated with RSV immunoprophylaxis (Table S14). However, we did replicate 16 of these 30 CpG sites associated with allergic asthma and 48 out of 284 with asthma[34] in nasal epithelium in our study (Tables S15 and S16).

4 | DISCUSSION

This study shows that RSV prophylaxis by palivizumab at infancy has global and persistent effects on nasal DNA methylation patterns in childhood. We could observe such changes at global, single CpG site, and regional level. Using pathway analysis, we linked these immunoprophylaxis-associated global DNA methylation changes to viral response and viral budding genes.

We propose that prevention of RSV respiratory tract infection in early childhood leads to different timing and severity of early life RSV respiratory tract infection, which in turn affects local DNA methylation of relevant innate immune response genes. This was further evidenced by differential methylation of cg16834953 in the *NOD2* gene, which functions as a viral pattern recognition receptor and participates in inducing antiviral signaling.[35]

An important question is whether the epigenetic patterns we identified would lead to functional differences of these nasal cells, for example in response to other respiratory infections. An alternative explanation would be a direct effect of palivizumab on global gene methylation patterns, or microbiome changes in relation to early life RSV infections that in turn could affect nasal DNA methylation. To our knowledge, this is the first time that long-term methylation effects could be related to the timing of early life RSV infection. We performed our study within the framework of the randomized controlled MAKI trial, enabling us to infer causality rather than reporting an association, as can be done in observational cohorts. As we expect all children to have had RSV respiratory tract infections at the age of six years, we interpret our findings on changes in palivizumab-related DNA methylation as an effect of timing and/or severity of early life RSV infection. However, this hypothesis needs further validation in future studies, focused on the timing and severity of early life RSV infection, preferentially in a longitudinal design. The low prevalence of documented RSV infection in the first year of life did not enable us to perform these analyses in this cohort (see Table 1).

We did not identify a direct link between the epigenetic markers associated with RSV immunoprophylaxis and asthma at age 6 years in global, single-site, and regional analyses, there was no strong overlap between the RSV immunoprophylaxis-associated gene set and previously published asthma-associated CpG sites. This is in line with the clinical findings in this cohort, reporting no strong association with early life palivizumab prevention and doctor's diagnosed asthma at age 6 years.[3] Much larger studies are needed to identify a potential link between RSV immunoprophylaxis, epigenetic changes, and asthma.[36]

Despite the overall robustness and biological plausibility of our study findings, there are some limitations to consider. First, there is a lack of independent replication studies. To the best of our knowledge, there are no suitable data or cohorts available for replication of

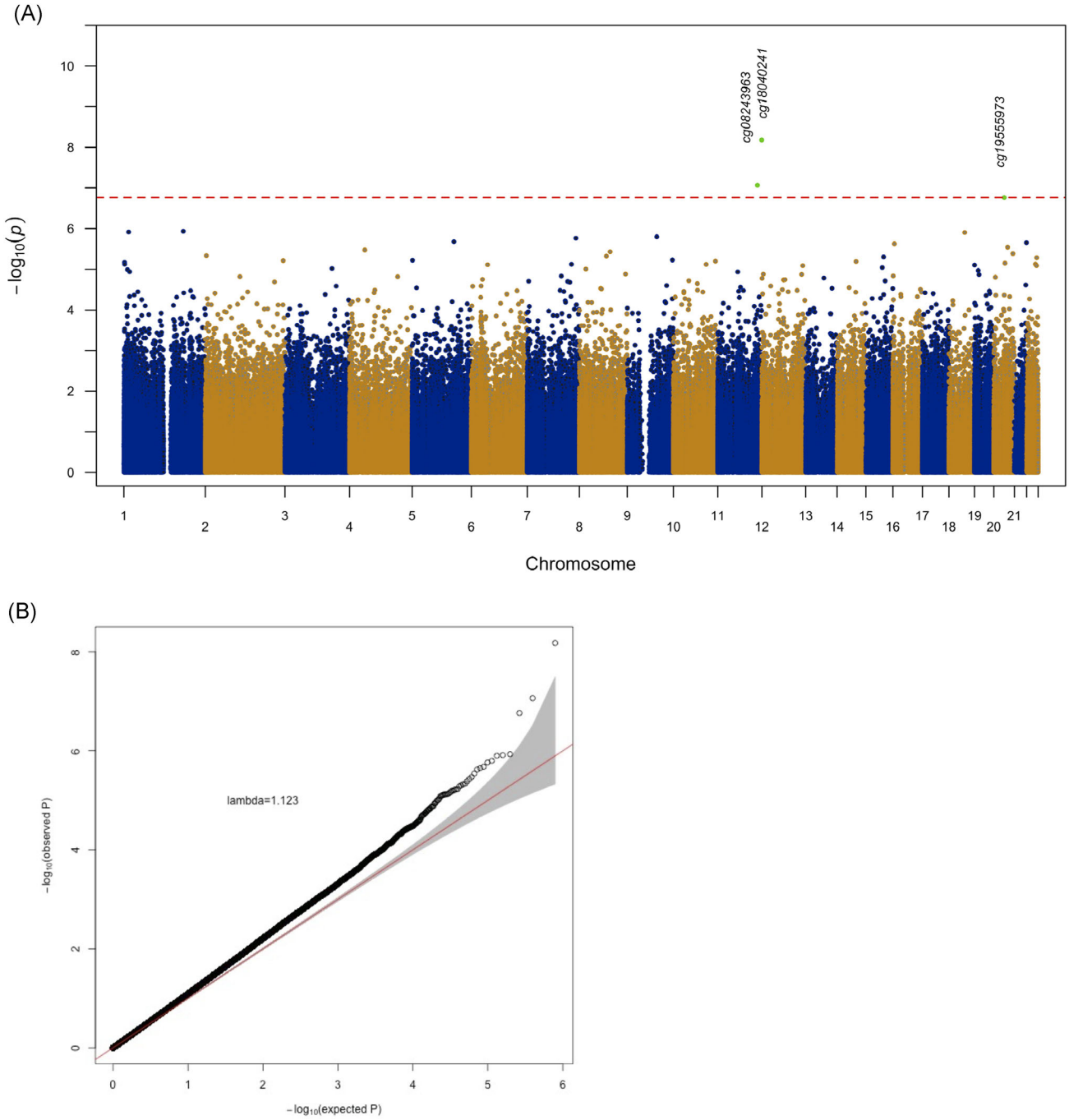

FIGURE 3 (A) Manhattan plot of 790,437 CpG sites for differentially methylation with early life RSV prophylaxis by palivizumab. *P* values (*y*-axis) correspond to the significance of the difference in methylation between prevention and no prevention subject. The red line corresponds to the *p* value FDR = 0.05 threshed. (B) A quantile–quantile (QQ) plot displays the experimentally observed *p* values (vertical axis) as well as the expected *p* values of a null distribution (horizontal axis). The observed *p* value is obtained from the association between CpG sites methylation and RSV prophylaxis. The model to test the association is methylation ~RSV prophylaxis + age at nasal brush sample + sex + maternal smoking + batch, with correction of two surrogate variables. The gray area represents the 95% concentration band. FDR, false discovery rate; RSV, respiratory syncytial virus [Color figure can be viewed at wileyonlinelibrary.com]

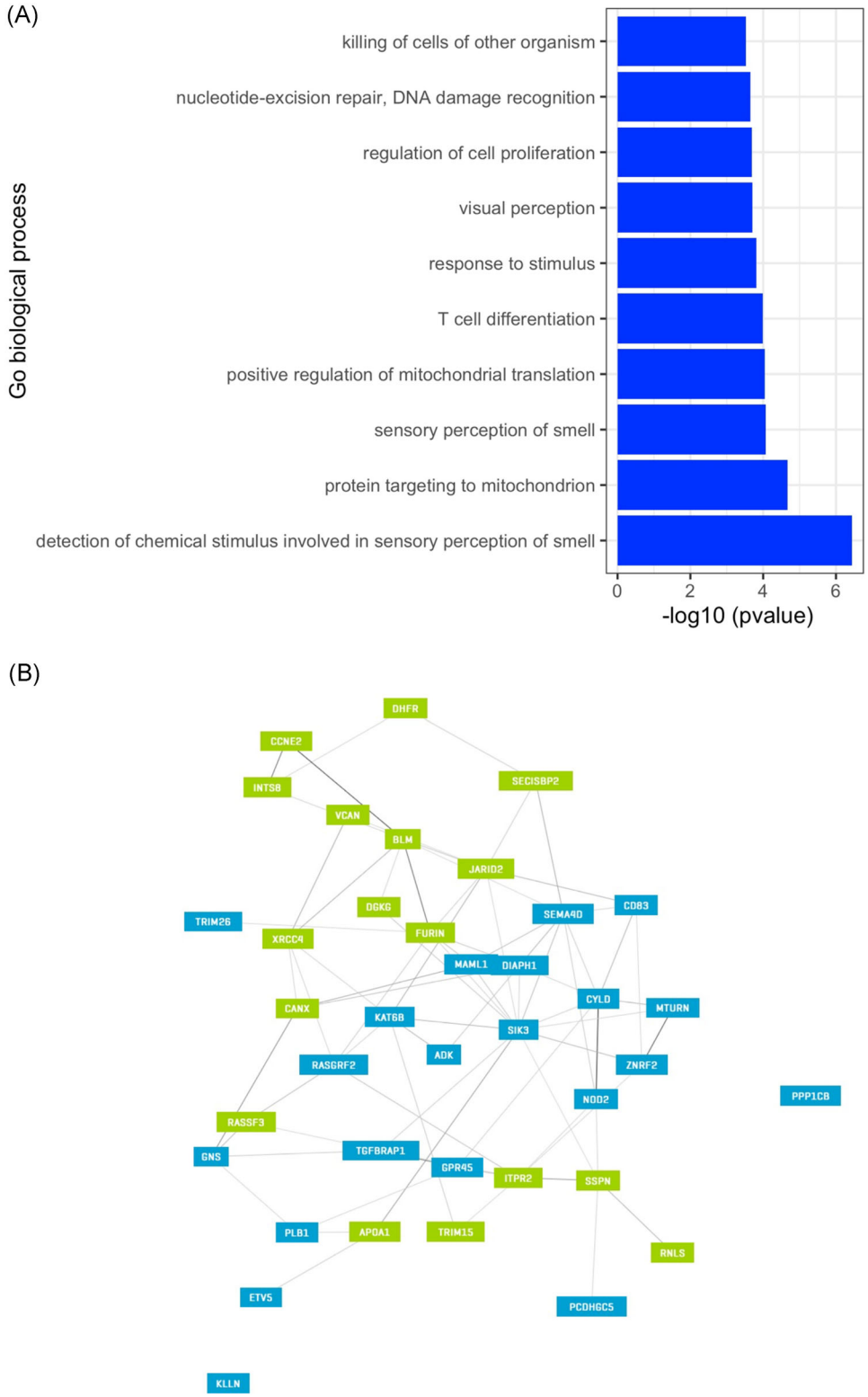

FIGURE 4 Pathway enrichment analysis of genes that significantly contribute to PC1. (A) Pathway analysis by using Gene network analysis and Go-biological process databases. (B) There are two clusters (blue and green) in this network. PC, principal component [Color figure can be viewed at wileyonlinelibrary.com]

our findings. The study by O'Brien et al.[37] did not include epigenetic studies in the long-term follow-up (personal communication). Second, there is no gold standard to define cell composition in nasal brushes, so we used surrogates variables[24] to represent cell types. We additionally modeled cell type by ReFACTor, and show that global results are strongly driven by cell-type composition. However, since we lack cell type composition models based on DNA methylation, this still remains a data-driven correction. We suggest that nasal cell-type annotation based on DNA methylation may provide further insight into the identity of the cells responsible for the epigenetic signature related to RSV-immunoprophylaxis. Third, our study was single-blinded. Fourth, we could not directly confirm that these methylation changes in nasal epithelium regulated gene expression, which should be part of future studies. Fifth, although we studied over 800,00 CpG sites of the human methylome, this still represents about 3% of all human CpG sites. Further work confirming these differentially methylated sites and detailed assessment of the associated regions using pyrosequencing should be performed. Sixth, other factors may also associate with DNA methylations, such as age,[38] cigarette smoke,[39] air pollution,[40] or other respiratory infections. Seventh, global DNA methylation analyses are exploratory and must be interpreted with caution as they are not conclusive. However, given that our analysis was embedded in a randomized controlled clinical trial, it is very reasonable to assume that potential confounders are distributed equally across the two treatment arms; and that the results were driven by the RSV Immunoprophylaxis.

In conclusion, infant RSV prophylaxis persistently changes nasal epithelial DNA methylation at least until age 6 years, and these methylation profiles are linked to host sensory perception, epidermal growth factor receptor signaling, and adaptive immune responses.

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CONFLICT OF INTERESTS

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ETHICS STATEMENT

The MAKI trial was approved by the ethical committee Medisch Ethische Toetsingscommissie (METC) of the Universitair Medisch Centrum Utrecht (July 21, 2008), and all study participants provided written consent.

AUTHOR CONTRIBUTIONS

Louis J. Bont and Gerard H. Koppelman conceived and designed the study; Cheng-Jian Xu, Cancan Qi, and Rolf Vedder performed statistical analyses; Cheng-Jian Xu drafted the manuscript and interpreted the data. Nienke M. Scheltema, Louis J. Bont, and Gerard H. Koppelman contributed to the manuscript writing. Nienke M. Scheltema, Laura B. C. Klein, Elisabeth E. Nibbelke, Cornelis K. van der Ent, and Louis J. Bont obtained informed consent and clinical information. All authors reviewed and provided comments to the final manuscript (Cheng-Jian Xu, Nienke M. Scheltema, Cancan Qi, Rolf Vedder, Laura B. C. Klein, Elisabeth E. Nibbelke, Cornelis K. van der Ent, Louis J. Bont, and Gerard H. Koppelman). All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The epigenetic data sets generated and analyzed are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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