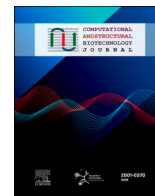


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Research Article

Methylation and transcriptomic profiling reveals short term and long term regulatory responses in polarized macrophages

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ABSTRACT

Macrophage plasticity allows the adoption of distinct functional states in response to environmental cues. While unique transcriptomic profiles define these states, focusing solely on transcription neglects potential long-term effects. The investigation of epigenetic changes can be used to understand how temporary stimuli can result in lasting effects. Epigenetic alterations play an important role in the pathophysiology of macrophages, including their trained innate immunity, enabling faster and more efficient inflammatory responses upon subsequent encounters to the same pathogen or insult. In this study, we used a multi-omics approach to elucidate the interplay between gene expression and DNA-methylation, to explore the potential long-term effects of diverse polarizing environments on macrophage activity. We identified a common core set of genes that are differentially methylated regardless of exposure type, indicating a potential common fundamental mechanism for adaptation to various stimuli. Functional analysis revealed that processes requiring rapid responses displayed transcriptomic regulation, whereas functions critical for long-term adaptations exhibited co-regulation at both transcriptomic and epigenetic levels. Our study uncovers a novel set of genes linked to the long-term effects of macrophage polarization. This discovery underscores the potential of epigenetics in elucidating how macrophages establish long-term memory and influence health outcomes.

1. Introduction

Macrophages are a versatile cellular component of the immune system and are one of the first lines of defense of the body against pathogens or tissue damage [1]. The plasticity of macrophages allows them to adopt distinct functional states in response to microenvironmental cues [2]. Traditionally, macrophages have been classified into two distinct activation states representing opposite ends of a functional spectrum. Classically activated macrophages, induced by stimuli such as bacterial lipopolysaccharide (LPS) and interferon-gamma (IFN γ), exhibit pro-inflammatory (PI) functions [3]. Conversely, alternatively activated macrophages, triggered by factors like interleukin-4 (IL-4), display anti-inflammatory (AI) properties [3]. This spectrum of activation allows macrophages to exhibit functional diversity, playing crucial roles in pathophysiological processes and responses to foreign materials [4,5]. This functional diversity and macrophage plasticity is tightly regulated by transcriptional reprogramming, where macrophage phenotypes are

defined by distinct gene expression profiles [6–8]. On top of this, epigenetic shifts are increasingly recognized as directing the transcriptional differences observed between macrophages in polarized states [9–12]. Epigenetic modifications, the most stable and persistent of which being DNA methylation [13,14], bridge the gap between transient stimuli and long-lasting cellular responses, even after the initial stimulus has subsided [11,15–17]. Coupled with transcriptional and metabolic reprogramming, these epigenetic modifications can prime innate immune cells to respond differently to subsequent environmental cues [18], leading to an altered response to further challenges [19–21].

Integrating transcriptomic and DNA methylation data allows us to scrutinize how macrophage polarization leads to long-term reprogramming. This combined analysis can shed light on whether biological or synthetic insults can induce long-lasting epigenetic imprinting, while also revealing co-regulatory pathways and genes critical for macrophage responses [22,23]. While toxicology has traditionally focused on phenotypic endpoints such as cytotoxicity, the field is increasingly

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embracing toxicogenomics to elucidate the mechanisms of action of an expanding arsenal of synthetic chemicals used in human activity. THP-1 macrophages, a well-established cell model for innate immunity testing in traditional toxicology (OECD Environment, Health and Safety Publications Series on Testing & Assessment No. 360), are now being explored for their utility in toxicogenomic safety assessments. For example, previous work using THP-1 macrophages has integrated DNA methylation changes and gene expression changes to investigate the effect of carbon nanotube exposure both in vitro and in vivo, showing genes and pathways regulated at both levels linked to the outcome of lung fibrosis [17, 24]. Other studies focusing on polarized macrophages have also explored different types of epigenetic control such as posttranslational histone modifications and chromatin accessibility [6,10,11] and whilst there have been explorations of general DNA methylation dynamics in immune cells [25,26], studies looking at differences in methylation patterns between distinctly polarized macrophage states are still missing.

In this study, we investigated how macrophage polarization may lead to long-term changes that prepare these innate immune cells for future challenges. We employed a multi-omics approach, integrating DNA methylation profiling with transcriptomic analysis of polarized macrophages. Macrophages were polarized into distinct phenotypes using established exposures. LPS and IFN γ were used to generate PI macrophages (M(LPS-IFN γ)) [27]. While IL-4 and interleukin-13 (IL-13) were used to create AI macrophages (M(IL4-IL13)) [4]. We identified and functionally characterized the set of genes that are differentially expressed or epigenetically modified in these M(LPS-IFN γ) and M(IL4-IL13) macrophages. LPS-IFN γ triggered a significantly higher number of differentially expressed genes (DEGs) compared to IL4-IL13. At the epigenetic level, however, both stimuli predominantly affected the same genes, demonstrating a common core set of genes that are differentially methylated, regardless of the environmental cue, at each end of the polarization spectrum. We also identified a smaller subset of genes displaying exposure specific epigenetic regulation, showing differentially polarized macrophages also have the potential for distinct regulatory mechanisms at the level of DNA methylation changes. Finally, in both polarized states, we found that immune and metabolic processes were regulated by both transcriptomic and methylation changes. This shows parallels with previous studies that have established a link between metabolic rewiring and epigenetic reprogramming in trained immunity [28–31]. However, further studies would be needed to elucidate the precise role of the genes implicated in this study, during the shaping of macrophage trained immunity. Our approach explored both short-term and long-term macrophage regulatory strategies, informing our understanding of macrophage population responses across the polarization spectrum in pathophysiological conditions and following chemical exposures.

2. Materials and methods

2.1. THP-1 cell culture

THP-1 cells (ATCC TIB-202, USA) were cultured in RPMI-1640 (Gibco, USA) supplemented with 10 % FBS (Gibco, USA) (culture media). Cells were cultured in 75 cm² flasks at a density $< 1 \times 10^6$ cells/ml. Cells were differentiated in 12 well plates, with 500,000 cells/well (127,000 cells/cm²), in the culture media supplemented with 30.9 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, USA) for 48 h. After PMA differentiation, cells were exposed to two different cytokines cocktails and fresh media for 24 h, 48 h, and 72 h. To simulate a PI environment the cells were exposed to LPS (Merck) 10 pg/ml [32] and IFN γ (Sigma-Aldrich, USA) 20 ng/ml [32–34]; to simulate an AI environment, the cells were exposed to IL-13 (Sigma-Aldrich, USA) 20 ng/ml and IL-4 (Sigma-Aldrich, USA) 20 ng/ml [32–34].

2.2. Collection of RNA

Cells were lysed on the plate and the RNeasy mini kit (Qiagen, Germany) was used as per the vendor's instructions. The quality of the RNA samples was verified using Bioanalyzer 2100 (Agilent, USA), with RNA 6000 Nano Kit (Agilent, USA) following the vendor's instructions. The RIN values for each sample exceeded 9.0.

2.3. RNA-sequencing

Quantity and quality of the RNA samples was assessed with quality checks as follows: preliminary quality control was performed on 1 % agarose gel electrophoresis to test RNA degradation and potential contamination, sample purity and preliminary quantitation and RNA integrity were measured using Bioanalyzer 2100 (Agilent Technologies, USA). For library preparation, the Novogene NGS RNA Library Prep Set (PT042) was used. The mRNA present in the total RNA sample was isolated with magnetic beads of oligos d(T)25 using polyA-tailed mRNA enrichment. Subsequently, mRNA was randomly fragmented and cDNA synthesis using random hexamers and reverse transcription was performed. Once first chain synthesis was finished, the second chain was synthesised with the addition of an Illumina buffer (non-directional library preparation). Together with the presence of dNTPs, RNase H and polymerase I from E. Coli, the second chain was obtained by Nick translation. The resulting products then underwent purification, end-repair, A-tailing and adapter ligation. Fragments of the appropriate size were enriched by PCR, where indexed P5 and P7 primers (Illumina) were introduced, and final products were purified. The library was checked with Qubit 2.0 and real-time PCR for quantification and bioanalyzer Agilent 2100 for size distribution detection. Quantified libraries were pooled and sequenced on the Illumina Novaseq X platform, according to effective library concentrations and data amounts using a paired-end 150 strategy (PE150). RNA-seq data have been submitted to NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE273627.

2.4. RNA-sequencing data analysis: data collection and pre-processing

The “.fastq” format raw files were used for the quality assessment of all RNA-Seq datasets, conducted using FastQC v0.11.7. Trimming of reads, including removal of low-quality ends and adapters, was performed using cutadapt v4.4_dev with Python 3.8.10 with default parameters. Subsequently, the trimmed and adapter-clipped raw reads underwent a second round of quality checks using FastQC v0.11.7. The RNA sequencing reads were aligned to the human reference genome assembly GRCh38 using the HISAT2 algorithm and its corresponding genome indexes. Transcript abundance was determined using the featurecounts function from the Rsubread v1.34.4 R package with Rstudio version 1.1.453, using Ensembl annotation version 105 (<https://www.ensembl.org>). Non-expressed and lowly expressed genes were filtered out by applying a proportion test, as implemented in the NOISeq Bioconductor package.

2.5. RNA-sequencing: Differential expression analysis

The DESeq2 v1.24.0 R package was employed for normalization and differential expression analysis [35], we conducted a comparison for differential gene expression at each time point between the treatments and the control (e.g. M(LPS-IFN γ)_24h vs control_24h, M(LPS-IFN γ)_48h vs control_48h, etc.), as well as for the differential expression of genes among control groups over time (e.g. control_48h vs control_24h). Differentially expressed genes (DEGs) are determined by comparing samples from the treatment group to untreated samples the genes are then filtered for adjusted p-value FDR < 0.01 and absolute log₂FC > 0.58 (Supplementary Table 1). As our cell population comprised of monocyte-derived macrophages, we took precautions to exclude any

variations in gene expression linked to macrophage differentiation. Our focus remained solely on assessing the impact of the stimulation cocktail and we wanted to mitigate the potential impact of macrophage differentiation on our analysis. Consequently, genes displaying differential expression over time among the control groups (e.g. control_48h vs control_24h, control_72h vs control_24h) were omitted from the analysis.

2.6. DNA-methylation: data collection and pre-processing

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen). The quality and the concentration of the DNA samples were verified using Nanodrop2000. Genomic DNA was treated with sodium bisulfite using EZ-96 DNA methylation kit (Zymo Research, cat no.: D5004), following the manufacturer's standard protocol. Assessment of levels of DNA methylation of known CpG regions and promoters across the genome was done with Infinium MethylationEPIC v2.0 Kit (Illumina, Inc.) and Illumina iScan. In brief, following bisulfite conversion, approximately 500 ng of the bisulfite-converted DNA per sample was used for methylation analysis. The initial quality control and identification of signal intensities for each probe were performed with Illumina GenomeStudio Software. Methylation data have been submitted to NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE273627.

Methylation data were analyzed in R following the workflow previously described by Maksimovic *et al.* [36]. Raw methylation files were uploaded together with the metadata and microarray annotation file. Illumina methylation data is usually obtained in the form of Intensity Data (IDAT) files, a proprietary format that is generated by the scanner and stores summary intensities for each probe on the array. The raw intensity signals were read into R from the IDAT files using the “read.metharray.exp” function of the minfi package v1.46 [37]. The function creates an RGChannelSet object that contains all the raw intensity data, from both the red and green color channels, for each of the samples. The quality of the dataset was evaluated first by calculating the detection p-value for every CpG in every sample and then generating a quality control report using the “qcReport” function of the minfi package [37]. CpG probes were filtered by removing probes with a detection p-value higher than 0.05 in any sample. Further filtering was applied to remove probes for CpGs located on the sex chromosomes or those containing single nucleotide polymorphisms. To minimize the unwanted variation between samples, data were normalized using the “preprocessQuantile” function of the minfi package. Once the data had been filtered and normalized, the M-values and beta values were calculated. M-values have better statistical properties and are thus better for use in statistical analysis of methylation data whilst beta values are easy to interpret and are thus better for displaying data. M-values were obtained using the “getM” function from the minfi package [37], while the beta values were obtained using the function “getBeta” from the same package. Probe wise differential methylation analysis was performed using the M-values between each treatment and its control for each time point. The analysis was performed on the matrix of M-values using the limma package [38], obtaining moderated t-statistics and associated p-values for each CpG site, which has been further corrected with the FDR method. Given the genome-wide nature of the analysis and the inherent uncertainty associated with interpreting beta values, a stringent p-value threshold of 9×10^{-8} was employed to have a more robust statistical significance [39].

2.7. DNA-methylation: probe annotation

At the epigenetic level, probes showing differential methylation ($FDR < 9 \times 10^{-8}$) between the control group over time were excluded. After pinpointing these probes, the GREAT software [40] was used for CpG annotation to genes ($FDR < 0.05$). Specifically, each gene was allocated a regulatory domain extending to the midpoint between its transcription start site (TSS) and the nearest gene's TSS, limited to 1000 kb

(Supplementary Figure 1). To ensure robust annotation, we defined posthoc regions, where only genes with at least three significant CpGs annotated were considered as differentially methylated genes (DMGs). The overall methylation level of DMGs is calculated as a weighted average of the methylation values of its constituent CpG sites, with weights determined by the proximity of each CpG to the TSS (Supplementary Table 2). To further elucidate the regulatory context of our CpGs, we mapped the genomic location of CpG sites within the genes of interest, using the biomaRt package v2.38 (Supplementary Figure 2).

2.8. Functional annotation analysis

Pathway enrichments were performed using the “gost” function of the gProfiler package [41]. Lists of official genes were offered as input after being converted to gene symbols. Gene Ontology (GO) terms were enriched using all known genes as the statistical domain scope of the analysis. GO terms were considered significantly enriched with an FDR-adjusted p-value < 0.05 (Supplementary Table 3–4). When comparing the functional annotation of the two phenotypes (e.g. M(LPS-IFN γ) and M(IL4-IL13)), the most significant terms are categorized into four groups: (a) unique to M(LPS-IFN γ), (b) unique to M(IL4-IL13), (c) shared and most significant in M(LPS-IFN γ), and (d) shared and most significant in M(IL4-IL13). This categorization will allow the identification of functionally distinct terms associated with each phenotype.

3. Results and discussion

3.1. Differential methylation patterns in polarized macrophages are numerically similar despite transcriptomic divergence

To evaluate perturbations in the transcriptome of macrophages treated with either LPS and IFN γ (M(LPS-IFN γ)), or IL4 and IL13 (M(IL4-IL13)) and to assess whether this polarization resulted in distinct gene expression profiles, a differential expression analysis was performed. We identified 5753, 6209, and 5021 DEGs in M(LPS-IFN γ), compared to untreated samples, at 24 h, 48 h, and 72 h, respectively (Fig. 1 A in red). In M(IL4-IL13), the number of DEGs identified at 24 h, 48 h, and 72 h was 1527, 2309, and 1916, respectively (Fig. 1A in green) (Supplementary Table 1). Therefore, M(LPS-IFN γ) macrophages exhibited over twice as many DEGs compared to M(IL4-IL13) at all time points, revealing a divergence in gene expression between PI and AI macrophages consistent with previous findings [42–44]. As LPS and IFN γ simulate the acute immune response to infection, while IL4 and IL13 are expressed during resolution of the acute response, these results suggest that the transcriptomic regulation of more genes is required for maintaining the acute PI state compared to the AI state. When examining the differential methylation results, the M(LPS-IFN γ) did not exhibit any statistically significant DMGs at 24 h but showed 3595 DMGs at 48 h and 5247 DMGs at 72 h (Fig. 1B in red). Similarly, the M(IL4-IL13) phenotype showed a differential methylation in only 11 genes at 24 h, but 2935 DMGs at 48 h and 5241 genes at 72 h (Fig. 1B in green) (Supplementary Table 2).

At each individual time point (24 h, 48 h, 72 h), the total number of DEGs significantly diverged between PI and AI conditions (Fig. 1A). In contrast, while DMGs increased from 24 h to 72 h, the number of DMGs at each individual time point is approximately the same in each condition (Fig. 1B). A subset of genes exhibited sustained differential expression changes across all three time points (24 h, 48 h, and 72 h), specifically 3378 genes for M(LPS-IFN γ) and 835 M(IL4-IL13), indicating a degree of persistent variation in gene expression induced by both stimuli (Fig. 1C). The majority of the genes that showed initial differential methylation at 24 h and 48 h time points were maintained up to 72 h (Fig. 1D). Unlike the rapid fluctuations observed in gene expression, DNA methylation patterns are far more stable over short experimental time periods and take longer to shift in response to an exposure [45–48]. While the influence of LPS and IFN γ on the

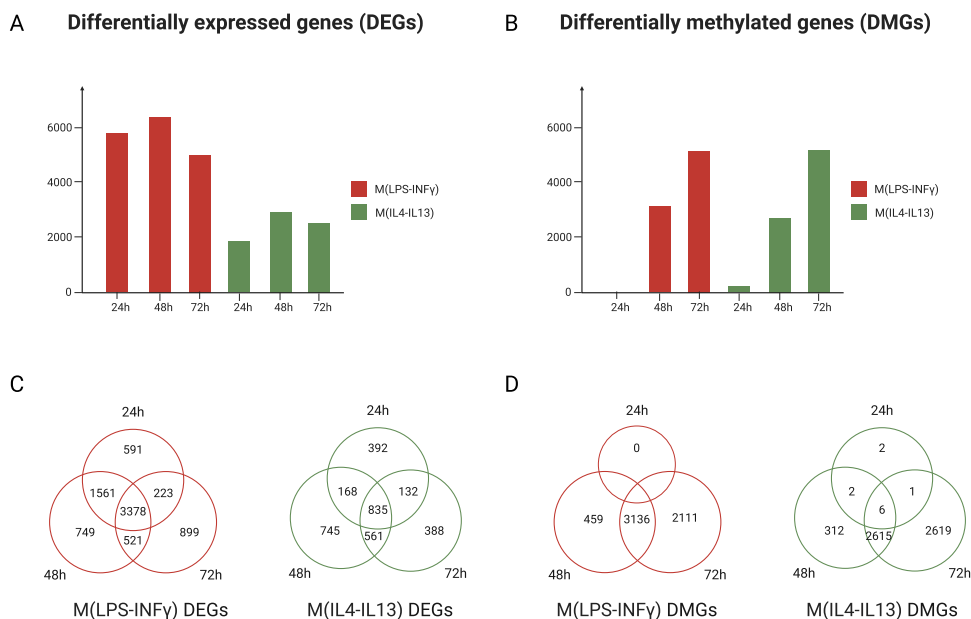


Fig. 1. (A) Number of differentially expressed genes (DEGs) and (B) differentially methylated genes (DMGs) at time points 24 h, 48 h and 72 h of pro-inflammatory stimulation (in red) and anti-inflammatory stimulation (in green). (C-D) Venn diagrams of DEGs and DMGs in time in the pro-inflammatory phenotype (in red) and anti-inflammatory phenotype (in green) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

transcriptome was considerably larger compared to IL4 and IL13, this degree of difference was not seen at the epigenetic level. The number of DMGs under the two conditions remained similar at each timepoint. Establishing a direct causal relationship between CpG methylation and gene expression is complex [49,50]. Contrary to earlier assumptions, the correlation between DNA methylation in gene regulatory regions and transcriptional repression [51,52], as well as the relationship between

gene body methylation and active transcription [53,54], is now understood to be more intricate. The precise mechanisms underlying these associations remain elusive, and factors such as transcription factor binding, influenced by gene-specific methylation patterns, contribute to this complexity [55,56]. These complexities preclude a straightforward correlation between CpG methylation status and gene expression. With that said, we included the direction of methylation and expression

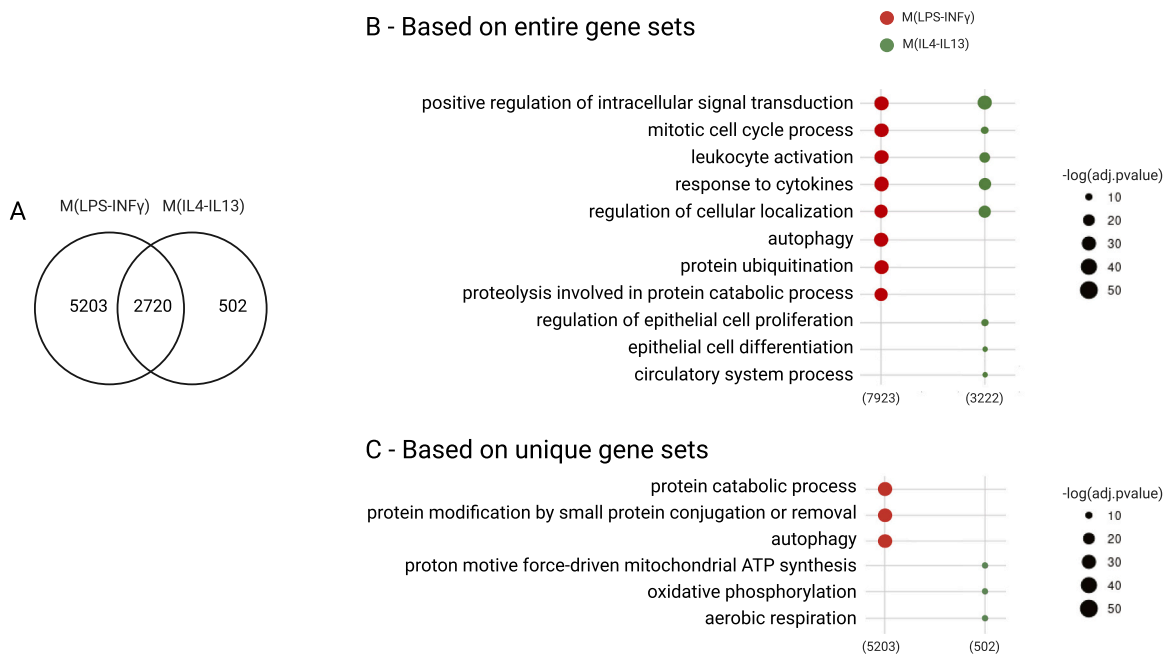


Fig. 2. (A) Number of DEGs ($FDR < 0.01$; $abs(\log_2FC) > 0.58$) between macrophages exposed to LPS-IFN γ and IL4-IL13. (B) Gene ontology biological processes enrichment ($FDR < 0.05$; terms with number of genes $5 < N < 1000$) was performed with the entire set of genes associated with each phenotype; the plotted terms are the three most significant ontologies in the category of shared and unique pathways. For the common pathways, the most significant three for each phenotype have been selected. (C) The enrichment analysis was performed within the gene set exclusive to each phenotype, not shared between the two (i.e. 5203 and 502 respectively for M(LPS-IFN γ) and M(IL4-IL13)); the plotted terms are the three most significant ontologies in the category of unique pathways. The color code utilizes red for terms enriched in M(LPS-IFN γ) and green for the ones enriched in M(IL4-IL13). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

changes for genes exhibiting differential regulation at both the transcriptomic and methylation levels in [Supplementary Figure 3](#). To understand the functional implications of the observed differences in gene regulation, functional annotation of the DEGs and DMGs was performed.

3.2. Functional annotation of transcriptome shows polarized phenotypes

To gain a deeper understanding of the biological processes affected by our polarization protocol, enrichment analysis was performed to functionally annotate the DEGs. Enrichment analysis of the DEGs at 24 h, 48 h, and 72 h, revealed for each condition, M(LPS-IFN γ) or M(IL4-IL13), the top 5 significant enriched terms remain consistent across all three time points. This suggested sustained functional changes across 72 h of continuous exposure ([Supplementary Figure 4](#)). Therefore, we focused on the combined set of DEGs from all three time points. A total of 7923 genes exhibited differential expression at the union of all three time points in M(LPS-IFN γ), compared to unexposed controls. For M(IL4-IL13), 3222 DEGs were identified across all 3 time points compared to unexposed controls. The comparison of DEGs between M(LPS-IFN γ) and M(IL4-IL13) revealed 2720 genes commonly differentially expressed, indicating a subset of genes responsive to both stimuli ([Fig. 2A](#)). To understand the directionality of these expression changes, we calculated the median fold change across all three time points in both exposures. This analysis identified 2075 genes exhibiting concordant regulation (with expression changing in the same direction) between M(LPS-IFN γ) and M(IL4-IL13), while 645 genes displayed discordant regulation between M(LPS-IFN γ) and M(IL4-IL13) ([Supplementary Table 4](#)). These 645 discordantly regulated genes were enriched in functional categories associated with cytokine signaling and inflammatory pathways ([Supplementary Table 5](#)). These enrichment results align with the established roles of LPS-IFN γ and IL4-IL13 in driving distinct macrophage phenotypes with contrasting cytokine profiles and inflammatory responses [3].

We conducted a gene ontology enrichment analysis on all 7923 DEGs found for M(LPS-IFN γ) and then also for all 3222 DEGs for M(IL4-IL13), revealing both common and distinct terms associated with each phenotype ([Fig. 2B](#)). Consistent with the use of immune signaling cytokines and LPS for polarization, functional enrichment analysis revealed immune pathway activation (e.g., “responses to cytokines”, “leukocyte activation”) in both M(LPS-IFN γ) and M(IL4-IL13) ([Fig. 2B](#)). Notably, M(LPS-IFN γ) uniquely displayed enrichment of autophagy, protein ubiquitination, and proteolysis pathways, suggesting enhanced antigen presentation and a PI state, and reflecting the role of these processes in responding to pathogens [57–59]. In contrast, M(IL4-IL13) macrophages displayed unique enrichment of pathways associated with cell proliferation and differentiation ([Fig. 2B](#)). This suggests a functional role in tissue repair and remodeling, potentially through the release of growth factors like TGF β 1 and platelet-derived growth factor (PDGF), which promote epithelial cell proliferation and fibroblast activation [60–62]. 5203 and 502 genes displayed unique differential expression in M(LPS-IFN γ) and M(IL4-IL13) respectively ([Fig. 2A](#)). To avoid the masking of distinct functions in M(LPS-IFN γ) and M(IL4-IL13) by the substantial overlap in their DEGs, a further enrichment analysis focused on genes exclusive to each phenotype (i.e., 5203 for M(LPS-IFN γ) and 502 for M(IL4-IL13)), highlighting environment-specific transcriptional signatures. This analysis revealed distinct metabolic profiles associated with each macrophage phenotype ([Fig. 2C](#)). Terms related to macromolecule and protein breakdown were seen for M(LPS-IFN γ), potentially reflecting activity seen in macrophages responding to pathogens by degrading foreign material [63,64]. Conversely, the AI phenotype (M(IL4-IL13)) displayed enrichment of oxidative phosphorylation terms, suggesting a metabolic shift. This aligns with reports of M(LPS-IFN γ) favoring glycolysis and M(IL4-IL13) relying more on oxidative phosphorylation, highlighting the metabolic reprogramming that accompanies macrophage polarization [65–68].

Taken together, this transcriptomic analysis indicated successful

polarization of macrophages in response to LPS-IFN γ and IL4-IL13. The DEGs identified in M(LPS-IFN γ) and M(IL4-IL13) displayed functional annotations consistent with PI and AI phenotypes, respectively. The M(LPS-IFN γ) group showed enrichment of terms related to inflammation, autophagy, and immune cell activation, characteristic of the PI phenotype. In contrast, the M(IL4-IL13) group displayed enrichment of terms associated with oxidative phosphorylation metabolism and tissue repair pathways, aligning with an AI phenotype.

3.3. Polarized macrophages show common and distinct epigenetic regulation

Through the examination of epigenetic modifications in polarized macrophages, we aimed to identify the DNA methylation changes specific to PI or AI macrophage phenotypes. Deciphering the unique epigenetic signatures associated with polarized macrophage states offers valuable insights into the potential long-term impact of this polarization on immune function. Epigenetic regulation plays a significant role in trained immunity, a process where innate immune cells, like macrophages, exhibit memory that alters response to future encounters [69–72]. While previous research has explored epigenetic modification in polarized macrophages by investigating changes in the activity of DNA methyltransferases and histone modifiers [10,12,73], our study takes a novel approach by directly identifying sets of common and unique DMGs associated with the polarized macrophage states.

A progressive increase in DMGs was observed over time in both M(LPS-IFN γ) and M(IL4-IL13) ([Fig. 1B](#)). As shifts in DNA methylation statuses are slower compared to gene expression fluctuations [14,45,47], genes that did not display persistent methylation status were filtered out (i.e. genes showing methylation status change at 24 h and 48 h that are not seen at 72 h, were omitted as false positives). For the sake of completeness, a list of genes excluded from the analysis based on the applied criteria is provided in [Supplementary Table 6](#). This approach yielded 4856 and 4899 DMGs in the cells stimulated with M(LPS-IFN γ) and M(IL4-IL13), respectively. In total, there were 4419 DMGs shared between the two phenotypes, 437 uniquely differentially methylated in M(LPS-IFN γ), and 480 uniquely differentially methylated in M(IL4-IL13) ([Fig. 1A](#)). These results suggest that, regardless of the polarization phenotype, macrophages display a core set of DNA methylation changes, indicating a potential adaptation mechanism that macrophages undergo no matter what environmental challenge is faced. These conserved epigenetic modifications may represent a fundamental state, allowing flexible responses to various environmental cues. Together our DNA methylation results show the presence of a dual regulatory strategy: a larger common core set of DMGs that shift regardless of which exposure is faced, and a second smaller set of DMGs that respond to the specific stimulus given.

Functional analysis of the genes displaying DNA methylation changes (i.e. 4856 genes in M(LPS-IFN γ) and 4899 in M(IL4-IL13)) revealed shared enrichment for DNA damage response and non-coding RNA (ncRNA) regulation ([Fig. 3B](#)). While LPS exposure is known to induce genotoxic stress via TLR activation [66,67], DNA damage is not expected in M(IL4-IL13). Analysis of genes within the “DNA damage response” GO term revealed key genes including TNF, PARP1, and PARP2, which are not primarily involved in oxidative stress or DNA damage pathways, but instead contribute to immune response modulation [74–76]. Therefore, the enrichment of the “DNA damage response” term in M(IL4-IL13) may instead reflect preparation for future immune challenges. The presence of ncRNA regulation potentially indicates epigenetic reprogramming, as ncRNA involvement in modulating immune responses and shaping long-lasting epigenetic modifications has previously been demonstrated [77–80]. Our analysis also revealed a small set of distinct functional terms associated with each macrophage phenotype, suggesting an epigenetically imprinted response to specific environmental cues. M(LPS-IFN γ) exhibited enrichment for terms related to chromatin and histone modifications.

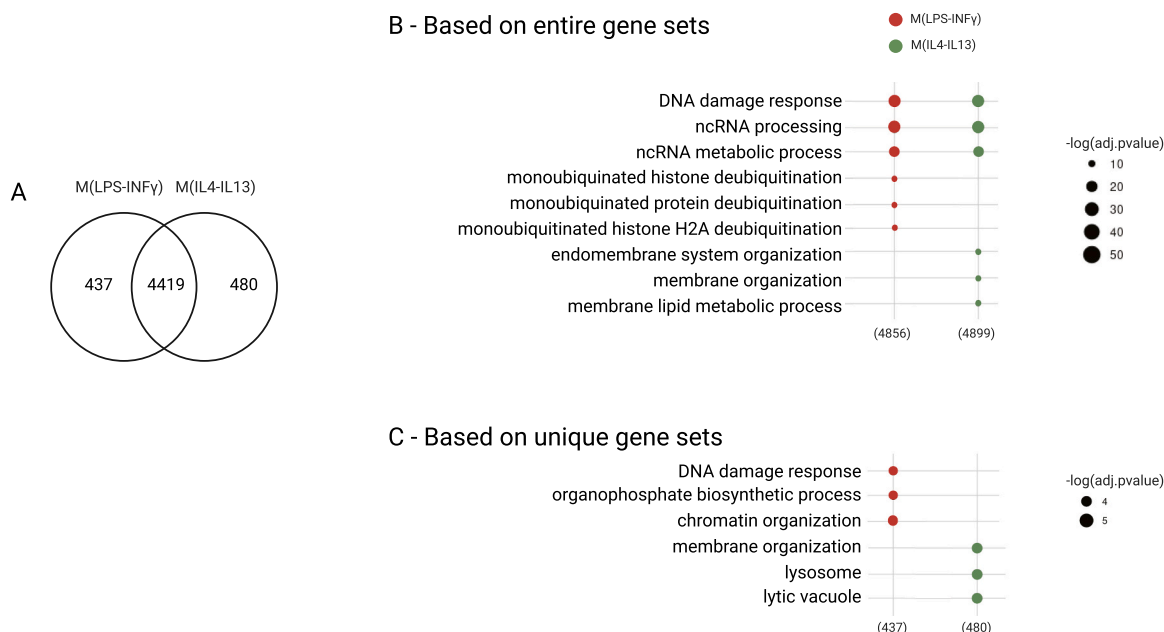


Fig. 3. (A) Number of differentially methylated genes (DMG) (FDR<0.05) between macrophages exposed to pro-inflammatory stimuli M(LPS-IFN γ) and anti-inflammatory stimuli M(IL4-IL13). (B) Gene ontology biological processes enrichment (FDR<0.05; terms with the number of genes 5 < N < 1000) was performed with the entire set of genes associated with each phenotype; the plotted terms are the three most significant ontologies in the category of shared and unique pathways. For the common terms, the most significant three for each phenotype have been selected. (C) The enrichment analysis was performed within the gene set exclusive to each phenotype not shared between the two (i.e. 437 and 408 respectively for M(LPS-IFN γ) and M(IL4-IL13)); the plotted terms are the three most significant ontologies in the category of unique pathways. The color code utilizes red for terms enriched in M(LPS-IFN γ) and green for the ones enriched in M(IL4-IL13). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LPS stimulation is known to increase metabolites including cytosolic acetyl-CoA, which can facilitate histone acetylation at promoters and enhancers of LPS target genes such as IL6 and IL12B [81–84]. Therefore, metabolic reprogramming induced by LPS exposure can directly influence chromatin structure and gene expression through epigenetic modifications. In contrast, M(IL4-IL13) showed enrichment for “membrane organization” terms and lysosomal/lytic compartments (Fig. 3B-C). Previous studies have demonstrated that lysosomal compartments play a crucial role in immune response and pathogen degradation [85,86]. Moreover, lysosomal structural remodeling has been shown to adapt to immune challenges and regulate antigen processing [87]. Our enrichment analysis suggests a potential role for epigenetic mechanisms in orchestrating the long-term adaptations of lysosomal function and immune regulation. However, further research is necessary to fully elucidate the precise mechanisms involved.

Our analysis of the DNA methylation landscape in M(LPS-IFN γ) and M(IL4-IL13) macrophages revealed the presence of a dual regulatory strategy. Firstly, a larger core set of DMGs is regulated regardless of the initial stimulus, and likely regulates macrophage functions that are common across different polarization states. This shared core suggests epigenetic priming that is stimulus-independent. Secondly, an environmental-specific DMG strategy exists that is specific to either PI or AI polarization. In both strategies, these DMGs provide a long-lasting epigenetic signature imprinted by the initial environmental signal that may extend beyond the immediate effects and potentially influence the long-term fate and function of the cells.

3.4. Integrated analysis of transcriptomic and epigenetic alteration unveils distinct regulatory mechanisms of macrophage activity in polarized states

After confirming effective polarization via transcriptome analysis and observing both commonalities and differences in DNA methylation changes between M(LPS-IFN γ) and M(IL4-IL13), a multi-omics approach was employed to gain a comprehensive understanding of environment-induced alterations. Unlike transcriptional changes, which often follow

an ‘impulse-like’ kinetic, epigenetic modifications can persist even after the initial stimulus fades [46,88]. This enduring nature of epigenetic alterations makes them better long-term indicators of exposure-induced changes as compared to analyzing transcriptional changes alone [28–31]. To develop a contextualized view of the regulatory relationships between dynamic and immediate transcriptomic regulation and long-term epigenetic regulation post exposure in polarized states of macrophage activation, we combined analysis of both transcriptomic and DNA methylation data layers.

Following the enrichment analysis of DEGs and DMGs in each phenotype, the ontologies were categorized into three groups: transcriptomic-specific, epigenetic-specific, and shared regulation (enriched in both transcriptomic and epigenetic datasets). From each group, the 10 most significant terms were selected and grouped based on broader biological processes (i.e. *cell death*, *signaling*, *cell-ECM interaction*, *developmental process*, *cell cycle*, *cellular response to stimulus*, *immune system process*, *metabolic process*, *translation and protein modifications*, *gene expression regulation*) (Fig. 4). Enriched term analysis revealed distinct regulatory patterns between transcriptional and epigenetic mechanisms. Processes such as *cell death*, *signaling*, and *cell-ECM interaction* were primarily transcriptionally regulated. In contrast, collagen fibril organization was regulated at the epigenetic level in both macrophage phenotypes. Protein production, metabolic processes (including autophagy and peptide metabolism), and DNA repair displayed differential regulation between the two macrophage populations. While M(LPS-IFN γ) macrophages exhibited both transcriptional and epigenetic regulation of these processes, M(IL4-IL13) macrophages predominantly showed epigenetic regulation. Notably, immune-related functions were regulated at both levels in both macrophage phenotypes (Fig. 4).

It is perhaps unsurprising that apoptosis and cell-extracellular matrix interactions were regulated primarily through gene expression changes, as these processes necessitate rapid transcriptional adaptations to ensure precise control and adaptability in quickly changing cellular environments [89–91]. In contrast, the process of organizing collagen fibers was only regulated by epigenetic changes. This might suggest a role for



Fig. 4. Integration of transcriptomic and epigenetic data by combined analysis of pathway enrichment of DEGs and DMGs. The top 10 most significant terms are presented for each phenotype, categorized into transcriptomics, epigenetics, or common terms. The p-value of common terms was evaluated with average FDR. Different colors (light blue, orange, red) distinguish these categories. The enriched pathways were grouped under broader biological processes (cell death, signaling, cell-ECM interaction, developmental process, cell cycle, cellular response to stimulus, immune system process, metabolic process, translation and protein modifications, gene expression regulation). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

epigenetic priming in macrophage-fibroblast interaction during the resolution phase of inflammation, as macrophages encounter a collagen-rich matrix [92]. In the PI phenotype, terms that involve protein production were regulated at the transcriptome level. This aligns with the established role of rapid transcriptional changes and increased protein production for a robust response during acute inflammation [93, 94], further supported by the higher number of DEGs observed upon LPS-IFN γ exposure (Fig. 1A). In the M(IL4-IL13) population there was no acute stress response, observed as a lack of transcriptomic changes of DNA repair mechanisms, which is likely due to the absence of LPS-induced stress. DNA repair mechanisms were however, altered at the epigenetic level in M(IL4-IL13), potentially indicating that these macrophages are preparing for future stress challenges, and this priming may lead to a more efficient future response [95]. Immune terms in both M(LPS-IFN γ) and M(IL4-IL13) were controlled at both the transcriptomic and epigenetic level, suggesting rapid transcriptional changes for an initial immune response, as well as epigenetic modifications for long-term immune memory regardless of the initial challenge or stimulus. In fact, the observed epigenetic modifications of immune genes, coupled with a metabolic shift seen at the transcriptional level (Section 3.2), suggests a potential priming effect in both M(LPS-IFN γ) and M(IL4-IL13) macrophage populations. Given the established link between metabolic reprogramming and epigenetic alterations in trained immunity [22,23,84,85], we hypothesize that the identified epigenetic changes may underpin a functional state capable of enabling accelerated

responses to subsequent challenges. With that said, further experiments would be necessary to conclusively demonstrate training and the persistence of epigenetic modifications post-stimulus.

4. Conclusion

This study investigated the long-term reprogramming induced by macrophage polarization, potentially establishing an epigenetic memory for subsequent challenges. This study supports the growing use of toxicogenomics and multi-omics analysis to understand how different exposures can cause short-term effects and long-term epigenetic changes in immune cells. By employing multi-omics analysis, our study unveiled co-regulatory pathways and key genes with potential relevance for toxicogenomic safety assessments. Transcriptomic analysis revealed distinct gene expression profiles between polarized macrophage states, demonstrating the functional diversity that can be achieved by macrophages in response to specific challenges. By contrast, DNA methylation changes in polarized macrophages displayed more similarity when compared to the distinct transcriptomic profiles. This finding suggests the existence of a core set of genes that undergo epigenetic modifications, regardless of the initial environmental cue. Future experiments should measure DNA methylation changes in this newly identified core set of genes across diverse stimuli, to determine if these genes represent key regulators of macrophage plasticity and memory formation. Furthermore, a deeper investigation into the methylation status of this

core set of genes, and how it precisely alters their expression, is required to fully understand the functional significance of these observations.

Our multi-omics analysis indicated distinct and common regulatory patterns in PI and AI phenotypes. While some processes exhibited coordinated changes at both the transcriptome and epigenome levels, others displayed regulation solely at the epigenetic level. This observation prompts further investigation into the mechanisms driving these epigenetic modifications and the potential absence of a corresponding transcriptomic priming event. Our analysis showed that processes requiring rapid responses, such as motility and adhesion, exhibited regulation primarily at the transcriptomic level. Whereas functions including protein production, metabolism, and immune regulation, displayed co-regulation at both transcriptomic and epigenetic levels. The epigenetic regulation of these processes may well play a pivotal role in imprinting a long-term memory for these macrophage functions, and the corresponding metabolic shifts observed at the transcriptomic level suggests the possible establishment of trained immunity. Overall, this study revealed the dynamics of transcriptomics and DNA methylation changes during macrophage polarization. The newly identified core set of epigenetically modified genes presents an opportunity for novel therapeutic targets and can inform future toxicogenomic safety assessments designed to predict long-term health consequences of environmental exposures. To further elucidate the underlying mechanisms of macrophage polarization, future multi-omics studies that incorporate not only transcriptomics and epigenomics, but also metabolomics analysis, along with secondary exposures, are merited.

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CRediT authorship contribution statement

Giorgia Migliaccio: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Jack Morikka:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Giusy del Giudice:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Maaret Vaani:** Writing – review & editing, Investigation. **Lena Möbus:** Writing – review & editing, Supervision. **Angela Serra:** Writing – review & editing, Visualization, Supervision. **Antonio Federico:** Writing – review & editing, Supervision, Investigation. **Dario Greco:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors utilized Google Gemini to improve the grammar and clarity of the text. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

RNA-seq and methylation data have been submitted to NCBI Gene Expression Omnibus (GEO) database under the series accession number GSE273627.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.csbj.2024.08.018](https://doi.org/10.1016/j.csbj.2024.08.018).

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