

DNA demethylation regulates gene expression in IgE-activated mouse mast cells

To the Editor

Mast cells (MCs) have a major detrimental impact on various allergic conditions. MCs express the high affinity receptor for IgE (FcεRI) and are sensitized by the binding of antigen-specific IgE to such receptors. Upon encounter of specific antigen, FcεRI cross-linking occurs, which leads to degranulation and to activated transcription of genes coding for pro-inflammatory cytokines and chemokines.^{1,2}

Epigenetics refers to the effects on gene transcription that are unrelated to changes in DNA sequence. Major epigenetic mechanisms include DNA methylation, histone modification, and effects of noncoding RNAs.^{3–6} Generally, DNA methylation of gene promoters leads to gene repression whereas DNA demethylation typically leads to activated gene transcription. Considering the major effects of IgE receptor cross-linking on gene transcription in MCs, it appears plausible that epigenetic mechanisms may have an impact on such processes, but this has received relatively little attention.^{7,8} To provide additional insight into this issue, we here investigated whether IgE-mediated MC activation affects global DNA methylation in MCs and whether modulation of DNA methylation/demethylation affects gene transcription.

We used fully mature mouse peritoneal cell-derived MCs with a phenotype similar to that of human mast cells⁹ (Figure S1A). MCs were sensitized with DNP-specific IgE, followed by IgE receptor cross-linking by the exposure to DNP-conjugated human serum albumin. This led to substantial MC degranulation (Figure 1A and Figure S1). To assess the effect of IgE receptor cross-linking on global DNA methylation, we used an ELISA based on a monoclonal antibody to 5-methylcytosine. As seen in Figure 1B, IgE receptor cross-linking caused a substantial drop in global DNA methylation, which was most prominent 2 hours after IgE receptor cross-linking. Next, we assessed the effects of DNA methyltransferase (5-azacitidine; inhibitor of DNMT enzymes) and demethylase (Octyl-α-hydroxyglutarate [2-HG]; inhibitor of Ten-eleven translocation [TET] enzymes) inhibitors on gene expression in MCs. 2-HG was nontoxic for MCs at concentrations up to 150 μmol/L, and 5-azacitidine was nontoxic up to 10 μmol/L (Figure 1C–D). Next, we tested the effects of 5-azacitidine and 2-HG (at nontoxic concentrations) on the expression of *Il6*, *Nr4a3*, *Il3*, and *Tnf*. IgE receptor cross-linking induced an upregulated expression of all these genes. As shown in Figure 1E, the expression of *Il6* was profoundly attenuated by the DNA demethylase inhibitor

and, conversely, inhibition of DNA methyltransferase activity potentiated the *Il6* expression. Similarly, the expression of *Il3* was blunted by DNA demethylase inhibition and potentiated by inhibition of DNA methyltransferase activity (Figure 1F). Neither *Tnf* nor *Nr4a3* expression was significantly repressed by DNA demethylase inhibition (Figure 1G–H). The expression of classical MC markers (*Mcpt4*, *Mcpt6*) was not affected by interference with DNA demethylase/methyltransferase activity (Figure S2).

To obtain deeper insight into how DNA demethylase inhibition affects gene transcription pathways in MCs, we performed an unbiased transcriptomic analysis, using the AmpliSeq platform. As shown in Figure S3, IgE-mediated MC activation caused upregulated expression of numerous genes. These included several pro-inflammatory genes, transcription factors, growth factors, and receptors. After interference with DNA demethylation, the expression of a substantial number of IgE-induced genes was blunted (altogether 126 genes; genes with ≥ fourfold repression are highlighted in Figure 2). These included *Mrgprx1*, *S1pr2* (coding for sphingosine-1-phosphate receptor 2), *Ccl3*, *Myc*, and *Il6*. DNA demethylase inhibition also caused an increased expression of several genes in the IgE-activated MCs, many of which having anti-inflammatory properties (eg, *Gdf9*, *Hspa1a* (HSP72), *Wnt4*, *Egr1*, *Tnfrsf2*, and *Shb*). DNA demethylase inhibition also affected the expression of numerous genes at baseline conditions. Among the genes that were induced by DNA demethylase inhibition under baseline conditions, many were also highly induced under conditions of IgE-mediated MC activation. These included *Hspa1a*, *Hspa1b*, *Mrgprb8*, *Cnnm4*, *Mospd4*, *Egr1*, *Klf4*, and *Elil2* (Figure S4). In addition, several genes were downregulated by DNA demethylase inhibition under baseline conditions. Of these, several were also highly downregulated (≥fourfold) by DNA demethylase inhibition in IgE-activated MCs (eg, *S1pr2*, *Lhpl2*, and *Nef2*).

To confirm that findings from the transcriptome analysis were replicated with an independent method, we used qPCR analysis where we focused on selected genes: *Mrgprx1*, *Myc*, and *Ccl3*. We also assessed the expression of *Mrgprx2*, a close relative to *Mrgprx1*. As seen in Figure S5, the qPCR analysis confirmed a profound upregulation of *Mrgprx1* in response to IgE-mediated MC activation and also confirmed that DNA demethylase inhibition suppressed the stimulatory effect of IgE receptor cross-linking on *Mrgprx1* expression. A small stimulatory effect of IgE receptor cross-linking on *Mrgprx2* expression

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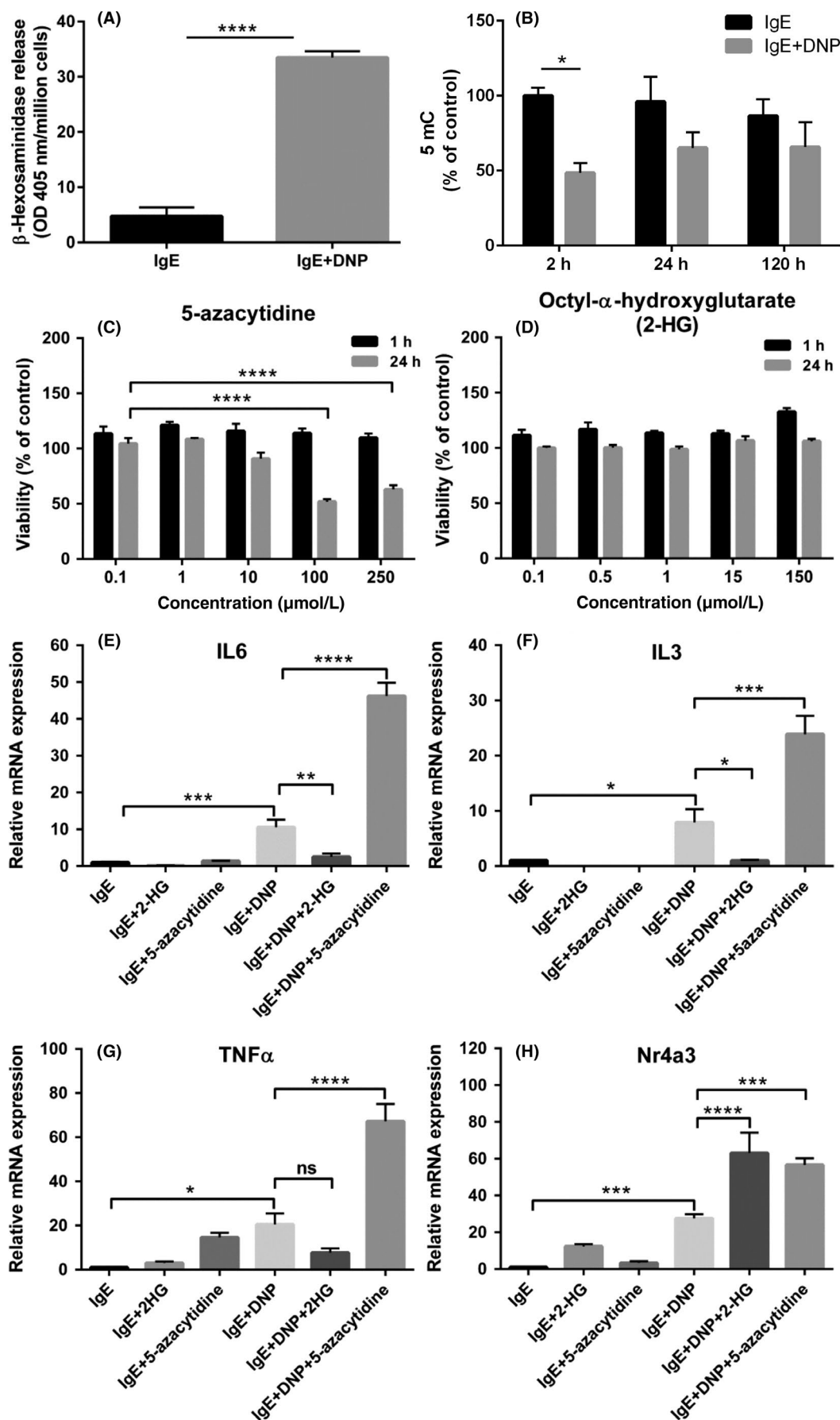


FIGURE 1 IgE receptor cross-linking causes MC degranulation, decreased global DNA methylation, and effects on gene expression in MCs. Effects on gene expression are modulated by inhibition of DNA demethylase and DNA methyltransferase activity. A, Release of β -hexosaminidase activity from MCs (1.5×10^6 cells) activated by IgE receptor cross-linking. Results are given as mean values $\pm$ SEM ($n = 6$); *** $P \leq .001$. B, MCs (2×10^6 cells) were activated by IgE receptor cross-linking. At the time points indicated, cells were recovered followed by purification of DNA and analysis of DNA methylation levels by ELISA. Results are given as mean values $\pm$ SEM ($n = 6$); * $P \leq .05$. C, 5-azacitidine (DNMT inhibitor) and (D) 2-HG (TET inhibitor) at the indicated concentrations were assessed for effects on cell viability in MCs. E-H, Effect of 5-azacitidine and 2-HG on gene expression in IgE-activated MCs. MCs (1.5×10^6 cells) were activated by IgE receptor cross-linking (IgE + DNP-HSA). As controls, MCs were sensitized with anti-DNP IgE but not subjected to IgE receptor cross-linking (IgE). Inhibitors were added 1h before initiating IgE receptor cross-linking. Two hours after IgE receptor cross-linking, cells were recovered, followed by RNA isolation and qPCR analysis for expression of *Il6* (E), *Il3* (F), *Tnf* (G) and *Nr4a3* (H). Results are given as mean values $\pm$ SEM ($n = 3$) and are relative to the expression of the housekeeping gene (Hprt). * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$ **** $P \leq .0001$

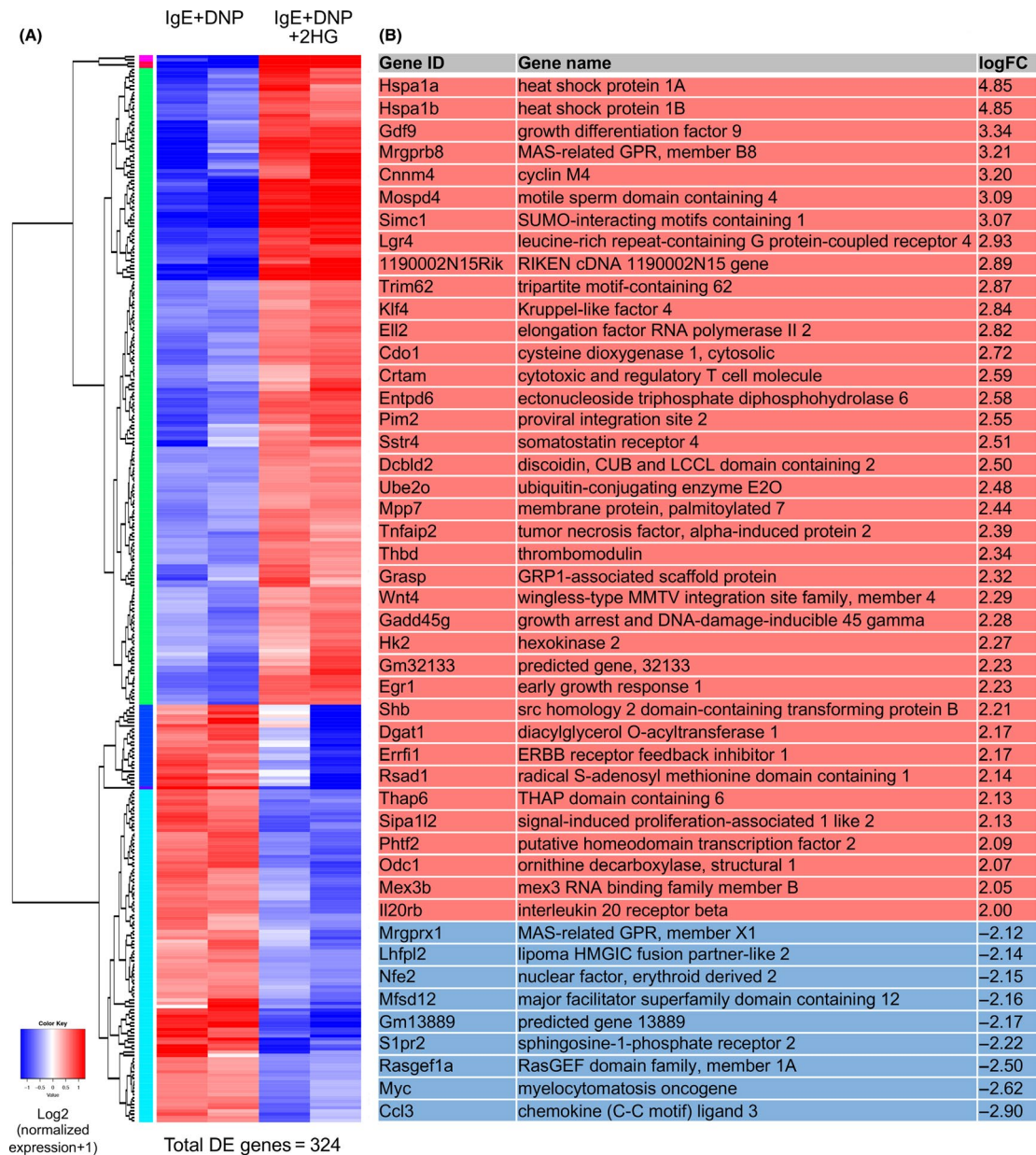


FIGURE 2 Effect of DNA demethylase blockade on gene expression in IgE-activated primary MCs. MCs (1.5×10^6 cells) were activated for 2 h by IgE receptor cross-linking $\pm$ DNA demethylase inhibitor (2-HG), followed by RNA isolation and transcriptome analysis. Differential gene expression analyses were performed using edgeR. A, Log2-normalized expression of genes with significant differential expression between activated MCs (IgE + DNP) and activated MCs $\pm$ DNA demethylase inhibitor (IgE + DNP+2-HG). The side color shows genes clustered by hclust. Among 324 differentially expressed genes, 198 genes were upregulated by 2-HG whereas 126 genes were downregulated. B, List of top genes differentially expressed by |log FC| ≥ 2 (red: upregulated, blue: downregulated)

was also seen, and this effect was blocked by DNA demethylase inhibition. The qPCR analysis also confirmed the induction of *Ccl3* and *Myc* by IgE receptor cross-linking, as well as the inhibitory effects of DNA demethylase blockade on the induction of these genes (Figure S5).

Altogether, our findings reveal that gene transcription events occurring downstream of IgE receptor cross-linking in MCs can be regulated by epigenetic mechanisms. Notably, interference with DNA demethylation blunted only a fraction of those genes that were induced by IgE receptor cross-linking. When examining the nature of the genes that were downregulated by DNA demethylase blockade, we found that many of these have pro-inflammatory properties (eg, *Myc*, *S1pr*, *Il6*, and *Il3*). DNA demethylase inhibition also caused upregulated expression of numerous genes in the IgE-activated MCs. Intriguingly, many of these latter genes can have anti-inflammatory functions, for example, *Hspa1a* (HSP72), *Wnt4*, *Egr1*, *Shb*, *Crtam*, and *Tnfrsf2*. Hence, our findings suggest that interference with DNA demethylation in IgE-activated mouse MCs may dampen selected pro-inflammatory pathways and, at the same time, potentiate various anti-inflammatory events. We may therefore propose that interference with epigenetic mechanisms can represent a novel strategy for intervening with pathologies mediated by IgE-activated MCs. However, future studies will be needed to extrapolate the findings to the effects on primary human mast cells. Moreover, such strategies should be adopted with caution, given the possibilities of detrimental side effects associated with global inhibition of DNA demethylation/methylation.

KEYWORDS

DNA demethylase, DNA methylation, epigenetics, mast cells, transcriptome

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CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Supplementary Materials and Methods

Reagents

Octyl- α -hydroxyglutarate (2-HG) was from Cayman chemical (Ann Arbor, Michigan), 5-azacytidine was from Sigma (Saint Louis, MO). The chromogenic substrate S-2288 was from Chromogenix (Milano, Italy). RNase A was Sigma (Saint Louis, MO).

Cells

Peritoneal cell-derived MCs (PCMCs) were developed from C57BL/6 mice as described previously [1, 2]. Cultures of 4-8 weeks were used for all experiments. The animal experiments were approved by the local ethical committee (Uppsala Animal Ethics Committee; Dnr 5.8.18-05357/2018). Cells were stained with toluidine blue as described [3].

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) was performed as described previously [3], using the primers specified in Table 1. Gene expression levels were presented relative to the house-keeping gene (*Hprt* and/or *Gapdh*) and normalized to IgE-sensitized PCMCs.

Cytotoxicity test

PCMCs (at 10^6 cells/ml) were incubated with serial dilutions of inhibitors (ranging from 0.1 to 250 μ M for 1h or for 24h. Cell viability was assessed using the Cell Titer-Blue® cell viability assay (Promega-Invitrogen, Carlsbad, CA).

Isolation of DNA

QiAmp DNA Mini Kit (Qiagen, Hilden, Germany) was used to isolate DNA from PCMCs. To obtain RNA-free genomic DNA we added 0.4 mg of RNase A/sample to the isolation procedure. DNA purity and concentrations were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and the ND-1000 V3.7.0 program.

DNA methylation assay

DNA methylation measurements were performed using the MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, Farmingdale, NY) using 100 ng of genomic DNA/sample. Absorbance was determined with a microplate reader: Tecan Infinite 200 (Tecan Austria, Grödig, Austria) and the Magellan V. 6.6 software.

IgE receptor crosslinking and tryptase activity measurements

IgE receptor crosslinking and measurement of tryptase activity was performed as described previously [3, 4]. Briefly, cells were sensitized with 0.1 mg/mL IgE anti-DNP (Sigma) overnight. After IgE incubation, the cells were washed with PBS three times and resuspended in fresh BMMC medium and stimulated with DNP-Human Serum Albumin (HAS; Sigma) at 0.5 mg/mL. Cells were recovered by centrifugation (400 x g; 7 min) at 2h, 24h and 120h after IgE receptor crosslinking, for further analysis. For the 24h and 120h time points, cells were washed 2h after initiating IgE receptor crosslinking, followed by further incubation in fresh medium.

AmpliSeq transcriptome analysis

Total RNA was isolated using the Direct-zol RNA MiniPrep kit (The Epigenetics Company, Irvine, CA). cDNA libraries were created and amplified using the Ion AmpliSeq™ Transcriptome Mouse Gene Expression Kit (Life Technologies, Carlsbad, CA) following the protocol provided by the manufacturer. Sequencing was performed on an Ion S5™ XL Sequencer (Thermo Fisher Scientific, Waltham, MA). Generated data had read lengths with average of 98-110 bp and high mapping (95% of aligned bases). Normalized expression values generated by Torrent Suite™ Software (version 5.10.1) were used for downstream differential expression analyses using the edgeR package [5] implemented in R (version 3.5.0) (<https://www.R-project.org/>). Genes that, at least in three samples, had counts per million (CPM) > 1 were analyzed. By this selection, only genes with sufficient expression values were analyzed in order to enable downstream analyses. Pairwise comparisons were performed between IgE (sensitized; non-activated) vs. IgE+DNP (activated), IgE+DNP (activated) vs. IgE+DNP+2-HG (activated + DNA demethylase inhibitor), and IgE (sensitized; non-activated) vs. IgE+2-HG (sensitized; non-activated + DNA demethylase inhibitor). A false discovery rate (FDR) threshold ($p \leq 0.05$) and log fold-change ($\log_{2}FC \geq 1$) were used to prioritize genes of interest. For clustering, dendrogram and heatmap generation, heatmap.2 from the R package gplots3.0.1.1 (<https://cran.r-project.org/web/packages/gplots/index.html>) were used. Gene ontology (GO) and pathway enrichment analyses were conducted using DAVID (Database for Annotation Visualization and Integrated Discovery) [6] and the obtained results were visualized by R (version 3.5.0) (<https://www.R-project.org/>).

Statistical analyses

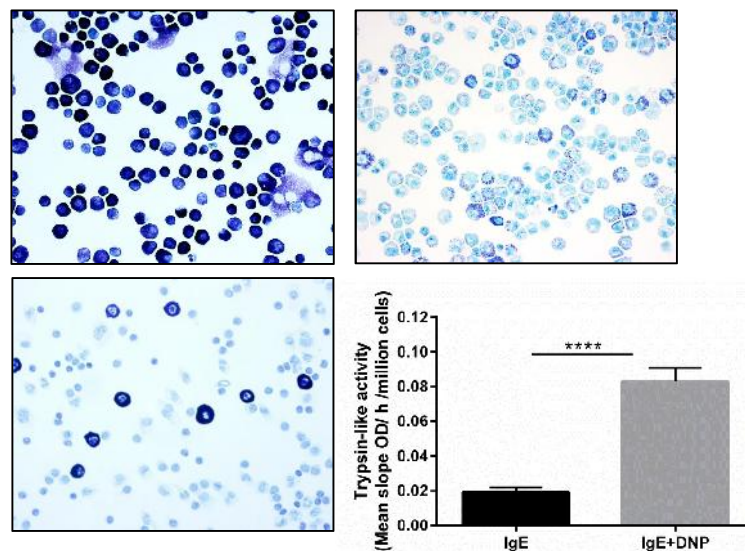
The shown data represent mean values $\pm$ standard error of the mean (SEM). Statistical analyses were performed with the GraphPad Prism 7.0 software, and either unpaired t-test (β -hexosaminidase release, trypsin-like activity, DNA methylation assay), 2-way ANOVA with multiple comparison (cytotoxicity of 5-azacytidine) or one-way ANOVA with multiple comparison (qPCR). Differences were considered significant if the P-values were 0.05 or less.

Table 1. Primers used for qPCR

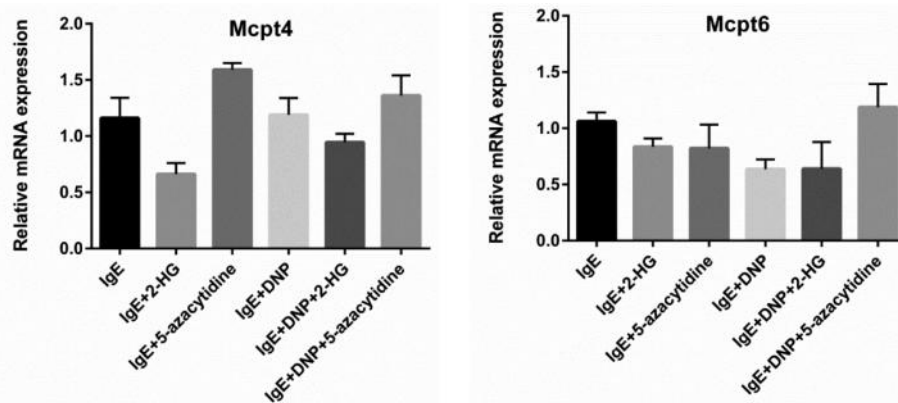
Target Gene	Forward Sequence (5'→3')	Reverse Sequence (5'→3')
<i>Mcpt4</i>	GCA GTC TTC ACC CGA ATC TC	CAG GAT GGA CAC ATG CTT TG
<i>Mcpt6</i>	AGA ACC AGG GCT GTG CTG TCT	AGA GGG AGC CAC ACA ATG CAA
<i>Il6</i>	AGA CAA AGC CAG AGT CCT TCA GAG A	TAG CCA CTC CTT CTG TGA CTC CAG C
<i>Il3</i>	ACC CAC CGT TTA ACC AGA ACG	TGG ACA GGT TTA CTC TCC GAA
<i>Tnf</i>	CCA CAT CTC CCA GAA AA	AGG GTC TGG GCC ATA GAA CT
<i>Nr4a3</i>	TCA CCA TCA CCA TCA TCA CCA	AAG GCG GAG ACT GCT TGA AGT
<i>Hprt</i>	GAT TAG CGA TGA TGA ACC AGG TTA	GAC ATC TCG AGC AAG TCT TTC AGT C
<i>Mrgprx1</i>	TCT CAT CCC ACG ACA CAG AAT	AGC CAG AGT ACA ATG GTG TTT C
<i>Mrgprx2</i>	CAG TGG AAG GGA TCT AAG AGT GG	GGT ACA AGA CAC GAC TTC ACA A
<i>Ccl3</i>	TTC TCT GTA CCA TGA CAC TCT GC	CGT GGA ATC TTC CGG CTG TAG
<i>Myc</i>	ATG CCC CTC AAC GTG AAC TTC	GTC GCA GAT GAA ATA GGG CTG
<i>Gapdh</i>	CTC CCA CTC TTC CAC CTT CG	CCA CCA CCC TGT TGC TGT AG

References

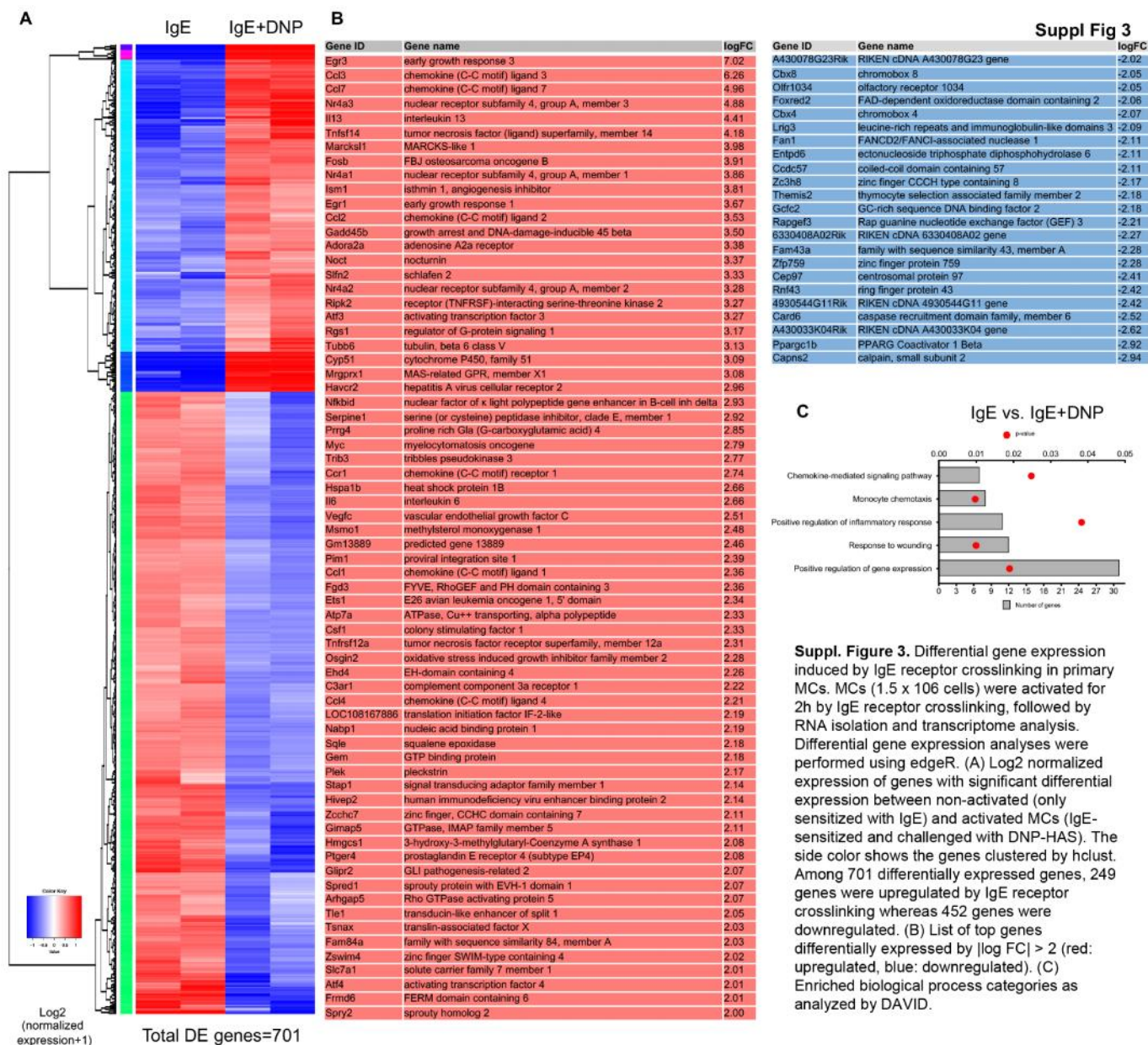
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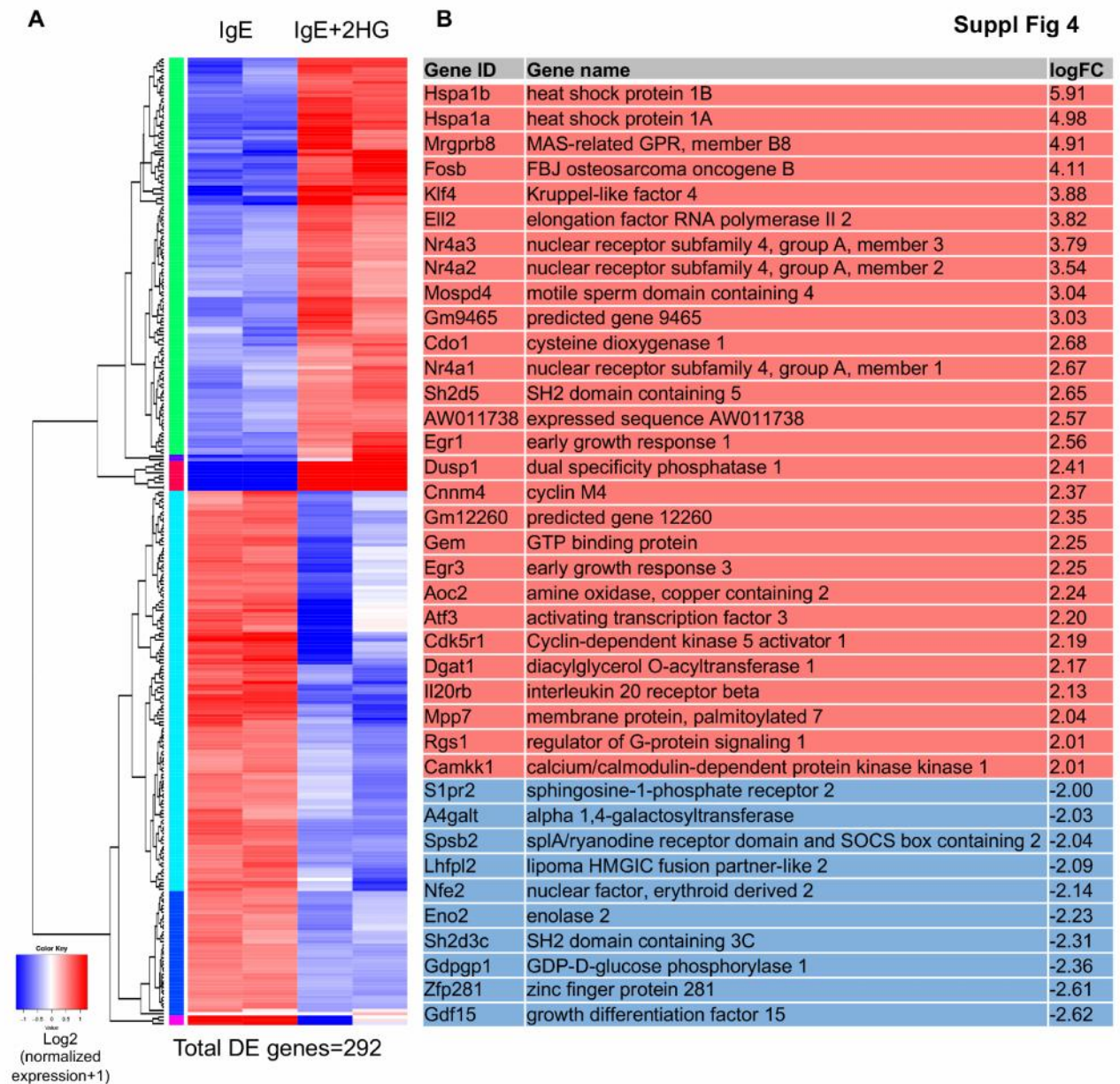


Suppl. Fig. 1. Morphology and IgE-mediated activation of MCs. Left panels: Toluidine blue staining of cytopsin slides prepared from MCs (upper panel), freshly isolated mouse peritoneal cell suspensions (middle; MCs are indicated by arrows; remaining cells represent macrophages and lymphocytes) and bone marrow-derived MCs (lower panel). Note the mature phenotype of MCs in comparison with bone marrow-derived MCs, and note that the morphology of the MCs is indistinguishable from the freshly isolated mouse peritoneal MCs. Right panel: Release of trypsin (trypsin-like activity; lower panel) from MCs (1.5×10^6 cells) activated by IgE receptor crosslinking. Results are given as mean values $\pm$ SEM ($n = 6$); *** $p \leq 0.001$.

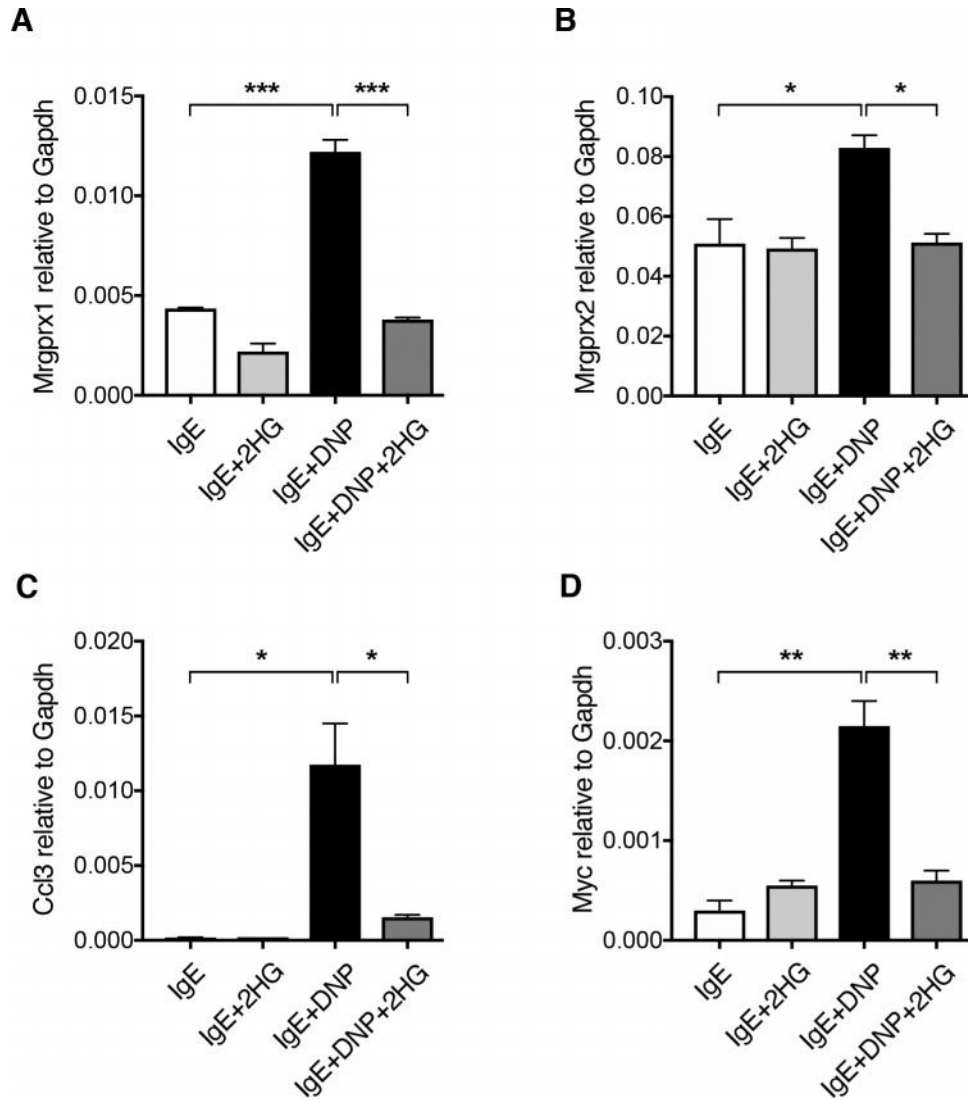


Suppl. Fig. 2. Effect of 5-azacytidine and 2-HG on expression of MC markers in IgE-activated MCs. MCs (1.5×10^6 cells) were activated by IgE receptor crosslinking (IgE + DNP-HSA). As controls, MCs were sensitized with anti-DNP IgE but not subjected to IgE receptor crosslinking (IgE). Inhibitors were added 1h before initiating IgE receptor crosslinking. Two hours after IgE receptor crosslinking, cells were recovered, followed by RNA isolation and qPCR analysis for expression of *Mcpt6* and *Mcpt4*. Results are given as mean values $\pm$ SEM ($n = 3$), and are relative to expression of the house keeping gene (Hprt).





Suppl. Figure 4. Effect of DNA demethylase blockade on gene expression in primary MCs at baseline conditions. MCs (1.5 x 10⁶ cells) were cultured $\pm$ DNA demethylase inhibitor (2-HG), followed by RNA isolation and transcriptome analysis. Differential gene expression analyses were performed using edgeR. (A) Log2 normalized expression of genes with significant differential expression $\pm$ 2-HG. Among 292 differentially expressed genes, 131 genes were upregulated by 2-HG whereas 161 genes were downregulated. (B) List of top genes differentially expressed by $|\log FC| > 2$ (red: upregulated, blue: downregulated).



Suppl. Figure 5. DNA demethylase blockade inhibits the IgE-induced expression of Mrgprx1, Mrgprx2, Ccl3 and Myc in primary MCs. MCs (1.5×10^6 cells) were cultured $\pm$ DNA demethylase inhibitor (2-HG), followed by RNA isolation and qPCR analysis for the expression of the indicated genes. Results are given as mean values $\pm$ SEM ($n = 2$), and are relative to expression of the house keeping gene (Gapdh). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ **** $p \leq 0.0001$.