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SUPPORTING INFORMATION

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

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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 cells[9] (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).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 ± 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 ± 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 ± SEM (n = 3) and are relative to the expression of the housekeeping gene (Hprt). *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$ ****$P \leq .0001$

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*, *Tnfaip2*, 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 *Ell2* (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*, *Lhfpl2*, and *Nef2*).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 ± 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 ± 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)

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 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 *Tnfaip2*. 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

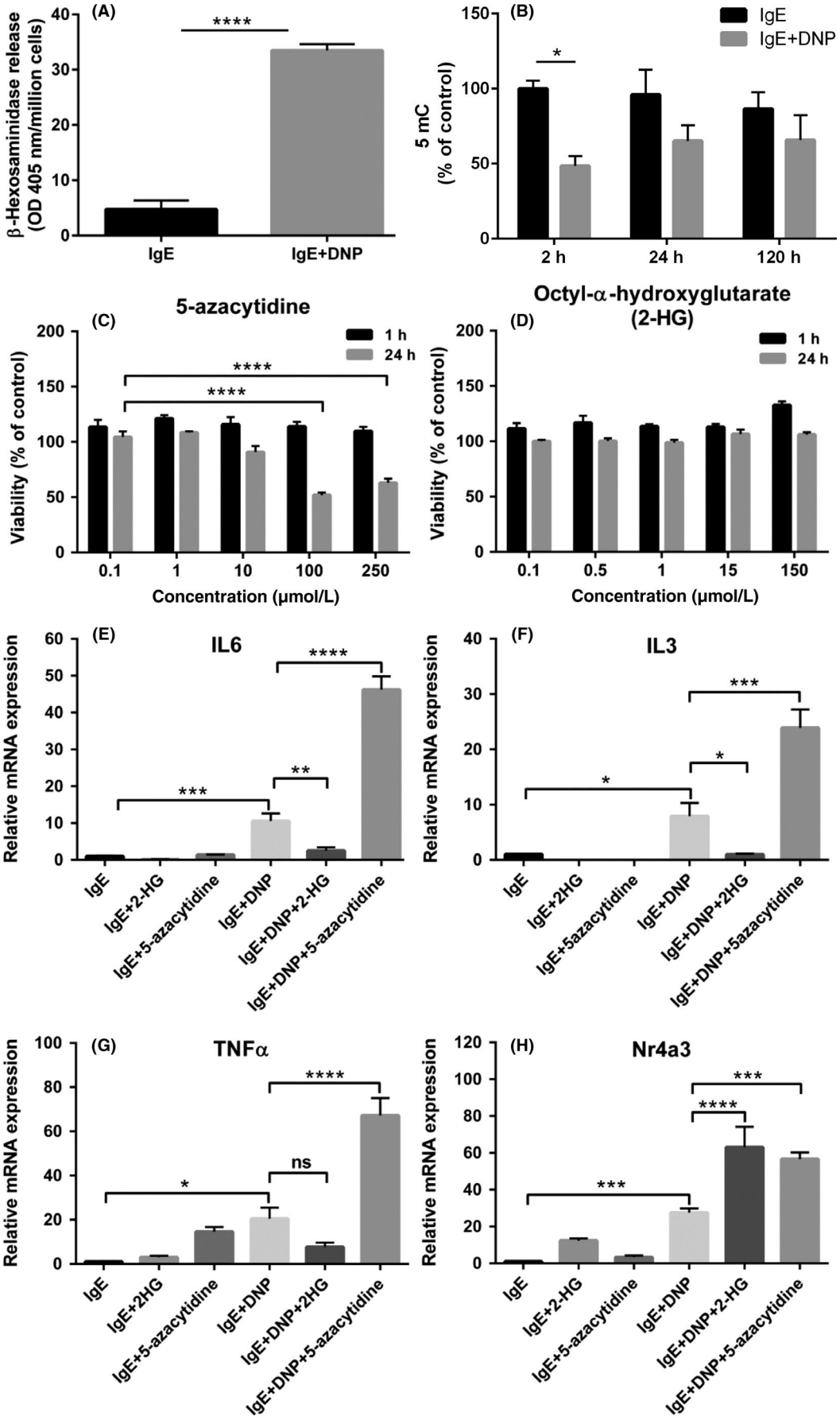
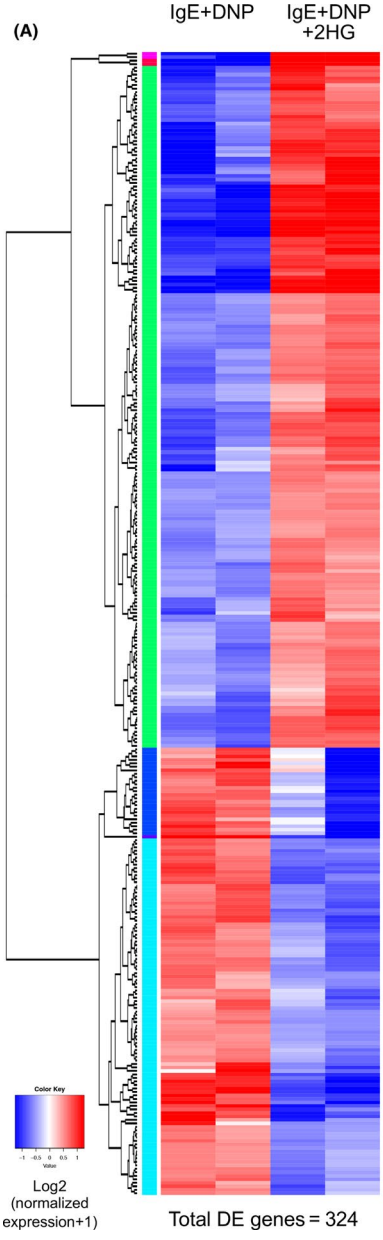

(A)
β-Hexosaminidase release (OD 405 nm/million cells)
IgE
IgE+DNP
****
(B)
5 mC (% of control)
IgE
IgE+DNP
*
2 h
24 h
120 h
(C)
5-azacytidine
Viability (% of control)
1 h
24 h
****
****
Concentration (μmol/L)
(D)
Octyl-α-hydroxyglutarate (2-HG)
Viability (% of control)
1 h
24 h
Concentration (μmol/L)
(E)
IL6
Relative mRNA expression
IgE
IgE+2-HG
IgE+5-azacytidine
IgE+DNP
IgE+DNP+2-HG
IgE+DNP+5-azacytidine
(F)
IL3
Relative mRNA expression
IgE
IgE+2HG
IgE+5azacytidine
IgE+DNP
IgE+DNP+2HG
IgE+DNP+5azacytidine
(G)
TNFα
Relative mRNA expression
ns
IgE
IgE+2HG
IgE+5-azacytidine
IgE+DNP
IgE+DNP+2HG
IgE+DNP+5-azacytidine
(H)
Nr4a3
Relative mRNA expression
IgE
IgE+2-HG
IgE+5-azacytidine
IgE+DNP
IgE+DNP+2-HG
IgE+DNP+5-azacytidine

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 ± 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 ± 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 ± SEM (n = 3) and are relative to the expression of the housekeeping gene (Hprt). *$P \leq .05$; **$P \leq .01$; ***$P \leq .001$ ****$P \leq .0001$



(B)

Gene ID	Gene name	logFC
Hspa1a	heat shock protein 1A	4.85
Hspa1b	heat shock protein 1B	4.85
Gdf9	growth differentiation factor 9	3.34
Mrgprb8	MAS-related GPR, member B8	3.21
Cnnm4	cyclin M4	3.20
Mospd4	motile sperm domain containing 4	3.09
Simc1	SUMO-interacting motifs containing 1	3.07
Lgr4	leucine-rich repeat-containing G protein-coupled receptor 4	2.93
1190002N15Rik	RIKEN cDNA 1190002N15 gene	2.89
Trim62	tripartite motif-containing 62	2.87
Klf4	Kruppel-like factor 4	2.84
Ell2	elongation factor RNA polymerase II 2	2.82
Cdo1	cysteine dioxygenase 1, cytosolic	2.72
Crtam	cytotoxic and regulatory T cell molecule	2.59
Entpd6	ectonucleoside triphosphate diphosphohydrolase 6	2.58
Pim2	proviral integration site 2	2.55
Sstr4	somatostatin receptor 4	2.51
Dcbld2	discoidin, CUB and LCCL domain containing 2	2.50
Ube2o	ubiquitin-conjugating enzyme E2O	2.48
Mpp7	membrane protein, palmitoylated 7	2.44
Tnfaip2	tumor necrosis factor, alpha-induced protein 2	2.39
Thbd	thrombomodulin	2.34
Grasp	GRP1-associated scaffold protein	2.32
Wnt4	wingless-type MMTV integration site family, member 4	2.29
Gadd45g	growth arrest and DNA-damage-inducible 45 gamma	2.28
Hk2	hexokinase 2	2.27
Gm32133	predicted gene, 32133	2.23
Egr1	early growth response 1	2.23
Shb	src homology 2 domain-containing transforming protein B	2.21
Dgat1	diacylglycerol O-acyltransferase 1	2.17
Errfi1	ERBB receptor feedback inhibitor 1	2.17
Rsad1	radical S-adenosyl methionine domain containing 1	2.14
Thap6	THAP domain containing 6	2.13
Sipa1l2	signal-induced proliferation-associated 1 like 2	2.13
Phtf2	putative homeodomain transcription factor 2	2.09
Odc1	ornithine decarboxylase, structural 1	2.07
Mex3b	mex3 RNA binding family member B	2.05
Il20rb	interleukin 20 receptor beta	2.00
Mrgprx1	MAS-related GPR, member X1	−2.12
Lhfpl2	lipoma HMGIC fusion partner-like 2	−2.14
Nfe2	nuclear factor, erythroid derived 2	−2.15
Mfsd12	major facilitator superfamily domain containing 12	−2.16
Gm13889	predicted gene 13889	−2.17
S1pr2	sphingosine-1-phosphate receptor 2	−2.22
Rasgef1a	RasGEF domain family, member 1A	−2.50
Myc	myelocytomatosis oncogene	−2.62
Ccl3	chemokine (C-C motif) ligand 3	−2.90

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 ± 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 ± 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)

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.

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Is pollen-food syndrome a frequent comorbidity in adults with irritable bowel syndrome?

To the Editor,

Irritable bowel syndrome (IBS) affects up to 10% of UK adults, 50% of whom may also have seasonal allergic rhinitis (SAR) and thus an increased risk of developing pollen-food syndrome (PFS) if sensitized to birch tree pollen.[1-3] In an exploratory prospective controlled cohort study, we compared the prevalence of PFS in IBS subjects from a secondary care clinic diagnosed using the Rome IV criteria,[4] with that of an age and gender-matched control group with