



Evaluation of 5-methylcytosine and 5-hydroxymethylcytosine as potential biomarkers for characterisation of chemical allergens



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ABSTRACT

Epigenetic regulation of gene expression plays a pivotal role in the orchestration of immune responses. Chemical allergens form two categories: skin sensitizing chemicals associated with allergic contact dermatitis, and chemicals that cause sensitization of the respiratory tract and occupational asthma. In mice these are characterized by different T helper (Th) cell responses. Changes in DNA methylation in particular have been implicated in the *in vivo* responses to chemical allergy. As such it was hypothesised that differentially methylated regions (DMR) may provide candidates biomarkers of chemical allergy. To examine this, mice were exposed to 2,4-dinitrochlorobenzene (DNCB; a contact allergen) or trimellitic anhydride (TMA; a respiratory allergen). DNA from draining lymph nodes was processed for methylated (5mC) and hydroxymethylated (5hmC) DNA immunoprecipitation (MeDIP/hMeDIP) then selected DMR analysed by qPCR. We describe a number of DMRs which, by combined analysis of 5mC and 5hmC, differentiate between responses induced by DNCB and those by TMA. Furthermore, these changes in methylation are specific to the draining lymph node. The *Gmpr* DMR is suggested as a possible biomarker for contact allergen-induced immune responses; it is characterised by divergent levels of 5mC and 5hmC in DNCB-treated mice only. In contrast, the *Nwc* DMR was characterised by divergent 5mC and 5hmC specifically in response to TMA, highlighting its possible utility as a biomarker for responses induced by chemical respiratory allergens. These data not only represent novel analysis of 5hmC in response to chemical allergy *in vivo*, but with further investigation, may also provide a possible basis for differentiation between classes of chemical allergens.

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1. Introduction

A wide variety of chemicals is able to induce allergic sensitisation resulting in allergic disease. Chemical allergy can take two main forms; skin sensitisation resulting in allergic contact dermatitis, and sensitisation of the respiratory tract associated with rhinitis and asthma. Both forms of allergy represent

important occupational and environmental health issues (Kimber et al., 2011). Many hundreds of chemicals are known to cause skin sensitisation, whereas chemicals that have been implicated as inducing sensitisation of the respiratory tract and occupational asthma are fewer in number. Contact allergens can be identified *in vivo* using the local lymph node assay (LLNA) where activity is measured as a function of induced proliferative responses in regional lymph nodes (Basketter et al., 2002; Kimber et al., 2002), and more recently using validated *in vitro* approaches (Natsch et al., 2013). In contrast, there are presently no validated methods for the identification and characterisation of chemical respiratory allergens (Kimber et al., 2014). There are differences in the nature of immune responses elicited by these two types of chemical allergen, both in experimental animals and in humans. Studies in mice have revealed that contact allergens are characterised by a selective type 1 T cell response, associated with interferon (IFN)- γ production by CD4⁺ Thelper (Th) 1 and CD8⁺ cytotoxic T cell (Tc)

Abbreviations: 5hmC, 5-Hydroxymethylcytosine; 5mC, 5-Methylcytosine; AOO, Acetone:olive oil; DMR, Differentially methylated region; DNCB, 2,4-Dinitrochlorobenzene; hMeDIP, Hydroxymethylated DNA immunoprecipitation; IFN, Interferon; IL, Interleukin; IP, Immunoprecipitated; LLNA, Local lymph node assay; MeDIP, Methylated DNA immunoprecipitation; Tc, Cytotoxic T cell; Tet, Ten eleven translocation; Th, T helper; TMA, Trimellitic anhydride.

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1 cells (Dearman et al., 2005, 1996), and interleukin (IL)-17 production contributed by CD4⁺ Th17 cells (Hayes et al., 2011; He et al., 2009). Conversely, chemical respiratory allergens induce in mice a preferential Th2-type immune response, characterised by IL-4, IL-5 and IL-13 production by CD4⁺ Th2 cells (Ban and Hettich, 2005; Betts et al., 2002; Dearman et al., 1996). The use of cytokine fingerprinting, wherein profiles of cytokines expressed by cultured draining lymph node cells from exposed mice, has facilitated the categorisation of chemicals allergens as favouring either skin sensitisation or sensitisation of the respiratory tract (Dearman et al., 2003).

Recently, some progress has been made in the characterisation of the role played by epigenetic modifications in the regulation of immune responses to chemical allergens in mice (Chapman et al., 2014). Changes in the epigenome, including DNA methylation and histone modification, reflect interplay between the environment and gene expression. DNA methylation, the addition of a methyl group to cytosine, is one of the most well studied epigenetic markers (Bird, 2002). In the context of immune function, DNA methylation is now known to play a key role in T cell development and lineage commitment (Kanno et al., 2012), and is now being explored as a marker of immunological disease. Recently, it has been shown that differences in the DNA methylome, but not genome-wide gene expression patterns, are able to identify those individuals with seasonal allergic rhinitis (Nestor et al., 2014). Additionally, diisocyanate-induced occupational asthma is associated with a higher degree of IFN- γ (*IFNG*) promoter methylation compared with non-asthmatic counterparts (Ouyang et al., 2013). Many key DNA methylation-dependent changes in gene expression occur too rapidly for passive demethylation during DNA replication to be sufficient. This active removal of DNA methylation is facilitated by a family of enzymes; ten eleven translocation (Tet) enzymes (Pastor et al., 2013). In particular, Tet2 is required for *in vitro* Th1 and Th17 polarisation, but not Th2 polarisation (Ichiyama et al., 2015). The Tet enzymes progressively oxidise 5-methylcytosine (5mC) to a number of different products, including 5-hydroxymethylcytosine (5hmC). Mechanistically, 5hmC has been correlated with the release of 5mC silencing and depending on the location within the gene body, active gene transcription (Tsagaratou et al., 2014). The detection of 5hmC and its correlation with gene expression suggests that it may now be regarded as a stable epigenetic marker (Ichiyama et al., 2015; Tsagaratou et al., 2014). In T cell lineages derived *in vitro*, peaks of 5hmC at conserved non-coding sequences and promoters for *Ifng*, *Il4* and *Il17* correlated with the expression of these genes by Th1, Th2 and Th17 cells, respectively (Ichiyama et al., 2015).

The fact that DNA methylation plays an important role in the orchestration of immune responses prompted us to explore alterations in 5mC and 5hmC levels during the acquisition in mice of allergic sensitisation to chemicals (Moggs et al., 2012). We have shown previously that chronic exposure of mice to either the reference contact allergen 2,4-dinitrochlorobenzene (DNCB), or to the reference chemical respiratory allergen trimellitic anhydride (TMA), under conditions that induce polarised immune responses, is associated with alteration of the 5mC methylome in allergen-activated draining lymph node cells (Chapman et al., 2014). We have here investigated in greater detail a number of chemical allergen-induced differentially methylated regions (DMR) with respect to both 5mC and 5hmC levels. Collectively these data show that 5mC and 5hmC patterns induced in chemical allergen activated draining lymph nodes are associated with selective immune activation, and are absent from non-involved tissues, such as spleen. For some DMR changes in 5mC are also accompanied by changes in 5hmC, suggesting active regulation of DNA methylation in the draining lymph node.

2. Methods

2.1. Animals

Young adult (6–12 weeks old) female BALB/c strain mice, obtained from the specific pathogen free breeding unit (Harlan Olac, Bicester), were used for all experiments. Food (Beekay Rat and Mouse Diet No1 pellets; B and K Universal, Hull, UK) and water were available *ad libitum*. Ambient temperature was maintained at $21 \pm 2^\circ\text{C}$ and relative humidity was $55 \pm 10\%$ with a 12 h light/dark cycle. All procedures were approved by the UK Home Office and carried out in compliance with the Animals (Scientific Procedures) Act 1986 under a Home Office granted project licence.

2.2. Sensitisation to chemical allergen

The chemical allergens DNCB (97% pure) and TMA (99% pure) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Chemicals were dissolved in 4:1 (v/v) acetone:olive oil (AOO). All solutions were freshly prepared prior to use. Groups of mice ($n=5$ or 3) received 50 μl of 10% TMA or 1% DNCB in AOO on each shaved flank. Control animals were treated concurrently with vehicle (AOO) alone. Five days later, this treatment was repeated. After a further 5 days, 25 μl of chemical or vehicle alone was applied to the dorsum of both ears daily for three consecutive days. Thirteen days after the initiation of exposure, draining (auricular) lymph nodes were excised, pooled on an experimental animal basis and immediately transferred to DNA digestion buffer as described below. Spleens were also taken from selected mice and treated in the same way.

2.3. DNA extraction and preparation

Genomic DNA from mouse lymph nodes and spleen were extracted by overnight proteinase K digestion (Sigma) in lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM ethylenediamine tetraacetic acid [EDTA] pH 8.0, 0.5% sodium dodecyl sulphate) prior to RNaseA treatment, phenol-chloroform extraction and ethanol precipitation. Genomic DNA was sonicated (S220 Focused-ultrasonicator, Covaris, USA) to produce DNA fragments ranging in size from 300 to 1000 bp, with a mean fragment size of 500 bp. Fragment size was confirmed by separation on a 1% agarose gel.

2.4. Methylated DNA immunoprecipitation (MeDIP) assay

For each sample, 6 μg of fragmented DNA (input) was denatured for 10 min at 95°C and immunoprecipitated for 3 h at 4°C with 15 μg monoclonal antibody against 5mC (Eurogentec, Seraing, Belgium) in a final volume of 500 μl immunoprecipitation buffer (10 mM sodium phosphate [pH 7.0], 140 mM NaCl, 0.05% Triton X-100). This mixture was incubated with 60 μl of magnetic Dynabeads M-280 Sheep Anti-Mouse IgG (Invitrogen, Paisley, UK) for 2 h prior to washing off all unbound fragments three times with 1 ml immunoprecipitation buffer. Washed beads were then resuspended in 250 μl of digest buffer (50 mM Tris [pH 8.0], 10 mM EDTA, 0.5% sodium dodecyl sulfate) and incubated with proteinase K for 3 h at 50°C . The immunoprecipitated methylated DNA was recovered by phenol–chloroform extraction and ethanol precipitation then resuspended in 20 μl Tris EDTA (TE).

2.5. Hydroxymethylated DNA immunoprecipitation (hMeDIP) assay

For each sample, 2 μg of fragmented DNA (input) was denatured for 10 min at 95°C and immunoprecipitated for 3 h at 4°C with 1 μg polyclonal antibody against 5hmC (Active Motif, La Hulpe, Belgium) in a final volume of 500 μl immunoprecipitation

buffer. This mixture was incubated with 20 µl of magnetic Dynabeads Protein G (Invitrogen) for 1 h at 4 °C prior to washing off all unbound fragments three times with 1 ml immunoprecipitation buffer. Washed beads were then resuspended in 250 µl of digest buffer and incubated with proteinase K for 3 h at 50 °C. The immunoprecipitated hydroxymethylated DNA was recovered by phenol-chloroform extraction and ethanol precipitation then resuspended in 20 µl TE.

2.6. qPCR of selected regions

qPCR was performed using Fast SYBR[®] Green Master Mix (Applied Biosystems; Grand Island, NY, USA) with 250 nM of forward and reverse primers, according to the manufacturer's instructions. Primers were designed using the region defined by the DMR probe coordinates (Table 1) to produce a product 50–200 bp in size. PCR products were quantified against a standard curve of genomic DNA. To calculate enrichment, the amount of PCR product recorded for input DNA was compared with that recorded for the immunoprecipitated (IP; enriched) DNA of the particular sample to give the %input/IP. The ratio of allergen vs. vehicle (AOO) was calculated using the %IP/input for each individual allergen treated sample compared with the average AOO %IP/input. When the ratio of allergen vs. vehicle >1 the DMR is designated as hypermethylated, when the ratio is allergen vs. vehicle <1 the DMR is designated as hypomethylated. The methylation status of the selected DMR examined by qPCR is listed in Table 1.

2.7. Quality control (QC) and amplification of MeDIP and hMeDIP products

For QC of immunoprecipitation, 10 µl aliquots of IP DNA were taken and diluted to 100 µl in TE. For MeDIP QC, highly methylated control regions (the *H19* imprinted control region [*H19* ICR] and a promoter associated with an intracisternal A-particle element [*IAP*]) and poorly methylated regions (an unmethylated control sequence [*CSa*] with no CpG and the glyceraldehyde 3-phosphate dehydrogenase promoter [*Gapdh*]) as reported in Lempiäinen et al., 2011 were used. For hMeDIP QC, the *IAP* and *Gapdh* promoters were used as a region with low hydroxymethylation and the testis expressed gene 19.1 promoter (*Tex19.1*) and *H19* promoter as regions rich in hydroxymethylation (Thomson et al., 2012). For further qPCR analysis, 50 ng of input DNA and 10 µl of IP DNA was

subjected to whole genome amplification using the WGA2: GenomePlex Complete Whole Genome Kit (Sigma) and tested for linear amplification by qPCR. Amplified DNA was then purified by passing through GenElute[™] PCR Clean-Up columns (Sigma), diluted to 5 ng/µl and analysed by qPCR as described previously. Each sample was considered to have passed QC if enrichment was >100% and depletion <100% for 3 or more of the regions tested. Differences between study groups were analysed by one-way ANOVA, followed by Tukey's multiple comparison test.

2.8. Statistical analysis

Data was found to be non-normally distributed using the Shapiro–Wilk test. Therefore non-parametric testing was used to determine if there were statistical differences between means. Kruskal–Wallis test was used to determine if there were differences in the means of the %IP/input of AOO, DNCB and TMA, followed with Dunn's multiple comparison tests. Comparisons of the ratio of AOO to DNCB vs. the ratio of AOO to TMA were performed using Mann–Whitney test. Differences between allergen treated lymph node and spleen 5mC or 5hmC were analysed by two-way ANOVA followed by Tukey's multiple comparisons test. The analysis of 5mC vs. 5hmC was performed on paired samples so the Wilcoxon matched pairs signed rank test was used. When described, the mean %IP of input or ratio of treated to control is followed by plus/minus the standard error of the mean. Significant differences were reported as follows: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

3. Results

As described previously (Chapman et al., 2014), mice were exposed topically to the reference contact allergen DNCB or the reference respiratory sensitiser TMA. This exposure regimen is known to induce immune responses characterised by selective Th1/Tc1 or Th2 polarisation, respectively (Dearman et al., 2005). Following allergen treatment, auricular lymph nodes (study 1, *n* = 5 and study 2, *n* = 3) and spleen tissue (study 3, *n* = 5) were removed. To assess changes in DNA methylation and hydroxymethylation, immunoprecipitated was used to enrich for 5mC or 5hmC respectively. Both the immunoprecipitated fractions and an aliquot of the input DNA fraction were amplified before the effectiveness of the enrichment was assessed by qPCR (Fig. 1).

Table 1
DMR identified in genome-wide array.

DMR ID	DMR location	DNCB vs. AOO		TMA vs. AOO		Refseq ID	Location	%GC	No. CpG	CpG freq.
		<i>p</i> Value	Δ Meth	<i>p</i> Value	Δ Meth					
<i>CD40lg</i>	chrX:54465947–54466425	0.0002	0.59 Hyper	ns		NM_011616	1st intron	40%	4	0.21
<i>Cxcr1</i>	chr18:74375668–74377099	0.0065	–0.46 Hypo	0.0009	–0.47 Hypo	NM_028868	Upstream to 1st intron	59%	98	0.85
<i>Dap12</i>	chr7:31199133–31199514	0.0081	–0.57 Hypo	ns		NM_011662	1st intron to 2nd intron	47%	5	0.28
<i>Fam208a</i>	chr14:28243377–28243798	0.0014	0.61 Hyper	ns		NM_001114879	1st intron	37%	6	0.4
<i>Gmpr</i>	chr13:45604789–45605346	0.0016	0.42 Hyper	ns		NM_025508	1st intron	49%	6	0.12
<i>Ifng</i>	chr10:117879416–117879784	0.0037	0.51 Hyper	0.0029	0.77 Hyper	NM_008337	1st intron to 3rd exon	38%	1	0.07
<i>Il12b</i>	chr11:44214537–44214869	0.0002	0.49 Hyper	0.0068	0.46 Hyper	NM_008352	1st intron	43%	3	0.19
<i>Il13</i>	chr11:53445522–53445858	ns		0.0088	–0.42 Hypo	NM_008355	4th exon/3rd intron	53%	7	0.3
<i>Il25</i>	chr14:55552457–55552798	0.0006	0.45 Hyper	ns		NM_080729	2nd intron	42%	3	0.2
<i>Nwc</i>	chr2:101460755–101461402	ns		0.0017	–0.64 Hypo	NM_026592	1st intron	45%	5	0.16
<i>Rnf125</i>	chr18:21103000–21103339	0.0039	–0.48 Hypo	ns		NM_026301	Upstream to 1st intron	52%	7	0.31

Abbreviations: DMR, differentially methylated region; Δ Meth, change in methylation and methylation status (hypo or hypermethylated with respect to AOO) is indicated by Hypo or Hyper, respectively; ns, not significant. CpG frequency was calculated by observed/expected where observed 'no. CpG × DMR length' and expected 'no. C × no. G'. Using the least stringent definition, >50% GC% and >0.6 frequency of CpG defined a CpG island (2009). *Cxcr1* was therefore defined as a CpG island. *Dap12* is also known as Tyrobp and *Fam208a* is also known as D14abb1e. The *Nwc* DMR covers a Rag2 DNase Hypersensitive 1 site; HS1. DMR selected for further study are highlighted in grey.

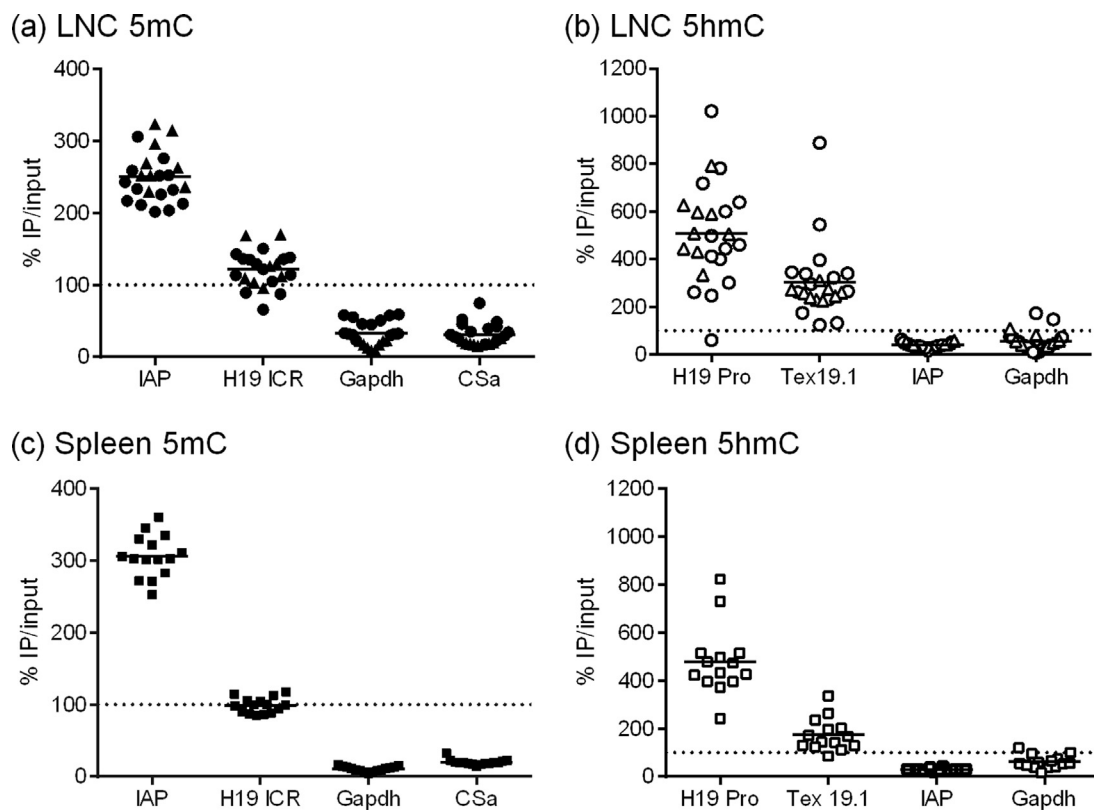


Fig. 1. Enrichment for methylated and hydroxymethylated DNA.

BALB/c strain mice were exposed topically to DNCB, TMA or to AOO. Thirteen days following initiation of treatment, draining (auricular) lymph nodes (a and b, $n = 5$, study 1 ●/○ or $n = 3$, study 2 ▲/△) were harvested and pooled on an individual animal basis. In a separate study (c and d, $n = 5$, study 3 ■/□), spleen tissue was harvested and processed on an individual animal basis. DNA was prepared, MeDIP (a and b; closed symbols) and hMeDIP (c and d; open symbols) were performed and both genomic input DNA and IP enriched DNA samples were amplified by whole genome amplification. Enrichment of methylated DNA (a, c) was confirmed by qPCR for highly methylated control regions (IAP promoter and H19 ICR) and unmethylated Gapdh promoter and CpG poor CSa region. Enrichment of hydroxymethylated DNA (b, d) was confirmed by qPCR for the hydroxymethylated regions H19 and *Tex19.1* promoters, the methylated IAP promoter and non-hydroxymethylated Gapdh promoter. The relative enrichment of each region in the IP sample was compared to the corresponding input fraction. Data are displayed as %IP/input for each individual animal. No change compared with input is illustrated by the dashed line at 100%.

3.1. Robustness of MeDIP and hMeDIP

It was important to determine the effectiveness of the immunoprecipitation for both methylated and hydroxymethylated DNA, to assess the robustness of the method with respect to variation between experiments. Thus, following MeDIP, enrichment of highly methylated control regions (IAP promoter and H19 ICR) and depletion of unmethylated Gapdh promoter and CpG-poor CSa region were analysed (Lempiäinen et al., 2011). For the hMeDIP, enrichment of the hydroxymethylated regions H19 and *Tex19.1* promoters, and depletion of the methylated IAP promoter and non-hydroxymethylated Gapdh promoter, were assessed (Thomson et al., 2012). Following whole genome amplification, comparison of the immunoprecipitated 5mC fraction to the input (non-enriched) fraction (%IP/Input) showed that in lymph node tissue (Fig. 1a) the controls for 5mC enrichment, IAP promoter and H19 ICR, were enriched by $251 \pm 7\%$ and $121 \pm 5\%$, respectively. In contrast, the methylation-poor controls, Gapdh and CSa were markedly depleted, with a mean 5mC %IP/input of $33 \pm 3\%$ and $31 \pm 3\%$. For spleen tissue (Fig. 1c) a similar pattern was observed with a somewhat higher enrichment at the IAP promoter, mean $306 \pm 8\%$, and robust depletion at Gapdh and CSa, $11 \pm 1\%$ and $20 \pm 1\%$ 5mC IP/Input, respectively. However, there was limited enrichment of H19 ICR, showing similar levels to the input fraction, $99 \pm 3\%$ 5mC IP/input. Overall, both the lymph node (Fig. 1a) and spleen (Fig. 1c) tissue showed the expected pattern of enrichment and depletion for methylated and methylation poor regions confirming that there

was robust enrichment and limited non-specific immunoprecipitation.

The effectiveness of the lymph node hMeDIP, post whole genome amplification, is shown in Fig. 1b. Comparison of the immunoprecipitated 5hmC fraction to the input fraction (%IP/input), demonstrated a more profound enrichment at the hydroxymethylated H19 ($507 \pm 44\%$) and *Tex19.1* ($303 \pm 32\%$) promoters than the corresponding positive controls for the MeDIP. However, compared to 5mC there was considerably more inter-animal variation in levels of 5hmC. There was a single sample from the DNCB group which failed to show H19 promoter enrichment (60% 5hmC IP/input). Despite this, good enrichment at the *Tex19.1* promoter (345% 5hmC IP/input) was recorded for this sample so it was included in the data set for further analyses. There was effective depletion of the IAP promoter ($40 \pm 2\%$ 5hmC IP/input), and the non-methylated, Gapdh promoter ($56 \pm 9\%$ 5hmC IP/input) for the majority of samples. The two samples with $>100\%$ 5hmC IP/input at the Gapdh promoter displayed good depletion at IAP ($<50\%$ 5hmC IP/input) and were therefore included in subsequent analyses. In contrast to the more marked spleen 5mC enrichment, enrichment of 5hmC in this tissue (Fig. 1d) was lower than that found in lymph node tissue; the promoter of H19 and *Tex19.1* displayed enrichments of $479 \pm 39\%$ and $174 \pm 18\%$ 5hmC IP/input, respectively. Furthermore, depletion of 5hmC was recorded at IAP and Gapdh, with %IP/input of $29 \pm 1\%$ and $61 \pm 8\%$ 5hmC IP/input, respectively. Thus, both the lymph node (Fig. 1b) and spleen tissue (Fig. 1d) produced the expected levels of 5hmC enrichment and

depletion for the appropriate markers. In addition, the same patterns of 5mC and 5hmC enrichment were observed in the pre-amplified samples (data not shown), demonstrating that there was limited amplification bias for methylated/hydroxymethylated or unmethylated/unhydroxymethylated fragments.

As two independent animal studies generated the lymph node data, it was important to compare the extent of enrichment between the two experiments. For the lymph node MeDIP (Fig. 1a), there was significantly greater enrichment in study 2 than in study 1 at the *IAP* locus ($271 \pm 11\%$ vs. $238 \pm 8\%$ 5mC IP/input, $p < 0.01$) whilst for the *Gapdh* locus, significantly greater depletion was recorded for study 2 compared with study 1 ($17 \pm 2\%$ vs. $42 \pm 3\%$ 5mC IP/input, $p < 0.05$). There were no differences between enrichment at the other regions. Likewise, although there was considerably more inter-animal variation in the hMeDIP, there were no significant differences between the two studies (Fig. 1b). The difference in 5mC enrichment between two studies is not surprising given the stochastic nature of the whole genome amplification as well as the technical variation between immunoprecipitations. In order to control for this inter-study variation, data were therefore expressed as the ratio to the average corresponding study AOO %IP/input.

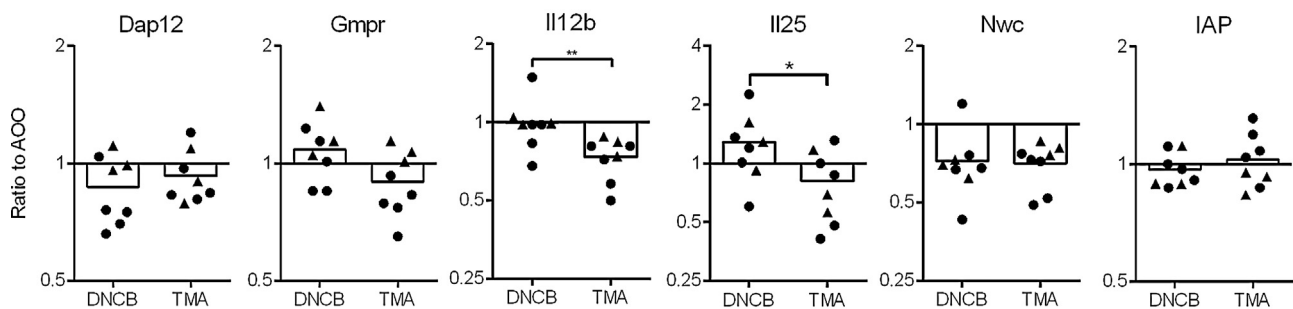
3.2. Tissue specific changes in 5-methylcytosine

Differentially methylated regions were chosen from our previously published genome wide array (Chapman et al., 2014) based on the following criteria. All DMR had to show sufficient

robustness to fall within a cut-off of $p < 0.01$ and a difference in absolute methylation in the genome wide array of >0.4 for at least one allergen treatment compared to the AOO control. The initial set of DMR selected contained nine DMR identified in DNCB vs. AOO and five DMR identified in TMA vs. AOO (Table 1). As described previously (Chapman et al., 2014), DNCB treatment resulted in a greater number of robust DMR and thus contributed a greater proportion of those tested. The initial set, shown in Table 1, were subjected to a further round of selection based on their reproducibility by qPCR in an independent repeat study. Differentially methylated regions that failed to show reproducibility in the repeat study displayed either limited or no significant change in methylation compared to AOO when measured by qPCR. The five chosen are highlighted in grey. All but one of these remaining DMR were associated with DNCB treatment (*Dap12*, *Gmpr*, *Il12b*, *Il25*). The *Nwc* DMR was associated with TMA treatment and the *Il12b* DMR was associated with both treatments in the genome wide array. Interestingly, all chosen DMR were located in either the first or the second intron of the gene with which they were associated. Additionally, none of these DMR could be classified as CpG islands; for all DMR the observed to expected ratio of CpG was less than 0.6 for all DMR and CG% $< 50\%$ (Illingworth and Bird, 2009).

Methylation patterns were assessed by qPCR at the selected DMR in two independent studies (a), study 1 ($n = 4/5$) and study 2 ($n = 3$) to confirm concordance with the genome wide array. The methylated control region *IAP* was included as a negative control for allergen induced changes in methylation as in lymph node tissue this showed no significance differences in 5mC %IP/input

(a) Lymph node



(b) Spleen

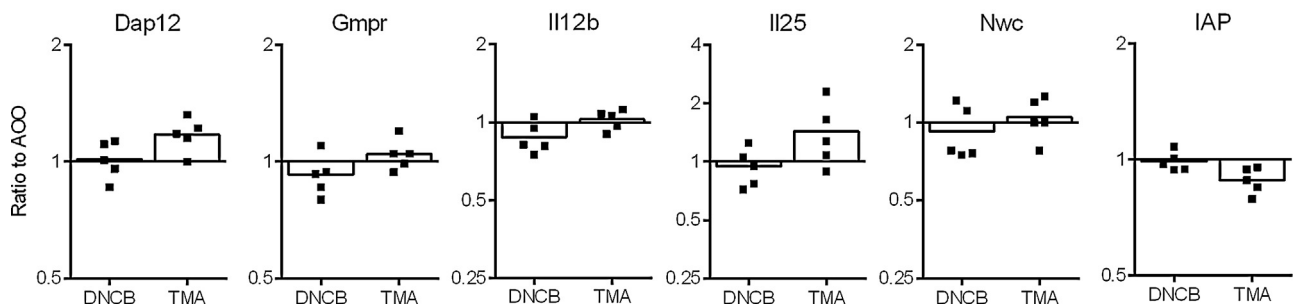


Fig. 2. Allergen induced tissue specific changes in DNA methylation.

Selected DMR located in *Dap12*, *Gmpr*, *Il12b*, *Il25*, *Nwc* and in a negative control region (*IAP*) were analysed by real-time quantitative PCR (qPCR) following methylated DNA immunoprecipitation (MeDIP). Mice were treated topically with DNCB, TMA or the vehicle control (AOO). Thirteen days after the initiation of exposure, draining (auricular) lymph nodes (a, pooled on an individual animal basis) and spleen (b) were collected. For lymph node tissue (a) two independent studies are shown, study 1 (●) $n = 5$ for all groups except AOO ($n = 4$) and study 2 (▲) $n = 3$. Spleens were collected in study 3 (■, $n = 5$). Methylated DNA was immunoprecipitated (IP) by MeDIP then subjected to whole genome amplification. An equivalent amount of input DNA was also amplified. Using qPCR, the relative enrichment of each DMR was compared to the paired input fraction for each individual animal (%IP/input). The ratio of DNCB or TMA methylation to the control (average AOO value of the concurrent study) was calculated using %IP/input and is displayed as individual animals on a logarithmic scale. The column represents the mean of both studies combined. The horizontal line at 1 represents no change in methylation compared with the concurrent AOO control. Differences in methylation between DNCB and TMA groups were deemed significant if $p < 0.05$ (Mann–Whitney test, * $p < 0.05$, ** $p < 0.01$).

between AOO, DNCB and TMA treatments. To account for any changes in baseline methylation and technical variations between MeDIP and whole genome amplification, changes in methylation were expressed as a ratio of %IP/input for allergen compared to the concurrent AOO control.

Whilst the *Dap12* and *Nwc* DMR (a) displayed similar decreases in methylation compared to AOO in response to both allergens, significantly divergent patterns of methylation between allergens were recorded at *Il25* ($p < 0.05$). The *Il25* DMR was hypermethylated in DNCB but was hypomethylated in TMA (1.28 ± 0.18 vs. 0.81 ± 0.12). Following DNCB treatment, methylation at the *Dap12*, *Gmpr* and *Il25* DMR in the draining lymph node tissue (a) correlated with the genome wide array results (Table 1). The *Nwc* DMR, hypomethylated in TMA compared to AOO (%IP/Input $p < 0.05$), also correlated well with the genome wide array. The *Il12b* DMR (hypermethylated following exposure to both DNCB and TMA in the genome wide array) failed to follow the same patterns in the qPCR analyses in the repeat studies (Fig. 2a); in fact TMA was significantly more hypomethylated than DNCB (0.74 ± 0.05 vs. 1.00 ± 0.08 , $p < 0.001$). Importantly, spleen tissue isolated from allergen-treated mice showed no significant differences in 5mC between allergen treatments (Fig. 2b). The tissue specificity of allergen-induced changes in lymph node 5mC was most apparent in the TMA-treated mice. For all DMR with the exception of *Gmpr*, significant differences between the lymph node methylation and spleen methylation were recorded (*Dap12*: 0.93 ± 0.05 vs. 1.17 ± 0.05 $p < 0.05$, *Il12b*: 0.74 ± 0.05 vs. 1.03 ± 0.04 $p < 0.01$, *Il25*: 0.81 ± 0.12 vs. 1.44 ± 0.25 $p < 0.05$, *Nwc*: 0.71 ± 0.05 vs. 1.05 ± 0.08 $p < 0.01$). In contrast, there were no

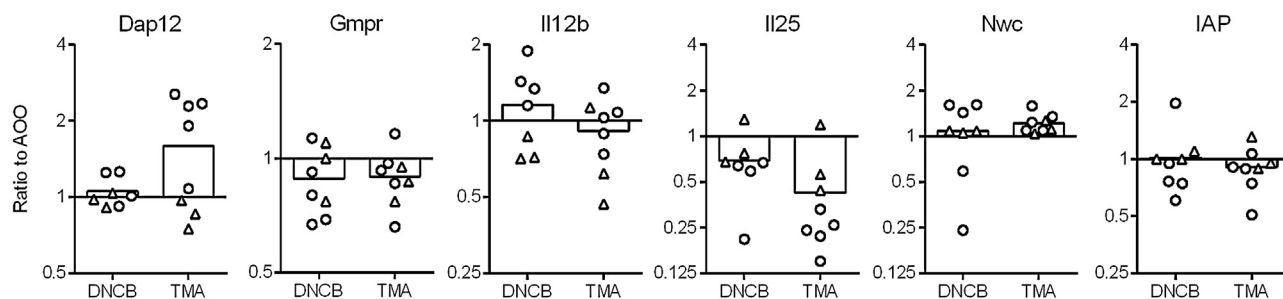
significant differences in methylation between lymph node and spleen 5mC derived from DNCB-treated mice.

3.3. DNA hydroxymethylation as a biomarker

In addition to analysing levels of 5mC, we investigated allergen-induced changes in 5hmC in the same tissues. Following allergen treatment, patterns of 5hmC at the selected DMR in lymph node tissue from study 1 ($n = 4/5$) and study 2 ($n = 3$) (Fig. 3a) and spleen tissue from study 3 ($n = 5$) (Fig. 3b) were assessed by hMeDIP and qPCR. As previously, the control region *IAP* was included as a negative control for allergen-induced changes in 5hmC. Baseline changes and technical variation in the immunoprecipitation and whole genome amplification step were controlled for by calculating the change in 5hmC as the ratio of %IP/input, then allergen treatment was compared with the concurrent AOO control.

In general, there was a wider degree of variation (both within and between experiments) and changes were more extensive for hydroxymethylation compared with methylation, particularly for tissue derived from TMA-treated mice. Thus, in lymph node tissue (Fig. 3a) for the control region, *IAP*, hydroxymethylation was equivalent to AOO for both allergens (5hmC ~ 1) whereas *Dap12* and *Il25* demonstrated large and variable changes in 5hmC in response to TMA; 5hmC at *Dap12* increased while at *Il25* 5hmC decreased considerably, although such changes did not reach statistical significance. In contrast to methylation status, differences between the two independent studies were much more apparent at the level of 5hmC, despite being normalised to the concurrent vehicle control. This was particularly apparent for

(a) Lymph node



(b) Spleen

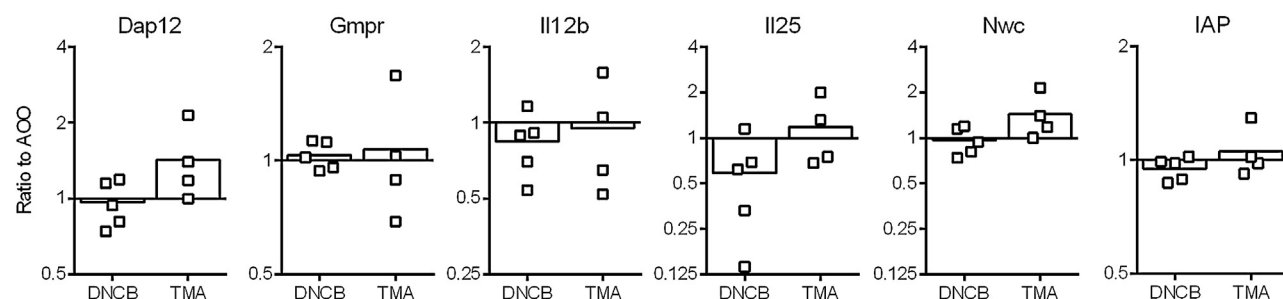


Fig. 3. Allergen induced changes in DNA hydroxymethylation.

Selected DMR located in *Dap12*, *Gmpr*, *Il12b*, *Il25*, *Nwc* and in a negative control region (*IAP*) were analysed by qPCR following hydroxymethylated DNA immunoprecipitation (hMeDIP). Mice were treated topically with DNCB, TMA or the vehicle control (AOO) (Study 1 (○) $n = 5$ for all groups except AOO ($n = 4$) and study 2 (△) $n = 3$). Thirteen days after the initiation of exposure, draining (auricular) lymph nodes (a, pooled on an individual animal basis) and spleen (b, □, Study 3 $n = 5$) were collected. Hydroxymethylated DNA was immunoprecipitated (IP) by hMeDIP then subjected to whole genome amplification. An equivalent amount of input DNA was also amplified. The relative enrichment of each DMR was compared to the paired input fraction for each individual animal (%IP/input). The ratio of DNCB or TMA hydroxymethylation to the control (AOO) was calculated using %IP/input and displayed as individual animals on a logarithmic scale. The column represents the mean of both studies combined. The horizontal line at 1 represents no change in hydroxymethylation compared with the concurrent AOO control, differences in hydroxymethylation between DNCB and TMA groups were deemed significant if $p < 0.05$ (Mann–Whitney test).

Dap12; whilst 5hmC in study 1 (Fig. 3a; open circles) was significantly increased in TMA over DNCB tissue ($p < 0.05$), yet study 2 (Fig. 3a; open triangles) failed to show differential responses. The lack of divergent responses in study 2 was also recorded for *Il12b* and *Il25*; both showed significant differences between DNCB and TMA in study 1 only ($p < 0.05$). Consistent with methylation levels in spleen, there was no difference between allergen treatments at the level of 5hmC in spleen tissue (Fig. 3b). For *Il25* there were significantly divergent patterns of 5hmC between lymph node and spleen tissue with TMA-derived tissue (0.42 ± 0.12 vs. 1.18 ± 0.3 , $p < 0.05$), but there were no differences in 5hmC between the two tissues at the remaining DMR following TMA treatment or at any of the DMR after exposure to DNCB.

3.4. Comparison between 5-methylcytosine and 5-hydroxymethylcytosine

As 5hmC is a product of DNA demethylation and more recently, as an marker of active gene expression, a reciprocal relationship between 5mC and 5hmC is expected to be observed, with higher levels of 5hmC there is relatively low DNA methylation, and vice versa (Guo et al., 2014; Thomson et al., 2013). The levels of 5mC and 5hmC in the draining lymph node tissue were therefore compared for each allergen (Fig. 4). As previously, the negative control *IAP* (Fig. 4f) showed no difference between 5mC and 5hmC, regardless of allergen treatment. In contrast, there was a significant difference between 5mC and 5hmC for all other DMR tested, for one or both

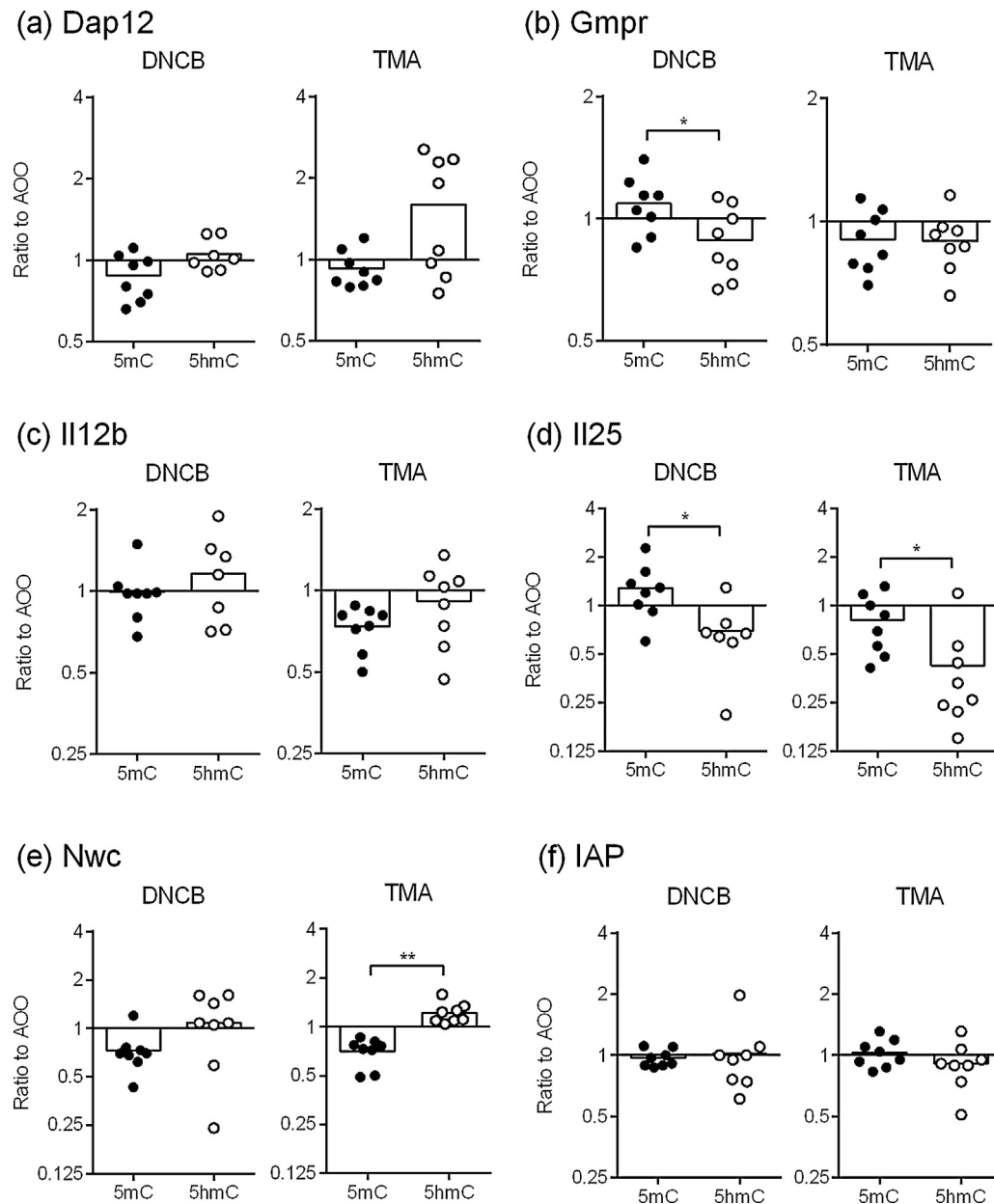


Fig. 4. Comparison of levels of methylation vs. hydroxymethylation.

Selected DMR located in *Dap12*, *Gmpr*, *Il12b*, *Il25*, *Nwc* and in a negative control region (*IAP*) were analysed by qPCR to compare methylation and hydroxymethylation. Mice were treated topically with DNCB, TMA or the vehicle control (AOO). Thirteen days after the initiation of exposure draining (auricular) lymph nodes were collected and pooled on an individual animal basis. Each sample was subjected to MeDIP and hMeDIP. Using qPCR, the relative enrichment of methylation or hydroxymethylation at each DMR was compared to the paired input fraction (%IP/input). The ratio of DNCB or TMA hydroxymethylation to the control (AOO) was calculated using %IP/input and displayed as individual animals on logarithmic scale ($n=8$). The column represents the mean and the horizontal line at 1 represents no change in methylation compared with the concurrent AOO control. Levels of methylation (●) were compared to levels of hydroxymethylation (○) by Wilcoxon matched paired signed rank tests (* $p < 0.05$, ** $p < 0.01$).

allergen treatments, with the exception of *Il12b* and *Dap12*. Treatment with DNCB resulted in significantly divergent levels of 5mC and 5hmC in the draining lymph node tissue at *Gmpr* (Fig. 4b) and *Il25* (Fig. 4d). At the *Gmpr* DMR, there were small but robust, reciprocal changes in 5mC and 5hmC (1.09 ± 0.06 vs. 0.89 ± 0.06 , $p < 0.05$), while *Il25* was characterised by a relatively large increase in 5mC and a decrease in 5hmC (1.28 ± 0.18 vs. 0.69 ± 0.12 , $p < 0.05$). Treatment with TMA resulted in a somewhat different profile of methylation/hydroxymethylation. Significantly divergent levels of 5mC and 5hmC at *Nwc* (Fig. 4e, $p < 0.01$) and *Il25* (Fig. 4d, $p < 0.05$) were recorded. At *Nwc*, TMA treatment resulted in a decrease in 5mC (0.70 ± 0.11) and in this case an increase in 5hmC compared to AOO (1.22 ± 0.06). Interestingly, *Il25* showed a reduction in both 5mC (0.81 ± 0.12) and 5hmC (0.42 ± 0.12) in response to TMA (compared to AOO), although the difference between the extent of loss of 5mC and 5hmC reached statistical significance ($p < 0.05$).

4. Discussion

There are tests available for the prospective identification of contact allergens, including the currently preferred *in vivo* method the LLNA (Basketter et al., 2009; Kimber et al., 2002), and more recently validated *in vitro* methods (Basketter et al., 2013). However, such tests fail to discriminate between contact and respiratory allergens (Dearman et al., 2013), and there are currently no validated methods for chemical respiratory allergens identification. Although cytokine fingerprinting goes some way towards addressing this (Dearman et al., 2003), there remains a need for innovative new approaches for the characterisation and categorisation of chemical allergens (Kimber et al., 2011). Consistent with this, the aim of investigations described here was to identify stable biomarkers of contact and/or chemical respiratory allergens based on epigenetic markers, and in particular DNA methylation. Immune responses in mice to the two reference chemical allergens, DNCB and TMA, were investigated, building on DMRs that were identified in a previous genome-wide screen of DNA methylation in allergen-treated lymph node tissue (Chapman et al., 2014; Moggs et al., 2012). It was hypothesised that, due to an important role for epigenetic mechanisms in maintaining lineage commitment, changes in DNA methylation would be more stable and robust than induced alterations in gene expression or protein production that commonly display variations in kinetics and the magnitude of response. This rationale is supported by the fact that methylation of intragenic regions, where our selected chemical allergen-modulated DMR are located, can exhibit differential effects on gene expression relative to DNA methylation at promoter sites (Jones, 2012).

Having identified a number of potential DMR from the genome wide analysis, the initial check point was concordance with previous work (Chapman et al., 2014). For the majority of DMR, patterns of 5mC matched those observed in the genome wide study, the only exception being *Il12b*. It was anticipated that the DMR at *Il12b*, which encodes the p40 subunit of IL-12 and IL-23, would be hypermethylated in response to treatment with both allergens, whereas in fact it was found to be hypomethylated in response to TMA. This discrepancy may be due to differences in DMR identified in the genome wide methylation array (>400 bp), compared with the region covered by the PCR primers (75–150 bp) (Chapman et al., 2014). Additionally, it could be that a key CpG was missed, or that there are low or variable levels of methylation across the DMR. Bisulfite sequencing (BS-seq), to identify individual CpG methylation, may therefore be used to confirm these DMR (Krueger et al., 2012). Although BS-seq is commonly described as the 'gold standard' for methylation it is unable to

distinguish between 5mC and 5hmC (Huang et al., 2010; Suzuki and Bird, 2008). Recent advances have expanded on BS-seq to enable detection of 5mC and 5hmC individually. Further oxidation of 5hmC to 5fC in oxidative BS-seq (Booth et al., 2013) or 5hmC glycosylation and Tet-assisted BS-seq (Yu et al., 2012) can be used to specifically identify 5mC or 5hmC, respectively. The combination of these two methods would provide quantitative and reproducible conformation of the biomarkers suggested in this study.

The lack of concordance may also be attributed to biological variation and/or technical variation in enrichment and amplification efficiency. Although for each study expected patterns of T cell polarisation were confirmed by analysis of cytokine protein production the magnitude of responses varied (data not shown). Furthermore, the stochastic nature of immunoprecipitation and genome wide amplification could also result in variation in enrichment, and at CpG dense regions there is the opportunity for amplification bias (Jia et al., 2010). This may be a key consequence for the low-CpG density regions analysed here. The decision to analyse changes in 5mC and 5hmC as a ratio of the concurrent control minimised the impact of this variation between studies.

It was also of interest to determine if changes in DNA methylation were restricted to the draining lymph nodes, or whether similar patterns could be detected in other lymphoid tissue distant from the site of exposure, specifically in the spleen. The initial immune activation is anticipated to be focused primarily within local lymph nodes draining the site of exposure to chemical allergen, rather than in distant lymphoid tissue. As predicted, there were no significant effects of chemical allergen in the spleen for the selected DMR. In addition, changes in 5mC in lymph node tissue were generally significantly different to the patterns recorded in the spleen.

Having established concordant methylation patterns for the selected DMR, subsequent experiments addressed whether the differences in 5mC in response to the two allergens were sufficiently robust to find use as a potential biomarker of allergen exposure. However, for all DMR except *Il25* and the non-concordant *Il12b*, this was not the case. The reason for this might be that both types of sensitiser induce local inflammation, and that both activate dendritic cells and are immunogenic causing both T and B lymphocyte activation and proliferation (Kimber et al., 2011). Indeed, examination of genome wide differences in mRNA expression following topical exposure of mice to various chemical allergens identified regulation of similar families of genes. Among the genes that were found to display comparable expression patterns between contact and respiratory allergens were those known to play important roles in development of adaptive immune responses, such as *Cd83* and *Cd51* (associated with mature dendritic cells and activated macrophages, respectively) (Adenuga et al., 2012).

As discussed above, the only concordant DMR with differential 5mC between DNCB and TMA treatments was in *Il25*. Despite this, the data suggest that analysis of methylation changes alone is insufficient to differentiate between DNCB and TMA. For this reason levels of 5-hydroxymethylcytosine, an intermediate of oxidative DNA demethylation that can be associated with specific transcriptional states, were measured. Due to the often mutually exclusive nature of 5mC and 5hmC the question addressed was whether 5hmC levels might provide confirmation of changes (a reduction or a gain) in the levels of 5mC. Hydroxymethylcytosine is also a key epigenetic modification in its own right; the presence of 5hmC (and the absence of 5mC) may even be sufficient for transcriptional activation in some cases (Sérandour et al., 2012). The presence of 5hmC is associated with poised/activated promoters and regulatory regions, as well as in the gene bodies

of highly expressed genes (Sérandour et al., 2012; Stroud et al., 2011).

This is the first occasion on which levels of 5hmC have been determined *in vivo* in chemical allergy. In this experiment there was a lack of divergent responses to DNCB and TMA with respect to 5hmC levels. However, when combined with changes in 5mC levels, comparisons of the relationship between 5mC vs. 5hmC provided the identification of 3/5 discriminatory DMR. These showed a strong association with chemical allergen exposure, with some being exclusive to either DNCB or TMA. The DMR at *Nwc*, a conserved gene located in the *Rag* locus (Cebrat et al., 2008) showed divergent patterns of 5mC and 5hmC in response to TMA. This DMR was also characterised by patterns suggesting active transcription (increased 5hmC). In the UCSC Genome browser this region is characterised by H3K4me1 alone in spleen tissue; when combined with 5hmC enrichment this suggests that the *Nwc* DMR could be a poised regulatory region (Calo and Wysocka, 2013; Yu et al., 2012).

The *Gmpr* DMR shows an association with DNCB when changes in both 5mC and 5hmC are taken into account; *Gmpr* is predicted to help maintain the balance of available nucleotides within a cell. While both allergens induce lymphocyte proliferation, there may be differential requirements between allergens for nucleotides and therefore *Gmpr* expression. It is also possible that this DMR is affecting gene regulation further afield (Stadler et al., 2011).

While DMR at *Gmpr* and *Nwc* were specific to DNCB and TMA respectively, at *Il25* divergent 5mC patterns were significantly different from 5hmC levels for both allergens. Interleukin-25 is an IL-17 family member that is characteristically found in Th2 related immunopathology, including helminth infection (Fallon et al., 2006) and allergic asthma (Ballantyne et al., 2007). In the Th2-mediated response to TMA, decreased methylation and even lower hydroxymethylation levels may suggest a demethylation of the DMR to allow *Il25* expression. Although DNCB is associated with Tc1/Th1 polarisation (Dearman et al., 2005), the polarisation is not absolute, with some Th2 cell activation in what is thought to be a regulatory role (Xu et al., 1996) suggesting a possible role for the similar methylation patterns that were observed for DNCB. A more detailed analysis of both DNA methylation and gene expression within Tc1/Th1 vs. Th2 immune cell subsets would therefore be of interest.

Two of the selected DMR, at *Gmpr* and *Nwc*, show potential as biomarkers for contact and chemical respiratory allergens, respectively, due to their association with the reference contact allergen DNCB or the reference respiratory allergen TMA. The next stage in confirming the suitability of DNA methylation as a biomarker would be to assess the relationship with a range of chemical allergens. Although there are relatively few known respiratory chemical allergens compared with contact allergens, there are several chemical families including, for instance, the diisocyanates and chloroplatinate salts (Dearman and Kimber, 2001). In addition, focusing on a specific cell population, rather than the mixed lymph node cell population would likely increase the magnitude and reliability of changes in methylation. However, this requires additional manipulation and may be limited by cell number in minority populations. Additionally, an important part of progressing these DMR as potential markers for chemical allergens, or for the quality of the immune response is to determine whether the methylation patterns result in functional effects. However, such analyses will be complicated by the kinetics of mRNA expression. It is also worth noting that changes in DNA methylation do not preclude gene expression but rather modulate the “permissiveness” of gene expression. Despite this, even if DMR functional effects are unclear, sufficiently high levels of DMR

selectivity and sensitivity would still be of potential utility as a biomarker for chemical allergy.

In conclusion, for the first time we link changes in DNA hydroxymethylation to exposure to chemical allergens *in vivo*. Furthermore, the analysis of a combination of DNA methylation and hydroxymethylation status of a number of DMR could, in theory and following confirmation with other classes of chemical allergens, provide an attractive basis for distinguishing between preferential contact allergens, and those that favour sensitisation of the respiratory tract.

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