Chemical Allergen Induced Perturbations of the Mouse Lymph Node DNA Methylome

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Epigenetic regulation of gene expression plays a pivotal role in the orchestration of immune responses and may determine the vigor, quality, or longevity of such responses. Chemical allergens can be divided into 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 cell responses. To explore the regulation and maintenance of these divergent responses, 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 DNA immunoprecipitation followed by hybridization to a whole-genome DNA promoter array. 6319 differently methylated regions (DMRs) were identified following DNCB treatment, whereas 2178 DMRs were measured following TMA treatment, with approximately half of the TMA DMRs common to DNCB. When limited to promoter region-associated DMRs, 637 genes were uniquely associated with DNCB-induced DMRs but only 164 genes were unique to TMA DMRs. Promoter-associated DMRs unique to either DNCB or TMA were generally hypomethylated whereas DMRs common to both allergens tended to be hypermethylated. Pathway analyses highlighted a number of immune-related pathways, including chemokine and cytokine signaling. These data demonstrate that chemical allergen exposure results in characteristic patterns of DNA methylation indicative of epigenetic regulation of the allergic response.

Key words: DNA methylation; chemical allergy; mouse lymph node; epigenetic.

ABBREVIATIONS

AOO acetone:olive oil
CGI CpG island
CSA unmethylated control sequence
DAVID Database for Annotation, Visualization and Integrated Discovery
DMR differently methylated region
DNCB 2,4-dinitrochlorobenzene
EASE Expression Analysis Systematic Explorer
EDTA ethylenediamine tetra-acetic acid
GWA genome-wide assay
H19 H19 imprinted control region
IAP intracisternal A-particle element
IFN-γ interferon gamma
IP immunoprecipitated
KEGG Kyoto Encyclopedia of Genes and Genomes
LN lymph node
MeDIP Methylated DNA Immunoprecipitation
qPCR quantitative polymerase chain reaction
SDS sodium dodecyl sulphate
Tc cytotoxic T cell
Th helper T cell
TMA trimellitic anhydride
TSS transcription start site
Treg regulatory T cell
WGA whole genome amplification

Chemical allergy remains an important health issue and there is a continuing need for improved characterization of the relevant immunological and toxicological mechanisms. Chemical allergy has two main clinical manifestations. Skin sensitization, resulting in allergic contact dermatitis, is the most common manifestation of immunotoxicity in humans and many hundreds of chemicals have been implicated as contact allergens (Kimber et al., 2011). In addition, there are some chemicals, fewer in number, known to cause sensitization of the respiratory tract associated with occupational rhinitis and asthma (Isola et al., 2008).

There is considerable interest in the cellular and molecular events required for the initiation and maintenance of allergic sensitization to chemicals, and the differences that exist between skin sensitization and sensitization of the respiratory tract (Boverhof et al., 2008; Kimber et al., 2011). A body of evidence from studies in experimental animals demonstrates that differences in response to skin and respiratory tract sensitizing chemicals are associated with different classes of T cells. Rodent studies have revealed that the development of skin sensitization is favored by polarized T helper (Th) 1 and cytotoxic...
T (Tc) 1 responses (Dearman et al., 2005; Honda et al., 2013). More recently, another population of helper T cells, Th17, has been shown to play important roles (Peiser, 2013). In humans, allergic contact dermatitis is also associated with Th1/Tc1-type cells (Martin, 2012). In contrast, in rodents and in humans the limited evidence available suggests that sensitization to chemical respiratory allergens is commonly associated with selective Th2-type immune responses (Dearman et al., 2005; Kimber and Dearman, 2005; Mameessier et al., 2007). Interestingly, the ability of these different classes of chemical allergens to provoke divergent T-cell responses is independent of the route of exposure (skin vs. inhalation) that results in the acquisition of sensitization. Thus, skin exposure to respiratory allergens has been shown to result in preferential Th2 cell activation (Kimber and Dearman, 2002). Indeed, the possibility that, in an occupational setting, sensitization or priming to chemical respiratory allergens may result from skin contact is increasingly acknowledged (Redlich, 2010). These polarized adaptive immune responses are believed to be driven by differential cytokine expression and dendritic cell function (Fukuyama et al., 2009).

What is not clear, however, is the extent to which allergic sensitization to chemicals is subject to regulation by epigenetic mechanisms, and whether polarized immune responses induced by exposure to chemical allergens are supported by changes in gene expression mediated by DNA methylation and other epigenetic mechanisms. These epigenetic mechanisms can play important roles in orchestrating immune responses (Moggs et al., 2012) and it is well established that certain epigenetic mechanisms, in particular the silencing of gene expression by DNA methylation, are implicated in T-cell development and differentiation (Cuddapah et al., 2010; Wilson et al., 2009; Yeo and Fearon, 2011). Furthermore, epigenomic profiling has shown that modulation of DNA methylation is a feature of normal T-cell memory and effector function (Hashimoto et al., 2010). Disruption of these methylation patterns, for example by knocking out DNA methyltransferase 1 (responsible for re-establishing methylation following replication), leads to lethal autoimmunity as a consequence of loss of suppressive regulatory T cell (Treg) function (Wang et al., 2013). Of greater potential relevance to chemical allergy are the observations that in a mouse model of asthma, and in subjects with occupational asthma to disocyanates, there is down-regulation of interferon-γ (IFN-γ) expression secondary to methylation of the ifng locus (Brand et al., 2012; Ouyang et al., 2013), reduced expression of this cytokine favoring the development of Th2 selective immune responses.

Recent advances in epigenomic profiling technologies provide new opportunities to gain mechanistic insights into the molecular basis of long-lasting cellular perturbations within functional immune cell populations that underlie aberrant or exaggerated immune responses following exposure to xenobiotics. In addition, profiling the molecular changes associated with sensitization to chemical allergens may identify novel biomarkers for allergic responses to chemicals and drugs. Here, we have used a well-established mouse model for chemical allergen induced polarization of T-cell responses to investigate epigenetic mechanisms of gene regulation during T-cell polarization in vivo (Dearman et al., 2005). We have characterized chemical allergen induced perturbations of the mouse lymph node (LN) DNA methylome following skin exposure to a reference contact allergen (2,4-dinitrochlorobenzene, DNCB) or to a reference respiratory allergen (trimellitic anhydride, TMA). Allergen-activated LN exhibited unique patterns of DNA methylation that include molecular pathways associated with polarized immune responses. These data provide novel insights into the pharmacodynamics of chemical allergy and the molecular regulation of immune cell polarization in vivo.

MATERIALS AND METHODS

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; BandK Universal, Hull) and water were available ad libitum. Ambient temperature was maintained at 21 ± 2°C and relative humidity was 55 ± 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 license.

Sensitization to chemical allergen. The chemical allergens DNBC (97% pure) and TMA (99% pure) were obtained from Sigma Aldrich (St Louis, MO). Chemicals were dissolved in 4:1 (vol/vol) acetone:olive oil (AOO). All solutions were freshly prepared prior to use. Groups of mice (n = 5 for MeDIP-array and n = 3–5 for subsequent studies) received 50 µl of 10% TMA or 1% DNBC 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) LNs were excised, pooled on an experimental animal basis, and immediately snap frozen in liquid nitrogen for storage until processing for the MeDIP-array. In subsequent experiments, LNs were excised into RNA later (Life Technologies, Grand Island, NY). The schema for dosing is outlined in Figure 1.

Methylated DNA immunoprecipitation (MeDIP) assay. Genomic DNA from mouse LN tissue samples was extracted by overnight proteinase K digestion (Sigma) in lysis buffer (50mM Tris-HCl pH 8.0, 10mM ethylenediamine tetra-acetic acid [EDTA] pH 8.0, 0.5% sodium dodecyl sulphate) prior to RNaseA digestion, phenol-chloroform extraction, and ethanol precipitation. Genomic DNA was sonicated (Bioruptor, Diagenode, Liège, Belgium) to produce DNA fragments ranging in size...
from 300–1000 bp, with a mean fragment size of 500 bp. 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 μl mouse monoclonal antibody against 5-methylcytidine (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 M-280 protein G Dynabeads (Invitrogen, Paisley) for 2 h prior to washing all unbound fragments three times with 1 ml immunoprecipitation buffer. Washed beads were then resuspended in 250 μl of lysis buffer and incubated with proteinase K for 2 h at 50°C. Methylated DNA was immunoprecipitated (IP) then recovered by phenol-chloroform extraction and ethanol precipitation and resuspended in 20 μl Tris EDTA. For qPCR analysis, 10 μl aliquots of IP DNA were taken and diluted to 100 μl in Tris EDTA with each qPCR reaction using 2 μl of diluted DNA. For the MeDIP-array, 50 ng of input DNA and 10 μl of IP DNA was subjected to whole genome amplification (WGA) 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) and eluting into Tris EDTA. For each sample, 6 μg of amplified IP and input material was sent to Roche Nimblegen (Reykjavik, Iceland) for Cy3 and Cy5 labeling and hybridization on 2.1M Deluxe mouse promoter tiling arrays (MeDIP-array).

Processing of Nimblegen promoter arrays. 2.1M Deluxe Mouse Promoter Arrays (mm9 build) (Roche Nimblegen) contain 2,056,330 unique probes of 50–70 bp length with approximately 50 bp spacing distributed over 21,562 tiled regions spanning 52,016 annotated transcription start site (TSS) regions over 20,718 unique genes. In addition, these arrays cover 15,963 annotated CpG islands (CGIs) over both promoter and “non-promoter” (inter-/intra-genic) regions. Signals for each probe of the 5mC-enriched samples (Cy5 labeled) were compared to input samples (Cy3 labeled) to generate log2 (IP/input) scores (fold changes). These log2 scores were then normalized by subtracting the weighted median log2 score from each array and scaling each array for the same median absolute deviation using the Limma package in R/Bioconductor (Smyth and Speed, 2003). Subsequent analyses were carried out in the Refiner Genome and Analyst modules of the Expressionist software package (Version 7.0, Genedata AG, Basel). Using the Sliding-Window ANOVA Activity from Refiner Genome (Genedata Expressionist Refiner Genome Reference Manual, Version 7.0), genomic ranges with a minimum length of 400 bp and a higher than usual variance (threshold: p-value of at least 100 on the phred scale) were identified and then exported to Analyst for further analysis. Group-wise t-tests were performed to define significant differentially methylated regions (DMRs) between DNCB versus AOO, TMA versus AOO, and DNCB versus TMA, with a cut-off of p < 0.01. DMRs are expressed as the difference of the average log2 fold methylation value of the indicated conditions (i.e., Delta log2 5mC [treated – control]) calculated by log2 treated/IP/input – log2 control/IP/input. The 5mC raw data files have been deposited with Gene Expression Omnibus with accession number GSE54768.

Statistical, consistent, and pathway analyses. DMRs (probe coordinates) were exported to EXCEL and then mapped to RefSeq (Pruitt et al., 2012) gene TSS using the probe coordinates and the University of California Santa Cruz (UCSC) Genome Browser with mouse genome assembly MM9 (Waterston et al., 2002). An individual gene may have several associated DMRs. Each DMR was then considered based on the proximity to the nearest TSS and grouped as follows: intra-genic (+250 bp to +3 kb), TSS region (–250 bp to +250 bp), promoter (–1 kb to –250 bp), upstream (–2 kb to –1 kb), or inter-genic (–8 kb to –2 kb) (as in Thomson et al., 2012 and Fig. 3a). Consistency analysis was performed using Venn diagrams generated with BioVenn (Hulsen et al., 2008). Analysis was performed on the whole genome-wide data sets, and then on promoter-associated DMRs only (those DMRs that were within –1000 bp to +250 bp of a TSS). Following on from the consistency analysis, promoter-associated DMRs were separated into those unique to either DNCB or TMA treatment or those common to both treatments. These DMRs were further split on the basis of methylation status and pathway analysis was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009) and annotated with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The pathway analysis is limited to genes with an annotated function; as such genes of unknown function or hypothetical genes are not included in the analysis. Cut-offs were set to EASE (Expression Analysis Systematic Explorer) score p < 0.1, based on a modified Fisher’s exact test, to determine if submitted genes were significantly more enhanced than the background population, which in this case was the mouse genome. The fold enrichment of genes within the pathway was also calculated against the genome-wide background as described in Huang et al. (2009).

Testing DMRs using real-time qPCR. Quality control of MeDIP was performed by qPCR of highly methylated control regions (H19 imprinted control region [H19], intracisternal A-particle element [IAP]), and CpG-poor regions (unmethylated control sequence [CSA], intergenic3 [interg.3]) as reported in Lempiäinen et al. (2011). Testing was performed using Fast SYBR Green Master Mix (Applied Biosystems, Grand Island, NY) with 500 nM of forward and reverse primers, according to the manufacturer’s instructions. Primers were designed using the region defined by the DMR probe coordinates 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 IP (enriched) DNA of the particular sample to give the %input/IP. The ratio of allergen...
versus vehicle (AOO) was calculated using the %IP/input for each individual allergen-treated sample compared with the average AOO %IP/input. For technical validation of the MeDIP-array, primer sets targeting a selection of the most significantly hypermethylated DMRs (n = 5) and significantly hypomethylated DMRs (n = 5) were designed for both DNCB and TMA. When the ratio of allergen versus vehicle >1, the DMR is designated as hypermethylated; when the ratio of allergen versus vehicle <1, the DMR is designated as hypomethylated. The methylation status of further selected genes/DMRs examined by qPCR are listed in Table 1.

RESULTS

DNCB and TMA Treatment Cause Genome-Wide Changes in Mouse LN DNA Methylation

Mice were exposed topically to the reference contact allergen DNCB or to the reference respiratory allergen TMA under conditions that have been shown previously to result in selective polarized Th1 and Th2 type immune responses, respectively (Cumberbatch et al., 2005; Dearman et al., 2005). Thirteen days after the initiation of exposure to allergen, or to vehicle AOO alone, draining (auricular) LNs were removed, as described in the schema in Figure 1a, and stored in liquid nitrogen. DNA was prepared on an individual animal basis (n = 5 per group) and MeDIP performed. Enrichment before (data not shown) and after WGA (Fig. 1b) was assessed by real-time qPCR directed against known methylated/non-methylated regions (Lempiäinen et al., 2011; Weber et al., 2005). This analysis demonstrated that there was linear amplification with no bias toward heavily methylated/non-methylated regions (data not shown). These quality control measures also demonstrated that there was appropriate enrichment of highly methylated control regions H19 and IAP versus CpG-poor regions (CSA and intergenic3) for individual samples derived after WGA, with the exception of one of the TMA-treated tissue samples that displayed lower levels of enrichment for IAP than the other samples (Fig. 1b). The MeDIP fraction and input fraction were then analyzed by MeDIP-array to profile methylation in response to exposure to either DNCB or TMA. The array covered −8 kb to +3 kb of over 20,718 unique gene promoters, 15,693 CGIs, and 510 miRNA promoters. DMRs were selected based on comparisons of the average log2 fold methylation in the vehicle (AOO) treated tissue (i.e., ∆ log2 5mC [treated – control]), or on comparisons between the two reference allergens, identified as statistically significant by Student’s t-test (p < 0.01). A visual representation of DMR identification is shown in Supplementary figure S2, which illustrates individual regions associated with a selection of DMRs for all three treatment groups. Each DMR was then assigned a methylation status either in relation to vehicle-treated tissue or to the opposing allergen-treated tissue. The scatter plots illustrate the distribution of the DMRs for each comparison and whether the region is hypo- or hyper-methylated with respect to the comparator (Fig. 1c, TMA vs. AOO; Fig. 1d, DNCB vs. AOO; Fig. 2a, DNCB vs. TMA). The majority of hypermethylated loci were found between 0.4 and 0.8 and the hypomethylated loci between −0.2 and −0.8. Consistent with the expected high degree of cell-type and tissue-specificity for DNA methylation profiles, the mouse LN DNA methylome was distinct from a previously reported mouse liver DNA methylome that was profiled using the same promoter microarray platform (Lempiäinen et al., 2011). Further characterization of the tissue-specificity of allergen-induced DNA methylation changes would require assessment of selected DMR in both immune and non-immune tissues isolated concurrently from allergen-treated mice.

Interestingly, treatment with DNCB resulted in a higher frequency of DMRs than did treatment with TMA (taking into account both hyper- and hypomethylated loci), with 2178 compared with 6319 DMRs and a slight bias toward hypermethylation (~1.5:1, p < 0.01). When DNCB-treated tissue was compared directly with TMA-treated tissue (Fig. 2a), approximately fourfold more hypermethylated loci were observed (therefore these regions are hypomethylated following TMA treatment). This suggests that when a locus is differentially methylated between DNCB- and TMA-treated tissue, it is likely to be hypermethylated in response to DNCB and hypomethylated in response to TMA. Consistency analysis of all DMRs (regardless of methylation status; p < 0.01; Fig. 2b) indicated that there were similar numbers of DMRs unique to TMA as there were common to both TMA and DNCB (1074 vs. 976; p < 0.01). Treatment with DNCB induced a larger number of DMRs, of which approximately a sixth were common to both allergens (5284 vs. 976; p < 0.01). Treatment with TMA resulted in 121 DMRs (p < 0.01) that were significantly different from both DNCB and AOO, that is, unique to TMA. This is in contrast to DNCB-treated tissue that displayed fewer DMRs that were significantly different to both TMA and AOO (52, p < 0.01). The seven DMRs that appeared in all three lists were significantly different (p < 0.01) both between allergens and between the two allergens and AOO control. The methylation status of each of the DMRs identified in the consistency analysis (Fig. 2b) is illustrated as a heatmap (Fig. 2c), and demonstrates that there are a number of DMRs for which there are marked differences in methylation between the two allergens, which in some cases leads to a shift in methylation status, i.e., from hypo- to hypermethylated. The heatmap also revealed that there were many qualitative similarities between the allergens, although the fact that these are not captured in the consistency analysis shows that there are also many commonalities between the allergens with respect to induced changes in methylation status.

Prior to technical validation, data generated by the MeDIP-array were searched for known Th1/Th2-related cytokine and transcription factor genes. The master transcription factor T-bet (encoded by tbx21) is responsible for increased expression of ifng and inhibition of il4 and gata3 in Th1 cells. In contrast, Gata3 is responsible for increased expression of il4 and inhi-
bition of ifng in Th2 cells. In DNCB versus AOO comparisons, tbx21 and ifng were associated with hypomethylated DMRs and hypermethylated DMRs, respectively. Whereas gata3 and il4 were not differently methylated in response to TMA, il13 was associated with a hypomethylated DMR. The robustness of the DMRs identified in the MeDIP-array was then validated technically using qPCR directed against a selection of DMRs for each allergen, chosen on the basis of the degree of statistical significance of the changes recorded in the MeDIP-array (Supplementary fig. S1). For each allergen, five primer sets were designed against hypermethylated DMRs (1–5) and five against hypomethylated DMRs (6–10); for details see Supplementary table S1. Of the 10 DMRs chosen for DRCB, only one primer set (primer set 10) failed to show the expected methylation pattern (Supplementary fig. S1a). In contrast, the DMRs chosen for validation of the TMA data displayed more variable levels of methylation when assessed by qPCR (Supplementary fig. S1b). In general, the hypermethylated loci (1–5) performed as expected whereas only one of the hypomethylated loci expressed a marked change (6–10).

Genomic Distribution and Methylation Status of Allergen-Induced DMRs

In addition to the changes in methylation, the MeDIP-array data also included the genomic coordinates of each DMR. These coordinates were used to link each DMR to any gene(s) in close proximity. Following from this, the genes mapped to all significant DMRs (p < 0.01) were annotated with their TSS. The proximity of a DMR to a TSS was then used as the basis for location analysis. Approximately 30% of genes represented by the array were associated with one or more DMRs (TMA vs. AOO 9.4% [1837 DMRs, 1949 genes], DRCB vs. AOO 25% [3370 DMRs, 5283 genes], and DRCB vs. TMA 1.2% [236 DMRs, 254 genes]). These DMRs were then further classified on the basis of their location in relation to the TSS of all proximal genes, as described in the schema in Figure 3a (Thomson et al., 2012). Using Chi² analysis, the expected distribution of DMRs across the gene body was compared with the observed distribution; all were found to be significantly different (Chi² test, p < 0.05). For each of the comparisons, over 51% of genes, more than expected, had a DMR located in the inter-genic region (8 kb to 2 kb upstream of the TSS), with a further 22–27% found in the intra-genic region (250 bp to 3 kb downstream of the TSS) (Fig. 3b). When either allergen was compared with AOO, approximately 7% of DMRs were located in the promoter region, which was somewhat more than would be expected. A further 5–7% of genes associated with either DRCB versus AOO or TMA versus AOO had DMRs in the TSS region (−250 bp to +250 bp of the TSS), again more than predicted. In contrast, for DRCB versus TMA, there were fewer than expected gene-associated DMRs in the TSS region (3%).

Next, in order to focus on DMRs that were most likely to directly affect gene expression, analyses were limited to DMRs associated with one or more gene promoter regions: those within −1000 bp and +250 bp of the TSS (Fig. 3c and d). As shown in Figure 3c, nearly 40% (100) of the gene promoter-associated

### TABLE 1

<table>
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<tr>
<th>Gene</th>
<th>Description</th>
<th>Refseq ID</th>
<th>Distance to TSS (bp)</th>
<th>DRCB</th>
<th>TMA</th>
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<td>( \Delta ) Meth</td>
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</tr>
</tbody>
</table>

**Notes.** Following MeDIP-array analysis of chemical allergen induced changes in DNA methylation, a number of DMRs (p < 0.01; Student’s t-test and a \( p < 0.05 \)) were associated with hypomethylated and hypermethylated DMRs, respectively. The data also included the genomic coordinates of each DMR. These coordinates were used to link each DMR to any gene(s) in close proximity. Following from this, the genes mapped to all significant DMRs (p < 0.01) were annotated with their TSS. The proximity of a DMR to a TSS was then used as the basis for location analysis. Approximately 30% of genes represented by the array were associated with one or more DMRs (TMA vs. AOO 9.4% [1837 DMRs, 1949 genes], DRCB vs. AOO 25% [3370 DMRs, 5283 genes], and DRCB vs. TMA 1.2% [236 DMRs, 254 genes]). These DMRs were then further classified on the basis of their location in relation to the TSS of all proximal genes, as described in the schema in Figure 3a (Thomson et al., 2012). Using Chi² analysis, the expected distribution of DMRs across the gene body was compared with the observed distribution; all were found to be significantly different (Chi² test, p < 0.05). For each of the comparisons, over 51% of genes, more than expected, had a DMR located in the inter-genic region (8 kb to 2 kb upstream of the TSS), with a further 22–27% found in the intra-genic region (250 bp to 3 kb downstream of the TSS) (Fig. 3b). When either allergen was compared with AOO, approximately 7% of DMRs were located in the promoter region, which was somewhat more than would be expected. A further 5–7% of genes associated with either DRCB versus AOO or TMA versus AOO had DMRs in the TSS region (−250 bp to +250 bp of the TSS), again more than predicted. In contrast, for DRCB versus TMA, there were fewer than expected gene-associated DMRs in the TSS region (3%).

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DMRs induced by TMA were also common to DNCB. In contrast, DNCB treatment resulted in nearly four times as many uniquely differently methylated promoter regions compared with TMA treatment. Interestingly, there were no common DMRs between the DNCB versus TMA and TMA versus AOO comparisons, but changes in methylation at seven promoter regions associated with DNCB were significantly different to both AOO and TMA. The differently methylated promoter regions were then segregated on the basis of methylation status (Fig. 3d). The 100 differently methylated promoter regions common to both allergens showed a bias toward hypermethylated DMRs. In contrast, the differently methylated promoter regions unique to either TMA or DNCB treatment showed a bias toward hypomethylation at promoter regions.

**The Methylation Status of Individual DMRs is Biologically Reproducible**

In addition to the technical validation of the MeDIP-array (see Supplementary fig. S1), and in order to assess the biological reproducibility of the DMRs identified, qPCR was used to investigate methylation at individual loci in tissue taken from independent repeat experiments. The DMRs were chosen from the MeDIP-array on the basis of either a large change in methylation (>0.5) following allergen exposure (CGr, plekhm3, acot7, tex10, and dll4) or likely contribution to the polarized immune response (Il13 and Il25) (see Table 1 for gene specific data with respect to identity, statistical significance, ∆methylation, and assigned methylation status). As well using the same DNA input and MeDIP fractions from the MeDIP-array (study 1, n = 5 DNCB/AOO,
FIG. 2. Comparison of DNA methylation changes induced by TMA and DNCB treatment. Data are displayed as a scatter plot, illustrating the distribution of the average methylation status of each DMR (Student’s $t$-test, $p < 0.01$) showing the number of significant hyper- and hypomethylated DMRs for (a) DNCB versus TMA. Hypomethylation is illustrated in blue, hypermethylation in red. The number of unique DMRs identified following treatment with either TMA or DNCB and the overlap of DMRs between allergens was determined by consistency analysis with a cut-off of $p < 0.01$ (b) (Venn diagrams generated using BioVenn; Hulsen et al., 2008). All DMRs ($p < 0.01$) following TMA and DNCB treatment (Venn diagram (b)) were clustered in a heatmap as the average change in methylation ($\Delta \log 2 \, 5mC \, \text{[treated – control]}$) for TMA or DNCB compared with the AOO control ($\log 2 \, \text{treated/IP} / \log 2 \, \text{control/IP}$). Hierarchical clustering of these DMRs is shown on the y-axis. Blue represents hypomethylation (min. $-1.37$ $\Delta \log 2 \, 5mC \, \text{[treated – control]}$) whereas red represents hypermethylation (max. $+1.55 \Delta \log 2 \, 5mC \, \text{[treated – control]}$) compared with the AOO control.

For DNCB versus AOO (Fig. 4b), DMRs associated with CGI, plekhm3 and il25 were hypermethylated in the MeDIP-array and this pattern was reproducible across the three studies when analyzed by qPCR. Likewise, for TMA versus AOO (Fig. 4d), a consistent pattern was seen for DMRs associated with plekhm3, acot7, tex10, and dlg4 (hyp) between the MeDIP-array and qPCR analyses. In contrast, il13 was not detected as a hypomethylated locus when all three studies were combined. Interestingly, like tex10, DMRs that were significantly hypomethylated in TMA were also hypomethylated in DNCB,

$n = 4$ TMA), two independent biological repeat studies (study 2, $n = 5$; study 3, $n = 3$) were analyzed (Fig. 4). Although for some of the DMRs (tex10 and acot7, for example, Fig. 4a and c), there was considerable variation in baseline methylation of AOO samples between, but not within, experiments (shown as %IP/input for vehicle and allergen), the effect of allergen was still consistent across experiments. Thus, significant hypomethylation of tex10 in DNCB samples and of acot7 in TMA samples was recorded. Although in both of the examples illustrated, study 1 (the tissue from the MeDIP-array) displayed the lowest levels of methylation for AOO, this study did not show routinely low methylation status across all genes.

In order to reduce the effect of different vehicle control group methylation baselines in the three studies, the change in methylation was expressed as a ratio of %IP/input for allergen to AOO for the complete data set. This enabled direct comparison of methylation changes across all three studies shown for DNCB versus AOO (Fig. 4b) and TMA versus AOO (Fig. 4c). For DNCB versus AOO (Fig. 4b), DMRs associated with CGI, plekhm3 and il25 were hypermethylated in the MeDIP-array and this pattern was reproducible across the three studies when analyzed by qPCR. Likewise, for TMA versus AOO (Fig. 4d), a consistent pattern was seen for DMRs associated with plekhm3 (hyper) and acot7, tex10, and dlg4 (hypo) between the MeDIP-array and qPCR analyses. In contrast, il13 was not detected as a hypomethylated locus when all three studies were combined. Interestingly, like tex10, DMRs that were significantly hypomethylated in TMA were also hypomethylated in DNCB,
FIG. 3. Genomic distribution DMR induced by exposure to DNCB or TMA. Each DMR (p < 0.01) identified in the MeDIP-array as displayed in Figures 1 and 2 was annotated based on proximity to the TSS of nearby genes. Further analysis was then restricted to those DMRs that were located within −8 kb to +3 kb of a TSS. These DMRs were further grouped by location as illustrated schematically in (a). DMRs were categorized as intra-genic (+250 bp to +3 kb), TSS region (−250 bp to +250 bp), promoter (−1 kb to −2 kb) or inter-genic (−2 kb to −8 kb). Data are displayed for TMA versus AOO, DNCB versus AOO, and DNCB versus TMA (b). The distributions were compared by Chi2 test and were found to be significantly different from the expected distribution (p < 0.05). The total number of gene-associated DMRs (p < 0.01) for each comparison are indicated above the bars in parentheses. Subsequent analyses were restricted to promoter-associated DMRs (within −1000 bp and +250 bp of TSS) (c and d). Consistency analysis was performed on these DMRs (c) to determine the numbers of DMRs that were common to both or unique to either DNCB or TMA treatment (Venn diagram generated using BioVenn; Hulsen et al., 2008). The DMRs that were in common, and those unique to either DNCB or TMA, were then separated by methylation status (d), classified as hypomethylated or hypermethylated with respect to AOO.

despite not reaching the p < 0.01 cut-off in the MeDIP-array. This suggests that there may be a higher proportion of DMRs which are common between allergens that did not reach the level of significance in the MeDIP-array.

Pathway Analysis of Genes with Promoter-Associated DMRs

Genes with hypo/hypermethylated promoter regions (p < 0.01) were uploaded to DAVID for functional analysis (Huang et al., 2009). Then, KEGG pathway analysis was used to visualize pathways that were enriched by either hyper- or hypomethylated genes, as this strategy provided the largest coverage of genes (Table 2). Despite this, only a maximum of 28% of the genes were annotated in the KEGG pathways due to the presence of genes of unknown function within the MeDIP-array. Twenty-six percent (78) of the hypermethylated and 27% (107) of the hypomethylated genes for DNCB and 28% (34) of the hypermethylated and 20% (25) of the hypomethylated genes for TMA (considering for each allergen both unique and shared genes) were annotated in KEGG pathways. Genes common to both allergens were also considered separately: 27% (13) of the hypermethylated and 24% (9) hypomethylated genes were annotated in KEGG pathways. The genes that were not annotated in the KEGG pathway included many not yet associated with published biological pathways and hypothetical or predicted genes. Given the increased number of differently methylated promoter regions uniquely associated with DNCB treatment, it is unsurprising that DNCB is associated with many more KEGG pathways than genes unique to TMA treatment or common to both allergens. As such, changes in methylation at the promoters of genes associated with DNCB treatment are associated with a number of immune-related pathways. Enrichment of genes in the “cytokine-cytokine receptor interaction” pathway (9 genes, 2.7-fold enrichment) and “chemokine signaling” pathways (7 genes, 2.8-fold enrichment) is associated with hypermethylated promoter-associated DMRs in response to DNCB. Further promoter-associated DMRs hypermethylated in response to DNCB are associated with general biosynthesis and cell growth pathways including “steroid hormone biosynthesis” (5 genes, 8.2-fold enrichment), “androgen and estrogen metabolism” pathways (4 genes, 8.9-fold enrich-
FIG. 4. Selected DMR analyzed by quantitative PCR. A number of gene or CpG island (CGI) associated DMRs identified in the genome-wide screen were analyzed by real-time quantitative PCR (qPCR). Tissue was derived from three independent studies; study 1 (•) corresponds with the MeDIP-array, the same DNA being used for both (n = 5 except for TMA = 4 due to insufficient sample); study 2 (■) (n = 5) and study 3 (▲) (n = 3) are independent biological repeats. Data are displayed as the percentage of immunoprecipitated methylated DNA (IP) versus non-immunoprecipitated DNA (input) for (a) tex10 and (c) acot7 in DNCB-treated tissue (a) or TMA-treated tissue (c) in comparison with the control (AOO) and analyzed by two-way ANOVA. The ratio of either (b) DNCB versus AOO or (d) TMA versus AOO was calculated using %IP/input for each mouse for selected genes (see Table 1 for detail). Data from all three studies were then collated and are displayed as Tukey’s box and whisker plots; outliers are shown as •. The status of each gene in the MeDIP-array (Hyper [hypermethylated] p < 0.01; Hypo [hypomethylation] p < 0.01; ns [not significant]) is indicated on the horizontal axis.

DISCUSSION

There is increasing evidence that epigenetic regulation of gene expression plays a pivotal role in the orchestration of immune responses. This includes modulation of the selective development of functional subpopulations of Th cells, such as long-lived memory T lymphocytes, the immunoregulatory activity of Treg cells, monocyte and dendritic cell function, and long-term immunosuppression following sepsis (Carson et al., 2011). In particular, functional subpopulations of CD4+ Th cells, CD8+ Tc cells, and Treg cells determine the quality, vigor, and longevity of immune and allergic responses (Moggs et al., 2012).

Here we have used an in vivo mouse model of chemical allergen induced sensitization to investigate epigenetic mechanisms of gene regulation, the aim being to explore whether such mechanisms are influential in the development of chemical allergy. These data provide novel insights into the pharmacodynamics of chemical allergy and the molecular regulation of the in vivo polarization of immune responses. We have characterized, for the first time, genome-wide DNA methylation changes associ-
TABLE 2

KEGG Pathway Analysis of Promoter-Associated DMRs

<table>
<thead>
<tr>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>Ease p-value</th>
<th>Fold enrichment</th>
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<tr>
<td><strong>DNCB hypermethylated DMRs</strong></td>
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<tr>
<td>Renal cell carcinoma</td>
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<td>Steroid hormone biosynthesis</td>
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<td>22</td>
<td>7.3</td>
<td>0.0130</td>
<td>1.7</td>
</tr>
<tr>
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<td><strong>DNCB hypomethylated DMRs</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Vascular smooth muscle contraction</td>
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<td>1.8</td>
<td>0.0230</td>
<td>3.1</td>
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<td>0.0330</td>
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<tr>
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<td>-</td>
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Notes. Promoter-associated DMRs (within −1000 bp and +250 bp of TSS; p < 0.01) were split into groups depending on their methylation status following either TMA or DNCB treatment as determined in the MeDIP-array. These included DMRs that are common to both allergens. These groups of DMRs were then analyzed functionally using Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang et al., 2009). The analysis criteria of a minimum of two hits per pathway and an Expression Analysis Systematic Explorer (EASE) score of p < 0.1 were applied. The fold enrichment of genes identified in each pathway, in relation to their overall abundance, was also calculated. A fold enrichment of >1.5 is considered to be of significance (Huang et al., 2009). Pathways that were identified for each grouping of DMR, with the number (count) and the percentage (%) of genes from the pathway found in the list, are shown.

aGnRH, gonadotropin-releasing hormone,

bns, not significant.

ated with the initiation of selective immune responses in mouse whole LN populations following skin exposure to a reference contact allergen (DNCB) and a reference respiratory allergen (TMA), revealing unique patterns of DNA methylation associated with divergent T-cell polarization in vivo. Changes were observed in the relative frequency of T and B cells in allergen-activated LNs compared with vehicle controls, with an increase in the percentage of B cells (15% to 45%) and a concomitant reduction in the frequency of T cells (80% to 50%) (data not shown). Importantly, however, there were no differences in the proportion of T and B cells in LNs responding to DNCB or TMA (Goutet et al., 2005). There are changes in the absolute numbers of these cells due to proliferation of allergen-specific cells and the influx of other, non-specific, cells into the LN, which may contribute to some of the observed differences in DNA methylation patterns. Despite these caveats, allergen-specific alterations to the LN DNA methylome have been identified, and these changes are profound enough to be detected despite the changing background of other cell types. Experimental conditions known to provoke divergent immune responses were utilized and the cytokine secretion profiles were confirmed in parallel experiments (data not shown).

As expected, treatment with DNCB altered methylation of the archetypal Th1 genes, ifng and tbx21, with methylation occurring downstream and demethylation upstream of their TSS, respectively. Additionally, the key Th2 gene, il13, was associated with demethylation of a region downstream of its TSS following treatment with TMA. The presence of these DMRs suggests that DNA methylation may modulate expression of these genes. Although it is not feasible to describe every DMR in detail, a number of promoter-associated DMRs unique to DNCB (within −1000 bp to +250 bp of TSS) have been shown to map to functionally relevant genes. For example, a reduction in il27 promoter region methylation was recorded; IL-27 can either promote Th1 responses or exert immunosuppressive functions by inhibiting Th17 cell differentiation and converting activated T cells to Tr1 cells (Yoshida et al., 2009). Methylation of the il27 promoter may be associated with reduced gene expression as there is a known association between silenced gene expression and methylation of the promoter region. However, this would need to be confirmed with concurrent mRNA expression data.

One of the most striking observations is the difference in the number of stastically robust DMRs associated with the different allergens (four times as many for DNCB compared with TMA). It has been demonstrated that there are both genetic and epi-
genetic differences, particularly at the level of DNA methylation, between BALB/c and other mouse strains (Schilling et al., 2009). BALB/c strain mice are known to display a Th2 bias, with high baseline levels of IgE, and are able to quickly and efficiently mount vigorous Th2 responses in order to expel parasitic worm infections (Cliffe and Grencis, 2004). In addition, in the context of chemical allergy, BALB/c strain mice respond very strongly to respiratory allergens in contrast to other mouse strains (De Vooght et al., 2010). Given the preferential Th2 background of BALB/c strain mice, we hypothesize that in order for DNCB to generate a type-1 response in BALB/c mice, alteration of epigenetic markers needs to be more robust than those changes that lead to a type-2 response to TMA. It may be interesting to examine epigenetic changes in more than one mouse model, particularly if differing levels of baseline methylation are demonstrated. This may have further implications with respect to mouse model selection for epigenetic studies of other immune endpoints.

The higher frequency of DMRs associated with DNCB treatment also impacted on pathway analyses. Differently methylated promoter regions were split by their methylation status for pathway analysis, but it should be noted that when hypo- and hypermethylated genes were combined, similar pathways were enriched (data not shown). The number of differently methylated genes associated with KEGG pathways was relatively low compared with the total number of genes highlighted in the study. This is due to the presence of genes of unknown function in the MeDIP-array that are not attributed to any KEGG pathways. As expected, various immune-related pathways were enriched following DNCB treatment. DMRs in the promoter region of the chemokines ccl11, ccl7, cxc1, cxc15, and ccr6 were all hypermethylated, highlighting possible modulation of gene expression in both chemokine and cytokine signaling pathways. Of the non-immune pathways associated with DNCB, many, including all the cancer-related pathways, featured genes also associated with the MAPK signaling pathway, for example, nras and cdc42. Given that MAPK signaling is a very common cellular pathway, those pathways in which only these genes are identified are likely to be non-specific leads. Although the two classes of chemical allergens provoke divergent qualities of immune response, many more general pathways will be regulated in common as both allergens induce T lymphocyte proliferation and activation. For example, enrichment of the “regulation of actin cytoskeleton” pathway by hypermethylated genes has been observed in response to both allergens, suggesting common modulation of gene expression. The presence of these common DMRs is further illustrated by the comparisons shown in the heatmap, although these are often not as statistically robust in TMA-treated tissue. Interestingly, the “olfactory receptor” pathway was associated with both DNCB- and TMA-induced hypermethylation. All of these DMRs are associated with olfactory receptor genes, G-protein coupled receptors that are normally expressed in sensory organs. The significance of these DMRs for LN responses to chemical allergens is not yet clear.

The model and approaches described here also provide an opportunity to explore whether epigenetic mechanisms provide a basis for long-lasting effects on the immune system as well as initial stimulation of a particular quality of immune response. That is, whether exposure to a particular chemical allergen results in epigenetic programming of preferential patterns of T-lineage deployment that influence the development and quality of subsequent immune responses. The 13-day treatment protocol used herein is associated with a polarized response at the protein level. This suggests that these observed epigenetic changes are associated with a memory/recall reaction. It is likely that these epigenetic marks are relatively stable, given that methylation patterns and gene expression have been set for a specific phenotype, but further experimentation is needed to determine the stability and longevity of these marks.

In conclusion, chemical allergens induce specific and unique perturbations in the mouse LN methylome. This not only encompasses alterations in DNA methylation at known immune-related gene promoter regions, suggesting that DNA methylation performs a functional role in the modulation of chemical allergy, but also highlights a number of DMRs for further study in the context of xenobiotic responses.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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