Chronic exposure to water pollutant trichloroethylene increased epigenetic drift in CD4⁺ T cells

Aim: Autoimmune disease and CD4⁺ T-cell alterations are induced in mice exposed to the water pollutant trichloroethylene (TCE). We examined here whether TCE altered gene-specific DNA methylation in CD4⁺ T cells as a possible mechanism of immunotoxicity. **Materials & methods:** Naive and effector/memory CD4⁺ T cells from mice exposed to TCE (0.5 mg/ml in drinking water) for 40 weeks were examined by bisulfite next-generation DNA sequencing. **Results:** A probabilistic model calculated from multiple genes showed that TCE decreased methylation control in CD4⁺ T cells. Data from individual genes fitted to a quadratic regression model showed that TCE increased gene-specific methylation variance in both CD4 subsets. **Conclusion:** TCE increased epigenetic drift of specific CpG sites in CD4⁺ T cells.

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Although genetics clearly contributes to autoimmune disease, environmental stressors appear to provide an even larger role in disease etiology [1]. However, characterizing the events by which environment stressors initiate and maintain the long-term immune alterations associated with disease etiology has been challenging. This is due in part to the fact that autoimmune diseases represent long-term processes that encompass relapsing-remitting and secondary progressive forms [2].

We have been studying the autoimmunity generated by chronic exposure to the industrial solvent and common water pollutant trichloroethylene (TCE). Human TCE exposure (both occupational and environmental) has been linked to several autoimmune diseases (scleroderma, systemic lupus erythematosus, hepatitis and diabetes [3–11]) and hypersensitivity disorders [12–14]. By using a female MRL^{+/+} mouse model, we have shown that chronic exposure to TCE during adulthood

promotes autoimmune hepatitis that corresponds to idiopathic autoimmune hepatitis in humans, that is, T-cell infiltration of the liver and development of antibodies specific for liver microsomal proteins [15,16]. TCE exposure appears to predominantly impact CD4⁺ T cells, with effects on cellularity manifested as increased percentages of effector/memory CD4⁺ T cells with altered cytokine production profiles [15,17].

Autoreactivity in CD4⁺ T cells is becoming increasingly associated with epigenetic alterations. For example, aberrant DNA methylation in CD4⁺ T cells has been noted in idiopathic autoimmune diseases, including primary biliary cirrhosis, Sjogren's syndrome, systemic lupus erythematosus and juvenile idiopathic arthritis [18–20]. Most of these alterations involve demethylation of genes that encode for cytokines, chemokines or adhesion molecules that promote autoimmunity, such as *CXCR3*, *LTA*, *IL32*, *IRF7*, *HERVE*, *ITGAL* (*CD11a*), *PERFORIN*,

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CD70, and *CD40L* [21–24]. The events that initiate these disease-related epigenetic alterations are not clear, but in some cases appear to involve a reduction in DNA methyltransferase activity [24]. A more direct illustration of the role of epigenetic alterations in autoreactivity is the finding that CD4⁺ T cells exposed to the demethylating agent 5-azacytidine decreased *Dnmt1* expression, increased expression of numerous genes and became autoreactive in adoptive transfer [25].

We have some evidence that TCE exposure can generate epigenetic alterations. Particularly susceptible targets for epigenetic alterations include retrotransposons and associated metastable epialleles. These genes are usually silenced by hypermethylation, and perinatal environmental exposures have been shown to impact their epigenetic marks and subsequent gene expression [26]. Along these lines, tail tissue samples from mice perinatally exposed to lead showed increase methylation in the *Cabp*^{JAP} metastable epiallele [27]. Although it is likely that developmental exposure to toxicants would have a more robust and long-term effect on epigenetic alterations, adult exposures could also be capable of altering DNA methylation. We have reported alterations in the expression of intracisternal A-particle transposon in CD4⁺ T cells from autoimmune-prone MRL^{+/+} mice exposed to TCE during adulthood [28]. However, this previous examination of TCE-induced effects of epigenetics was indirect, and confined to global DNA methylation. The current study was designed as an important extension of the earlier experiment, and examined the impact of adult TCE exposure on the methylation of specific CpG sites in the promoters of genes important for CD4⁺ T-cell function. Individual variability in terms of DNA methylation can be both age- and cell type-specific [29]. Consequently, we examined gene-associated CpG sites in both naive and effector/memory CD4⁺ T cells isolated from MRL^{+/+} mice every 6 weeks during a 40 week exposure to TCE. CpG site-specific increases or decreases in methylation induced by TCE were tested using a probabilistic model and quadratic regression models.

In addition to a measurable TCE-induced increase or decrease in specific gene-associated DNA methylation, we also examined the impact of TCE on the subtle changes in DNA methylation known as ‘epigenetic drift’. Epigenetic drift has been attributed to a stochastic process that generates an ongoing series of local methylation variations due to reduced stringency in epigenetic maintenance that occurs over time [30]. However, there is evidence that these stochastic events are constrained by genetic polymorphisms and environmental exposures that impact the local activity of DNA methyltransferases and DNA demethylases [31,32]. The identities of different environmental stressors that

impact epigenetic drift are still being defined, but appear to include immunotoxicants such as lead [27], and tobacco smoke [33]. Epigenetic drift may play a role in the age-dependent increase in interindividual variability in the DNA methylation of specific CpG sites [29], as well as the discordant DNA methylation patterns in monozygotic twins [34]. It may also explain the progressive increase in methylation heterogeneity observed in immortalized cells cultured *in vitro* [30]. If exposure to toxicants, such as TCE, promotes epigenetic drift with cumulative effects on DNA methylation and associated gene expression, this phenomenon could conceivably, over time, generate a population of CD4⁺ T cells with an autoreactive phenotype.

Materials & methods

Ethics statement

All work was approved by the Animal Care and Use Committee at the University of Arkansas for Medical Sciences, and conformed to the USDA Animal Welfare Act and Regulations.

Mouse treatment

Eight week-old female MRL^{+/+} mice (Jackson Laboratories, ME, USA) were exposed to TCE as previously described [28]. The mice (8–9 mice/treatment group/time point) received 0 or 0.5 mg/ml TCE in their drinking water for 4, 10, 16, 22, 28, 34 or 40 weeks. Mice received drinking water and food (Harlan 7027) *ad libitum*.

CD4⁺ T cells

Mice were sacrificed at different time points, and splenic CD4⁺ T cells were isolated using Dynabeads FlowComp Mouse CD4 kit (Invitrogen, ThermoFisher Scientific, MA, USA). The CD4⁺ T cells were then further separated into CD62L^{lo} or CD62L^{hi} CD4⁺ T-cell populations using Dynabeads M-280 Streptavidin (Invitrogen) conjugated with biotinylated anti-CD62L antibody (eBiosciences, 13-0621-85, CA, USA). The resulting CD62L^{hi} CD4⁺ T cells (naive CD4⁺) or CD62L^{lo} CD4⁺ T cells (effector/memory CD4⁺ T cells) were stimulated with immobilized anti-CD3 antibody and anti-CD28 antibody for 18 h as described [35], and the activated CD4⁺ T cells were frozen for subsequent examination of DNA methylation. The CD4⁺ T cells were activated *in vitro* prior to the assessment since it is the DNA methylation state of activated CD4⁺ T cells that has the most functional significance. To ensure sufficient cells for the analyses each sample of CD4⁺ T cells used in the study originated in an equal number of pooled spleen cells from two to three mice resulting in four samples per time point within each treatment group.

DNA methylation

Targeted bisulfite next-generation sequencing (NGS) was performed at the Molecular Cytogenetics and Epigenetics facility of Columbia University. Amplicons were generated on a Fluidigm Access Array and sequenced using an Illumina MiSeq platform. Sequence alignment to chromosomal locations (mouse genome build mm10) and DNA methylation levels were determined using Bismark [36,37]. The degree of methylation is expressed as the percentage of total cytosines methylated at individual CpG sites. For most CpG sites, hundreds of reads per sample were used for each percentage methylation determination. In all cases, at least ten reads/CpG site/sample were used. The location of the gene-specific CpG sites analyzed relative to transcription start sites is shown in [Figure 1](#). Only the CpG sites with methylation data in this study are shown. Some intervening CpG sites are not included for technical reasons such as too few reads.

Statistics

The data are presented as means $\pm$ standard deviations. The threshold for statistical significance was set at $p < 0.05$. Differences between experimental groups were tested first with analysis of variance, and where the F test was significant, subsequent pairwise contrasts were tested using a two-sample t-test. In order to test the difference between TCE and control mice in terms of DNA methylation variance, quadratic regression models including the quadratic and linear interaction between groups (TCE vs control) and average methylation were fit to the methylation data within each gene and cell type (effector/memory and naive). A partial group F-test was used to test the significance of the quadratic and linear interaction terms. If significantly different, then the relationship between methylation variability and average methylation, as indicated by the parabola shape, is significantly different between TCE and control mice. Furthermore, the quadratic terms of average methylation in both TCE and control groups were tested for significance. If the coefficient was significant, then the quadratic regression model was a good fit to describe the hypothesized parabolic relationship.

Modeling epigenetic drift

Methylation data from biological samples has been modeled using a binomial distribution to help overcome issues associated with unstable variance estimation [38,39]. Using a similar approach in the current study, the methylation process at each CpG site was assumed to be a Bernoulli process with exactly two possible outcomes: ‘methylation’ and ‘demethylation’, with methylation occurring with a probability p and

demethylation with a probability of $1-p$ where $p \in [0,1]$ is the success (methylation) parameter of the process.

Considering the number of individual CpG sites examined the fraction methylated, f , would approximate the probability, p . Under these assumptions, the predicted variance in M , the methylation state of a given CpG site, would follow the relation:

$$\text{var}[M] = f \cdot (1 - f)$$

For the purposes of the present investigation, this form was generalized slightly to the following:

$$\text{var}[M] = (1 - \alpha) \cdot f \cdot (1 - f)$$

where α is a parameter to be determined from the data. It is indicative of the departure from a strict Bernoulli process; the larger the value, the greater the departure from a random or probabilistic methylation variance. The software used was Python 2.7.8 and Statsmodels 0.6.1.

Results

Gross changes induced by chronic trichloroethylene exposure

Similar to previous studies, TCE enhanced the age-dependent increase in the number of CD62L^{lo} effector/memory CD4⁺ T cells in the spleen ([Figure 2](#)). Also similar to earlier studies, chronic 40-week exposure to TCE did not inhibit weight gain. TCE exposure from water consumption (0.5 mg/ml TCE) was weight-dependent, but averaged 40–50 mg/kg/day. The current 8-h Permissible Exposure Limit (established by the Occupational Safety and Health Administration [OSHA]) for TCE is 100 ppm or approximately 76 mg/kg/day.

DNA methylation of CpG sites associated with cytokines in CD4⁺ T cells

Targeted bisulfite NGS was used to examine TCE- and time-dependent changes in DNA methylation in CD4⁺ T cells. Since DNA methylation patterns can vary considerably among different immune cell types (e.g., CD4⁺ T cells vs CD8⁺ T cells vs B cells) [29], the analysis was conducted using separate populations of naive and effector/memory CD4⁺ T cells collected at 6-week intervals during the 40-week TCE exposure. It has been reported that the majority of differentially methylated genes in CD4⁺ T cells do not contain promoter CpG islands [40]. Consequently, we did not focus on CpG islands, but instead targeted regions where methylation of individual CpGs has been shown to regulate gene expression. The 16 genes examined were selected because they have previously been shown to be regulated by DNA methylation, or were shown above to be differentially expressed in association with TCE exposure.

Figure 1. Location of assayed CpG sites in the selected genes. The bent arrow designates the transcription start site and the direction of transcription. Parentheses indicate chromosome location of the genes. TSS: Transcription start site.

Time-dependent DNA methylation levels of individual CpG sites associated with ten representative genes are illustrated in Figure 3A–D. These include 15 CpG sites proximal to the transcription start site for *Ifng*. CpG sites in the *Ifng* promoter displayed consistently low-to-medium (under 40%) levels of DNA methylation regardless of CD4⁺ T cell subset, mouse age or TCE exposure. In contrast, CpG sites in the exon and intron uniformly demonstrated intermediate-to-high (over 60%) levels of DNA methylation. When examined individually, none of the CpG sites associated with *Ifng* demonstrated a consistent time-dependent increase or decrease in methylation in either naive or effector/memory CD4⁺ T cells.

Time-dependent changes in the DNA methylation of CpG sites associated with other functionally important genes were also compared in both effector/memory and naive CD4⁺ T cells from control and TCE-treated mice (Figure 3A & B). Several sites in the promoter for *Il4* displayed low-to-mid range levels of DNA methylation. The level of DNA methylation at these sites in the *Il4* promoter was higher in naive CD4⁺ T cells, but DNA methylation at those sites was not consistently altered by age or TCE treatment in either naive or effector/memory CD4⁺ T cells. DNA methylation at sites in the *Il5* promoter was consistently high in both CD4⁺ T-cell subsets at all time points, and the same was true for CpG sites associated with the *Il17* gene (Figure 3A & B). In terms of the *Tnf* promoter, CpG sites distal to the transcription start site were uniformly hypomethylated, while those proximal to the transcription site mostly displayed medium-to-high levels of DNA methylation. This methylation profile for the *Tnf* promoter sites did not vary regardless of CD4⁺ T-cell subset, TCE treatment, or age of the mouse.

Several CpG sites in both the promoter and first exon of *Lif* displayed variable mid-range levels of DNA methylation, which were maintained for the duration of the study (Figure 3A & B). In the *Cdkn1a* gene, sites in a CpG island in the promoter were hypomethylated, while those CpG sites in the first exon were hypermethylated (Figure 3C & D). Once again, the DNA methylation profile for *Cdkn1a* was not impacted by age or differentiation state. CpG sites in the promoter and first exon of *Ctla4* were mostly hypomethylated with a few more distal to the transcription start site showing higher levels of DNA methylation. Several other sets of CpG sites were either consistently hypermethylated (*Faim2*) or hypomethylated (*Dnmt2*, *Dnmt3a*, *Pdcd1*, *Pdcd2*, *Pdcd4* and *Myc*) (data not shown). These profiles were tightly maintained in both CD4⁺ T-cell subsets for the duration of the 40-week exposure. Thus, with the exception of *Il4*, the methylation profiles of the CpG sites examined were very similar when com-

Figure 2. Impact of chronic trichloroethylene exposure. Female MRL+/+ mice were exposed to TCE (0.05 mg/ml) for 4–40 weeks. (A) TCE exposure was determined based on water consumed and bodyweight. (B) The numbers of naive (CD62L^{hi}) and effector/memory (CD62L^{lo}) CD4⁺ T cells in spleens after 4 or 40 weeks of TCE exposure is described here.

*Significantly different ($p < 0.05$) compared with control values.

TCE: Trichloroethylene.

paring naive and effector/memory CD4⁺ T cell that had been activated *in vitro*. In addition, the general methylation profiles remained stable over time, and no consistent increases or decreases in DNA methylation of individual CpG sites could be attributed to TCE exposure.

Effects of trichloroethylene on DNA methylation variance

The mean DNA methylation levels of the individual gene-associated CpG sites examined were relatively stable. However, methylation is subject to intra-individual changes, that is, epigenetic drift. We asked whether exposure to TCE could alter methylation variance in samples matched for cell type and age. Toward this end a simple probabilistic model was developed.

Figure 3. Mean methylation of gene-associated CpG sites in both effector/memory and naive CD4⁺ T cells. Average DNA methylation at CpG sites in multiple cytokine genes in effector/memory (A) and naive (B) CD4⁺ T cells, as well as other functionally important genes in effector/memory (C) and naive (D) CD4⁺ T cells, from control or TCE-treated mice was determined. Although this data was collected at all seven time points, only the results from the 4- and 40-week time points are shown. The arrows point in the direction of transcription. The CpG sites are designated by chromosome position. TCE: Trichloroethylene.

Figure 3. Mean methylation of gene-associated CpG sites in both effector/memory and naive CD4⁺ T cells (cont.).

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Figure 4. Modeling variance of DNA methylation. Mean inter-sample methylation variance at all the CpG sites for all the genes examined, except *Ifng*, at all time points in naive and effector/memory CD4⁺ T cells from control or TCE-treated mice. The solid line and dashed lines represent model (Equation 2) that fits to the naive and memory cell data, respectively, while the dotted line represents predictions based on Equation 1. TCE: Trichloroethylene.

This model used cumulative data from all the CpG sites examined across all time points with separate analyses conducted for both cell types and treatments. Intron, exon and promoter data were grouped together. Differences in α between different sets of data are expected to reflect differences in how the methylation process is mediated. Results from these parameter estimation studies are shown in Figure 4. Table 1 presents the resulting values for α , along with the number of data points, n , and the coefficient of determination, R^2 , and the 95% CI.

As indicated in Figure 4, total methylation variance detected in both naive and effector/memory CD4⁺ T cells, regardless of TCE treatment, was substantially lower than the maximal possible variance that would occur if the degree of methylation was random. The α values indicate that naive CD4⁺ T cells from both control and TCE-treated mice experienced more

constraints (greater departure from random) on methylation variance than effector/memory CD4⁺ T cells. However, exposure to TCE decreased the control on DNA methylation variance (i.e., decreased the departure from random) found in naive CD4⁺ T cells. Thus, DNA methylation patterns in CD4⁺ T cells appear to be fairly rigid with natural constraints that prevent totally random methylation and demethylation. However, small but significant alterations in methylation variance, indicating less constraint and greater randomness, are associated with differentiation stage and TCE exposure.

Effects of trichloroethylene on DNA methylation variance of individual genes

To examine how well the gene-associated CpG sites conformed to the model described above, the intersample variance within a treatment group and CD4⁺

Table 1. Cumulative values of α derived from methylation variance.		
CD4 T-cell subset	Control	Treatment
Naive	0.742	0.693
	$n = 354$; $R^2 = 0.81$; 95% CI (0.731–0.754)	$n = 349$, $R^2 = 0.82$; 95% CI (0.679–0.707)
Memory	0.642	0.627
	$n = 354$; $R^2 = 0.80$; 95% CI (0.625–0.659)	$n = 349$, $R^2 = 0.80$; 95% CI (0.609–0.644)
<small>n: Number of data points; R^2: Coefficient of determination</small>

Figure 5. Methylation variance at gene-specific CpG sites in naive and effector/memory CD4⁺ T cells. Scatter plots show the correlation between the mean intersample variance of DNA methylation at specific CpG sites (across all time points) and the mean DNA methylation observed at the same specific sites in effector/memory **(A)** and naive **(B)** CD4⁺ T cells. Each point represents a specific CpG site. TCE: Trichloroethylene.

Figure 5. Methylation variance at gene-specific CpG sites in naive and effector/memory CD4⁺ T cells (cont.)

T-cell subset was calculated as the statistical variance of methylation values across individual gene-specific CpG sites over all time points.

Multiple linear regression analyses showed that intersample methylation variance at the CpG sites examined in effector/memory and naive CD4⁺ T cells correlated with distance to either end of the 0–100% methylation scale (**Figure 5A & B**). As expected, the most variance was observed at those CpG sites with mid-

range levels of DNA methylation. TCE-induced differences in the pattern of this relationship, as indicated by the significance of quadratic and linear interaction terms of group and average methylation, were observed in some genes, but not all (Table 2). Specifically, in effector/memory CD4⁺ T cells of *Cd70*, *Tnf*, *Dnmt3a* and *Ctla4* genes, and naive CD4⁺ T cells of *Cd70*, *Tnf*, *Dnmt3a*, *Il4*, *Lif* and *Cdkn1a* genes, a significant difference in parabolic shape was observed between

Table 2. Quadratic regression models for DNA methylation variance and average methylation at individual CpG sites in CD4⁺ T cells.

Genes	Q or L fit		p-value of interaction terms	Difference between control and TCE?
	Control	TCE		
Effector/memory				
<i>Cd70</i>	Q	Q	<0.001	Y
<i>Lif</i>	Q	Q	0.21	N
<i>Tnf</i>	Q	Q	<0.01	Y
<i>Il4</i>	Q	Q	0.46	N
<i>Cdkn1a</i>	Q	Q	0.05	N
<i>Dnmt3a</i>	Q	Q	<0.001	Y
<i>Ctla4</i>	Q	Q	0.02	Y
<i>Ifng</i>	L	L	0.32	N
Naive				
<i>Cd70</i>	L	Q	<0.001	Y
<i>Lif</i>	Q	Q	0.01	Y
<i>Tnf</i>	Q	Q	<0.001	Y
<i>Il4</i>	Q	Q	0.04	Y
<i>Cdkn1a</i>	Q	Q	<0.001	Y
<i>Dnmt3a</i>	Q	Q	<0.001	Y
<i>Ctla4</i>	Q	Q	0.06	N
<i>Ifng</i>	L	L	0.16	N
L: Linear; TCE: Trichloroethylene; Q: Quadratic.				

L: Linear; TCE: Trichloroethylene; Q: Quadratic.

TCE and control mice. Thus, TCE exposure altered the relationship between mean CpG methylation and methylation variance.

For the *Ifng* gene in effector/memory CD4⁺ T cells, the intersample variance of individual CpG sites did not correlate with average percent methylation in the same way as the other genes (Figure 5A). Comparing genes where the full range of average methylation was observed (0–100%), all genes followed a parabolic shape, except for *Ifng*, where a quadratic term of average methylation is tested to be nonsignificant. In other words, methylation of the CpG sites associated with *Ifng* appeared to be regulated differently from those of other genes in which epigenetic marks were largely determined by distance to either end of the mean methylation scale.

Discussion

The analysis examined DNA methylation in individual subsets of effector/memory and naive CD4⁺ T cells from female MRL+/+ mice during a 40-week exposure to TCE. Using this approach, adult exposure to TCE was not shown to induce consistent changes, either hypo- or hypermethylation of specific CpG sites

in effector/memory or naive CD4⁺ T cells. It should be noted that one limitation of the methods used is that insufficient NGS data were obtained for some gene regions so that not all of the desired CpG sites could be included, and for that reason, one entire gene, *Il2*, was excluded from the analysis. Another limitation was the need to pool spleen cells from 2–3 mice in order to obtain sufficient numbers of both effector/memory and naive CD4⁺ T cells. Although this eliminated possible confounding effects that might result from different percentages of the two subsets in a sample, it may have decreased the sample size/time point that would be necessary to detect subtle consistent changes in DNA methylation of individual CpG sites.

Although a relatively low exposure to TCE during adulthood did not consistently alter the methylation profile of specific CpG sites, TCE did alter intersample variability. With the exception of *Ifng*, when all other genes were combined in the probabilistic model, TCE increased variance in naive CD4⁺ T cells. A similar trend was seen in effector/memory CD4⁺ T cells, although it did not reach statistical significance. Analysis of variance of individual genes also demonstrated that TCE significantly altered DNA methylation

variance of *Cd70*, *Tnf* and *Dnmt3a* in both naive and effector/memory CD4⁺ T cells.

Most CpG sites of the genes examined displayed either 0–20% or 80–100% methylation. This pattern was very stable over time, and variation in the DNA methylation of a particular site was largely due to stochastic effects related to mean methylation of the site. Thus, variance for sites that averaged 0–20% or 80–100% methylation was uniformly low. The highest variance in DNA methylation was detected at CpG sites where the average methylation was intermediate rather than low or high. This phenomenon has been described by others, and is related to the fact that methylation is measured on a finite scale that ranges from 0 to 100%. A mean methylation of 50% could encompass values at both ends of the scale with potentially large variance, while a mean methylation of 95% could only be achieved if the lowest values were close to the highest values, that is, encompassed low variance. The fact that CpG sites with intermediate levels of methylation experience the most variance may have important mechanistic implications, in that such sites may be particularly susceptible to extrinsic factors with the capacity to either increase or decrease DNA methylation.

Similar to our findings, Jacoby *et al.* found that the highest variability in DNA methylation for eight immune genes examined in human CD4⁺ T cells occurred at CpG sites where average methylation levels were intermediate rather than high [29]. In terms of specific cell types, they noted that the interindividual variability for human adult blood CD4⁺ T cells was significantly less than CD8⁺ T cells and significantly more than CD14⁺ monocytes. Considerable variability was also noted in unseparated peripheral blood mononuclear cells (PBMC), and was associated with relative levels of different cell populations, most notably the percentage of CD56⁺ natural killer cells. PBMC represent the human tissue most often used for routine evaluation of immune function including CD4⁺ T cells. Although they do demonstrate methylation variance based on cellularity, PBMC have been shown to be more constrained than buccal epithelial cells in terms of the range of methylation variance and the number of highly variable CpG sites [41]. Thus, relatively speaking, DNA methylation variance seems to be tightly controlled in human CD4⁺ T cells. The murine CD4⁺ T cells examined in the current study were also very stable over time, and seem to reflect constraints on random methylation and demethylation, even at those CpG sites with mid-range levels of methylation. However, even within these constraints there appears to be a dynamic role for DNA methylation in CD4⁺ T cells

that results in a certain level of interindividual methylation variance susceptible to alterations by TCE exposure. Therefore, even adult exposure to certain toxicants may have the capacity to subtly alter DNA methylation by increasing methylation variance.

The CpG sites in the *Ifng* promoter were the only ones examined that did not conform to the model, in which methylation variability was linked to mean methylation levels. This finding is perhaps not surprising considering the evidence demonstrating the susceptibility of these CpGs to external factors. Theoretically, DNA methylation patterns in most mature cells are mitotically stable. However, in the case of the immune system it is clear that extrinsic factors can modulate methylation in mature CD4⁺ T cells. These factors include the cytokines that drive differentiation from naive CD4⁺ T cell to Th1 or Th2 cell, and in many cases these factors target methylation of *Ifng*. Proximal CpGs in the *Ifng* promoter are hypomethylated in mature naive and differentiated Th1 cells, but are hypermethylated in differentiated Th2 cells [42,43]. It has been reported that DNA methylation in a relatively small region of the *Ifng* promoter (that includes the -53, -205 and -297 CpG sites) controls promoter-driven *Ifng* expression during CD4⁺ T-cell differentiation [42,44]. In addition to the cytokines secreted during Th2 cell differentiation, certain environmental events such as adult exposure to diesel exhaust or developmental exposure to polycyclic aromatic hydrocarbons can alter epigenetic marks in the *Ifng* promoter [25,45,46]. Low to moderate levels of methylation on the *Ifng* promoter such as we report here following TCE exposure may allow the gene to remain ‘poised’ for varying levels of transcription without being silenced.

Conclusion

In this study we examined for the first time gene-specific and time-dependent changes in DNA methylation in CD4⁺ T cells from female MRL+/+ mice exposed as adults for 4–40 weeks to TCE in drinking water. TCE exposure increased DNA methylation variance in both naive and CD4⁺ T cells over the 40-week time course. Thus, adult exposure to the immunotoxicant TCE increased methylation variance in the promoters of genes important for CD4⁺ T cell function, thereby providing a possible mechanism for TCE immunotoxicity.

Future perspective

Stochastic alterations in CpG methylation overlaid by genetic and extrinsic factors that control DNA methyltransferases and DNA demethylases are thought to work together to generate a ‘normal’ age-specific pattern of

DNA methylation variance. This pattern is tissue-specific, and in humans is often assessed using peripheral blood lymphocytes, including CD4⁺ T cells. Variations in the shape of the pattern measured in peripheral blood CD4⁺ T cells may be useful in detecting increased susceptibility to immune pathology. Studies are needed to determine in which genes alterations in DNA methylation variance are most predictive of future autoimmune diseases.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Both naive and effector/memory CD4⁺ T cells were isolated from the spleens of female MRL^{+/+} mice exposed as adults to trichloroethylene (TCE) in drinking water (0.5 mg/ml) for 4, 10, 16, 22, 28, 34 or 40 weeks.
- DNA methylation of individual CpG sites associated with 17 different genes was examined in both populations of CD4⁺ T cells.
- No consistent increase or decrease in the methylation of specific CpG sites was associated with mouse age or TCE treatment.
- When the CpG sites were considered cumulatively, TCE did decrease the tight DNA methylation control found in CD4⁺ T cells.
- Examination of individual genes showed methylation variance correlated with distance to either end of the 0–100% methylation scale; TCE disrupted this correlation in several genes important in both naive and effector/memory CD4⁺ T cells.
- Unlike the CpG sites for all the other genes examined, DNA methylation variance and average DNA methylation were not correlative for *Ifng*.
- TCE altered DNA methylation variance in genes important for CD4⁺ T-cell function.

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