Chapter 2
Trichloroethylene and Autoimmunity in Human and Animal Models

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Abstract Based on likelihood of exposure and potential health impact trichloroethylene (TCE) is consistently ranked 16th out of 275 chemicals on the annual CERCLA (Comprehensive Environmental Response, Compensation, and Liability Act) list of hazardous substances. Although environmental contact with TCE in the water, air or soil, is generally thought to be risk-free, there is evidence that chronic exposure to TCE at levels too low to be overtly toxic can generate autoimmune diseases including lupus, scleroderma, and autoimmune liver disease. This chapter examines human exposure data. It also discusses the mechanistic information that has been provided by animal studies, and identifies some important gaps in our understanding. Since human exposure to TCE will continue for the foreseeable future, we need to understand and prevent the autoimmune-promoting effects of this toxicant.

Keywords Autoimmune disease • Immunotoxicity • $CD4^+$ T cells

2.1 Introduction to Autoimmune Disease

The immune system is supposed to be restricted to recognizing and attacking foreign antigens such as disease-causing micro-organisms. If the immune system instead attacks self-antigens chronic incurable disorders characterized as autoimmune diseases occur. There are over 80 different autoimmune diseases, and at least one for every organ system in the body. The NIH estimates up to 23.5 million Americans have at least one type of autoimmune disease. In comparison, cancer

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affects up to 9 million and heart disease up to 22 million. The most prevalent of the more than 80 autoimmune diseases identified include Type 1 diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, Sjogren's Syndrome, and the several types of autoimmune thyroid disease. Most of these diseases are found much more often (3–10 fold) in women. Some autoimmune diseases are life-threatening; all are debilitating and require lifelong medical care.

There is much we do not know about autoimmune disease. We have been most successful at identifying the type of immune pathology (e.g. autoantibody vs T cell-mediated) associated with a particular autoimmune disease, and sometimes characterizing the specific autoantigens targeted. This information has been used to classify autoimmune diseases as type II, III or IV hypersensitivity reactions. However, this is only somewhat useful, since many autoimmune diseases involve more than one type of immune pathology. Even if we can document the type of immune pathology associated with a particular autoimmune disease, we don't know what initiates this pathology. Studies involving identical twins have provided some useful hints in this regard. Even though autoimmune diseases as a group affect between 5 and 8 % of the population in the US, the incidence for any one autoimmune disease is relatively rare. Thus, the fact that the concordance rate for developing a particular autoimmune disease in identical twins is much higher than the general population demonstrates the involvement of genetic susceptibility (He et al. 2001). On the other hand, the finding that the concordance rate is not 100 %, and is indeed usually much less than 50 % for any autoimmune disease, demonstrates that environmental factors also contribute to disease etiology. The environmental contribution to autoimmune disease is a relatively vague and wide-ranging concept that has come to include lifestyle (e.g. diet) and history of bacterial and/or viral infection. It also includes exposure to environmental chemicals which impact the immune system. One such chemical, trichloroethylene (TCE), will be examined here for its contribution to autoimmune disease. The current state of knowledge will be outlined as will the information gaps that need to be filled.

2.2 TCE and Autoimmunity/Hypersensitivity in Humans

As noted by a National Research Council report evidence on human health hazards from TCE exposure, either occupational or environmental, has strengthened in recent years (Committee on Human Health Risks of Trichloroethylne 2006). One of the predominant non-cancer outcomes associated with TCE exposure in humans is immunotoxicity, most notably the development of hypersensitivity responses. Although not all types of TCE-induced hypersensitivity has been classified as autoimmune, at least some of the hypersensitivity responses induced by TCE clearly mimic idiopathic autoimmune diseases.

The links between autoimmune disease and TCE were originally described in humans exposed to the chemical at work. Going back to the 1970s numerous case reports have correlated sometimes fatal systemic or localized sclerosis or diffuse

fasciitis with industrial TCE exposure (Czirjak et al. 1994; Flindt-Hansen and Isager 1987; Karamfilov et al. 2003; Lockey et al. 1997; Pralong et al. 2009; Saihan et al. 1978; Waller et al. 1994). Systemic sclerosis, also known as scleroderma, is an autoimmune disease of normally unknown etiology. The autoimmune response targets connective tissue of the skin, internal organs and the walls of blood vessels. It is characterized by alterations of the microvasculature and by massive deposition of collagen and other matrix substances in the connective tissue. At least three case control studies of men or women with scleroderma identified TCE exposure in occupational or hobby settings as a likely risk factor (Diot et al. 2002; Garabrant et al. 2003; Nietert et al. 1998). Possible mechanisms by which TCE triggers scleroderma are not known.

Scleroderma is not the only autoimmune disease associated with TCE exposure. A cohort study of people living near a TCE-contaminated Superfund Site in New York demonstrated an increased prevalence of the autoimmune disease primary biliary cirrhosis (Ala et al. 2006). In another study TCE-exposed individuals from metal industries were shown to have increased urine levels of N-acetyl-beta-D-glucosaminidase, a marker of autoimmune lupus nephritis (Brogren et al. 1986). Case reports from around the world have also linked chronic occupational TCE exposure to sometimes fatal non-viral hepatitis that is worsened by rechallenge (Anagnostopoulos et al. 2004; Joron et al. 1955; McCunney 1988; Pantucharoensri et al. 2004; Schattner and Malnick 1990). Although the rechallenge exacerbation of this TCE-induced hepatitis suggests an immune component, this aspect of the disease was not tested.

There are several studies which have linked TCE exposure to the generation of autoantibodies, biomarkers of an autoimmune response if not actual autoimmune disease. Between 1964 and 1979 domestic water supplies in East Woburn, MA, were unknowingly contaminated with industrial solvents, with TCE as the main volatile organic found (267 ppb). Five years after the wells were closed individuals from East Woburn demonstrated increased numbers of total T cells (both $CD4^+$ and CD8) and increased incidence of anti-nuclear antibodies compared to controls (Byers et al. 1988). A cohort study of individuals exposed to TCE in contaminated well water in Arizona demonstrated significantly increased levels of anti-nuclear antibodies and increased ARA (American Rheumatism Association) scores for lupus (Kilburn and Washaw 1992). A recent serological proteome analysis showed that sera from patients with active TCE-induced hypersensitivity, unlike control sera, contained antibodies specific for several ontologically diverse self antigens including NM23 (nucleoside disphosphate kinase), and lactate dehydrogenase B (Liu et al. 2009). Interestingly, although TCE appeared to increase the levels of specific autoantibodies, it has also been shown to decrease serum levels of total IgG and IgM (Zhang et al. 2013). The mechanism by which TCE exposure activates specific antibodies or alters total immunoglobulin, and their functional significance, remains to be determined.

Even if overt autoimmune pathology was not revealed (in many cases not examined) other epidemiological studies have demonstrated TCE-induced immunotoxicity. Data collected from subjects who had worked at least 3 years in the in the

printing industry showed that levels of TCE in the breathing zone and levels of a TCE metabolite in urine correlated with increased serum levels of T cell-derived cytokines IL-2 and IFN-γ and decreased levels of IL-4 (Iavicoli et al. 2005). TCE has also been shown to induce a hypersensitivity disorder that targets the skin and liver (Bond 1996; Xu et al. 2009a). The number of patients suffering from occupational TCE-related severe skin disorders has been increasing in areas where TCE is still widely used as a solvent, including the Philippines, Taiwan, Singapore, and the Guangdong Province, China. The clinical manifestations are different from irritating contact dermatitis caused by TCE defatting action. Instead, the subjects experience a relatively long period of exposure before disease onset, rash, fever, lymphadenopathy, liver dysfunction and recurrence after just minimal re-exposure (Nakajima et al. 2003). The TCE-induced dermatitis is considered to be a T cell-mediated type IV hypersensitivity disease. Although the pathology appears to be immune mediated, it is not clear whether the immune response is directed toward self. More information about this type of TCE-induced hypersensitivity will be provided in Chap. 3.

2.3 Xenobiotics and Autoimmunity in Animal Models

Defining toxicant exposure as a risk factor for a particular type of human disease, autoimmune or otherwise, is difficult. Many times people do not realize they have been in contact with a particular chemical such as TCE, and there are often few if any biomarkers of exposure. In addition, since people are never exposed to a single chemical how do you accurately assess the contribution of a single toxicant? These challenges make it difficult to define a direct cause and effect relationship between toxicant exposure and autoimmune disease. This has led to the popularity of animal models in which toxicant exposure can be controlled and monitored. Several animal models have been used to test the immunotoxicity of environmental chemicals. When testing chemicals such as TCE that are thought to inappropriately stimulate rather than suppress the immune system animal models with a genetic susceptibility to hypersensitivity are often selected. This is designed to mimic the similar ill-defined predisposition thought to be important for human idiopathic disease, and to increase the likelihood that toxicant-induced hypersensitivity can be detected.

There are several well-characterized mouse strains that are genetically predisposed to develop autoimmune disease. In some cases the diseases occur spontaneously, and in some cases they need to be triggered by administration of antigen or mitogen. Of the mouse models that develop disease spontaneously the most widely studied include NOD mice (type 1 diabetes), BXD1/TyJ (rheumatoid arthritis) and MRL/lpr, NZBWF1/J and BXSB/MpJ mice (lupus). Several of these models have been used to test the role of xenobiotics in autoimmune disease etiology. A recent excellent review describes the different animal models, and discusses environmental agents that have been shown to trigger or exacerbate autoimmune disease in these models (Germolec et al. 2012).

2.4 TCE-Induced Autoimmunity in Mice

2.4.1 Disease Characterization

In terms of TCE, its capacity to promote autoimmunity has been studied most extensively in the model consisting MRL+/+ mice. MRL+/+ mice are related to MRL/lpr mice which have a defect in Fas expression and spontaneously develop lupus within 3–4 months of age. Due to the rapidity of disease development in MRL/lpr mice, it can be difficult to test whether exposure to a toxicant exacerbates the response. In contrast to MRL/lpr mice, the genetically-similar but not identical MRL+/+ mice have normal Fas expression and spontaneously develop a relatively mild lupus-like disease late in life (50 % mortality at 17 months). MRL+/+ mice can also spontaneously develop other autoimmune disorders such as Sjogren's syndrome and T cell-infiltrating pancreatitis (Qu et al. 2002; Skarstein et al. 1997). The basis for the autoimmune predisposition in MRL+/+ mice is not known. Before they reach 1 year of age most female MRL+/+ mice do not exhibit autoimmune tissue pathology and indications of autoimmunity are minor. Thus, young adult female MRL+/+ mice, with their propensity for autoimmunity but absence of overt disease, make a good model to test whether TCE can boost autoimmunity.

In our initial study we expected TCE to accelerate the development of lupus in young adult female MRL+/+ mice. Instead, adding TCE at concentrations lower than sanctioned occupational exposure to drinking water at for 26 or 23 weeks generated a T cell-mediated liver disease commensurate with human idiopathic AIH (Griffin et al. 2000c). The TCE-induced AIH in the MRL+/+ mice was associated with several alterations in $CD4^+$ T cells, an immune subset that play a large role in driving autoimmune disease. One such alteration included decreased sensitivity to activation-induced apoptosis (Gilbert et al. 2006). Activation-induced apoptosis is supposed to keep $CD4^+$ T cells in check and thus help prevent autoimmune disease. This process occurs when autoreactive $CD4^+$ T cells repeatedly stimulated with self antigen co-express death receptors such as Fas as well as the ligand for the death receptor (e.g. FasL). Cross-linking of death receptors on the surface of susceptible T cells promotes the release of active caspase-8 thereby initiating apoptosis (Crispe 1994; Kischkel et al. 1995). Activation-induced cell death is widely believed to help the host protect itself against repeated stimulation and expansion of autoreactive $CD4^+$ T cells (Green et al. 2003; Marrack and Kappler 2004; Van Parijs et al. 1998).

Supporting the important protective effects of activation-induced apoptosis is the fact that defects in this process has been linked to the development of several idiopathic autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and rheumatoid arthritis in both humans and mice (Bona et al. 2003; Kovacs et al. 1996; Semra et al. 2002; Sneller et al. 1997; Szodoray et al. 2003; Waiczies et al. 2002). On the other hand, therapies that facilitate Fas-mediated T-cell apoptosis can ameliorate autoimmune disease (Hong et al. 1998; Nishimura-Morita et al. 1997; Zhou et al. 1999). Events such as TCE exposure that inhibit this protective

mechanism could thus promote autoimmunity by promoting the expansion of autoreactive $CD4^+$ T cells. Perhaps because of its ability to decrease susceptibility to apoptosis TCE exposure in MRL+/+ mice also increased expansion of an activated/memory population ($CD62L^{lo}$ and/or $CD44^{hi}$) of $CD4^+$ T cells that produced more of the pro-inflammatory cytokine IFN-γ (Griffin et al. 2000c). Compared to naïve $CD4^+$ T cells activated/memory $CD4^+$ T cells have been shown to have a more robust effector function and cytokine production. Thus, work in our laboratory showed that chronic exposure of female MRL+/+ mice to TCE in drinking water induced T cell-mediated autoimmune hepatitis in association with several alterations in $CD4^+$ T cells that align with increased autoreactivity.

Other laboratories have also studied the effects of TCE on autoimmune disease in the MRL+/+ mouse model. A series of important studies conducted by researchers at the University of Texas at Galveston showed that long term exposure to TCE (0.5 mg/ml) in drinking water increased production of lupus-associated autoantibodies as well as promoting the generation of autoimmune hepatitis (Cai et al. 2008; Khan et al. 1995). The autoantibodies induced by TCE encompassed nuclear proteins as well as lipid peroxidation products (Khan et al. 2001; Wang et al. 2007). More about these TCE-induced antibodies will be described in Chap. 4.

2.4.2 Need for Metabolism

The toxicity of many chemicals requires their metabolism. TCE can be metabolized by a glutathione-dependent pathway in the kidney. However, in both mice and humans the majority of TCE absorbed into the circulation is metabolized by an oxidative pathway in the liver (Lipscomb et al. 1996). In this pathway cytochrome P450s (CYPs) rapidly converts TCE to trichloroacetaldehyde (TCAA; also known as chloral), which in solution is in equilibrium with trichloroacetaldehyde hydrate (TCAH ;also known as chloral hydrate) (Fig. 2.1). Once formed, TCAA and TCAH are converted to trichloroacetic acid (TCA), or trichloroethanol (TCOH) which is excreted as the alcohol glucuronide [see review(Lash et al. 2000)]. This later pathway is regulated by alcohol dehydrogenase that works to convert TCOH back to aldehyde. Thus, the level of TCAH depends on the activity of several metabolizing enzymes, all of which display considerable genetic variation in both humans and mice. For example, MRL+/+ mice have much higher levels of alcohol dehydrogenase than C3H/HeJ mice (Teichert-Kuliszewska et al. 1988), and may therefore be expected to have an increased steady-state level of TCAH if exposed to the chemical.

It appears that many of the $CD4^+$ T cell modulating effects of TCE are in fact induced by its metabolite TCAH. It was shown that immune dysfunction induced by TCE in MRL+/+ mice could be blocked by suppressing the activity of CYP2E1 (Griffin et al. 2000a). Similarly, MRL+/+ mice exposed to TCAH instead of TCE in their drinking water developed the same alterations in $CD4^+$ T cells as mice exposed to the parent compound (Blossom et al. 2007b). In humans, TCE-induced

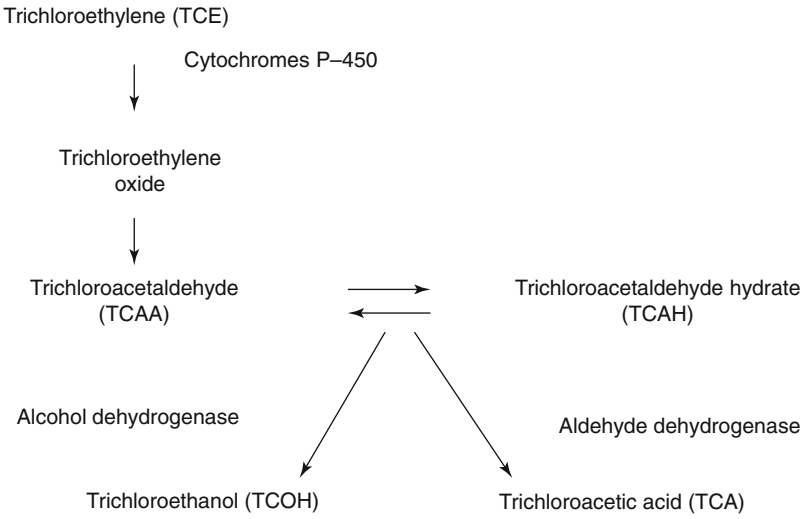

Fig. 2.1 Metabolism of trichloroethylene

hypersensitivity dermatitis has been linked to single nucleotide polymorphisms of TCE metabolizing enzymes CYP2E1 and CYP1A1 (Xu et al. 2009b). CYP2E1 was also shown to be the main enzyme involved in TCE-induced hepatoxicity in mice (Ramdhan et al. 2008). Taken together, it seems likely that similar to many of its other toxic effects TCE-induced immunotoxicity requires its metabolism.

2.4.3 Mechanisms of Immune Alteration

How TCE, seemingly via TCAH, alters $CD4^+$ T cell function is not clear. However, the structure of TCAH may provide a clue. As an aldehyde TCAH has the capacity to form a chemical reaction known as Schiff base, a transient covalent bond between nucleophiles on proteins (e.g. amino group on lysine) and electrophilic carbonyl carbons of aldehydes. As it turns out, Schiff base formation is the foundation for some of the stimulatory interactions that normally occur between specific molecules on the $CD4^+$ T cell surface and associated ligands on the surface of accessory cells such as dendritic cells or endothelial cells (Chen et al. 1997). These interactions between $CD4^+$ T cell and accessory cells are crucial for many aspects of $CD4^+$ T cell activation and effector function. The role of Schiff base formation in these interactions means that certain small Schiff-base-forming compounds may be able to bypass the need for ligand-bearing accessory cells and co-stimulate $CD4^+$ T cells directly. One such compound, tucaresol, is being clinically tested as a drug capable of stimulating T cells to combat neoplasia and opportunistic infection (Charo et al. 2004; Rhodes et al. 1995). The ultimate effect of Schiff base formation on $CD4^+$ T

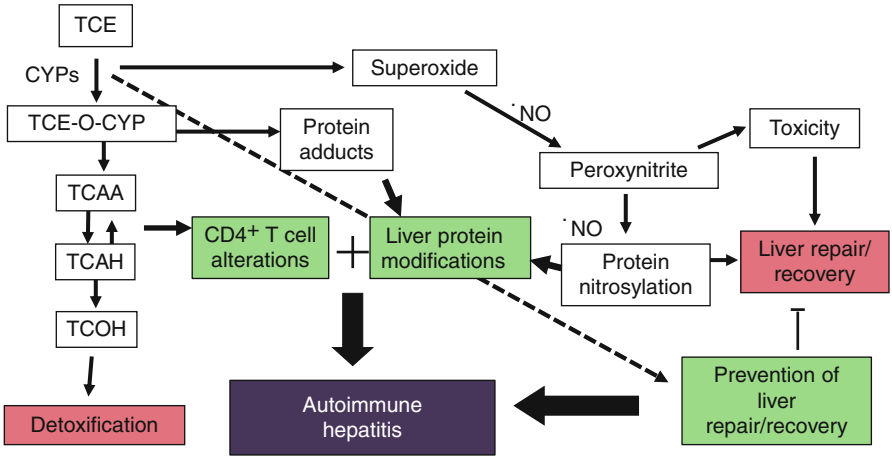

Fig. 2.2 Possible mechanism of TCE-induced autoimmune hepatitis

cells may depend on the existing baseline immune response; in immunosuppressed individuals this event may be beneficial, while in individuals with a predisposition for hypersensitivity, it may be enough to trigger autoimmune disease.

The stimulatory Schiff base-forming compounds identified thus far are aldehydes, similar to TCAH. Schiff base formation by TCAH should be a major reaction because of the electron withdrawing of the three chloro groups on the adjacent carbon. *In vitro* experiments demonstrated that TCAH could form a functionally-active Schiff base with molecules on the surface of $CD4^+$ T cells (Gilbert et al. 2004). This interaction triggered signaling events in the $CD4^+$ T cells similar to those initiated by interaction with ligand-bearing accessory cells. A better understanding of the signaling events triggered in $CD4^+$ T cells by this chemical interaction is required. This includes identifying the molecules on the $CD4^+$ T cell surface that are altered by the TCAH-induced Schiff base formation. In addition, the possibility that these signaling events encompass epigenetic alterations by TCE in $CD4^+$ T cells is being studied, and will be discussed in more detail in Chap. 10.

2.4.4 Liver Events

Although TCE appeared to induce $CD4^+$ T cells alterations commensurate with autoimmunity, it was not clear why the pathology targeted the liver instead of some other organ in the MRL+/+ mice. Simultaneous events in the liver such as nitrosative/oxidative stress and/or adduct formation which are induced by TCE may represent a second requirement for disease pathology that involves protein modification (Fig. 2.2). It has been proposed that formation of chemically-modified self-proteins capable of triggering an immune response represents a mechanism by which chemicals could initiate autoimmunity.

Very early in its metabolism in the liver (prior to TCAH formation) TCE produces a highly reactive intermediate (TCE-O-CYP) that can form adducts with nearby proteins. TCE has been shown to form adducts with a number of liver proteins, most predominantly CYP2E1 (Halmes et al. 1997). Some of these adducted proteins are immunogenic; antibodies specific for TCE-protein adducts have been found in TCE-treated MRL+/+ mice (Griffin et al. 2000b). We have demonstrated a time-dependent increase in the repertoire of liver microsomal proteins recognized by antibodies in the sera of TCE-treated MRL+/+ mice as compared to age-matched untreated MRL+/+ mice. Interestingly, the antibodies in the sera recognized liver microsomal protein from control mice. This indicated that even if chemically-altered liver protein was required to initiate the autoimmune response, the resulting antibody reaction recognized non-modified liver protein. The liver protein epitopes targeted by the TCE-induced autoantibodies, and the specificity of the $CD4^+$ T cells that promote the autoantibody production remains to be determined.

TCE-induced nitrosative/oxidative stress may also increase the immunogenicity of liver proteins. Nitrosative/oxidative stress occurs when the generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) is not balanced by the appropriate detoxification by various antioxidant compounds (e.g. glutathione or vitamin E) and enzymes (e.g. glutathione peroxidase). One important consequence of nitrosative/oxidative stress is the generation of the superoxide anion which can interact with another free radical nitric oxide to form the extremely reactive peroxynitrite. Peroxynitrite can trigger a variety of cellular responses ranging from lipid peroxidation, protein tyrosine nitration, DNA damage and cell death. Tissue damage associated with increased levels of inducible nitric oxide synthase (iNOS) (an enzyme which produces nitric oxide), and/or the accumulation of nitrotyrosine residues has been found in a variety of autoimmune diseases in humans, including autoimmune hepatitis (Pemberton et al. 2004; Sanz-Cameno et al. 2002). Similarly, both iNOS and nitrotyrosine accumulation in the liver have been found in TCE-treated mice (Wang et al. 2007). In addition, investigators have shown that sera from TCE-treated MRL+/+ mice contain antibodies specific for lipid peroxidation-derived aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Khan et al. 2001). More about these antibodies, and the $CD4^+$ T cells that support their production, will be discussed in Chap. 4.

2.5 Susceptibility Factors for Toxicant-Induced Autoimmunity

It has not been possible for researchers to define a single causative agent for idiopathic autoimmune disease. Even those linked to contact with a particular toxicant only occur in a fraction of people with the seemingly same level of exposure. Consequently, etiology is largely suspected of having several contributing factors

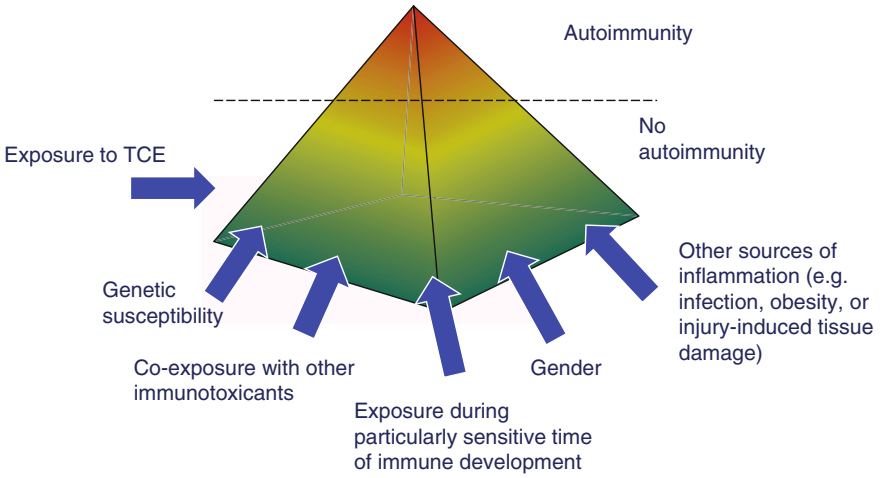

Fig. 2.3 Possible contributors to TCE-induced autoimmune disease

which may be additive or synergistic in nature. Defining these factors, and estimating their relative contribution to autoimmunity is proving to be a huge challenge. Some of the susceptibility factors that appear to contribute to TCE-induced autoimmune disease (Fig. 2.3) are described below.

2.5.1 Genetics

As mentioned above the high concordance rate for identical twins developing the same autoimmune disease demonstrates the importance of genetic susceptibility in human idiopathic autoimmune disease (Jarvinen and Aho 1994; Tomer and Davies 1997). If one assumes that some idiopathic autoimmunity is actually triggered by undetected chemical exposure this implies that genetics also plays a role in toxicant-induced autoimmune disease and other forms of hypersensitivity. Genetic predisposition has also been found in certain hypersensitivity disorders such as asthma, atopic eczema, drug hypersensitivity and food allergies that represent a response to exogenous irritants (Dreskin 2006; Pirmohamed 2006; Mohrenschlager et al. 2006; Meurer et al. 2006). The mechanism for this increased sensitivity is not known.

Aside from MRL+/+ mice, other strains of mice [i.e. (NZBxNZW)F1, and female BXSB] with ill-defined genetic patterns that make them "autoimmune-prone", have been used to test the disease-promoting capacity of xenobiotics. The insecticide chlordecone was shown to accelerate the development of lupus in (NZBxNZW)F1 mice, but had no effect in non-autoimmune-prone BALB/c mice (Sobel et al. 2006). Similarly, exposure to low doses of mercuric chloride has been shown to promote autoimmunity in female BXSB mice, but not in MHC-compatible non-autoimmune-prone C57BL/6 mice (Pollard et al. 2001). Thus, a propensity

for autoimmunity appears to be a requirement for at least some types of xenobiotic-induced autoimmunity in mice.

The need for genetic susceptibility in TCE-induced autoimmunity in mice is still being debated. Keil et al. reported that chronic exposure to TCE in drinking water slightly increased renal pathology in non-autoimmune-prone B6C3F1 mice but not autoimmune-prone NZBWF1 mice (Keil et al. 2009). Similarly, TCE was shown to increase serum levels of lupus-related autoantibodies (i.e. anti-ds and anti-ss DNA) at only early time points in NZBWF1 mice, but at multiple time points in B6C3F1 mice. However, the levels of autoantibodies and renal pathology at every time point were higher in the untreated NZBWF1 mice than in the TCE-treated B6C3F1 mice. Since the levels of lupus-associated autoantibodies increase spontaneously in most lupus-prone strains of mice as they age, a high baseline response may mask a TCE-induced effect on this type of autoantibodies. Regardless of the effect of TCE in the NZBWF1 mice, the immunotoxicity of the pollutant could be observed in non-autoimmune-prone mice.

Although we have not examined the effects of TCE itself in non-autoimmune-prone mice we have examined whether its metabolite TCAH required a genetic predisposition to be effective. Chronic exposure to TCAH induced autoimmunity and $CD4^+$ T cell alterations in female MRL+/+ mice, but had much more modest effects when added to the drinking water of non-autoimmune-prone but genetically-related female C3H/HeJ mice (Blossom et al. 2007a). This result would suggest that at least in terms of some parameters, an autoimmune-predisposition increased susceptibility to TCE immunotoxicity.

In order to better define a possible autoimmune-predisposition to TCE we performed a transciptomic analysis comparing unstimulated splenic $CD4^+$ T cells from untreated female age-matched MRL+/+ and C3H/HeJ mice. Table 2.1 provides a list of some of the most robustly altered functionally significant genes flagged in the transcriptomic analysis, and confirmed by qRT-PCR. Also included in Table 2.1 are qRT-PCR results for genes examined as specificity controls for the flagged genes, or because they that had been identified through other assays. The expression levels of some genes were not surprising; a mutation in *Tlr1* is a hallmark of C3H/HeJ mice. Not as predictable, $CD4^+$ T cells from female MRL+/+ mice expressed higher constitutive levels of *Stra6*, which acts as a high-affinity cell-surface receptor for the complex comprised of retinol (main metabolite of Vitamin A) and retinol-binding protein. Stra6 removes retinol from the complex and transports it across the cell membrane where it has been shown to promote $CD4^+$ T cell differentiation and recruitment to inflammatory sites (Pino-Lagos et al. 2011). $CD4^+$ T cells from the MRL+/+ mice also expressed higher baseline levels of *Spp1*, a gene that encodes for pro-inflammatory cytokine osteopontin (OPN). Interestingly, quantitative trait loci (QTL) analysis conducted by others revealed that the locus for susceptibility to lupus nephritis in MRL mice corresponded to the OPN gene, and that allelic polymorphism of OPN caused the functional differences in antibody production between MRL and C3H strains (Miyazaki et al. 2005).

Also flagged by the transcriptomic analysis was the differential expression of a member of the tumor necrosis factor receptor superfamily (Tnfrsf). The $CD4^+$ T

Table 2.1 Differential gene expression in T cells from MRL+/+ and C3H/HeJ mice

Gene ID	Gene name	Gene description	Transcriptomics fold change (p-value)	qRT-PCR fold change ± SD
Splenic CD4⁺ T cells				
NM_030682	*Tlr1*	Toll-like receptor 1	11.221 (0.0001)	
NM_009291	*Stra6*	Stimulated by retinoic acid gene 6	9.0145 (0.0002)	1.89 ± 0.30
NM_009263	*Spp1*	Secreted phosphoprotein 1; osteopontin	7.6511 (0.0001)	4.18 ± 1.34
NM_011838	*Lynx1*	Ly6/neurotoxin 1	5.2374 (0.0001)	13.24 ± 6.52
NM_013599	*Mmp9*	Matrix metallopeptidase 9	4.6446 (0.0007)	5.34 ± 0.51
NM_007399	*ADAM10*	A disintegrin and metalloproteinase domain-containing protein 10		1.14 ± 0.26
NM_178589	*Tnfrsf21*	Tumor necrosis factor receptor superfamily, member 21	0.3198 (0.0001)	0.32 ± 0.10
NM_013869	*Tnfrsf19*	Tumor necrosis factor receptor superfamily, member 19	16.406 (0.0001)	10.88 ± 5.70
NM_178931	*Tnfrsf14*	Tumor necrosis factor receptor superfamily, member 14		1.79 ± 0.88
	Lap	Intracisternal A particle		0.04 ± 0.02
Y12713	*Muerv*	Murine endogenous retrovirus		1.1 ± 0.18
NM_010066	*Dnmt1*	DNA methyltransferase 1		1.05 ± 0.26
NM_007872	*Dnmt3a*	DNA methyltransferase 3 alpha		0.83 ± 0.35
Thymocytes				
NM_013869	*Tnfrsf19r*	Tumor necrosis factor receptor superfamily, member 19		3.24 ± 0.52
NM_054039	*Foxp3*	Forkhead box P3		1.85 ± 0.44
NM_009646	*Aire*	Autoimmune regulator		1.51 ± 0.26

All shaded results were statistically different from results obtained from control CD4+ T cells or thymocytes

cells from MRL+/+ mice expressed comparatively lower levels of *Tnfrsf21*, a gene that encodes for a protein known as death receptor 6 (DR6). Interestingly, a decrease in DR6 in CD4⁺ T cells has been shown to enhance proliferation and production of IL-2 (Liu et al. 2001), effects which may enhance expansion and effector function of autoreactive CD4⁺ T cells. *Tnfrsf14* was not differentially expressed in CD4⁺ T cells from MRL+/+ and C3H/HeJ mice. On the other hand, *Tnfrsf19* was highly increased (>10 fold) in CD4⁺ T cells from MRL+/+ mice. The protein encoded by *Tnfrsf19* is highly expressed during embryonic development, but in adults is primarily expressed in pulmonary and ductal epithelium, but can be detected in lymphocytes. The functional significance of its increased expression in the CD4⁺ T cells

from MRL+/+ mice is not known. However, the cell-inappropriate expression of *Tnfrsf19* suggests some kind of epigenetic mechanism.

Several epigenetic mechanisms have been shown to regulate $CD4^+$ T cell activity and autoimmune pathology in MRL/lpr mice (Pan et al. 2010; Sawalha and Jeffries 2007; Yang et al. 2013). Although distinct from the MRL+/+ mice used to test the effects of TCE, MRL/lpr are closely related. Consequently, as a preliminary look at epigenetics in our model we compared expression of the retrotransposon *Iap* (intracisternal A particle) in the $CD4^+$ T cells from MRL+/+ mice and C3H/HeJ mice. Since *Iap* expression is largely dependent on DNA methylation, its expression is often used as an indirect measurement of this epigenetic process. As shown in Table 2.1 expression of *Iap* was dramatically suppressed in $CD4^+$ T cells from the MRL+/+ mice. This finding suggests that epigenetics plays a role in at least some of the differential gene expression in $CD4^+$ T cells from the MRL+/+ and C3H/HeJ mice. The effects of DNA methylation on susceptibility to toxicant-induced autoimmunity is currently being investigated in and will be described in more detail in Chap. 10.

Taken together, the results demonstrated that $CD4^+$ T cells from MRL+/+ mice differentially express several genes which may make them more likely to be autoreactive. Defining the relative contribution of these alterations to the autoimmune-prone phenotype of the MRL+/+ mice, not to mention determining how they impact the response to TCE exposure, constitutes an important challenge.

2.5.2 Gender

Aside from an ill-defined genetic predisposition autoimmune disease in humans is also regulated by sex. At least 75 % of people with autoimmune disease are women, with the male/female ratios varying among disease. For example, type 1 diabetes is found in both sexes at about the same ratio, while most thyroid autoimmune diseases (e.g. Graves' disease) occur ten times more often in women. In most mouse models of spontaneous lupus nephritis females are more susceptible. In the MRL+/+ mouse model both sexes develop lupus, but the disease is more robust in females which die at about 73 weeks of age compared to males which die at about 93 weeks of age. There is not much known about the role of sex differences in toxicant-induced autoimmune disease in humans. This is largely due to the fact that in epidemiology studies the particular exposure being studied is often gender biased, either toward men (e.g. occupational exposure to toxicants such as silica) or women (e.g. exposure to cosmetics). Animal studies have also been conducted primarily using females except in those few cases in which autoimmunity primarily occurs in males (e.g. BXSB mice). A recent review highlights what is known about gender differences in autoimmunity induced by chemical exposure (Pollard 2012).

In terms of TCE, very little is known about sex-specific immunotoxicity. With regard to adult exposure one meta-analysis of case–control studies concluded that although scleroderma affected women predominantly, among subjects with

occupational exposure to solvents (which included TCE) men were at higher risk for developing the disease (Kettaneh et al. 2007). Developmental exposure to TCE at 14,000 ppb reportedly decreased all thymic T cell subsets in male but not female MRL+/+ mice (Peden-Adams et al. 2008). Developmental exposure to lower levels of TCE has been shown to induce subtle differences in double-negative lineage thymocytes in male and female MRL+/+ mice (Blossom and Doss 2007). How the sex-specific thymocyte alterations induced by developmental TCE exposure impact the peripheral immune phenotype during the lifespan of the mice is not clear. Based on the paucity of information, it is currently impossible to predict whether TCE does in fact induce sex-specific alterations in immune function. This represents a gap in the knowledge base that needs to be filled.

2.5.3 Age of Exposure

Most epidemiological studies of immunotoxicity have focused on adult occupational contact with the particular chemical since it is easier to document and often involves relatively higher exposure levels. However, the developing immune system is especially sensitive to environmental perturbation. A recent review compared early vs adult exposure to several immunosuppressive toxins including lead and tributyltin in animal models (Luebke et al. 2006). In all cases sensitivity was greater if exposure occurred during development. In fact, immune suppression in developmentally exposed offspring often occurred at doses that were ineffective in adults. Developmental sensitivity to toxicants has also been found in humans. For example, prenatal exposure to polychlorinated biphenyls decreased the immune response to standard immunizations (Heilmann et al. 2006). Prenatal exposure to polybrominated diphenyl ethers produced a persistent decrease in lymphocyte numbers (Leijs et al. 2009). Aside from immune suppression, there is increasing evidence that adult onset autoimmunity can be triggered by pre- and early post-natal toxicant exposure to environmental factors such as cigarette smoke or organochlorines (Colebatch and Edwards 2011; Langer et al. 2008).

Developmental exposure to TCE is not uncommon; one study showed that 100 % of breast milk samples from 4 US urban areas had detectable levels of TCE (Pellizzari et al. 1982). TCE exposure is also a possible concern for bottle-fed infants because they ingest more water on a bodyweight basis than adults. Gestational and early-life TCE exposure has primarily been examined for its neurotoxicity rather than immunotoxicity (Gist and Burg 1995). However, children continuously exposed for 3–19 years beginning *in utero* to a water supply contaminated with solvents [with TCE being the predominant toxicant (267 ppb)] had altered ratios of T cell subsets and increased levels of autoantibodies (Byers et al. 1988). Autoimmune disease was not assessed. Blossom et al. have shown that continuous exposure to TCE in mice (gestation, lactation and early life) generated $CD4^+$ T cell alterations and early signs of tissue inflammation (Blossom and Doss 2007). More information

about the effects of developmental exposure to TCE is available in the Chap. 7. The published experiments concerning developmental effects of TCE on immunotoxicity in MRL+/+ mice to TCE were not extended past 6–8 weeks of age, and although they detected early signs of liver inflammation, they did not assess actual autoimmune disease. The experiments to make such an assessment are currently underway in our laboratory.

2.5.4 Toxicant Co-exposure

In addition to genetics, gender and age of the immunotoxic response to TCE may also be influenced by chemical co-exposure. Anyone exposed to TCE, either as an adult or during development, is also exposed to other chemicals with defined or potential immunotoxicity. Co-exposure to another immunotoxicant with additive or synergistic effects may promote autoimmunity at concentrations that would be harmless for either chemical alone. One such toxicant is mercury.

Human exposure to mercury (#3 CERCLA Priority List of Hazardous substances) is common due to its existence as a natural element and its anthropogenic release from industrial use. Blood mercury analyses in the 1999–2000 National Health and Nutrition Examination Survey for 16–49 year old women showed that approximately 8 % of women in the survey had blood mercury concentrations greater than 5.8 μg/L (which is a blood mercury level equivalent to the current RfD). TCE and mercury are often found together; 44 % of the active Superfund sites on the National Priorities List contaminated with TCE also list mercury as a contaminant. In addition, since both are listed in the top 20 chemicals of the almost 300 chemicals on the CERCLA list based in part on the likelihood of human contact, co-exposure is probable. Mercury has been implicated as a co-factor in systemic human autoimmunity, where studies showed that mercury exposure increased levels of anti-nucleolar antibodies (Cooper et al. 2004; Gardner et al. 2010). The ability of mercury to promote autoimmunity has been especially well-documented in mice where it promotes autoantibodies specific for fibrillarin and other nuclear antigens such as chromatin, and induces immune complex-mediated lupus nephritis (Hultman et al. 1996; Pollard et al. 2001).

A recent study examined the combined effects of mercury and TCE exposure on the induction of autoimmune disease in adult female MRL+/+ mice. Mice received either 0, 0.1 or 2.0 mg/ml TCE in their drinking water for 8 weeks. Some mice were injected sc twice per week for 8 weeks with 40 μg $HgCl_2$. Exposure to TCE alone at these concentrations for only 8 weeks was not expected to induce autoimmune hepatitis. And indeed the livers of mice exposed to $HgCl_2$ or TCE alone exhibited no significant pathology. However, based on cumulative scores of mononuclear cell infiltration, fibrosis, and hepatocellular enlargement, liver pathology in mice exposed to $HgCl_2$ and either 0.1 or 2.0 mg/ml TCE was significantly increased from that of control mice, indicating the early stages of autoimmune hepatitis. In addition, TCE and heavy metals have been shown by others to have an additive effect on

antioxidant endpoints (Tabrez and Ahmad 2011), an effect that can promote immunotoxicity.

Documenting the ability of TCE to augment the activity of other known or suspected immunotoxicants is needed to accurately evaluate the role of TCE in promoting seemingly idiopathic autoimmune disease. This however, represents a large challenge. There seems to be little consensus in how to study mixtures regardless of the outcome measured. Should we use a labor-intensive and expensive full-factorial design in a mouse model starting with TCE-containing binary mixtures and expanding to include other immunotoxicants? Or, should we study the murine effects of complex TCE-containing mixtures selected because they include those found most commonly in ground water or Superfund sites? Alternatively, should we identify the chemical mixtures to which a particular patient subset (e.g. children recently diagnosed juvenile autoimmune hepatitis) are most commonly exposed, and test those mixtures for immunotoxicity in mice? The fact that there are pros and cons associated with all of these approaches should not preclude selecting one to further this important area of toxicity assessment.

2.6 Challenges

Both epidemiological studies and work with animal models provide evidence that TCE exposure contributes to autoimmunity. However, as mentioned in the text there are several aspects of TCE-induced immunotoxicity that remain to be explored. Studying the ability of a toxicant such as TCE to promote autoimmune disease is complicated by the difficulty of determining exposure in humans, and by the contribution of risk factors such as ill-defined genetic susceptibility, differential sensitivity based on sex or age, and possible augmentation or antagonism by co-exposure to other environmental triggers (e.g. additional chemicals, diet, infections, changes in the microbiome or obesity). Similarly, determining which of the TCE-induced alterations in $CD4^+$ T cells (e.g. susceptibility to apoptosis, expansion of memory/activated populations, and skewing of cytokine production) are actually required for pathology represents another challenge. Nevertheless, in view of the increased prevalence of certain autoimmune diseases in humans, and the widespread nature of TCE exposure, determining the contribution of the later to the former should remain a priority. Once the mechanism(s) of action have been more clearly defined it should be possible to circumvent at least some of these processes, and thereby decrease the likelihood of TCE-induced autoimmunity. For example, dietary interventions that combat DNA methylation could be used until TCE remediation is more advanced. The changes induced in $CD4^+$ T cells by often undetected TCE exposure may make the host more sensitive to a variety of hypersensitivity/autoimmune responses. Instead of focusing on a few specific syndromes such as scleroderma we should consider the possibility that TCE and other immunotoxic chemicals contribute to a wide array of idiopathic chronic inflammatory diseases.

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