REGULATORY ROLES FOR NATURAL KILLER T CELLS AND TOLL-LIKE RECEPTORS IN MERCURY-INDUCED AUTOIMMUNITY

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ABSTRACT

The development of autoimmune diseases is frequently linked to exposure to environmental factors such as chemicals, drugs or infections. In the experimental model of metal-induced autoimmunity, administration of subtoxic doses of mercury (a common environmental pollutant) to genetically susceptible mice induces an autoimmune syndrome with rapid anti-nucleolar antibody production and immune system activation. Regulatory components of the innate immune system such as NKT cells and TLRs can also modulate the autoimmune process. We examined the interplay among environmental chemicals and NKT cells in the regulation of autoimmunity. Additionally, we studied NKT and TLR ligands in a tolerance model where pre-administration of a low dose of mercury in the steady state renders animals tolerant to metal-induced autoimmunity. We also studied the effect of Sphingomonas capsulata, a bacterial strain that carries both NKT cell and TLR ligands, on metal-induced autoimmunity. Overall, NKT cell activation by synthetic ligands enhanced the manifestations of metal-induced autoimmunity. Exposure to S. capsulata exacerbated autoimmunity elicited by mercury. Although the synthetic NKT cell ligands that we used are reportedly similar in their ability to activate NKT cells, they displayed pronounced differences when co-injected with environmental agents or TLR ligands. Individual NKT ligands differed in their ability to prevent or break tolerance induced by low-dose mercury treatment. Likewise, different NKT ligands either dramatically potentiated or inhibited the ability of TLR9 agonistic oligonucleotides to disrupt tolerance to mercury. Our data suggest that these differences could be mediated by the modification of cytokine profiles and regulatory T cell numbers.
The mechanisms by which a heavy metal with an elementary chemical structure induces autoimmunity are unknown. Herein we show that mercury administration results in release of endogenous ligands that activate TLR7, an innate immune receptor implicated in the development of systemic autoimmunity. Moreover, our results suggest that fine specificity of autoantibodies recognizing RNA-containing snoRNPs could be a consequence of TLR7 activation.
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This thesis is dedicated to my parents, Lakshmi and Vasu. I could not have done this without your incredible support.
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CHAPTER 1

INTRODUCTION

Environmental link to autoimmune disease

Autoimmune diseases have a complex multifactorial etiology. A strong genetic component exists, however environmental factors can play a considerable role in the breakdown of tolerance. Heavy metals can exert remarkable changes upon cell physiology, and have the capacity to modulate the function of multi-cellular networks such as the immune system. Certain heavy metals such as zinc, copper and manganese are required for normal physiological processes, while others including lead, arsenic, cadmium and mercury are abundant environmental pollutants and can adversely affect human health. Of the various heavy metals that pose health risks, mercury (Hg) stands out because of its extensive distribution in the environment and the vast spectrum of effects that it can induce.

Types and sources of mercury

Mercury is a natural component of our environment, constituting 0.5 parts per million of the earth’s crust. It is found in three main forms: elemental mercury or quicksilver (Hg\textsubscript{0}), inorganic mercury (Hg\textsuperscript{+} and Hg\textsuperscript{2+}), and organic (such as methyl, ethyl and phenyl) mercury (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Inorganic mercury is derived from oxidization of elemental mercury (Agency for
Toxic Substances and Disease Registry [ATSDR], 1999) while the organic derivatives are produced by biomethylation of inorganic mercury compounds by microorganisms in aquatic sediments and soils (Agency for Toxic Substances and Disease Registry [ATSDR], 1999).

Between 2700 and 6000 tons of elemental mercury are released per year to the biosphere through degassing from the earth’s crust and oceans (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Additionally, humans have historically used mercury and its compounds for medicinal, cosmetic and occupational purposes. The first known use of mercury, in the form of cinnabar as the pigment in red ink, occurred in China (Clarkson et al., 2007). Alchemists used liquid metallic mercury to dissolve precious metals such as gold and silver, which could be recovered in pure form following evaporation of the metal. This property has enabled its use in gold mining in river basins, a practice still widely used in some areas of the world (Clarkson et al., 2007). Mercury was also used in the treatment of syphilis in Europe soon after its transmission from maritime personnel returning from the New World (Clarkson et al., 2007). Mercury-containing compounds have long been used as antiseptics and preservatives (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). Mercury-containing amalgams have been used in tooth fillings since the early 19th century (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). The industrial era ushered in widespread usage of mercury, owing to its many useful physical properties (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). Anthropogenic release of
mercury greatly expanded following industrialization (Swain et al., 2007), although growing awareness of the health hazards has lead to efforts to curtail some of most hazardous usage of this heavy metal and to place restrictions on its disposal (Swain et al., 2007). Nonetheless, anthropogenic activities continue to add to the environmental mercury burden, with industrial waste dumping, combustion of fossil fuels, mining and smelting activities releasing up to 2000–3000 tons of mercury to the environment (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Although efforts have been made to replace mercury in various household products with suitable alternatives (Swain et al., 2007), elemental mercury is still found in some, such as thermometers, barometers, batteries, light bulbs and certain electrical switches (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Swain et al., 2007). These devices can break during use or disposal, releasing mercury into the indoor air. Mercury poisoning following an indoor spill is rare, but is reported occasionally. For example, three children were hospitalized in Michigan (United States) as a result of mercury poisoning, following spillage of a small vial of the chemical in their bedroom a few months prior to detection of symptoms (Swain et al., 2007; Taueg et al., 1992). Elemental mercury has been identified at more than 700 hazardous waste sites nationwide in the United States (Agency for Toxic Substances and Disease Registry [ATSDR], 1999); release of mercury vapor from incineration of solid wastes is another source of contamination. An important source of elemental mercury exposure in the human population is dental amalgams, which typically contain about 50% elemental mercury. Occupational exposure to mercury vapor is also a concern, for example in gold mining populations (Clarkson, 2001; da Costa et al. 2008), in chloralkali plant workers (Mniszek,
2001), and dental personnel (Brownawell et al., 2005). Inorganic mercury salts are also found in a variety of commercial and consumer products such as fungicides, skin lightening creams, paints and some tattoo dyes (Agency for Toxic Substances and Disease Registry [ATSDR], 1999).

Elemental mercury can be oxidized to the inorganic form in the air and can be transported to water or soil in rain or snow (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). The inorganic species may also enter water or soil from the weathering of mercury-containing rocks, from industrial release of water contaminated with the heavy metal, or from incineration of mercury-containing municipal garbage (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Other sources for release into water bodies include local gold mining operations (Clarkson, 2001) or use of mercury-containing fungicides (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Inorganic mercury compounds thus accumulate in soils and aquatic sediments, and are biomethylated by various microbes (bacteria, phytoplankton in the ocean, and fungi) to generate methyl mercury (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Methyl mercury bioaccumulates up the food chain, reaching the highest levels in the top fish predators. Additional sources of organic mercury also exist. Some organic mercury compounds are still used as antibacterials. These products include ethylmercurithiosalicylate (thimerosal) and phenylmercuric nitrate, which are also used in small amounts as preservatives in some medicines (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Thimerosal has also been used as a preservative in vaccines, a controversial usage which generated public concerns over
whether it contributed to the development of autism. Although no links between thimerosal in vaccines and autism has been found, despite extensive research, it was withdrawn from use in pediatric vaccines in the United States in 1999 (Ball et al., 2001). However, it is still present in various adult vaccines, including the influenza, hepatitis B and tetanus toxoid vaccines (National Advisory Committee on Immunization (NACI), 2007).

Today, the most widespread human exposures to mercury are to mercury vapor released from amalgam tooth fillings, thimerosal (ethyl mercury) as a preservative in vaccines, and methyl mercury in fish (Clarkson, 2001; Clarkson et al., 2007; Clarkson et al., 2003). Exposure risks from other sources are confined to certain sectors including occupationally-exposed workers (Brownawell et al., 2005; Clarkson, 2001; da Costa et al., 2008) and people using mercury-containing skin lightening creams, a practice widespread in several parts of the world (Agency for Toxic Substances and Disease Registry [ATSDR], 1999).

**Absorption and conversion of different forms**

Though human populations are mainly exposed to organic and elemental mercury, both forms are converted to the inorganic species *in vivo* (Clarkson et al., 2007). Elemental mercury is poorly absorbed via the skin and is not absorbed via the oral route unless the skin or mucosal barrier is compromised (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). However, inhaled mercury vapor is extremely well absorbed, entering the bloodstream directly from the lungs (Agency for Toxic Substances
and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). Elemental mercury, being lipid soluble, is rapidly dispersed within the body. It can traverse cell membranes without hindrance, and crosses blood-brain and placental barriers (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). Within the cell, it is oxidized by the hydrogen-peroxide-catalase pathway to yield inorganic mercury (Clarkson, 2001; Clarkson et al., 2007). In the brain, the inorganic mercury attaches to the selenide species of selenium to form mercuric selenide, which is insoluble and retained in brain tissue for years (Clarkson, 2001). Most of the elemental mercury is converted to the inorganic form and then excreted via the urine and feces. Some removal also occurs in the exhaled breath (Clarkson et al., 2007).

Inorganic mercury compounds generally do not vaporize at room temperatures like elemental mercury. If inhaled, they are poorly absorbed (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Following ingestion of inorganic mercury, generally less than 10% is absorbed through the intestinal tract; however, up to 40% may enter the body through the stomach and intestines in some instances (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Some inorganic mercury can enter the body through the skin, but this route is less efficient than ingestion (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Once in the blood stream, it disperses to all tissues. Inorganic mercury accumulates mostly in the kidneys and does not readily traverse blood-brain or placental barriers (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). It is excreted in the urine or feces over a period of several weeks or months. Excretion can also occur in breast milk (Agency for Toxic
Substances and Disease Registry [ATSDR], 1999). A small amount of the inorganic mercury can be converted to elemental mercury and exhaled as vapor (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson, 2001; Clarkson et al., 2007).

In contrast to inorganic mercury, organic mercury compounds are most easily absorbed through the gastrointestinal tract (Clarkson, 2001). The efficiency of absorption via the skin differs among different organic species; methyl mercury is poorly absorbed but other forms of organic mercury (in particular dimethyl mercury) can rapidly enter the body through the skin (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Entry of dimethyl mercury via this route resulted in the death of a chemistry professor following an accidental spillage (Siegler et al., 1999). Organic mercury compounds may evaporate slowly at room temperature and vapors are extremely efficiently absorbed (Agency for Toxic Substances and Disease Registry [ATSDR], 1999). Once in the bloodstream they bind to thiol-containing proteins (Clarkson et al., 2007) and rapidly disperse to all parts of the body. Organic mercury compounds easily cross the blood-brain and placental barriers (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson, 2001). Organic mercury is converted to the inorganic form in the body at various sites (Clarkson et al., 2007); notably, the conversion rate for different species is variable.

Humans are mainly exposed to methyl mercury from fish and ethyl mercury from thimerosal. Conversion of ethyl mercury is faster than conversion of methyl mercury (Clarkson, 2001; Magos et al., 1985). Like elemental mercury, excretion of methyl
mercury occurs in the inorganic form, via bile and the feces (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Clarkson et al., 2007). Excretion in the inorganic form is slow, and is prolonged over a period of many months. Elimination of ethyl mercury occurs mainly via the feces (Clarkson, 2001). The kinetics of elimination from the body is unknown. Ethyl mercury is probably retained for a shorter time within the body than methyl mercury, but quantitative data are lacking (Clarkson, 2001).

Health hazards posed by mercury

The effects of mercury intoxication first came to light following contamination of local waters in Minamata, Japan. Chisso, an acetaldehyde manufacturing factory in the area, used inorganic mercury salts as catalysts. Some of the mercury was chemically converted to methyl mercury compounds. Between 1932 and 1968, methyl mercury was continually released in wastewaters into the Minamata Bay. By 1956, the local population began showing manifestations of what became known as Minamata disease, which was eventually attributed to mercury intoxication (Elhassani, 1982). Ethyl mercury compounds had been used safely and effectively as fungicides for several decades, however cases of mercury poisoning occurred following misuse in rural Iraq (Clarkson and Strain, 2003; Jalili and Abbasi, 1961) and China (Clarkson and Strain, 2003; Zhang, 1984). These incidents clearly brought to light the toxic effects of mercury. Organic mercury compounds are primarily toxic to the central nervous system. Methyl mercury damages DNA (Sager et al., 1984) and impairs mitosis (Hammond, 1971). Due to its ability to disrupt neuronal migration (Hammond, 1971), it is especially toxic during
all developmental phases. Inorganic mercury is primarily nephrotoxic (Daston et al., 1984). The development of arrhythmias and cardiomyopathy has been linked to toxic levels of mercury (Agency for Toxic Substances and Disease Registry [ATSDR], 1999; Frustaci et al., 1999; Jalili and Abbasi, 1961). The net results of mercury intoxication include cerebral palsy, seizures, mental retardation, deafness, blindness and ultimately death ([WHO] World Health Organization, 1990). Cases of large-scale mercury poisoning of human populations have thankfully been rare, however, humans are constantly exposed to low, subtoxic levels of the metal. The neurotoxic and nephrotoxic effects of mercury, which develop following exposures to high doses, are consistent and develop in all individuals and animal models exposed. Toxic doses of mercury (especially the organic species) induce cell death in the immune system (Descotes, 1986; Havarinasab and Hultman, 2005), resulting in immunosuppression, but in instances of organic mercury poisoning this would be overshadowed by the more life-threatening neurotoxic effects. However, mercury can profoundly affect the immune system at concentrations well below that required to damage the central nervous system and the kidneys. Depending on the species of mercury, either immunosuppression (organic mercury) or immunostimulation (inorganic mercury) can result (Havarinasab and Hultman, 2005). Additionally, inorganic mercury can disrupt normal tolerance and induce autoimmunity. Importantly, these effects induced by low levels of mercury are controlled by genetic elements and differ widely among individuals.
Rodent models of inorganic mercury-induced autoimmunity

Druet and colleagues first observed that repeated administration of low doses of Hg$^{2+}$ (in the form of HgCl$_2$) induced a membranous glomerulonephritis in Wistar rats (Bariety et al., 1971). Subsequent studies demonstrated that subtoxic doses (1 mg/kg body weight) in susceptible rat strains induced an autoimmune syndrome with polyclonal B and T cell activation, increased serum immunoglobulin levels, autoantibody production, and renal immune complex deposition and glomerulonephritis (Druet et al., 1978; Druet et al., 1988). The autoantibodies produced possess a variety of specificities, including anti-DNA, anti-phospholipid, anti-glomerular basement membrane, anti-laminin 1 and anti-thyroglobulin (Marriott et al., 1994; Pusey et al., 1990). Additionally, susceptible strains develop widespread tissue injury including necrotizing vasculitis in the gut (Mathieson et al., 1992). The Hg$^{2+}$-induced autoimmune disease is self-limiting, with most symptoms resolving after 4–5 weeks. This attenuation of the serological and clinical manifestations occurs even if the HgCl$_2$ injections are continued. Following this resolution phase, a resistance phase takes place. At this point, the animals are resistant to further induction of autoimmunity by HgCl$_2$ treatment. This resistance is mediated by suppressor CD$^+$ T cells (Bowman et al., 1984; Castedo et al., 1993; Mathieson et al., 1991; Pelletier et al., 1990).

HgCl$_2$ also induces autoimmune manifestations in certain mouse strains. Goter-Robinson and colleagues (Goter Robinson et al., 1984) first observed that HgCl$_2$ elicited autoantibody production in outbred Swiss ICR mice. Subsequent studies in a number of inbred strains demonstrated that the mercury-induced syndrome in mice includes
polyclonal expansion of T and B lymphocytes, with increases in serum IgG1 and IgE, autoantibody production and a mild glomerulonephritis associated with immune complex and complement deposition in the kidneys (Bagenstose et al., 1999a; Hultman et al., 1992; Hultman et al., 1993; Pollard et al., 2005). An important distinction between autoimmunity in mice and rats is that autoantibody production in mice is highly specific, being mostly directed towards nucleolar antigens (Hultman et al., 1992; Hultman et al., 1989; Monestier et al., 1994; Pollard et al., 1997; Pollard et al., 2000b). However, low levels of poorly defined anti-chromatin and anti-histone antibodies also develop (Hultman et al., 1992). The polyclonal B cell activation with increase in serum immunoglobulins is transient and resolves by 4 weeks after the start of treatment; however antinucleolar autoantibodies can persist for months after the cessation of treatment (Hultman et al., 1996). Unlike rats, which show a suppressor CD8+ T cell-mediated resistance phase, autoimmunity can be re-induced in mice by administration of mercury. Importantly, mercury-induced autoimmunity in both rats (Goldman et al., 1991) and mice (Hultman et al., 1992) is under stringent genetic restriction and different rodent strains show varying degrees of susceptibility or resistance.

**Direct effects of mercury on cells**

To better understand the mode of action of mercury *in vivo*, it is necessary to examine the direct effects on cells. Hg^{2+} is a very potent thiol binding agent (Oram et al., 1996); a significant part of the effects it induces are attributable to its ability to bind to thiol-containing molecules. Mercury also shows substantial affinity for amines, phosphoryl, carboxyl, and hydroxyl groups (Passow et al., 1961). Thus mercury has a
capacity to bind to a wide range of biological molecules and affect their function. Numerous studies have examined the in vitro effects of mercury in cell lines and primary cell cultures across a range of concentrations. The picture emerging from these is complex and often contradictory, as the effect of mercury is both dose- and cell type-dependent. At higher concentrations, it is cytotoxic. Numerous studies have examined the mode of mercury-induced cell death, however there are some disagreements in the reports (Ben Ozer et al., 2000) attributable to differences in concentrations and species of mercury used, and different cell types studied. Numerous groups (Araragi et al., 2003; Kim and Sharma, 2003; Kim and Sharma, 2004; Lash et al., 2007; Yole et al., 2007) have reported that mercury induces cell death in various cell lines and primary cell cultures, by apoptosis or necrosis or a mixture of the two, in a time and dose-dependent manner. Moreover, mercury-induced cytotoxicity has been demonstrated in cells of the immune system, including B cells, T cells and monocytes (Kim and Sharma, 2004; Shenker et al., 1992; Shenker et al., 1993a). However, as previously mentioned, autoimmunity develops following administration of low, sub-toxic amounts of the heavy metal; at lower concentrations, mercury is no longer cytotoxic, instead it can exert immunomodulatory, mitogenic and anti-apoptotic effects.

Effect on Glutathione: Binding of mercury to an abundant and important cellular thiol, glutathione (GSH) plays an important part in its cytotoxic and immunomodulatory effects. Mercury uptake, accumulation and toxicity is dependent on the cellular level of GSH (Baggett and Berndt, 1986; Berndt et al., 1985; Burton et al., 1995; De Ceaurriz et al., 1994; Girardi and Elias, 1993; Lash et al., 1999; Zalups and Lash, 1997). Shenker
and colleagues (Shenker et al., 1993b) observed a positive correlation between cellular levels of GSH and resistance to cytotoxic and immunotoxic effects of mercury. Conversely, mercury affects the cellular GSH levels; Hultberg and colleagues observed that low concentrations of Hg^{2+} (up to 50 µM) increased total cellular GSH levels in the HeLa cell line suggesting that, at low concentrations, mercury increased GSH synthesis (Hultberg et al., 1998). Another study (Woods and Ellis, 1995) reported an early upregulation of GSH synthesis following mercury exposure and suggested that this enhancement was linked to Hg^{2+}-induced free radical generation (Woods and Ellis, 1995). In contrast to these studies demonstrating a low dose Hg^{2+}-induced up regulation of GSH, the majority of reports have shown that Hg^{2+} exposure, in a time- and dose-dependent manner will deplete intracellular glutathione stores (Lash et al., 2007; Mondal et al., 2005; Shenker et al., 1993b). This effect has been demonstrated in many cell types, including T cells, B cells and monocytes (Shenker et al., 1993b).

**Modulatory effects:** Mercury can bind to sulphhydryl group–containing surface molecules and cause receptor aggregation leading to deregulated cell-signaling. Nakashima and colleagues (Nakashima et al., 1994) reported that high, rapidly-cytotoxic doses (over 100 µM) of mercury caused aggregation of surface CD4, CD3, CD45 and Thy-I molecules and resulted in dramatic recruitment and activation of the protein tyrosine kinase (PTK) p56lck, thus deregulating lymphocyte signal transduction pathways. McCabe, Rosenspire and colleagues observed that non-toxic doses (0.1-1 µM) similarly upregulated PTK activation in splenocytes and a mouse B cell lymphoma cell line, showing that low doses can also deregulate normal lymphocyte signal transduction
pathways (McCabe et al., 1999). Low doses of mercury inhibited LPS-induced (Kim et al., 2002) as well as TNF-α- and IFN-γ-induced (Tian and Lawrence, 1996) nitric oxide (NO) production in macrophages and IL-1β-induced NO production in pancreatic islet β cells (Eckhardt et al., 1999). Furthermore, Kim and colleagues demonstrated that the inhibition of NO production is due to a mercury-induced downregulation of NF-κB nuclear translocation (Kim et al., 2002). Downmodulation of LPS-induced NF-κB translocation and DNA binding has been observed by other researchers in mercury-treated kidney epithelial cells (Dieguez-Acuna et al., 2001). This study demonstrated that mercury directly decreased NF-κB activation by impairing IκBα proteolysis. In contrast to its effect on NF-κB, mercury activated p38 mitogen-activated protein kinase (p38MAPK) and synergistically increased LPS-induced p38MAPK phosphorylation. Mercury also induces a dose–dependent activation of protein kinase C (Badou et al., 1997; McCabe et al., 1999; Yeh et al., 2004). This activity may be linked to the mercury-induced increase in intracellular calcium (Ca^{2+}) levels (Badou et al., 1997; McCabe et al., 1999; Yeh et al., 2004). The ability of mercury to increase intracellular Ca^{2+} levels has also been demonstrated in rat splenic T cells (Tan et al., 1993). Additionally some Ca^{2+} blockers inhibit mercury-induced cytotoxicity, suggesting the involvement of Ca^{2+} in mercury-induced cell death (Gassó et al., 2001). Mercury-induced intracellular Ca^{2+} elevation increases reactive oxygen species (ROS) (Kim and Sharma, 2003) and lipid peroxidation of mitochondrial membranes (Lund et al., 1993).

**Mechanisms of tolerance disruption:** Defects in apoptosis have been linked to the development of autoimmunity. CD95 (also called Fas) is a transmembrane death
receptor belonging to the TNF/nerve growth factor family. CD95 is important in the maintenance of peripheral tolerance; the CD95-CD95L signaling contributes substantially to the elimination of peripheral lymphocytes after an immune response, by inducing the so-called ‘activation-induced cell death’ (AICD) (Alderson et al., 1995; Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Sartorius et al., 2001). Defects in this pathway can give rise to lupus-like and rheumatoid-like autoimmune disorders in mice and humans (Martin et al., 1999; Watanabe-Fukunaga et al., 1992; Zhang et al., 2001). CD95 activation results in clustering of the receptor, followed by recruitment of Fas-associated protein with death domain (FADD) and procaspase 8 to form a death-inducing signaling complex (DISC) (Algeciras-Schimnich et al., 2002; Chinnaiyan et al., 1995; Kischkel et al., 1995; Scaffidi et al., 1999). The formation of DISC results in auto-proteolytic cleavage of pro-caspase 8 to generate caspase 8, which initiates the apoptosis cascade by cleaving and activating effector caspases (Algeciras-Schimnich et al., 2002; Scaffidi et al., 1999). In contrast to the many studies that demonstrate mercury-induced apoptosis at high doses, McCabe and colleagues showed that low doses (in the range of 5-10 µM) of mercury attenuated CD95-induced apoptosis (Whitekus et al., 1999; Ziemba et al., 2005). Jurkat T cells or Hut cells cultured in the presence of a CD95 agonist or CD95 demonstrated increased survival and continued to proliferate in presence of mercury (Whitekus et al., 1999; Ziemba et al., 2005). Mercury did not interfere with agonist-induced CD95 receptor aggregation, but disrupted the CD95 signaling pathway by preventing proper recruitment of the adaptor protein FADD into the DISC. Mercury only blocked CD95-induced cell death, but not TNF-α- or ceramide-6-induced cell death.
In addition to its inhibitory effects on apoptosis, low doses of mercury can also promote cell proliferation. *In vitro* studies have revealed that mercury elicits lymphoproliferation in a number of species including guinea pigs, rats, rabbits (Pauly et al., 1969), humans (Caron et al., 1970) and mice (Jiang and Moller, 1995; Pollard and Landberg, 2001). The concentration range that induces lymphoproliferation *in vitro* is very narrow (Berger and Skinner, 1974; Jiang and Moller, 1995; Pollard et al., 2005). A study of different mouse strains has revealed a genetic control of the process (Jiang and Moller, 1995). A low concentration (10 μM) induced a strong *in vitro* proliferative response in splenocytes of mouse strains susceptible to mercury-induced autoimmunity, while inducing little or no proliferation in splenocytes of resistant mice (Jiang and Moller, 1995).

Taken together, these studies suggest that following exposure to low doses of mercury, potentially autoreactive lymphocytes may escape immune suppression that normally occurs in peripheral lymphoid organs. Inhibition of apoptotic activities necessary for the maintenance of peripheral T cell homeostasis may then set the stage for development of autoimmune disease in genetically predisposed individuals (Bleesing et al., 2000; Hayashi and Faustman, 2003; Lenardo, 2003; Ziemba et al., 2005).

A limiting factor in extrapolating these *in vitro* effects to those induced *in vivo* is rapid dispersal of mercury within the body. Autoimmunity can be induced in a number of ways, including subcutaneous injection of mercury. Concentrations at the injection site are likely to be cytotoxic, and will cause local cell death and inflammation.
Following dispersion, immune cells possibly encounter mercury at a range of sub-toxic concentrations, which will modulate cytokine production. The autoimmune process elicited by mercury could be attributable to both the cytotoxic and the immunomodulatory effects induced by different concentrations.

**Genetic control of heavy metal-induced autoimmunity**

Development of the HgCl$_2$-induced autoimmune syndrome in rats is dependent on MHC class II genes of the rat RT-1 locus; inbred strains carrying the RT-1$^b$ haplotype are highly susceptible, RT-1$^{a, c, b, f, k}$ haplotypes confer intermediate susceptibility, while the RT-1$^l$ haplotype confers resistance (Druet et al., 1977; Goldman et al., 1991). Mercury-induced autoantibody production in mice is also under stringent genetic control, with susceptibility mapping to the I-A region of the MHC class II locus (Hansson and Abedi-Valugerd, 2003; Hultman et al., 1992; Mirtcheva et al., 1989; Robinson et al., 1986). A number of investigators have extensively investigated the effect of the H-2 haplotype on mercury-induced autoantibody production and have demonstrated that H-2$^a$ mice are most susceptible, H-2$^q$ and H-2$^f$ mice have intermediate susceptibility (Hansson and Abedi-Valugerd, 2003; Hultman et al., 1992), while H-2$^a$, H-2$^b$ and H-2$^d$ mice are resistant (Hultman et al., 1992; Mirtcheva et al., 1989; Robinson et al., 1986). Sobel and colleagues investigated the mechanism by which H-2 alleles control autoantibody production (Hanley et al., 1997; Hanley et al., 1998). They reported that F1 offspring of MHC-congenic susceptible H-2$^a$ and resistant H-2$^b$ mice, which co-dominantly expressed both I-A$^a$ and I-A$^b$ gene products, were resistant to mercury-induced antinucleolar autoantibody production (Hanley et al., 1998). These results are in contrast to other
autoimmune models wherein MHC class II heterozygosity either enhances autoimmunity or modestly affects antibody titers (Hanley et al., 1997; Hanley et al., 1998; Hirose et al., 1986; Nygard et al., 1993). The authors further observed that resistance to autoantibody production was not merely the result of lower expression of the susceptible I-A<sup>s</sup> molecule, but was caused by co-expression of the I-A<sup>b</sup> molecule itself, on the otherwise responsive B cells (Hanley et al., 1997). However, non H-2 genes also control development of the heavy metal-induced autoimmune syndrome (Hansson and Abedi-Valugerdi, 2003; Hultman et al., 1992; Hultman et al., 1993; Mirtcheva et al., 1989; Robinson et al., 1986; Stiller-Winkler et al., 1988). Mouse strains bearing the same H-2 haplotype show varying degrees of overall susceptibility to mercury. H-2<sup>d</sup> mice do not develop autoantibodies following mercury administration, although different mouse strains bearing this haplotype show greatly varying susceptibility to mercury-induced autoimmune disease. For example, BALB/c (H-2<sup>d</sup>) mice are highly susceptible to both lymphoproliferation and immune complex glomerulonephritis, B10.D2 (H-2<sup>d</sup>) mice are susceptible to lymphoproliferation, but develop less severe immune complex glomerulonephritis than BALB/c mice, while DBA/2 (H-2<sup>d</sup>) mice are resistant to both lymphoproliferation and glomerulonephritis (Hultman et al., 1992; Hultman et al., 1993; Stiller-Winkler et al., 1988). Of the various inbred mouse strains studied, A.SW (H-2<sup>s</sup>) mice are the most susceptible, while DBA-2 (H-2<sup>d</sup>) mice are resistant to all features of mercury-induced autoimmunity. Although specific susceptibility genes predisposing to mercury-induced autoimmunity have yet to be identified, some progress has been made. Pollard and colleagues (Kono et al., 2001) investigated the genetic basis of resistance to mercury in the DBA/2 strain and mapped the resistance to loci on mouse chromosomes 1
and 7. The locus on chromosome 1, designated Hmr1 (heavy metal resistance), overlaps with other lupus-predisposing loci. Strikingly, all of the lupus-prone strains, except for the MRL-lpr, have susceptibility loci that overlap with the Hmr1 interval (Bickerstaff et al., 1999; Bolland and Ravetch, 2000; Clynes et al., 1998; Majeti et al., 2000; Prodeus et al., 1998; Roths et al., 1984). The other DBA/2 resistance locus, on chromosome 7, encompasses a region that overlaps with the NZW susceptibility locus, Sle2 or Lbw5 (Kono et al., 1994; Pollard and Landberg, 2001; Rozzo et al., 1996; Silbergeld et al., 2005) and the CD22 gene, which has been linked to lupus-like disease (O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1996).

**Mechanisms underlying autoantibody production**

One of the most striking aspects of the autoimmune syndrome in the mouse is the specificity of autoantibodies directed towards nucleolar antigens. These autoantibodies predominantly recognize fibrillarin, a 34 kDa basic protein of the small nucleolar ribonucleoprotein particles (snoRNPs) (Hultman et al., 1989). A subset of autoantibodies recognize other protein components in snoRNPs (Monestier et al., 1994; Yang et al., 2001), although these protein components have not yet been identified. The anti-fibrillarin response found in mercury-treated mice is very similar to the anti-fibrillarin response diagnostic of a subset of patients suffering from systemic sclerosis (Kindas-Mugge, 1989), which also shows association with certain MHC class II alleles (Arnett et al., 1996). Two other heavy metals, silver and gold also elicit highly specific anti-fibrillarin autoantibodies in a MHC class II-restricted manner (Havarinasab et al., 2007; Hultman et al., 1994; Hultman et al., 1995b; Robinson et al., 1986).
The mechanisms by which heavy metals like mercury, silver and gold disrupt tolerance and induce autoimmune manifestations have been extensively investigated, but no definitive model has emerged. An important issue is why nucleolar antigens are specifically targeted in this process. Silver salts have been used since the early 1900s as histological stains and they display particular affinity for the nucleolus (Ochs, 1998; Rosen and Casciola-Rosen, 1998). A number of studies have offered evidence suggesting that mercury may specifically target fibrillarin, modifying it to reveal cryptic epitopes which activate T cells (Chen et al., 2002; Kubicka-Muranyi et al., 1995; Kubicka-Muranyi et al., 1996; Pollard et al., 1997). Chen and colleagues observed that mercury induces a redistribution of fibrillarin resulting in its colocalization with nucleoplasmic proteasomes (Chen et al., 2002). This effect was observed both in cell lines treated in vitro with mercury and in splenocytes from mercury-injected mice. In contrast, other nucleolar proteins such as nucleolin or B23 (which are not targeted during mercury-induced autoimmunity) did not colocalize with proteasomes after mercury exposure. Pollard and colleagues demonstrated that treatment with cytotoxic doses (40 µM) of mercury resulted in modification of molecular and antigenic properties of fibrillarin, and mutations of cysteines in the fibrillarin sequence to arginine abolished this effect (Pollard et al., 1997). Furthermore, mercury-induced cell death in macrophages produced a novel 19 kDa peptide of fibrillarin which was not found following cell death induced by other stimuli (Pollard et al., 2000a). Immunization with this peptide, but not intact fibrillarin, induced production of anti-fibrillarin autoantibodies with some of the properties of the HgCl₂-induced anti-fibrillarin response. Evidence suggesting that Hg-
induced modification of fibrillarin played a role in the autoimmune process came from a study which demonstrated that CD4 T cells from mercury-treated B10.S mice exhibited a strong proliferative response to mercury-complexed fibrillarin, but responded to a lesser degree to normal fibrillarin (Kubicka-Muranyi et al., 1995). Furthermore, T cells obtained from naïve mice did not respond to mercury-treated fibrillarin (Kubicka-Muranyi et al., 1995). Collectively, these studies suggest that direct mercury-induced modification of fibrillarin and mercury-induced alterations in fibrillarin processing play a role in breaking tolerance to this protein.

Although the initial T cell response is restricted to mercury-modified fibrillarin, it can then spread to epitopes present on native fibrillarin. T cells isolated from B10.S mice treated short-term with mercury vigorously responded to mercury-complexed fibrillarin, but not to the native configuration of the protein. In contrast, T cells isolated from B10.S mice treated long-term with mercury showed equal responses to both mercury-modified and native fibrillarin (Kubicka-Muranyi et al., 1996), suggesting that, although mercury-modification of fibrillarin is required for the initial loss of tolerance, epitope spreading subsequently occurs.

The standard model of antigen processing and presentation holds that endogenous antigens (such as fibrillarin) are processed by proteasomes via a non-lysosomal pathway and presented to CD8+ T cells in the context of cell surface MHC class I molecules, whereas exogenous antigens typically are processed by the lysosomal pathway and presented to CD4 T cells in the context of cell surface MHC class II molecules (Germain
and Margulies, 1993; Yewdell and Bennink, 1990). However mercury-induced autoantibody production is MHC class II restricted. The theory that mercury-induced antinucleolar autoantibody production is due to abnormal fibrillarin presentation, which is thought to be via the class I pathway, does not reconcile with this. An increasing number of studies have provided evidence that the two major antigen-processing and presentation pathways are less restrictive than originally recognized. Endogenous antigens are presented in the context of MHC class II molecules to activate CD4+ T cells, and exogenous antigens that are phagocytosed by antigen-presenting cells can gain access to the MHC class I pathway (Albert et al., 1998; Chen and Mikecz, 2005; Guermonprez et al., 2003; Malnati et al., 1992; Munz et al., 2000). Although the cellular mechanisms allowing such crisscross pathways are not clear, chaperones such as heat shock proteins might mediate both cross-presentation pathways for exogenous antigens on MHC class I molecules, and for endogenous antigens on MHC class II molecules (Heath and Carbone, 2001; Reed, 2000). Interestingly, mercury increases the levels of both cytoplasmic and nucleocytoplasmic heat shock proteins (Bauman et al., 1993; Brkljacic et al., 2007; Goering et al., 1992; Goering et al., 2000), allowing for the possibility that in mercury-treated cells, alternately processed or mercury-modified fibrillarin epitopes are presented by MHC class II molecules to CD4+ T cells, breaking T cell tolerance to this self protein.

**Molecular and cellular mechanisms of mercury-induced autoimmunity**

**Role of T cells:** As mentioned earlier, mercury induces a strong lymphoproliferation of splenocytes from mice susceptible to autoimmunity, while inducing little or no proliferation in splenocytes from mercury-resistant mouse strains.
Studies by Jiang (Jiang and Moller, 1995) and Pollard (Pollard and Landberg, 2001) showed that it was splenic T cells but not B cells that proliferate in response to mercury. Pollard and colleagues further observed that only mature T cells proliferated in response to mercury; thymocytes exposed to the heavy metal did not show increased proliferation (Pollard and Landberg, 2001). This ability is controlled by non-MHC class II genes, as demonstrated by studies in two mouse strains, BALB/c and DBA/2, which share the same MHC class II haplotype (H-2d) but show opposite responses to the metal (Jiang and Moller, 1995). Additionally, when splenocytes were taken from mercury-susceptible, high-responder strains, both CD4+ and CD8+ T cells proliferated in response to mercury. However, only the CD8+ T cells from mercury-resistant, low-responder mice proliferated in the presence of the heavy metal (Jiang and Moller, 1995). Additionally, adherent cell-induced T cell proliferation could be blocked by anti-MHC class II antibodies, but not by anti-MHC class I antibodies (Hu et al., 1997b). Interestingly, the ability of T cells to respond to mercury showed T cell receptor restriction; only T cells bearing certain Vβ chains, such as Vβ6, 8, 10 and 14 in the BALB/c strain, and Vβ6, 7 and 14 in the SJL strain, could proliferate in response to mercury (Jiang and Moller, 1996).

Pollard and colleagues also reported that the proliferation response was costimulation-dependent, as it was inhibited by antibodies to adherent cell-derived IL-1 and to a lesser extent by antibodies to CD40L, CD80 and CD86 (Pollard and Landberg, 2001). Collectively these studies suggest that adherent cells (macrophages) induce T cell proliferation in a class II-restricted manner in response to mercury-induced antigens. Additionally, Hu and colleagues (Hu et al., 1997a) showed that in experiments where
mercury exposure was limited and excess mercury was removed by washing prior to pulsing with $[^3]H$ thymidine, proliferation was increased in splenocytes from both high and low responder mice, with a greater activation of CD4 T cells in both groups. This study suggests that, although mercury can induce proliferation at a narrow range of low concentrations, increasing concentrations have a suppressive effect.

Pelletier and colleagues observed that BN rats deficient in T cells did not develop autoimmunity following mercury treatment (Pelletier et al., 1987). Hultman and colleagues also made the same observation in mercury-susceptible athymic mice or mice that were CD4$^+$ T cell depleted (Hultman et al., 1995a), establishing an essential role for T cells in mercury model. HgCl$_2$ exposure rapidly increases expression of cell surface markers of T cell activation and proliferation including CD25 (IL-2 receptor) and the transferrin receptor CD71 (Johansson et al., 1997). Additionally Pelletier and colleagues showed that T cells from susceptible HgCl$_2$-treated BN rats transferred disease to untreated recipients (Pelletier et al., 1988). Layland and colleagues similarly demonstrated that CD4$^+$CD25$^-$ T cells from mercury-treated mice induced autoantibodies in naïve animals (Layland et al., 2004).

The specificity of T cells in mercury-induced autoimmunity is unclear, although autoreactive clones have been identified in both mercury-treated rats (Rossert et al., 1988) and mice (Kubicka-Muranyi et al., 1995). Autoreactive T-helper cell populations in rats included self-MHC class II I-A reactive clones (Rossert et al., 1988). This study (Rossert et al., 1988) showed T cell autoreactivity to be mercury-independent, as
autoreactive T cells could be activated by B cells from untreated mice, and could transfer
disease to naïve mice without accompanying treatment with mercury (Rossert et al.,
1988). In mercury-treated mice, T cell autoreactivity is directed towards several
nucleolar targets, including fibrillarin (Kubicka-Muranyi et al., 1995). Somewhat unlike
rats, autoreactive T cell clones identified in mercury treated mice recognized both normal
fibrillarin as well as mercury-modified fibrillarin (Kubicka-Muranyi et al., 1995).

Susceptibility to mercury-induced autoimmunity correlates with a T cell-intrinsic
ability to proliferate in response to the heavy metal; in vitro studies have shown that T
cells from susceptible animals have a strong capacity to proliferate in response to
mercury and that cognate T cell-adherent cell interactions are required for this. On the
other hand, T cells from mercury-resistant strains showed little or no ability to proliferate
in response to mercury in vitro (Jiang and Moller, 1995). Kubicka-Muryani and
colleagues (Kubicka-Muranyi et al., 1993) demonstrated the same phenomenon in vivo.
They transferred irradiated splenocytes from either susceptible or resistant mercury-
treated donors accompanied by injections of HgCl₂ or PBS into the footpads. The
mercury injection in the recipients was optimized to induce proliferation, but was lower
than that required to induce autoimmunity. The authors reported that this protocol
induced a mercury-dependent proliferation of popliteal lymph node cells only in the
susceptible strains, but not the resistant strains. In agreement with the in vitro (Jiang and
Moller, 1995; Pollard and Landberg, 2001) observations, the authors found that it was the
CD4⁺ T cells that proliferated in response to mercury, and further demonstrated that these
T cells could proliferate in response to mercury stored in killed peritoneal cells from
mercury-injected donors. Both the in vitro (Jiang and Moller, 1995; Pollard and Landberg, 2001) and in vivo (Kubicka-Muranyi et al., 1993) studies suggest that the antigen-presenting cell inducing the T cell proliferation are macrophages, which accumulate (Warfvinge et al., 1994) mercury following in vivo administration.

The other factor determining susceptibility or resistance initially appeared to be T cell polarization towards either Th1 or Th2. A study by van Vliet and colleagues (van Vliet et al., 1993) suggested that polarization towards Th1 or Th2 in the mercury model may be MHC-controlled. This study compared T cell responses in mercury-susceptible B10.S (H-2s) or mercury-resistant B10.D2 (H-2d) mice, and showed that although CD4 T cell activation occurred in both strains, the proportion of IL-4 mRNA-expressing T cells was greater in the strain with the susceptible H-2s haplotype (van Vliet et al., 1993) and that mercury had an immunostimulatory effect in these mice. The study further demonstrated that mercury induces a mild IFN-γ dependent immunosupression of the antibody response following sheep erythrocyte injection only in the resistant mouse strain. Thus, HgCl₂ in resistant mouse strains appears to induce more IFN-γ, while in susceptible strains a stronger IL-4 response is generated. Some rat studies supported this finding, showing increases of IL-4 and IFN-γ mRNA in splenocytes of mercury-treated susceptible BN rats (Gillespie et al., 1996) as well as slight increases in IL-12 mRNA levels in spleen and lymph nodes from these animals (Mathieson and Gillespie, 1996). In these studies, HgCl₂ failed to increase cytokines in resistant LEW rats (Gillespie et al., 1996). Additionally, resistant LEW rats showed higher baseline levels of IL-12 (Mathieson and Gillespie, 1996) and IFN-γ (Gillespie et al., 1996) mRNA than BN rats,
supporting the theory that an increased Th2 response is one of the determining factors for susceptibility. Additional evidence for this theory came from a study which reported that an antibody to the Th1-associated marker OX221 before administration of HgCl$_2$ lead to exacerbation of the autoimmune syndrome in susceptible BN rats (Mathieson et al., 1993). Additionally, self-MHC class II-reactive T cells that are of the Th1 type suppress development of autoimmunity in this strain (Druet and Pelletier, 1996). However, although Th1 polarization \textit{in vivo} correlates to resistance to mercury-induced autoimmunity, knockout studies showed that Th1 cytokines are required in this model (Kono et al., 1998) (discussed below).

How might mercury modulate the Th1/Th2 balance? Mercury is a strong thiol binder; it depletes cellular GSH, as has been demonstrated in B cells, T cells and macrophages exposed to mercury \textit{in vitro} (Shenker et al., 1993b). Sulfhydryl compounds have many important biologic functions, including maintenance of intracellular redox balances (Meister and Anderson, 1983). GSH is required for Concanavalin A (Con A)-mediated induction of IFN-\(\gamma\)-producing cells \textit{in vitro} (Van der Meide et al., 1993). Furthermore, N-acetyl-L-cysteine, a GSH precursor, inhibits IL-4 and IgE production by human T cells \textit{in vitro} (Jeannin et al., 1995), whereas \textit{in vivo} depletion of GSH in mice results in decreased IFN-\(\gamma\) production and increased IL-4 production \textit{in vitro} (Peterson et al., 1998). A recent study showed that biologically relevant concentrations of the organic mercury compound, thimerosal inhibited LPS-induced TNF-\(\alpha\), IL-6, and IL-12p70 secretion from human monocyte-derived dendritic cells (DCs), while having no effect on IL-10 production. The thimerosal-exposed DCs increased Th2 (IL-5 and IL-13) and
decreased Th1 (IFN-\(\gamma\)) cytokine secretion from T cells. The authors further showed that thimerosal-induced glutathione depletion was responsible for these effects (Agrawal et al., 2007). Thimerosal is rapidly broken down to yield inorganic Hg\(^{2+}\) \textit{in vivo}. Therefore, Hg\(^{2+}\)-induced depletion of intracellular GSH in cells of the immune system could be underlying the stronger Th2 response elicited in HgCl\(_2\)-treated mice.

\textit{Role of B cells:} Many of the apparent immune responses to mercury (autoantibody production and polyclonal increase in serum IgG1 and IgE levels) are humoral, suggesting that mercury strongly affects B cells. However, there does not appear to be a direct effect; Pollard and colleagues observed that B cells do not directly proliferate in response to mercury \textit{in vitro}, unlike mature T cells (Pollard and Landberg, 2001). However, in some mouse strains, mercury administration induces B cell proliferation \textit{in vivo} (Johansson et al., 1998). Additionally, treatment with mercury results in strong induction of germinal centers in the spleen of susceptible A.SW mice.

As mentioned previously, mercury-induced antinucleolar autoantibody production is strictly controlled by the H-2 locus genes. Hultman and colleagues (Johansson et al., 1998) studied the effect of both H-2 and mouse strain-specific non H-2 genes on the B cell response to mercury. A.SW and SJL mice bearing the H-2\(^{s}\) haplotype (which confers maximal susceptibility) develop an early, strong, but transient B cell activation with production of Th1- as well as Th2-regulated immunoglobulin isotypes with broad (polyclonal) specificities, in addition to antinucleolar autoantibody production (Johansson et al., 1998). BALB/c mice bearing the H-2\(^{d}\) haplotype (which does not support
antinucleolar reactivity) developed a moderate but short-lived B cell activation involving mainly Th2-regulated isotypes, and persisting antinuclear autoantibodies (ANA) of the IgG class, while DBA/2 mice (also H-2^d) developed only a minimal B cell response without autoantibodies (Johansson et al., 1998). This study demonstrated that, although the B cell autoantibody response is strongly linked to the MHC haplotype, the ability to proliferate depends on both MHC and non-MHC genes. The authors also observed that in the susceptible H-2^s strains, the mercury-induced increase in splenic B cells was preceded by an upregulation of the proliferation marker CD71 (Johansson et al., 1998). Additionally, the A.SW strain also showed an increased expression of CD23 (low-affinity IgE receptor) (Johansson et al., 1998) on B cells after 7–14 days of mercury treatment, which coincides with the increase in IL-4-producing cells in this strain. Prigent and colleagues (Prigent et al., 1995b) demonstrated an IL-4-driven CD23 upregulation on B cells following mercury administration in BN rats.

A study from our laboratory examined whether mercury might increase survival of autoreactive B cells in the periphery. An important checkpoint in B cell ontogeny is the T1-T2 transition in the secondary lymphoid organs. Significant cell losses occur at this point, suggesting that this step is a selection checkpoint where self-reactive B cells may be eliminated (Allman et al., 2001). B cell activation factor of the tumor necrosis family (BAFF) is a key survival factor for B cells, required for the T1-T2 transition in the periphery. BAFF is expressed (in both membrane-associated and secreted forms) by monocytes, macrophages, dendritic cells, B cells and to a lesser extent, T cells (Chu et al., 2007; Hahne et al., 1998; Litinskiy et al., 2002; Mackay et al., 2007; Nardelli et al.,
Elevated levels of BAFF are observed in sera and target organs of mouse models that develop systemic lupus erythematosus (SLE) (Mackay et al., 2007). Moreover, blockade of BAFF using a fusion protein of one of its ligands, TACI, ameliorated manifestations of SLE (Gross et al., 2000). We examined whether emergence of autoreactive B cells in susceptible mice was related to induction of BAFF by mercury (Zheng et al., 2005). We found that treatment with mercury increased serum BAFF levels only in susceptible A.SW mice, but not in resistant C57BL/6 and DBA/2 mice (Zheng et al., 2005). Self-reactive B cells constitute a fairly significant proportion (5–20%) of the naïve B-cell repertoire in most individuals (Wardemann et al., 2003). In normal individuals, autoreactivity is kept in check by peripheral tolerance mechanisms, such as anergy. Anergic B cells show greater dependence than naïve B cells on BAFF for survival, and over-expression of BAFF attenuates apoptosis and promotes B cell survival. Thus, BAFF-mediated rescue of anergic self-reactive B cells from competitive elimination, as well as attenuation of apoptosis of self reactive lymphocytes, could play an important role in mercury-induced breakage of tolerance. Supporting this theory, BAFF blockade (using TACI-Ig), greatly reduced the mercury-induced autoantibody response (Zheng et al., 2005).

_Mercury-induced cytokine production:_ A number of studies have examined cytokine production following mercury treatment _in vitro_, however a clear and comprehensive picture has yet to emerge. Overall, treatment of lymphocytes with mercury modulates production of several cytokines, including IL-1, IL-2, IL-4, TNF-α and IFN-γ (Badou et al., 1997; Hu et al., 1997a; Hemdan et al., 2007). A number of
studies demonstrate that in vitro mercury exposure induces Th2 cytokines in susceptible mice and rats, while more Th1 cytokines are induced in resistant animals. Prigent and colleagues (Prigent et al., 1995b) showed that 5 μM HgCl₂ treatment strongly induced IL-4 mRNA in splenocytes and purified T cells from mercury susceptible BN rats but not in those from resistant LEW rats. On the other hand, IFN-γ was induced in splenocytes from both strains. Olivera and colleagues (Oliveira et al., 1995) demonstrated a role for mast cells in the production of IL-4. Moreover, mast cells from BN rats, but not LEW rats, showed an induction of IL-4 following in vitro treatment with HgCl₂. Additionally, exposure of a mast cell line to HgCl₂ enhanced both IL-4 mRNA and its promoter activity. Oxidative stress by hydrogen peroxide mimicked the effects of mercury in enhancing IL-4 promoter activity, suggesting that mercury-induced IL-4 production could be due, in part, to its free radical-inducing effects (Wu et al., 2001). Hu and colleagues demonstrated that, like lymphoproliferation (Hu et al., 1997a), cytokine production was also affected by exposure to Hg²⁺. They compared cytokine production from splenocytes following continuous treatment with 10 μM HgCl₂, or after pretreatment of cells followed by washing to removing excess mercury. The continuous presence of 10 μM HgCl₂ in vitro induced IL-2 and IFN-γ production, but no IL-4 production from cells of both susceptible H-2^s and resistant H-2^b mice. In contrast, pretreatment of cells with mercury followed by washing resulted in IL-4 production in cells of susceptible and resistant mice (Hu et al., 1997a). A recent study by Sack and colleagues demonstrated that HgCl₂ exposure (at concentrations below 5 μM) increased IL-4 and IL-10 production and decreased IFN-γ, TNF-α and IL-6 production in monoclonal antibody (anti-CD3/-CD8/-CD40)-activated human PBMCs (Hemdan et al., 2007). Another study showed that
subtoxic concentrations (5-20 µM) of HgCl$_2$ increased TNF-α expression by regulating the p38 pathway in murine macrophages *in vitro* (Kim and Sharma, 2004). Overall, while reports of the potentiating effect of mercury on IL-4 production are more consistent, considerable variation exists with effects reported on other cytokines (Hemdan *et al.*, 2007; Kim and Sharma, 2004).

Cytokines elicited by *in vivo* mercury administration can be examined at two levels: cytokines induced locally at the site of injection, and those induced at a systemic level. Pollard and colleagues examined local cytokine production and showed that a single mercury exposure elicits increased expression of proinflammatory cytokines such as TNF-α, IL-1, IL-6 as well as IL-10 at the site of injection, while IFN-γ is detected at a later time point, after a second injection of mercury (Pollard *et al.*, 2004; Pollard *et al.*, 2005).

T-helper cell polarization is a crucial determinant of an immune response. Th1 and the recently discovered Th17 cells play a pivotal role in several cell-mediated autoimmune diseases (Bettelli *et al.*, 2007; Druet *et al.*, 1995). Th2 cells, on the other hand, have been implicated in autoimmune syndromes involving B cell dysfunction, such as SLE (Mok and Lau, 2003). A number of investigators examined the importance of Th1 or Th2 pathways in the mercury model by studying disease development following cytokine blockade or recombinant cytokine administration and by using knockout mice. Studies of the Th1-inducing cytokine IL-12 yielded the contradictory results of either protection (Bagenstose *et al.*, 1998a) or exacerbation (Haggqvist and Hultman, 2003) of
mercury-induced autoimmunity. Many lines of evidence suggest that Th2 polarization correlated with susceptibility to mercury, but IL-4 knockout mice still showed antinucleolar autoantibody (of the IgG2a and IgG2b subclasses) in response to mercury, showing that IL-4 is not required for loss of tolerance to nucleolar antigens (Bagenstose et al., 1998b). Although the polyclonal increases in serum IgG1 and IgE were abrogated in the IL-4−/− animals, they still showed polyclonal IgG2a and IgG2b antibodies at levels comparable with wild type animals, indicating that IL-4 is mainly needed for class switch to IgG1 and IgE isotypes. Additionally, Kono and colleagues observed comparable levels of immunoglobulin and complement deposits in the kidney in IL-4−/− animals (Kono et al., 1998). These investigators also examined the development of mercury-induced autoimmunity in IFN-γ−/− mice and found that the antinucleolar autoantibody production was drastically reduced (Kono et al., 1998). It should be noted that very low levels of antinucleolar reactivity was seen in these mice, indicating that mercury-induced breakage of tolerance can still occur in the absence of IFN-γ, although it is severely attenuated. Furthermore, both complement and immunoglobulin deposits in the kidney were virtually absent in IFN-γ−/− mice, indicating that this cytokine modulates the development and severity of mercury-induced autoimmune disease. Another study (Pollard et al., 2005) further examined the contribution of various genes, including those which induced IFN-γ expression, such as IL-12, signal transducer and activator of transcription-4 (STAT-4), and interleukin-1 converting enzyme (ICE). IL-12 plays a significant role in Th1 immunity, including IFN-γ production. STAT-4 is an essential component of the IL-12 signaling pathway (Pollard et al., 2005; Szabo et al., 2003). ICE (also known as caspase-1) cleaves pro-IL-18 to produce active IL-18 which synergizes
with IL-12 to augment IFN-γ production (Dinarello, 1998). Interestingly, the study showed that perturbation of these genes did not affect development of mercury-induced autoimmunity, beyond a decrease in autoantibody levels found in IL-12 p35−/− mice (Pollard et al., 2005). On the other hand, perturbation of genes which regulated IFN-γ function (IFN-γR, IRF-1), significantly affected development of mercury-induced autoimmunity, suggesting that IL-1, IL-18 (acting via ICE) and STAT-4 are not absolutely essential for initiating IFN-γ production, but may augment IFN-γ responses (Pollard et al., 2005; Szabo et al., 2003). Low levels of IFN-γ, present initially during mercury exposure, may be sufficient to induce autoimmunity in mercury-susceptible animals (Pollard et al., 2005). This study also examined the requirement of other cytokines for the development of autoimmune disease. TNF receptor deficiency had little effect, but IL-6−/− mice showed suppression of polyclonal B cell responses and loss of anti-chromatin reactivity, while retaining the ability to make antinucleolar autoantibodies. In contrast, the thimerosal-induced antinucleolar autoantibody production is lost in IL-6 knockout animals (Havarinasab et al., 2005).

Role of costimulation: Following antigen encounter, the nature of the immune response is determined by the integration of both positive and negative costimulatory signals delivered between innate and adaptive immune cells. A positive stimulus results in activation of the immune system with pro-inflammatory responses predominating, while a negative stimulus may result in the induction of tolerance towards the antigen. The B7-CD28 stimulatory signals are required for the initial activation of naïve T cells, while the B7-CTLA-4 inhibitory signal is an essential negative regulator following T cell
activation. On activation, CD4+ T cells express CD40L and promote B cell activation, antibody production, germinal center formation and isotype switching via the CD40-CD40L interactions (Durie et al., 1994; Snanoudj et al., 2006). In addition to the B7-CD28 and the B7-CTLA-4 signaling, T cell activation can be modulated by numerous other costimulatory pathways (Snanoudj et al., 2006). Cognate T cell–antigen presenting cell (APC) interactions, as well as T cell-B cell interactions, are required for mercury-induced breaking of tolerance. A number of investigators have also shown that both positive and negative costimulatory pathways play important roles during this process. Biancone and colleagues (Biancone et al., 1996) showed that CD40-CD40L blocking by an anti-CD40L monoclonal antibody (mAb), or B7-CD28 blocking by a recombinant CTLA-4 fusion protein abrogated the development of the autoimmune syndrome. A previous study (Bagenstose et al., 2002) from our laboratory also examined the effect of B7 blockade by administering antibodies to B7.1 and B7.2. In agreement with Biancone and colleagues, we found that blockade of both B7 ligands abrogated the effect of mercury. Individual ligand blockade using either B7.1 or B7.2 mAbs had no effect on IgG1 or IgE hypergammaglobulinaemia, indicating redundancy between the two ligands for induction of polyclonal B cell activation. However, B7.1 blockade completely inhibited mercury-induced antinucleolar autoantibodies, while B7.2 mAb had only a partial inhibiting effect, suggesting that B7.1 signaling is more important in the mercury-induced autoimmune process.

The Inducible Costimulator molecule (ICOS) is a positive costimulatory receptor also belonging to the CD28 family. Expression of ICOS occurs on activated, but not
resting, T cells (Hutloff et al., 1999). The ligand for ICOS, B7h (Swallow et al., 1999), is constitutively expressed on unstimulated B cells and is inducible on macrophages and peripheral blood-derived dendritic cells (Ling et al., 2000; Swallow et al., 1999; Yoshinaga et al., 1999). ICOS-deficient mice show defective T cell activation and proliferation and surprisingly, enhanced susceptibility to experimental autoimmune encephalomyelitis (Dong et al., 2001). A lack of ICOS in these mice also results in severely defective T cell-dependent B cell responses, germinal center formation and Ig class switching, including IgE (McAdam et al., 2001; Tafuri et al., 2001). These studies indicate a complex role for ICOS in the regulation of the autoimmune process. A study from our laboratory showed that blockade of the ICOS-B7h pathway by an anti-ICOS mAb decreased mercury-induced autoimmune manifestations, as indicated by the almost complete suppression of antinucleolar autoantibody production and a significant reduction in IgE production (Zheng et al., 2004).

4-1BB, a costimulatory molecule belonging to the TNFR superfamily, is expressed by T cells, including regulatory T cells (T\textsubscript{reg}s) and Natural Killer T (NKT) cells, as well as by NK cells, monocytes, DCs, and macrophages (Croft, 2003; Vinay and Kwon, 1998; Watts, 2005). It binds a high affinity ligand, 4-1BB ligand (4-1BBL), present on a variety of APCs (Croft, 2003; Vinay and Kwon, 1998; Watts, 2005). Several studies suggest that 4-1BB strongly enhances Th1 signaling. However the consequences of 4-1BB engagement are quite complex; both stimulatory and suppressive functions have been attributed to 4-1BB engagement on different cell types (Vinay and Kwon, 1998). Vinay and colleagues (Vinay et al., 2006) investigated the effects of 4-
1BB blockade, and observed that this treatment strongly inhibits manifestations of mercury-induced autoimmunity, irrespective of whether it was given before or after onset of symptoms. Furthermore, they showed that this treatment elicited massive IFN-γ production by CD4+ T cells, expansion of CD4+ and CD8+ T cells, and resulted in a dramatic depletion of B cell subsets, including B1, B2, marginal zone and follicular B cells. Interestingly, B cell depletion could be attributed to increased IFN-γ levels, as a neutralizing antibody restored B cells in this model. This finding is in agreement with older studies which attributed resistance to mercury-induced autoimmunity to an increased Th1 skewing (Druet and Pelletier, 1996; Gillespie et al., 1996; Mathieson et al., 1993; Prigent et al., 1995a; van Vliet et al., 1993), although the need for IFN-γ to break tolerance and establish autoimmunity in this model has been well demonstrated by knockout studies (Kono et al., 1998; Pollard et al., 2005).

The contribution of negative costimulatory receptors has also been explored. CTLA-4, a molecule highly homologous to CD28, also binds B7.1 and B7.2, but with a much higher affinity. CTLA-4 is expressed on T cells following activation and may act as a terminator of T cell responses. Zheng and colleagues examined the effect of CTLA-4 blockade using an anti-CTLA-4 mAb and showed this treatment exacerbated HgCl2-induced autoimmunity both in the early and late phases of the syndrome in susceptible A.SW mice (Zheng and Monestier, 2003). Interestingly, CTLA-4 blockade in mercury-resistant DBA/2 mice resulted in antinucleolar antibody production in these animals (Zheng and Monestier, 2003).
Fcγ receptors, which bind the Fc region of IgG antibodies, are widely expressed on cells of the hematopoietic system. They perform an important costimulatory function; Fcγ receptors can be either inhibitory (FcγRIIB in mice) or stimulatory (FcγRI, III and IV in mice) (Ravetch and Bolland, 2001). Stimulatory FcγR signaling augments the immune response by enhancing antigen presentation and immune complex-induced maturation of dendritic cells (Ravetch and Bolland, 2001). On the other hand, inhibitory FcγRIIB engagement inhibits B cell activation and plasma cell survival (Ravetch and Bolland, 2001). Deficiency of stimulatory FcγR expression results in protection in experimental models of autoimmune disease (Ravetch and Bolland, 2001), whereas gene polymorphisms that reduce expression or function of the inhibitory FcγRIIB are associated with increased susceptibility to autoimmune disease (Ravetch and Bolland, 2001). Hultman and colleagues demonstrated regulatory roles for both inhibitory and stimulatory Fcγ receptors in mercury-induced autoimmunity. Deficiency of inhibitory FcγRIIB enhances mercury-induced hyperimmunoglobulinemia (Martinsson and Hultman, 2006; Martinsson et al. 2008; Zheng and Monestier, 2003), while mice deficient in stimulatory FcγR expression demonstrated reduced autoantibody production (Martinsson et al., ) and immune complex deposition (Martinsson and Hultman, 2006).

**Induction of tolerance to mercury**

The regular regimen of mercury administration results in the development of an antigen-specific autoimmune response, accompanied by a non-specific polyclonal activation of the immune system. Interestingly, this response can be prevented by pre-administration of a low dose of mercury given intraperitoneally to naïve animals.
Beyond the finding that T\textsubscript{regs} play a role in tolerance induction and maintenance (Yan Zheng, unpublished observations), an in-depth understanding of this process is lacking.

**Relevance of mercury-induced autoimmunity**

As human populations are pervasively exposed to low levels of mercury, the question arises whether such exposures would trigger autoimmune disease similar to that seen in rodent models. There are several reports of immune dysfunction in occupationally-exposed humans. T cell lymphoproliferation (Moszczynski et al., 1995) and anti-laminin autoantibodies (Lauwerys et al., 1983) were detected in some individuals occupationally exposed to mercury vapors. Another study reported a correlation between urinary mercury levels, severity of disease and the presence of serum anti-fibrillarin autoantibodies in scleroderma patients (Arnett et al., 2000). Silbergeld and colleagues demonstrated that Amazonian populations exposed to mercury via gold mining exhibited increased levels of antinuclear and antinucleolar autoantibodies (Silbergeld et al., 2005).

Autoimmune diseases show a complex multifactorial etiology wherein one or more environmental components, in combination with underlying genetic susceptibility, could promote autoimmune disease. It is possible that mercury, in combination with other environmental factors or an underlying genetic predisposition, promotes or exacerbates autoimmune disease. This is borne out by studies in experimental animal models. Stronger autoimmune responses have been reported in (NZB x NWZ)F1 mice compared to non-autoimmune prone mice strains following exposure to mercury (al-
Balaghi et al., 1996). Another study compared the effect of mercury on C57BL/6 and autoimmune-prone BXSB mice (Pollard et al., 2001), neither of which is susceptible to typical mercury-induced autoimmunity. Mercury accelerated the onset of systemic autoimmune disease only in BXSB mice, the strain with the autoimmune-prone background. Furthermore, the disease induced was more consistent with spontaneous lupus than with typical mercury-induced autoimmunity (Pollard et al., 2001). A study by Via and colleagues showed that exposure to low-dose mercury severely exacerbated disease in a mouse model of lupus-like chronic-graft-versus-host disease (GVHD) (Via et al., 2003). Furthermore, low-dose mercury exposure increases the severity and prevalence of experimental autoimmune myocarditis (induced by immunization with cardiac myosin peptide in adjuvant) in A/J mice, a strain resistant to typical mercury-induced autoimmunity (Silbergeld et al., 2005). Collectively, these studies support the theory that exposure to low, subtoxic levels of mercury could synergize with other risk factors (genetic or environmental) to promote or exacerbate autoimmune disease.

In summary, although mercury possesses an elementary chemical structure, it can have profound and intricate effects on the immune system. The most remarkable of these effects is its ability to induce in susceptible animals a complex autoimmune syndrome. The ultimate mechanisms by which mercury can elicit autoimmune disease remain unknown. Elucidating these mechanisms would help us understand how environmental factors contribute to the development of autoimmunity in humans.
NKT cells: Powerful Regulators of the Innate Immune System

Overview

First described over 20 years ago, NKT cells represent a unique lineage of immune cells in that they demonstrate characteristics of both the innate and the adaptive immune systems. They are defined as a distinct population expressing both NK cell-lineage and T cell-lineage markers that exclusively recognize glycolipid antigens presented by a non-polymorphic molecule CD1d (Bendelac et al., 2007; Wilson and Delovitch, 2003). This population has been identified in several species, including mice and humans. Their antigen presenting molecule CD1d has been extremely well conserved in mammalian species (Porcelli and Modlin, 1999), suggesting an important evolutionarily-conserved function for these cells. NKT cells resemble activated memory T cells, expressing CD69 and high levels of CD44, even in germ-free mice. NKT cells have the capacity to be the first responders during an immune response; their activation is an extremely rapid process that robustly activates the immune system. Due to this ability, they have attracted immense interest as immunomodulators in infection, tumor immunology and autoimmunity.

NKT cell classification based on TCR usage

Although all NKT cells have the ability to react to CD1d, further classification is done based on their TCR sequence. A majority of NKT cells (termed classical or type 1 NKT cells) bear a ‘canonical’ TCR with a Vα14 to Jα18 TCR rearrangement (Vα14i) in mice (Lantz and Bendelac, 1994) and an orthologous Vα24-Jα18 TCR chain (Vα24i) (Exley et al., 1997; Lantz and Bendelac, 1994) in humans. As the complementarity-
determining region 3 (CDR3) loop is encoded in a germline configuration, the \( \alpha \)-chain is invariant in sequence and is co-expressed with a limited set of randomly-rearranged \( V\beta \)-chains, predominantly \( V\beta 8.2 \) in mice and \( V\beta 11 \) in humans (Arase et al., 1992; Hayakawa et al., 1992) generate a semi-invariant TCR. A small subset of NKT cells however bear randomly rearranged, variant TCRs and are termed Type II NKT cells (Berzofsky and Terabe, 2008). Importantly, Type I and Type II NKT cells recognize distinct glycolipid targets (Berzofsky and Terabe, 2008). This study examines only the classical or Type I NKT cell population, which for simplicity will be referred to as ‘NKT cells’.

**NKT cell ligands**

The first identified NKT cell agonist was an \( \alpha \)-branched galactosylceramide originally recognized for its potent anti-tumor activity (Kobayashi et al., 1995). This glycolipid, which was extracted from a marine sponge *Agelas mauritianus*, was slightly modified for optimal efficacy to produce a compound termed KRN7000, commonly referred to as \( \alpha \)-Galactosyl Ceramide (\( \alpha \)-GalCer) (Kobayashi et al., 1995). \( \alpha \)-GalCer is an unusual ligand in that its sugar moiety is linked in the \( \alpha \)-anomeric position, while mammalian glycolipids typically exist in the \( \beta \)-anomeric form. This glycolipid is an extremely potent stimulator of mouse, and to a lesser extent, human NKT cells. The affinity of interaction between CD1d-\( \alpha \)GalCer and mouse TCRs is one of the highest ever recorded for natural TCR/ligand pairs with a Kd \(~100\) nM, while that between the CD1d-\( \alpha \)-GalCer and human TCR is lower, being in the range of \(~7\)nM (Bendelac et al., 2007; Cantu et al., 2003; Gadola et al., 2006; Liu et al., 2005d). Notably, \( \alpha \)-GalCer mainly
activates the Type 1 or classical NKT cells; α-GalCer-loaded CD1d tetramers are used to identify this population in both mice and humans (Benlagha et al., 1999).

Extensive efforts have focused on uncovering the physiological relevance of this lineage. The evidence now points to NKT cells being an important part of the innate immune defense against microbes. CD1d<sup>−/−</sup> mice or J<sub>α</sub>18<sup>−/−</sup> mice exhibit increased susceptibility to infection with *Streptococcus pneumoniae, Pseudomonas aeruginosa, Borrelia burgdorferi, Leishmania donovani, Leishmania major* and *Trypanosoma cruzi* and also to viruses such as *Herpes Simplex Virus* (HSV)-1 and HSV-2 (Bendelac et al., 2007; Tupin et al., 2007). Several viruses, including Kaposi’s sarcoma-associated herpesvirus, vaccinia virus, lymphocytic choriomeningitis virus and Human Immune deficiency virus (HIV), downregulate CD1d expression (Tupin et al., 2007). Some limited progress has been made in identifying microbial NKT cell ligands. α-galactosyldiacylglycerols expressed by gram-negative LPS-negative *Borrelia burgdorferi* resemble α-galactosylceramide and could directly stimulate NKT cells. Purified phosphatidylinositolmannoside PIM4, a mycobacterial membrane phospholipid elicited cytokine production from a fraction of mouse and human NKT cells. Additionally, purified phospholipids originally extracted from tumors, such as phosphatidylinositol, phosphatidylethanolamine, and phosphatidylglycerol, weakly stimulated some V<sub>α</sub>14 NKT hybridomas. Human melanomas overexpress the ganglioside GD3 and immunization with the human melanoma SK-MEL-28 was reported to expand a very small subset of V<sub>α</sub>14 NKT cells *in vivo* (Bendelac et al., 2007; Tupin et al., 2007).
However, these ligands are weak agonists of only a fraction of NKT cells and their physiological relevance has been hard to establish (Bendelac et al., 2007).

Although the presence of a powerful NKT agonist (α-GalCer) in a marine sponge has been puzzling, recent studies show that this lipid, with its unusual α-linkage closely resembles α-linked glycosphingolipids (GSLs) in cell walls of many members of the class of α-proteobacteria, one of the most ubiquitous classes of Gram-negative bacteria on Earth. α-proteobacteria exhibit a wide range of lifestyles, from free-living to obligate intracellular pathogens, and are found in marine and soil environments. Some members, such as the Rickettsiales are pathogenic, with tick-borne pathogens such as Rickettsia and Ehrlichia, agents of the plague epidemic typhus, Rocky Mountain spotted fever, and other severe febrile and typhus-like syndromes. Interestingly, many α-proteobacteria, including Ehrlichia lack LPS expression. Other example of LPS-negative bacteria include Sphingomonas, a ubiquitous bacterium found in soil and marine (e.g., sponges and corals) environments. Instead of LPS, its cell wall contains α-glycuronylceramide ligands of NKT cells (Mattner et al., 2005). Sphingomonas was detected by PCR in stool samples of 25% of healthy human beings (Bendelac et al., 2007) and can cause acute infections, particularly in immunocompromised individuals. Intriguingly, it has been implicated in the etiopathlogy of primary biliary cirrhosis, a chronic autoimmune disease targeting intrahepatic bile ducts (Mattner et al., 2008). In contrast to the other microbial agonists identified thus far, Sphingomonas GSLs strongly activate the entire NKT cell population in mice and humans. Keeping in mind the striking structural
similarity to α-GalCer, it is possible that original sponge extracts with NKT cell-activating ability contained contaminations of *Sphingomonas* bacteria.

Another area of intensive focus has been the identification of self ligands for NKT cells. Studies by Bendelac and colleagues identified isoglobotrihexosylceramide (iGB3) as an endogenous ligand for this subset (Zhou *et al.*, 2004). iGb3 is a weaker ligand than α-GalCer or Sphingomonas GSLs. It is required for NKT cell development in the thymus, as β-hexosaminidase-B-deficient mice, which lack the ability to degrade the precursor of iGb3 in the lysosome, exhibited a 95% decrease in thymic NKT cell production (Zhou *et al.*, 2004).

**Mechanisms of NKT cell activation**

Direct NKT cell activation by glycolipid ligands with or without cytokines: The effector functions of NKT cells can be initiated by TCR-CD1d-loaded glycolipid interaction. α-GalCer or *Sphingomonas* GSLs are powerful agonists, efficiently activating NKT cells by themselves. Weaker ligands like iGb3, on the other hand, are unable to induce full activation. However, their effect can be strengthened by IL-12 from DCs, resulting in robust NKT cell activation.

Indirect NKT cell activation by endogenous ligands and cytokines: *Salmonella typhimurium*, an organism which does not bear NKT cell-stimulating glycolipids, can activate this subset by a novel combination of cytokine- and endogenous antigen-mediated activation (Mattner *et al.*, 2005). Salmonella-derived lipopolysaccharide (LPS)
stimulates Toll-like receptors (TLRs) on DCs and induces IL-12 release. NKT cells are activated by the combination of IL-12 produced by LPS-stimulated DCs and recognition of endogenous antigen iGB3 presented by CD1d (Tupin et al., 2007). It is unclear whether LPS can upregulate iGb3 presentation by DCs.

Indirect NKT cell activation by cytokines: NKT cells can be activated by IL-12 and IL-18 produced by DCs that have been activated by E. coli LPS, even in the absence of TCR engagement by CD1d-presented glycolipids (Nagarajan and Kronenberg, 2007). Thus, NKT cell activation could occur downstream of TLR engagement.

Effects of NKT cell activation

Rapidly following activation, NKT cells produce a slew of cytokines, including IFN-γ, IL-4, IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, TNF-α, TGF-β and GM-CSF. They also produce chemokines and upregulate CD40L expression (Bendelac et al., 2007; Matsuda et al., 2008; Wilson and Delovitch, 2003). CD40-CD40L cross-linking, in combination with cytokines such as IFN-γ, upregulate costimulatory and MHC molecule expression on DCs promoting their ability to initiate adaptive immunity. Importantly, NKT cell activation elicits IL-12 production from DCs which in turn enhances NKT cell activation and cytokine production (Bezbradica et al., 2006; Tomura et al., 1999). Propagation of this reaction involves the activation of NK cell cytolysis and IFN-γ production (Carnaud et al., 1999; Kitamura et al., 1999).

NKT cells in autoimmune disease
NKT cells are powerful first-responders in an immune response, and can guide the response in either a pro-inflammatory, suppressive or a humoral direction. They have hence been highly scrutinized in fields of tumor biology, autoimmunity and allergy. Interest in the role of NKT cells in autoimmune disease was sparked by reports of numerical and functional defects in NKT cell population associated with autoimmunity, in the NOD mouse model, and also in human patients suffering from rheumatoid arthritis (RA), SLE, and Scleroderma and Sjögren’s syndrome (Yu and Porcelli, 2005). The effect of NKT cell activation (mostly by α-GalCer) has been extensively studied; however the picture emerging is increasingly contradictory. NKT cell activation can ameliorate, but in many cases, also exacerbate various autoimmune diseases (Bendelac et al., 2007; Wilson and Delovitch, 2003; Yu and Porcelli, 2005). Skewing towards a particular T helper subset is observed in most autoimmune syndromes. α-GalCer-activated NKT cells produce both Th1 and Th2 cytokines and thus have the inherent capacity to direct the immune response in either direction.

**TLR7 and 9: Role in autoimmune disease**

The innate immune system has evolved to recognize a conserved set of molecular structures as foreign ‘danger signals’. These structures, called pathogen-associated molecular patterns (PAMPs), are recognized by innate immune receptors designated as ‘pathogen recognition receptors’ (PRRs). Different classes of PRRs exist, including two classes of cytosolic receptors, retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) (Kumagai et al., 2008). The best characterized family of PRRs is the TLR family, so
named for their homology for a Drosophila protein toll. The mammalian TLR family now consists of 13 members with each TLR having its intrinsic signaling pathway and inducing specific biological responses against microorganisms (Mitchell et al., 2007).

The ‘danger’ hypothesis proposed by Polly Matzinger postulated that the adaptive immune system evolved to respond not to infection but also to non-physiological cell death, damage or stress. According to this idea, abnormal cell death is a potential threat to the organism whether it is caused by an infection or other pathological process, and could be a universal sign of danger. Indeed it has now known that cells contain a number of molecules which can be classified as ‘damage-associated molecular patterns’ or DAMPs that can also activate the innate immune system via conserved PRRs. DAMPs ligands have been identified for most of the TLRs, except TLR5 and TLR11 (Kono and Rock, 2008). In most of the cases, the same TLR is activated by distinct PAMPs and DAMPs, for example, TLR4 recognizes bacterial LPS and also a number of mammalian DAMPs such as High-mobility group box 1 protein (HMGB1) and heat shock proteins (Kono and Rock, 2008). However certain structurally similar PAMPs and DAMPs are recognized by the same TLRs. TLR7 and 9 were originally identified as receptors specific for bacterial and viral RNA and DNA, but now are shown to also recognize host nucleic acids or associated proteins.

Expression of TLR9 is restricted to pDCs and B cells in humans whereas in mice myeloid DCs and macrophages also express TLR9 (Krug, 2008). Bacterial and viral DNA sequences containing hypomethylated CpG motifs are potent stimulators of TLR9
Following identification of specific TLR9-stimulatory sequences in bacteria, synthetic oligodeoxynucleotides (ODNs) stabilized by a nuclease resistant phosphorothioate backbone have constructed and used as adjuvant mimics of bacterial DNA. The optimal motif for stimulation of mouse cells is GACGTT, and GTCGTT is the optimal motif for stimulation of human cells (Krieg, 2002). ODNs in which the entire backbone is phosphorothioate-linked are sometimes referred to as type B ODNs (also known as type K ODNs) and mainly stimulate B cells and monocytes. ODNs with a mixed phosphodiester–phosphorothioate backbone can also be synthesized; in this case, the CpG motif is flanked by self-complementary bases to form a stem–loop structure that is capped at the 3' end by a poly(G) tail. These ODNs can be referred to as type A ODNs (also known as type D ODNs) and are particularly effective inducers of interferon-α production by plasmacytoid DCs (pDCs) (Verthelyi and Zeuner, 2003). Type C ODNs contain structured elements of both type A ODNs and type B ODNs, and stimulate both B cells and pDCs effectively (Hartmann, 2003). In comparison, purified mammalian genomic DNA has poor adjuvant activity, most probably reflecting the lack of hypomethylated CpG motifs, as well as the presence of sequences that inhibit effective activation of TLR9 by agonist motifs (Gursel, 2003; Stacey, 2003).

TLR7 is expressed in both human and murine pDCs, myeloid DCs and B cells (Krug, 2008). Expression of this receptor in B cells is strongly upregulated by IFN-α (Marshak-Rothstein, 2006). It was first identified as the receptor responsible for the activity of anti-viral and anti-tumor activity of low molecular weight imidazoquinolines
imiquimod and resiquimod (also known as R-848) (Hemmi et al., 2002), as well as guanine analogues such as loxoribine (Heil et al., 2003). Subsequently, guanosine or uridine rich single-stranded (ss) RNA was identified as a natural ligand for this receptor (Diebold et al., 2004a). Importantly, TLR7 is also activated by RNA sequences in mammalian U1 snRNPs (Diebold et al., 2004b; Savarese et al., 2006).

A remarkably high proportion of the autoantibodies that are commonly associated with systemic autoimmune diseases, such as SLE, scleroderma and Sjögren's syndrome bind DNA, RNA or macromolecular complexes that contain DNA or RNA (Marshak-Rothstein, 2006). These autoimmune diseases are each associated with particular autoantibody reactivities: SLE, with antibodies that react with doublestranded (ds) DNA, nucleosomes and the protein Sm; Scleroderma, with antibodies that react with Topoisomerase I, fibrillarin and centromeres; and Sjögren’s Syndrome, with antibodies that react with the RNA-binding proteins Ro and La (Marshak-Rothstein, 2006).

The reasons behind this preferential targeting are not well understood, but increasing evidence suggests that activation of TLR7 and 9 (expressed on pDCs or B cells) by endogenous ligands (RNA or DNA) released by dying cells could induce a pro-inflammatory response with increased IFN-α production, which could, in part, contribute to the break in tolerance resulting in autoimmune disease (Marshak-Rothstein, 2006; Marshak-Rothstein and Rifkin, 2007). IFN-α has been strongly implicated in the various systemic autoimmune diseases, particularly SLE. The expression of many of the genes that are induced by IFN-α is higher in patients with SLE, and this gene-expression
pattern, as determined by DNA-microarray analysis, is referred to as the IFN signature. Many of its effects of this cytokine can contribute to break in tolerance allowing the development of autoimmunity (Banchereau et al., 2004; Marshak-Rothstein, 2006). For example, IFN-α promotes myeloid DC maturation and antigen presentation. Mature DCs can then activate autoreactive T helper cells and cytotoxic effector CD8+ T cells. IFN-α can also contribute to the availability of autoantigen by potentiating cell death; it increases the sensitivity of target cells to cytotoxic effector mechanisms and also increases cytotoxic capacity of NK cells and CD8+ T cells expression, through upregulation of expression of granzyme B, CD95 ligand and TRAIL (Tumour-Necrosis-Factor-related apoptosis-inducing ligand). Additionally it potentiates B cell function, promoting plasmablast and/or plasma-cell differentiation and switching to the pathogenic immunoglobulin isotype IgG2a, and upregulating expression of TLR7 and the TLR adaptor protein MyD88 (myeloid differentiation primary-response gene 88). Additionally, IFN-α also influences the functional properties of plasmacytoid DCs directly, increasing the responsiveness of plasmacytoid DCs to RNA- and/or DNA-containing immune complexes.

The proposed delivery mechanisms of the self nucleic acid-containing structures to the TLRs located in intracellular endosomal compartments include FcγR-mediated endocytosis of immune complexes (containing IgG autoantibodies complexed with self-RNA or -DNA) occurring in pDCs, or by internalization of autoreactive BCRs bound to DNA- or RNA-containing macromolecular complexes (Marshak-Rothstein, 2006).
NKT cells recognize microbial glycolipids and can activate the immune system during infection. The innate immune system uses pattern recognition receptors such as the TLRs to respond to infection. Both NKT cells and TLRs powerfully stimulate the innate immune system resulting in a proinflammatory response, although in the case of NKT cell stimulation, the results are more complicated, capable of inducing a suppressive response as well. Autoimmune diseases have a complex but ill-understood etiology. It is accepted that inflammation, infection, environment and cell death may individually contribute to the initiation and perpetuation of autoimmunity. However it is likely that human autoimmunity arises due to combinations of these factors. Interactions between infection (which can cause inflammation and cell death) and environmental factors, (which can elicit cell death, inflammation or immunomodulatory effects) have complex consequences, yet such interactions are rarely studied.

We examined the interplay between innate immune components that respond to infection and an environmental pollutant, mercury. We explore the effects of *Sphingomonas* bacteria (which bear both NKT cell and TLR agonists), or combinations of synthetic NKT agonists and a TLR9 ligand with mercury in the development of autoimmunity.
A large portion of the autoantibodies in systemic autoimmune diseases recognize nucleic acids or associated proteins. This is true of the mercury-induced syndrome as well, where autoantibodies recognize fibrillarin and other protein components of RNA-containing snoRNPs. Recent studies by Marshak-Rothstein and others indicate that TLR7 and TLR9 could play a role in the pathogenesis of certain systemic autoimmunities and also suggest that the autoantibody specificities in such syndromes could be due to TLR-mediated recognition of self-nucleic acids. We examined whether similar mechanisms operate in the mercury model, supporting the theory that autoimmunity arises due to inappropriate recognition of self by the innate immune system.
CHAPTER 3
MATERIALS AND METHODS

Mice

A.SW (H-2<sup>s</sup>) mice were obtained from The Jackson Laboratory and maintained in our animal facilities. Congenic C57BL/6.SJL mice (H-2<sup>s</sup>) were originally obtained from the Jackson laboratory and are bred and maintained in our animal facility. All of the mice used in our experiments were at least 2 months old.

In vivo treatments

HgCl<sub>2</sub> administration

Mice were injected 3 times a week subcutaneously (s.c.) with 30 μg HgCl<sub>2</sub> (Sigma, St. Louis, MO) in 100 μl sterile PBS during the first week. This dose of HgCl<sub>2</sub> corresponds to approximately 1.0 mg/kg body weight of mice, which is considered subtoxic to mice. Mice were bled once a week retro-orbitally for up to 4 weeks after the start of treatments. The blood samples were incubated at 37°C for 40 minutes, followed by incubation at 4°C for 1.5 hours. Coagulated blood was removed using a wood applicator. Samples were then centrifuged at 8000 rpm for 5 minutes. The separated serum was transferred to 0.65 ml Eppendorf tubes and stores at -20°C for future analysis.

Glycolipid synthesis and administration
Two synthetic variants of α-GalCer, PBS 57 and 4-deoxy α-GalCer (Figure 1) were used in this study. PBS 57 was synthesized by Dr. Paul B. Savage (Brigham Young University) and was kindly provided to us by Dr. Albert Bendelac (University of Chicago). 4-deoxy α-GalCer was synthesized by Dr. Amy Howell (University of Connecticut). Stock solutions of both glycolipids were originally prepared in 100 % DMSO at a concentration of 1 mg/ml. Stock solutions of 4-deoxy α-GalCer were heated at 90º C for 15 minutes to ensure dissolution, prior to aliquoting and storage at -20º C. Prior to use, thawed stock solutions were diluted 1:50 in PBS (PBS 57) or PBS 0.5 % Tween 20 (4-deoxy α-GalCer). 100 µl or 150 µl of the diluted stock (2 or 3 µg of glycolipids) were injected i.p. injection into mice as indicated. Control groups received equivalent volumes of PBS 2% DMSO or PBS 2% DMSO 0.5 % Tween 20.

**Heat killed bacteria preparation and administration**

*Sphingomonas capsulata* (ATCC 14666) was a kind gift from Dr. Bendelac. Cultures were heat killed for 2 hours at 74ºC. Cells were washed after the procedure by centrifugation and taken up in PBS. 0.1 µl of bacterial suspension (equivalent to $10^7$ bacterial CFUs) was injected i.p. at indicated timepoints.

**TLR9 stimulation**

Immunostimulatory Type B ODN CpG 1826 (TCCATGACGTTCTGACGTT) on a phosphorothioate background was obtained from Oligos Etc (Willsonville, OR). A stock solution was prepared at 2 mg/ml and diluted 1:1 in PBS prior to use. A.SW mice
were injected s.c. in the flank with 100 µg CpG 1826 (in 100 µl) or vehicle (PBS) at the indicated timepoint.

**TLR7 and 9 inhibition**

Inhibitory ODNs immunoregulatory DNA sequences (IRS) 661 5'-TGCTTGCAAGCTTGCAAGCA-3', IRS 869 5'-TCCTGGAGGGGTGT-3' and IRS 954 5'-TGCTCCTGGAGGGGTGT-3' on a nuclease-resistant phosphorothioate backbone were synthesized as described (Barrat et al., 2005; Duramad et al., 2005). Underlined motifs are responsible for TLR7 inhibition (Barrat et al., 2005), while the motif in italics has TLR9 inhibitory activity (Duramad et al., 2005). ODNs were kindly supplied to us by Dr. Franck Barrat (Dynavax Technologies Corporation, Berkeley, CA). Mice received indicated amounts of ODNs (suspended in sterile PBS) intraperitoneally at indicated timepoints. Control groups received equivalent amounts of a control ODN 532 or PBS.

**Flow Cytometry**

**NKT cells**

Tetramer Preparation: Biotinylated, recombinant CD1d molecules were incubated with PBS 57 (at a 2:1 ratio) in PBS for 2 hrs at 37° C. Free PBS 57 was removed by centrifugation dialysis in a Microcon YM-30 tube (Millipore). Tetramers were generated by mixing PBS 57-loaded monomers with fluorochrome-labeled streptavidin (streptavidin–APC, BD Biosciences, San Jose, CA) at a 5:1 ratio. The resulting solution was incubated for 30 minutes at 37° C. The CD1d molecules were a kind gift from Dr.
Albert Bendelac (University of Chicago) or were obtained from the NIH tetramer facility (Emory University). NKT cell staining: Spleens were mashed in sterile PBS using nylon mesh bags. Cells were then strained through a sterile 40 μm cell strainer (BD Biosciences, San Jose, CA) to obtain a single cell suspension. RBC lysis was performed using a sterile 0.165 M NH₄Cl for 2-5 minutes at RT. Nonspecific staining was blocked using 1 μg anti-mouse CD16/32 (2.4G2) (BD Biosciences, San Jose, CA) and 100 μg pure streptavidin (Pierce Protein Research Products, Rockford, IL) per million cells. Splenocytes were incubated with APC-conjugated PBS 57-loaded or unloaded CD1d tetramers (45 minutes at 37°C) and then stained for 30 minutes at 4°C with PE-labeled anti-mouse B220 (RA3-6B2) (BD Biosciences, San Jose CA) to negatively gate B cells. Cells were then washed once 1X with FACS buffer and fixed with 4 % paraformaldehyde (Electron Microscopy Sciences, Hatfield PA). NKT cell population data analyses were conducted using a dual laser FACS Aria or a FACSCanto (BD Biosciences, San Jose, CA). Flow cytometry data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR) or FACSDiva™ Software (BD Bioscience, San Jose, CA).

\[ T_{reg}s \]

Spleens were mashed in sterile PBS using nylon mesh bags. Cells were then strained through a sterile 40 μm cell strainer (BD Biosciences, San Jose, CA) to obtain a single cell suspension. RBC lysis was performed using a sterile 0.165 M NH₄Cl for 2-5 minutes at RT. Nonspecific staining was blocked using 1 μg antimouse CD16/32 (2.4G2) (BD
Figure 1: Structure of \( \alpha \)-GalCer and two closely related synthetic variants PBS 57 and 4-deoxy \( \alpha \)-GalCer, that were used to activate NKT cells in this study.
Biosciences, San Jose, CA). Splenocytes were incubated with FITC-conjugated anti-mouse CD4 (GK1.5) (BD Biosciences, San Jose, CA) and PE-conjugated anti-mouse CD25 (3C7) (BD Biosciences, San Jose, CA) at 4°C, followed by overnight permeabilization using a Cytoperm/Cytofix kit (eBioscience, San Diego, CA), followed by staining with APC-conjugated anti-mouse Foxp3 (FJK-16s) (eBioscience, San Diego, CA). Data analyses were conducted using a dual laser FACSCalibur or a FACSCanto (BD Biosciences San Jose, CA). Flow cytometry data were analyzed using FlowJo software (Treestar) or FACSDiva™ Software (BD Bioscience, San Jose, CA).

**Ex vivo splenocyte stimulation**

Splenocytes were suspended at 1.5 x 10^6 cells/ml in 6 well plates and stimulated with 20 ng/ml PMA (Biomol. International L.P., Plymouth Meeting, PA) and 500 ng/ml ionomycin (Tocris Bioscience, Ellisville, MO). In some experiments, splenocytes were suspended in 24 well plates and stimulated with plate-bound anti-CD3 mAb (coated in PBS at 3 µg/ml) and anti-CD28 mAb (coated in PBS at 2 µg/ml). Anti-CD3 and anti-CD28 mAbs were obtained from BioXCell (West Lebanon, NH). Cell culture supernatants were assayed at 24, 48 or 96 hrs for cytokine release by sandwich ELISA.

**Immunofluorescence**

Serum ANoA titers were determined by indirect immunofluorescence. Sera diluted in PBN (PBS containing 1% BSA and 0.02% sodium azide) were incubated with HEp-2 slides (Antibodies, Inc., Davies CA) for 30 min in a moist chamber at room temperature. Sera were removed by rinsing with PBS. Slides were dipped twice in PBS
followed by two 5 minute washes in PBN. ANoA were detected with FITC-conjugated goat anti-mouse IgG, IgG1 or IgG2a antibodies (Southern Biotechnology Associates, Birmingham, AL). FITC-conjugated antibodies diluted 1:40 in PBN were added to each well (30 µl/well). The slides were then incubated for 30 minutes in the dark, in a moist chamber at room temperature. Slides were then washed as before and mounted with a glass coverslip using Flouramount G (Southern Biotechnology Associates, Birmingham AL). The inverse of the highest serum dilution at which nucleolar fluorescence could be detected was defined as the ANoA titer.

ELISA Protocols

ELISA for Total Serum IgG1 and IgG2a

Total serum IgG1 and IgG2a levels were determined using a sandwich ELISA (enzyme-linked immunosorbant assay). For quantification of IgG1 and IgG2a levels, 96 well polystyrene plates (BD Biosciences San Jose, CA) were coated overnight with goat anti-mouse Igκ (Southern Biotechnology associates, Birmingham, AL) diluted 2 µg/ml in carbonate buffer. Following 3 washes with PT (PBS 0.05 % Tween 20) buffer, wells were blocked with PBTN (PBS 1% BSA + 0.02 % NaN₃ + 0.05 % Tween 20) for 30 minutes. Sera diluted 1/50,000 in PBTN were added to the wells and incubated overnight at 4°C. Samples were washed out 7X with PT and AP (Alkaline Phosphatase)-coupled goat anti-mouse IgG1 or IgG2a (Southern Biotechnology associates, Birmingham AL) diluted 1/4000 in PBTN was added. After incubation for 1 hour at room temperature, plates were washed 6X with PT and 1X with AP buffer (10 mM diethanolamine + 0.05 mM MgCl₂ in dH₂O). p-Nitrophenylphosphate (PNPP) solution
in AP buffer (1 mg/ml) was then added and allowed to develop for 20 minutes. Absorbance values were read at 405 nm. Antibody levels in the samples were extrapolated from a standard curve generated using varying concentrations (3.15-400 ng/ml) of ASWU1 (IgG1) or ASWA3 (IgG2a) previously purified in our laboratory (Monestier et al., 1994).

**ELISA for Serum IgE**

96 well polyvinylchloride plates (BD Biosciences San Jose, CA) were coated overnight with a rat anti-mouse IgE (Clone R35-72 BD Pharmingen, San Diego, CA) diluted 2 µg/ml in carbonate buffer. Following 3 washes with PT buffer, wells were blocked with PBTN for 120 minutes. Sera diluted 1/100 in PBN were added to the wells and incubated overnight at 4°C. Samples were washed out 7X with PT and a 2 µg/ml solution of a biotinylated rat-anti mouse IgE (R35-72) (BD Pharmingen, San Diego, CA) added. After incubation for 60 minutes at room temperature, plates were washed 7X with PT and streptavadin-AP (SAP) (Southern Biotechnology Associates, Birmingham AL) diluted 1:2000 in PBTN was added to the wells. After a 30 minute incubation at room temperature, plates were washed out 6X with PT and 1X with AP buffer (10mM diethanolamine + 0.05 mM MgCl₂ in dH₂O). p-Nitrophenylphosphate (PNPP) solution in AP buffer (1 mg/ml) was then added and allowed to develop for 60-120 minutes. Absorbance values were then read at 405 nm. Antibody levels in the samples were extrapolated from a standard curve generated using varying concentrations (3.15-400 ng/ml) of purified mouse IgE (IgE-3) (BD Biosciences, San Diego CA).
Matched capture (clones 11B11 and AN-18) and biotinylated detection (clones BVD6-24G24 and XMG1.2) antibodies for IL-4 and IFN-γ respectively were obtained from BD Biosciences (San Jose, CA). 96 well MaxiSorp plates (Thermo Scientific Nunc-Immuno™) were coated with a 2 μg/ml solution of capture antibody in carbonate buffer. Following an overnight incubation at 4°C, plates were washed 3X with PT, then blocked with PFT (PBS +10 % Fetal Bovine Serum + 0.05 % Tween 20) for 1 hour at RT. Following a 3X PT wash, undiluted supernatants (for IL-4 ELISA) or 1/10 diluted supernatants (for IFN-γ ELISA) were added and plates were incubated overnight at 4°C. Samples were washed out 8X with PT, followed by addition of detection antibody (diluted to 1 μg/ml) in PFT. After a 60 minute incubation at room temperature, plates were washed out 7X followed by addition of SAP (diluted 1:3000 in PFT) for 60 minutes at room temperature. Plates were then washed 6X with PT and 1X with AP buffer (10mM diethanolamine + 0.05 mM MgCl₂ in dH₂O). p-Nitrophenylphosphate (PNPP) solution in AP buffer (1mg/ml) was then added and allowed to develop for 30 minutes (IFN-γ) or for 180 minutes (IL-4). Absorbance values were read at 405 nm. Cytokine levels in the samples were extrapolated from a standard curve generated using varying concentrations (15.625-4000 pg/ml) of recombinant cytokines (BD Biosciences, San Jose CA)

Matched capture (clone JES5-2A5) and biotinylated detection (clone JES5-16E3) antibodies were obtained from BD Biosciences (San Jose, CA). 96 well polystyrene
MaxiSorp plates (Thermo Scientific Nunc-Immuno™) were coated with a 4 μg/ml solution of capture antibody in a 0.2 M Sodium Phosphate buffer (pH 6.5). Following an overnight incubation at 4°C, plates were washed 3X with PT, then blocked with PFT (PBS + 10 % Fetal Bovine Serum + 0.05 % Tween 20) for 30 minutes at RT. Following a 3X PT wash, undiluted supernatant was added and plates were incubated overnight at 4°C. Samples were washed out 8X with PT, followed by addition of detection antibody (1 μg/ml) in PFT. After a 60 minute incubation at room temperature, plates were washed out 7X followed by addition of SAP (diluted 1:3000 in PFT) for 60 minutes at room temperature. Plates were then washed 6X with PT and 1X with AP buffer (10 mM diethanolamine + 0.05 mM MgCl₂ in dH₂O). p-Nitrophenylphosphate (PNPP) solution in AP buffer (1 mg/ml) was then added and allowed to develop for 180 minutes. Absorbance values were read at 405 nm. IL-10 levels in the samples were extrapolated from a standard curve generated using varying concentrations (15.625-4000 pg/ml) of recombinant IL-10 (BD Biosciences, San Jose CA)

**Statistical Analyses**

For all experiments in the NKT cell project, the dependent variables (flow cytometry counts, cytokine levels, etc.) were treated as continuous variables for all analyses. Means, standard deviations, and number of observations were presented for each variable. The experiments used a two factor randomized design (group, time period). The null hypothesis was that there would be no difference between groups or time periods. Prior to analysis, all data were tested for normality using the Shapiro-Wilk test (Armitage and Berry, 1994). The data was significantly non-normal for all variables.
In order to apply ANOVA methods, a “normalized-rank” transformation was applied to the data (Conover and Iman, 1981; Harter, 1961). The rank-transformed data was analyzed using a generalized linear model ANOVA followed by multiple comparisons to detect significant mean differences between groups and periods. Differences between means (rejection of the null hypothesis) were considered significant if the probability of chance occurrence was $\leq 0.05$ using two-tailed tests. Statistical analyses were carried out using SAS v9.1 software (SAS Institute, Cary, NC). For the TLR blockade experiments, analyses were conducted using the GraphPad Prism Software (Version 5.0, GraphPad Software Inc, San Diego, CA). All groups were compared using a two-way ANOVA (without repeated measures) with the Bonferroni post-tests to compare replicate means by row.
CHAPTER 4

RESULTS

Differential effects of NKT cell activation by synthetic ligands

Effect of NKT cell activation on mercury-induced autoimmunity in C57BL/6.SJL mice

The C57BL/6.SJL mouse strain is moderately mercury-susceptible, and requires several weeks of mercury administration to induce autoimmunity. We examined whether NKT cell activation could accelerate autoimmunity in this strain. We used 2 synthetic derivatives of α-GalCer, PBS 57 and 4-deoxy α-GalCer to activate NKT cells. Briefly, mice received only 3 injections of HgCl₂ with or without 4-deoxy α-GalCer or PBS 57 (Figure 2). Increase in serum immunoglobulins IgG1 and IgE in mercury-administered mice was negligible and was not further enhanced by NKT cell activation by either ligand (Figures 3 and 4). The group receiving mercury alone showed little or no autoantibody production (Figures 3 and 4). However, NKT cell activation by 4-deoxy α-GalCer significantly potentiated anti-nucleolar autoantibody production of both the IgG1 (p<0.001) and the IgG2a (p<0.0001) isootypes (Figure 3), while NKT cell activation by PBS 57 strongly potentiated IgG2a autoantibodies (p<0.0001), while having a modest effect on IgG1 autoantibodies (Figure 4). NKT cell activation by both ligands thus increased susceptibility to mercury-induced autoimmunity in this mouse strain.
Protocol 1:

2 µg 4-deoxy α-GalCer or PBS 57 or vehicle i.p.

Time (Days) 0 2 3 4 7

30 µg HgCl₂/ vehicle s.c.

Figure 2. Time course for NKT cell activation with HgCl₂ treatment. C57/BL6.SJL mice (n=3 or 4) received subcutaneous injections of 30 µg HgCl₂ in 100 µl of sterile PBS three times during the first week. In addition groups of mice received injections of 2 µg 4-deoxy α-GalCer in 100 µl of PBS 2 % DMSO 0.5 % Tween 20 or 2 µg PBS 57 in 100 µl of PBS 2 % DMSO. Respective controls received 100 µl of PBS 2 % DMSO 0.5 % Tween 20 or PBS 2% DMSO. NKT cell ligands or corresponding vehicle was injected intraperitoneally.
Figure 3. NKT cell activation by 4-deoxy α-GalCer increases susceptibility to mercury-induced autoimmunity in C57BL/6.SJl mice. Groups of mice received HgCl$_2$ and NKT cell ligand 4-deoxy α-GalCer as described in Protocol 1 (Figure 2). ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1) or µg/ml ± SD (IgE). **p<0.001, ***p<0.0001 vs. HgCl$_2$-treated group.
Figure 4. NKT cell activation by PBS 57 increases susceptibility to mercury-induced autoimmunity in C57BL/6.SJL mice. Groups of mice received HgCl₂ and NKT cell ligand PBS 57 as described in Protocol 1 (Figure 2). ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1) or µg/ml ± SD (IgE). *p<0.05, ***p<0.0001 vs HgCl₂-treated group.
Effect of NKT cell activation on mercury-induced autoimmunity in A.SW mice

We next assessed the role of NKT cells in A.SW mice, a strain highly susceptible to mercury-induced autoimmunity. Briefly, mice received 3 injections of HgCl\(_2\) with or without the NKT cell ligand, as described in Protocol 2 (Figure 5). A.SW mice in response to mercury produced anti-nucleolar autoantibodies and also showed an increase in serum immunoglobulins (Figure 6). No manifestations of autoimmunity were observed when NKT cell ligands were administered alone (data not shown). Activation of NKT cells modulated mercury-induced autoimmunity but surprisingly, the 2 ligands had strikingly different effects. NKT cell activation by 4-deoxy \(\alpha\)-GalCer, but not PBS 57 (Figure 6) significantly increased IgG2a autoantibody production compared to mercury-treated controls. Both ligands dramatically increased IgE levels relative to the mercury-treated controls, although this effect was more pronounced with PBS 57 (Figure 6).

Mercury potentiates PBS 57-induced NKT cell population expansion

We examined the effect of dual administration of mercury and glycosphingolipids on the NKT cell population. A.SW mice received HgCl\(_2\) with or without NKT cell ligands (as shown in Figure 5), or NKT cell ligands alone, at the same time points. We first examined the effect of PBS 57. In mice that received mercury only, NKT cells represented 0.37 \% of the splenocytes (10.48 x 10\(^4\) NKT cells/spleen), similar to values in untreated mice (not shown). As expected with an NKT cell ligand, PBS 57 Significantly (p<0.05) expanded the NKT cell population, increasing the percentage to 1.23 \%
Protocol 2

Figure 5. Time course for NKT cell activation with HgCl$_2$ treatment. A.SW mice (n=5) received subcutaneous injections of 30 µg HgCl$_2$ in 100 µl of sterile PBS three times during the first week. In addition groups of mice received 2 µg 4-deoxy α-GalCer in 100 µl of PBS 2 % DMSO 0.5 % Tween 20 or 2 µg PBS 57 in 100 µl of PBS 2 % DMSO at the indicated timepoints. NKT cell ligands or vehicle were injected intraperitoneally.
Figure 6. Differential effects of NKT cell activation on mercury-induced autoimmunity in A.SW mice. Groups of mice received HgCl₂ with either NKT cell ligand as described in Protocol 2 (Figure 5). ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1 and IgG2a) or µg/ml ± SD (IgE). *p<0.05, **p<0.001 ***p<0.0001 vs. HgCl₂-treated group.
Figure 7: PBS 57-induced NKT cell expansion is further potentiated by mercury co-administration. A.SW (n=3) mice received HgCl₂ (30 μg) or vehicle on days 0, 2 and 4 with or without 2 μg of A) PBS 57 or B) 4-deoxy α-GalCer on days 0 and 2. On day 5, splenocytes were stained with empty or PBS 57-loaded APC-conjugated CD1d tetramers and PE-conjugated anti-B220 mAb to negatively gate B cells. Percentages (top) and absolute numbers (bottom) of NKT cells are expressed as mean ± SD. #p<0.05 vs. HgCl₂–treated group * p< 0.05 vs. PBS 57–treated group.
Interestingly, co-administration of mercury significantly (p<0.001) potentiated the PBS 57-induced expansion of NKT cells, further increasing it to 2.33 % (205 x 10^4 NKT cells/spleen) (Figure 7).

We next examined the effect of 4-deoxy α-GalCer in combination with mercury. 4-deoxy α-GalCer also expanded (p<0.05) the NKT cell population, increasing it to 1.34 % (144.4 x 10^4 NKT cells/spleen). However, the NKT cell population in mice receiving 4-deoxy α-GalCer and mercury was not significantly different from that in mice receiving 4-deoxy α-GalCer alone (Figure 7). Thus, unlike PBS 57, the 4-deoxy α-GalCer-induced NKT cell expansion was not further potentiated by co-administration of mercury.

**NKT cell activation prevents tolerance induction**

Previous studies in our laboratory demonstrated that genetically susceptible mice can be rendered tolerant to mercury by a single pre-injection of a lower dose of HgCl$_2$ (Yan Zheng, unpublished observations). We investigated whether NKT cell activation can prevent the induction of tolerance. A.SW mice received PBS 57, 4-deoxy αGalCer, or vehicle four hours prior to administration of a low dose (3 µg) of HgCl$_2$. One week later, all mice received 3 injections of the regular dose (30 µg) of HgCl$_2$ (Figure 8). Following challenge with the standard course of mercury, tolerized mice showed low titers of autoantibodies and low serum immunoglobulin (Figure 9) levels. Pre-activation of NKT cells with PBS 57 prevented tolerance establishment and significantly increased
Protocol 3

3 μg 4-deoxy α-GalCer / PBS 57 or vehicle i.p
(4 hrs prior to low dose HgCl₂ injection)

Day -7

low dose (3 μg) HgCl₂ i.p.

0

30 μg HgCl₂ s.c.

2

4

Figure 8. Time course for tolerance induction with NKT cell pre-activation. A.SW mice (n=5) received 3 μg 4-deoxy α-GalCer in 150 μl of PBS 2 % DMSO 0.5 % Tween 20 or 3 μg PBS 57 in 150 μl of PBS 2 % DMSO on day -7. The control group received 150 μl of PBS 2 % DMSO 0.5 % Tween 20. 4 hours later, all groups received a low dose (3 μg) HgCl₂ in 200 μl PBS. All injections on day -7 were administered intraperitoneally. One week later, all groups were challenged with 3 injections of 30 μg HgCl₂ in 100 μl PBS subcutaneously.
Figure 9. NKT cell activation by PBS 57, but not 4-deoxy α-GalCer, prevents tolerance establishment. A.SW mice (n=5) were pre-administered NKT cell agonists prior to tolerance induction treatment as shown in Protocol 3. All mice were then challenged with three injections of 30 µg HgCl₂ on days 0, 2 and 4. ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1) or µg/ml ± SD (IgE). *p<0.05, **p< 0.001 ***p<0.0001 vs. HgCl₂-treated group.
IgG1 (p<0.05) and IgG2a (p<0.0001) autoantibodies and serum IgE (p<0.05) levels (Figure 9). In contrast, pre-activation of NKT cells by 4-deoxy α-GalCer was not effective at preventing tolerance induction; mice treated with this ligand showed no increase in serum IgE levels or IgG1 autoantibodies and only a mild increase in IgG2a autoantibodies at week 3 (Figure 9).

**NKT cells can potentiate or antagonize TLR activation**

Our results demonstrate that NKT cell activation can prevent tolerance establishment. We next investigated whether NKT cell activation could break established tolerance. *In vivo* exposure to microbial NKT ligands is likely to involve co-exposure to various TLR ligands. To study the interplay between NKT cell ligands and TLR ligands in this system, we challenged tolerized mice with the NKT cell ligands given alone or in combination with a TLR9 ligand, a stimulatory Class B ODN CpG 1826. Briefly, ASW mice received a low dose of mercury intraperitoneally. One week later all mice received the standard regimen of mercury with or without the NKT cell ligands, CpG 1826 or combinations of these, at the indicated timepoints (Figure 10). In agreement with earlier reports (Liu *et al.*, 2005), we observed that NKT cell activation synergized with CpG 1826 to increase serum immunoglobulin levels (Figure 11).

On the other hand, striking differences were observed when we examined autoantibody production. NKT cell activation alone by either ligand had a mild effect on autoantibody levels (Figure 12), although 4-deoxy α-GalCer administration resulted in higher IgG2a titers than PBS 57. In contrast to this, TLR9 activation was more effective
Protocol 4

Figure 10. Time course of NKT cell ligand administration in combination with a TLR9 agonist to tolerized animals. A.SW mice (n=5) received a low dose (3 µg) HgCl$_2$ in 200 µl PBS intraperitoneally on day -7. Some groups received three intraperitoneal injections of 2 µg of either NKT ligand alone or in combination with a single subcutaneous injection of 100 µg CpG ODN 1826 in 100 µl PBS on day 0. One group received CpG 1826 alone at day 0 along with injections of NKT cell ligand-vehicle (PBS 2 % DMSO 0.5 % Tween 20) on days -2, 0 and 2. A control-tolerized group received NKT cell ligand-vehicle and TLR9 agonist-vehicle (PBS) at the respective time points. Starting day 0, all groups received 3 injections of 30 µg HgCl$_2$ in 100 µl PBS subcutaneously.
Figure 11. Both NKT ligands synergize with TLR9 to disrupt tolerance controlling polyclonal B cell activation. Groups of tolerized mice, mercury-challenged mice received either NKT cell ligand (alone or in combination with CpG 1826) or CpG 1826 alone, as described in Protocol 4 (Figure 10). Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1 and IgG2a) or µg/ml ± SD (IgE).
Figure 11. NKT cell ligands can either synergize with or antagonize TLR9-induced breakage of tolerance controlling autoantibody production. Groups of tolerized mice, mercury-challenged mice received either NKT cell ligand (alone or in combination with CpG 1826) or CpG 1826 alone, as described in Protocol 4 (Figure 10). ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. *p<0.05 vs. CpG 1826-treated group.
at breaking tolerance. It had a stronger effect on the autoantibody production, especially of the IgG2a isotype (Figure 12). When both an NKT cell ligand and a TLR9 ligand were administered, 4-deoxy α-GalCer synergized with CpG 1826 and potentiated IgG1 (p<0.05) autoantibody production (Figure 12). Interestingly, PBS 57 had the opposite effect, significantly (p<0.05) inhibiting TLR9-induced restoration of IgG1 and IgG2a autoantibodies (Figure 12). Thus, NKT cell activation can either synergize with or antagonize TLR9-induced autoantibody production, depending on the activating NKT ligand. Therefore, PBS 57 (administered without mercury) prevents induction of tolerance but when given with regular dose of mercury, antagonizes TLR9-induced breaking of tolerance.

Effect of NKT cell activation with mercury administration on regulatory T cells

CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells are required to induce tolerance in our model (unpublished observations). Previous studies have demonstrated that NKT cell activation can modulate the T_{reg} population (Cava et al., 2006; Liu et al., 2005). We examined whether mercury could affect the crosstalk between NKT cells and T_{regs}. Briefly, A.SW mice received either NKT cell ligand alone or in combination with mercury, or mercury alone using the injection schedule shown in Protocol 2 (Figure 4). We observed that, as previously described (Liu et al., 2005), NKT cell activation moderately increased T_{reg} numbers in the spleen (Figure 13). Interestingly, mercury very strongly potentiated this effect, increasing T_{reg} numbers in mice treated with either NKT ligand (Figure 13) although, this effect was significantly (p<0.05) stronger in PBS 57-
Figure 13. Mice receiving PBS 57 and mercury show increased regulatory T cells. Groups of A.SW mice (n=3) received 3 injections of 30 μg HgCl₂ in 100 μl of PBS or PBS alone on days 0, 2 and 4. Additionally some groups received injections of 4 μg PBS 57 or 4-deoxy α-GalCer or equivalent volumes of NKT cell ligand vehicle (PBS 2 % DMSO 0.05 % Tween 20) on days 0 and 2. On day 8, mice were sacrificed and splenocytes were stained with antibodies to surface CD4 and CD25 and for intracellular Foxp3. Both percentages and absolute numbers of regulatory T cells are shown.
treated compared to 4-deoxy α-GalCer-treated animals. However, the NKT cell ligands and mercury strongly synergize to increase overall splenocyte numbers. Hence, the increase in T_{reg} numbers could be a reflection of the increase in the total splenocyte number. When the percentage of T_{regs} was examined, we found that treatment with mercury, NKT cell ligands, or the combination of the two decreased the T_{reg} frequency in the spleen (Figure 13). There was no difference among mice receiving NKT cell ligands or mercury alone. However, the percentage of T_{regs} was significantly (p<0.05) higher in mice that received PBS 57 with mercury, compared to mercury-treated animals. On the other hand, the mice receiving 4-deoxy α-GalCer with mercury had a significantly lower frequency of T_{regs} than the group which received PBS 57 with mercury (p<0.001) or the mice that received mercury alone (p<0.05) (Figure 13). Thus, in mercury-administered animals, PBS 57 increases T_{reg} frequency, while 4-deoxy α-GalCer lowers it.

Co-administration of mercury and PBS 57 increases IL-10 and IL-4 production

We also examined cytokine production by splenocytes from the same experimental groups. Briefly, splenocytes collected at day 8 after start of treatments were stimulated in vitro with PMA/ionomycin. Supernatants were analyzed for IFN-γ, IL-4 and IL-10 levels. There were no significant differences in cytokine production among mice receiving either NKT cell ligand (Figure 14). However, administration of mercury significantly (p<0.05) increased IL-4 and IL-10 production in PBS 57-treated, but not 4-deoxy α-GalCer-treated animals (Figure 14). On the other hand, IFN-γ levels were similar between mice given either NKT ligand, with or without mercury (Figure 14).
Figure 14. Mice receiving PBS 57 and mercury show Th2 cytokine production. Splenocytes from same animals were also stimulated with PMA/inomycin. Culture supernatants collected at 48 and 96 hours were analyzed for IFN-γ, IL-4 and IL-10 levels by sandwich ELISA as described in Materials and Methods. Cytokine levels are expressed in pg/ml ± SD. *p < 0.05 vs. PBS 57-treated group.
These results suggest that the PBS 57-induced (but not 4-deoxy α-GalCer-induced) cytokine secretion is altered by mercury, with increased skewing towards Th2 cytokine production. Collectively these results indicate that, in the presence of mercury, 4-deoxy α-GalCer-induced NKT cell activation induces fewer Tregs and decreased Th2 cytokine production when compared to NKT cell activation by PBS 57.

**PBS 57 modulates the Treg population in tolerized, CpG 1826-challenged mice**

NKT cell ligands when administered with CpG 1826 induce opposing effects on the autoantibody production (Figure 12). As Tregs downregulate autoantibody production in tolerized animals (unpublished observations), we examined the Treg population in tolerized mice challenged with NKT cell ligands and CpG 1826 following protocol 4 (Figure 10). Mice were sacrificed on day 8, the time point at which they become positive for anti-nucleolar reactivity. We noted that the absolute number of splenocytes was reduced in mice receiving PBS 57 with CpG 1826 (247.3 x 10^6) compared to mice receiving CpG 1826 alone (350.6 x 10^6) or CpG 1826 with 4-deoxy α-GalCer (340 x 10^6) (Figure 15A). Furthermore the total number of CD4+CD25+ cells was decreased in mice receiving PBS 57 with CpG 1826, compared to mice receiving CpG 1826 alone or with 4-deoxy α-GalCer (Figure 15B). Although the frequency of Tregs (CD4+CD25+Foxp3+ cells) in these 3 groups was similar (Figure 15C), the frequency of Teffectors (CD4+CD25+ Foxp3- cells) was greatly reduced when mice received PBS 57 with CpG 1826 (Figure 15D). The ratio of Tregs to Teffectors is shown in Figure 15E. Interestingly, treatment with CpG1826 significantly (p<0.05) decreased this ratio compared to control-tolerized or
A. number of splenocytes (x 10^6)

B. CD4+CD25+ cells as percentage of splenocytes

C. CD4+CD25+Foxp3+ cells as percentage of splenocytes

D. CD4+CD25+Foxp3- cells as percentage of splenocytes

E. CD4+CD25+Foxp3+ / CD4+CD25+Foxp3-
Figure 15. PBS 57 restores balance between T$_{\text{reg}}$ and T$_{\text{effector}}$ cell populations in the spleen. A.SW mice (n=3) received a single low dose (3 µg) of HgCl$_2$ on day -7. Animals then received either NKT cell ligand (2 µg) with CpG 1826 (100 µg) at the indicated timepoints. All groups received 30 µg HgCl$_2$ on days 0, 2 and 4. At day 8, mice were sacrificed and splenocytes were stained with antibodies to surface CD4 and CD25 and for intracellular Foxp3. A) Absolute numbers of cells per spleen following RBC lysis. B) Percentage (right) and absolute numbers (left) of splenic CD4$^+$CD25$^+$ cells. C) Percentage (right) and absolute numbers (left) of T$_{\text{reg}}$. D) Percentage (right) and absolute numbers (left) of T$_{\text{effector}}$. E) Ratio of T$_{\text{reg}}$: T$_{\text{effector}}$. *p<0.05 vs. PBS 57 $^+$ CpG 1826-treated and vehicle-treated groups.
untreated animals (3.61 vs. 8.77 or 8.44). Co-administration of 4-deoxy α-GalCer (3.37) maintained this decrease but co-administration of PBS 57 restored the ratio to 9.33, similar to the value in control-tolerized mice (8.77).

**Increased Th1 cytokine production in mice receiving 4-deoxy α-GalCer with CpG 1826**

We also examined cytokine production from splenocytes obtained from the same experimental groups. Briefly, splenocytes collected at day 8 were stimulated *in vitro* with PMA/ionomycin or with plate-bound anti-CD3 and anti-CD28 mAbs. Supernatants were analyzed for IFN-γ, IL-4 and IL-10 levels. As expected, splenocytes from CpG 1826-treated mice produced less IL-4 and IL-10 compared to tolerized controls. Surprisingly, co-treatment with 4-deoxy α-GalCer increased IL-4 (p<0.05) levels relative to the CpG 1826-treated group, although differences in IL-10 levels did not reach statistical significance (p=0.105). However administration of PBS 57 with CpG 1826 significantly (p<0.05) increased both IL-4 and IL-10 production (Figure 16).

We observed robust IFN-γ production from splenocytes from untreated or control-tolerized mice. Surprisingly, splenocytes from tolerized mice treated *in vivo* with CpG 1826 made very little IFN-γ following anti-CD3 and anti-CD28 stimulation (Figure 16). The implications of this finding will have to be further investigated. Interestingly, 4-deoxy α-GalCer, but not PBS 57, significantly (p<0.05) increased IFN-γ levels compared to animals receiving CpG 1826 alone (Figure 16).
Figure 16. Effect of NKT cell and TLR ligands on cytokine production. A.SW mice received a single low dose (3 µg) of HgCl$_2$ on day -7. Animals then received either NKT cell ligand (2 µg) with CpG 1826 (100 µg) using the injection schedule described in Protocol 4 (Figure 10). All groups received 30 µg HgCl$_2$ on days 0, 2 and 4. On day 8, mice were sacrificed and splenocytes were stimulated with PMA/ionomycin or anti-CD3 + anti-CD28 mAbs. Culture supernatants collected at indicated timepoints were analyzed for IFN-γ (following anti-CD3 + anti-CD28 stimulation) or IL-4 and IL-10 (following PMA/ionomycin stimulation) by sandwich ELISA as described in Materials and Methods. Cytokine levels are expressed in pg/ml ± SD. *p<0.05 vs. PBS 57 + CpG 1826-treated and CpG 1826−treated groups, #p<0.05 vs. CpG 1826-treated group.
NKT cell ligand bearing bacteria exacerbate mercury-induced autoimmunity

Recent work has identified a number of microbes expressing cell wall lipids capable of activating NKT cells (Kinjo et al., 2005; Kinjo and Kronenberg, 2005; Kumar et al., 2000). Among these are gram-negative, LPS-negative α-proteobacteria of the Sphingomonas strain that express glycosylceramides recognized by both human and mouse NKT cells (Bendelac et al., 2007; Mattner et al., 2005; Tupin et al., 2007).

To test whether exposure to such microbes had an effect on autoimmune disease induced by mercury, groups of A.SW mice received a heat-killed suspension of S. capsulata, with or without HgCl$_2$ injections (Figure 17). Administration of bacteria alone did not induce any manifestations of autoimmunity, however the group receiving both bacteria and mercury demonstrated significantly increased autoantibody titers and serum IgG2a production (Figure 18) and moderately increased IgE levels (Figure 18). Thus, when compared to animals receiving mercury only, the heat-killed bacteria potentiated the autoimmunity-inducing effect of mercury.

TLR7 in mercury-induced autoimmunity

TLR7 activation occurs following HgCl$_2$ administration

TLR7 and 9 have been implicated in the pathogenesis of SLE, a systemic autoimmune disease with certain similarities to mercury-induced disease. Mammalian or viral DNA nucleic acids contain motifs that inhibit the activation of TLR7 and 9. ODNs incorporating these motifs that block the activation of TLR7, TLR9 or both have been
Protocol 5

Figure 17. Time course for heat killed bacteria administration and HgCl₂ treatment.
A.SW mice (n=5) received subcutaneous injections of 30 μg HgCl₂ in 100 μl of sterile PBS or 100 μl of sterile PBS alone three times during the first week. In addition groups of mice received 100 μl of a heat killed Sphingomonas capsulata suspension or 100 μl of sterile PBS intraperitoneally on days 0 and 2.
Figure 18. NKT cell ligand-bearing bacteria exacerbate mercury-induced autoimmunity. Groups of A.SW mice received HgCl\textsubscript{2} and heat killed *Sphingomonas capsulata* as described in Protocol 5 (Figure 15). ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1 and IgG2a) or µg/ml ± SD (IgE). *p<0.05, ***p<0.0001 vs. HgCl\textsubscript{2}-treated group.
Figure 19. Time course of administration of individual or combined ODN inhibitors of TLR7 and 9 in non-tolerized mercury-administered animals. Groups of A.SW mice (n=5) received 6 injections of 100 μg of a combined TLR7 and 9 inhibitor (IRS 954), a TLR7 inhibitor (IRS 661) or a TLR9 (IRS 869) inhibitor in 200 μl PBS intraperitoneally at the indicated timepoints. The control group was administered 100 μg ODN 532 and vehicle (PBS). All mice received 3 injections of 30 μg HgCl₂ in 100 μl of PBS subcutaneously on days 0, 2 and 4.
Figure 20. TLR7 engagement occurs following HgCl$_2$ administration. A SW mice received ODN inhibitors of TLR7, 9 or both in combination with mercury administration. ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1 and IgG2a) or µg/ml ± SD (IgE). *p<0.05 for TLR7/9/combined blockade groups vs. control group; **p<0.001 for TLR7/combined blockade group vs. control group; $p<0.05 for combined blockade group vs. control group.
synthesized (Barrat et al., 2005). We examined whether blockade of these receptors by inhibitory ODNs would affect development of heavy metal-induced autoimmunity. Groups of A.SW mice received 6 injections of IRS 661 (a TLR7 inhibitor), IRS 869 (a TLR9 inhibitor) or IRS 954 (a combined TLR7 and 9 inhibitor) at the indicated timepoints (Figure 19). Another group of mice received 3 injections of IRS 532 (a control ODN) or PBS. We have previously verified that this control oligo did not affect mercury-induced autoimmunity. All groups received 3 injections of 30 µg HgCl₂ during the first week (Figure 19). TLR7 inhibition or combined inhibition significantly (p<0.001) increased serum IgE levels compared to the control group (Figure 20). Blockade of either TLR significantly decreased (p<0.05) serum IgG2a at one time point (Figure 20). Both TLR7 and TLR9 blockade moderately decreased autoantibodies at week 2, although statistical significance (p<0.05) was attained by the group receiving the combined inhibitor. Overall, these findings suggest that TLR7 and possibly, TLR9 engagement occurs following mercury administration.

**TLR7 engagement is required for establishment of tolerance to mercury**

We also examined the contribution of TLR7 and 9 to tolerance establishment by low dose HgCl₂. A.SW mice were administered 100 µg of IRS 661 (a TLR7 inhibitor), IRS 869 (a TLR9 inhibitor) or IRS 954 (a combined inhibitor) or control ODN 532 intraperitoneally (Figure 21). Four hours later, all mice received the tolerizing 3 µg dose of HgCl₂. One week later, all mice were challenged with the regular regimen of mercury
Figure 21 Time course of administration of individual or combined ODN inhibitors of TLR7 and 9 prior to tolerance induction. Groups of A.SW mice (n=5) received 100 µg of a combined TLR7 and 9 (IRS 854), TLR7 (IRS 661) or TLR9 (IRS 869) inhibitor, or control ODN 532, on day -7. 4 hours later, all mice received a low dose (3 µg) of HgCl₂. One week later all groups received 3 injections of 30 µg HgCl₂ on days 0, 2 and 4.
Figure 22. TLR7, but not TLR9 engagement is required for establishment of antigen-specific tolerance. ASW mice received individual or combined ODN inhibitors or TLR7 or 9 followed by low dose HgCl$_2$ injection. All mice were challenged with the regular regimen of mercury administration one week later. ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers $\pm$ SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml $\pm$ SD (IgG1 and IgG2a) or $\mu$g/ml $\pm$ SD (IgE). $^*p<0.05$ vs. control group.
administration. Mice administered individual or combined inhibitors showed no increase in serum immunoglobulins (Figure 22) in response to mercury challenge, indicating that TLR7 or 9 engagement is not required for establishment of non-specific tolerance to mercury. TLR9 blockade by IRS 869 did not prevent tolerization towards autoantibody production; interestingly, autoantibody titers were slightly lower in mice administered IRS 869 compared to control-tolerized mice (Figure 22). This finding is in agreement with the results demonstrating that TLR9 engagement is effective at breaking tolerance (Figure 12). Strikingly, TLR7 blockade prevented tolerization to autoantibody production, with 3 out of 5 mice administered IRS 661 demonstrating robust autoantibody production. Surprisingly, this effect was lost when TLR7 blockade was combined with TLR9 blockade, suggesting opposing roles for TLR7 and TLR9 during the process of tolerance establishment to mercury.

**Dosage-dependent effect of TLR7 inhibitor**

We observed robust antibody production in only 3 out of 5 mice administered 100 µg IRS 661 (Figure 22). It is possible that a high dose (100 µg) could persist in the system for up to a week after administration, resulting in undesired TLR7 inhibition at the time of mercury challenge (day 0). We wanted to optimize the dosage so as to achieve inhibition only at the time of tolerance establishment. Efficient TLR7 inhibition has been observed using as low as 10 µg IRS 661 (F. Barrat personal communication). We hence tested a range of doses of IRS 661. Groups of A.SW mice received a single injection of
Figure 23. The 40 µg dose of the TLR7 inhibitor is most efficient at preventing the induction of antigen-specific tolerance. A.SW mice (n=5) received indicated amounts of ODN inhibitor IRS 661 followed by low dose HgCl$_2$ injection. All mice were challenged with the regular regimen of mercury administration one week later. ANoA were detected by immunofluorescence as described in the Materials and Methods section and are expressed as serum titers ± SD. Serum immunoglobulin levels were measured by ELISA as described in the Materials and Methods section and are expressed in mg/ml ± SD (IgG1 and IgG2a) or µg/ml ± SD (IgE). **p<0.001 vs. vehicle-treated group. #p<0.05 vs. 100 µg IRS 661-administered group
10 μg, 40 μg or 100 μg IRS 661, followed by low dose mercury administration. One week later all mice were challenged with the regular regimen of mercury. The 10 μg dosage was insufficient to prevent tolerance establishment. The 40 μg dose of inhibitor was optimal at preventing tolerance, inducing high titers (≥4000) in 5 out of 5 animals.

Interestingly, increasing the dose to 100 μg significantly (p<0.05) decreased the efficacy of the treatment, although a moderate potentiation of autoantibody production was still observed (Figure 23). This experiment demonstrates that amount of the inhibitor is an important factor in determining the effect observed and also confirms our observation that TLR7 engagement is required for the establishment of antigen-specific tolerance to mercury.
CHAPTER 5
DISCUSSION

NKT cells in mercury-induced autoimmunity

We examined in this study the effects of NKT cell activation on mercury-induced autoimmunity. The two synthetic NKT cell ligands PBS 57 and 4-deoxy α-GalCer differentially modulated mercury-induced autoimmunity (Table 1). When tested in a model of tolerance induction, NKT cell activation prevented tolerance establishment but was by itself insufficient to break established tolerance. To disrupt tolerance, NKT cells required an additional signal, which was in this case provided by a TLR9 agonist. In addition, we observed that different NKT ligands can either synergize with or antagonize TLR9-induced breakage of tolerance. Lastly, bacteria bearing NKT cell ligands have an exacerbating effect on our model of chemically-induced autoimmunity.

The outcome of NKT cell activation in autoimmunity depends on a variety of factors, including the mouse strain studied (Jahng et al., 2001; Yang et al., 2003), the timing of administration of NKT cell activating ligand (Coppieters et al., 2007), and the NKT cell activating ligand itself (Miyamoto et al., 2001; Pal et al., 2001). Our results emphasize the importance of the mouse strain and the activating ligand. In the
<table>
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<tr>
<th>NKT agonist</th>
<th>Standard Hg Challenge without tolerance</th>
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<td>Vehicle</td>
<td><em>anti-nucleolar autoantibody production and polyclonal B cell activation</em></td>
<td><em>Low Titters of anti-<em>nucleolar</em> autoantibodies; polyclonal activation abrogated</em></td>
<td><em>Breaks tolerance and potentiates autoantibody production; Decreases ratio of T\text{regs}:T\text{effectors}; Tilts balance in favor of effector T cells in the spleen</em></td>
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<tr>
<td>PBS 57</td>
<td><em>Does not exacerbate mercury-induced autoantibodies; Increases splenic T\text{regs}, Th2 bias and IL-10 production</em></td>
<td><em>Prevents tolerance establishment when administered prior to tolerogenic Hg injection</em></td>
<td><em>Inhibits CpG1826-induced potentiation of autoantibodies; Restores splenic balance in favor of T\text{regs}, increases Th2 bias and IL-10 production</em></td>
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<tr>
<td>4-deoxy α-GalCer</td>
<td><em>Exacerbates mercury-induced autoantibodies; Decreases splenic T\text{regs}</em></td>
<td><em>No effect when administered prior to tolerogenic Hg injection</em></td>
<td><em>Potentiates CpG 1826-induced autoantibody production; Increases Th1 bias, maintains CpG-induced decrease in ratio of T\text{regs}: T\text{effectors}</em></td>
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<tr>
<td><em>Sphingomonas capsulata</em></td>
<td><em>Strongly exacerbates autoimmunity</em></td>
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Table 1: Overview of interplay between NKT cell agonists, a TLR9 agonist and mercury. *In vivo* results are italicized, *ex vivo* observations are in the regular font; ND— not done. Note: Unless otherwise mentioned, NKT agonists were administered with the regular Hg challenge.
C57BL/6.SJL strain, both NKT cell ligands potentiated development of autoimmunity. However, in the A.SW strain of mice, NKT cell activation has profoundly different results depending on the glycolipid used to stimulate them. 4-deoxy α-GalCer consistently exacerbated mercury-induced autoantibody production while PBS 57 either had no effect on or downregulated autoantibody levels. Thus, NKT cells are able to modulate heavy metal-induced autoimmunity differently depending on the ligand that activates them. These results are surprising as both ligands are derivatives of the widely used NKT cell ligand α-GalCer with earlier studies indicating that their immunostimulatory properties are comparable to the parental ligand (Liu et al., 2006; Sidobre et al., 2004). We expanded our study by comparing the two ligands in a model of tolerance induction. Since microbial infections can expose the immune system simultaneously to both NKT cell ligands and TLR ligands, we examined interplay between two NKT cell ligands and a TLR9 ligand, CpG 1826 in the breakage of established tolerance. 4-deoxy α-GalCer-induced NKT cell activation strongly synergized with TLR9 stimulation to break established tolerance. This result is in agreement with a recent study wherein co-exposure to a NKT cell ligand, α-GalCer and various TLR ligands resulted in enhancement of DC maturation with increased T cell and B cell responses, suggesting that synergy between NKT ligands and different TLR ligands is a generalized phenomenon (Hermans et al., 2007). Surprisingly, our results showed that NKT cell activation can also antagonize TLR signals. NKT cell activation by PBS 57 antagonized the effects of TLR9 stimulation, reducing TLR9-induced autoantibody production.
Since the two NKT ligands had opposite effects on autoantibody production when administered with mercury, we examined the in vivo effects of dual administration of NKT ligands and mercury. In mercury-exposed animals, PBS-57 induced NKT cell activation increased the frequency of T\text{regs}, while 4-deoxy α-GalCer-induced NKT cell activation had the opposite effect. Moreover, in the tolerance model, PBS 57 but not 4-deoxy α-GalCer, restored the balance between T\text{regs} and T\text{effectors} which had been disrupted by TLR9 stimulation. The importance of this balance has been highlighted by a recent study which demonstrated that a decrease in the ratio of T\text{regs} to T\text{effectors} correlated with progression of disease in nonobese diabetic (NOD) mice (Tang et al., 2008). Similarly, our study demonstrates that a treatment which disrupts tolerance decreases this ratio and suggests that this balance can be modulated by interplay between NKT cell agonists and a TLR9 ligand.

PBS 57 increased Th2 skewing in mercury-treated mice, whereas 4-deoxy α-GalCer increased Th1 skewing in tolerized mice challenged with CpG 1826 and mercury. Since autoantibody production in the mercury model is dependent on IFN-γ, but not IL-4 these results suggest an explanation as to why PBS 57 negatively regulates mercury-induced autoantibody production and why 4-deoxy α-GalCer and PBS 57 have opposing effects on CpG 1826-induced autoantibody production in tolerized mice. Strikingly, as mercury increased Th2 skewing in PBS 57-treated, but not 4-deoxy α-GalCer-treated animals these results also suggest that mercury, a common environmental pollutant can modify the outcome of NKT cell activation by certain agonists.
We also examined effects of the NKT cell activation in a model of tolerance induction. Current models of tolerance induction hypothesize that antigen presentation in the steady state by DCs induces peripheral tolerance (Steinman et al., 2003; Steinman and Nussenzweig, 2002). The nature of these tolerogenic DCs is however unclear. Certain studies suggest that ‘tolerogenic’ DCs are phenotypically immature, while maturation imparts the ability to be immunogenic (Inaba et al., 2000; Miyamoto et al., 2001; Schuurhuis et al., 2000). However, in certain cases, phenotypically mature DCs can be tolerogenic (Lutz and Schuler, 2002; Menges et al., 2001; Reis e Sousa, 2006; Rutella et al., 2006). Menges and colleagues showed that DCs matured with repeated injections of TNF-α induce antigen-specific protection of mice against EAE (Menges et al., 2001). Activation of NKT cells induces phenotypic maturation of dendritic cells, causing upregulation of CD40, CD80 and CD86 costimulatory molecules and MHC class II antigen-presenting molecules (Fujii et al., 2003). This study (Reis e Sousa, 2006) and others (Steinman et al., 2003; Steinman and Nussenzweig, 2002) suggest that NKT cell activation could produce immunogenic DCs. In contrast, studies that examined the role of NKT cells in two models of tolerance have shown that this cell population is necessary for tolerance establishment (Roelofs-Haarhuis et al., 2004; Sonoda et al., 2007). In a model of tolerance establishment following oral administration of the contact allergen nickel, CD4⁺ NKT cells producing IL-4 and IL-10, but not IFN-γ were required for the induction of tolerogenic APCs (Roelofs-Haarhuis et al., 2004). Somewhat in contrast to these findings, we demonstrate that NKT cell activation was sufficient to prevent establishment of tolerance to mercury. In our experiment, NKT cell activation by PBS 57, but not 4-deoxy α-GalCer, prevented tolerance establishment. PBS 57 is also a
stronger NKT cell agonist than 4-deoxy α-GalCer. It is a more potent inducer of several cytokines, including IL-17 (unpublished observations). A recent study has demonstrated that IL-17 can block establishment of oral tolerance (Ehirchiou et al., 2007). Further studies are required to elucidate the mechanisms involved in PBS 57-induced prevention of tolerance.

A principal evolutionary role for NKT cells has been identified as antimicrobial defense (Bendelac et al., 2007; Tupin et al., 2007) this cell type is required for clearance of a large number of microbes (Arase et al., 1992; Kinjo et al., 2005; Kinjo and Kronenberg, 2005; Kumar et al., 2000; Nieuwenhuis et al., 2002). Bacterial, viral and parasitic infections have been implicated in the development and exacerbation of autoimmune diseases (Christen and von Herrath, 2005). A number of other studies have shown that exposure to chemicals (drugs or heavy metals) can also trigger or exacerbate autoimmune disease (Christen and von Herrath, 2005; Ehirchiou et al., 2007; Pollard KM et al., 2001; Via et al., 2003). However, the effects of infections and chemicals on autoimmune disease have for the most part been studied separately, whereas human patients are likely to be exposed to both factors. Hence in this study we tested the effect of a commonly dispersed chemical and an infectious agent on autoimmunity. NKT cell ligand-bearing bacteria of the Sphingomonas strain are abundant soil microbes and have been detected in the stools of 25% of healthy individuals (Bendelac et al., 2007). Although contact levels vary among individuals, mercury exposure is virtually universal because of its natural release in the environment, abundance as a pollutant and presence in dental amalgams, cosmetics, preservatives, fumigants and vaccine preparations.
(Bagenstose et al., 1999b; Pelletier et al., 1994). As previously demonstrated, normally maintained immune tolerance is broken by exposure to mercury. Administration of both mercury and bacteria induced pronounced anti-nucleolar reactivity and exacerbated the heavy metal-induced autoimmunity. Unlike many bacterial species which primarily activate innate immunity via TLR signaling, *S. capsulata* bears triggers for both NKT cells and TLRs. These dual stimuli may have contributed to exacerbation of autoimmune manifestations.

Certain synthetic NKT ligands, such as OCH or the C-glycoside analogue of α-GalCer, induce strongly different effects, owing to structural differences which result in highly polarized cytokine production. In contrast, PBS 57 and 4-deoxy α-GalCer have both been described as similar to α-GalCer. However, our study demonstrates that these NKT agonists, in combination with immunomodulator mercury, yield strikingly different effects. There could be several explanations for this. PBS 57 is more soluble due to the amide and the double bond in the acyl chain. Differential solubility can affect loading of lipids on to CD1d which in turn can modulate the uptake and presentation of CD1d-expressing APCs (Zajonc et al., 2005). Additionally, decrease in acyl chain length (Goff et al., 2004) and increased unsaturation (Yu et al., 2005) increase Th2 cytokine production. The latter findings may represent explanations as to why more IL-4 is detected with PBS 57. Mercury is an immunomodulatory agent that increases skewing towards Th2 (Rowley and Monestier, 2005). It is possible that the combination of the two agents results in increased Th2 skewing, and this could be, in part, contributing to the regulatory effects of PBS 57 in mercury-administered A.SW animals. Our study
demonstrates that an environmental agent with immunomodulatory capacity can strongly influence the effect of NKT cell activation. This observation serves as a cautionary note when considering NKT cell activators as therapeutics in patients who may be simultaneously exposed to various environmental agents.

**TLR7 in mercury-induced autoimmunity**

Apoptosis is considered a non-inflammatory process, while necrosis is inflammatory. However, insufficient clearance of apoptotic cell debris results in accumulation of cells undergoing secondary necrosis. Release of DAMPs (including HMGB1, DNA and RNA-containing complexes, heat shock proteins, uric acid and others) from necrotic cells can initiate an inflammatory response (Kono and Rock, 2008). Thus, defects in apoptosis or in the clearance of apoptotic debris are likely contributing factors to the development of systemic autoimmune diseases. Other contributing factors can include infection and environmental exposure. Synergy between these factors is possible. Environmental factors linked to autoimmune disease include microbes, chemicals, drugs, pollutants and physical factors such as ultraviolet (UV) radiation (Kuhn and Bijl, 2008). Infection or environmental exposures may raise the apoptotic burden; for example viruses (Dockrell, 2001) or UV radiation (Kuhn and Bijl, 2008) can directly or indirectly increase cell death. Additionally, infections can provide adjuvants that can increase the immunogenicity of apoptotic cells (Rovere-Querini *et al*., 2005).

The mechanisms by which mercury induces a break in tolerance are unknown. Mercury at very low concentrations can inhibit apoptosis, but at higher concentrations
can induce cell death, by apoptosis, necrosis or a mixture of the two (Liu et al., 2005a). We investigated the effect of TLR7 and TLR9 inhibition in mercury-induced autoimmunity. Our preliminary results indicate that inhibition of TLR7 increases mercury-induced IgE production, while having a mild inhibitory effect on mercury-induced IgG2a. Taken together with recent report which directly demonstrates that TLR7 activation decreases IgE production (Shen et al., 2008), this finding suggests that HgCl₂ administration elicits endogenous ligands for TLR7. However, the development of autoimmunity despite TLR7 inhibition indicates that several other pathways are involved.

Mercury treatment can cause the release of more than one DAMP. For example, HMGB-1 release was observed in serum of mercury-treated A.SW mice (unpublished observations). HgCl₂ also causes release of heat shock proteins and elevates intracellular Ca²⁺ and ROS, all of which can also play a role in stimulating the immune system.

Additionally, we have examined only the effect of short term TLR7 inhibition. To better gauge the importance of TLR7 activation for the development of the autoimmune response, both the protocol of administration and the dosage of the inhibitor have to be optimized. Examination of the complete abrogation of TLR7 signaling by use of TLR7 knockout mice bred onto a mercury-susceptible background is also of interest.

Autoantibodies found in mercury-treated mice specifically recognize fibrillarin and other proteins found in RNA-containing snoRNPs (Goyer, 1990; Hultman et al., 1989; Monestier et al., 1994). We explored the possibility that TLR7 activation by endogenous RNA played a role in determining this specificity. Tolerance induction by
low dose mercury suppresses the polyclonal B cell activation as well as the antigen-specific anti-nucleolar autoantibody response. Interestingly, TLR7 inhibition at the time of the low-dose HgCl$_2$ injection prevented tolerization towards only the anti-nucleolar autoantibody response, suggesting that engagement of this receptor is required for establishment of antigen-specific tolerance. Low dose HgCl$_2$ administration induces T$_\text{regs}$, which, following antigenic expansion by HgCl$_2$, protect naïve animals from mercury-induced autoimmunity (Yan Zheng, unpublished observations). Examining the protective capacity of adoptively transferred T$_\text{regs}$ from TLR7 inhibitor-administered mice will help reveal whether TLR7 engagement is required for the induction of protective T$_\text{regs}$. 
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