Published in final edited form as:

Toxicol Appl Pharmacol. 2007 December 15; 225(3): 238–250.

Exposure to inorganic mercury *in vivo* attenuates extrinsic apoptotic signaling in *Staphylococcal aureus* enterotoxin B stimulated T-cells

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Abstract

The heavy metal mercury (Hg) is known to have immunomodulatory properties affecting lymphocyte signal transduction, death receptor signaling and autoimmunity. In this study we tested the hypothesis that Hg exposure would attenuate T-cell activation and caspase 8 and 3 activity in response to antigenic stimuli. To test this hypothesis, BALB/cJ mice were exposed to 10 mg/L mercuric chloride (HgCl₂) in their drinking water for two weeks followed injection with 20µg of the *Staphylococcal aureus* enterotoxin B (SEB) superantigen. Eighteen hours after SEB challenge, there was a statistically significant reduction in caspase 8 and caspase 3 enzyme activity in the SEB reactive Vβ8+ T-cells. The attenuated caspase activity in Hg exposed mice persisted for 48 hours after exposure. Moreover, activation of caspase 8 and caspase 3 was reduced by more than 60% in CD95 deficient MRL/MpJ-*Fas*^{lpr} mice demonstrating caspase 8 and 3 activation in response to SEB is CD95 dependent. In addition to the effects of Hg on caspase activity, expression of the T-cell activation marker CD69 was also attenuated in SEB reactive Vβ8 T-cells in Hg-exposed mice. Moreover, CD69 expression in MRL/MpJ-*Fas*^{lpr} mice was also reduced. Taken together the caspase and CD69 data support a role for CD95 in promoting a proapoptotic and activated state in SEB responsive T-lymphocytes and this state is attenuated by the autoimmune potentiating environmental agent mercury.

Keywords

T-cells; Autoimmunity; Superantigens; Apoptosis

INTRODUCTION

Autoimmune disorders are defined by a loss of immune system homeostasis resulting in aberrant activation of lymphocytes with specificity directed against self antigens. This breakdown of self tolerance yields activated lymphocytes that may infiltrate target organs, leading to inflammation, and tissue damage. It has long been suspected that disruption of

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immune system homeostasis leading to autoimmune disorders is mediated by interactions between environmental factors and the genome. Moreover, while neither a specific environmental factor or genetic susceptibility may be enough to induce autoimmune disease, the combination of both at the inappropriate time may be sufficient to drive either the initiation or progression of disease.

During the last two decades considerable attention has been directed toward identifying and understanding genetic risk factors which may lead to a break down of self tolerance. In comparison to the genetic risk factors, far fewer advances have been made in identifying environmental agents and their mechanisms of initiation and progression toward autoimmune etiologies. Nevertheless, a growing body of literature is providing evidence that exposure to metals, particularly in the context of specific genetic variants, may increase the risk for disrupting immune system homeostasis (Lawrence and McCabe, 2002). For example the metals beryllium and cobalt have been associated with chronic inflammatory diseases (Poticchio *et al.*, 1997; Fontenot *et al.*, 2001). Additionally, the heavy metals gold and mercury (Hg) have been implicated in metal-induced autoimmune disease in humans (Verwilghen *et al.*, 1992; Bagenstose *et al.*, 1999; te Boekhorst *et al.*, 1999). Hg in particular has been demonstrated to play a significant role in the initiation and progression of autoimmune disease in animal models (Monestier *et al.*, 1994; Hultman *et al.*, 1995; Takeuchi *et al.*, 1995; Hultman *et al.*, 1996; Pollard *et al.*, 1997; Bagenstose *et al.*, 1999; Pollard *et al.*, 2000). In these models, MHC as well as non-MHC genes have been identified as susceptibility factors for Hg-induced autoimmunity (HgIA). In addition to genetic factors involved in HgIA, our laboratory has demonstrated that low doses of Hg interfere with CD95 dependent signaling in human T-cells (Whitekus *et al.*, 1999; McCabe *et al.*, 2003; McCabe *et al.*, 2005; Ziemba *et al.*, 2005). These findings implicate CD95 signaling as a specific cellular target of interference by Hg, and this disruption of CD95 signaling may contribute to disease.

CD95 (Fas, APO-1), is a critical regulator in maintaining immune system homeostasis and tolerance, by mediating apoptosis after interaction with Fas ligand (FasL). Genetic lesions of Fas in both mouse and humans have clearly identified this receptor as a key mediator in regulating lymphocyte homeostasis (Bidere *et al.*, 2006). Humans with Fas mutations present with lymphoproliferation, peripheral expansion of CD4-CD8- $\alpha\beta$ -cells and impaired lymphocyte apoptosis in a disorder known as autoimmune lymphoproliferative syndrome (ALPS) (Rieux-Laucat *et al.*, 2003; Sneller *et al.*, 2003). These findings are consistent with observations made in mouse models where CD95 is mutated spontaneously in the *lpr* mutation (Altman, 1994), or by gene targeted mutations (Adachi *et al.*, 1995; Adachi *et al.*, 1996). In the *lpr* mutation, a transposable insertion sequence is inserted into the second intron of CD95 resulting in aberrant mRNA transcript splicing and consequently, translation of a truncated protein (Adachi *et al.*, 1993). The truncated protein is blocked from entering the plasma membrane efficiently, and thus surface expression is found at very low levels, inhibiting CD95 dependent signal transduction (Adachi *et al.*, 1993).

CD95 is a member of the TNF super-family of receptors and initiates apoptotic signaling when the CD95 trimer or possibly larger order multimer complexes are triggered and/or clustered by the CD95 ligand (Krueger *et al.*, 2003). Immediately after activation, CD95 recruits the intracellular adaptor protein FADD (Fas associated death domain) and apoptotic initiator caspase 8 to form the death inducing signaling complex or DISC. Caspase 8 exists in the cytosol as a proenzyme and clustering by the DISC leads to autoprocessing, releasing the highly active form of caspase 8 (Bidere *et al.*, 2006). Downstream targets of the DISC include the pro-apoptotic factors Bid and the effector caspase 3 (Krueger *et al.*, 2003; Bidere *et al.*, 2006). Activation of caspase 3 leads to caspase signal amplification and eventually cellular death.

Considering the critical role that CD95 plays in regulating lymphocyte homeostasis and tolerance, and given the modulatory role Hg plays on CD95 signaling, we asked if Hg – intoxication could affect T-cell activation and cellular death *in vivo*? To address this question, we evaluated the role of Hg in T-cells stimulated by the *Staphylococcal aureus* enterotoxin B (SEB) superantigen. SEB is classified as a superantigen due to its ability to stimulate naive T-cells by directly crosslinking the alpha chain of MHC class II with the CDR2 loop of the V β 8 T-cell antigen receptor (TCR) (Kawabe and Ochi, 1990; Marrack *et al.*, 1990; Muraille *et al.*, 1997). After systemic exposure to SEB, V β 8 T-cells undergo rapid proliferation and expansion, transient expression of the early activation antigen CD69, followed by cell death (Vasseur *et al.*, 1999). Pro-apoptotic factors are induced very rapidly after SEB challenge, however, death is actually refractory to cell proliferation. It has been demonstrated in SEB stimulated T-cells, that activation induced cell death (AICD) does not occur for at least 48 hours after SEB challenge despite rapid induction of pro-apoptotic factors (Yang *et al.*, 1998). The consequences of this delayed AICD are to allow for expansion of effector T-cells in order to clear the toxin. Therefore, the SEB model system provides a useful tool for assessing Hg-induced alterations in death receptor signaling, rapidly after T-cell activation, but prior to antigen clearance.

Our results demonstrate that SEB activates caspase 8 and caspase 3 signaling within 12 hours of administration, and that Hg-intoxication reduces the number of T-cells capable of activating caspases. Moreover, we show that the induction of caspase 8 and caspase 3 activity is CD95 dependent. These data illustrate the modulatory properties of Hg on T-cell activation and survival *in vivo*. Given the critical role for AICD in regulating T-cell homeostasis and self-tolerance, these findings that Hg-intoxication affects death receptor signaling have implications for establishment and progression of autoimmunity.

MATERIALS AND METHODS

Experimental animals

Six to eight week old female BALB/cJ mice, MRL/MpJ, and MRL/MpJ-*Fas^{lpr}* were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were randomized into treatment groups at the time of arrival and allowed to acclimate for one week after arrival at the University of Rochester. Mice were maintained on a 12-h dark, 12-h light cycle and were provided food (5001 rodent diet; Purina Mills, St. Louis, MO) and water *ad libitum*. All mice were housed and cared for according to protocols approved by the University Committee for Animal Resources (UCAR) at the University of Rochester.

Chemicals and treatment protocol

Mercuric chloride (HgCl₂), was obtained from Sigma-Aldrich Chemicals (St. Louis, MO), and a stock solution of 5 g/L HgCl₂ in double distilled Milli-Q purified water was prepared. After acclimation, 10 mg/L HgCl₂ in Milli-Q purified (Millipore, Billerica, MA) autoclaved water, or the purified and autoclaved water alone, was administered to BALB/cJ mice for a period of two weeks prior to challenge with SEB. Five mg of SEB (Sigma-Aldrich Chemicals) was diluted in Ca⁺⁺ and Mg⁺⁺ free Dulbeccos Phosphate Buffered Saline (Gibco Invitrogen Cell Culture; Carlsbad, CA) to yield a stock solution of 200 μ g/ml SEB. Two weeks after exposure to HgCl₂ in their drinking water, mice were challenged with 20 μ g of SEB in 0.1 ml of PBS, (or PBS alone) by i.v. tail vein injection at 12, 18, 21, 24, 48, 72, or 96 hours prior to euthanasia. At the appropriate time, mice were euthanized by CO₂ anesthesia, followed by cervical dislocation.

Mercury exposure and analysis of mercury burden

BALB/cJ mice were exposed to 10 mg/L mercuric chloride in milliQ purified and autoclaved drinking water for a duration of two weeks. Control mice received milliQ purified, autoclaved water alone. To determine if the dosing protocol resulted in Hg intoxication, the levels of Hg per gram of kidney tissue was measured. Kidneys are well known to be the principle target organ of deposition and accumulation of Hg in exposed animals and individuals (Warfvinge *et al.*, 1995; Clarkson and Magos, 2006). Individual kidneys were homogenized and digested, followed by analysis using cold vapor atomic absorption and results are reported from Table 1. Using this method, it was determined that mice exposed to Hg registered nearly 1000 fold higher levels of Hg in their kidneys than did mice drinking Milli-Q purified water alone (Table 1, $p \leq 0.01$). In addition to the kidney analysis, Hg measurements were also made in homogenized pellets of RBC depleted splenocytes. As reported in Table 1, the level of Hg associated with ten million cells is forty percent higher in mice exposed to Hg in their drinking water than control mice ($p \leq 0.05$).

Lymphocyte and splenocyte isolation

After euthanasia, spleen and inguinal lymph nodes (draining nodes for kidney) were removed, cleaned of fat and connective tissue and placed in cold Hanks Balanced Salt Solution (HBSS; Gibco Invitrogen Cell Culture), supplemented with 5% FBS. Cell suspensions were created by crushing the respective organ between the ends of frosted slides, and rinsing with additional HBSS. Lymphocytes were pelleted at 900 x g for 6 minutes, resuspended in 1 ml cold HBSS and cell yield enumerated using a Coulter Z1 series particle counter (Beckman-Coulter, Fullerton, CA).

Splenocyte suspensions were pelleted, and depleted of Red blood cells (RBC) by treatment with Red Blood Cell Lysis Buffer (Sigma-Aldrich). Cell yield was enumerated as described above.

Detection of caspase 8 and caspase 3 activity

One to two million lymphocytes or splenocytes from each mouse were aliquoted into individual wells in a 96 well plate. Cells were pelleted, and resuspended in 40 μ l of either the caspalux L1D2 caspase 8 fluorogenic substrate (OncoImmunin, Gaithersburg, MD), or phiphilux G1D2 caspase 3 fluorogenic substrate (Oncoimmunin). After one hour incubation in a humidified, 5% CO₂, 37°C incubator, cells were washed, pelleted, and prepared for flow cytometry surface staining. Active caspase 8 and caspase 3 enzymes cleave the lipophilic substrate, resulting in ≥ 10 fold increase in fluorescence intensity detectable in the FITC channel of the flow cytometer.

Antibodies and lymphocyte staining for flow cytometry

The following monoclonal fluorochrome-conjugated antibodies were used at predetermined saturating concentrations for staining freshly isolated lymphocytes: CD95 (clone Jo2) conjugated to FITC, V β 8 TCR (clone F23.1) conjugated to phycoerythrin (PE), V β 6 TCR (clone RR4-7) conjugated to Biotin, CD69 (clone H1.2F3) conjugated to PE-Cy7, CD8 (clone 5H10) conjugated to Allophycocyanin (APC), and CD4 (clone L3T4) conjugated to APC-Alexa fluor 750. All antibodies were purchased from BD Pharmingen, (San Diego, CA), with the exception of CD4 and CD8 which were purchased from CalTag-Invitrogen, (Carlsbad, CA).

After incubation with the appropriate fluorogenic caspase substrate, cells were stained with FITC, PE, PE-Cy7, APC, APC-Alexa Fluor 750 and Biotin conjugated antibodies for 20

minutes on ice. Cells are then washed, and stained with Streptavidin conjugated to PerCP (BD Pharmingen).

After lymphocyte staining, cells were acquired on a BD LSR II flow cytometer. Approximately $2-5 \times 10^5$ events per sample were acquired and data analyzed using FlowJo software (Treestar, Ashland, OR), and visualized using biexponential scaling (Herzenberg *et al.*, 2006).

Statistics

For each experiment, three to five mice per group were analyzed individually. Each time point was analyzed on at least two separate independent experiments. Results are presented as the mean $\pm$ SEM with the appropriate number of mice indicated in the figure legend. A one-way analysis of variance was performed for each experiment using JMP statistical software (SAS, Cary, NC). Comparisons between the means were performed using Student's t-test and $p \leq 0.05$ were considered statistically significant.

RESULTS

Mercury intoxication attenuates the amplitude of the primary SEB response

Our laboratory has previously established that low concentrations of HgCl_2 inhibit CD95 dependent apoptosis by interfering with the formation of the death inducing signaling complex (DISC) in Jurkat and Hut-78 T-cells (McCabe *et al.*, 2003). Given the importance of apoptosis in regulating peripheral tolerance, the CD95 data suggest a possible mechanism of how Hg may contribute to development of systemic autoimmunity. In order to translate our earlier biochemical findings to an *in vivo* model system, we hypothesized that Hg exposure *in vivo*, impairs death receptor signaling in activated T-cells. The implications of impairment of death receptor signaling are an increased risk of developing autoimmune disease, particularly in genetically prone individuals. To test this hypothesis *in vivo*, we assayed for caspase 8 and caspase 3 activation in response to the SEB superantigen at early time points after exposure. The SEB model was employed in this manner, in order to determine the effect Hg has on T-cell activation and death receptor signaling during the initial phases of an immune response prior to antigen clearance. Using the SEB model we can compare T-cell expansion and activation induced cell death between SEB responsive $\text{V}\beta 8^+$ T-cells and SEB non-responsive $\text{V}\beta 6^+$ T-cells in either Hg-intoxicated, or non-intoxicated control mice.

As shown in figure 1, administration of SEB resulted in the predicted expansion of $\text{V}\beta 8$ T-cells by 48–72 hours in both the spleen and inguinal lymph nodes, followed by a rapid depletion of activated T-cells returning to near control numbers around 96 hours. By 12 days after injection, cell numbers of all populations return to baseline (data not shown). Treatment with the PBS alone had no effect on $\text{V}\beta 8$ cell number, or expansion in the presence or absence of Hg at 18, 24, 48 and 96 hours (data not shown). As shown in figure 1, Hg intoxication has little to no effect on the termination phase of the response (i.e. >72 hours). If Hg is interfering with CD95 signaling, then normal cell loss in response to SEB with or without Hg is not surprising given reports that the activation induced cell death (AICD) phase in response to SEB is CD95 independent (Hildeman *et al.*, 1999; Hildeman *et al.*, 2002). Rather, the termination phase in response to SEB has been shown to be dependent on reactive oxygen species and the pro-apoptotic protein BIM (Hildeman *et al.*, 1999; Hildeman *et al.*, 2002). Nevertheless, the number of $\text{V}\beta 8^+$ T-cells is slightly reduced during the peak expansion phase of the SEB response in Hg intoxicated mice compared with non-exposed mice. Although there are approximately 20% fewer $\text{CD4}^+ \text{V}\beta 8^+$ cells in the spleen, and 20% fewer $\text{CD8}^+ \text{V}\beta 8^+$ lymph node T-cells 48 hours after SEB exposure these data do not achieve statistical significance. Additionally, lymph node $\text{CD4}^+ \text{V}\beta 8^+$ cell numbers are reduced by nearly 40% in Hg intoxicated SEB challenged

mice 72 hours later ($p \leq 0.01$) (figure 1). There were no other differences between Hg – exposed and control mice after 96 hours (data not shown).

Mercury intoxication impairs SEB dependent caspase activation

Given the subtle effects of Hg observed on SEB dependent T-cell expansion, we hypothesized that the window where potential modulation occurs is brief. Moreover, induction of pro-apoptotic genes occurs rapidly after T-cell activation in response to SEB, while actual apoptosis is relatively insensitive to the initial gene up-regulation (Yang *et al.*, 1998). Given the potentially rapid kinetics of pro-apoptotic signals and the known effects of Hg on CD95 (Whitekus *et al.*, 1999; McCabe *et al.*, 2003; McCabe *et al.*, 2005), we assessed caspase 8 and caspase 3 activation in response to SEB at early time points after superantigen challenge. Prior to CD95 ligation, the cysteine protease caspase 8 exists in the cytosol as a pro-enzyme. Subsequent to CD95 ligation and DISC formation, the inhibitory domain of caspase 8 is cleaved (Bidere *et al.*, 2006). Often referred to as the initiator caspase, caspase 8 then cleaves intracellular substrates including the effector caspase, caspase 3, which promotes apoptosis. In order to measure caspase activity in cells, we incubated splenocytes or lymphocytes in the presence of lipophilic fluorogenic caspase substrates possessing the IETDGI or DEVDGI target peptide sequences for caspase 8 and caspase 3 respectively (Komoriya *et al.*, 2000). After substrate incubation, cells in which an active caspase enzyme is present will cleave the target peptide sequence, releasing the fluorophore that is detectable in the FITC channel of the flow cytometer.

Figure 2 demonstrates the approach taken for analyzing caspase 8 and caspase 3 activity in different populations of T-cells, and it shows that Hg-intoxication attenuates the activation of these proteases in response to SEB stimulation. Splenocytes are first gated on CD4 or CD8, followed by discrimination based on the surface expression of the V β 8 or V β 6 TCR. Importantly, there were no significant differences in the distribution of T-cells within the CD4, CD8, V β 8 or V β 6 TCR subsets at the early time points (less than 24 hours) following exposure to either SEB, HgCl₂ or both. Subsequently, the activity of the appropriate caspase enzyme is quantified by applying the Overton multiple comparison algorithm to the FITC fluorescence detected in the V β 8+ and V β 6+ TCR populations. The Overton algorithm calculates the cumulative mean fluorescence intensity (MFI) difference between overlaid experimental (V β 8) and control (V β 6) histograms, yielding an unbiased percentage of positive cells (Overton, 1988). Caspase 8 and caspase 3 activity is negligible in resting T-cells obtained from control PBS challenged mice and no difference is observed in Hg intoxicated mice. However, caspase 8 and caspase 3 are maximally induced 18 to 24 hours after SEB challenge in V β 8+ T-cells when compared to non-responsive V β 6+ T-cells with nearly 60 to 70% of the cells expressing activated caspase 3 or caspase 8 proteases, respectively. In support of our hypothesis, intoxication with Hg significantly reduces the percentage of both CD4+ and CD8 + V β 8+ T-cells in the spleen and lymph node with activated caspase 8 and caspase 3 enzymes (Figure 2 and 3). Specifically, 68.7 ± 1.31 % of CD4+ V β 8+ splenocytes have activated caspase 8 enzyme activity 24 hours after SEB challenge compared with 62.5 ± 1.31 % in Hg intoxicated mice (Figure 3; $p \leq 0.01$). CD8+ V β 8+ splenocytes peaked six hours earlier with 71.1 ± 1.7 % of these cells expressing activated caspase 8 compared with 61.5 ± 1.7 % of CD8 T-cells obtained from SEB-challenged Hg-intoxicated mice (Figure 3; $p \leq 0.01$). As shown in figure 3, Hg intoxication impairs the peak activation of both caspases 8 and 3, and the level of activation is consistently and significantly lower between 18 to 48 hours after SEB challenge. By 72 hours after SEB exposure, there is no difference between Hg – exposed and control mice, and by 96 hours, caspase activity has returned to baseline levels as determined from PBS-injected controls (data not shown). In terms of the kinetics of death receptor signaling and caspase activation, these data make it clear that the critical window occurs within the first 48 hours of SEB exposure when the antigen is still present. These kinetic data are not surprising

given that the SEB toxin is known to be cleared within 24 hours of exposure (Vabulas *et al.*, 1996).

Mercury intoxication attenuates early SEB dependent T-cell activation

In addition to the known effects of Hg on CD95 dependent signaling, inhibition of TCR dependent signal transduction by Hg has also been observed (Ziemba *et al.*, 2006), suggesting a possible effect of Hg on lymphocyte activation. In order to determine if Hg intoxication has an effect on T-lymphocyte activation *in vivo*, we compared induction of the early T-cell activation marker CD69 in response to SEB in the presence or absence of Hg. As has been previously reported, CD69 induction occurs rapidly after exposure to SEB (Vasseur *et al.*, 1999). Moreover, we found that CD69 induction is highly correlated with caspase 8 and caspase 3 activity 18 hours after SEB exposure as shown in figure 4A. In Hg exposed mice, both the percentage and absolute number of CD69+ T-lymphocytes with activated caspase 8, or caspase 3 is reduced by more than 30% 18 hours after SEB challenge (Figure 4). Additionally, the MFI of CD69 in the V β 8+ T-cell population is significantly reduced in Hg exposed mice (figure 4B and 4C). The reduction of CD69 MFI is most likely attributable to an accumulation of CD69 negative cells in Hg exposed mice, rather than a general decrease in CD69 antigen density within the total population (figure 4B and 4C).

Despite the strong correlation between CD69 expression and caspase activity, the two events are dissociated temporally. Specifically, CD69 expression achieves maximal levels 12 hours after SEB (figure 4C) and in the reference (Vasseur *et al.*, 1999), whereas maximal caspase activity is not seen until 18–24 hours. Moreover, caspase activity is unaffected by Hg 12 hours after SEB, whereas CD69 expression levels in the CD4+ T-cells are significantly depressed by Hg at the same time (figure 4B; $p < 0.01$).

Caspase 8 and 3 activation following SEB challenge is CD95 dependent

Pro-caspase 8 cleavage and activation is largely dependent on the clustering of the Fas receptors on T-cells by FasL expressed on APCs and other T-cells. Pro-caspase 8 exists in the cytoplasm as a zymogen awaiting CD95 clustering, bringing the Fas associated death domain (FADD) in proximity to procaspase 8 resulting in its cleavage and release of the active caspase 8 cysteine protease. Recent work has proposed caspase 8 may be activated independently of CD95, in response to TCR activation (Misra *et al.*, 2005). To test this possibility, we analyzed caspase 8 and caspase 3 activity in the *lpr* mouse model, where CD95 signaling is impaired due to an insertion into the second intron of CD95, causing altered splicing and premature termination of the transmembrane receptor (Adachi *et al.*, 1993).

CD95 mutant MRL/MpJ-*Fas^{lpr}* or wild type control MRL/MpJ mice, were injected with 20 μ g SEB in PBS or PBS alone and T-cell and caspase activation analyzed 18, 24, or 48 hours later. As shown in figure 5, more than 60% of V β 8+ T-cells possess activated caspase 8 and 3 in response to SEB in the control MRL/MpJ mice. However, in MRL/MpJ-*Fas^{lpr}* mice, only about 25% of the CD4+V β 8+ T-cells show detectable caspase activation 18 hours after exposure. Additionally, the amount of caspase 8 and 3 activity is significantly reduced in the CD95 mutant mice at all three time points in lymph node and splenic CD4+ and CD8+ T-cells (figure 6; $p < 0.01$). These data are consistent with the hypothesis that CD95 is required for caspase cleavage immediately after T-cell activation by SEB.

In addition to investigating the role of CD95 in caspase 8 and 3 activation, we also examined the potential contribution of CD95 to T-cell activation by comparing CD69 expression in SEB-exposed MRL/MpJ or MRL/MpJ-*Fas^{lpr}* mice. As shown in figure 7, 18 hours after SEB challenge, 59% of CD4+V β 8+ T-cells from the MRL/MpJ mice are CD69+caspase 8+. In comparison to the wild type, only 29% of CD4+V β 8+ T-cells from the MRL/MpJ-*Fas^{lpr}*

express the early activation marker along with caspase 8 activity. In addition to the deficiency of activated and pro-apoptotic T-cells in the MRL/MpJ-*Fas^{lpr}* mice, MFI of CD69 within the V β 8 T-cell subset is significantly reduced in the CD95 defective animals by nearly 65%, 18 hours after SEB challenge (Table II). The decrease in MFI in MRL/MpJ-*Fas^{lpr}* mice is most likely accounted for by the large cohort of T-cells which fail to induce CD69 in response to SEB (Figure 7). Nevertheless, CD69 expression levels were considerably higher in SEB exposed MRL/MpJ-*Fas^{lpr}* compared to the PBS control group. These data suggest CD95 is required for promoting the activated pro-apoptotic state of V β 8 T-cells in response to SEB.

While the SEB induced caspase activation is impaired in mice expressing a defective CD95 receptor, total caspase activity is elevated in all T-cell subsets from mice expressing the MRL/MpJ-*Fas^{lpr}* mutation. (figures 5 and 7). For the experiments involving SEB-challenged BALB/cJ mice, caspase 8 and 3 activation was measured by comparing the fluorescence of a caspase 8 or 3 substrate between the V β 8 and V β 6 T-cell populations in each experimental animal. However, in the CD95 defective mice, where background levels of caspase activity are higher in all T-cells, (as measured in PBS controls, figure 7 and data not shown), the cumulative effect of SEB on caspase activation might appear diluted in the MRL/MpJ-*Fas^{lpr}* mice. To test this possibility, we compared caspase 8 activation in V β 8 T-cells using the Overton comparison, between PBS-treated and SEB-treated MRL/MpJ or MRL/MpJ-*Fas^{lpr}* mice directly. For this comparison, we still found that CD95 was required for maximal caspase 8 activation with more than 60% of MRL/MpJ V β 8 T-cells possessing activated caspase 8 compared with approximately 40% of V β 8 T-cells from MRL/MpJ-*Fas^{lpr}* mice (figure 7B). It is still possible that the elevated caspase activation observed in the CD95 defective model is confounding the comparison. Consequently, we compared caspase 8 activation between V β 8 T-cells from MRL/MpJ mice and V β 8 T-cells from MRL/MpJ-*Fas^{lpr}* mice stimulated with SEB. As shown in figure 7, the fluorescence from the caspase 8 substrate is higher in V β 8 T-cells from the MRL/MpJ control mice than in the MRL/MpJ-*Fas^{lpr}* V β 8 T-cells. These data show that despite elevated baseline caspase activity in the MRL/MpJ-*Fas^{lpr}* mice, maximal induction of caspase activity in response to SEB requires expression of a functional CD95 receptor.

DISCUSSION

Evidence of HgIA exists in humans and rodent models, although the precise mechanism(s) of induction or exacerbation of disease are poorly understood. Nevertheless, both MHC and non-MHC loci have been implicated as autoimmunity susceptibility factors in the pathogenesis of HgIA (Bagenstose *et al.*, 1999; Lawrence and McCabe, 2002). Research from our laboratory demonstrating that Hg attenuates CD95 dependent apoptosis suggests an additional mechanism by which Hg could contribute to an autoimmune disease state. Specifically, it was demonstrated that HgCl₂ treatment attenuated apoptosis in human T-cells stimulated with an agonistic anti-Fas antibody (Whitekus *et al.*, 1999). Moreover, it was demonstrated that this attenuation of apoptosis by HgCl₂ occurred by disruption of the DISC and dissociation of preassembled CD95 oligomers (McCabe *et al.*, 2003; McCabe *et al.*, 2005; Ziemba *et al.*, 2005). CD95, a member of the TNF superfamily of cell surface receptors, exists in the plasma membrane as either individual monomers, or as preassembled trimers and multimers. Ligation with CD95L expressed on other cells is thought to mediate a conformational change in the CD95 multimer resulting in the recruitment and/or activation of the Fas associated death domain (FADD) protein and procaspase 8 on the cytoplasmic side of the plasma membrane (Bidere *et al.*, 2006). Activated CD95, FADD and procaspase 8 comprise the multiprotein complex commonly referred to as the DISC, thus, Hg appears to modulate one of the earliest stages of CD95 dependent signaling.

We have extended our previous study by demonstrating that Hg-intoxication modulates CD95 signaling in activated T-cells *in vivo*. Furthermore, the characteristic increase in CD95 levels

in response to SEB occurs rapidly (≤ 12 hours) and equivalently, in Hg-exposed and non-exposed mice (data not shown), indicating the modulatory effect of Hg must be on the signaling capability of CD95 and the DISC. While evidence exists that caspase 8 may be activated in T-cells subsequent to TCR stimulation (Kennedy *et al.*, 1999; Misra *et al.*, 2005), our data in the MRL/MpJ-*Fas^{lpr}* model demonstrate a requirement of CD95 for maximal activation of caspase 8 in response to SEB challenge. Moreover, in cases of TCR dependent activation, caspase 8 remains as a proenzyme targeting the I κ B kinase (IKK) complex for degradation with little to no activation of caspase 3 (Su *et al.*, 2005). Our data showing activation of both caspase 8 and caspase 3 following SEB exposure is therefore consistent with ligation of CD95 and DISC formation.

Given the inhibitory effects of Hg on caspase activation and death receptor signaling *in vivo*, one might have predicted that V β 8 cell numbers would have been elevated in response to SEB compared with control mice. Surprisingly, this was not the case, and in fact, V β 8 cell numbers were slightly decreased in the Hg exposed mice. Nevertheless, these data are consistent with previously published work demonstrating that the percentage of V β 8 T-cells is actually reduced in response to SEB in mice possessing genetic mutations in death receptor complex proteins (Hildeman *et al.*, 2002). Moreover, we observed approximately 30% fewer V β 8 T-cells in MRL/MpJ-*Fas^{lpr}* mice 48 hours after SEB exposure compared to the WT controls (data not shown). Taken together, these data suggest that inhibition of death receptor signaling does not necessarily result in more cells following activation. Indeed, it appears the opposite effect may be the case, at least in response to a single antigenic challenge.

Our results also find reduced MFI of CD69 in both Hg – treated mice and CD95 deficient MRL/MpJ-*Fas^{lpr}* mice after SEB exposure. In both cases, the decrease of CD69 MFI is attributable to an increase in a population of T-lymphocytes which fail to induce CD69 in response to SEB. Taken together, these data could be explained in the context of recent studies implicating CD95L as a costimulatory molecule during T-cell activation (Sun *et al.*, 2006). Alternatively, Hg may have a direct effect on T-cell activation independent of CD95. Indeed, we have previously shown that Hg can modulate TCR dependent signal transduction (Ziemba *et al.*, 2006), suggesting a mechanism where TCR activation in response to an antigen such as SEB could be affected by Hg.

Our findings that Hg modulates T-cell activation, death receptor signaling and caspase activation *in vivo*, provide additional insight as to the mechanisms of HgIA. Numerous studies implicate CD95 as a critical regulator of immune system homeostasis and autoimmunity both in mouse and humans (Siegel *et al.*, 2003; Bidere *et al.*, 2006). Indeed there are several important parallels between the biochemical abnormalities seen in autoimmune lymphoproliferative syndrome (ALPS) patients and the alterations we observe both with Hg-exposed and MRL/MpJ-*Fas^{lpr}* mice. For instance, in ALPS patients, a significant reduction in FADD and caspase 8 recruitment to the DISC after Fas crosslinking is often observed (Martin *et al.*, 1999; Siegel *et al.*, 2003). Additionally, individuals who carry the identical CD95 gene mutation as the ALPS patients have been identified, however, these individuals do not present with an autoimmune condition (Bleesing *et al.*, 2001). Interestingly, the defect in CD95 dependent apoptosis at the cellular level is fully penetrant in these healthy individuals, suggesting other factors outside of the CD95 loci are required for developing disease (Bleesing *et al.*, 2001). If disruption of peripheral tolerance requires multiple factors in order to establish and/or sustain an autoimmune disease, then understanding how environmental agents may interact with genetic factors to disrupt immune system homeostasis is critical.

Based on our previously published findings that Hg attenuates CD95 dependent signaling in T-cells, we wanted to test the hypothesis that Hg exposure *in vivo*, modulates death receptor mediated signaling in activated T-cells. In the current study, we chose to use SEB as a model

of death receptor mediated signaling because a large population of T-cells are directly activated during the initiation phase of the immune response with proapoptotic signaling known to occur concomitantly to the T-cell expansion phase. Moreover, because SEB is rapidly cleared within 24 hours of exposure (Vabulas *et al.*, 1996), antigen persistence is not the issue it would be in an autoimmune state, thus avoiding potential confounding factors caused by inflammation and/or signals from other cells. Given the large population of activated T-cells with a proapoptotic phenotype in SEB exposed mice, this model was judged to be a good initial system to test whether Hg exposure would modulate death receptor signaling *in vivo*. Consequently, our findings of deregulated caspase 8 and caspase 3 activation in SEB responsive T-cells by low dose of Hg – exposure are consistent with our hypothesis that death receptor mediated signals are attenuated by Hg *in vivo*. Considering that regulation of cell death is critical for maintaining peripheral tolerance and immune system homeostasis, these data have profound implications in the understanding of autoimmune disease. However, because SEB activation of T-cells occurs in the absence of antigen processing, co-receptor engagement and without costimulatory signals, extrapolation to other infection and AICD models should be made with caution at this time. Nevertheless, the finding that Hg can modulate death receptor signaling in a system where the antigen is only present for at most 24 hours, has profound implications in an autoimmune condition where the activating antigen persists. In an individual with a genetic predisposition to developing autoimmune disease, and where the persistence of antigen is a factor, any compound such as Hg, which modulates cell death processes in T-cells, could be the difference between maintenance of peripheral tolerance and systemic autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Dr. B. Paige Lawrence for critical reading of this manuscript; the University of Rochester vivarium staff for advice and technical assistance with animal studies; and Dr. Sally Quataert and Matthew Cochran of the Human Immunology Center at the University of Rochester for advice and technical assistance with flow cytometry.

This work was supported by National Institutes of Health Grant RO1-ES12403 (to MJM), RO1-ES11000 (to AJR), Center grant ES01247 (University of Rochester), and Training grant T32-ES07026 (University of Rochester).

References

- Adachi M, Suematsu S, Kondo T, Ogasawara J, Tanaka T, Yoshida N, Nagata S. Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat Genet* 1995;11:294–300. [PubMed: 7581453]
- Adachi M, Suematsu S, Suda T, Watanabe D, Fukuyama H, Ogasawara J, Tanaka T, Yoshida N, Nagata S. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc Natl Acad Sci U S A* 1996;93:2131–2136. [PubMed: 8700897]
- Adachi M, Watanabe-Fukunaga R, Nagata S. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* 1993;90:1756–1760. [PubMed: 7680478]
- Altman A. Abnormal antigen receptor-initiated signal transduction in lpr T lymphocytes. *Semin Immunol* 1994;6:9–17. [PubMed: 7513195]
- Bagenstose LM, Salgame P, Monestier M. Murine mercury-induced autoimmunity: a model of chemically related autoimmunity in humans. *Immunol Res* 1999;20:67–78. [PubMed: 10467984]
- Bidere N, Su HC, Lenardo MJ. Genetic disorders of programmed cell death in the immune system. *Annu Rev Immunol* 2006;24:321–352. [PubMed: 16551252]
- Bleesing JJ, Brown MR, Straus SE, Dale JK, Siegel RM, Johnson M, Lenardo MJ, Puck JM, Fleisher TA. Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. *Blood* 2001;98:2466–2473. [PubMed: 11588044]

- Clarkson TW, Magos L. The toxicology of mercury and its chemical compounds. *Crit Rev Toxicol* 2006;36:609–662. [PubMed: 16973445]
- Fontenot AP, Newman LS, Kotzin BL. Chronic beryllium disease: T cell recognition of a metal presented by HLA-DP. *Clin Immunol* 2001;100:4–14. [PubMed: 11414740]
- Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR. Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* 2006;7:681–685. [PubMed: 16785881]
- Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, Kappler J, Marrack PC. Reactive oxygen species regulate activation-induced T cell apoptosis. *Immunity* 1999;10:735–744. [PubMed: 10403648]
- Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J, Marrack P. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 2002;16:759–767. [PubMed: 12121658]
- Hultman P, Johansson U, Dagnaes-Hansen F. Murine mercury-induced autoimmunity: the role of T-helper cells. *J Autoimmun* 1995;8:809–823. [PubMed: 8824708]
- Hultman P, Turley SJ, Enestrom S, Lindh U, Pollard KM. Murine genotype influences the specificity, magnitude and persistence of murine mercury-induced autoimmunity. *J Autoimmun* 1996;9:139–149. [PubMed: 8738957]
- Kawabe Y, Ochi A. Selective anergy of V beta 8+, CD4+ T cells in Staphylococcus enterotoxin B-primed mice. *J Exp Med* 1990;172:1065–1070. [PubMed: 1976734]
- Kennedy NJ, Kataoka T, Tschopp J, Budd RC. Caspase activation is required for T cell proliferation. *J Exp Med* 1999;190:1891–1896. [PubMed: 10601363]
- Komoriya A, Packard BZ, Brown MJ, Wu ML, Henkart PA. Assessment of caspase activities in intact apoptotic thymocytes using cell-permeable fluorogenic caspase substrates. *J Exp Med* 2000;191:1819–1828. [PubMed: 10839799]
- Krueger A, Fas SC, Baumann S, Krammer PH. The role of CD95 in the regulation of peripheral T-cell apoptosis. *Immunol Rev* 2003;193:58–69. [PubMed: 12752671]
- Lawrence DA, McCabe MJ Jr. Immunomodulation by metals. *Int Immunopharmacol* 2002;2:293–302. [PubMed: 11811932]
- Marrack P, Blackman M, Kushnir E, Kappler J. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J Exp Med* 1990;171:455–464. [PubMed: 2303780]
- Martin DA, Zheng L, Siegel RM, Huang B, Fisher GH, Wang J, Jackson CE, Puck JM, Dale J, Straus SE, Peter ME, Krammer PH, Fesik S, Lenardo MJ. Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia. *Proc Natl Acad Sci U S A* 1999;96:4552–4557. [PubMed: 10200300]
- McCabe MJ Jr, Eckles KG, Langdon M, Clarkson TW, Whitekus MJ, Rosenspire AJ. Attenuation of CD95-induced apoptosis by inorganic mercury: caspase-3 is not a direct target of low levels of Hg2+ *Toxicol Lett* 2005;155:161–170. [PubMed: 15585371]
- McCabe MJ Jr, Whitekus MJ, Hyun J, Eckles KG, McCollum G, Rosenspire AJ. Inorganic mercury attenuates CD95-mediated apoptosis by interfering with formation of the death inducing signaling complex. *Toxicol Appl Pharmacol* 2003;190:146–156. [PubMed: 12878044]
- Misra RS, Jelley-Gibbs DM, Russell JQ, Huston G, Swain SL, Budd RC. Effector CD4+ T cells generate intermediate caspase activity and cleavage of caspase-8 substrates. *J Immunol* 2005;174:3999–4009. [PubMed: 15778357]
- Monestier M, Losman MJ, Novick KE, Aris JP. Molecular analysis of mercury-induced antinucleolar antibodies in H-2S mice. *J Immunol* 1994;152:667–675. [PubMed: 8283044]
- Muraille E, De Smedt T, Andris F, Pajak B, Armant M, Urbain J, Moser M, Leo O. Staphylococcal enterotoxin B induces an early and transient state of immunosuppression characterized by V beta-unrestricted T cell unresponsiveness and defective antigen-presenting cell functions. *J Immunol* 1997;158:2638–2647. [PubMed: 9058796]
- Overton WR. Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry* 1988;9:619–626. [PubMed: 3061754]
- Pollard KM, Lee DK, Casiano CA, Bluthner M, Johnston MM, Tan EM. The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. *J Immunol* 1997;158:3521–3528. [PubMed: 9120314]

- Pollard KM, Pearson DL, Bluthner M, Tan EM. Proteolytic cleavage of a self-antigen following xenobiotic-induced cell death produces a fragment with novel immunogenic properties. *J Immunol* 2000;165:2263–2270. [PubMed: 10925315]
- Potolicchio I, Mosconi G, Forni A, Nemery B, Seghizzi P, Sorrentino R. Susceptibility to hard metal lung disease is strongly associated with the presence of glutamate 69 in HLA-DP beta chain. *Eur J Immunol* 1997;27:2741–2743. [PubMed: 9368635]
- Rieux-Laucat F, Fischer A, Deist FL. Cell-death signaling and human disease. *Curr Opin Immunol* 2003;15:325–331. [PubMed: 12787759]
- Siegel RM, Muppidi J, Roberts M, Porter M, Wu Z. Death receptor signaling and autoimmunity. *Immunol Res* 2003;27:499–512. [PubMed: 12857993]
- Sneller MC, Dale JK, Straus SE. Autoimmune lymphoproliferative syndrome. *Curr Opin Rheumatol* 2003;15:417–421. [PubMed: 12819469]
- Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M. Requirement for caspase-8 in NF-kappaB activation by antigen receptor. *Science* 2005;307:1465–1468. [PubMed: 15746428]
- Sun M, Ames KT, Suzuki I, Fink PJ. The cytoplasmic domain of Fas ligand costimulates TCR signals. *J Immunol* 2006;177:1481–1491. [PubMed: 16849454]
- Takeuchi K, Turley SJ, Tan EM, Pollard KM. Analysis of the autoantibody response to fibrillarin in human disease and murine models of autoimmunity. *J Immunol* 1995;154:961–971. [PubMed: 7529293]
- te Boekhorst PA, Barrera P, Laan RF, van de Putte LB. Hepatotoxicity of parenteral gold therapy in rheumatoid arthritis: a case report and review of the literature. *Clin Exp Rheumatol* 1999;17:359–362. [PubMed: 10410273]
- Vabulas R, Bittlingmaier R, Heeg K, Wagner H, Miethke T. Rapid clearance of the bacterial superantigen staphylococcal enterotoxin B in vivo. *Infect Immun* 1996;64:4567–4573. [PubMed: 8890208]
- Vasseur F, Le Campion A, Pavlovitch JH, Penit C. Distribution of cycling T lymphocytes in blood and lymphoid organs during immune responses. *J Immunol* 1999;162:5164–5172. [PubMed: 10227988]
- Verwilghen J, Kingsley GH, Gambling L, Panayi GS. Activation of gold-reactive T lymphocytes in rheumatoid arthritis patients treated with gold. *Arthritis Rheum* 1992;35:1413–1418. [PubMed: 1472120]
- Warfvinge K, Hansson H, Hultman P. Systemic autoimmunity due to mercury vapor exposure in genetically susceptible mice: dose-response studies. *Toxicol Appl Pharmacol* 1995;132:299–309. [PubMed: 7785057]
- Whitekus MJ, Santini RP, Rosenspire AJ, McCabe MJ Jr. Protection against CD95-mediated apoptosis by inorganic mercury in Jurkat T cells. *J Immunol* 1999;162:7162–7170. [PubMed: 10358162]
- Yang Y, Kim D, Fathman CG. Regulation of programmed cell death following T cell activation in vivo. *Int Immunol* 1998;10:175–183. [PubMed: 9533445]
- Ziemba SE, Mattingly RR, McCabe MJ Jr, Rosenspire AJ. Inorganic mercury inhibits the activation of LAT in T-cell receptor-mediated signal transduction. *Toxicol Sci* 2006;89:145–153. [PubMed: 16251484]
- Ziemba SE, McCabe MJ Jr, Rosenspire AJ. Inorganic mercury dissociates preassembled Fas/CD95 receptor oligomers in T lymphocytes. *Toxicol Appl Pharmacol* 2005;206:334–342. [PubMed: 16039944]

Abbreviations used in this paper

Hg	mercury
HgIA	mercury induced autoimmunity
DISC	death inducing signaling complex

SEB	Staphylococcal aureus enterotoxin B
AICD	activation induced cell death
MFI	mean fluorescence intensity
ALPS	autoimmune lymphoproliferative syndrome

Figure 1.

Time course of V β 8 T-cell expansion and deletion in response to SEB stimulation. BALB/cJ mice were first exposed to 10 mg/L HgCl₂ in drinking water (filled in shapes) or pure drinking water alone (open shapes) for a period of two weeks. Subsequently, mice were injected iv with 20 μ g SEB in PBS by tail vein injection, and mice were euthanized at various time points after injection. The spleen and lymph nodes were removed, cell suspensions made and surface phenotype assessed by flow cytometry. The absolute cell numbers for T-cells bearing the V β 8 T-cell antigen receptor (circles), or V β 6 (triangles) are shown for both CD4 and CD8 populations in the lymph node and spleen. Data are expressed as the mean $\pm$ SEM pooled from three independent experiments with 5 mice per group in each experiment.

Figure 2.

SEB selectively induces activation of caspase 8 and caspase 3 in Vβ8+ T-cells. Splenocytes from PBS or SEB challenged BALB/cJ mice and PBS or SEB challenged mice exposed to 10 mg/L HgCl₂ were stained for CD4, CD8, Vβ8 TCR, Vβ6 TCR and incubated with a fluorogenic caspase 8 or caspase 3 substrate detectable by flow cytometry. Representative splenocyte samples are shown to demonstrate how data were analyzed and that there was no significant effect on T-cell distribution following exposure to either SEB, HgCl₂ or both. Cells were first gated based on surface expression of either CD4 or CD8, followed by Vβ8 versus Vβ6 within each gate (CD4+ T-cells are shown in the second column). The percentage of cells with activated caspase 8 or caspase 3 is determined by calculating the maximal difference in fluorescence between Vβ8 T-cells and Vβ6 T-cells, using the Overton algorithm in the FlowJo analysis software. Shaded histograms represent fluorescence within CD4+Vβ6+ cells compared with solid lines indicating fluorescence of CD4+Vβ8+ T-cells. The number above the cursor represents the percentage of cells expressing caspase enzyme activity for each representative sample.

A. Caspase 8

B. Caspase 3

Figure 3.

Hg intoxication attenuates activation of caspase 8 and 3 enzymes. The percentage of cells with active Caspase 8 (figure 3A) and caspase 3 (figure 3B) was monitored 12-48 hours after SEB exposure in CD4 and CD8 T-cells in the lymph node (top panels) and spleen (bottom panels). The percentage of cells with activated caspase enzymes were determined by flow cytometry as described in figure 2. Data are presented as the mean $\pm$ SEM and are representative of three independent experiments with 5 mice per group. An * denotes statistical significance between control (open circles) and Hg intoxicated mice (dark circles) with $p \leq 0.01$. Both caspase 8 and caspase 3 activity was always less than 5% in PBS challenged mice at all time points and thus are not shown on these graphs.

Figure 4.

Hg intoxication attenuates SEB – induced T-cell activation. Eighteen hours after SEB exposure, splenocytes were removed, gated on CD4+Vβ8+ or CD8+Vβ8+ T-cells, and CD69 expression versus caspase 8 or caspase 3 activity was determined. In panel 4A, CD69+ caspase 8+ or caspase 3+ cells were considered activated T-cells with a pro-apoptotic phenotype and gates were established based on CD69 and caspase fluorescence in PBS challenged mice. The percentage of CD69+ caspase+ cells was used to calculate the absolute splenic number of these cells shown in panel B and C. In B, absolute cell number ($\times 10^6$) for CD4+ caspase 8 or CD4 + caspase 3 are shown with the mean $\pm$ SEM for 5 mice per group. Within CD4+Vβ8+ subset, CD69 expression was measured. The dark line represents SEB – exposed control mice, and

the shaded histogram represents the SEB – exposed Hg group. MFI of CD69 in each group over time is shown next to the CD69 histogram with open circles representing controls and filled in circles representing Hg – exposed mice. In C, data for the CD8+ splenic T-cells is shown. An * denotes statistical significance with $p \leq 0.01$.

Figure 5.

A genetic defect in CD95 attenuates SEB – induced activation of caspase 8 and caspase 3. MRL/MpJ or MRL/MpJ-Fas^{lpr} mice were injected with PBS or SEB and lymph nodes and spleens removed 18 hours later. CD4 versus CD8 profiles for splenocytes are shown 18 hours after SEB. Within the CD4 T-cell population the distribution of T-cells bearing either Vβ8+ or Vβ6+ T-cells is shown. Cells were first gated based on surface expression of either CD4 or CD8, followed by Vβ8 versus Vβ6 within each gate (CD4+ T-cells are shown in the second column). The percentage of cells with activated caspase 8 or caspase 3 is determined by calculating the maximal difference in fluorescence between Vβ8 T-cells and Vβ6 T-cells, using the Overton algorithm in the FlowJo analysis software. Shaded histograms represent fluorescence within CD4+Vβ6+ cells compared with solid lines indicating fluorescence of CD4+Vβ8 T-cells. The number above the cursor represents the percentage of cells expressing caspase enzyme activity for each representative sample.

A. Caspase 8

B. Caspase 3

Figure 6.

CD95 deficient mice fail to activate caspase 8 and caspase 3 enzyme activity upon SEB challenge. MRL/MpJ or MRL/MpJ-*Fas^{lpr}* mice were injected with PBS or SEB and lymph nodes and spleens removed 18, 24, or 48 hours later and caspase activity determined as shown in figure 5. In A, Caspase 8 activity is shown for CD4 and CD8 lymphocytes and splenocytes. Open triangles identify data from the MRL/MpJ and filled triangles identify the MRL/MpJ-*Fas^{lpr}*. In B, caspase 3 activity is shown. Data are presented as the mean $\pm$ SEM with 3 mice per group. An * indicates data are statistically significant with $p \leq 0.01$.

Figure 7.

CD95 is required for promoting the activated pro-apoptotic state of Vβ8 T-cells in response to SEB. A. Representative flow cytometry plots show CD69 versus caspase 8 (left four panels) or caspase 3 (right four panels) in CD4⁺ MRL/MpJ or MRL/MpJ-*Fas^{lpr}* splenocytes in response to PBS (top panels) or SEB (bottom panels) 18 hours later. B. Higher background levels of caspase activity in CD4⁺ T-cells do not account for decreased caspase activity observed in MRL/MpJ-*Fas^{lpr}*. In the top histogram, the percentage of cells with activated caspase 8 was measured by comparing CD4⁺Vβ8⁺ T-cells from PBS (shaded histogram) and SEB treated MRL/MpJ-*Fas^{lpr}* mice (solid line). In the bottom histogram, the percentage of cells with activated caspase 8 was compared in CD4⁺Vβ8⁺ T-cells from MRL/MpJ mice (solid line) and MRL/MpJ-*Fas^{lpr}* mice exposed to SEB (shaded histogram).

Table 1 Tissue concentration of Hg after two week exposure to 10 mg/L HgCl₂ in drinking water.^a

Tissue (units)	n per group	Hg-Exposed	Control
Kidney (µg/g)	25–30	22.34 ± 0.61, ^{bc}	0.03 ± 0.002
Spleen (µg/g)	4	0.44 ± 0.01 ^d	N.D. ^e
Splenocytes (ng/10 ⁷ cells)	10	1.0 ± 0.01, ^{cf}	0.6 ± 0.07

^a Mice were exposed to 10 mg/L HgCl₂ in Milli-Q purified and autoclaved water or Milli-Q, autoclaved water alone for a period of two weeks.^b Data are presented as the mean ± SEM.^c Statistically different with p ≤ 0.01.^d Data are presented as the mean ± SEM.^e Not detectable (i.e. <0.00075 µg/aliquot).^f 1 × 10⁷ RBC depleted splenocytes were pelleted and homogenized, followed by Hg analysis by cold vapor atomic absorption. Data are presented as the mean ± SEM.

Table 2MFI of CD69 is reduced in CD95 deficient SEB-exposed mice^a.

	MRL/MpJ Vβ8	MRL/MpJ-<i>Fas</i>^{lpr} Vβ8	MRL/MpJ Vβ6	MRL/MpJ-<i>Fas</i>^{lpr} Vβ6
18 hr PBS	1237 ± 1316 ^b	2327 ± 1316	703 ± 167	1512 ± 167 ^c
18 hr SEB	20874 ± 760	7423 ± 760 ^d	1806 ± 96	1711 ± 96
24 hr SEB	9683 ± 760	4613 ± 760 ^d	1408 ± 96	1696 ± 96

^aThe MFI of CD69 was measured on the BD LSR II flow cytometer in CD4+ Vβ8+ or CD4+ Vβ6+ splenocytes from MRL/MpJ or MRL/MpJ-*Fas*^{lpr} injected with either 20μg SEB in PBS or PBS alone and euthanized at the indicated times.

^bThe data are presented as the MFI of total CD69 fluorescence within the CD4+ Vβ8+ or CD4+ Vβ6+ population ± SEM with 3 mice per group.

^cStatistically different from the wild type Vβ6+ population with p≤0.01.

^dStatistically different from the wild type Vβ8+ population with p≤0.01.