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β_2 -Microglobulin is Required for the Full Expression of Xenobiotic-Induced Systemic Autoimmunity

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Abstract

Mercury exposure in both humans and mice is associated with features of systemic autoimmunity. Murine HgCl₂-induced autoimmunity (mHgIA) requires MHC Class II, CD4⁺ T-cells, co-stimulatory molecules, and interferon- γ (IFN γ), similar to spontaneous models of systemic lupus erythematosus (SLE). β_2 -Microglobulin (*B2m*) is required for functional MHC Class I molecules and the neonatal Fc receptor (*FcRn*). Deficiency of *B2m* in lupus-prone strains is consistently associated with reduced IgG levels, but with variable effects on other manifestations. Here, we examined the role of *B2m* in mHgIA and show that, in the absence of β_2 -microglobulin, mercury-exposed mice failed to exhibit hypergammaglobulinemia, had reduced anti-nucleolar autoantibodies (ANoA), and had a lower incidence of immune complex deposits in splenic blood vessels, while, IgG anti-chromatin autoantibodies and renal immune deposits were largely unaffected. Subclass analysis of the IgG anti-chromatin, however, revealed a significant reduction in the IgG₁ subtype. Examination of IFN γ , IL-4, and IL-2 in exposed skin, draining lymph nodes, and spleen following mercury-exposure showed reduced IL-4 in the spleen and skin in *B2m*-deficient mice, consistent with the lower IgG₁ anti-chromatin levels, and reduced IFN γ expression in the skin. These findings demonstrate how a single genetic alteration can partially but significantly modify the clinical manifestations of systemic autoimmunity induced by exposure to xenobiotics.

Keywords

autoantibodies; autoimmunity; cytokines; immune deposits; mercury; β_2 -microglobulin; mouse; xenobiotic

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Declaration of Interest

The authors report no conflicts of interest. The authors are alone responsible for the content and writing of the paper.

Introduction

Exposure to mercury has been identified as an environmental trigger in the induction of autoimmunity in humans (Abreu Velez et al., 2003; Cooper et al., 2004; Silva et al., 2004) including production of autoantibodies, pro-inflammatory cytokines (Gardner et al., 2010), and membranous nephropathy (Li et al., 2010). Animal model studies have contributed significantly to our understanding of the systemic autoimmunity induced by this environmental agent (Vas and Monestier, 2008; Schiraldi and Monestier, 2009; Pollard et al., 2010). The features of murine mercury-induced autoimmunity (mHgIA) include lymphadenopathy, hypergammaglobulinemia, humoral autoimmunity, and immune-complex disease (Pollard and Hultman, 1997; Vas and Monestier, 2008; Schiraldi and Monestier, 2009; Pollard et al., 2010). HgIA is dependent on functional major histocompatibility (MHC) Class II, CD4⁺ T-helper cells (Hultman et al., 1995) and co-stimulatory molecules (Pollard et al., 2004). MHgIA is also dependent upon interferon (IFN)- γ (Kono et al., 1998) that is predominantly secreted by CD4⁺ and CD8⁺ T-cells and natural killer (NK) cells (Boehm et al., 1997). NK cell function is reduced following mercury exposure (Santarelli et al., 2006), but NKT cell activation exacerbates mHgIA (Vas et al., 2008) and CD8⁺ T cells proliferate in response to HgCl₂ (Pollard and Landberg, 2001; Jiang and Moller, 1995). However it is unclear if absence of MHC Class I restricted cells contributes to development of disease.

β_2 -microglobulin (*B2m*) is critical for cell surface expression of MHC Class I molecules (Zijlstra et al., 1989) and deficiency leads to a loss of several cell types that require Class I for development, including CD8⁺ T-cells and NK1.1⁺ T-cells (Zijlstra et al., 1990; Bendelac et al., 1994). *B2m* is also critical for neonatal Fc receptor (FcRn) function (Israel et al., 1995). FcRn facilitates the transfer of maternal IgG from mother to fetus in humans and from gut to blood in suckling rodents, and, in adults, protects IgG from degradation (Ahouse et al., 1993; Israel et al., 1995; Roopenian and Akilesh, 2007). Absence of FcRn in *B2m*-deficient mice results in increased catabolism of IgG (Israel et al., 1996) and consequently reduced half-life and serum levels of IgG (Ghetie et al., 1996; Christianson et al., 1997). It has been argued that FcRn inhibitors could be used to reduce autoantibody levels in autoimmune disease (Low and Mezo, 2009).

β_2 -Microglobulin deficiency prevents hypergammaglobulinemia in SLE (systemic lupus erythematosus)-prone mice, and reduces specific antibody responses to T-dependent and T-independent antigens (Christianson et al., 1997). Hypogammaglobulinemia was argued to be the result of the reduced half-life of IgG in *B2m*-deficient mice, rather than reduction in B-cells, CD8⁺ T-cells, or NK1.1⁺ cells (Christianson et al., 1997). A β_2 -Microglobulin deficiency, however, has produced variable effects on murine systemic autoimmunity. For example, NZB.*B2m*^{-/-} mice showed no change in levels of anti-DNA autoantibodies, but the incidence of anti-erythrocyte autoantibodies was reduced and their onset delayed (Chen et al., 1997). In one study in MRL/*Fas*^{lpr} mice, this deficiency reduced all measured indicators of systemic autoimmunity (including hypergammaglobulinemia, autoantibodies and glomerulonephritis; Christianson et al., 1996), while another study showed no effect of *B2m* deficiency on autoantibody production (Ohteki et al., 1995). A lack of β_2 -microglobulin in C57BL/6 mice carrying the *lpr* mutation decreased (Maldonado et al., 1995), increased (Giese and Davidson, 1995), or did not change (Mixer et al., 1995) autoantibody levels. The only consistent effect of a β_2 -microglobulin deficiency in mice carrying the *lpr* mutation was a profound reduction in B220⁺CD4⁻CD8⁻ T-cells (B220⁺ double negative [DN] T-cells), consistent with the derivation of most DN T-cells from the CD8⁺ T-cell subset (Koh et al., 1995). MHC Class I expression is important for their accumulation. In contrast, *B2m* deficiency exacerbated autoimmunity in NZBWF1 mice resulting in increased anti-DNA autoantibodies, more severe proteinuria, and decreased

survival (Mozes et al., 2005). Thus, *B2m* appears to play different roles in lupus-like disease depending on background genetic susceptibility.

To define the role of β_2 -microglobulin in xenobiotic induction of systemic autoimmune disease we have examined its contribution to mHgIA. In the absence of β_2 -microglobulin, mercury-exposure did not elicit the characteristic hypergammaglobulinemia and certain disease manifestations, including anti-nucleolar autoantibodies (ANoA) and vascular-immune deposits in the spleen, but not kidney immune deposits, were reduced. The IgG subclass response to chromatin and cytokine expression in skin and lymphoid tissues were also altered by *B2m* deficiency. Thus, *B2m* plays a significant disease-promoting role for some, but not all, of the autoimmune manifestations induced by mercury exposure.

Materials and Methods

Mice

C57BL/6 mice with targeted disruption of the β_2 -microglobulin gene (B6.129P2-*B2m*^{tm1Unc}) were obtained from The Jackson Laboratory (Bar Harbor, ME). Subsequent breeding and maintenance were performed under specific-pathogen-free conditions at The Scripps Research Institute Animal Facility (La Jolla, CA). All studies involving animals were reviewed and approved by The Scripps Research Institute Animal Research Committee. Animal rooms were kept at 68–72°F and 60–70% humidity, with a 12 hr/12 hr light-dark cycle, and sterilized cages were replaced each week with fresh water and food to which the mice had access *ad libitum*.

H-2^s haplotype *B2m*^{−/−} mice were generated by first back-crossing homozygous knockout mice (H-2^b haplotype) for two generations to B10.SeCD1 (H-2^s) mice. The (*B2m*^{−/−} × B10.S)F1 × B10.S N2 offspring were then typed for H-2^s and the disrupted β_2 -microglobulin gene. Mice, homozygous for H-2s and heterozygous for the β_2 -microglobulin gene, were intercrossed to create +/+, +/-, and -/- β_2 -microglobulin H-2^s mice. H-2 typing was performed by PCR of genomic DNA using the D17Mit16 primers (Research Genetics, Huntsville, AL) that are polymorphic for H-2^b and H-2^s haplotypes. β_2 -microglobulin wild-type and knockout genes were typed by PCR of genomic DNA (35 cycles of 94°C/20 sec, 60°C/45 sec, 72°C/75 sec). Three primers (M β_2 m-1, 3'-GAAAACCCCTCAAATTCAAGTATACTCA-5'; M β_2 m-2, 5'-GACGGTCTTGGGCTCGGCCATACT-3'; neomycin, 5'-TCGAATTCGCCAATGACAAGACGCT-3') were used as previously described (Khare et al., 1996). M β_2 m-1 and M β_2 m-2 were used to detect the undisrupted gene (400 basepair fragment) and neomycin and β_2 m-1 the disrupted gene (280 basepair fragment).

Mercury Treatment

Unless noted otherwise, mice were injected subcutaneously with 40 μ g mercuric chloride (HgCl₂; Mallinckrodt Baker Inc, Phillipsburg NJ) twice per week for 2 or 4 wk in phosphate-buffered saline (pH 7.4, PBS) as previously described (Pollard et al., 2004) and euthanized (by Halothane [Halocarbon Labs, River Edge, NJ] anesthesia prior to cervical dislocation) 3 d after the final injection in the 2 or 4 wk regimens. Controls received PBS alone. Use of HgCl₂ was approved by The Scripps Research Institute Department of Environmental Health Services.

Autoantibody Determination

Anti-nuclear (ANA) and anti-nucleolar antibodies (ANoA)—ANA and ANoA were detected as previously described (Pollard et al., 2004) using HEp-2 cell slides (Bion Enterprises, Park Ridge, IL). Briefly, sera were diluted 100-fold in PBS containing 0.5%

bovine serum albumin (BSA), 0.1% bovine gammaglobulin (BGG), 0.001% gelatin, and 0.05% Tween-20 prior to assay. Goat anti-mouse IgG-FITC (Caltag Laboratories, South San Francisco, CA), diluted 100-fold in PBS containing 0.5% BGG, 0.1% BSA, and 0.05% Tween-20, was used as a detecting reagent. Intensity of fluorescence was scored from 0 – 4+. ANoA in mHgIA are MHC-restricted and directed against components of the small nucleolar ribonucleoprotein particles (snoRNP) (Yang et al., 2001). Comparative titration of IgG anti-snoRNP antibodies was achieved by quadruple dilutions of serum starting at 1/40.

Anti-chromatin antibodies—Anti-chromatin antibodies were detected using ELISA (Burlingame and Rubin, 1990). Briefly, sera were diluted 100-fold prior to assay, and chromatin bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Caltag Laboratories) diluted 2000-fold. Anti-chromatin monoclonal antibody 1D12 (Kotzin et al., 1984) was used as a positive control. Comparative titration of IgG anti-chromatin antibodies was achieved by doubling dilutions of serum starting at 1/100. The IgG subclass of anti-chromatin antibodies was determined using serum dilutions of 1/200 and a saturating (1/1000) dilution of subclass detecting reagent. HRP-Conjugated anti-IgG₁ and IgG_{2b} antibodies were purchased from Caltag Laboratories, anti-IgG_{2a}^{a+b} from Pharmingen (San Diego, CA), and anti-IgG₃ from Southern Biotechnology Associates (Birmingham, AL).

Serum Immunoglobulin Quantitation

Serum IgG₁ and IgG_{2a} levels were quantified using ELISA (Pollard et al., 1999). Briefly, ELISA plates were coated with 200 µl of 2 µg/ml goat anti-mouse kappa light chain antibody (Caltag Laboratories) diluted in PBS and incubated overnight at 4°C. The plates were then post-coated for 1 hr with 0.1% gelatin in PBS followed by three washes with PBS-0.05% Tween-20. Sera were diluted in serum diluent (Bell et al., 1992) and then added (in duplicate) to dedicated wells on the plates; the plate was then incubated with shaking for 2.5 hr, and then washed three times with PBS-0.05% Tween-20. HRP-Conjugated goat anti-mouse IgG₁ or IgG_{2a} antibodies (Caltag Laboratories) were then diluted in anti-Ig diluent and added to the wells; the plates were then incubated with shaking for 90 min. Following three washes with PBS-0.05% Tween-20 and four washes with PBS, ABTS substrate solution was added to each well and the associated optical density (OD) in each well measured at 405 nm in a Spectra Max Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Serum IgG₁ and IgG_{2a} concentrations were ultimately calculated by extrapolation from the linear portion of the standard curves generated with each plate using serial dilutions of polyclonal mouse reference sera containing predetermined levels of Ig isotypes (The Binding Site, Birmingham, UK).

Immunopathology

Sections of kidney and spleen were prepared and stained for direct immunofluorescence as previously described (Kono et al., 1998). Briefly, 4–5-µm thick cryostat sections were fixed in ethanol and incubated with serial dilutions of FITC-conjugated goat antibodies to IgG (γ chain-specific) and C3 (Southern Biotechnology Associates). The endpoint titer for immune deposits was defined as the highest dilution of antibody at which specific fluorescence could be detected. Vessel wall deposits were graded on a 0–4⁺ scale. The slides were examined without knowledge of treatment or other results.

In vivo Cytokine Responses

To measure cytokine production following exposure to *B2m*^{+/+} and *B2m*^{-/-} mice were injected subcutaneously (SC) twice per week for 2 wk with 40 µg HgCl₂ in PBS or PBS alone. Post-mortem, the spleen, superficial cervical lymph nodes (draining lymph nodes),

and a 1.2 cm diameter section of skin overlying the site of mercury injection was harvested from each mouse. Total RNA from these tissues was then isolated and purified using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). Contaminant DNA was removed using a DNase treatment (Amplification Grade DNase I, Invitrogen) at 25°C for 15 min. Final RNA content in each sample was then determined with a ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). An aliquot of the RNA (2 µg) was then denatured at 65°C for 5 min, placed on ice, and reverse-transcribed (in a total volume of 21 µl) using random hexamers, dNTPs, RNase inhibitor (RNaseOUT; Invitrogen), and 200 U of SuperScript III reverse transcriptase (Invitrogen). The resulting cDNA was diluted 10-fold in nuclease-free water (Nuclease-Free Water, Ambion Inc. Austin, TX), and stored at -80°C.

Levels of interferon (IFN)-γ, interleukin (IL)-4, and IL-2 in samples were measured by real-time PCR using primer and probe sequences as previously described (Giulietti et al., 2001). Spleen and lymph node cDNA were analyzed as previously described and levels expressed relative to cyclophilin A (Cauvi et al., 2007). Levels of cytokines in cDNA prepared from skin were analyzed using iQ SYBR-Green (Bio-Rad, Hercules, CA) and copy number related to total RNA concentration (Bustin, 2000). PCR amplifications were performed in a total volume of 20 µl containing 20 mM Tris-HCl, 50 mM KCl, 200 µM dNTPs, 0.50 U iTaq DNA polymerase, 3 mM MgCl₂, and 10 nM SYBR-Green I fluorescein. The reaction conditions were 94°C for 5 min, followed by 55 cycles of 20 sec at 94°C and 30 sec at 60°C; the melt curve used 70 cycles of 10 sec at 60 + 0.5°C. All PCR reactions were performed using an iCycler iQ (Bio-Rad).

Flow Cytometry

Flow cytometry was performed on single cell suspensions obtained from harvested spleens as previously described (Cauvi et al., 2007) using complete RPMI 1640 media (Whittaker M.A. Bioproducts, Walkersville, MD) containing 10% FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO), 10 mM HEPES (pH 7.2; Flow Laboratories, Irvine, Scotland), 1% non-essential amino acids (Irvine Scientific, Santa Ana, CA), 20 mM sodium carbonate (Fisher Scientific Company, Fair Lawn, NJ), and 20 µg/ml gentamicin sulfate (Irvine Scientific). After washing in complete RPMI 1640 and then PBS containing 0.04% horse serum (Gemini Bio-Products, Woodland, CA), cells were incubated in PBS/0.04% horse serum with Fc Block and then stained with combinations of anti-NK1.1-FITC, Thy1-PE, anti-B220-PerCP, anti-CD3-APC, anti-CD4-FITC, or anti-CD8α-PerCP for 30 min at 4°C, or appropriate isotype controls. All antibodies were from BD PharMingen (San Diego, CA). Cells were analyzed on a dual-laser FACScalibur flow cytometer (BD Biosciences, San Jose, CA). A minimum of 20,000 events was acquired for each sample; all data were analyzed using CellQuest Pro software (BD Biosciences).

Statistics

Unless otherwise noted, all data is expressed as mean ± SD. Groups were compared by two-tailed single-factor analysis of variance (ANOVA), unpaired t-test, or Mann-Whitney U test as appropriate. A p-value less than 0.05 was considered significant.

Results

Characteristics of *B2m*^{-/-} Mice

Mice used in this study were produced by mating H-2^{s/s} (B10.SeCD1) mice heterozygous for the β₂-microglobulin gene. *B2m*^{+/+}, *B2m*^{+/-}, and *B2m*^{-/-} littermates were genotyped by PCR and all offspring appeared healthy. Flow cytometric analyses of T-cells from various lymphoid organs revealed the expected absence of CD8⁺ T-cells in both spleen and lymph

nodes of $B2m^{-/-}$ mice (data not shown). A β_2 -microglobulin deficiency also resulted in a 1.8-fold reduction in serum IgG₁ ($p = 0.0043$) and 7.5-fold reduction of IgG_{2a} ($p < 0.0001$) compared to values associated with wild-type littermates (Table 1).

Features of Xenobiotic-Induced Systemic Autoimmunity in β_2 -Microglobulin-Deficient Mice

Exposures to HgCl₂ elicited hypergammaglobulinemia, ANoA and anti-chromatin auto-antibodies, and immune-complex deposits in wild-type mice ($B2m^{+/+}$) as described previously (Kono et al., 1998; Pollard et al., 2004) and in $B2m^{+/-}$ mice (Tables 1 and 2). In contrast, the HgCl₂ did not elicit a significant increase in hypergammaglobulinemia in $B2m^{-/-}$ mice and their levels of IgG₁ ($p < 0.0001$) and IgG_{2a} ($p < 0.0001$) were markedly lower than that of mercury-exposed wild-type mice or $B2m^{+/-}$ (Table 1). Strikingly, after mercury exposure, IgG₁ serum concentrations were 6-fold lower in $B2m^{-/-}$ than in wild-type mice, and 10-fold lower for IgG_{2a}. In contrast, $B2m^{-/-}$ mice had increased autoantibody responses to both ANoA and chromatin (Table 1). Indeed, all three groups of HgCl₂-exposed mice showed significant and similar increases in IgG anti-chromatin autoantibodies (Table 1). However, although all mice developed IgG ANoA following mercury exposure (Table 1), the geometric mean of IgG ANoA titers was over 4-fold greater for $B2m^{+/+}$ compared to $B2m^{-/-}$ mice (1540 vs. 340; $p < 0.025$) (Figure 1A). This reduction of ANoA in mercury-exposed $B2m^{-/-}$ mice was supported by comparison of the intensities of fluorescence for ANoA at a dilution of 1/100, which for $B2m^{-/-}$ and $B2m^{+/+}$ mice was $1.3 (\pm 1.1)$ vs. $2.8 (\pm 0.6)$, respectively ($p = 0.003$, Figure 1B).

On pathologic examination, exposure to mercury resulted in significant and similar titers of IgG and C3 deposits in the glomeruli of $B2m^{+/+}$, $B2m^{+/-}$, and $B2m^{-/-}$ mice (Table 2). Similarly, IgG and C3 deposits in kidney vessels were found in all three groups, but with only one or two animals positive in any group. In contrast, $B2m^{+/-}$ and $B2m^{-/-}$ mice had reduced deposits of IgG and C3 in splenic vessels when compared to wild-type mice (Table 2). Thus, $B2m$ -deficiency affects some features of systemic autoimmunity induced by mercury exposure.

Titers and Subclasses of Anti-Chromatin Autoantibody in β_2 -Microglobulin-Deficient Mice

The presence of elevated autoantibody levels despite the lack of hypergammaglobulinemia in mercury-exposed $B2m^{-/-}$ mice was an unexpected finding (Table 1). To confirm this, the anti-chromatin autoantibody response was further analyzed by titration (Figure 2) and subclass content (Table 3). IgG anti-chromatin titration curves for $B2m^{-/-}$ and $B2m^{+/+}$ mice were essentially identical (Figure 2), suggesting that either chromatin specific IgG autoantibody levels and/or their affinity did not differ between the two groups. Measurement of IgG subclass specific anti-chromatin autoantibodies showed that although $B2m^{-/-}$ mice had a significantly lower IgG₁ anti-chromatin autoantibody response compared to wild-type littermate controls ($p = 0.0006$), the levels of IgG_{2a}, IgG_{2b}, and IgG₃ anti-chromatin autoantibodies were not significantly different between the two groups (Table 3). Thus, $B2m$ is required for the IgG₁, but not overall anti-chromatin antibody production induced by mercury.

In Vivo Production of Cytokines in β_2 -Microglobulin-Deficient Mice

Reduction of IgG₁, but persistence of IgG_{2a}, anti-chromatin autoantibodies in $B2m^{-/-}$ mice suggested that differential expression of T_H1/T_H2 cytokines might contribute to the humoral autoimmune response. To examine this possibility, cytokine expression was determined after 2 wk of mercury exposure (when humoral responses are evident) by real-time PCR in the spleen, draining lymph nodes, and skin overlying the site of mercury injection (Figure 3).

In the spleen, mercury exposure resulted in increased IL-4 ($p = 0.012$) in wild-type mice as described previously (Kono et al., 1998), but no changes in IL-2 or IFN γ (Figure 3A). In contrast, splenic IL-2 production was elevated in saline-treated $B2m^{-/-}$ mice compared to that in both wild-type mice ($p = 0.033$) and mercury-exposed $B2m^{-/-}$ ($p < 0.038$); however, there was no difference in IL-2 expression between the mercury-exposed wild-type and $B2m^{-/-}$ mice. In $B2m^{-/-}$ mice, levels of splenic IFN γ or IL-4 were not changed by mercury exposure.

In lymph nodes, $B2m$ deficiency resulted in a significant reduction in IFN γ regardless of whether mice were exposed to mercury or PBS ($p = 0.049$). The levels of IL-2 and IL-4 were not significantly different in $B2m^{-/-}$ and wild-type mice, with or without mercury exposure (Figure 3B).

In the skin, there was a significant increase in IFN γ in wild-type mice following mercury-exposure ($p = 0.0012$); in striking contrast, levels in $B2m^{-/-}$ mice did not increase and were significantly reduced compared to those in wild-type hosts ($p = 0.0021$) (Figure 3C). IL-4 levels were also significantly elevated in wild-type mice after mercury exposure ($p < 0.05$) and, to a lesser degree, increased in $B2m^{-/-}$ mice ($p = 0.027$). IL-2 expression was not significantly affected by mercury exposure in wild-type or $B2m^{-/-}$ mice. These findings indicate that $B2m$ deficiency affects cytokine levels primarily in mercury-exposed skin where it is associated with significant reductions in both IL-4 and IFN γ .

Discussion

In this study, we investigated the role of $B2m$ in xenobiotic-induced autoimmunity by comparing murine mercury-induced autoimmunity (mHgIA) in $B2m^{-/-}$ and wild-type mice. A β_2 -microglobulin deficiency markedly reduced mercury-induced hypergammaglobulinemia, including that for IgG $_1$ and IgG $_{2a}$ isotypes, lowered levels of ANoA (but not anti-chromatin), preferentially reduced levels of IgG $_1$ isotype anti-chromatin, and reduced IL-4 and IFN γ cytokine expression at the site of mercury exposure; in contrast, this status did not affect the incidence and severity of glomerular immune complex deposits. Thus, $B2m$ is required for some of the features of mHgIA.

Previous studies have shown that both β_2 -microglobulin (Spriggs et al., 1992) and neonatal Fc receptor (FcRn) (Roopenian et al., 2003)-deficient mice have reduced levels of IgG subclasses and that this was associated with significantly shorter half-lives for all IgG subclasses, excepting IgG $_{2b}$ (Israel et al., 1996). In other studies, $B2m$ - and $FcRn$ -deficient mice were also shown incapable of vigorous IgG immune responses against foreign antigens. Thus, β_2 -microglobulin-deficient mice exhibited reduced antibody responses for all IgG subclasses to vaccinia virus infection and TNP-KLH immunization (Spriggs et al., 1992) and $FcRn$ deficiency substantially reduced the anti-DNP response irrespective of the IgG subclass (Roopenian et al., 2003). Since β_2 -microglobulin is required for a functional FcRn (Roopenian and Akilesh, 2007), the lower immunoglobulin levels in both $B2m$ - and $FcRn$ -deficient mice are believed to be from the absence of FcRn-mediated recycling of IgG (Lencer and Blumberg, 2005). In our studies, defective FcRn function is also likely responsible for the overall reduction in serum IgG associated with $B2m$ deficiency and may also explain the reduction in ANoA. Interestingly, however, total IgG anti-chromatin autoantibody levels after mercury exposure in $B2m$ -deficient mice were, in contrast, not reduced and only the IgG $_1$ isotype was significantly lowered despite dramatic reductions in both the polyclonal IgG $_1$ and IgG $_{2a}$ serum concentrations. Furthermore, kidney immune complex deposits were not reduced in $B2m^{-/-}$ mice exposed to mercury despite significantly lower IgG concentrations. In MRL/Fas^{lpr} mice IgG1 is selectively dependent upon $B2m$ (Christianson et al, 1996) and NZB. $B2m^{-/-}$ mice have reduced IL-4 (Chen et al, 1997)

suggesting that the *B2m*-dependent reduction of IgG1 in mHgIA may reflect changes in cytokine expression.

Previous studies have shown that although the cytokine and autoantibody isotype profile in mHgIA is T_H2 -like with greatest elevations in IL-4, IgE, and IgG₁, the development of humoral autoimmunity and tissue pathology in mHgIA paradoxically requires the presence of IFN γ , but not IL-4 (Pietsch et al., 1989; Ochel et al., 1991; Kono et al., 1998). Here we observed that *B2m* deficiency resulted in a significant reduction in anti-chromatin IgG₁, but not other IgG isotypes, suggesting the possibility that the cytokine profile might be directed away from the characteristic T_H2 response (Ochel et al., 1991). Consistent with this, significant increases in IL-4 mRNA in the spleen and exposed skin were seen in wild-type mice exposed to mercury, while *B2m*^{-/-} mice had no or smaller increases. We also observed a marked increase in IFN γ mRNA in mercury exposed wild-type mice, but only in exposed skin, while IFN γ did not increase in *B2m*^{-/-} mice, supporting the notion that increased IFN- γ is necessary for full expression of disease (Kono et al., 1998). Although the cells responsible for IFN- γ expression were not determined in this study they likely include MHC Class I restricted T cells and NK cells (Boehm et al., 1997; Sun and Lanier, 2008). Thus, *B2m*, is necessary for full expression of IL-4 and IFN γ following mercury exposure and the predominant T_H2 response. Its role in overall autoantibody production is less certain as *B2m*-deficiency did not affect total IgG anti-chromatin titers or severity of kidney immune complex deposits.

While the absence of β_2 -microglobulin and changes in cytokine expression can be argued to have contributed to the IgG subclasses of the autoantibody responses, they do not explain the persistence of the anti-chromatin, but reduction in the ANoA response. This difference suggests a role for an antigen specific mechanism in maintaining circulating anti-chromatin antibody. Both DNA and nucleosomes have been detected in the blood of mice (Bjorkman et al., 2003) and humans (Williams et al., 2001; Chen et al., 2007) with systemic autoimmunity, presumably a byproduct of cell death (Pisetsky, 2004, 2007). The immune-complexes formed upon interaction of circulating nucleosomes with anti-chromatin autoantibodies would be well-suited to bind Fc γ R and activate immune effector cells through endocytosis of the DNA-containing complexes and engagement of nucleic acid-binding toll-like receptors (TLRs) (Nimmerjahn and Ravetch, 2008), thereby maintaining the anti-chromatin response. By contrast, the ANoA response may be less effective in generating immune complexes and Fc γ R-mediated activation, as the release from dying cells of soluble fibrillar (the major antigenic component of ANoA) is accompanied by proteolytic cleavage and loss of antigenicity (Pollard et al., 1997, 2000). Less effective Fc γ R-mediated activation, in addition to increased IgG catabolism, might explain the selective reduction in the ANoA response.

Despite reduced immunoglobulin levels, *B2m*-deficient mice exposed to mercury retained glomerular immune deposits, but had reduced deposits in vessels of the kidney compared to wild-type mice. Other indices of kidney function (viz. nephritis, proteinuria) may be impacted by *B2m* as noted in studies with idiopathic models (Chan et al., 2001; Christianson et al., 1996) however mercury toxicity can make such studies challenging. Even a single exposure to HgCl₂ results in tubular necrosis and proteinuria (Rumbeiha et al., 2000) and exposure in C57BL/6 mice, which do not develop renal immune deposits (Hultman et al., 1992), leads to proteinuria (Piacenza et al., 2009). Deficient Fc γ Rn expression in the kidneys of β_2 -microglobulin-deficient mice might have contributed to the glomerular pathology observed in mHgIA. It has been proposed that in glomerulonephritis, IgG may be deposited at the basolateral side of the podocyte (Haymann et al., 2000; Roopenian and Akilesh, 2007). The absence of Fc γ Rn in *B2m*-deficient mice might be expected to limit transcytosis of multimeric IgG containing complexes via podocytes, leading to immune deposits even in the

presence of a reduced IgG half-life. A persistence of anti-chromatin autoantibodies may also contribute to glomerular deposition because of the binding of antibody-nucleosome complexes to glomerular basement components (Kramers et al., 1994; van Bruggen et al., 1997).

Single gene deletions can influence expression of mHglA in different ways ranging from no effect, to suppression of one or more features of disease, or suppression of all features of disease (Pollard et al., 2003, 2005). This study provides the additional observation that lack of β_2 -microglobulin leads to modulation of the autoantibody profile, a feature that has been observed for single gene deficiencies in other models of autoimmunity. The most notable being that observed in lupus-prone mice deficient in Toll-like receptors resulting in preferential effects on IgG subclasses and autoantibodies against DNA or RNA containing autoantigens (Baccala et al., 2007; Marshak-Rothstein and Rifkin, 2007; Kono et al., 2009). Differences in autoantibody profiles have also been found in xenobiotic-induced autoimmunity following pristane injection of IL-6deficient mice (Richards et al., 1998). In contrast to β_2 -microglobulin deficiency, absence of IL-6 led to a lack of anti-chromatin but persistence of anti-snRNP antibodies. The discordance of autoantibody profiles in mercury-exposed β_2 -microglobulin-deficient mice is further evidence that multiple molecular pathways influence the autoantibody profiles of systemic autoimmunity.

Conclusion

These studies are the first to demonstrate that β_2 -microglobulin is required for the full development of xenobiotic-induced systemic autoimmunity, with mercury-exposed *B2m*-deficient mice having significantly lower levels of polyclonal IgG and certain autoantibodies, reduced expression of IL-4 and IFN γ , but yet similar serum concentrations of IgG anti-chromatin and amounts of kidney immune complex deposits. These studies show that *B2m* is a potential genetic modifier of xenobiotic-induced systemic autoimmunity and document the complex manner in which genetic variation can alter autoimmune responses.

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Figure 1. ANA and ANoA in HgCl₂-exposed *B2m*^{+/+} and *B2m*^{-/-} mice
Mice were injected with HgCl₂ (40 µg in 100 µl PBS) twice per week for 4 wk before determination of serum IgG ANoA by indirect immunofluorescence on HEP-2 cells. **(A)** Endpoint titer of IgG ANoA in *B2m*^{+/+} and *B2m*^{-/-} mice. The geometric mean [and range] of IgG ANoA titers differed significantly between *B2m*^{-/-} and *B2m*^{+/+} mice (340 [160–640] vs. 1540 [160–2560]; *p* < 0.025). **(B)** Fluorescence intensity of IgG ANA and ANoA in *B2m*^{+/+} (*n* = 9), *B2m*^{+/-} (*n* = 8) and *B2m*^{-/-} (*n* = 8) mice at a serum dilution of 1/100. ANoA autoantibodies (as mean ± SEM) differed significantly between *B2m*^{-/-} (*n* = 8) and *B2m*^{+/+} (*n* = 9) mice (1.3 ± 1.1] vs. 2.8 ± 0.56]; *p* = 0.0027).

Figure 2.

Titration of IgG anti-chromatin antibodies in HgCl₂-exposed $B2m^{+/+}$ and $B2m^{-/-}$ mice. Mice were injected with HgCl₂ (40 µg in 100 µl PBS) twice per week for 4 wk before determination of serum IgG anti-chromatin antibodies using ELISA. Titration consisted of serial 2-fold dilution of individual sera from 1/100 to 1/3200. OD₄₀₅ values represent the geometric mean at each serum dilution for $B2m^{+/+}$ (n = 9) and $B2m^{-/-}$ (n = 6) mice. $B2m^{+/+}$ ■; $B2m^{-/-}$ □.

Figure 3.

IFN γ , IL-2, and IL-4 expression in the (A) spleen, (B) draining lymph node, and (C) skin of HgCl₂-exposed $B2m^{+/+}$ and $B2m^{-/-}$ mice. Mice were injected subcutaneously with 40 μ g HgCl₂ in phosphate buffered saline (PBS) (solid bars) or with PBS alone (open bars) for 2 wk. Total RNA was prepared and quantitative real-time polymerase chain reaction was performed on reverse-transcribed RNA. The mRNA level was normalized using cyclophilin A for spleen and lymph node. For skin samples cytokine mRNA levels were related to total RNA concentration. Values represent the mean and SEM of 4 mice per group.

Table 1

Immunoglobulin levels and autoantibodies in $B2m^{+/+}$, $B2m^{+/-}$, and $B2m^{-/-}$ mice following 4 wk of mercury exposure^a.

$B2m$	No.	Treatment	Immunoglobulin Level		
			IgG ₁ (mg/ml)	IgG _{2a} (mg/ml)	Anti-Chromatin OD ₄₀₅
+/+	8	PBS	0.250 ± 0.060	0.113 ± 0.025	0.05 ± 0.02
	9	HgCl ₂	1.106 ± 0.292	0.208 ± 0.080	0.73 ± 0.46
+/-	10	PBS	0.269 ± 0.092	0.091 ± 0.034	0.07 ± 0.09
	8	HgCl ₂	1.079 ± 0.256	0.182 ± 0.078	0.46 ± 0.29
-/-	8	PBS	0.130 ± 0.107	0.015 ± 0.003	0.04 ± 0.06
	8	HgCl ₂	0.174 ± 0.165	0.021 ± 0.007	0.75 ± 0.57

^aValues are expressed as mean (± 1SD); p-values were determined for comparisons between PBS and HgCl₂ data within each group, and between HgCl₂ data between groups. NS = not significant.

IgG₁, PBS vs. HgCl₂ $B2m^{+/+}$, $p < 0.0001$; $B2m^{+/-}$, $p < 0.0001$; $B2m^{-/-}$, NS.

IgG_{2a}, PBS vs. HgCl₂ $B2m^{+/+}$, $p < 0.006$; $B2m^{+/-}$, $p < 0.004$; $B2m^{-/-}$, NS.

Anti-chromatin Ab, PBS vs. HgCl₂ $B2m^{+/+}$, $p < 0.001$; $B2m^{+/-}$, $p < 0.001$; $B2m^{-/-}$, $p < 0.004$.

IgG₁, HgCl₂ $B2m^{+/+}$ vs. $B2m^{-/-}$, $p < 0.0001$; $B2m^{+/-}$ vs. $B2m^{-/-}$, $p < 0.0001$.

IgG_{2a}, HgCl₂ $B2m^{+/+}$ vs. $B2m^{-/-}$, $p < 0.0001$; $B2m^{+/-}$ vs. $B2m^{-/-}$, $p < 0.0001$.

Anti-chromatin Ab, HgCl₂ $B2m^{+/+}$ vs. $B2m^{-/-}$, NS; $B2m^{+/-}$ vs. $B2m^{-/-}$, NS.

Table 2

Immunopathological changes in $B2m^{+/+}$, $B2m^{+/-}$, and $B2m^{-/-}$ mice following 4 wk of mercury exposure^a

$B2m$	No.	Treatment	Kidney Immunopathology			Spleen Immunopathology		
			Glomerular IgG	Glomerular C3	Vessel IgG	Vessel C3	Vessel IgG	Vessel C3
$+/+$	8	PBS	0	30 ± 60	0	0	0	0
$+/+$	9	HgCl ₂	978 ± 726	676 ± 485	0.45 ± 0.88	0.11 ± 0.33	1.33 ± 0.87	0.89 ± 0.55
$+/-$	10	PBS	90 ± 224	120 ± 222	0.06 ± 0.18	0	0.06 ± 0.18	0.06 ± 0.18
$+/-$	8	HgCl ₂	710 ± 512	790 ± 548	0	0	0.25 ± 0.46	0.19 ± 0.26
$-/-$	8	PBS	0	0	0	0	0	0
$-/-$	8	HgCl ₂	760 ± 559	560 ± 468	0.25 ± 0.71	0.06 ± 0.18	0.44 ± 1.05	0.25 ± 0.71

^aValues are expressed as mean (± 1 SD); p-values were determined for comparisons between post-PBS and post-HgCl₂ data within each group, and between post-HgCl₂ data between groups.

Glomerular IgG, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, $p < 0.001$; $B2m^{+/-}$, $p < 0.001$; $B2m^{-/-}$, $p < 0.001$.

Glomerular C3, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, $p < 0.001$; $B2m^{+/-}$, $p < 0.005$; $B2m^{-/-}$, $p < 0.001$.

Kidney Vessel IgG, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, NS; $B2m^{+/-}$, NS; $B2m^{-/-}$, NS.

Kidney Vessel C3, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, NS; $B2m^{+/-}$, NS; $B2m^{-/-}$, NS.

Splenic Vessel IgG, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, $p < 0.005$; $B2m^{+/-}$, NS; $B2m^{-/-}$, NS.

Splenic Vessel C3, post-PBS vs. post-HgCl₂ $B2m^{+/+}$, $p < 0.005$; $B2m^{+/-}$, NS; $B2m^{-/-}$, NS.

Splenic Vessel IgG, post-HgCl₂ $B2m^{+/+}$ vs. $B2m^{-/-}$, $p = 0.0266$; post-HgCl₂ $B2m^{+/+}$ vs. $B2m^{+/-}$, $p = 0.0133$.

Splenic Vessel C3, post-HgCl₂ $B2m^{+/+}$ vs. $B2m^{-/-}$, $p = 0.0234$; post-HgCl₂ $B2m^{+/+}$ vs. $B2m^{+/-}$, $p = 0.0089$.

Table 3

IgG subclass of anti chromatin antibodies in $B2m^{+/+}$ and $B2m^{-/-}$ mice following 4 wk of mercury exposure.

IgG Subclass of Anti-Chromatin Antibodies (OD ₄₀₅)				
	IgG1	IgG2a	IgG2b	IgG3
$B2m^{+/+}$	0.63 ± 0.21	1.02 ± 0.64	1.67 ± 1.51	0.03 ± 0.02
$B2m^{-/-}$	0.19 ± 0.10	0.60 ± 0.36	1.32 ± 0.98	0.08 ± 0.16
p value	p = 0.0006	p = 0.24	p = 0.96	p = 0.29