Lack of Plasma Protein Hemopexin Dampens Mercury-Induced Autoimmune Response in Mice

Sharmila Fagoonee, Cristiana Caorsi, Mirella Giovarelli, Meredin Stoltenberg, Lorenzo Silengo, Fiorella Altruda, Giovanni Camussi, Emanuela Tolosano and Benedetta Bussolati


http://www.jimmunol.org/content/181/3/1937

References

This article cites 29 articles, 10 of which you can access for free at: http://www.jimmunol.org/content/181/3/1937.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Lack of Plasma Protein Hemopexin Dampens Mercury-Induced Autoimmune Response in Mice¹

Sharmila Fagoonee,²* Cristiana Caorsi,†‡ Mirella Giovarelli,†‡ Meredin Stoltenberg,¶
Lorenzo Silengo,* Fiorella Altruda,* Giovanni Camussi,§§ Emanuela Tolosano,³* and Benedetta Bussolati²,3,4

Several factors affect the autoimmune response, including iron-dependent modulation of T cells. Hemopexin is the plasma protein with the highest binding affinity to heme. It mediates heme-iron recovery in the liver, thus controlling heme-iron availability in peripheral cells. The aim of the present study was to investigate the role of hemopexin in the progression of an autoimmune response. To this end, we chose a mouse model of mercury-induced autoimmunity and evaluated the susceptibility of hemopexin-null mice to mercury treatment compared with wild-type controls. In this study we show that lack of hemopexin dampens mercury-induced autoimmune responses in mice. Hemopexin-null mice produced fewer antinuclear autoantibodies and had reduced deposits of immune complexes in the kidney after mercuric chloride treatment compared with wild-type mice. These features were associated with a reduction in activated T cells and lower absolute B cell number in spleen and impaired IgG1 and IgG2a production. In contrast, in hemopexin-null mice the response to OVA/CFA immunization was maintained. In addition, hemopexin-null mice had reduced transferrin receptor 1 expression in T cells, possibly due to the increase in heme-derived iron. Interestingly, CD4⁺T cells isolated from mercury-treated hemopexin-null mice show reduced IFN-γ-dependent STAT1 phosphorylation compared with that of wild-type mice. Our data suggest that hemopexin, by controlling heme-iron availability in lymphocytes, modulates responsiveness to IFN-γ and, hence, autoimmune responses. The Journal of Immunology, 2008, 181: 1937–1947.

¹Address correspondence and reprint requests to Dr. Sharmila Fagoonee, University of Turin, Via Nizza 52, Turin, Italy. E-mail address: sharmila.fagoonee@unito.it or Dr. Benedetta Bussolati, Molecular Biotechnology Center, Via Nizza 52, 10126 Turin, Italy. E-mail address: benedetta.bussolati@unito.it
²E.T. and B.B. contributed equally to this work.
³Abbreviations used in this paper: HO, heme-oxygenase; ANA, antinuclear antibody; CO, carbon monoxide; HgCl₂, mercuric chloride; Hx-null, hemopexin-null; IC, immune complex; TIR1, transferrin receptor 1; Treg, T regulatory cell.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00
of circulating hemopexin may increase the peripheral cell availability of heme with the consequent activation of HO-1, which is known to exert an immunomodulatory and cytoprotective effect (9).

To investigate the role of hemopexin in autoimmune response, we chose the well-established mouse model of mercury-induced autoimmunity characterized by the production of antinuclear Abs (ANA) and immune complex (IC) deposition in the kidney (10). We evaluated the susceptibility of Hx-null mice to mercury treatment compared with wild-type controls.

We show that the lack of hemopexin dampens mercury-induced autoimmune responses in mice. Hx-null mice produced less ANA and had reduced deposits of IC in the kidney after mercuric chloride (HgCl2) treatment compared with wild-type mice. Hx-null mice had a reduction in activated T cells and a reduced number of B lymphocytes in the spleen after treatment with HgCl2 but not after OVA/CFA immunization, pointing to a specific defect in the autoimmune response. Interestingly, Hx-null mice had reduced transferrin receptor 1 (TfR1) expression in T cells under basal conditions possibly dependent on higher heme-derived iron in transferrin receptor 1 (TfR1) expression in T cells under basal conditions possibly dependent on higher heme-derived iron in these mice. Finally, we demonstrated that IFN-γ-induced activation of STAT1 in CD4+ T cells isolated from HgCl2-treated Hx-null mice was impaired compared with that of wild-type mice.

We suggest that hemopexin, by controlling heme-iron availability to lymphocytes, may participate in the control of autoimmunity by modulating IFN-γ responsiveness.

Materials and Methods

Mice and treatment with HgCl2

Hx-null mice were generated as previously described (8) and were in a genetic 129/Sv background (H-2b). All mice (age matched and 6–8 wk old at the beginning of each experiment) used were housed in our animal facilities, with a 12-h dark/light cycle and access to standard laboratory chow and tap water ad libitum. At least five animals were used for each experimental point. Hx-null and wild-type mice were injected with a dose of 1.6 mg of HgCl2 (Sigma-Aldrich) per kilogram of body weight s.c. every third day for 4 wk. Additional groups of mice were treated for 6 wk (prolonged treatment), 4 days, or 1 day. Nontreated mice were used as controls in all experiments.

Collection of blood, hearts, skin, spleens, and kidneys

After the 4-wk treatment, mice were bled retroorbitally under anesthesia and sacrificed. Their hearts, skin, spleens, and kidneys were removed aseptically and embedded in optimal cryo-preserving tissue compound (OCT) (Miles), snap frozen, and stored at −80°C until sectioning or inclusion in paraffin after formalin fixation. Blood samples were allowed to clot at 37°C, and then centrifuged at 3,000 rpm (Microfuge 18 centrifuge; Beckman Coulter) for 10 min at room temperature. The serum obtained was then kept at −20°C until further use.

Detection of ANA by immunofluorescence

The serum levels of IgG ANA were assayed by using indirect immunofluorescence. Briefly, 4-μm-thick cryostat sections of liver were fixed in ice-cold acetone, allowed to dry, and incubated with serum from HgCl2-treated mice of both genotypes at serial dilutions followed by FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) for 45 min at 37°C. The sections were incubated in cold acetone followed by fixing in 3.5% parafomaldehyde containing 2% sucrose as previously described (11). After saturation with PBS-BSA, the sections were blocked by adding cold PBS and the cells were fixed and processed for intracellular staining vs 30,000 cells for surface staining. PE-, FITC-, and allophycocyanin Abs (BD Biosciences) were used as controls in all experiments.

Intracellular staining of cytokines and flow cytometry

Spleens were dissected and splenocytes were flushed gently out from the spleens into DMEM by using a needle. Single cell suspensions were prepared by pipetting several times and subjecting to a Ficoll gradient. The lymphocytes were recovered, resuspended, counted, and adjusted to a density of 1×10⁶ cells/ml.

The mAbs used for the detection of cell surface markers and intracellular cytokines are documented in Table I. Briefly, the isolated spleen and blood cells were washed with PBS-BSA (0.25%), stained with the appropriate cell surface Ag or the relevant control for 30 min at room temperature, and then washed again and resuspended in PBS-BSA. For intracellular staining, cells were fixed in 4% paraformaldehyde solution for 10 min at 4°C, permeabilized with TBS plus 1% Triton X-100, and stained with anti-IL-4, anti-IFN-γ, or anti-IL-17 conjugated with PE for 30 min at room temperature. Allophycocyanin-conjugated Foxp3 staining was performed by using a Foxp3 staining set (e Bioscience; Insight Biotechnology) according to the manufacturer’s instructions. To measure the levels of phosphorylated STAT1, cells were fixed immediately after stimulation with 4% paraformaldehyde, permeabilized with ice-cold methanol followed by sodium citrate and then simultaneously incubated with the anti-phospho(Tyr701) STAT1-PE or anti-IgG-PE and CD4-allophycocyanin Abs (BD Biosciences). Cells were analyzed on a FACS (BD Biosciences). Seventy thousand cells were analyzed at each experimental point for intracellular staining vs 30,000 cells for surface staining. PE-, FITC-, and allophycocyanin-conjugated anti-IgGs were used as isotype controls where appropriate.

Treatment of splenocytes with IFN-γ ex vivo

Mice were treated with HgCl2 for 4 days and 1×10⁶ splenocytes were treated with IFN-γ (100U/ml) for 15 min at 37°C. The reaction was blocked by adding cold PBS and the cells were fixed and processed for anti-phospho(Tyr701) STAT1-PE and CD4 staining and flow cytometry analysis. IFN-α (100U/ml) was used as control.

Activation of T cells ex vivo

To analyze cytokine production by T cells ex vivo, 2×10⁶ splenocytes were plated in a 24-well, flat-bottom plates containing 1 ml of RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin and either PMA (10 ng/ml, final concentration) or ionomycin (500 ng/ml) and monensin (2 μM) or with monensin (Sigma-Aldrich) alone as control.

Following incubation for 4 h at 37°C, the cells were harvested, washed, and prepared for flow cytometry. The percentages of IL-4- and IFN-γ-expressing CD4+ T cells were determined in the spleens of mice under basal conditions and after treatment with HgCl2.

Autometallographic mercury tracing

To trace mercury deposits (i.e. mercury–sulfur nanocrystals) in tissues, autometallographic silver enhancement was performed (13, 14). Briefly, gelatin-coated, paraffin-included sections of tissues were placed in an autometallographic developer containing a mixture of gum arabic, citrate buffer (pH 3.7), hydroquinone, and silver lactate and incubated at 26°C for 60
Table 1. Monoclonal antibodies employed to detect cell surface markers and intracellular cytokines during flow cytometry

<table>
<thead>
<tr>
<th>Raised in</th>
<th>Specificity</th>
<th>Conjugated with</th>
<th>Isotype Form</th>
<th>Purchased from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Mouse CD4</td>
<td>FITC</td>
<td>IgG2b</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD8</td>
<td>PE</td>
<td>IgG2b</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD25</td>
<td>PE</td>
<td>IgG2b</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Hamster</td>
<td>Mouse CD69</td>
<td>PE</td>
<td>IgG1</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD44</td>
<td>FITC</td>
<td>IgG1</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD19</td>
<td>PE</td>
<td>IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD4</td>
<td>PE</td>
<td>IgG2b</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD71</td>
<td>FITC</td>
<td>IgG2a</td>
<td>Abcam</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse IL-4</td>
<td>PE</td>
<td>IgG2b</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse IFN-γ</td>
<td>PE</td>
<td>IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse FoxP3</td>
<td>Allophycocyanin</td>
<td>IgG2a</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Hamster</td>
<td>Mouse CD11b</td>
<td>FITC</td>
<td>IgG2b</td>
<td>Serotec</td>
</tr>
<tr>
<td>Hamster</td>
<td>Mouse CD11c</td>
<td>PE</td>
<td>IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse F4/80</td>
<td>PE</td>
<td>IgG2a</td>
<td>Serotec</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse IL-17</td>
<td>PE</td>
<td>IgG1</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Rat</td>
<td>Mouse CD4</td>
<td>Allophycocyanin</td>
<td>IgG2a</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Mouse</td>
<td>Mouse STAT1 (Y701)</td>
<td>PE</td>
<td>IgG2a</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Rat</td>
<td>Unknown</td>
<td>FITC</td>
<td>IgG2a</td>
<td>Dako</td>
</tr>
<tr>
<td>Rat</td>
<td>Unknown</td>
<td>PE</td>
<td>IgG2a</td>
<td>Dako</td>
</tr>
<tr>
<td>Rat</td>
<td>Unknown</td>
<td>Allophycocyanin</td>
<td>IgG2a</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

* Negative isotype control.

Analysis of kidney functionality

Creatinine was determined on a Beckman creatinine analyzer (Beckman Instruments). After treatment for 4 wk with HgCl₂, mice were individually housed for 24 h in metabolic cages and urine was collected for proteinuria determination with the Biuret assay.

Statistical analysis

Results are expressed as mean ± SE. Statistical analyses were performed using an unpaired Student’s t test.

Results

HgCl₂-induced ANA production and IC deposition was reduced in Hx-null mice

Control 129/Sv mice and Hx-null 129/Sv mice were treated with HgCl₂ every three days for 4 wk. After that time, the appearance of an autoimmune disease was evaluated by production of ANA and glomerular deposition of IC.

ANA were barely detectable in both Hx-null and wild-type mice under basal conditions. After HgCl₂ treatment, the titers of ANA were significantly reduced in the Hx-null mice compared with the wild-type mice after treatment with HgCl₂ for 4 wk. The absence of IC deposits in the Hx-null mice was also confirmed by electron microscopy (Fig. 1, O and Q). After 4 min. After several washings, the sections were counterstained with cresyl violet and examined under a light microscope.

Passive Arthus reaction

For cutaneous Arthus reactions, mice were injected i.v. with 200 µl of a mixture of 20 mg/ml OVA containing 2.5% Evans blue dye (Sigma-Aldrich), followed by intradermal injection of 60 µg/30 µl rabbit IgG anti-OVA Ab (Cappel) 30 min later. Skin was harvested 4 h later. To assess for edema, the diameter of extravascular Evans blue dye on the reverse side of the skin at site of injection was measured directly. Skin was fixed in 10% formalin and paraffin-included sections were stained with H&E and the numbers of infiltrating neutrophils and mast cells were determined.

Immunization with OVA/CFA and measurement of serum IgG by ELISA

Mice were immunized with a single i.p. injection of 0.2 mg of OVA in CFA. Ten days later, blood was taken and serum was prepared to measure IgG Abs to OVA by ELISA. Briefly, 96-well plates were coated with a 2% solution of OVA in 0.1 M Tris-HCl buffer. Serum samples were serially diluted in Tris buffer containing 2% bovine γ-globulin (Sigma-Aldrich) to block nonspecific binding of mouse globulins to the microtitration trays. The samples were incubated at 75 l/well for 1 h, the wells were washed five times and treated with Fc-specific, peroxidase-conjugated goat Abs to mouse IgG (Cappel). After incubation at room temperature for 1 h, the wells were washed five times and treated with o-phenylenediamine (100 µl/well) for 10 min in darkness. The enzymatic reaction was stopped with 2N H₂SO₄, and absorbance was measured at 490 nm.

Western blots and Abs

T cells were purified from the spleens of mice under basal conditions with CD90⁺ microbeads (Miltenyi Biotec) and using MidiMACS magnetic columns (Miltenyi Biotec) as per the manufacturer’s instructions. The column was removed from the magnetic field and the CD90⁺ fraction was collected. Cell numbers and viability were determined using trypan blue exclusion.

Brieﬂy, total protein extracted from purified T cells was separated by electrophoresis on 10% SDS-polyacrylamide gels, blotted onto nitrocellulose membrane (Whatman), and incubated overnight with 1/1000 anti-TR1 polyclonal Ab (Zymed Laboratories). Membranes were then incubated with 1/5000 HRP-conjugated goat anti-rabbit IgG and revealed by ECL on a ChemiDoc system (Bio-Rad).
4 wk of treatment with HgCl₂, no increase in serum creatinine levels were observed in Hx-null and wild-type mice compared with basal levels, nor was there any difference in proteinuria (not shown). Prolonged treatment (6 wk) with the heavy metal salt did not further alter the autoimmune response of the two genotypes (not shown).

**FIGURE 1.** Mercuric chloride (HgCl₂)-induced antinuclear Ab production and immune complex deposition was reduced in Hx-null mice after 4 wk of treatment. A, Quantification of serum ANA. ANA titers were reduced in HgCl₂-treated Hx-null mice compared with wild-type mice (**, p < 0.01). No differences were noted in basal ANA levels. Levels of circulating ANA were measured by indirect immunofluorescence using cryostatic liver sections. Data are expressed as the mean serum titer ± SE. B–M, Direct immunofluorescence of IC deposits. Representative photomicrographs of renal tissue of the wild-type mice showed generalized and diffuse granular deposits of IgG (B and C) and C3 (E and F) in the glomerular capillary walls and mesangium. Similar deposition of IgG (K) and C3 (L) was detected in the muscle capillaries of wild-type mice. In Hx-null mice, no deposits of IgG (D) and C3 (G) were observed in the kidney. No IgG deposits were also present in the cardiac muscle (M). Representative photomicrographs of renal tissue under basal conditions are shown for wild-type (H and I) and Hx-null (J) mice. N–Q: Electron microscopy analysis of kidney. Representative photomicrographs showing presence of dense deposits (arrows) in the mesangial matrix (M) and in the glomerular capillary wall of wild-type mice (N and P). No deposits were observed in the mesangium or in the glomerular capillary walls of Hx-null mice (O and Q). The data are representative of three separate experiments (n = 4/genotype/experiment). Original magnification: B, E, H, and K, ×150; C, D, F, G, I, J, L, and M, ×400; N and O, ×6000; P and Q, ×12,000.
These data indicate that Hx-null mice were protected from HgCl2-induced autoimmune disease. Thus, experiments investigating defects in general immune responses, mercury delivery, or autoimmune pathways in Hx-null mice compared with wild-type mice were conducted.

Similar tissue distribution of mercury as revealed by autometallography

Because hemopexin is capable of binding divalent metal cations, e.g., Fe2+, Zn2+, and Co2+, the possibility of differential distribution of mercury to various tissues between wild-type and Hx-null mice could not be ruled out (15). Autometallography was applied. The autometallographic technique is based on silver enhancement of quantum dots resulting from the following: 1) the metabolism of toxic metals in animals and humans: 2) in vivo, in vitro, and immersion-created zinc-sulfur/zinc-selenium nanocrystals; and 3) metal ions liberated from metal implants and particles (14). Wild-type (Fig. 2B) and Hx-null (Fig. 2C) mice showed similar intense autometallographic mercury staining in the renal tubules of mercury-exposed kidneys after 4 wk of treatment vs the unstained sham control (A). Thus, plasma hemopexin did not control the distribution of mercury to the tissues after HgCl2 injection in mice.

Characterization of spleen cell populations following HgCl2 injections

We subsequently analyzed, by flow cytometry, absolute spleen cell populations under basal conditions and after HgCl2 treatment. Under basal conditions, we did not find any statistically significant differences either in the total number of splenocytes or in specific cell populations. After treatment with HgCl2 for 4 wk there was an increase in the absolute cell number of the spleen cells of both genotypes, but this was only statistically significant in wild-type mice (Fig. 4A). Flow cytometry analysis of the spleen cell populations revealed a significant increase in CD4+ T cells in both Hx-null and wild-type mice (Fig. 4B). No significant alterations were noted in CD8+ T cell population (Fig. 4C).

Other spleen populations, i.e., B-cells, macrophages, and dendritic cells, were measured by flow cytometry using, respectively,
anti-CD19, anti-CD11b, or anti-F4/80 and anti-CD11c Abs (Fig. 4, D and G). There was an increase in APC number in both wild-type and Hx-null mice after HgCl₂ treatment. Interestingly, the increase in CD19⁺ B cells was statistically significant in wild-type mice but not in the Hx-null ones (Fig. 4D). Macrophages (E and F) did not change while the increase in CD11c⁺ dendritic cells (G) was statistically significant in wild-type controls only. Macrophages did not change significantly after HgCl₂ injections (Fig. 4, E and F), whereas absolute dendritic cell number increased significantly in both genotypes (Fig. 4G).

Splenic regulatory T cells (Treg) expressing CD4⁺CD25⁺FoxP3⁺ markers, which participate in the regulation of autoreactive T cells, were also analyzed. There was no significant increase in the percentage of splenic Treg in wild-type and Hx-null mice after HgCl₂ treatment for 4 wk vs the respective basal levels. Results in Table II show the percentage of CD4⁺⁰ T cells expressing CD25 and the percentage of CD4⁺CD25⁺ cells expressing FoxP3.

To investigate the activation status of CD4⁺ T cells, the expression of CD69 (early activation marker) and CD44 (memory T cell marker) were analyzed 1 day and 4 wk, respectively, after treatment with HgCl₂. There was a statistically significant increase in the number of CD4⁺CD69⁺ (Fig. 5A) and CD4⁺CD44high (Fig. 5B) T cells in wild-type mice compared with basal levels, but not in Hx-null mice.

Analysis of Th1 and Th2 cytokine production in splenic CD4⁺ T lymphocytes

Because the Th1/Th2 dichotomy might account for the different consequences after HgCl₂ injection, the role of Th1/Th2 cytokines in this model was investigated on isolated splenocytes. There was no significant increase in production of IFN-γ or IL-4 (representative of Th1- and Th2-responses, respectively) by CD4⁺ T cells in wild-type mice after treatment with HgCl₂. However, there was a statistically significant increase in the number of IFN-γ⁺ T cells in Hx-null mice after treatment with HgCl₂.

Table II. Flow cytometric evaluation of Treg population

<table>
<thead>
<tr>
<th>Markers</th>
<th>Wild-Type Mice</th>
<th>Hx-Null Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>HgCl₂ Treated</td>
</tr>
<tr>
<td>CD4/CD25</td>
<td>8.56 ± 0.58</td>
<td>9.227 ± 0.25</td>
</tr>
<tr>
<td>CD4/CD25/FoxP3</td>
<td>5.68 ± 0.49</td>
<td>4.66 ± 0.11</td>
</tr>
</tbody>
</table>

* The percentage of CD4⁺ T cells expressing CD25 as well as the percentage of CD4⁺CD25⁺ cells expressing FoxP3 are shown under basal conditions and after 4 wk of treatment with HgCl₂. Values represent mean percentage ± SE of five separate experiments (n = 4/genotype/experiment).
either genotype after treatment with HgCl$_2$ for 4 wk (Table III) as shown by flow cytometry experiments. Ex vivo treatment of isolated splenocytes with PMA and ionomycin induced IL-4 and IFN-$\gamma$ expression in CD4$^+$ T cells in both Hx-null and wild-type mice before and after treatment with HgCl$_2$ (data not shown). To confirm the cytokine profile, quantitative RT PCR was performed on spleen total RNA of 10-day HgCl$_2$-treated mice from both genotypes using assays-on-demand probes for IL-4 and IFN-$\gamma$ (Applied Biosystems). Again, no difference in Th1 and Th2 cytokine expressions was noted (not shown). Yet another CD4$^+$ T cell subset, Th17, implicated in immune regulation, was analyzed in the spleen, but no increase in IL-17-producing CD4$^+$ cells was noted after HgCl$_2$ treatment in both Hx-null and wild-type mice compared with nontreated mice (Table III).

![FIGURE 5. Expression of activation markers on CD4$^+$ T cells after treatment with HgCl$_2$. Mice were treated as described in Materials and Methods. A, Percentages of splenic CD4$^+$CD69$^+$ T cells evaluated 1 day after treatment with HgCl$_2$ showed a significant increase in wild-type mice only (*, $p < 0.05$). B, Percentages of splenic CD4$^+$CD44$^{hi}$ T cells evaluated 4 wk after treatment with HgCl$_2$ showed a significant increase in wild-type mice only with respect to basal level. The difference in CD4$^+$CD44$^{hi}$ T cells between HgCl$_2$-treated wild-type and Hx-null mice was statistically significant (*, $p < 0.05$). Bars represent mean $\pm$ SE of three separate experiments ($n = 4$/genotype/experiment).](http://www.jimmunol.org/)

Table III. Flow cytometric analysis of cytokine expression by CD4$^+$ T cells in wild-type and Hx-null mice under basal conditions and after treatment with HgCl$_2$ for 4 wk was performed as described in Materials and Methods$^a$

<table>
<thead>
<tr>
<th>Responder</th>
<th>Untreated</th>
<th>HgCl$_2$ Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>Wild-Type</td>
<td>Hx-Null</td>
</tr>
<tr>
<td>CD4/IL-4</td>
<td>1.81 $\pm$ 0.61</td>
<td>1.12 $\pm$ 0.62</td>
</tr>
<tr>
<td>CD4/IFN-$\gamma$</td>
<td>13.94 $\pm$ 3.34</td>
<td>13.58 $\pm$ 3.43</td>
</tr>
<tr>
<td>CD4/IL-17</td>
<td>1.00 $\pm$ 0.35</td>
<td>1.41 $\pm$ 0.68</td>
</tr>
</tbody>
</table>

$^a$Th1, Th2, and Th17 cells were analyzed for the production of IFN-$\gamma$, IL-4, and IL-17, respectively. The percentages of CD4$^+$ T cells expressing the different cytokines are shown. Values represent mean percentage $\pm$ SE of three separate experiments ($n = 4$/genotype/experiment).

![FIGURE 6. Evaluation of total IgG1, IgG2a, and IgE from serum after treatment with HgCl$_2$. The levels of total IgG1, IgG2a, and IgE from serum were measured as described in Materials and Methods section. A and B, Analysis at different time points after HgCl$_2$ injections showed that there was no increase in IgG1 (A) and IgG2a (B) production in Hx-null mice compared with wild-type controls. The increase in serum IgG1 and IgG2a in HgCl$_2$-treated wild-type mice was statistically significant after 4 days and returned to basal levels after 4 wk of treatment (*, $p < 0.05$; **, $p < 0.01$). C, Serum IgE significantly increased in both genotypes 10 days after HgCl$_2$ treatment and returned to basal levels 4 wk later (*, $p < 0.05$; **, $p < 0.01$). No significant differences in IgE were noted between wild-type and Hx-null mice. Values represent mean $\pm$ SE of three separate experiments ($n = 4$/genotype/experiment).](http://www.jimmunol.org/)

Determination of serum levels of IgG1, IgG2a, and IgE after HgCl$_2$ injections

IgG1, IgG2a, and IgE were measured from the serum of mice treated for different time periods with HgCl$_2$ as parameter for cell activation. Interestingly, there was a statistically significant increase in IgG1 and IgG2a after 4 days of treatment in wild-type mice but not in Hx-null mice (Fig. 6, A and B). In contrast, a statistically significant increase in IgE 10 days after HgCl$_2$ injection was seen in both genotypes (Fig. 6C). Four weeks after treatment, these IgS returned to baseline levels.

Spleen cell characterization following OVA/CFA injection

To analyze whether the differences in absolute spleen cell numbers and T cell activation status observed between wild-type and Hx-null mice were specific to HgCl$_2$ injection, we also characterized...
the response to OVA/CFA immunization. Ten days after treatment with OVA/CFA, there was a similar increase in T and B lymphocytes in both genotypes, the increase being more pronounced in the B cell population (Fig. 7A). Moreover, both CD4+ T cells and CD4+CD69+ T cells increased in the same manner following OVA/CFA treatment in Hx-null and wild-type mice (Fig. 7, B and C). There were no statistically significant changes in IL-4 and in IFN-γ in either genotype 10 days after OVA/CFA treatment (not shown).

Overall, OVA/CFA treatment revealed no differences between wild-type and Hx-null mice.

**Effect of IFN-γ treatment on CD4+ T lymphocytes isolated from HgCl2-treated mice**

To study whether an excess of unbound heme in Hx-null mice might affect iron homeostasis in T lymphocytes, we analyzed the expression of the heme-degrading enzyme HO-1 and that of TIR1 on T cells isolated from the spleens of Hx-null and wild-type mice. As expected, HO-1 expression was higher in the T cells of Hx-null mice than in those of wild-type animals, even if the difference did not reach statistical significance (not shown). Moreover, Hx-null splenic T cells showed a significantly reduced TIR1 expression compared with wild-type mice (Fig. 8, A and B). In agreement with Western blot data, flow cytometry analysis demonstrated a significantly lower TIR1 (CD71) expression in Hx-null CD4+ T cells compared with that of wild-type mice (Fig. 8C).

Based on previously reported results showing that iron may affect IFN-γ responsiveness through the TIR1-mediated down-regulation of IFN-γR2 expression at the cell membrane (16), we reasoned that an excess of heme in Hx-null mice might have the same effect. To test this hypothesis, we treated human ST4 T cells with increasing amounts of hemin and then verified their responsiveness to IFN-γ by measuring STAT1 phosphorylation following 15 min of incubation with IFN-γ. Interestingly, hemin at 2.5 and 5 μM significantly inhibited IFN-γ-induced STAT1 activation (not shown). Finally, STAT1 phosphorylation was measured in splenic CD4+ T cells. The endogenous levels of phosphorylated STAT1 in CD4+ T cells isolated from wild-type and Hx-null mice, measured by flow cytometry, were undetectable. We thus treated mice of both genotypes with HgCl2 for 4 days and then determined the responsiveness of splenic CD4+ T cells to IFN-γ by measuring STAT1 phosphorylation following 15 min of incubation with 100U/ml IFN-γ. Interestingly, Hx-null CD4+ T cells showed a statistically significant reduction in the activation of STAT1 after IFN-γ treatment compared with that of wild-type mice. CD4+ T cells from both genotypes displayed the same levels of STAT1 phosphorylation in response to IFN-α, which was used as a control (Fig. 9).

**Discussion**

In this study, we show that 129/Sv wild-type mice elicited an autoimmune response to s.c. injection of the heavy metal mercury as reflected by ANA production and IgG and C3 deposits in several organs. Intriguingly, the absence of hemopexin in mice of the same strain dampened the autoimmune manifestations. Hx-null mice produced significantly less autoantibodies as judged by lower ANA titers in serum and had reduced IC deposits in the kidneys and in other organs compared with wild-type mice after chronic treatment with HgCl2.

The differences in the autoimmune response cannot be ascribed to differences in mercury distribution, inflammatory response, or the ability to produce Abs after challenge with an exogenous Ag. This led to the conclusion of a specific defect in the autoimmune response to the chronic injection of HgCl2 in the knockout mice. Because mercury is a lymphoproliferative agent (17), the absolute cell numbers of the different splenic populations were determined in the two genotypes before and after 4 wk of treatment. There was a significant increase in CD4+ T cells in both genotypes after HgCl2 injections. The APCs also increased in number in both genotypes, but the increase was significantly larger.
in wild-type mice with respect to basal level, especially in CD19^+ B cells and CD11c^+ dendritic cells compared with Hx-null mice. In contrast, after OVA/CFA treatment, a similar increase in absolute T and B cell number was observed in wild-type and Hx-null mice, demonstrating that the difference in B cell proliferation after HgCl₂ treatment was specific to this autoimmune stimulus. The reduced increase in the number of B cells in HgCl₂-treated Hx-null mice with respect to wild-type mice might explain why Hx-null mice produce significantly fewer autoantibodies.

Moreover, Hx-null mice failed to activate CD4^+ T cells as demonstrated by the lack of induction of CD4^+CD69^+ and CD4^+CD44^high T cell populations. Again, this impairment is specific to the autoimmune stimulus, because T cell activation after OVA/CFA treatment is similar in Hx-null and wild-type mice. This notion is supported by previous observations in mice deficient for CD28 and CD40 ligand (18). These mice exhibited reduced numbers of CD4^+CD44^high T cell after HgCl₂ injections and had complete lack of all features of mercury-induced autoimmunity. Due to the reduced activation of T cells, Hx-null T and B lymphocytes might have weaker interactions which could justify the lower production of autoantibodies after mercury exposure.

The percentage of CD4^+CD25^+FoxP3^+ Treg or CD4^+CD17^+ T cells did not change after chronic HgCl₂ injections in the respective genotypes, thus excluding the involvement of Treg or Th17 populations in the pathogenesis of mercury-induced autoimmunity in our models.

Cytokines produced by CD4^+ T cells have been shown to play crucial roles in the development of mercury-induced autoimmune manifestations (19). This point was addressed by our present studies by analyzing IFN-γ and IL-4 expression, assessed as representative cytokines of the Th1 and Th2 subsets, respectively. The expression of IFN-γ and IL-4 in CD4^+ T cells was similar in Hx-null and wild-type mice under basal conditions and did not change after HgCl₂ treatment. Thus, another recently identified T cell subset, Th17, was analyzed. Th17 participates in various diseases like rheumatoid arthritis and multiple sclerosis (20). CD4^+ T cell-specific production of IL-17 in the spleen was measured by flow cytometry, and no induction in the production of IL-17 was noted in either genotype compared with mice under basal conditions. These results show that the lack of hemopexin does not influence the expression of IL-4, IFN-γ, and IL-17 in the CD4^+ T cells, at least in the 129/Sv genetic background, after chronic treatment with HgCl₂.

In contrast, measurements of total IgG isotypes and IgE in serum at different times after HgCl₂ treatment showed a transient and significant increase in IgG1 and IgG2a as well as IgE after 10 days, indicating both Th1- and Th2-type (Th0) responses in wild-type mice. This is in concordance with data from other groups showing that both types of responses are involved in the pathogenesis of mercury-induced autoimmunity (21). Hx-null mice had no change in serum IgG1 or IgG2a levels but had a significant increase in IgE levels, pointing to a Th2-type response in these mice after treatment with HgCl₂. These experiments suggested that Hx-null mice, after HgCl₂ injections, lack a response to IFN-γ, which is crucial for class switching to IgG2a (22).

In accordance with this, Kono and coworkers have shown that cell responsiveness to IFN-γ especially in the early phase of disease induction, rather than IL-4/IFN-γ imbalance, is crucial in determining susceptibility to mercury-induced autoimmunity (23). In agreement with this view, IFN-γ-null as well as IFN-γr-null or IFN regulatory factor-1-null mice showed significant protection against mercury-induced autoimmune lesions (17).

Recently, it has been shown that iron regulates T lymphocyte sensitivity to IFN-γ. In particular, iron uptake mediated by a transferrin receptor delivers a signal that leads to IFN-γR2 internalization, thus attenuating activation of the IFN-γ/STAT1 pathway.
In this study we show that Hx-null T cells have significantly reduced TfR1 expression compared with wild-type mice. Because TfR1 is mainly regulated posttranscriptionally by iron through the iron regulatory protein system (24), our data indicate that Hx-null T cells are indeed iron overloaded compared with those of wild-type mice, likely because these take up the excess heme. In agreement with this, a slight increase in HO-1 in Hx-null T lymphocytes was observed. The increase in HO-1 activation in lymphocytes has been previously reported to suppress the autoimmune response and to limit T cell activation (25). The interplay between TfR1 and IFN-γR2 during CD4⁺ T cell activation is not yet clear, but it has been shown that TfR1 can physically associate with TCR in the immunological synapse (26, 27) and that TCR engagement also induces a rapid copolarization of IFN-γ immunological synapse (26, 27) and that TCR engagement also

In conclusion, our data show a dampened activation of CD4⁺ T cells in Hx-null mice that could be responsible for reduced autoimmune response after mercury exposure. This possibly led to reduced B cell proliferation and differentiation and a lower amount of autoantibody production and hence reduced the amount of systemic IC deposits in Hx-null mice.

Thus, hemopexin modulation may be a new potential therapeutic tool in the treatment of autoimmune disorders.

Acknowledgments

We thank Nanni Passerini for creatinine measurement and proteinuria evaluation, Laura Morando for performing transmission electron microscopy, and Veronica Fiorito for help with some flow cytometry experiments. Valerie Poli, Franco Novelli, and Gabriella Regis are acknowledged for providing ST4 cells and for helpful advice on STAT1/IFN-γ experiments.

Disclosures

The authors have no financial conflict of interest.

References


