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

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

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 progress 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.

A

utoimmune diseases are characterized by unknown etiology and may involve ∼20% of the human population (1). Representative diseases are systemic lupus erythematosus, rheumatoid arthritis, scleroderma, and type I diabetes mellitus, and the hallmark of the autoimmune disease is the production of highly specific autoantibodies that recognize molecules conserved throughout evolution (2). Several factors affect the autoimmune response, including iron-dependent modulation of T cells. Studies in rodents have shown that iron deficiency can lead to thymocyte depletion in cortical and medullary regions of the thymus and a reduced number of spleen T lymphocytes (3). In contrast, rats loaded with iron have been reported to have an increase in CD8+ cells. However, the relationship between iron overload and the immune response is controversial, as other studies have shown that transfusional iron overload in thalassemia intermedia patients is associated with a blunted response of T cells to mitogens and with decreases in circulating CD4+ cells and CD4/CD8 ratios (4). Finally, on investigating the effect of iron status on murine systemic lupus erythematosus, Leiter et al. have found that iron supplementation leads to more severe renal disease and increases mortality (5).

Several genes involved in iron handling have been associated with T cell abnormalities, including the hemochromatosis gene HFE and the iron transporter NRAMP1 (6). In particular, the latter has been linked to rheumatoid arthritis, juvenile rheumatoid arthritis, and multiple sclerosis. More recently, the haptoglobin α2 polypeptide has been found to be over-expressed in the serum of systemic lupus erythematosus patients (7). Haptoglobin is an acute phase plasma protein with high binding affinity for hemoglobin and prevents hemoglobin-derived iron loss. The overexpression in systemic lupus erythematosus patients of the haptoglobin α2 polypeptide, which has a higher iron binding capacity than the other variant, α1, suggests that heme-iron availability may modulate the autoimmune response.

Hemopexin (∼10–20 μM in plasma) is a 60-kDa, circulating, class I, acute phase reactant with the highest binding affinity to heme (Kd < 1 pM). It binds free heme and mediates its uptake in liver cells through receptor-mediated endocytosis. Heme is then catalyzed by heme oxygenase (HO)4 into biliverdin, carbon monoxide (CO), and iron. In this way, hemopexin is thought to prevent heme-iron loss and the proinflammatory effects of free heme. Studies in vivo on hemopexin-null (Hx-null) mice have confirmed this view. Indeed, Hx-null mice recover less efficiently than wild-type controls after an acute hemolytic stress and accumulate iron in proximal tubular cells of the kidney (8). In parallel, the lack

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3 E.T. and B.B. contributed equally to this work.

4 Abbreviations 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.

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of circulating hemopexin may increase the peripheral cell avail-
ability 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 treat-
ment 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 chlo-
ride (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 (TIR1) expression in T cells under basal
conditions possibly dependent on higher heme-derived iron in
these mice. Finally, we demonstrated that IFN-γ-induced activa-
tion 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 availabil-
ity 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 (pro-
longed 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 retro-orbitally under anesthesia
and sacrificed. Their hearts, skin, spleens, and kidneys were removed asep-
tically under aseptic conditions. Kidney, heart, and skin tissues were
snap frozen, and stored at -80°C until further use.

Detection of ANA by immunofluorescence

The serum levels of IgG ANA were assayed by using indirect immuno-
fluorescence. 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-conju-
gated goat anti-mouse IgG (Sigma-Aldrich) for 45 min at 37°C. The
titrates were expressed as the reciprocal value of the highest serum dilution
that gave an unequivocal positive reaction.

Detection of deposits of IgG and C3 in kidneys, muscles, hearts, and skin

The presence of glomerular deposits of IgG and C3 Abs was detected by
direct immunofluorescence performed on mouse tissues rapidly frozen in
liquid nitrogen. Cryostat sections, nonincubated in cold acetone fol-
lowed by fixing in 3.5% paraformaldehyde containing 2% sucrose as pre-
viously described (11). After saturation with PBS-BSA, the sections were
incubated with FITC-conjugated goat anti-mouse IgG or anti-mouse C3
(Sigma-Aldrich). Muscle, skin and heart were treated similarly. Immune
deposits were analyzed using a Zeiss fluorescence microscope.

Serum Ig levels

Serum IgE levels were determined using an immunoassay for mouse IgE
(OPT EIA mouse IgE set; BD Pharmingen). Anti-mouse IgE mAb (cap-
ture) was coated on 96-well plates (Falcon) and incubated overnight.
Standard and serum (diluted 1:10) were incubated followed by detection with
biotinylated anti-mouse IgE, avidin-HRP, and tetramethylbenzidine sub-
strate (Sigma-Aldrich). Serum IgG1 and IgG2a levels were determined using
an ELISA kit (BD Pharmingen) according to manufacturer’s instruc-
tions. Sera were diluted 1/100 in blocking buffer. Absorbance values were
measured at 450 nm.

Electron microscopy

Transmission electron microscopy was performed using Karnovsky’s fix-
ative with osmium tetroxide-postfixed tissues embedded in epoxy resin
according to standard procedures (12). Ultrathin sections were stained with
uranyl acetate and lead citrate and examined with a Jeol JEM 1010 electron
microscope.

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 pre-
pared by pipetting several times and subjecting to a Ficoll gradient. The
lymphocytes were recovered, resuspended, counted, and adjusted to a
density of 1 x 10⁶ cells/ml.

The mAbs used for the detection of cell surface markers and intracel-
larular cytokines are documented in Table I. Briefly, the isolated spleen
and blood cells were washed with PBS-BSA (0.25%), stained with the appro-
priate cell surface Ab or the relevant control for 30 min at room temper-
ature, and then washed again and resuspended in PBS-BSA. For intracel-
ular 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 (eBioscience; Insight Biotechnology) ac-
cording to the manufacturer’s instructions. To measure the levels of phos-
phorylated STAT1, cells were fixed immediately after stimulation with 4%
paraformaldehyde, permeabilized with ice-cold methanol followed by sa-
ponin, and then simultaneously incubated with the anti-phospho(Tyr³³³),
STAT1-PE or anti-IgG-PE and CD4-allophycocyanin Abs (BD Bio-
sciences). Cells were analyzed on a FACS (BD Biosciences). Seventy
thousand cells were analyzed at each experimental point for intracel-
ular 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 x 10⁶ splenocytes were
exposed 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(Tyr³³³)-STAT1 and CD4 staining and flow cytometry analy-
ysis. IFN-α (100U/ml) was used as control.

Activation of T cells ex vivo

To analyze cytokine production by T cells ex vivo, 2 x 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) simultaneously. 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, au-
ometallographic silver enhancement was performed (13, 14). Briefly, gel-
atin-coated, paraffin-included sections of tissues were placed in an au-
ometallographic developer containing a mixture of gum arabic, citrate buffer
(pH 3.7), hydroquinone, and silver lactate and incubated at 26°C for 60
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 Abs (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 microtiter trays. The samples were incubated at 75 °C for 1.5 h at room temperature. The wells were then washed five times and incubated 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 α-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-CD19 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).

**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 only 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 (Fig. 1, A and B). Generalized and diffuse granular deposits of IgG were observed by immunofluorescence in the glomerular capillary walls and mesangium and in the basal parts of the proximal, distal, and collecting tubules of the wild-type mice (Fig. 1, C and D). C3 deposition was also present in the glomerular capillary walls, mesangium, Bowman’s capsule, and tubular basement membrane (Fig. 1, C and D). A similar deposition of IgG was detected in the cardiac, skin (not shown), and muscle capillaries (Fig. 1, E and F).

Electron microscopy, dense deposits were seen in the glomerular capillary wall and mesangial matrix of the renal glomeruli of wild-type mice (Fig. 1, C and D). In contrast, the Hx-null mice showed little or no deposition of immune complexes or complement in the kidney (Fig. 1, D and E). Heart, skin (not shown), and muscle capillaries (Fig. 1, A and L) were not affected by electron microscopy, dense deposits were seen in the glomerular capillary wall and mesangial matrix of the renal glomeruli of wild-type mice (Fig. 1, C and D). In contrast, the Hx-null mice showed little or no deposition of immune complexes or complement in the kidney (Fig. 1, D and E). Heart, skin (not shown), and muscle capillaries (Fig. 1, A and L) were not affected by electron microscopy.

<table>
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<td>Allophycocyanin</td>
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<td>eBioscience</td>
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* Negative isotope control.
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).
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 (Fig. 2A). Thus, plasma hemopexin did not control the distribution of mercury to the tissues after HgCl₂ injection in mice.

**Similar inflammatory responses, vascular permeability, and immune reactions to foreign Ag in wild-type and Hx-null mice**

To investigate whether Hx-null mice had defects in inflammatory processes and/or vascular permeability, passive Arthus reaction was performed. In this reaction, OVA plus Evans blue was injected in the tail vein of mice of both genotypes and anti-OVA Abs were injected s.c. in two sites. There was no difference in inter-genotype vascular permeability as evaluated by Evans blue dye diffusion (wild-type mice: 11 mm ± 0.1; Hx-null mice: 10 mm ± 0.2). In addition, a dense leukocyte infiltration was observed at the site of anti-OVA injection in both wild-type (Fig. 3A) and Hx-null mice (Fig. 3B). These data indicate that the inflammatory response due to leukocyte activation by immune deposit formation in the skin was maintained in the Hx-null mice.

Moreover, Hx-null mice immunized with OVA were able to produce anti-OVA Abs (Fig. 3C) at titers similar to those of wild-type mice. Thus, Hx-null mice were able to respond to a foreign Ag administration by producing Abs, suggesting a specific defect in autoantibody production after mercury exposure.

**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). A significantly larger increase in the number of APCs occurred in wild-type mice after treatment vs Hx-null ones. The increase in CD19+ B cells (D) was statistically significant in wild-type controls only. Macrophages (E and F) did not change while the increase in CD11c+ dendritic cells (G) was statistically significant in both genotypes (***, p < 0.001). Data represent mean percentage ± SE of five separate experiments (n = 4/genotype/experiment).

Table II.  Flow cytometric evaluation of Treg population

<table>
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<th>Hx-Null Mice</th>
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<td>CD4/CD25</td>
<td>8.56 ± 0.58</td>
<td>9.227 ± 0.25</td>
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</table>

*a 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).

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 the untreated wild-type and Hx-null mice.
three separate experiments (Table III). Flow cytometric analysis of cytokine expression by CD4
expression was noted (not shown). Yet another CD4
applied Biosystems). Again, no difference in Th1 and Th2 cytokine
, IFN-
was statistically significant (\( p < 0.05 \)). Bars represent mean ± SE of three separate experiments (\( n = 4 \)/genotype/experiment).

either genotype after treatment with HgCl2 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 HgCl2 (data not shown). To confirm the cytokine profile, quantitative RT PCR was performed on spleen total RNA of 10-day HgCl2-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 expression 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 HgCl2 treatment in both Hx-null and wild-type mice compared with nontreated mice (Table III).

### 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 HgCl2 for 4 wk was performed as described in Materials and Methods

<table>
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<th></th>
<th>Untreated</th>
<th>HgCl2 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-Type</td>
<td>Hx-Null</td>
</tr>
<tr>
<td>CD4/IL-4</td>
<td>1.81 ± 0.61</td>
<td>1.12 ± 0.62</td>
</tr>
<tr>
<td>CD4/IL-17</td>
<td>13.94 ± 3.34</td>
<td>13.58 ± 3.43</td>
</tr>
<tr>
<td>CD4/IFN-( \gamma )</td>
<td>1.00 ± 0.35</td>
<td>1.41 ± 0.68</td>
</tr>
</tbody>
</table>

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

### FIGURE 5. Expression of activation markers on CD4\(^+\) T cells after treatment with HgCl2. Mice were treated as described in Materials and Methods. A, Percentages of splenic CD4\(^+\)CD69\(^+\) T cells evaluated 1 day after treatment with HgCl2 showed a significant increase in wild-type mice only (\( \ast , p < 0.05 \)). B, Percentages of splenic CD4\(^+\)CD44\(^{high}\) T cells evaluated 4 wk after treatment with HgCl2 showed a significant increase in wild-type mice only with respect to basal level. The difference in CD4\(^+\)CD44\(^{high}\) T cells between HgCl2-treated wild-type and Hx-null mice was statistically significant (\( \ast , p < 0.05 \)).

### FIGURE 6. Evaluation of total IgG1, IgG2a, and IgE from serum after treatment with HgCl2. 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 HgCl2 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 HgCl2-treated wild-type mice was statistically significant after 4 days and returned to basal levels after 4 wk of treatment (\( \ast , p < 0.05 \); **, \( p < 0.01 \)). C, Serum IgE significantly increased in both genotypes 10 days after HgCl2 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 ± SE of three separate experiments (\( n = 4 \)/genotype/experiment).

### Determination of serum levels of IgG1, IgG2a, and IgE after HgCl2 injections

IgG1, IgG2a, and IgE were measured from the serum of mice treated for different time periods with HgCl2 as parameter for B 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 HgCl2 injection was seen in both genotypes (Fig. 6C). Four weeks after treatment, these IgEs 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 HgCl2 injection, we also characterized
Role of Hemeopexin in Autoimmunity

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⁺CD69⁺ and CD4⁺CD44high 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 TfR1 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 TfR1 expression compared with wild-type mice (Fig. 8, A and B). In agreement with Western blot data, flow cytometry analysis demonstrated a significantly lower TfR1 (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 TfR1-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 hemeopexin 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.
null mouse CD4\(^+\) T cells after OVA/CFA treatment is similar in wild-type and Hx-null mice. OVA/CFA treatment is specific to this autoimmune stimulus. The reduced increase in the number of B cells in HgCl\(_2\)-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 HgCl\(_2\), 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 HgCl\(_2\) 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-\(\gamma\) and IL-4 expression, assessed as representative cytokines of the Th1 and Th2 subsets, respectively. The expression of IFN-\(\gamma\) and IL-4 in CD4\(^+\) T cells was similar in Hx-null and wild-type mice under basal conditions and did not change after HgCl\(_2\) 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 cells did not change after chronic HgCl\(_2\) injections in the respective genotypes, thus excluding the involvement of Treg or Th17 populations in the pathogenesis of mercury-induced autoimmunity in our models.

In contrast, measurements of total IgG isotypes and IgE in serum at different times after HgCl\(_2\) treatment showed a transient and significant increase in IgG1 and IgG2a after 4 days as well as in IgE after 10 days, indicating both Th1- and Th2-type 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 HgCl\(_2\). These experiments suggested that Hx-null mice, after HgCl\(_2\) injections, lack a response to IFN-\(\gamma\), which is crucial for class switching to IgG2a (22).

In accordance with this, Kono and coworkers have shown that cell responsiveness to IFN-\(\gamma\) especially in the early phase of disease induction, rather than IL-4/IFN-\(\gamma\) imbalance, is crucial in determining susceptibility to mercury-induced autoimmunity (23). In agreement with this view, IFN-\(\gamma\)-null as well as IFN-\(\gamma\)R-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-\(\gamma\). In particular, iron uptake mediated by a transferrin receptor delivers a signal that leads to IFN-\(\gamma\)R2 internalization, thus attenuating activation of the IFN-\(\gamma\)/STAT1 pathway.
The iron chelating agent desferroxamine up-regulates IFN-γR2 surface expression and reinstates IFN-γ/STAT1 activation. We have previously shown the importance of hemopexin in recuperating heme-derived iron from the circulation (8). In Hx-null mice, it is expected that heme-iron is not recovered by the liver but taken up by other tissues, including lymphocytes.

In this study we show that Hx-null T cells have significantly reduced TIR1 expression compared with wild-type mice. Because TIR1 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 TIR1 and IFN-γR2 during CD4+ T cell activation is not yet clear, but it has been shown that TIR1 can physically associate with TCR in the immunological synapse (26, 27) and that TCR engagement also induces a rapid copolarization of IFN-γR with TCR (28). Moreover, Regis et al. have recently demonstrated that TIR1 expression at the plasma membrane modulates the IFN-γR level, thus regulating the responsiveness of T cells to IFN-γ (16).

Importantly, ex vivo treatment of splenocytes with IFN-γ showed that there was a statistically significant reduction in STAT1 phosphorylation in CD4+ T lymphocytes isolated from HgCl2-treated Hx-null mice compared with that of HgCl2-treated wild-type mice. Moreover, in vitro treatment of human ST4 T cells with hemin followed by IFN-γ stimulation demonstrated that STAT1 phosphorylation is reduced, supporting our hypothesis that free heme might affect IFN-γ signaling.

Our data suggest that the blunted T cell activation in Hx-null mice is accounted for by their refractoriness to IFN-γ according to the model presented in Fig. 10. In wild-type mice, extracellular heme does not contribute to the intracellular iron pool because it is bound by hemopexin and transported to the liver. In Hx-null mice, unbound heme is taken up by the cell and, consequently, is metabolized by enzyme HO-1 into biliverdin, CO, and iron. The increase in the iron pool results in TIR1 mRNA degradation and lower protein expression at the plasma membrane. This causes the down-regulation of IFN-γR2 expression, inducing refractoriness of CD4+ T cells to IFN-γ.

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.

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Disclosures
The authors have no financial conflict of interest.

References


