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A Role for B Cell-Activating Factor of the TNF Family in Chemically Induced Autoimmunity

Yan Zheng,* Stefania Gallucci,‡ John P. Gaughan,† Jane A. Gross,* and Marc Monestier*1

After exposure to subtoxic doses of heavy metals such as mercury, H-2b mice develop an autoimmune syndrome consisting of the rapid production of IgG autoantibodies that are highly specific for nucleolar autoantigens and a polyclonal increase in serum IgG1 and IgE. In this study, we observe that HgCl2 administration in susceptible mice results in the elevated production of B cell-activating factor of the TNF family (BAFF) also known as BLYS, TALL-1, zTNF-4, THANK, and TNSF13B), a B cell growth factor belonging to the TNF family. A transmembrane activator and calcium-modulating and cyclophilin ligand interactor (TACI)-Ig fusion protein (which neutralizes both BAFF and a proliferation-inducing ligand (APRIL), another TNF family member) inhibited Hg-induced autoantibody or serum IgE production. These results are discussed in the context of the inhibitory effect of TACI-Ig on B cell maturation at the transitional stage. The Journal of Immunology, 2005, 175: 6163–6168.

Follow our productive Ig gene rearrangements in the bone marrow, newly emerging B cells in the periphery go through transitional stages associated with negative selection steps before becoming fully mature B cells (MB). Early reports described two transitional stages, transitional type 1 B cells (T1) and transitional type 2 B cells (T2), in the mouse (1). Certain members of the TNF superfamily and their receptors are critical for the development of the mammalian immune system, being involved in the organogenesis of lymphoid organs and the regulation of efficient adaptive immune responses (2). In recent years, two closely related TNF family members, B cell-activating factor of the TNF family (BAFF), also known as BLYS, TALL-1, zTNF-4, THANK, and TNSF13B (3–7) and a proliferation-inducing ligand (APRIL) (8), have been identified that are critical for immune system function (9). There are three currently identified receptors for BAFF and APRIL: B cell maturation protein (BCMA) (10), transmembrane activator and calcium-modulating and cyclophilin ligand interactor (TACI) (11), and BAFF receptor (BAFF-R; BR3) (12). Expression of BCMA and BAFF-R is thought to be restricted to B cells, whereas TACI is expressed by both B cells and activated T cells (9, 13, 14). Recently, Ng et al. (15) revisited BAFF-R expression and showed its expression on activated/memory subset of T cells. The individual role of these factors or their receptors is not fully elucidated, but mice treated with TACI-Ig (a soluble TACI fusion protein) and TACI-Ig transgenic mice have fewer transitional T2 and MB. Furthermore, TACI-Ig treatment inhibits disease progression of collagen-induced arthritis (16). In BLYS−/− mice, B cell maturation arrests at the transitional T1 stage (16, 17). However, APRIL−/− mice do not show abnormalities in T and B cell development and functions or in T cell-dependent or -independent humoral responses (18). These data indicate that APRIL may be dispensable and that BAFF is the major player for the development of the immune system. Research on the BAFF/BLYS system and its receptors has shown its crucial impact on the immune response and especially B cell survival (19).

Systemic autoimmune diseases result from a complex interaction between genetic and environmental factors. Mercury, an environmental pollutant, has been used in a wide range of commercial and medical products, which give rise to accidental and occupational exposures. In humans, chronic exposure to low doses of elemental mercury or mercuric salt can induce glomerulonephritis and proteinuria (20, 21), excessive T cell activation, increased level of serum IgE, and antinuclear Abs in the serum (22, 23). Some of these features are also observed in the HgCl2 model of murine autoimmune disease. Mouse or rat strains expressing certain MHC Ags are susceptible to the heavy metal induction of a complex autoimmune syndrome. In susceptible H-2b mice, subtoxic doses of HgCl2 induce an autoimmune syndrome characterized by production of anti-nucleolar Abs (ANoA) and increased serum levels of IgG1 and IgE. The increase in serum IgS peaks 2–3 wk after the beginning of the HgCl2 injections, whereas ANoA can persist for several months after the induction phase (24–26). In the present study, we observe that HgCl2 administration induces BAFF production only in susceptible A.SW mice. TACI-Ig (which neutralizes both BAFF and APRIL) administration prevents ANoA or IgE induction in HgCl2-treated mice.

Materials and Methods

Mice

A.SW/SnJ (H-2b), CS7BL/6 (H-2d), and DBA/2 (H-2b) mice were obtained from The Jackson Laboratory and maintained in our animal facilities. All of the mice used in our experiments were at least 2 mo old.

Recombinant TACI-Ig protein

The neutralizing TACI-Ig fusion protein and the control Hu-Ig (the Fc portion of human IgG1 produced for TACI-Ig) were prepared as described before (16).
**HgCl$_2$ and TACI-Ig treatment**

Mercury-induced autoimmunity was induced according to a standard protocol by three s.c. injections (30 μg of HgCl$_2$ in 100 μl of sterile PBS) every other day (27). In addition to HgCl$_2$, some groups of mice received TACI-Ig or control Hu-Ig. TACI-Ig injection schedules are depicted in Fig. 1. In all cases, fusion proteins were administered i.p., and mice received 100 μg of each protein in 500 μl of sterile PBS per injection.

**ELISA for mouse serum BAFF**

Serum BAFF levels were determined using an ELISA detection kit for soluble mouse BAFF (ApoTech). A 96-well ELISA plate was coated overnight at 4°C with 50 μl/well of a 5 μg/ml solution of mAb to BAFF (S8) diluted in PBS (pH 7.4). After extensive washes and a blocking step with 200 μl of PBS containing 5% FCS for 1 h at 37°C, a standard of recombinant mouse soluble BAFF diluted in PBS containing 0.5% FCS and mouse serum samples were added. After incubation at 37°C for 2 h, wells were extensively washed. Then 50 μl/well of a 2 μg/ml solution of biotinylated mAb to BAFF (IC9) diluted in PBS containing 0.5% FCS was added. After incubation for 1 h at 37°C, wells were extensively washed. A total of 50 μl/well of avidin-HRP conjugate (BD Pharmingen) diluted 1/500 was added for 30 min. After extensive washes, 100 μl/well 3,3',5,5'-tetramethylbenzidine substrate working solution (BD Pharmingen) was used as a substrate, and 20 min later, 100 μl/well stop solution containing 1 M phosphoric acid was added, and absorbance was measured at 450 nm subtracted by 570 nm in an ELISA reader.

**ANoA immunofluorescence**

Serum ANoA levels were determined by indirect immunofluorescence, as described previously (27). Sera diluted in PBN (PBS containing 1% BSA and 0.02% sodium azide) were incubated with Hep-2 slides (Antibodies, Inc.) for 30 min, and ANoA were detected with FITC-conjugated goat IgG1 (the main subclass in HgCl$_2$-induced autoimmunity) Abs (Southern Biotechnology Associates). The inverse of the highest serum dilution at which nucleolar fluorescence could be detected was defined as the ANoA titer.

**ELISA for mouse serum IgG1 and IgE**

Total serum IgG1 and IgE levels were determined using a sandwich ELISA as previously described (27).

**FIGURE 1.** Time course of HgCl$_2$ and TACI-Ig fusion protein injection protocols. In both Protocols 1 and 2, mice received s.c. injections of 30 μg of HgCl$_2$ in 100 μl of sterile PBS three times on days 0, 2, and 4. A.SW mice were treated via i.p. injection with 100 μg of TACI-Ig fusion protein or control Hu-Ig (the Fc portion of human IgG1) three times per week starting from day 0 for 2 wk (Protocol 1) or starting from day −7 for 3 wk (Protocol 2).

**Cell preparation and staining**

Suspensions of mouse splenocytes were prepared through perfusion of spleens with FACS buffer (PBS containing 0.5% BSA, 1 mM EDTA, and 0.05% sodium azide). Following lysis of RBCs with 0.165 M NH$_4$Cl, cells were washed then pretained for 10 min with rat anti-mouse CD16/CD32 (clone 2.4G2) Abs to block FcγR, and then incubated with optimal dilutions of the indicated Abs in Eppendorf tubes in a final volume of 50 μl. After 30 min on ice, cells were washed twice with FACS buffer then fixed in 1% formaldehyde in PBS.

**Abs and flow cytometric analyses**

Commercially obtained Abs used in these studies include PE-conjugated anti-mouse IgM (R6-60.2), FITC-conjugated anti-mouse CD121/CD35 (7G6), PerCP-Cy5.5-conjugated anti-mouse CD19 (ID3), and allophycocyanin-conjugated anti-mouse CD45R/B220 (RA3-6B2), all from BD Pharmingen. In parallel tubes, cells were stained with isotype control Abs to subtract nonspecific staining. Analyses were conducted on a dual laser flow cytometer (FACSCalibur; BD Immunocytometry Systems). All flow cytometry data were analyzed by CellQuest software (BD Immunocytometry Systems).

**Statistical methods**

Using SAS version 9.0 (SAS Institute), the dependent variables serum IgG1, IgE, and ANoA-IgG were treated as continuous variables for all analyses. Means, SDs, and number of observations are presented for each variable. The experimental unit was each individual mouse.

The experiment used a repeated measures design with each mouse evaluated at five periods. The null hypothesis was that there would be no difference between groups or week. Before analysis, all data were tested for normality using the Shapiro-Wilk test. The data were significantly non-normal for all variables. To apply ANOVA methods, a “normalized-rank” transformation was applied to the data. The rank-transformed data was analyzed using a mixed-model ANOVA for repeated measures followed by multiple comparisons to detect significant mean differences between groups at each week. Multiple pair-wise comparisons used the Bonferroni adjustment to maintain an experiment-wise type I error of 0.05 or less. Differences between means (rejection of the null hypothesis) were considered significant if the probability of chance occurrence was ≤0.05 using two-tailed test. Adjusted $p$ values are presented for each analysis.

**Results**

### 3.1 HgCl$_2$ treatment induces BAFF production only in susceptible A.SW mice

Because BAFF plays an essential role in B cell maturation and can affect immune tolerance (28), we investigated whether it contributes to the emergence of autoreactive B cells in HgCl$_2$-induced autoimmunity. To assess this question, we first measured BAFF serum levels in susceptible A.SW mice, as well as resistant C57BL/6 and DBA/2 mice (29), all of which received three HgCl$_2$ injections at days 0, 2, and 4. As expected, susceptible A.SW mice showed significant increases in serum IgG1, IgE, and ANoA levels, whereas resistant strains did not show any significant response (Fig. 2). Moreover, we observed steadily increasing serum BAFF levels exclusively in susceptible A.SW mice for several weeks even though HgCl$_2$ administration was only conducted between days 0 and 4 (Fig. 2). BAFF levels remained undetectable in HgCl$_2$-treated C57BL/6 and DBA/2 mice. As a control, A.SW mice also received injections with PBS alone, and BAFF level also remain undetectable by ELISA (data not shown).

### 3.2 TACI-Ig treatment during the induction phase reduces autoantibody production in HgCl$_2$-induced autoimmunity

To assess the effect of TACI-Ig in HgCl$_2$-induced autoimmunity, we initially conducted a series of experiments (protocol 1; Fig. 1) in which groups of susceptible A.SW mice received three HgCl$_2$ injections during the first week. TACI-Ig or control Hu-Ig was administered three times a week i.p. for 2 wk (from day 0) during the induction phase of the disease (see details in Fig. 1). Mice treated with both HgCl$_2$ and TACI-Ig fusion protein developed significantly lower ($p < 0.01$) ANoA IgG1 levels than mice...
3.3 TACI-Ig treatment before the induction phase reduces IgE production in HgCl₂-induced autoimmunity

Because the TACI-Ig regimen in protocol 1 did not affect the polyclonal induction of IgG1 and IgE, we administered TACI-Ig 1 wk (day −7) before the first injection of HgCl₂ at day 0 (protocol 2). TACI-Ig or control Hu-Ig was administered 3 times a week i.p. for 3 wk, whereas HgCl₂ injections were given at days 0, 2, and 4 (Fig. 1). This TACI-Ig treatment regimen significantly inhibited IgE induction in Hg-treated mice (p < 0.05 at week 2), although there were no significant differences between control and TACI-Ig groups with respect to serum IgG1 levels (Fig. 4). TACI-Ig pre-treatment somewhat inhibited ANoA production, but statistical significance (p < 0.001) was only observed at week 2 where ANoA levels had yet to appear in mice that received TACI-Ig (Fig. 4).

3.4 Effect of HgCl₂ and TACI-Ig on splenic B cell development

Because TACI-Ig blocks B cell maturation at the transitional stage, we also examined splenic B cell subpopulations to determine which subsets were affected by treatment with TACI-Ig and/or HgCl₂. Peripheral B cell maturation occurs in multiple discrete steps. In the model described by Loder et al. (1), transitional B cells are divided into two subsets, T1 (sIgM<sup>high</sup>CD21/35<sup>low</sup>) and T2 (sIgM<sup>high</sup>CD21/35<sup>high</sup>); T2 is envisioned to yield marginal zone B cells (MZ). We have examined the splenic B cell development according to this model following HgCl₂ and/or TACI-Ig treatment. Splenocytes from four groups of mice treated for 2 wks with PBS plus Hu-Ig (group I), HgCl₂ plus Hu-Ig (group II), TACI-Ig plus PBS (group III), or HgCl₂ plus TACI-Ig (group IV) were collected, stained with appropriate Abs, and examined by flow cytometry. By comparing group I and III, TACI-Ig treatment itself showed a dramatic loss of MB and T2/MZ B cells with accumulation of T1 B cells (Fig. 5 and Table I). These results confirmed previous reported data that blocking BAFF induces a specific arrest in B cell development at the immature transitional T1 stage and has dramatic effect on survival of MB cells (10, 16). Similar results were found when we compared group II and IV, in which both groups of mice received HgCl₂ administration. Consistent with previous study showing that HgCl₂ administration resulted in an increase in splenic B cell numbers by week 2 (27, 30), our HgCl₂ administration also increased absolute number of B cells, including MB, T2/MZ, and T1. More importantly, TACI-Ig still was able to reverse this HgCl₂-induced phenomenon, showing a normal absolute number of B cells (compared with group I) and a reduction in their transitional populations with a particularly pronounced decrease at the T1 to T2 transition (Fig. 5 and Table I).

Discussion

The complexity of the TNF family of proteins underlies the intricacy of their biological effects on the immune system. An example of this complexity is the association of BAFF (BLyS) and APRIL with three known receptors: TACI, BCMA, and BAFF-R (7, 12, 31). BAFF expression is particularly associated with macrophages, monocytes, and dendritic cells (4, 5, 32), whereas APRIL is expressed at low levels by lymphoid cells and at higher levels by some tumor cells (8). In contrast to TACI and BCMA, BAFF-R selectively binds BAFF, but not APRIL (12). BAFF and APRIL influence B cell maturation and survival at several levels. BAFF can stimulate B cells in vivo (5, 6, 33) and in vitro (7, 28), whereas APRIL acts as a costimulator for T cells (33) and enhances the survival of some tumors (8). BAFF transgenic mice produce autoantibodies and develop autoimmune disease manifestations reminiscent of human systemic lupus erythematosus and Sjögren’s syndrome (16, 28, 34–36). Several studies also have revealed the presence of increased BAFF levels in sera from patients with autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Sjögren’s syndrome (35, 37, 38). Consistent...
with these findings, we also observed that HgCl₂ treatment resulted in elevated levels of BAFF only in susceptible A.SW mice. The increase in serum BAFF continued for several weeks even though mice received only three HgCl₂ injections. Lesley et al. (39) have shown that, compared with naive B cells, anergic B cells have a greater dependence on BAFF for survival. Moreover, studies have shown that overexpression of BAFF attenuated apoptosis by changing the ratio between Bcl-2 family proteins in favor of cell survival, predominantly by reducing the proapoptotic Bak and increasing its antiapoptotic partners, Bcl-2 and Bcl-xL (40, 41). By rescuing anergic B cells from competitive elimination and accumulation of autoreactive lymphocytes from attenuation of apoptosis, the increase in BAFF serum levels in A.SW mice may thus play a critical role in the susceptibility to HgCl₂ observed in this strain.

In Sjögren’s syndrome, the presence of BAFF can be detected in tissue lesions (35). In human rheumatoid arthritis, the concentration of BAFF in the synovial fluid is greater than in the blood (38). It is therefore likely that inflamed tissue represents a major site of BAFF production. In susceptible animals, administration of HgCl₂ leads to immune-mediated tissue pathology including glomerulonephritis and vasculitis. In our studies, even though HgCl₂ was injected only from days 0 to 4, BAFF production increased for several weeks, suggesting that the inflammatory lesions contributed to BAFF production. The absence of BAFF in mice that are resistant to mercury and do not develop tissue lesions support this view. This production of BAFF during chemically mediated autoimmunity will result in increased systemic levels, further affecting B cells in secondary lymphoid organs and leading to increased autoantibody production. This view is supported by earlier studies showing that IFN-γ is required for autoantibody production in mercury-induced autoimmunity (42) and by observations showing
levels. When TACI-Ig was administered in a prevention regimen no significant differences in terms of serum total IgG1 and IgE susceptibility A.SW mice led to a disruption of autoimmunity, as and CD21 high-IgM high (T2/MZ). IgM-positive cells are shown. CD35 positive-IgM intermediate (M), CD21/CD35 low-IgM high (T1), development were calculated as percentages of live cells as follows: CD21/CD19, and allophycocyanin anti-CD45R/B220. Splenocytes were gated for forward light scatter-side scatter of live cells, laser FACSCalibur flow cytometer. Events (200,000) were analyzed. Left, Splenocytes were stained with PE anti-IgM, FITC anti-CD21/CD35, PerCP-Cy5.5 anti-CD19, and IgM-positive cells are shown. Right, The different stages of B cell development were calculated as percentages of live cells as follows: CD21/CD35 positive-IgM intermediate (M), CD21/CD35 low-IgM high (T1), and CD21 high-IgM high (T2/MZ). IgM-positive cells are shown.

that IFN-γ up-regulates BAFF production by macrophages and monocytes (32).

We used a TACI-Ig fusion protein, which neutralizes both BLyS and APRIL, to evaluate the role of these ligands and their receptors in our HgCl2-induced autoimmunity mouse model. The administration of TACI-Ig during the induction phase of HgCl2-treated susceptible A.SW mice led to a disruption of autoimmunity, as evidenced by significant lower ANoA levels, although there were no significant differences in terms of serum total IgG1 and IgE levels. When TACI-Ig was administered in a prevention regimen (7 days before HgCl2 injection), it significantly blocked IgE induction although its effect on ANoA was more transient than when given only during the induction phase. The limited effect of TACI-Ig on ANoA production in protocol 2 may be due to a neutralizing response of the mouse immune system toward the human TACI-Ig fusion protein as was reported in a recent study in murine lupus (43). Under both treatment protocols, the TACI-Ig treatment did not substantially change the serum IgG1 level. This suggests that this feature of HgCl2-induced autoimmunity is BAFF-independent and is consistent with previous findings that mice either transgenic for BAFF or injected with BAFF showed significantly increased levels of serum IgM and IgA, but not IgG (6, 34, 40). Previous studies of this model have also shown that different features such as ANoA production and polyclonal activation can be individually manipulated (44, 45). The present findings confirm that the various manifestations of Hg-induced autoimmunity are separately regulated.

After maturation in the bone marrow, newly formed B cells migrate to the secondary lymphoid organs (spleen and lymph nodes). These B cells do not possess all the characteristics of fully MBs, and they are referred to as transitional B cells. These transitional B cells are immature because they do not proliferate after surface IgM cross-linking. Turnover studies also indicate significant cell losses at the T1–T2 transition, suggesting that this step is a selection checkpoint where self-reactive B cells may be eliminated. Therefore, the transitional B cell stages represent important regulatory milestones in B cell development (46). The characterization of splenic B cells in both TACI-Ig-treated and TACI-transgenic mice revealed a specific arrest in B cell development at the immature transitional T1 stage (16). Indeed, we also observed a similar result, in which there is a dramatic decrease in cell numbers at the T1–T2 transition in mice treated with TACI-Ig.

As mentioned above, the role of BAFF in B cell activation has suggested a therapeutic use for TACI-Ig in the treatment of autoantibody-associated autoimmune diseases. Indeed, an earlier study has shown that TACI-Ig suppresses the production of anti-collagen Abs and disease in a model of collagen-induced arthritis (16). Our results in the Hg-induced autoimmunity model show that TACI-Ig can be effective at inhibiting autoimmune manifestations, but the specifics of this inhibition (IgE increase vs autoantibody production) vary with the schedule of administration of TACI-Ig.

Disclosures
The authors have no financial conflict of interest.

Table I. Percentage and numbers of B cell subsets in HgCl2 and/or TACI-Ig-treated mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>I (PBS + Hu-Ig)</th>
<th>II (HgCl2 + Hu-Ig)</th>
<th>III (PBS + TACI-Ig)</th>
<th>IV (TACI-Ig + HgCl2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells (%)</td>
<td>41.61 ± 0.94</td>
<td>42.68 ± 6.48</td>
<td>17.60 ± 0.10</td>
<td>29.44 ± 0.05</td>
</tr>
<tr>
<td>MB absolute numbers (×10⁶)</td>
<td>20.53 ± 0.02</td>
<td>37.01 ± 1.54</td>
<td>8.10 ± 1.34</td>
<td>21.96 ± 2.88</td>
</tr>
<tr>
<td>MB absolute numbers (%)</td>
<td>32.41 ± 1.27</td>
<td>34.44 ± 4.84</td>
<td>14.28 ± 0.25</td>
<td>22.52 ± 0.02</td>
</tr>
<tr>
<td>T2/MZ (%)</td>
<td>15.61 ± 0.37</td>
<td>30.12 ± 3.57</td>
<td>6.16 ± 1.18</td>
<td>15.09 ± 2.42</td>
</tr>
<tr>
<td>T2/MZ absolute numbers (%)</td>
<td>4.18 ± 0.40</td>
<td>4.56 ± 0.76</td>
<td>1.22 ± 0.11</td>
<td>1.84 ± 0.33</td>
</tr>
<tr>
<td>T2/MZ absolute numbers (×10⁶)</td>
<td>2.06 ± 0.15</td>
<td>3.95 ± 0.10</td>
<td>0.56 ± 0.05</td>
<td>1.36 ± 0.06</td>
</tr>
<tr>
<td>T1 (%)</td>
<td>0.90 ± 0.34</td>
<td>1.24 ± 0.81</td>
<td>2.02 ± 0.06</td>
<td>3.28 ± 0.60</td>
</tr>
<tr>
<td>T1 absolute numbers (%)</td>
<td>0.44 ± 0.17</td>
<td>1.02 ± 0.50</td>
<td>0.93 ± 0.18</td>
<td>2.42 ± 0.12</td>
</tr>
</tbody>
</table>

a The mean percentage ± SD of cells as a fraction of total splenocytes derived from two mice in each group are shown.
b The indicated splenic B cell subsets were gated as illustrated in Fig. 5.
References


