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supplemented with 10% fetal calf serum (Sigma), 5 × 10⁻⁵ M 2-mercaptoethanol, 0.1 mg/mL streptomycin, 100 U penicillin, and 2 mM 1-glutamine (Sigma). Cells were stimulated in 24-well Costar plates at a concentration of 4 × 10⁵ cells/mL with a final concentration of 4.0 µg/mL concanavalin A (ConA; Sigma). BPA was added at final concentrations ranging from 50 µM to 0.5 nM. Cells were incubated in a humidified chamber with 5% CO₂ at 37°C for 24 hr. Supernatants were harvested and analyzed by ELISA for IFN-γ.

After in vivo exposure to BPA, isolated splenic mononuclear cells were resuspended in complete media at a concentration of 4 × 10⁶ cells/mL and stimulated with either a final concentration of 4.0 µg/mL ConA or heat-killed Staphylococcus epidermidis. An overnight culture of S. epidermidis (optical density, 0.9 at 570 nm) was incubated at 60°C for 1 hr and added to cells at a final dilution of 1:20. Cells were incubated in a humidified chamber with 5% CO₂ at 37°C. After 24 hr, supernatants were harvested and assayed for IFN-γ production by ELISA. Optimal IL-10 levels were detected in ConA-stimulated supernatants by ELISA after 72 hr incubation.

Flow cytometric analysis of subsets. To determine the percentage of B cells and T-cell subsets, mononuclear splenic cells were stained immediately upon isolation. Briefly, cells were incubated on ice with FcBlock (PharMingen, San Jose, CA) and then stained with either phycoerythrin-conjugated anti-CD4 or FITC conjugated anti-CD8 monoclonal antibodies (all purchased from PharMingen and used at a final concentration of 2 µg/mL), anti-CD19 biotinylated monoclonal antibody followed by streptavidin-PE (Guava Technologies), or isotype-matched control antibodies (PharMingen). Live cells were gated using 7-AAD dye exclusion (Guava Technologies) and analyzed using a Guava personal flow cytometer and CytoAnalysis software (Guava Technologies).

Cytokine analysis by ELISA. Culture supernatants were assayed for cytokine production by ELISA following the manufacturer’s recommended protocol (Pierce-Endogen, Rockford, IL). In brief, 50 µL recombinant IFN-γ standards or 24-hr culture supernatants were added to each precoated well in duplicate. Plates were incubated for 2 hr at room temperature followed by the addition of biotinylated anti-IFN-γ antibody. After a 1-hr room-temperature incubation, the plate was washed, and streptavidin-horseradish peroxidase (HRP) solution was added to each well. The plate was incubated for 30 min and again washed. TMB (tetramethylbenzidine) substrate solution (Pierce-Endogen) was added, and the plates were developed in the dark for 30 min. Stop solution containing sulfuric acid was added, and absorbance was measured at 450 nm on an EL312e plate reader (Bio-Tek Instruments, Winooski, VT). Results were calculated from the standard curve using Excel (Microsoft Corporation, Redmond, WA).

To determine IL-10, we added 72-hr supernatants or recombinant IL-10 standards to each anti-mouse IL-10 precoated well in duplicate, which were incubated at room temperature for 3 hr. After a wash step, biotinylated anti-IL-10 antibody was added to each well and allowed to incubate for 1 hr at room temperature. The remainder of the procedure is identical to that outlined for IFN-γ.

IFN-γ antibody production by ELISA. Splenic mononuclear cells were isolated from
8- to 19-week-old female NZB/NZW mice before they displayed disease symptoms (< 100 mg/dL protein in urine) and incubated in complete media with 10.0 µg/mL lipopolysaccharide (LPS; Sigma) at 37°C in humidified air with 5% CO₂ for 72 hr. IgG2a antibody production was determined by isotype-specific ELISA. All antibodies were purchased from Southern Biotechnology (Birmingham, AL). Immulon 2 plates (Thermo Labsystems, Franklin, MA) were coated with 5.0 µg/mL goat anti-mouse IgG2a unlabeled antibody in PBS for 1 hr at 37°C. After washing, plates were blocked in PBS/0.05% Tween-20 (PBST) for 30 min at 37°C. Plates were incubated with either IgG2a standard or diluted supernatants from LPS-stimulated spleen cells for 2 hr at 37°C. After washes with PBST, 1 µg/mL biotinylated goat anti-mouse IgG2a antibody was added to each well and incubated for 1 hr at 37°C. Plates were washed with PBST, and HRP-avidin was added to each well and incubated for 1 hr at room temperature. After washing, the plates were developed using ABTS-peroxidase substrate (Bio-Rad, Hercules, CA) and read after 5 min at 37°C. Results of studies involving NZB/NZW mice were analyzed using the nonparametric Mann-Whitney U-test using SPSS 11 software for Macintosh (SPSS, Chicago, IL).

**Results**

**IFN-γ production decreases in BPA-treated mice.** Initially we tested the *in vitro* effects of the ER-binding molecule BPA on immune function using the estrogen-sensitive C57BL/6 strain. BPA induced a dose-dependent decrease in mitogen-stimulated IFN-γ secretion by splenocytes from both male and female 6-week-old C57BL/6 mice (Figure 1).

To investigate the effect of *in vivo* exposure to low doses of BPA on IFN-γ production, we fed C57BL/6 mice 2.5 µg BPA/kg bw daily for 7 days. This dose was based on previously published *in vivo* studies demonstrating reproducible effects from microgram doses of BPA per kilogram body weight and the detection of microgram levels of BPA in commonly used food containers (Brotorns et al. 1995; Gupta 2000). The mice were sacrificed 2–4 days after the last dose of BPA. There was a consistent and significant reduction of approximately 40% in ConA-stimulated IFN-γ production in male C57BL/6 mice treated *in vivo* with BPA (Figure 2). Control males produced 46,539 ± 6,837 pg/mL IFN-γ (n = 12) compared with 28,136 ± 4,506 pg/mL in BPA-fed males (n = 12; p < 0.01). Unstimulated cells produced undetectable levels of IFN-γ.

Female C57BL/6 mice in both treated and control groups consistently produced more IFN-γ than did the males in response to ConA stimulation (Figure 2). However, ConA-stimulated splenocytes from female C57BL/6 mice fed BPA for 7 days produced 28% less IFN-γ compared with untreated female controls (Figure 2; p < 0.05; paired Student’s *t*-test). Control females produced 57,438 ± 2,955 pg/mL IFN-γ compared with 41,592 ± 6,539 pg/mL in the BPA-fed group (p < 0.05). To test a more physiologic stimulus, splenocytes from an additional 12 female mice were incubated with heat-killed, gram-positive *S. epidermidis*. BPA exposure significantly reduced IFN-γ production by 24% (49,212 ± 3,819 pg/mL IFN-γ vs. 37,478 ± 3,264 pg/mL IFN-γ; p < 0.05; Figure 2).

**Lymphocyte distribution in C57BL/6 mice.** To determine whether the reduction in IFN-γ observed in BPA-fed mice was due to altered lymphocyte subset distribution, we analyzed C57BL/6 splenic mononuclear cells for expression of the T-cell subset markers CD4, CD8 and the B-cell marker CD19 by flow cytometry. Immediately upon isolation, the cells were stained with fluorescent monoclonal antibodies and analyzed by gating on live cells. Compared with control mice, both male and female BPA-treated mice showed a nonsignificant trend toward an increase in the percentage of CD19+ cells (Table 1). The T-cell subsets did not differ significantly between treated or control mice for either sex.

Although subset distribution did not differ with BPA treatment, C57BL/6 mice exposed to BPA for 7 days demonstrated a significant increase in total splenic mononuclear cells after separation by density centrifugation. The mean number of splenic mononuclear cells isolated from 32 BPA-fed mice was 51.7 ± 10⁶ ± 2.9 × 10⁶ cells compared with 40.8 ± 10⁶ ± 2.8 × 10⁶ cells in 32 age- and sex-matched control mice (p < 0.01, unpaired Student’s *t*-test).

**Cytokine changes in BPA-treated NZB/NZW mice.** IFN-γ contributes to disease progression in lupus (Hasegawa et al. 2002). After observing modulation of IFN-γ production in C57BL/6 mice, we were interested in studying the effects of BPA in NZB/NZW mice. Ten-week-old female NZB/NZW mice fed BPA at 5 weeks of age showed a significant reduction in ConA-stimulated IFN-γ production before the onset of proteinuria symptoms (Figure 3A). BPA-fed mice with < 100 mg/dL protein in urine produced an average of 16% less IFN-γ than did the control untreated mice (p < 0.05, Mann-Whitney). To determine whether the reduction in IFN-γ persisted over the course of several months, we also tested 8- to 16-week-old mice for evidence of proteinuria by tail bleeding and measuring protein levels in 24-h pooled urine samples before they displayed disease symptoms (< 100 mg/dL protein in urine). Male NZB/NZW mice fed BPA for 10 weeks produced 25% less IFN-γ than did the controls (Figure 3B; p < 0.05; Mann-Whitney). Histologic assessment of splenocytes from both control and BPA-fed mice by light microscopy revealed a significant reduction in the percentage of CD8+ cells (Table 1). In contrast, the percentage of CD4+ cells was not significantly different between control and BPA-fed mice.

**Table 1. Lymphocyte subset distribution in C57BL/6 and NZB/NZW mice.**

| CD4⁺ | CD8⁺ | CD19⁺ |
|------|------|-------|
| **C57BL/6 males** | **C57BL/6 females** | **NZB/NZW females** |
| Control (n = 6) | BPA (n = 6) | Control (n = 6) | BPA (n = 6) | Control (n = 12) | BPA (n = 12) |
| 26.7 ± 5.9 | 23.8 ± 5.3 | 22.4 ± 1.5 | 21.4 ± 2.0 | 32.1 ± 3.6 | 31.3 ± 3.4 |
| 19.9 ± 7.3 | 14.5 ± 2.6 | 15.5 ± 2.9 | 13.8 ± 2.9 | 17.9 ± 2.1 | 17.5 ± 1.4 |
| 33.0 ± 6.0 | 37.2 ± 6.5 | 38.6 ± 7.2 | 43.4 ± 4.2 | 34.7 ± 5.2 | 37.3 ± 5.2 |

*NZB/NZW mice analyzed before proteinuria developed (< 100 mg/dL protein in urine).
the splenocytes of 10 female NZB/NZW mice sacrificed at the onset of proteinuria. A dramatic 64% reduction in IFN-γ was observed in ConA-stimulated splenocytes from BPA-fed mice at disease symptom onset (Figure 3A). The BPA-treated NZB/NZW mice produced an average of 6,640 pg/mL IFN-γ versus 18,186 pg/mL in the controls, a difference approaching statistical significance ($p = 0.08$, Mann-Whitney).

IL-10 is a second cytokine that is both implicated in the development of lupus and modulated by estrogen (Kanda and Tamaki 1999; Yin et al. 2002). We analyzed whether in vivo BPA exposure could alter IL-10 levels in female NZB/NZW mice. Before disease symptom onset, at 10 weeks of age, both control and BPA-fed mice produced similar amounts of IL-10 (Figure 3B). Control NZB/NZW mice produced 276 ± 52 pg/mL versus 288 ± 59 pg/mL IL-10 in BPA-fed animals. As disease progressed, both control and BPA-fed mice produced higher levels of IL-10. Measuring IL-10 production at the time mice developed proteinuria demonstrated that the BPA-fed mice produced 32% less IL-10 than did controls (699 ± 114 pg/mL vs. 1,097 ± 146 pg/mL, $p < 0.05$).

**IgG2a production decreases in BPA-treated NZB/NZW mice.** In mice, IFN-γ plays a role in isotype switching to the complement-fixing antibody class IgG2a, contributing to the glomerulonephritis associated with lupus (Zeng et al. 2000). We were interested in seeing whether the decreased release of IFN-γ (Zeng et al. 2000) would result in altered IgG2a production. We measured IgG2a production in LPS-stimulated splenocytes from NZB/NZW female mice between 8 and 19 weeks of age by isotype-specific ELISA. All mice tested negative for proteinuria (< 100 mg/dL protein in urine). As shown in Figure 4, lymphocytes from BPA-treated animals ($n = 16$) produced significantly less IgG2a than age-matched controls ($n = 16$; $p < 0.05$, Mann-Whitney). The median IgG2a concentration was 10.4 ng/mL for BPA-fed mice compared with 23.0 ng/mL for untreated controls. The BPA-fed NZB/NZW mice produced a much narrower range of secreted IgG2a compared with the untreated control animals. The mean concentration of IgG2a for BPA-fed animals was 14.4 ± 3.2 ng/mL (including the one outlier; Figure 4) versus 29.0 ± 5.6 ng/mL in the untreated controls, a reduction of 50%.

To eliminate the possibility that the reduction of IgG2a in BPA-treated mice was due to a decreased percentage of B cells, we analyzed splenocytes for lymphocyte subsets by flow cytometry. The subset analysis was performed on the same NZB/NZW mice used in the IgG2a assays, with the exception of eight mice whose splenocytes were not stained. On average, BPA-fed mice had the same percentage of CD19+ cells compared with untreated mice (Table 1). As observed in the C57BL/6 mice, there was a significant increase in the total number of mononuclear cells isolated from the spleens of these BPA-treated mice ($n = 16$) by density centrifugation ($43 \times 10^6 \pm 2.9 \times 10^6$ vs. $34 \times 10^6 \pm 2.8 \times 10^5$, $p < 0.05$).

**Delay in disease onset observed in BPA-treated NZB/NZW mice.** To analyze whether in vivo BPA exposure modulates the course of lupus, we fed BPA to three separate groups of 5- to 6-week-old female NZB/NZW mice for 7 days. Each group consisted of five BPA-fed mice and five control mice. In each of the three experiments, a control NZB/NZW mouse was the first to develop proteinuria. Overall, female BPA-treated NZB/NZW mice showed an average delay of 7 weeks in the onset of proteinuria compared with untreated controls (Figure 5). The earliest onset of disease symptoms was at 26 weeks in a control mouse, whereas the earliest BPA-treated mouse to develop proteinuria was 33 weeks of age. On average, the mice treated with BPA remained symptom-free for 45 weeks compared with 38 weeks in control animals. Two of the BPA-fed mice showed no signs of proteinuria at 72 weeks of age. Seven days of oral BPA exposure at a young age appears to modulate the course of disease in female NZB/NZW mice.

**Discussion**

Although the biologic effects of BPA are not clearly understood, its ability to bind both ER isoforms makes it a potentially important modulator of immunity. Although estrogen increases IFN-γ production (Karpuzoglu-Sahin et al. 2001), we observed an inhibitory effect of BPA on IFN-γ secretion in both male and female C57BL/6 and female NZB/NZW mice. There is evidence that the outcome of transcriptional regulation at AP-1 or ERE sites is dependent both on the ER subtype involved and on the ligand (Mor et al. 2003; Pacch et al. 1997). For example, when tamoxifen is bound to ER-β, it regulates AP-1 sites in a manner opposite to that of estrogen (Pacch et al. 1997). Similarly, BPA may act differently than estrogen when bound to ER-α and/or ER-β, for example, by down-modulating the IFN-γ promoter.

In our studies, BPA acts in a protective manner in lupus-prone mice. It is likely that the decreased production of IFN-γ in BPA-fed mice contributed to a substantial reduction in isotype switching to IgG2a and to the prolonged symptom-free period we observed in BPA-fed NZB/NZW mice. Anti-dsDNA antibodies are a hallmark of disease in lupus-prone mice (Walker et al. 1996). Typically, anti-dsDNA antibodies of the IgG classes are not routinely detected until after 5 months of age (Yoshida et al. 2002). We were not able to detect IgG2a anti-dsDNA antibodies in the NZB/NZW mice used for antibody analysis because the mice were sacrificed between 8 and 19 weeks of age. Recently, we analyzed LPS-stimulated splenocytes isolated from 8-month-old female NZB/NZW mice that had been fed BPA for 1 week beginning at 5 weeks of age. All mice tested negative for proteinuria. BPA-fed mice demonstrated a 40% reduction in IgG2a anti-dsDNA antibodies as detected by ELISA (Sawai C. Unpublished data).

IL-10 is associated with lupus in both mice and humans (Gonzalez-Amaro et al. 1998; Llorente et al. 1995; Yin et al. 2002), yet its role in the disease appears to be complex. IL-10 may act as a regulatory cytokine that increases as a consequence of the disease process; alternatively, it may function as a contributing factor to disease (Moore et al. 2001). Knocking out the IL-10 gene in the lupus-prone MRL-Fas−/− mouse strain indicates that IL-10 plays a protective role early in disease; IL-10 is known to inhibit IFN-γ synthesis.
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