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# Phenotypic Analysis of Spleen, Thymus, and Peripheral Blood Cells in Aged C57Bl/6 Mice Following Long-Term Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin

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A mouse model was used to identify potential biomarkers of exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Female C57Bl/6 mice were treated weekly with 0.2 μg TCDD/kg body weight or vehicle for 14–15 months. Phenotypic analysis by flow cytometry identified the major cell subpopulations in the spleen, thymus, and peripheral blood as defined by the expression of CD4, CD8, B220, and Mac-1 molecules. These subpopulations were further characterized for the expression of I-A, Pgp-1, CD45RB, and/or T cell receptor antigens (CD3, $\alpha\beta$, $\gamma\delta$). A group of young (4 months old) mice was evaluated concurrently to document immunophenotype alterations associated with aging. Results showed several age-related changes in phenotype distribution in the spleen and blood, but not in the thymus, despite significant age-dependent thymic involution. The age-dependent changes in splenic phenotypes included a decreased frequency of $CD4^+$ cells and a major shift in the frequency distribution from naive T cells to effector and memory T cells as defined by Pgp-1 and CD45RB expression. These phenotypic changes in the spleen due to aging correlated with similar changes in the blood, providing preliminary support for the use of spleen cells as surrogates for blood in the development of biomarkers of immunotoxicity. Long-term exposure to a total cumulative dose 12–13 μg TCDD/kg body weight resulted in no overt toxicity, a 16-fold elevation of hepatic ethoxyresorufin-*O*-deethylase activity, and residue levels of 1.27 ± 0.16 ng TCDD/g abdominal fat. In comparison to the effects of aging, TCDD treatment produced relatively subtle changes in immunophenotypes. In the TCDD-treated thymus, the proportion of $CD4^-CD8^-$ cells was increased as was the proportion of $\gamma\delta^+$ thymocytes. These effects were very small but of interest in that similar thymic effects have been previously reported following prenatal exposure to TCDD. In the spleen, TCDD exposure did not alter the frequency of $CD4^+$ or $CD8^+$ T cells, B cells, or macrophages but significantly altered functionally discrete subpopulations within the T cell compartment. The most definitive change in TCDD-treated mice was a decrease in the frequency of memory T helper cells, defined as $CD4^+$ Pgp-$1^{hi}CD45RB^{lo}$, with a concomitant increase in the proportion of naive T helper cells identified as $CD4^+$Pgp-$1^{lo}CD45RB^{hi}$. These changes are consistent with the known immunosuppressive activity of TCDD. Thus, these results identify Pgp-1 and CD45RB as potential biomarkers of TCDD immunotoxicity.
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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has been the cause of much public concern due to its ubiquitous environmental distribution and toxic potency. The immunotoxic effects of TCDD and other related halogenated aromatic hydrocarbons (HAH) have been well documented in laboratory animals (reviewed by Thomas and Faith, 1985; Vos and Luster, 1989; Holsapple *et al.*, 1991; Kerkvliet and Burleson, 1994). However, no clear pattern of immunotoxicity has emerged from studies of humans exposed to HAH (Lu and Wu, 1985; Mocarelli *et al.*, 1986; Evans *et al.*, 1988; Stehr-Green *et al.*, 1989; Webb *et al.*, 1989). The lack of defined biomarkers of HAH immunotoxicity has hampered such clinical investigations.

The application of multiparameter flow cytometric analysis of cell surface antigens offers a promising approach to the field of immunotoxicology (Westermann and Pabst, 1990; Zola, 1992). However, in order to apply animal data to the assessment of human health risks for immunotoxicity, animal research needs to focus on the identification of biomarkers that are sensitive to environmental chemical exposure and measurable in humans. Ideally, these biomarkers would not only characterize the major lineage-specific immune cell populations (e.g., T cells, B cells, macro-

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phages) but would also assess the functional state of these cells. Recently, several monoclonal antibodies (mAbs) have become available that recognize cell surface molecules that are differentially expressed on naive, activated, proliferating and/or memory cells. Such molecules may be particularly useful as biomarkers of immunotoxicity.

In the present study, C57Bl/6 mice were exposed to a low dose of TCDD once a week for 14–15 months. Immunophenotypic analysis was performed on cells from the spleen, thymus, and peripheral blood to determine the major subsets as defined by the expression of CD4 or CD8 on T cells, B220 on B cells, and Mac-1 on macrophages and granulocytes. Thymocytes were further characterized by the expression of T cell receptor (TCR) antigens (CD3, $\gamma\delta$, $\alpha\beta$) based on the findings of Blaylock *et al.* (1992) that prenatal exposure to TCDD altered specific thymocyte expression of TCR antigens. The frequency of splenic T cells that expressed $\alpha\beta$ or $\gamma\delta$ TCR antigens was also characterized. Mac-1$^+$ and B220$^+$ splenic cells were examined for the expression of the activation antigen I-A. The effect of TCDD exposure on the patterns of Pgp-1 and CD45RB expression on T cell subpopulations was of particular interest for two reasons. First, differential expression of Pgp-1 and CD45RB has been shown to define functionally distinct naive, effector, and memory T cell subsets within the CD4$^+$ and CD8$^+$ T cell pools *in vivo* (Ernst *et al.*, 1990, 1993; Bradley *et al.*, 1991; Mobley and Dailey, 1992). Second, Pgp-1 and CD45RB are the murine analogs to the human markers CDw29 and CD45RA which have been shown to be altered by TCDD exposure in the marmoset (Neubert *et al.*, 1990, 1992, 1993).

In the present studies, we also addressed the feasibility of using mouse blood for phenotypic analysis and the correlation of any age- or TCDD-related changes in the spleen with similar changes in the blood. While rodent studies routinely use lymphocytes isolated from lymphoid organs (e.g., spleen, thymus, lymph nodes) for immunophenotyping, phenotypic analysis in humans is generally performed on lymphocytes isolated from the peripheral blood. Thus, potential biomarkers identified in the rodent by phenotypic changes in the lymphoid organs need to be validated for their correlation with similar changes in the peripheral blood.

## METHODS

***Animals and TCDD treatment.*** Female C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in cages with corn cob bedding and maintained on a 12-hr light/dark cycle at 22 ± 2°C. Wayne rodent chow and tap water were given *ad libitum*. TCDD (Cambridge Isotope Laboratories, Woburn, MA: code ED-901-C, purity >98%) was dissolved in anisole and diluted in peanut oil. Starting at 8 weeks of age, mice were administered 0.2 $\mu$g TCDD/kg body weight or vehicle by gavage once a week for 14–15 months. A single dose of 0.2 $\mu$g/kg body weight represents the lowest dose of TCDD that induces suppression of the primary antibody response to a T-dependent antigen (sheep red blood cells) in C57Bl/6 mice (Kerkvliet *et al.*, 1990; Kerkvliet and Brauner, 1990).

***N-methyl-N-nitrosourea (MNU) treatment.*** The original intent of this study was to evaluate the cocarcinogenic effect of TCDD in the MNU-induced thymic lymphosarcoma model (Joshi and Frei, 1970a,b; Frei *et al.*, 1978; Frank *et al.*, 1992). Mice were injected with a single dose of 35 mg MNU/kg body weight 3 weeks following the first weekly dose of TCDD or vehicle. Some mice were not injected with MNU and served as controls for MNU treatment. A dose of 35 mg MNU/kg body weight had been previously reported to induce thymic tumors in 35% of mice within 6 months (Joshi and Frei, 1970a). However, no animals developed palpable thymic tumors during a 13-month observation period. Prior to necropsy of the animals, the focus of the study was then reoriented to evaluate the effect of long-term exposure to a low dose of TCDD on lymphocyte phenotypes.

***Experimental design.*** Mice were killed by $CO_2$ asphyxiation in three separate trials following 14–15 months of chronic TCDD exposure. A group of young adult mice (4 months old) was also killed in each trial to evaluate age-associated alterations in lymphocyte phenotypes. To determine the possible influence of MNU treatment, all MNU-treated mice were evaluated in trials 1 and 2 and all mice not treated with MNU were evaluated in trial 3. Data from these three trials were extensively scrutinized by analysis of variance (ANOVA) modeling to discern any effect that could be attributed to MNU treatment and none was found. In addition, the particular lot of MNU used in this study also failed to induce thymic tumors in a subsequent experiment and was discarded as an ineffective preparation of MNU. Thus, we are convinced that the single injection of MNU early in the study does not confound the interpretation of the results reported here.

***Liver enzyme activity.*** Liver microsomes were prepared as previously described (Kerkvliet *et al.*, 1990). Total protein was determined using a bicinchoninic acid kit (Pierce, Rockford, IL). Cytochrome P450 content was measured by comparing the light absorption spectra of oxidized and reduced microsomal enzymes as described by Estabrook *et al.* (1972). The activity of ethoxyresorufin-*O*-deethylase (EROD) was determined using substrates and standards purchased from Molecular Probes (Eugene, OR) in a method adapted from Prough *et al.* (1978).

***Tissue residue analysis.*** Abdominal fat from vehicle- and TCDD-treated mice was frozen in liquid nitrogen, ground to a powder, and extracted using a soxhlet extractor. Prior to lipid removal, 5 ng $^{13}$C-labeled TCDD (Cambridge Isotope Laboratories) was added as an internal standard. Lipid was sequentially removed by chromatography using a 5% water-deactivated alumina column (Claeys and Inman, 1974) and a $H_2SO_4$ silica column (Lamparski *et al.*, 1979). Samples were fractionated on a 0.5% water-deactivated Florisil column (Bacon *et al.*, 1992) and analyzed using multiple ion detection gas chromatography/mass spectrometry. The limit of detection was 1 pg TCDD with a signal to noise ratio of 5.

***Blood.*** Blood was collected by cardiac puncture into syringes containing 5 U heparin/ml blood. Total numbers of red blood cells (RBC) and white blood cells (WBC) were determined using a Coulter counter. For differential cell counts, blood smears were prepared and stained with Gugol blue (The Gugol Stain Co., Long Island City, NY). The remaining blood cells were pelleted by centrifugation and RBC were removed using ammonium chloride/potassium lysing solution (Kruisbeek, 1992) prior to staining for flow cytometric analysis.

***Flow cytometric analysis.*** Single-cell suspensions were prepared from the spleen or thymus as previously described (Kerkvliet and Brauner, 1990). Spleen, thymus, and blood cells were incubated with mAbs (listed in Table 1) for 30 min on ice in 96-well round-bottom microplates (Flow Laboratories, Inc., McLean, VA) in the presence of anti-mouse FcR (Pharmingen) to block FcR-mediated binding of cytophilic Ig. Cy-Chrome–streptavidin (Pharmingen) was added as the second-step reagent for biotin-labeled mAbs. All samples were fixed in 1.0% paraformaldehyde for 20

**TABLE 1**

**Monoclonal Antibody (mAb) Combinations Used for Phenotyping Cells from the Spleen, Thymus, and Peripheral Blood Using Three-Color Flow Cytometry**

| | mAbs to cell surface antigens[a] | | |
|---|---|---|---|
| Cells | FITC[b] | PE[c] | CY-Chrome[d] |
| Spleen | CD4 (RM4-5) | CD45RB (C363.16A) | Pgp-1 (IM7.8.1) |
| | CD8 (53-6.7) | CD45RB (C363.16A) | Pgp-1 (IM7.8.1) |
| | CD4 (RM4-5) | $\alpha\beta$ TCR (H57-597) | $\gamma\delta$ TCR (GL3) |
| | CD8 (53-6.7) | $\alpha\beta$ TCR (H57-597) | $\gamma\delta$ TCR (GL3) |
| | Mac-1 (M1/70)[e] | B220 (RA3-6B2) | I-A$^b$ (AF6-120.1) |
| Blood | CD4 (RM4-5) | CD45RB (C363.16A) | Pgp-1 (IM7.8.1) |
| | CD8 (53-6.7) | CD45RB (C363.16A) | Pgp-1 (IM7.8.1) |
| | Mac-1 (M1/70) | B220 (RA3-6B2) | I-A$^b$ (AF6-120.1) |
| Thymus | CD8 (53-6.7) | CD4 (GK1.5)[f] | CD3 (145-2C11) |
| | CD8 (53-6.7) | CD4 (GK1.5) | $\alpha\beta$ TCR (H57-597) |
| | CD8 (53-6.7) | CD4 (GK1.5) | $\gamma\delta$ TCR (GL3) |

[a] mAb clone is indicated in parentheses.

[b] FITC (fluorescein)-labeled mAbs.

[c] PE (phycoerythrin)-labeled mAbs.

[d] CY-Chrome–streptavidin was used as a second-step reagent for biotinylated mAbs.

[e] Mac-1 was purchased from Boehringer–Mannheim Biochemicals (Indianapolis, IN).

[f] PE-CD4 was purchased from Becton–Dickinson (Mountainview, CA). All other mAbs were purchased from Pharmingen (San Diego, CA).

min. Appropriately labeled, isotypic-matched immunoglobulins were used as controls for nonspecific fluorescence.

Multicolor flow cytometric analysis was performed using an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) equipped with an argon ion laser operated at a wavelength of 488 nm at 300 mW. Fluorescent emissions were logarithmically amplified and electronically compensated to eliminate overlapping spectral emissions. All data were collected by listmode acquisition and analyzed using Gateway (Coulter) or Cyclops (Cytomation, Inc., Fort Collins, CO) software.

***Statistics.*** Mice were killed on 3 separate days in groups of two to four young mice, three to five aged vehicle-treated mice, and four to five aged TCDD-treated mice. Mice in trials 1 and 2 were treated with MNU. Mice in trial 3 were not treated with MNU. ANOVA modeling was performed for each response using SAS statistical software (Version 6.03, SAS Institute, Inc., Cary, NC). Significant TCDD effects ($p < 0.05$) were confirmed in ANOVA modeling that excluded the young mice from analysis. Bartlett's test was used to detect heterogeneity of variance with the alpha level set at 0.01.

Because of the potential confounding effect of MNU treatment, we were most interested in identifying treatment effects that were consistent across all three trials. The consistency of all treatment effects was assessed through graphical analysis and testing for trial-by-treatment interaction in a standard unbalanced two-factor ANOVA with each mouse as the experimental unit. When interaction effects were not significant at a $p$ value >0.20, a model with additive trial effects was used to test for treatment main effects (averaging across trials). Significant effects were explained using pairwise $t$ tests with the same model. When there was evidence that the treatment effect varied between trials (significant trial-by-treatment interaction), the effects were examined within each trial. For six responses (body weight; percentage CD4$^+$ spleen cells; percentage Mac-1$^+$ spleen cells; percentage CD4$^+$Pgp-1$^{lo}$CD45RB$^{hi}$ spleen cells; percentage CD8$^+$Pgp-1$^{hi}$CD45RB$^{hi}$ spleen cells; percentage CD8$^+$Pgp-1$^{lo}$CD45RB$^{hi}$ spleen cells), the mean square (MS) for treatment main effects was many times larger than the MS for interaction, and the significant interaction represented only a change in the magnitude of a treatment effect that was evident in each trial. For those cases, main effects are still useful summaries and are shown in the tables (Mead, 1988). Results summarized across trials are generally shown as least-squares means (SAS, 1990) from the model with additive trial and treatment effects.

## RESULTS

*P4501A1 induction.* Mice were treated weekly with 0.2 $\mu$g TCDD/kg body weight or vehicle for 14–15 months. Because the induction of hepatic cytochrome P4501A1 is considered one of the hallmarks of TCDD exposure (Whitlock, 1987), liver microsomes from vehicle- and TCDD-treated mice were analyzed for P450 content and EROD activity as indices of P4501A1 induction. EROD activity was elevated 16-fold in TCDD-treated mice (1.6 $\pm$ 0.10 nmol/min/mg) when compared to vehicle-treated mice (0.10 $\pm$ 0.01 nmol/min/mg). P450 content was also significantly increased in TCDD-treated mice (0.28 $\pm$ 0.01 nmol/mg) when compared to vehicle-treated mice (0.21 $\pm$ 0.02 nmol/mg).

*TCDD levels in fat.* Samples of abdominal fat from vehicle- and TCDD-treated mice were analyzed by gas chromatography/mass spectrometry for TCDD residues. Residue levels were <0.012 ng TCDD/gram fat (wet weight) in vehicle-treated mice and 1.27 $\pm$ 0.16 ng TCDD/gram fat in TCDD-treated mice.

*General toxicity.* Five animals died early in the study but none of these deaths were attributed to TCDD or MNU treatment. The surviving animals maintained good health throughout the 14- to 15-month study. Body weight was increased in the old mice compared to young mice and was not affected by TCDD exposure (Table 2). At the end of the study, the spleen of one aged vehicle-treated mouse (non MNU-treated) was grossly enlarged and covered with nodules of unknown origin; this mouse was excluded from further study.

*Lymphoid tissue cellularity.* As shown in Table 2, several age-related effects on lymphoid tissue cellularity were documented when aged vehicle-treated mice were compared to the young mice. Aging was associated with a significant increase in spleen weight and a significant reduction in the weight of the thymus. A decrease in thymic cellularity correlated with the marked reduction in thymic weight while spleen cellularity was unchanged despite the increase in splenic weight. Similar age-related changes were observed in TCDD-treated mice.

*Hematology.* As shown in Table 3, neither aging nor TCDD exposure significantly affected the total number of circulating RBC or WBC. However, both vehicle- and TCDD-treated old mice showed a significant increase in the

**TABLE 2**
**Changes in Spleen and Thymus Associated with Aging and Chronic Exposure to TCDD**

| Parameter | ANOVA $p$ value | Young | Vehicle | TCDD |
|---|---|---|---|---|
| Body weight (g) | <0.0001[c] | 20 ± 0.2[c] | 29 ± 1[d] | 28 ± 1[d] |
| Spleen | | | | |
| Weight (mg) | 0.0003 | 69 ± 2[c] | 91 ± 5[d] | 85 ± 3[d] |
| No. cells (×10⁻⁶) | 0.64 | 98 ± 6 | 100 ± 10 | 92 ± 4 |
| Thymus | | | | |
| Weight (mg) | <0.0001 | 44 ± 2[c] | 19 ± 1[d] | 18 ± 1[d] |
| No. cells (×10⁻⁶) | <0.0001[b] | 81 ± 5[c] | 26 ± 2[d] | 27 ± 2[d] |
| $N$ | | 12 | 9 | 13 |

*Note.* Mean ± SEM with variance calculated separately for each treatment (no evidence of trial effects and strong evidence of heterogeneity of variance for two of the parameters).

[a] Logarithmic transformation needed to stabilize variance; ANOVA and $t$ test $p$ values were similar on both scales.

[b] Heterogeneity not corrected by logarithmic transformation; $p$ values were similar on both scales.

[c,d] Within each row, values with different letters are statistically different at $p < 0.002$.

proportion of polymorphonuclear cells (PMNs) and a concomitant decrease in the proportion of lymphocytes in the peripheral blood when compared to young mice.

*Mononuclear cell phenotypes in spleen and blood.* The major subpopulations in the spleen were determined by the expression of CD4, CD8, B220, and Mac-1. As shown in Fig. 1, aged vehicle-treated mice had significantly fewer $CD4^+$ splenic T cells than young mice. No age-dependent changes were observed in the frequency of $CD8^+$, $B220^+$, or Mac-$1^+$ spleen cells. When compared to aged vehicle-treated mice, TCDD treatment had no effect on these major subpopulations (Fig. 1).

Like the spleen, an age-dependent decrease in the frequency of $CD4^+$ cells was seen in the peripheral blood (Fig. 1A). For individual mice, there was a high degree of correlation in the frequency of $CD4^+$ cells in the spleen and peripheral blood (Fig. 2). In addition, there was an age-related increase in the frequency of Mac-$1^+$ cells in the blood (Fig. 1D). Because PMNs have been shown to express low levels of Mac-1 (Springer *et al.*, 1979), the increase in Mac-$1^+$ cells in the blood most likely reflects the increase in PMNs enumerated in differentially stained blood smears (Table 3).

*I-A and TCR expression on spleen cells.* I-A expression on $B220^+$ and Mac-$1^+$ spleen cells was not altered due to aging or TCDD exposure (data not shown). Likewise, neither aging nor TCDD exposure significantly altered the frequency of T cells expressing $\alpha\beta$ or $\gamma\delta$ TCR antigens (data not shown).

*Splenic $CD4^+$ subsets defined by Pgp-1 and CD45RB expression.* As summarized in Table 4, aging had a dramatic effect on the subsets of $CD4^+$ cells defined by the differential expression of Pgp-1 and CD45RB. The majority of $CD4^+$ spleen cells from young mice expressed Pgp-$1^{lo}$CD45$RB^{hi}$ (the naive T helper ($T_H$) phenotype) while the majority of $CD4^+$ cells from aged vehicle-treated mice expressed Pgp-$1^{hi}$CD45$RB^{lo}$ (the preactivated/memory $T_H$ phenotype). These changes are easily discerned in the representative histograms of young and old mice illustrated in Fig. 3. The increase in the frequency of preactivated/memory $T_H$ cells and the decrease in the frequency of naive $T_H$ cells are well-documented effects of aging (Ernst *et al.*, 1990, 1993).

Age-dependent changes were also observed in $T_H$ cells from TCDD-treated mice. However, in comparison to vehicle-treated mice, there were fewer preactivated/memory $T_H$ cells and more naive $T_H$ cells in mice that had been treated with TCDD (Table 4).

*Splenic $CD8^+$ subsets defined by Pgp-1 and CD45RB expression.* Aging was also associated with dramatic changes in the expression of Pgp-1 and CD45RB on $CD8^+$ spleen cells (see Fig. 3 for representative histograms). As summarized in Table 5, the majority of $CD8^+$ spleen cells from young mice expressed the naive phenotype (Pgp-$1^{lo}$CD45$RB^{hi}$). A significant decrease was observed in the frequency of the naive phenotype in aged vehicle-treated mice with concomitant increases in the proportion of both Pgp-$1^{hi}$CD45$RB^{hi}$ (preactivated/memory cells) and Pgp-$1^{hi}$CD45$RB^{lo}$ (effector cells). These age-dependent alterations have been previously documented in mice (Ernst *et al.*, 1993).

The only difference between vehicle- and TCDD-treated mice was a very small but statistically significant decrease in the frequency of $CD8^+$ cells expressing the effector pheno-

**TABLE 3**
**Changes in Blood Associated with Aging and Chronic Exposure to TCDD**

| Parameter | ANOVA $p$ value | Young | Vehicle | TCDD |
|---|---|---|---|---|
| Blood[a] | | | | |
| RBC (×10⁻⁸/ml) | 0.089 | 8.2 ± 0.2 | 7.4 ± 0.2 | 7.6 ± 0.2 |
| WBC (×10⁻⁶/ml) | 0.47 | 6.0 ± 1.0 | 5.8 ± 1.0 | 7.9 ± 0.8 |
| $N$ | | 6 | 6 | 9 |
| Differentials (%) | | | | |
| Monocytes | 0.55 | 10 ± 2 | 12 ± 2 | 12 ± 2 |
| Lymphocytes | 0.043 | 77 ± 3[b] | 68 ± 3[c] | 70 ± 2[c] |
| PMNs | 0.012 | 10 ± 2[b] | 18 ± 2[c] | 15 ± 1[c] |
| Eosinophils | 0.86 | 2.8 ± 0.9 | 2.3 ± 0.9 | 2.2 ± 0.7 |
| $N$ | | 10 | 10 | 14 |

*Note.* Least squares mean ± SEM for untransformed data from ANOVA model with additive trial and treatment effects.

[a] No data from trial 1.

[b,c] Within each row, values with different letters are statistically different at $p < 0.05$.

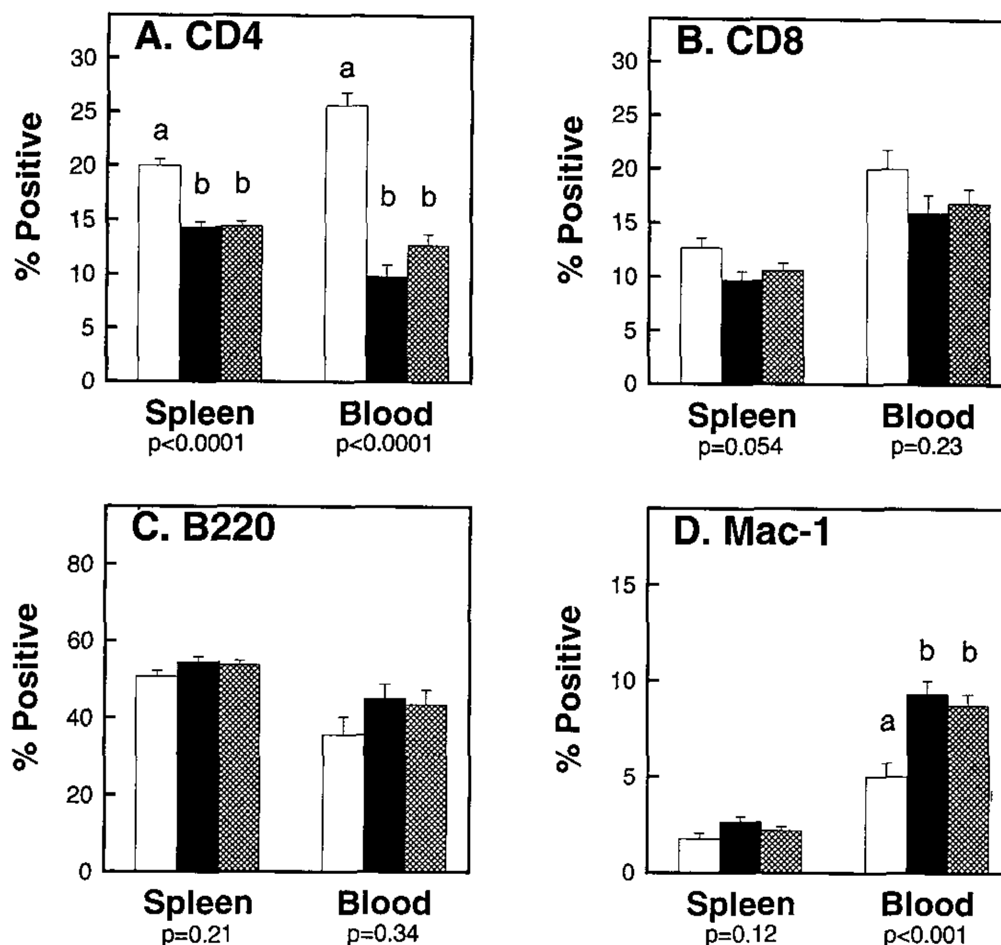


FIG. 1. Effect of TCDD treatment and aging on the cellular composition of the spleen and peripheral blood in mice. Each bar represents the least-squares mean ± SEM for untransformed data from the ANOVA model with additive trial and treatment effects. Open bars, 7–8 young mice; solid bars, 8–10 aged vehicle-treated mice; cross-hatched bars, 10–14 TCDD-treated mice. Blood cells were stained for the expression of B220 in a single study with 2–3 mice per group. ANOVA $p$ value is noted below $x$ axis. Bars with different letters represent statistically significant differences at $p < 0.0001$.

type. Because the magnitude of this effect was so small, its biological relevance is questionable.

*Peripheral blood lymphocyte subpopulations defined by Pgp-1 and CD45RB.* Due to technical difficulties with immunofluorescent staining, only data from trial 2 were available for analysis of Pgp-1 and CD45RB expression on $CD4^+$ and $CD8^+$ cells in the blood. However, despite the small sample size, the age-dependent changes observed in the spleen were also evident in the blood (Table 6). Figure 4 illustrates how well the age-dependent decrease in the proportion of naive $T_H$ cells ($CD4^+Pgp\text{-}1^{lo}CD45RB^{hi}$) in the spleen correlates with the blood for individual mice. These findings are presented as preliminary evidence due to the small sample size.

As in the spleen, TCDD treatment resulted in a small but statistically significant decrease in the frequency of $CD8^+$ cells in the blood that expressed the effector phenotype ($Pgp\text{-}1^{hi}CD45RB^{lo}$) (Table 6). Again, the biological relevance of such a small change is questionable.

*Thymic subpopulations.* As shown in Table 7, aging did not alter the relative proportions of the major thymic subpopulations as defined by CD4 and CD8 expression. When compared to vehicle-treated mice, chronic exposure to TCDD resulted in a small but statistically significant increase in the frequency of $CD4^-CD8^-$ thymic cells.

There were no apparent age-dependent changes in the expression of TCR antigens (CD3, $\alpha\beta$, $\gamma\delta$) on the four thymic subpopulations defined by CD4 and CD8 expression (data not shown). A very small but statistically significant ($p < 0.005$) increase was observed in the expression of $\gamma\delta$ TCR on all thymocytes from TCDD-treated mice (2.5 ± 0.2%) compared to vehicle-treated mice (1.5 ± 0.2%).

## DISCUSSION

The present study focused on the effect of long-term exposure to TCDD on various lymphocyte phenotypes in the spleen, blood, and thymus of C57B1/6 mice. Because aging

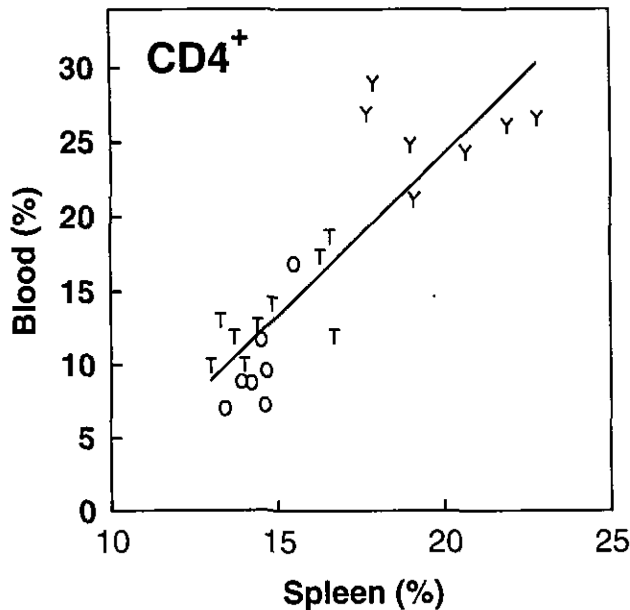


FIG. 2. Correlation between the proportion of $CD4^+$ cells in the spleen and blood. Y, seven young adult mice; O, seven aged vehicle-treated mice; T, nine TCDD-treated mice. If all mice are treated as one homogeneous group, then there is a highly significant correlation ($R = 0.86$; $p < 0.0001$). However, the strength of the correlation was primarily reflected by the aging effect. Within treatment groups, the vehicle- and TCDD-treatment groups had a strong correlation ($p = 0.028$ and $0.036$, respectively) but the young group did not ($p = 0.86$).

has been shown to alter immunophenotypes, we included a group of young mice (4 months old) to document the effects of aging. The age-dependent changes in splenic subsets largely support previous age-comparative studies in mice (Ernst *et al.*, 1990, 1993; Grossmann *et al.*, 1991; Callahan *et al.*, 1993). Changes with age in Pgp-1 and CD45RB expression on T cell subsets primarily represent a shift in the expression of naive cell phenotypes to activated and memory cell phenotypes, reflecting the history of exposure to environmentally encountered antigens. Similar age-dependent shifts in lymphocyte phenotypes were also seen in the peripheral blood, which are new data for the murine model. Despite the gross involution of the thymus in aged mice, there were no age-dependent changes in thymic subsets defined by CD4 and CD8 molecules or the expression of TCR antigens (CD3, $\alpha\beta$, $\gamma\delta$) on these subsets. The lack of age-dependent alterations in thymic subsets is supported by previous studies in C57Bl/6 mice (Dubiski *et al.*, 1989/1990).

To study the effect of long-term exposure to TCDD on various immune cell phenotypes, C57Bl/6 mice were given 0.2 μg TCDD/kg body weight on a weekly basis for 14–15 months. This dosing regimen resulted in a total cumulative dose of 12–13 μg TCDD/kg body weight. Exposure to this level of TCDD did not produce any overt sign of toxicity. Residue analysis showed 1.27 ± 0.16 ng TCDD/gram abdominal fat in TCDD-treated mice, which represented approximately 10% of the total dose administered. In a previous study, we reported that approximately 5% of a single dose of 5 μg TCDD/kg body weight was distributed to the abdominal fat in C57Bl/6 mice 2–4 days following exposure (Neumann *et al.*, 1992). Neubert *et al.* (1993) also found that rats initially treated with a loading dose of 1 μg TCDD/kg body weight and maintained on a weekly dose of 0.2 μg TCDD/kg body weight for 11 weeks had a level of 1.63 ± 0.22 ng TCDD/gram adipose tissue.

The induction of hepatic cytochrome P4501A1 as measured by increases in the activity of EROD or aryl hydrocarbon hydroxylase is considered one of the hallmarks of TCDD exposure (Whitlock, 1987). In this study, P450 content and EROD activity were significantly elevated in liver microsomes from TCDD-treated mice, documenting prolonged induction of P4501A1-associated enzyme activity with long-term TCDD exposure.

Chronic exposure to TCDD produced several subtle changes in thymic phenotypes as evidenced by small increases in the frequency of $CD4^-CD8^-$ (DN) thymocytes and thymocytes expressing $\gamma\delta$ TCR. These effects were quite small and of questionable biological relevance given the overall decrease in thymic cellularity associated with aging. However, they are intriguing from the perspective that similar changes have been previously reported in mice exposed prenatally to TCDD (Blaylock *et al.*, 1992; Fine *et al.*, 1989; Holladay *et al.*, 1991). An increase in the DN population was also seen in young adult mice treated with a single dose of 5 μg TCDD/kg body weight (Kerkvliet and Brauner, 1990).

In the spleen, chronic exposure to TCDD did not alter the frequencies of the major leukocyte subpopulations characterized by CD4, CD8, B220, and Mac-1 expression. This is not surprising given that TCDD is not cytotoxic even at relatively high concentrations *in vitro* (Knutson and Poland, 1980). Thus, analyses of these major subpopula-

TABLE 4
**Influence of Aging and TCDD Treatment on the Expression of Pgp-1 and CD45RB on Splenic $CD4^+$ T Cells**

| Subset | ANOVA *p* value | Percentage expressing phenotype | | |
|---|---|---|---|---|
| | | Young | Vehicle | TCDD |
| $Pgp\text{-}1^{hi}CD45RB^{lo}$ | <0.0001 | $18 \pm 1^a$ | $42 \pm 1^b$ | $36 \pm 1^c$ |
| $Pgp\text{-}1^{hi}CD45RB^{hi}$ | 0.36 | 18 ± 1 | 20 ± 1 | 21 ± 1 |
| $Pgp\text{-}1^{lo}CD45RB^{lo}$ | 0.89 | 20 ± 1 | 20 ± 1 | 19 ± 1 |
| $Pgp\text{-}1^{lo}CD45RB^{hi}$ | <0.0001 | $43 \pm 2^a$ | $18 \pm 2^b$ | $24 \pm 1^c$ |
| *N* | | 8 | 10 | 14 |

*Note.* Least squares means ± SEM for untransformed data from ANOVA model with additive trial and treatment effects.

[a,b,c] Within each row, values with different letters are statistically different at $p < 0.01$.

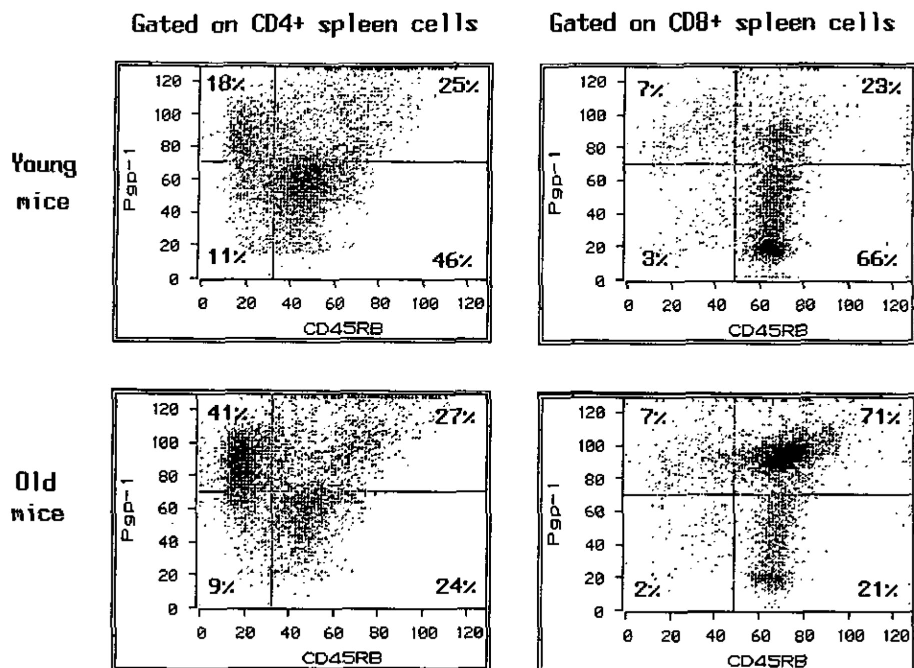


FIG. 3. Patterns of Pgp-1 and CD45RB expression on CD4$^+$ and CD8$^+$ spleen cells from young and aged vehicle-treated mice. Spleen cells were stained with FITC-CD4 or FITC-CD8, PE-CD45RB, and Cy-Chrome–streptavidin–biotin-Pgp-1 for three-color flow cytometric analysis. The correlated expression of Pgp-1 and CD45RB is shown in the two-parameter dot-plot histograms gated on either the CD4$^+$ or CD8$^+$ T cell population.

tions alone do not represent sensitive biomarkers of exposure to TCDD.

On the other hand, when CD4$^+$ T cells were further characterized by their differential expression of Pgp-1 and CD45RB, significant differences were seen between TCDD- and vehicle-treated mice. Specifically, fewer memory cells (Pgp-1$^{hi}$CD45RB$^{lo}$) and more naive cells (Pgp-1$^{lo}$CD45RB$^{hi}$) were found in TCDD-treated mice. These results are consistent with the known immunosuppressive activity of TCDD. Furthermore, even though the phenotypic changes induced by TCDD were modest, it is important to remember that the dose of TCDD was very low and the animals were not specifically challenged with antigen.

TABLE 5

**Influence of Aging and TCDD Treatment on the Expression of Pgp-1 and CD45RB on Splenic CD8$^+$ T Cells**

| Subset | ANOVA $p$ value | Percentage expressing phenotype | | |
|---|---|---|---|---|
| | | Young | Vehicle | TCDD |
| Pgp-1$^{hi}$CD45RB$^{lo}$ | 0.001[a] | 3 ± 1[b] | 8 ± 1[c] | 5 ± 1[d] |
| Pgp-1$^{hi}$CD45RB$^{hi}$ | 0.0001 | 30 ± 3[b] | 48 ± 2[c] | 49 ± 2[c] |
| Pgp-1$^{lo}$CD45RB$^{lo}$ | 0.59 | 4 ± 1 | 4 ± 1 | 3 ± 1 |
| Pgp-1$^{lo}$CD45RB$^{hi}$ | $<$0.0001 | 63 ± 3[b] | 40 ± 3[c] | 43 ± 2[c] |
| $N$ | | 8 | 10 | 14 |

*Note.* Least squares means ± SEM for untransformed data from ANOVA model with additive trial and treatment effects.

[a] $p$ values from analysis on logarithmic transformed data; on log scale, no evidence of treatment-by-trial interaction.

[b,c,d] Within each row, values with different letters are statistically different at $p < 0.0002$ for aging effects and $p = 0.02$ for TCDD effect.

TABLE 6

**Effect of Aging and TCDD Exposure on Pgp-1 and CD45RB Expression on T Cell Subsets in Peripheral Blood of Mice**

| Subset | ANOVA $p$ value | Percentage expressing phenotype | | |
|---|---|---|---|---|
| | | Young | Vehicle | TCDD |
| CD4$^+$ | | | | |
| Pgp-1$^{hi}$CD45RB$^{lo}$ | 0.002 | 5 ± 1[a] | 20 ± 2[b] | 18 ± 1[b] |
| Pgp-1$^{hi}$CD45RB$^{hi}$ | 0.087 | 11 ± 2 | 16 ± 1 | 15 ± 1 |
| Pgp-1$^{lo}$CD45RB$^{lo}$ | 0.99 | 19 ± 4 | 19 ± 1 | 19 ± 2 |
| Pgp-1$^{lo}$CD45RB$^{hi}$ | 0.005 | 65 ± 3[a] | 45 ± 2[b] | 49 ± 2[b] |
| CD8$^+$ | | | | |
| Pgp-1$^{hi}$CD45RB$^{lo}$ | 0.031 | 1 ± 0[a] | 4 ± 1[b] | 2 ± 1[a] |
| Pgp-1$^{hi}$CD45RB$^{hi}$ | 0.022 | 25 ± 3[a] | 41 ± 4[b] | 53 ± 5[b] |
| Pgp-1$^{lo}$CD45RB$^{lo}$ | 0.53 | $<$1 | $<$1 | $<$1 |
| Pgp-1$^{lo}$CD45RB$^{hi}$ | 0.033 | 74 ± 4[a] | 54 ± 5[b] | 45 ± 6[b] |
| $N$ | | 2 | 3 | 3 |

*Note.* Mean ± SEM for data from trial 2, using ANOVA model.

[a,b] Within each row, values with different letters are statistically different at $p < 0.005$ for age-related comparisons and $p < 0.04$ for TCDD-related comparisons.

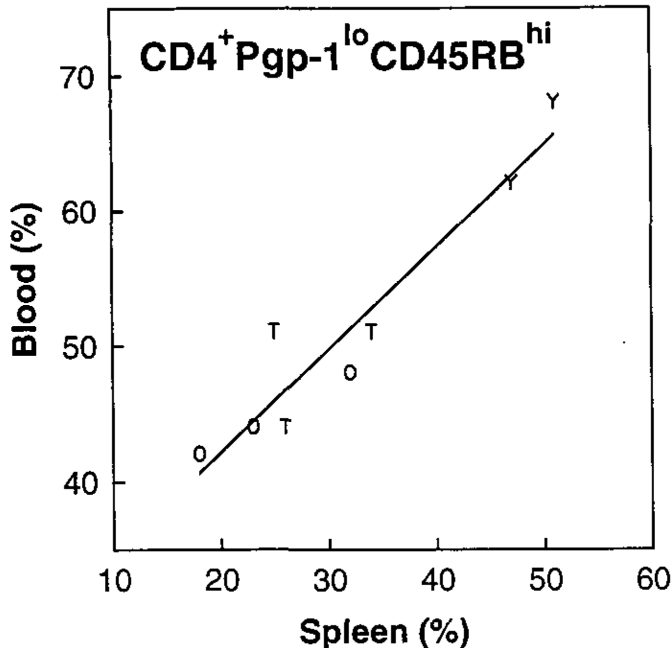


FIG. 4. The frequency of $CD4^+$ Pgp-$1^{lo}$CD45$RB^{hi}$ (naive $T_H$) cells in the spleen directly correlates with the frequency in the blood. Y, two young adult mice; O, three aged vehicle-treated mice; T, three TCDD-treated mice. If all mice are treated as one homogeneous group, then there is a highly significant correlation ($R = 0.95$; $p = .0002$). Because of the small sample size, there are not enough data to establish a 1:1 relationship within treatment groups. A similar correlation was observed for the proportion of $CD4^+$Pgp-$1^{hi}$CD45$RB^{lo}$ cells in the spleen and blood. In contrast, the correlation for the $CD4^+$Pgp-$1^{lo}$CD45$RB^{lo}$ and $CD4^+$Pgp-$1^{hi}$CD45$RB^{hi}$ subsets which were not altered by aging was not as strong ($R = 0.41$ and 0.59, respectively).

Alterations in $T_H$ cell subsets by TCDD are also consistent with the results of Neubert *et al.* (1990, 1992, 1993). They reported that long-term exposure to TCDD altered the ratio of helper–inducer/memory T helper cells ($CD4^+CDw29^+$) to suppressor–inducer/naive T helper cells ($CD4^+CD45RA^+$) in the peripheral blood of the *Callithrix jacchus,* the common marmoset. Interestingly, when marmosets were chronically treated with 0.3 ng TCDD/kg body weight, a significant increase was observed in the helper–inducer/memory T helper cell subset with a concomitant decrease in the suppressor–inducer/naive T helper cell subset. However, when these same animals were exposed for an additional 8 weeks to a higher dose of TCDD (1.5 ng/kg body weight), the inverse effect was found. Our findings in the mouse are similar to the findings in marmosets treated with 1.5 ng TCDD/kg body weight. Taken together, CD45RB, Pgp-1, or their analogues appear to represent potential biomarkers of TCDD exposure.

One of the main purposes of our study was to determine if phenotypic changes in spleen cells correlated with similar changes in blood cells. While rodent studies routinely use lymphocytes isolated from lymphoid organs (e.g., spleen, thymus, lymph nodes) for immunophenotyping, phenotypic analysis in humans is generally performed on lymphocytes isolated from venous blood. Extrapolation of animal data to human health risk assessment presumes that phenotypes in the spleen will reflect the phenotypes in the peripheral blood. Our study provides preliminary evidence that the age-related changes in spleen immunophenotypes are reflected by similar changes in the blood. The age-dependent decrease in the proportion of $CD4^+$ cells in the spleen correlated strongly with a similar decrease in the blood. Likewise, the age-related effects in the memory and naive $T_H$ phenotypes in the spleen correlated with comparable changes in the blood. However, due to the small sample size for the blood data, the significant TCDD effects observed in the expression of Pgp-1 and CD45RB on $CD4^+$ spleen cells could not be verified in the blood.

In conclusion, it is becoming increasingly apparent that no single marker can uncover the spectrum of phenotypic heterogeneity that exists within lymphoid cell subpopulations. On the basis of Pgp-1 and CD45RB expression, distinct T cell subsets can be differentiated within the $CD4^+$ and $CD8^+$ T cell pools that reflect their maturity and effector status (Ernst *et al.,* 1990, 1993; Bradley *et al.,* 1991; Mobley and Dailey, 1992). An increased frequency of mature effector or memory T cell phenotypes is normally associated with the aging process and most likely reflects (1) a decrease in the export of naive T cells from the atrophied thymus into the peripheral lymphoid tissues and (2) a longer history of antigenic exposure. Chronic TCDD exposure resulted in a decrease in the frequency of the effector and memory T cell phenotypes. Since these effects are consistent with both the thymotoxic and immunosuppressive activities of TCDD, Pgp-1 and CD45RB represent potential biomarkers of TCDD immunotoxicity.

TABLE 7
**Influence of Aging and Chronic Exposure to TCDD on the Relative Composition of the Thymus**

| Subset | ANOVA $p$ value | Percentage positive (%) Young | Vehicle | TCDD |
|---|---|---|---|---|
| $CD4^+CD8^+$ | 0.009 | $84 \pm 1^b$ | $82 \pm 1^{b,c}$ | $81 \pm 1^c$ |
| $CD4^-CD8^-$ | 0.002 | $5.3 \pm 0.7^b$ | $5.8 \pm 0.7^b$ | $8.2 \pm 0.5^c$ |
| $CD4^+CD8^-$ | 0.52 | $8.1 \pm 0.4$ | $8.5 \pm 0.3$ | $8.0 \pm 0.3$ |
| $CD4^-CD8^-$ | $0.24^a$ | $2.9 \pm 0.2$ | $3.5 \pm 0.2$ | $3.0 \pm 0.2$ |
| $N$ | | 8 | 9 | 14 |

*Note.* Least squares mean ± SEM for untransformed data from ANOVA model with additive trial and treatment effects.

[a] Logarithmic transformation needed to stabilize variance; $p$ values for ANOVA were similar on both scales.

[b,c] Within each row, values with different letters are statistically different at $p < 0.01$.

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