

Maternal Tributyltin Exposure Is Associated With Male-Biased Immune Dysregulation Across Generations

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Abstract

Tributyltin (TBT) is an environmental obesogen and endocrine-disrupting chemical that promotes adipogenesis and transgenerational metabolic dysfunction in mice. Although developmental TBT exposure has been linked to male-biased adiposity, insulin dysregulation, and hepatic pathology, its effects on the immune system remain unclear. We investigated whether maternal TBT exposure is associated with persistent immune dysregulation in F1 and F3 offspring. Peripheral blood immunophenotyping data generated by the German Mouse Clinic were reanalyzed in F1 offspring of control- and TBT-exposed dams. Maternal TBT exposure was associated with increased B-cell representation, reduced T-cell representation, an altered B-cell-to-T-cell balance, a reduced CD4/CD8 ratio, and decreased NK-cell frequencies. We then analyzed splenic immune-marker expression and circulating inflammatory cytokines in an independent transgenerational cohort. In F1 males, TBT exposure was associated with reduced expression of T-cell- and myeloid-associated markers and increased expression of inflammatory cytokines. Similar changes were observed in unexposed F3 male descendants of the TBT lineage, whereas F3 females showed no significant changes in the immune markers examined. Plasma TNF and IL-6 were increased in F1 and F3 males but not females. These findings identify the immune system as a potential target of developmental obesogen exposure and support a persistent, male-biased immune phenotype characterized by altered splenic immune-marker expression and elevated inflammatory cytokines across generations.

1 Introduction

2 Obesity and related metabolic diseases are increasingly recognized as disorders
3 programmed by early-life environmental exposures [1]. In addition to lifestyle and
4 genetics, mounting evidence supports the developmental origins of health and disease
5 (DOHaD) paradigm, which posits that perturbations during critical windows of
6 development can produce potentially adverse long-term metabolic outcomes [2, 3].
7 Among these perturbations, exposure to a subset of endocrine-disrupting chemicals
8 (EDCs), which are known as obesogens, has emerged as a key contributor to altered
9 energy balance, adipocyte fate, and systemic metabolism [1, 4-6].

10 Tributyltin (TBT) is a well-characterized obesogen that promotes adipogenesis in vitro
11 and in vivo by acting as an agonist of peroxisome proliferator-activated receptor gamma
12 (PPAR γ) and its heterodimeric partner retinoid X receptor (RXR) [7, 8]. TBT was widely
13 used as a biocide in marine antifouling paints and remains an environmental
14 contaminant of concern because of its persistence in aquatic systems and accumulation
15 in marine organisms [9]. As a result, dietary exposure can occur through contaminated
16 food, particularly seafood, as well as through contaminated water and other
17 environmental sources [10]. Importantly, TBT and related organotins can cross the
18 placental barrier, providing a route by which maternal exposure can directly affect the
19 developing fetus [11]. In addition to RXR/PPAR γ activation, TBT has been reported to
20 disrupt other endocrine pathways. For example, organotins can inhibit aromatase
21 activity, potentially reducing estrogen production from androgen substrates, and low-
22 dose TBT chloride has been reported to affect estrogen receptor- α signaling in MCF-7
23 cells [12]. These endocrine-disrupting actions broaden the potential relevance of TBT

1 beyond adipogenesis and suggest that developmental exposure may affect sexually
2 dimorphic endocrine and immune pathways. Previous work from our lab demonstrated
3 that prenatal and early-life TBT exposure increased fat depot size, hepatic steatosis,
4 and insulin resistance in directly exposed F1 and F2 mice, with transgenerational effects
5 observed in unexposed F3 and F4 generations [13-15]. Our prior work linked these
6 metabolic phenotypes with chromatin remodeling in multipotent mesenchymal stromal
7 stem cells (a.k.a., mesenchymal stem cells, MSCs), shifts in MSC lineage commitment
8 and germline epigenetic alterations [16, 17]. We also showed that TBT enhanced
9 macrophage inflammation and promoted lipolysis in co-cultured adipocytes via a
10 PPAR γ -dependent mechanism [18], highlighting the potential for immune–metabolic
11 disruption.

12 While the impact of TBT on immune system development and immune lineage
13 specification remains incompletely understood, a substantial body of literature
14 demonstrated immunotoxic and immunomodulatory effects of TBT in vertebrate and
15 cell-based models. A recent comprehensive review summarized studies showing that
16 TBT alters cytokine expression and immune cell viability both in vivo and in vitro,
17 thereby disrupting immune homeostasis [19]. Rodent studies reported thymic atrophy
18 and depletion of cortical thymocytes, suggesting impaired lymphoid development [20].
19 Human natural killer (NK) cells exhibited persistent suppression of cytotoxic function
20 following brief TBT exposure, including reductions in binding and lytic activity [21]. Ex
21 vivo studies on human peripheral immune cells showed that TBT dysregulated IL-1 β
22 secretion, with low TBT concentrations increasing IL-1 β secretion and higher TBT
23 concentrations blocking IL-1 β release via MAPK/NF- κ B-dependent pathways; secretion

1 of other cytokines, including IL-6 and TNF α , was also affected [22]. In aquatic vertebrate
2 models such as zebrafish, TBT exposure impaired innate immune function and
3 disrupted immune-related gene expression [23].

4 The immune system plays a central role in energy homeostasis and metabolic disease.
5 Chronic low-grade inflammation and altered immune cell composition contribute to
6 adipose dysfunction, insulin resistance, and hepatic pathology [24, 25]. Importantly,
7 PPAR γ not only governs adipogenesis but also regulates immune cell differentiation and
8 function, including macrophage polarization and regulatory T cell biology; this suggests
9 a mechanistic link between obesogen signaling and immune development [26]. Whether
10 early-life TBT exposure disrupts immune cell homeostasis in offspring, and if such
11 changes persist across generations, had not been investigated.

12 Here, we tested the hypothesis that maternal TBT exposure induces persistent immune
13 alterations in offspring. We analyzed peripheral immune cell profiles together with
14 splenic immune-marker expression and circulating inflammatory cytokines in F1 and F3
15 male and female offspring of F0 dams treated during pregnancy. We found that
16 maternal TBT exposure altered peripheral lymphocyte composition in F1 offspring and
17 was associated with reduced splenic immune-marker expression and elevated
18 circulating inflammatory cytokines in male descendants across generations. These
19 findings expand the known scope of TBT-induced developmental programming beyond
20 metabolism and adipogenesis and support the idea that the immune system is an
21 additional target of ancestral obesogen exposure.

22

Materials and Methods

Animal Model and Breeding Strategy

C57BL/6J mice were purchased from The Jackson Laboratory (Sacramento, CA) and housed in micro-isolator cages under a 12-hour light/dark cycle in a temperature- and humidity-controlled facility. Animals had ad libitum access to water and a standard chow diet (PicoLab 5053; 24.5% kcal from protein, 13.1% kcal from fat, and 62.3% kcal from carbohydrates). All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine, and conducted in accordance with institutional and federal ethical guidelines.

For the German Mouse Clinic (GMC) analysis, F1 male and female descendants of treated F0 dams were sent to the GMC at approximately 5 weeks of age, acclimatized for 2 weeks, and then subjected to their standard primary phenotyping workflow. GMC peripheral blood immunophenotyping was performed at approximately 7 weeks of age on four sex-by-treatment groups: control males (n = 15), TBT males (n = 15), control females (n = 15), and TBT females (n = 15). Each animal represented a biological replicate, and within each sex and treatment group, animals were derived from distinct litters. Treated and control offspring were not littermates. The GMC F1 animals were generated as a separate cohort from the F1 and F3 animals used for spleen qPCR and plasma ELISA analyses in the transgenerational study described below.

For the spleen qPCR and plasma ELISA analyses, a separate transgenerational experiment followed our established protocol, and materials were generated from our

recently published study [15]. Briefly, five-week-old female mice (F0) were exposed via drinking water to either 50 nM tributyltin (TBT) or vehicle control (0.1% DMSO) dissolved in 0.5% carboxymethyl cellulose (CMC) for 7 days prior to mating. Sires were unexposed. Treatment was paused during mating and resumed upon confirmation of pregnancy (vaginal plug = E0.5), continuing until postnatal day 21 (weaning). To avoid potential litter bias, only 1 male and 1 female per litter were randomly selected for analysis or breeding in each generation (F1–F3). Sibling mating was avoided. Descendants from each generation were maintained on standard chow without dietary intervention and tissues were collected at approximately 12 weeks of age. Estrous cycle stage was not monitored at the time of sample collection; therefore, female immune phenotypes were analyzed without stratification by cycle stage. For the spleen qPCR and plasma ELISA analyses, the F1 cohort was generated from 12 control F0 dams and 12 TBT-exposed F0 dams, contributing 12 control litters and 12 TBT litters. The F3 cohort was generated from 12 control-lineage litters and 12 TBT-lineage litters. Because only one male and one female were selected from each litter for analysis, each animal represented an independent litter within each sex and treatment group.

Flow Cytometry Analysis of Peripheral Blood Leukocytes (GMC Dataset)

Blood samples were collected from isoflurane-anesthetized mice by retrobulbar sinus puncture into heparinized tubes. The cell pellet was used for flow cytometric analysis of peripheral blood leukocytes. These analyses were performed as previously described. Briefly, peripheral blood leukocytes had been stained with fluorescence-conjugated monoclonal antibodies and analyzed using a 3-laser, 10-color Gallios flow cytometer (Beckman Coulter). Flow cytometry gating followed the standard GMC immunology

phenotyping workflow. Briefly, leukocytes were first identified within the CD45⁺ population, and major peripheral blood leukocyte subsets were then defined by lineage-marker expression. T cells were identified as CD3⁺ cells, with CD4⁺ and CD8⁺ T-cell subsets further defined within the T-cell compartment. Regulatory T cells were defined as CD4⁺CD25⁺ cells. $\gamma\delta$ T cells were defined as CD3⁺ $\gamma\delta$ TCR⁺ cells. B cells were identified as CD19⁺B220⁺ cells. NK cells were defined as NKp46⁺NK1.1⁺CD5⁻ cells, whereas NKT cells were defined as NKp46⁺NK1.1⁺CD5⁺ cells. Monocytes were defined as non-NK, non-granulocyte, CD11b⁺ cells. Additional subsets included T cells coexpressing CD62L or CD44, B cells coexpressing IgD and/or MHCII, and NK cells expressing CD11b. The GMC peripheral blood immunophenotyping dataset analyzed here has not been previously published, and no public accession number is available.

Spleen RNA Extraction and Quantitative PCR (qPCR) Analysis

Spleens were snap-frozen in liquid nitrogen immediately after dissection and stored at -80°C until further processing. For RNA extraction, approximately 20 mg of tissue was homogenized in TRIzol reagent (Thermo Fisher Scientific, MA) following the manufacturer-recommended protocol. Total RNA was recovered by chloroform extraction and isopropanol precipitation (Fisher Chemical, PA). RNA concentration and purity were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Complementary DNA (cDNA) was synthesized from 5 μ g of total RNA using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturer-recommended protocol. Quantitative PCR (qPCR) was performed using SYBR[™] Green PCR Master Mix (Thermo Fisher Scientific) on a Roche LightCycler 480 II system (Roche, Switzerland). Amplification specificity was verified

through melting-curve analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [27] and normalized to the expression of the housekeeping gene *Gapdh*. *Gapdh* was selected as the housekeeping gene based on its common use in mouse spleen qPCR analyses and its stable C_t values across sex, generation, and treatment groups in this dataset. Cycle threshold (C_t) values were determined using the second derivative maximum algorithm in the LightCycler software. All qPCR reactions were performed in triplicate, and data are presented as mean $\pm$ SEM. For fold-change calculations derived from the $2^{-\Delta\Delta C_t}$ method, standard propagation of error was used according to established procedures used in our previous studies [15]. A p -value < 0.05 was considered statistically significant. Primer sequences used in this study are listed in Supplementary Table 1 [28].

Splenic Protein Extraction and ELISA Analysis

Snap-frozen spleen tissues were homogenized in RIPA lysis buffer (25 mM Tris-HCl, pH 7.6; 150 mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS) containing protease inhibitor cocktail (Abcam, #ab201111) and centrifuged to collect the supernatant. Protein concentrations were measured using the Qubit Protein Assay Kit (A50668, ThermoFisher). Plasma and splenic protein levels of inflammatory cytokines were measured using TNF (Crystal Chem Cat# C67100, RRID:AB_3752618)) and IL-6 ELISA kits (Crystal Chem Cat# C66100, RRID:AB_3752619) following the manufacturer-recommended protocol. All ELISA samples and standards were run in duplicate. Cytokine concentrations were calculated from standard curves generated on each plate. No samples included in the final analyses were outside the assay range or below the limit of detection. No wells failed technical quality control; if a technical

1 replicate had failed, that well would have been excluded and the sample re-assayed
2 when sufficient material was available.

3 **Statistical Analysis**

4 Data are presented as mean $\pm$ SEM. Statistical significance was determined using
5 unpaired two-tailed Student's t-tests for comparisons between two groups. For
6 comparisons involving more than two groups, one-way or two-way ANOVA followed by
7 Tukey's post hoc test was used, as appropriate. For two-way ANOVA analyses, the main
8 effects of treatment and sex, as well as the treatment $\times$ sex interaction term, were
9 evaluated. When a significant interaction was detected, multiple-comparison post hoc
10 tests were used to compare treatment effects within sex; when the interaction term was
11 not significant, interpretation focused on the relevant main effects. Because only one
12 animal per sex from each litter was included in each analysis group, each animal
13 represented an independent litter within that sex and treatment group. Therefore, litter
14 was not included as an additional random effect in the statistical models. All analyses
15 were performed using GraphPad Prism (version 10; GraphPad Software, San Diego,
16 CA), and $p < 0.05$ was considered statistically significant.

Results

Altered Immune Cell Profiles in F1 Peripheral Blood Following Maternal TBT

Exposure

The German Mouse Clinic (GMC) provides comprehensive, standardized phenotyping across multiple physiological systems, including immunology, in large cohorts of mice. This platform enables unbiased detection of immune-related phenotypes that may not be apparent in smaller, single-laboratory studies. To leverage this resource, we analyzed previously unpublished peripheral blood immunophenotyping data generated by the GMC from a maternal TBT exposure cohort generated at UCI. These data provided an initial indication that developmental TBT exposure may be associated with altered immune cell distributions in offspring and motivated subsequent tissue-level analyses.

To assess whether maternal TBT exposure influenced circulating immune cell populations in F1 offspring, we reanalyzed raw GMC flow cytometry data from peripheral blood collected from F1 male and female mice whose F0 dams were exposed to vehicle (DMSO) or TBT during pregnancy. All animals were maintained on a standard chow diet without postnatal dietary intervention. Reanalysis revealed reproducible alterations in lymphoid cell composition in TBT-exposed offspring (Figure 1). In both males and females, the relative proportion of B cells was increased, whereas the proportion of T cells was decreased in the TBT group compared with controls (Figure 1A–D). These changes were further supported by analysis of the B cell-to-T cell relationship, which showed a shift toward B-cell predominance in TBT-exposed offspring of both sexes (Figure 1B, C). In addition, the CD4/CD8 ratio was reduced, particularly in

1 females, and NK cell frequencies were also decreased in TBT-exposed offspring of both
2 sexes (Figure 1E, F). Together, these findings indicate that maternal TBT exposure is
3 associated with altered peripheral lymphocyte composition in F1 offspring.

4 Because these analyses were performed on peripheral blood at a single developmental
5 time point, we interpreted the GMC findings as evidence of altered immune
6 homeostasis rather than overt pathology. We therefore performed follow-up analyses in
7 spleen and plasma to determine whether these immune alterations were associated
8 with tissue-level and systemic inflammatory changes and whether such effects persisted
9 across generations.

10 **Maternal TBT exposure alters splenic immune gene expression in F1 offspring in** 11 **a sex-dependent manner.**

12 To determine whether maternal TBT exposure affected immune homeostasis in
13 secondary lymphoid organs, we performed qPCR analysis of lineage-specific markers in
14 archived F1 spleen tissue collected from a recently completed transgenerational
15 experiment [15]. We measured expression of lineage-specific genes for T cells, B cells,
16 NK cells, and myeloid cells, as well as inflammatory cytokines.

17 In male offspring (Figure 2), we observed a significant reduction in expression of
18 mRNAs encoding multiple T cell associated genes in the TBT group, including *Cd3e*
19 ($p<0.05$), *Cd4* ($p<0.05$), and *Foxp3* ($p<0.001$). *Cd44* expression was not significantly
20 changed (Figure 2a-2d). These findings are consistent with reduced expression of
21 helper T-cell- and regulatory T-cell-associated markers in the male spleen. B cell
22 markers *Cd19* and *Cd79a* were unchanged (Figure 2e, 2f). NK cell marker *Nkg7* did not

change (Figure 2g), but expression of *Ifng*, a cytokine associated with NK and Th1 cell function, was significantly elevated ($p<0.05$) (Figure 2h), indicating a possible pro-inflammatory shift. Myeloid cell associated genes *Ly6c1*, *Cd11b*, and *Cd163* were significantly decreased, while *Cd14* remained unchanged (Figure 2i-2l). We also observed increased expression of inflammatory cytokines *Tnf* ($p<0.05$) and *Il6* ($p<0.001$), consistent with an inflammatory phenotype (Figure 2m,2n).

In contrast, female offspring (Figure 3) exhibited a distinct gene expression profile. Similar to males, *Cd3e*, *Cd4*, and *Foxp3* expression were reduced in TBT females ($p<0.05$), indicating reduced expression of T-cell-associated markers, including *Foxp3* (Figure 3a-3d). However, unlike in males, TBT-exposed females also showed significant reductions in B cell markers *Cd19* and *Cd79a* ($p<0.05$), indicating a possible broader suppression of adaptive immune components (Figure 3e,3f). NK cell marker genes were not significantly altered: *Nkg7* remained unchanged and *Ifng* was not detectable in either group (Figure 3g,3h). Among myeloid markers, only *Cd11b* was significantly elevated ($p<0.05$); other markers were unchanged (Figure 3i-3l). Expression of *Il6* was modestly but significantly elevated ($p<0.05$), whereas *Tnf* remained unchanged (Figure 3m,3n).

These data are consistent with a model in which maternal TBT exposure alters splenic immune cell gene expression in a sex-dependent manner. Male offspring exhibited decreased T cell and myeloid gene expression with concomitant increases in inflammatory cytokines; female offspring showed reductions in both T and B cell markers and limited pro-inflammatory activation. These findings suggest that maternal TBT exposure is associated with sex-dependent changes in splenic immune-marker

expression, which may intersect with previously reported sex-specific metabolic phenotypes.

Transgenerational effects of ancestral TBT exposure on splenic immune gene expression in F3 offspring.

To assess whether immune gene expression changes observed in prenatally-exposed F1 persisted in unexposed F3 descendants, we analyzed spleen samples from F3 male and female offspring. We measured the same lineage and inflammatory markers examined in F1 to determine whether any of the TBT-induced immune phenotypes were heritable.

In F3 male offspring (Figure 4), we found that expression of all T cell–associated genes *Cd3e*, *Cd4*, *Cd44*, and *Foxp3* was significantly downregulated in the TBT group ($p<0.05$ for all), mirroring the T cell suppression observed in F1 (Figure 4a–4d). Expression of B cell markers *Cd19* and *Cd79a* remained unchanged (Figure 4e,4f). NK cell genes *Nkg7* and *Ifng* were also unchanged (Figure 4). Interestingly, expression of myeloid markers *Cd11b*, *Cd14*, and *Cd163* was significantly decreased in the TBT group ($p<0.05$), whereas *Ly6c1* was unaffected. Expression of both inflammatory cytokine genes *Tnf* and *Il6* was significantly upregulated ($p<0.05$), suggesting that the pro-inflammatory phenotype persisted into the F3 generation.

In contrast, F3 female offspring (Figure 5) showed no significant changes in expression of any measured genes. Expression of *Cd3e*, *Cd4*, *Foxp3*, *Cd19*, *Cd79a*, *Nkg7*, *Ifng*, and all four myeloid markers (*Ly6c1*, *Cd11b*, *Cd14*, *Cd163*) did not change significantly.

1 Similarly, expression of inflammatory markers *Tnf* and *IL6* was not significantly different
2 between groups.

3 We infer from these results that alterations in splenic immune-marker expression persist
4 in F3 male descendants following ancestral TBT exposure. The persistence of reduced
5 T cell- and myeloid-associated gene expression together with elevated pro-inflammatory
6 cytokine expression is consistent with transgenerationally persistent immune
7 dysregulation in male descendants.

8 **TBT exposure increases circulating inflammatory cytokines in F1 and F3 male** 9 **offspring.**

10 To determine whether the immune alterations observed at the transcript level were
11 reflected in systemic inflammation, we measured circulating levels of pro-inflammatory
12 cytokines in plasma samples from F1 and F3 offspring using ELISA.

13 In F1 males, plasma levels of both TNF and IL-6 were significantly elevated in the TBT
14 group compared to controls (TNF: $p < 0.05$; IL-6: $p < 0.05$), indicating likely increased
15 systemic inflammation (Figure 6a–b). In contrast, F1 females did not show significant
16 changes in either cytokine (Figure 6c–d).

17 We observed a similar sex-specific pattern in F3 animals. Plasma TNF and IL-6 levels
18 were significantly increased in F3 males following ancestral TBT exposure ($p < 0.05$ for
19 both; Figure 6e–f), while no changes were detected in F3 females (Figure 6g–h).

20 These data indicate that ancestral TBT exposure is associated with persistent, male-
21 specific increases in circulating inflammatory cytokines. Together with the spleen gene

expression data, these findings support the possibility that in utero TBT exposure is associated with a heritable pro-inflammatory state in male descendants.

Discussion

Our analysis began with peripheral blood immunophenotyping data generated by the German Mouse Clinic (GMC), which revealed altered lymphocyte composition in F1 offspring following maternal TBT exposure. These unbiased observations, derived from standardized large-cohort phenotyping, suggested that early-life TBT exposure may be associated with altered immune homeostasis in peripheral blood and motivated further tissue-level investigation.

To determine whether immune alterations extended beyond peripheral blood and persisted across generations, we examined splenic immune-marker expression and circulating inflammatory cytokine levels in F1 and F3 offspring of F0 dams treated with TBT throughout pregnancy and lactation. We found that maternal TBT exposure was associated with reduced splenic expression of T cell- and myeloid-associated genes and with elevated TNF and IL-6 levels in F1 males, with similar patterns persisting in unexposed F3 male descendants. These effects were not observed in females.

Together, these findings indicate that developmental TBT exposure is associated with a heritable, male-specific pro-inflammatory phenotype, rather than only transient changes in circulating immune cell distributions.

Our findings are also consistent with prior work showing that TBT disrupts cytokine networks in vivo. Lawrence et al. reported that acute TBT exposure altered a wide range

1 of serum cytokines in BALB/c mice, including IFN γ , TNF α , IL-1 β , IL-2, IL-5, IL-7, IL-
2 12p40, IL-13, IL-15, MIP-1 β , RANTES, KC, and MIP-2 [29]. Some cytokines, such as
3 IFN γ and TNF α , were transiently elevated within hours of exposure, whereas others
4 (e.g., IL-2, IL-7, IL-15) were decreased at later time points [29]. These data
5 demonstrated that TBT can rapidly dysregulate both pro- and anti-inflammatory
6 signaling in serum. Our study extends these observations by showing that
7 developmental exposure is associated with long-lasting, male-biased alterations in
8 splenic immune-marker expression and inflammatory cytokines, with related effects
9 persisting into unexposed generations.

10 Our findings extend a substantial body of work showing that TBT is immunotoxic and
11 immunomodulatory in diverse systems. Previous studies reported thymic atrophy and
12 impaired T-cell development in rodents [20], suppressed NK cell cytotoxic function in
13 human immune cells [21], and altered cytokine secretion, including IL-1 β dysregulation,
14 in peripheral immune cells [22]. In aquatic models such as zebrafish, TBT reduced
15 lysozyme activity and IgM levels and disrupted immune gene expression [23]. A recent
16 review emphasized that TBT affects both innate and adaptive immune functions across
17 taxa [19]. Extending these prior reports of direct immunotoxicity, our study suggests that
18 prenatal TBT exposure is also associated with longer-lasting, male-biased alterations in
19 splenic immune-marker expression and inflammatory cytokine levels, with related
20 effects persisting into unexposed descendants. These findings support the possibility
21 that TBT not only exerts acute effects on immune function but may also contribute to
22 lasting transgenerational immune phenotypes.

1 These results extend previous work from our lab showing that ancestral TBT exposure
2 increased adiposity, impaired insulin signaling, and promoted hepatic pathology in a
3 male-biased manner [13, 30]. The current findings identify a parallel male-biased
4 immune phenotype characterized by reduced splenic T cell- and myeloid-associated
5 marker expression together with elevated *Tnf/Il6* expression and increased circulating
6 TNF and IL-6. Because chronic low-grade inflammation is closely linked to adipose
7 tissue dysfunction, insulin resistance, and liver pathology, these immune alterations may
8 represent an immune component of the broader male-biased metabolic phenotype
9 previously reported in this lineage. A study using a synthetic diet that modeled the 50th
10 percentile of US dietary consumption further demonstrated that TBT-lineage mice
11 exhibited elevated plasma cytokines, including TNF and IL-6, consistent with chronic
12 low-grade inflammation [15]. Our current findings build on these observations by
13 identifying the spleen as a potential site of altered immune homeostasis associated with
14 ancestral TBT exposure.

15 Although prior studies from our group identified epigenetic alterations associated with
16 TBT-induced metabolic phenotypes, the present study did not directly assess DNA
17 methylation, chromatin accessibility, or other epigenetic marks in immune cells.
18 Therefore, we interpret the current findings as evidence of immune phenotypes
19 associated with ancestral TBT exposure, rather than direct demonstration of epigenetic
20 inheritance in immune cells.

21 We also previously demonstrated that TBT promoted macrophage M1 polarization and
22 enhanced lipolysis in co-cultured adipocytes [18]. The observed decrease in *Cd14*,
23 *Cd11b*, and *Cd163* expression in spleens of F1 and F3 TBT males suggested that TBT

1 altered myeloid-associated gene expression and innate immune homeostasis in vivo.
2 These alterations may reflect reduced representation or altered functional state of anti-
3 inflammatory monocyte/macrophage populations, thereby contributing to systemic
4 inflammation.

5 T cell-associated genes, including *Cd3e*, *Cd4*, and *Foxp3*, were also consistently
6 suppressed in TBT males. The decrease in *Foxp3* expression raises the possibility that
7 developmental TBT exposure disrupts pathways related to regulatory T cell
8 maintenance or function. Because PPAR γ is required for Treg accumulation and anti-
9 inflammatory activity in adipose tissue [26, 31], it is plausible that RXR/PPAR γ signaling
10 during development contributes to this altered immune balance. This interpretation is
11 consistent with the increased *Tnf* and *Il6* expression and circulating cytokine levels
12 observed in F1 and F3 TBT males.

13 Future studies will be needed to define the cellular and molecular mechanisms
14 underlying these immune phenotypes. Important next steps include sorting splenic T cell
15 and monocyte/macrophage populations from F1 and F3 control- and TBT-lineage
16 animals, followed by DNA methylation, chromatin accessibility, and transcriptional
17 profiling to determine whether persistent immune alterations are associated with cell-
18 type-specific epigenetic changes. Functional assays will also be important, including
19 tests of regulatory T cell suppressive capacity and monocyte/macrophage cytokine
20 production, to determine whether the observed changes in immune-marker expression
21 correspond to altered immune cell function.

22 Importantly, these immune phenotypes were male-biased and persisted across
23 generations, mirroring the sex bias observed in TBT-associated metabolic phenotypes

[13, 14]. The mechanistic basis for this sex difference remains unclear, but several non-mutually exclusive mechanisms may contribute. First, sex hormones regulate both innate and adaptive immune responses, and developmental TBT exposure may interact with sexually dimorphic endocrine signaling during immune-system maturation [32]. Second, sex chromosome complement and sex chromosome-linked epigenetic regulation may contribute to baseline differences in immune-cell development or responsiveness to environmental exposures. Third, immune development itself is sexually dimorphic, including differences in T cell regulation, macrophage function, and inflammatory cytokine responses [33]. These mechanisms may help explain why male descendants showed persistent suppression of splenic T cell- and myeloid-associated markers together with elevated TNF and IL-6, whereas females exhibited weaker or non-persistent inflammatory changes. Importantly, the male-biased immune phenotype observed here does not indicate that females are generally insensitive to TBT. Previous studies revealed that TBT can disrupt female reproductive and endocrine physiology, including ovarian folliculogenesis, ovarian reserve, uterine morphology, and hypothalamic-pituitary-gonadal axis function [34, 35]. Thus, TBT effects are likely to be tissue-, endpoint-, developmental-window-, and generation-specific. In the present study, females exhibited several F1 spleen immune-marker changes, but these changes did not persist as a broad inflammatory cytokine phenotype in F3 females, suggesting that female TBT responses may follow mechanisms distinct from the male-biased immune-metabolic trajectory observed here. Future studies examining sex hormone signaling, sex chromosome-associated epigenetic regulation, and cell-type-specific immune function will be needed to define the basis of this male-biased phenotype.

1 These data raise the possibility that immune dysfunction may contribute to the “thrifty
2 phenotype” observed in previous studies [14]. Reduced *Foxp3* expression may reflect
3 impaired regulatory T cell abundance or function, while reduced myeloid-associated
4 markers may indicate altered monocyte/macrophage composition or activation state.
5 Together with elevated *Tnf* and *Il6* expression and increased circulating TNF and IL-6,
6 these changes are consistent with a shift toward chronic low-grade inflammation. Such
7 an inflammatory state could plausibly contribute to impaired insulin signaling, adipose
8 tissue dysfunction, and hepatic pathology, all of which were observed in our prior
9 metabolic studies [14, 15]. However, this remains to be shown, and the present study
10 does not directly test whether immune alterations cause these metabolic outcomes.
11 Therefore, we interpret these findings as supporting the possibility that immune
12 dysregulation may be one component of the broader phenotype induced by early-life
13 obesogen exposure.

14 Several limitations should be considered. First, the relationship between the immune
15 alterations reported here and the metabolic phenotypes described in our prior studies is
16 correlative. Although the male-biased increases in TNF and IL-6 and changes in splenic
17 immune-marker expression are consistent with chronic low-grade inflammation, the
18 present study does not directly test whether these immune changes cause the
19 increased adiposity, insulin resistance, or hepatic pathology observed in our published
20 study [14, 15]. Second, our spleen qPCR analyses were performed using bulk tissue
21 RNA. Therefore, reduced expression of lineage-associated markers such as *Foxp3*,
22 *Cd3e*, *Cd4*, *Cd11b*, or *Cd163* cannot distinguish between changes in immune cell
23 abundance and changes in gene expression within specific immune cell populations.

1 Third, immune readouts were assessed at a single time point in each generation, which
2 limits our ability to determine when these immune alterations first emerge, whether they
3 change with age, or how they relate temporally to metabolic phenotypes. Finally,
4 although prior studies from our group identified epigenetic alterations associated with
5 TBT-induced metabolic phenotypes [14, 17], the present study did not directly assess
6 DNA methylation, chromatin accessibility, histone modifications, or other epigenetic
7 marks in immune cells.

8 In conclusion, our findings suggest that the immune system is an important target of
9 TBT-induced developmental programming. The reduced expression of lymphoid- and
10 myeloid-associated markers together with elevated inflammatory cytokines in TBT-
11 exposed male descendants supports the idea that immune dysregulation is a
12 transgenerational, male-biased phenotype of environmental obesogen exposure. These
13 results broaden the known scope of TBT's effects and warrant further investigation into
14 how immune alterations intersect with metabolic outcomes.

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Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in the References.

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Figure 1. Maternal TBT exposure alters peripheral blood lymphocyte composition in F1 offspring.

Peripheral blood immunophenotyping data generated by the German Mouse Clinic were reanalyzed from raw flow cytometry tables and replotted. Immune cell populations are shown as percentages of total CD45⁺ leukocytes in F1 offspring of dams exposed to vehicle (DMSO) or tributyltin (TBT) during pregnancy. Data are presented separately for males and females. Shown are (A) B cells, the relationship between B-cell and T-cell percentages in (B) males and (C) females, (D) T cells, (E) the CD4/CD8 ratio, and (F) NK cells. Control males, TBT males, control females, and TBT females each included 15 animals. Each animal represents an independent biological replicate derived from a distinct litter. Each point represents an individual animal; horizontal bars in panels A, D, E, and F indicate mean $\pm$ SEM. Statistical significance in panels A, D, E, and F was assessed by two-way ANOVA evaluating the main effects of treatment and sex and the treatment $\times$ sex interaction, followed by Tukey's multiple-comparisons test where appropriate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$; ns, not significant.

Figure 2. Maternal TBT exposure alters splenic immune-marker expression in F1 male offspring.

Quantitative PCR analysis was performed using spleen tissue from F1 male offspring in the independent transgenerational spleen/plasma cohort following maternal exposure to vehicle (DMSO) or tributyltin (TBT) during pregnancy (n = 12 per group). Expression levels of lineage-associated and inflammatory genes were normalized to Gapdh and are presented relative to the mean of the DMSO control group. (A–D) T-cell markers, *Cd3e*, *Cd4*, *Cd44*, and *Foxp3*. (E–F) B-cell markers, *Cd19* and *Cd79a*. (G–H) NK-cell markers, *Nkg7* and *Ifng*. (I–L) Myeloid-associated markers, *Ly6c1*, *Cd11b*, *Cd14*, and *Cd163*. (M–N) Inflammatory cytokine genes, *Tnf* and *Il6*. Each point represents an individual animal; horizontal bars indicate mean $\pm$ SEM. Each animal was selected from a distinct litter; therefore, n represents both the number of animals analyzed and the number of independent litters contributing to each group. Statistical significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

Figure 3. Maternal TBT exposure alters splenic immune-marker expression in F1 female offspring.

Quantitative PCR analysis was performed using spleen tissue from F1 female offspring in the independent transgenerational spleen/plasma cohort following maternal exposure to vehicle (DMSO) or tributyltin (TBT) during pregnancy. Expression levels of lineage-associated and inflammatory genes were normalized to Gapdh and are presented relative to the mean of the DMSO control group. (A–D) T-cell markers, *Cd3e*, *Cd4*, *Cd44*, and *Foxp3*. (E–F) B-cell markers, *Cd19* and *Cd79a*. (G–H) NK-cell markers, *Nkg7* and *Ifng*. (I–L) Myeloid-associated markers, *Ly6c1*, *Cd11b*, *Cd14*, and *Cd163*. (M–N) Inflammatory cytokine genes, *Tnf* and *Il6*. Each point represents an individual animal;

horizontal bars indicate mean $\pm$ SEM. Up to 12 animals were analyzed per group. Each animal was selected from a distinct litter; therefore, n also represents the number of independent litters contributing to each group. Statistical significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant; n.d., not detected.

Figure 4. Ancestral TBT exposure is associated with persistent splenic immune-marker dysregulation in F3 male offspring.

Quantitative PCR analysis was performed using spleen tissue from F3 male offspring in the independent transgenerational spleen/plasma cohort derived from dams ancestrally exposed to vehicle (DMSO) or tributyltin (TBT) during pregnancy. Expression levels of lineage-associated and inflammatory genes were normalized to Gapdh and are presented relative to the mean of the DMSO control group. (A–D) T-cell markers, *Cd3e*, *Cd4*, *Cd44*, and *Foxp3*. (E–F) B-cell markers, *Cd19* and *Cd79a*. (G–H) NK-cell markers, *Nkg7* and *Ifng*. (I–L) Myeloid-associated markers, *Ly6c1*, *Cd11b*, *Cd14*, and *Cd163*. (M–N) Inflammatory cytokine genes, *Tnf* and *Il6*. Each point represents an individual animal; horizontal bars indicate mean $\pm$ SEM. Up to 12 animals were analyzed per group. Each animal was selected from a distinct litter; therefore, n also represents the number of independent litters contributing to each group. Statistical significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant; n.d., not detected.

Figure 5. Ancestral TBT exposure does not significantly alter splenic immune-marker expression in F3 female offspring.

Quantitative PCR analysis was performed using spleen tissue from F3 female offspring in the independent transgenerational spleen/plasma cohort derived from vehicle (DMSO) or tributyltin (TBT) lineages. Expression levels of lineage-associated and inflammatory genes were normalized to Gapdh and are presented relative to the mean of the DMSO control group. (A–D) T-cell markers, *Cd3e*, *Cd4*, *Cd44*, and *Foxp3*. (E–F) B-cell markers, *Cd19* and *Cd79a*. (G–H) NK-cell markers, *Nkg7* and *Ifng*. (I–L) Myeloid-associated markers, *Ly6c1*, *Cd11b*, *Cd14*, and *Cd163*. (M–N) Inflammatory cytokine genes, *Tnf* and *Il6*. Each point represents an individual animal; horizontal bars indicate mean $\pm$ SEM. Up to 12 animals were analyzed per group. Each animal was selected from a distinct litter; therefore, n also represents the number of independent litters contributing to each group. Statistical significance was determined using an unpaired two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant; n.d., not detected.

Figure 6. Maternal and ancestral TBT exposure are associated with increased circulating inflammatory cytokines in male offspring.

Enzyme-linked immunosorbent assay analysis was performed to measure plasma TNF and IL-6 concentrations in F1 and F3 offspring from the independent transgenerational spleen/plasma cohort derived from vehicle (DMSO) or tributyltin (TBT) lineages. (A–B) Plasma TNF and IL-6 concentrations in F1 males. (C–D) Plasma TNF and IL-6 concentrations in F1 females. (E–F) Plasma TNF and IL-6 concentrations in F3 males. (G–H) Plasma TNF and IL-6 concentrations in F3 females. Each point represents an individual animal; horizontal bars indicate mean $\pm$ SEM (n = 12 per group). Cytokine concentrations are expressed as pg per mg of total protein. Each animal was selected

1 from a distinct litter; therefore, n also represents the number of independent litters
2 contributing to each group. Statistical significance was determined using an unpaired
3 two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

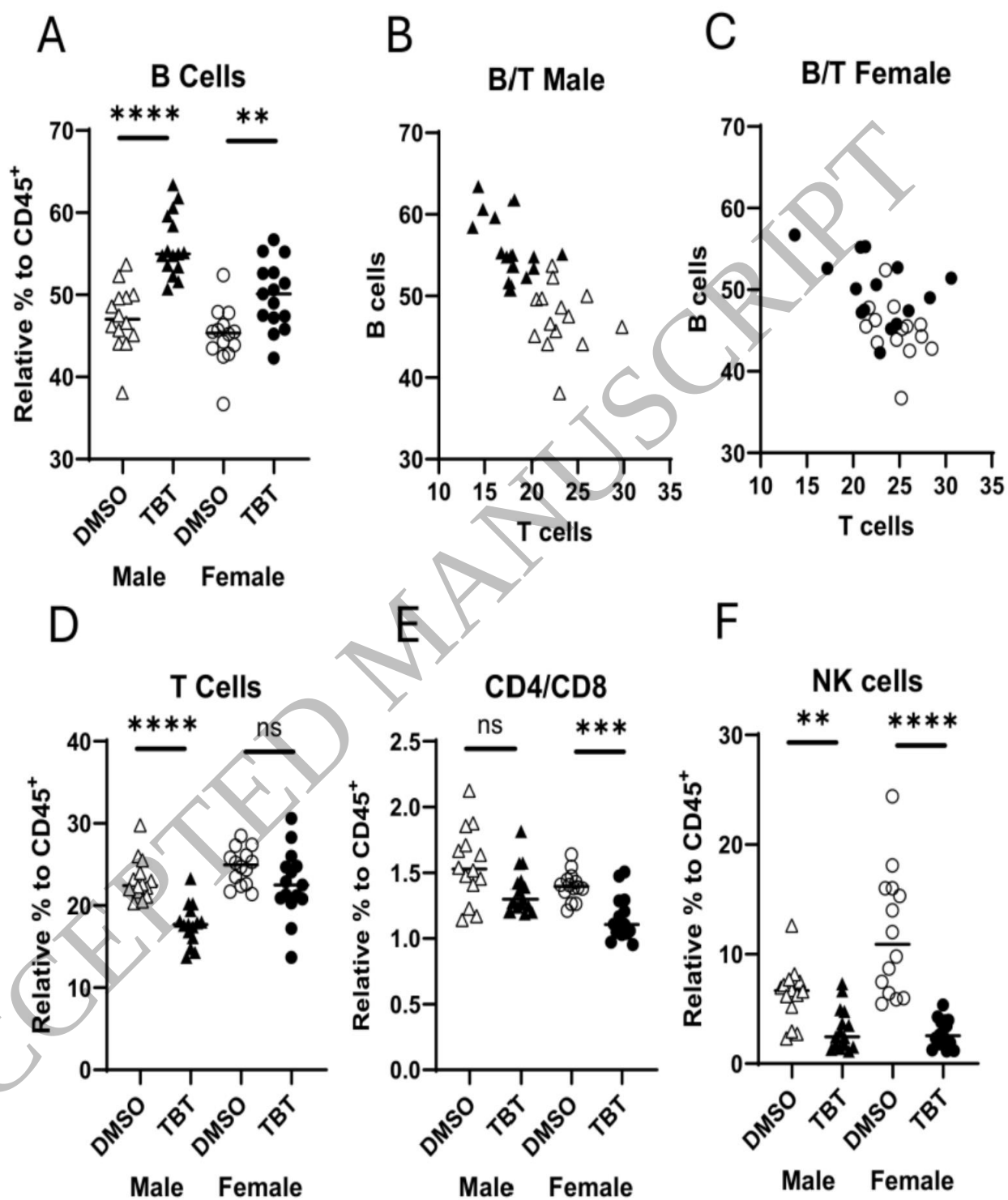


Figure 1
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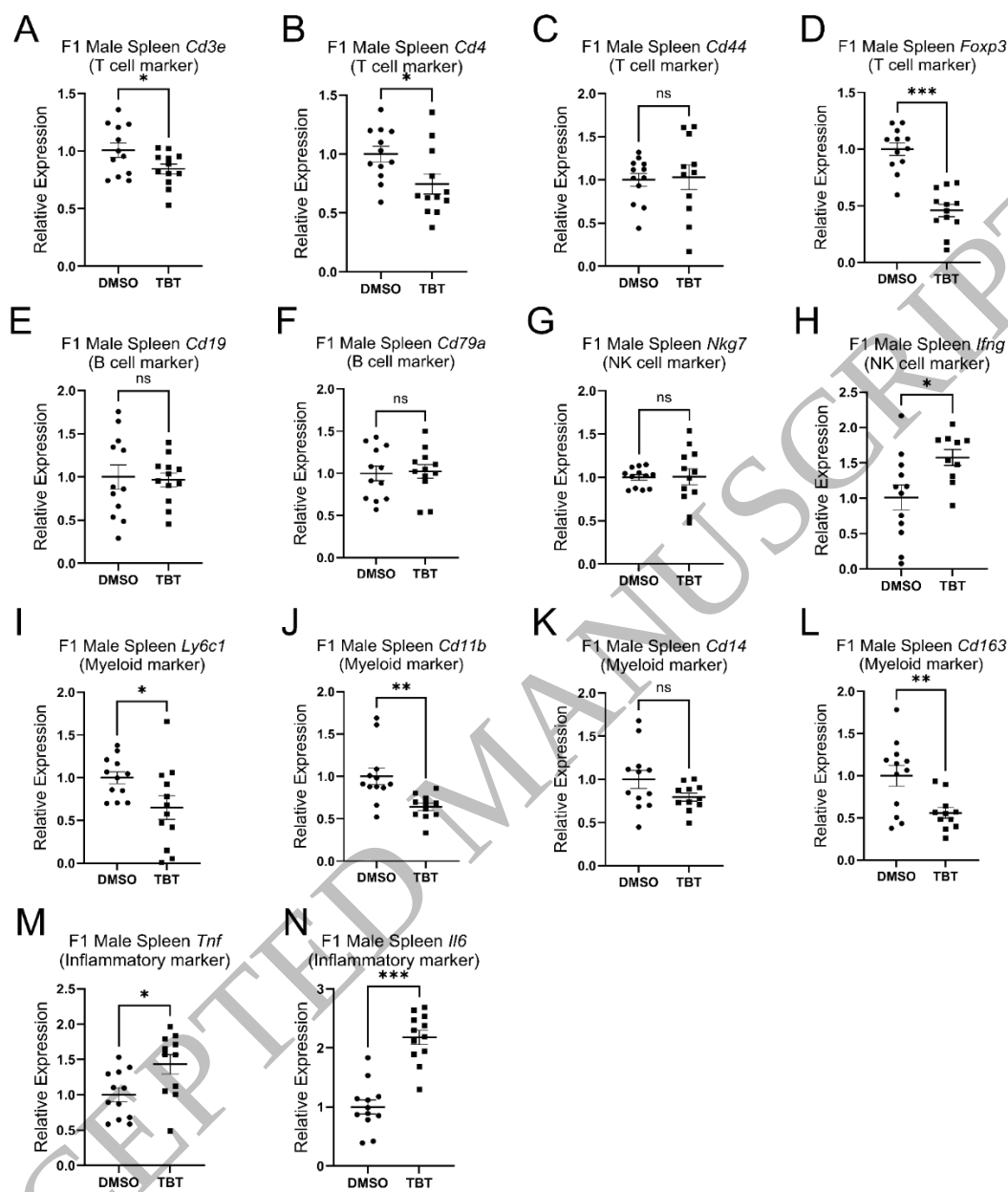


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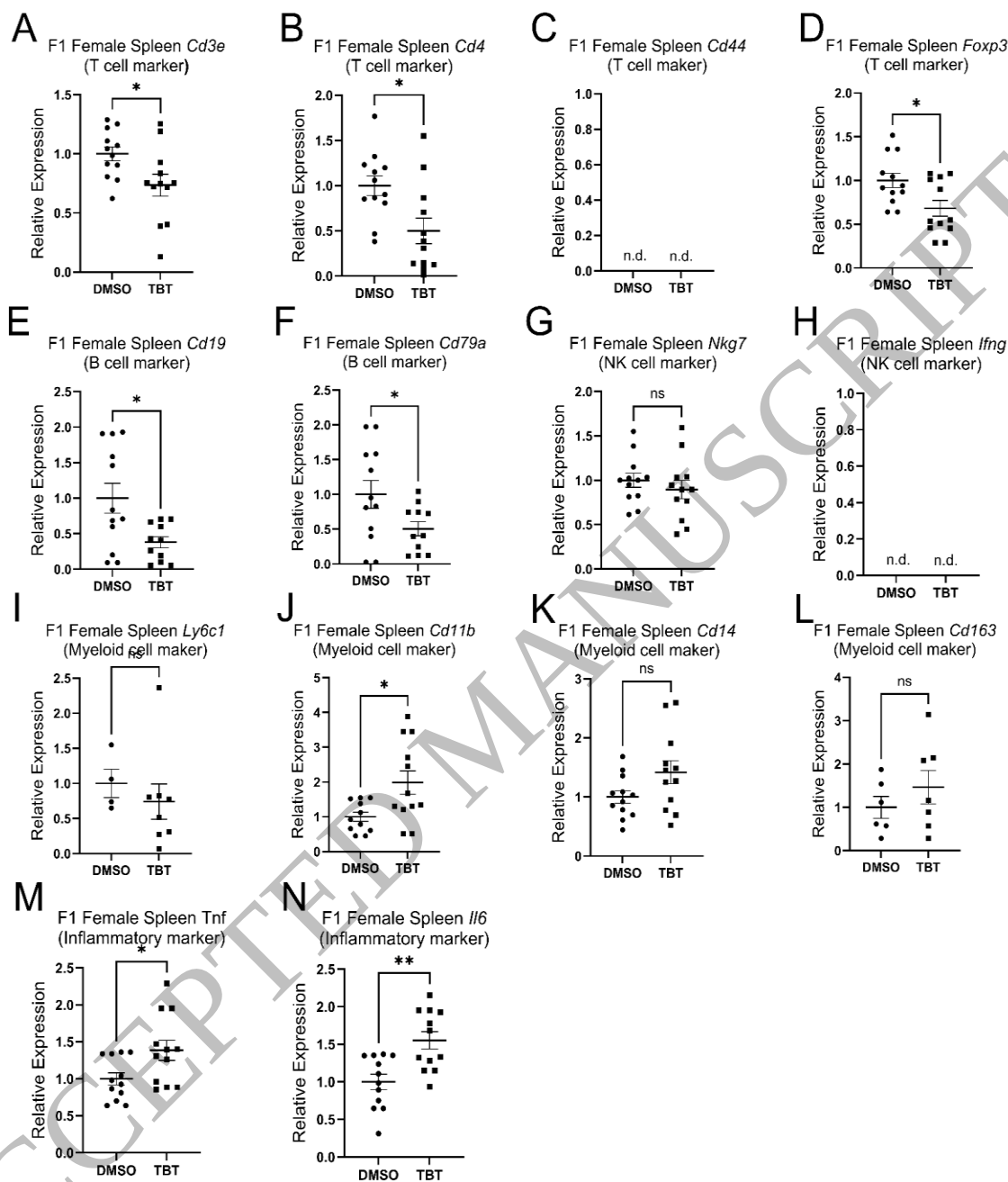


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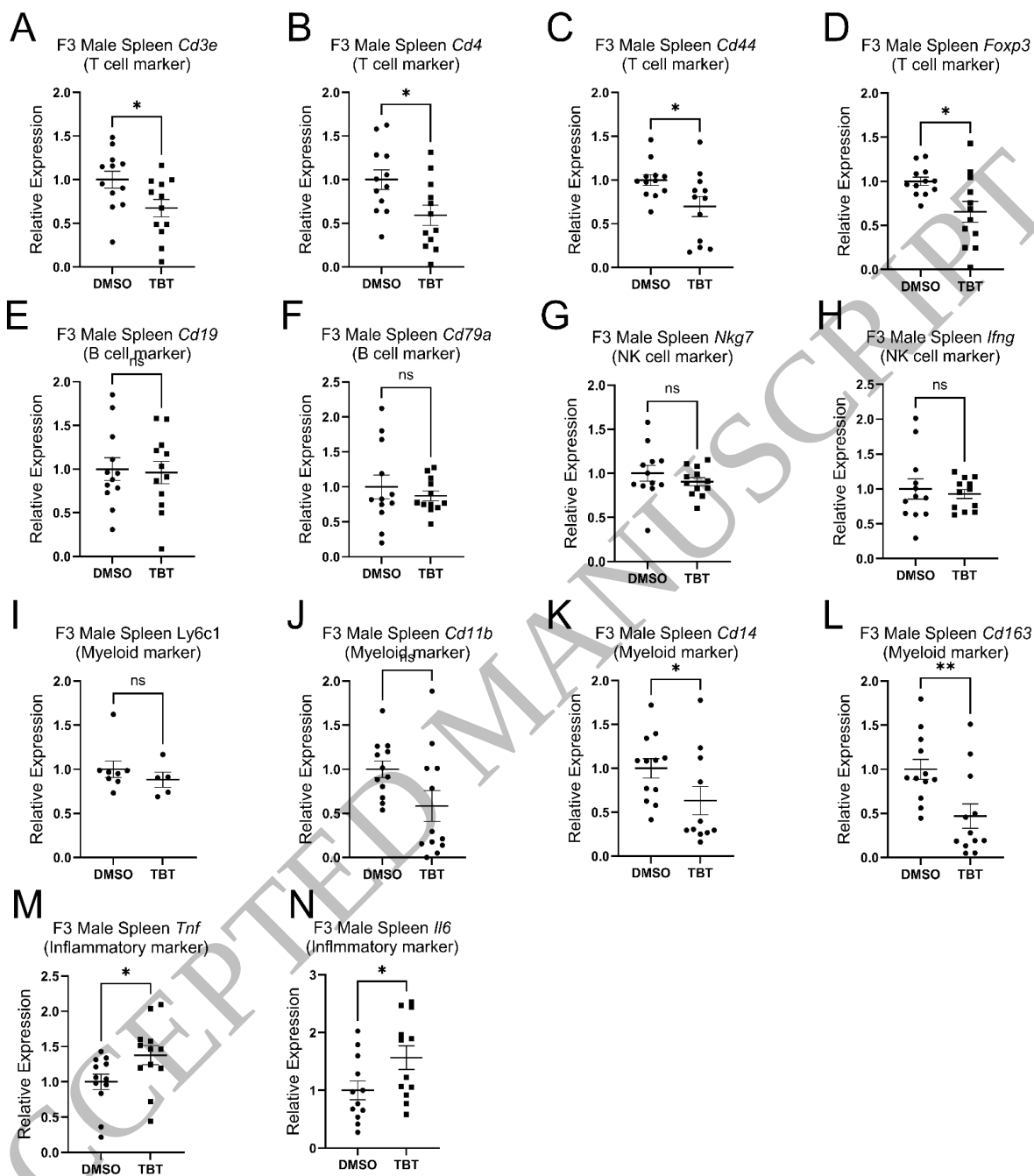


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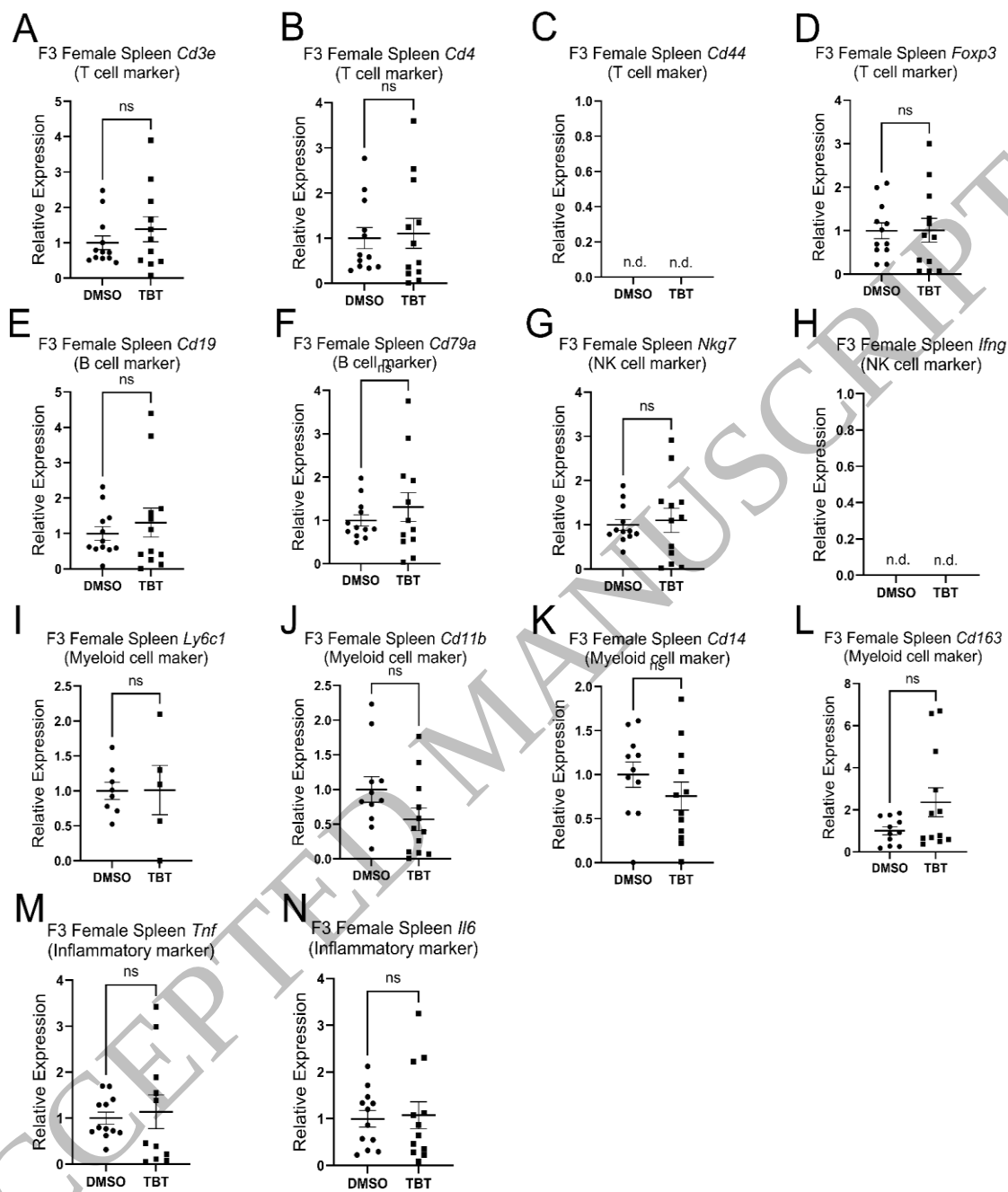


Figure 5
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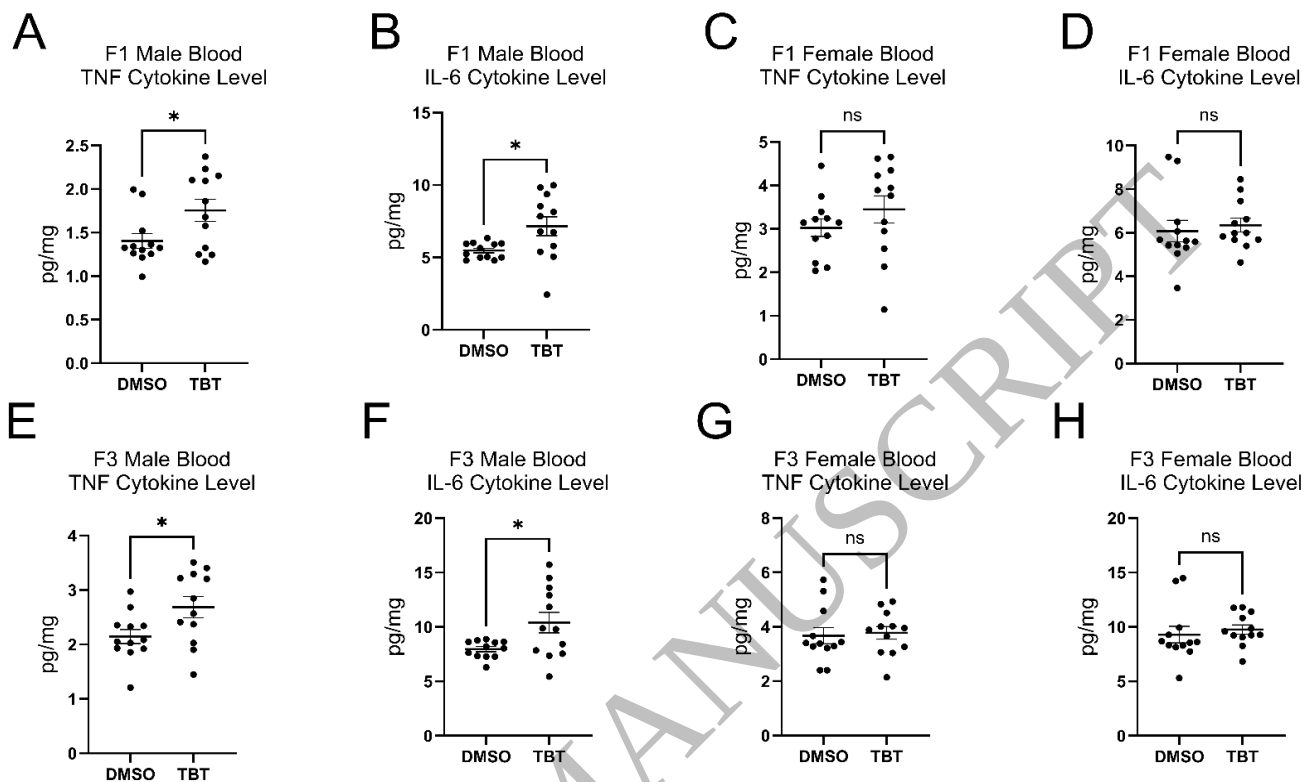


Figure 6
193x123 mm (x DPI)