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The Hidden Threat: Environmental Level of Organotin Compromises Immunity

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The Hidden Threat: Environmental Level of Organotin Compromises Immunity

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Abstract

Tributyltin (TBT) is an environmental contaminant whose immunotoxic potential has been increasingly recognized, despite an established safe exposure limit of 250 ng/kg/day. This study evaluated the immunological effects of acute TBT exposure at 100 ng/kg/day, a dose 2.5 times below the WHO threshold, on BALB/c mice. Male mice received TBT or vehicle (0.4% ethanol) by gavage for 14 days. Body and organ weights were monitored, and spleen and bone marrow cells were analyzed by flow cytometry (CD4, CD8, FOXP3, B220, IgM, CD11b). Total serum IgG, IgM and IgE were measured by ELISA, and *in vitro* cytokine secretion by anti-CD3-stimulated splenic T cells was assessed. TBT suppressed myeloid cells, B cells, and several T cell subsets peripherally, and impacted central B cell production. Serum immunoglobulin levels remained unchanged. However, IL-6, IL-10, and IL-2 secretion by stimulated T lymphocytes was significantly decreased. These findings indicate that

TBT at a dose below the WHO safe threshold induces significant immunosuppression in BALB/c mice, supporting the need to reassess the current acceptable daily intake.

Keywords: endocrine disrupting-chemical, tributyltin, immune system, immunoglobulins, cytokines, flow cytometry

Short Title: Effects of organotin on the immune system

Introduction

Tributyltin (TBT) is an organotin endocrine-disrupting chemical (EDC) whose environmental persistence and immunotoxic potential have been increasingly documented over the past three decades. Widely deployed in antifouling marine coatings from the 1960s onward, TBT leaches from painted surfaces into sediments and the water column, where its strong affinity for organic matter prolongs its environmental half-life for decades and, according to mathematical modelling, potentially up to a century [1, 2]. Despite a global ban on TBT-based antifouling paints enforced by the International Maritime Organization since 2008 [3], the compound remains detectable in marine sediments, seafood, and human tissues across all continents. Because TBT bioaccumulates and biomagnifies along marine food webs, dietary exposure, particularly through shellfish consumption, continues to represent a meaningful route of human contamination [4, 5]. The breadth of its toxic effects, spanning endocrine, metabolic, reproductive, and immune targets, has been comprehensively reviewed by our group [6]. Here we focus specifically on its immunotoxicity at an environmentally relevant dose.

The current acceptable daily intake (ADI) for TBT, set by the World Health Organization at 250 ng/kg/day, was derived by applying a 100-fold safety factor to rodent data in which dietary TBT caused reductions in thymic weight and function [7, 8]. This derivation has been challenged by subsequent findings demonstrating toxic effects at concentrations well below the ADI. In female rats, 100 ng/kg/day, a dose 2.5-fold lower than the ADI, was sufficient to disrupt the hypothalamic–pituitary–adrenal axis, producing measurable inflammation, oxidative stress, and fibrosis [9]. The same dose range induced renal impairment in a separate rodent study, characterised by reduced glomerular filtration rate, proteinuria, and kidney fibrosis [10]. Taken together, these observations suggest that the existing safety threshold may not be protective across all physiological systems, and that the immune system, which was the original basis of the ADI calculation, has not been formally re-evaluated under sub-threshold exposure conditions using modern immunological techniques.

The association between TBT and immunotoxicity is well established in aquatic species: thymic atrophy and lymphopenia have been reported in rainbow trout [11], and elevated TBT tissue burdens have been linked to fatal immunosuppression in bottlenose dolphins and sea otters [12, 13]. In mammals, *in vitro* studies using seal lymphocytes showed impaired NK cell activity, reduced T cell proliferation, and decreased macrophage phagocytosis at concentrations of 50–200 nM [14]. In human bone marrow cultures, nanomolar concentrations of TBT selectively suppressed B

lymphocyte populations [15, 16], while murine *in vitro* models have demonstrated TBT-driven apoptosis of Pro-B cells and inhibition of B cell differentiation [17]. The mechanisms involved span both innate and adaptive compartments and include alterations in cytokine secretion, cell viability, and lymphocyte development, as detailed in da Silva et al. [6].

Despite this body of work, most mammalian *in vivo* studies have employed doses several orders of magnitude above the WHO ADI or have focused on chronic exposure paradigms. Whether short-term exposure at a dose below the ADI, a scenario directly relevant to dietary contamination, is sufficient to alter immune cell populations and cytokine production in peripheral immune organs has not been directly addressed. The present study aimed to fill this gap by characterising the immunological effects of a 14-day oral exposure to 100 ng/kg/day TBT (2.5-fold below the WHO threshold) in male BALB/c mice, using multiparameter flow cytometry of spleen and bone marrow, serum immunoglobulin quantification, and *in vitro* cytokine measurement, to provide updated experimental evidence for reassessing the current acceptable daily intake.

Materials and Methods

Experimental animals and treatment

Adult male BALB/c mice weighing between 22 and 30 g (12 weeks old) were housed in polypropylene cages under controlled temperature and humidity conditions with a 12-h light/dark cycle and free access to water and food. The mice were divided into two groups. The tributyltin chloride group (TBT, n = 8 per independent experiment) was treated daily with tributyltin chloride (100 ng/kg/day of TBT diluted in a vehicle consisting of 0.4% ethanol) for 14 days by oral administration (gavage). The control group (Control, n = 8 per independent experiment) received the vehicle following the same protocol used for the TBT group. TBT was obtained from a commercial supplier (Sigma-Aldrich, São Paulo, Brazil, Catalog number T50202). On the 15th day after the treatment period ended, the animals were euthanized using Ketamine (180–300 mg/Kg) and Xylazine (10–30 mg/Kg) in overdose, administered intraperitoneally. The weights of control and TBT animals were assessed three times a week for the full period of the study.

Organ collection and weighting

The spleen and thymus of animals were collected and weighed on a precision balance following the euthanasia. The degree of changes in every organ was evaluated in each animal by determining the ratio of the organ's weight to the body weight of the animal.

Bone marrow cells recovery

After euthanasia of the animals, femurs were extracted using scissors and forceps. The bones were cleaned and placed in a Petri dish containing RPMI 1640 culture medium (Sigma). Subsequently, the epiphyses were excised, and the marrow cavity was manually flushed using a syringe and needle (6 mm × 0.25 mm). The cells were then centrifuged at 1200 RPM for 6 minutes and resuspended in ACK lysis buffer, where they were maintained for 2 minutes to lyse the red blood cells. Subsequently, PBS with fetal bovine serum was added to stop the lysis, and the cells were centrifuged

again and resuspended in RPMI 1640 culture medium. Viable cells were counted in the suspension using a Neubauer chamber, with a sample of cells diluted in 0.1% Trypan blue.

Spleen cells recovery

The spleen was dissociated using a sieve and syringe plunger in a Petri dish containing RPMI 1640 culture medium (Sigma). The cells were then centrifuged at 1200 RPM for 6 minutes and resuspended in ACK lysis buffer, where they were maintained for 2 minutes to lyse the red blood cells. Subsequently, PBS with fetal bovine serum was added to stop the lysis, and the cells were centrifuged again and resuspended in RPMI 1640 culture medium. Viable cells were counted in the suspension using a Neubauer chamber, with a sample of cells diluted in 0.1% Trypan blue.

Flow cytometry

For the characterization of distinct cell populations, various fluorophore-conjugated antibodies were employed to label specific molecules within each population analyzed. The antibodies used included anti-CD4 FITC (clone GK15, Biolegend), anti-CD8 PE (clone 53-6.7, Biolegend), anti-FOXP3 APC (clone FJK-16s, Thermofisher), anti-B220 PE (clone RA3-6B2, Biolegend), anti-IgM FITC (clone R6-60.2, BD Biosciences), and anti-CD11b FITC (clone M1/70, Thermofisher). Surface molecules were stained with the antibodies for 20 minutes on ice, shielded from light. For intracellular staining with anti-FOXP3, cells were treated with fixation and permeabilization solutions (Fixperm) for 30 minutes, followed by antibody dilution in permeabilization buffer (Permbuffer, e-Biosciences). Post-incubation with antibodies, cells were centrifuged and resuspended in PBS containing 3% fetal bovine serum and subsequently acquired on a flow cytometer (Facs Calibur). Fluorochromes associated with antibody molecules were detected as follows: FITC (Fluorescein Isothiocyanate) in the FL1 channel, PE (Phycoerythrin) in the FL2 channel, and APC (Allophycocyanin) in the FL4 channel of the Calibur cytometer. Data analysis was conducted using Summit software version 4.3. Following cytometer data acquisition, the absolute number of cells was calculated using the formula:

Total number of organ cells × Percentage of population cells obtained by cytometry / 100

Serum recovery

The animals were anesthetized with a mixture of 100 µL per 10 g of body weight, administered intraperitoneally. The mixture was prepared from an initial dilution containing 1 mL of Ketamine (Syntec) (10%), 0.5 mL of Xylazine (Syntec) (5%), and 8.5 mL of PBS or distilled water. Blood was collected via cardiac puncture on the 15th day. After resting for 2 hours at room temperature to allow clot formation, the sample was centrifuged under refrigeration (4°C) for 7 minutes at 2100 RPM and the material was then stored at −20°C.

In vitro cell treatment

Untreated BALB/c mice (12 weeks old) were euthanized, their spleens were removed and a cell suspension was obtained. The cells were then maintained in 24-well plates (1×10^6 cells with 2 mL of medium per well) for 72 hours. The culture medium was

prepared with RPMI 1640 medium, 10% fetal bovine serum (FBS), and penicillin (100 U/mL) (Sigma, São Paulo, Brazil). Experimental triplicates were performed for five points. In the control point, cells were cultured only in culture medium. In the TBT point, cells were cultured in medium with the addition of 10^{-4} M of TBT per well. In the anti-CD3 point, cells were cultured in culture medium with the addition of 1 µg/mL of anti-CD3 antibody (Invitrogen) as a stimulus for T lymphocyte activation. In the anti-CD3 plus TBT point, cells were cultured in medium with the addition of 1 µg/mL of anti-CD3 antibody (Invitrogen) and 10^{-4} M of TBT per well. In the ethanol point, cells were cultured in culture medium with the addition of the TBT diluent, 0.4% ethanol. After 72 hours, the supernatant was collected and kept at −20°C until the ELISAs were performed.

Analysis of cytokine and immunoglobulin modulation by TBT using Enzyme-Linked Immunosorbent Assay (ELISA)

The following Invitrogen kits were used in 96-well plates: IL-2 (catalog number 88-7024), IL-6 (catalog number 88-7064), IL-10 (catalog number 88-7105), IgG (catalog number 88-50400), IgM (catalog number 88-50470), and IgE (catalog number 88-50460). The entire procedure was carried out following the recommendations of the manufacturer. The absorbance of the samples was then measured using the Emax Plus plate reader at 450 nm. The standard curve was calculated using SoftMax Pro 6 software.

Statistical analysis of data

The results were expressed as the mean ± SEM. GraphPad Prism 5 software (GraphPad Software, Inc.) was used for statistical analysis. The normality test used was the Shapiro–Wilk test. Student's t-test was used for comparisons involving two variables. For multiple comparisons, ANOVA with Bonferroni post-test was employed. Statistical significance was set at $p < 0.05$.

Results

Absence of effect of *in vivo* tributyltin treatment on the weight of animals, spleen and thymus

To determine if the dose of 100 ng/kg/day was causing changes in the weight of the spleen and thymus, as well as potential alterations in animal body weight, given that TBT is described in the literature as having an obesogenic effect [18], the animal's weight during treatment and after euthanasia was monitored. No significant differences in the body weight of the animals (**Figure 1a**) or in the weight of their spleens (**Figure 1b**) and thymuses (**Figure 1c**) relative to their body weight were observed.

Effects of *in vivo* tributyltin treatment on bone marrow B cells

As shown in **Figure 2a**, was first defined as a region within the lymphoid population, referred to as the general gate for size (FSC-H) and granularity (SSC-H). From this, gates were delineated for the subpopulations of bone marrow B lymphocytes, including $B220^{+}IgM^{-}$, $B220^{+}IgM^{+}$, and $B220^{hi}IgM^{+}$ populations. Initially, no significant differences were found in the percentages of total lymphoid cells. However, a

reduction in the absolute number of these cells in TBT-treated animals (versus control) was observed (**Figure 2b and c**). Regarding the $B220^{+}IgM^{-}$ cell population, a significant decrease in both the percentage and absolute number of cells in TBT-treated animals was noticed (**Figure 2d and e**). This was not observed for the $B220^{+}IgM^{+}$ population, where no changes in either percentage or absolute cell number were detected (**Figure 2f and g**). Additionally, while no significant reduction in percentage for the $B220^{hi}IgM^{+}$ lymphocytes was observed, a significant decrease in their absolute number (**Figure 2h and i**) was detected.

Tributyltin has no *in vivo* effect on the myeloid population in the bone marrow

Subsequently, given the significant reduction observed in the bone marrow B lymphocyte population, the myeloid population in this organ was analyzed, using the general marker CD11b. Initially, we defined a general gate within the cell population based on size versus granularity. From this initial gate, the myeloid population was analyzed (**Figure 3a**). However, no significant differences in either the percentage (**Figure 3b**) or the absolute number (**Figure 3c**) was observed, suggesting that TBT does not affect the myeloid population in the bone marrow of the animals.

Effects of *in vivo* tributyltin treatment on spleen T lymphocytes

After analyzing the bone marrow, we proceeded to assess the impact of TBT on $CD4^{+}$ T cells, $CD8^{+}$ T and regulatory T cells in the spleen. To analyze these populations in the spleen, gates were defined as shown in **Figure 4**.

Firstly, no significant difference in the percentage of total splenic cells in the treated group (**Figure 4b**), but a significant decrease in the absolute number of these cells in the TBT-treated group compared to the control was observed (**Figure 4c**). Regarding $CD4^{+}$ T cells, there was no significant difference in the percentage of these cells between the control and treated groups (**Figure 4d**). However, TBT treatment led to a significant decrease in the absolute number of $CD4^{+}$ T cells (**Figure 4e**). Similarly, no significant difference in the percentage of $CD8^{+}$ cells was observed (**Figure 4f**), but there was a significant reduction in the absolute number of $CD8^{+}$ T cells in the TBT-exposed group (**Figure 4g**). Lastly, dual staining of $CD4^{+}$ T cells jointly with FOXP3 was performed and no significant difference in the percentage (**Figure 4h**), but a significant decrease in the absolute number of these cells was noted (**Figure 4i**). Together, these results demonstrate the immunomodulatory effect of TBT, reducing the absolute number of different T lymphocyte populations in the spleen of BALB/c mice while maintaining the proportion of these cells within this organ.

In vivo tributyltin treatment modulates B lymphocytes and myeloid cells in the spleen

In order to examine the $B220^{+}$ B lymphocyte and $CD11b^{+}$ myeloid cell populations in the spleen, a broad gate encompassing both types of cells was established as depicted in **Figure 5a**. Just as seen for the $CD4^{+}$, $CD8^{+}$, and $CD4^{+}FOXP3^{+}$ T lymphocytes, no notable variation in the proportion of $B220^{+}$ cells in the spleen was detected (**Figure 5b**). Nonetheless, there was a marked reduction in the absolute number of $B220^{+}$ cells in the TBT-treated group when compared to the untreated control group (**Figure 5c**). A similar pattern was seen in the myeloid group, with no

notable change in cell percentage (**Figure 5d**), but a significant decrease in the absolute number in the TBT group (**Figure 5e**).

Tributyltin does not alter the levels of total immunoglobulins in the serum of treated animals

To assess whether TBT not only decreases the population of B lymphocytes but also alters their ability to secrete immunoglobulins, serum from animals previously treated *in vivo* with TBT or untreated was collected and ELISA assays to evaluate the production of total IgE, IgM, and IgG were performed. However, no significant differences in total immunoglobulins in the serum of control and TBT-treated animals was observed (**Figure 6**).

Evaluation of the effects of tributyltin on cytokine production *in vitro*

Based on our findings regarding the immunosuppressive effects of TBT on immune cells, particularly T lymphocyte populations, *in vitro* cell cultures using splenocytes from untreated BALB/c mice were performed. Cells were exposed to TBT with and without stimulation by anti-CD3 antibodies *in vitro*. After 72 hours of culture, supernatants were collected and ELISAs were conducted to measure IL-6, IL-10, and IL-2 cytokine levels. These cytokines play pivotal roles in regulating the proliferation, maintenance, and activation of $CD4^+$, $CD8^+$, regulatory T cells, B cells, and myeloid cells, thereby modulating inflammatory responses and antibody production [19-24].

Our results indicated that exposure to TBT resulted in a significant reduction of pro-inflammatory cytokine IL-6 levels, irrespective of anti-CD3 stimulation, which normally activates the cell to produce the cytokine. Despite this activation, IL-6 levels were lowered to non-detectable levels in both cases (**Figure 7a**). This underscores the profound suppressive effect of 10^{-4} M TBT on IL-6 production. A similar result was observed in IL-10 and IL-2 production, where TBT exposure resulted in non-detectable levels of IL-10 and IL-2. However, upon anti-CD3 stimulation, IL-10 and IL-2 concentrations were still detectable in the anti-CD3 plus TBT point, though still modest (**Figures 7b** and **7c**).

Finally, this effect appears independent of the vehicle used as TBT solvent, as no significant difference was observed between the control and ethanol in any of the ELISAs performed (**Figure 7a–c**).

Discussion

The present study evaluated the immunological consequences of a 14-day oral exposure to 100 ng/kg/day TBT (a dose 2.5-fold below the WHO acceptable daily intake) in male BALB/c mice. Our findings collectively demonstrate that this sub-threshold exposure is sufficient to produce multifaceted immunosuppression, affecting both the central (bone marrow) and peripheral (spleen) immune compartments, and profoundly suppressing T lymphocyte cytokine secretion *in vitro*. These results reinforce the growing body of evidence indicating that the current WHO ADI of 250 ng/kg/day does not confer protection against immunotoxic effects and warrants reassessment [6].

No significant changes in body weight, spleen weight, or thymus weight were observed over the treatment period. The absence of an obesogenic effect is consistent with the dose- and duration-dependence of TBT-induced adipogenesis: studies documenting significant adipogenic changes have typically involved *in utero* exposure or longer treatment protocols [18, 25, 26]. Importantly, the preservation of macroscopic lymphoid organ weights does not rule out functional immune alterations at the cellular level, as our flow cytometry data demonstrate. This is a limitation of the morphological endpoints on which the original ADI was partly based [7, 8].

TBT exposure produced a significant reduction in the absolute number of total bone marrow lymphoid cells, with selective decreases in the Pro/Pre-B cell population ($B220^{+}IgM^{-}$) and the mature/recirculating B cell population ($B220^{hi}IgM^{+}$), while immature B cells ($B220^{+}IgM^{+}$) were unaffected. This pattern of vulnerability at the earliest and most mature B cell stages, with a spared intermediate compartment, suggests that TBT may simultaneously impair the bone marrow microenvironmental niche that sustains early progenitors and the survival signals available to returning mature B cells. This is consistent with Baker et al. [17], who demonstrated TBT-driven apoptosis of Pro-B cells and microenvironmental disruption in C57BL/6J mice at 100 nM *in vitro*, and with Carfi et al. [15, 16], who reported selective suppression of the B lymphocyte compartment in human bone marrow long-term cultures at 1 nM TBT via a PPARγ-independent mechanism.

At the peripheral level, TBT reduced the absolute number of $B220^{+}$ B lymphocytes in the spleen without altering their proportion within the total lymphoid gate. This pattern is consistent with *in vitro* data showing reduced differentiation, proliferation, and survival of mature human B cells at 100 nM TBT [27], and together with the bone marrow data indicates that TBT curtails B cell output centrally and constrains the peripheral mature pool simultaneously. A parallel reduction in the absolute number of splenic $CD11b^{+}$ myeloid cells was observed, again without a proportional shift, whereas no change was detected in the bone marrow myeloid compartment. The peripheral-specific effect on myeloid cells may reflect preferential susceptibility of tissue-resident or splenic macrophages to TBT-induced cell death. Ling et al. [28] demonstrated that TBT triggers apoptosis of bone marrow-derived macrophages via RIP1/RIP3-dependent necroptosis at 0.4–1.2 μM, and lower doses may selectively affect cells that have already egressed to peripheral organs. The subpopulation composition of the affected $CD11b^{+}$ compartment warrants further investigation with extended myeloid marker panels.

TBT treatment significantly reduced the absolute number of splenic $CD4^{+}$, $CD8^{+}$, and $CD4^{+}FOXP3^{+}$ T cells, while the proportions of each subset within the total lymphoid population were maintained. This reinforces the interpretation of a global reduction in splenic cellularity rather than preferential depletion of a single lineage. Decreased T lymphocyte numbers in the spleen following TBT exposure have been reported in C57BL/6 mice, with reductions in both naive and effector/memory fractions [29], although at doses substantially higher than those used here. The reduction in regulatory T cells ($CD4^{+}FOXP3^{+}$) merits particular attention: these cells are central to peripheral immune tolerance and their numerical decline has been mechanistically linked to heightened susceptibility to autoimmune pathology [30, 31]. This is further underscored by the preferential induction of apoptosis in regulatory T cells over Th2

lymphocytes by TBT [32], which may shift the immune balance toward dysregulated, pro-inflammatory responses even in the absence of overt organ pathology.

Despite the numerical depletion of both splenic B lymphocytes and bone marrow B cell precursors, total serum concentrations of IgE, IgM, and IgG remained unchanged in TBT-treated animals. This indicates that the residual B cell pool retains sufficient functional capacity to sustain baseline immunoglobulin output under homeostatic conditions [33]. The contrast with studies reporting TBT-associated elevations in antigen-specific IgE in allergen-sensitised C57BL/6 mice [34] and elevated intestinal IgM in zebrafish [35, 36] likely reflects the absence of antigenic challenge in our model. An immunisation protocol would be required to reveal whether the numerical B cell deficit translates to impaired antigen-specific humoral responses.

The most striking *in vitro* finding was a profound and broad suppression of IL-6, IL-10, and IL-2 secretion by splenic T lymphocytes exposed to TBT. Without anti-CD3 stimulation, concentrations of all three cytokines fell to undetectable levels. Upon TCR stimulation, IL-6 remained undetectable, while IL-10 and IL-2 were partially restored to low but measurable levels. The differential rescue of IL-10 and IL-2 by TCR co-stimulation, but not of IL-6, may reflect the broader cellular sources of IL-6, which is produced by macrophages, fibroblasts, and endothelial cells in addition to T cells [22, 23]. This broader origin would make IL-6 more comprehensively suppressed when all cell types are exposed to TBT simultaneously. In contrast, the partial preservation of IL-10 and IL-2 under TCR activation suggests that T cell-intrinsic production of these cytokines can be partially sustained when receptor signalling is intact, even in a TBT-suppressed environment [20, 21].

The concurrent suppression of IL-2, IL-6, and IL-10 provides a mechanistic framework for the cell population changes observed by flow cytometry. IL-2 is the principal growth and survival signal for T cells and is required for the maintenance of the regulatory T cell pool [19, 21, 37]. Its depletion would be expected to reduce the proliferative capacity and survival of all three T cell subsets detected here. The functional relevance of IL-2 loss is further supported by Odman-Ghazi et al. [38], who showed that TBT-induced impairment of NK cell cytotoxicity could be fully reversed by exogenous IL-2 addition, and by Lawrence et al. [39], who reported decreased serum IL-2 in BALB/c mice following TBT injection at 100 nM. IL-6 loss would remove a key differentiation and activation signal for both T and B lymphocytes, consistent with the reduced peripheral B cell numbers we observed. The suppression of IL-10, which ordinarily sustains regulatory T cell stability and modulates inflammatory tone [24], combined with the numerical reduction of $CD4^+FOXP3^+$ cells, creates a condition of impaired immunoregulation that may, over longer exposures, increase susceptibility to inflammatory and autoimmune conditions.

Not all of our results are consistent with published data. TBT at 2.5–200 nM stimulated IL-6 synthesis in human PBMCs [40, 41] and at 1–100 ng/L elevated IL-6 in zebrafish [42], while 100 nM TBT increased IL-10 and suppressed IL-12 in naive $CD4^+$ T lymphocytes from C57BL/6 mice [34, 43]. These discrepancies most likely reflect differences in concentration: the 10^{-4} M used in our *in vitro* experiment substantially exceeds nanomolar ranges used in other models and may engage a suppressive or cytotoxic mechanism rather than a stimulatory one. Differences in species, cell type, and the presence or absence of co-stimulatory signals are additional confounding factors. The divergence underscores that the immunological outcome of TBT exposure

is highly context- and concentration-dependent, and that experiments bridging the *in vivo* sub-ADI dose and the *in vitro* concentrations required for mechanistic studies remain a priority.

Collectively, these data show that a 14-day oral exposure to 100 ng/kg/day TBT induces significant immunosuppression in BALB/c mice, reducing B cell precursors and mature B cells in the bone marrow, and decreasing the absolute numbers of B cells, T cell subsets, and myeloid cells in the spleen, alongside a profound *in vitro* suppression of IL-6, IL-10, and IL-2 secretion. The absence of changes in body weight, organ weight, and basal serum immunoglobulins highlights that conventional toxicological endpoints would fail to detect this immunotoxic phenotype. These findings provide direct experimental support for reassessing the current WHO ADI for TBT, as exposure 2.5-fold below the accepted safe limit produced measurable and biologically significant alterations to the immune system of BALB/c mice within a short treatment window.

Statements and Declarations

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Author Contributions

MPT: conceptualization; JBG: conceptualization, project administration; LSP: conceptualization, methodology, investigation, supervision, project administration, funding acquisition; LMA: conceptualization, methodology, investigation, supervision, project administration, funding acquisition; RCS: methodology, investigation, writing – original draft, visualization; ROC: methodology, investigation, visualization; MLAC: methodology, investigation; MCSS: methodology, investigation; MSAS: methodology, investigation; MTSFS: methodology, investigation; writing – review and editing: all authors.

Ethical approval

All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU, and the National Research Council's Guide for the Care and Use of Laboratory Animals. The Ethics Committee for the Use of Animals (CEUA) of the Fluminense Federal University approved all procedures (number: 4248080420).

Competing interests

All authors declare no financial or non-financial competing interests.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Figure captions

Fig. 1 Organ and body weights of male BALB/c mice treated or not treated with TBT. (**a**) The average weight of animals in the control and TBT groups over the 14-day treatment period, represented in grams. $p = 0.9925$. (**b**) The ratio of spleen weight to the average body weight of the respective animal was calculated to normalize the observed results. $p = 0.5049$. (**c**) The ratio of thymus weight to the average body weight of the respective animal was calculated to normalize the observed results. $p = 0.5696$. The values are expressed as the mean ± SEM of 3 independent experiments (Control: n = 8, TBT: n = 8 per experiment). Statistics: Student's t-test. $p < 0.05$

Fig. 2 Analysis of $B220^{+}IgM^{+}$ or IgM^{-} and $B220^{hi}IgM^{+}$ cell populations in the bone marrow of male BALB/c mice. (**a**) Representative image of the gates defined in the control and TBT-treated animal groups. (**b**) Average percentage of total lymphoid cells in the bone marrow. $p = 0.1720$. (**c**) Average absolute number of total lymphoid cells. $p = 0.0372$. (**d**) Average percentage of $B220^{+}IgM^{-}$ cells. $p = 0.0009$. (**e**) Average absolute number of $B220^{+}IgM^{-}$ cells. $p = 0.0436$. (**f**) Average percentage of $B220^{+}IgM^{+}$ cells. $p = 0.0598$. (**g**) Average absolute number of $B220^{+}IgM^{+}$ cells. $p = 0.0994$. (**h**) Average percentage of $B220^{hi}IgM^{+}$ cells. $p = 0.8353$. (**i**) Average absolute number of $B220^{hi}IgM^{+}$ cells. $p = 0.0053$. Data are presented as mean ± SEM of 4 independent experiments (Control: n = 8, TBT: n = 8 per experiment). Statistical analysis: Student's t-test. $p < 0.05$, $p < 0.005$, $p < 0.0005$

Fig. 3 Analysis of $CD11b^{+}$ cell populations in the bone marrow of male BALB/c mice. (**a**) Representative image of the gates defined in the control and TBT groups. (**b**) Average percentage of $CD11b^{+}$ cells in the bone marrow. $p = 0.9985$. (**c**) Average absolute number of $CD11b^{+}$ cells in the bone marrow. $p = 0.4245$. The values are expressed as mean ± SEM of 3 independent experiments (Control: n = 8, TBT: n = 8 per experiment). Statistics: Student's t-test

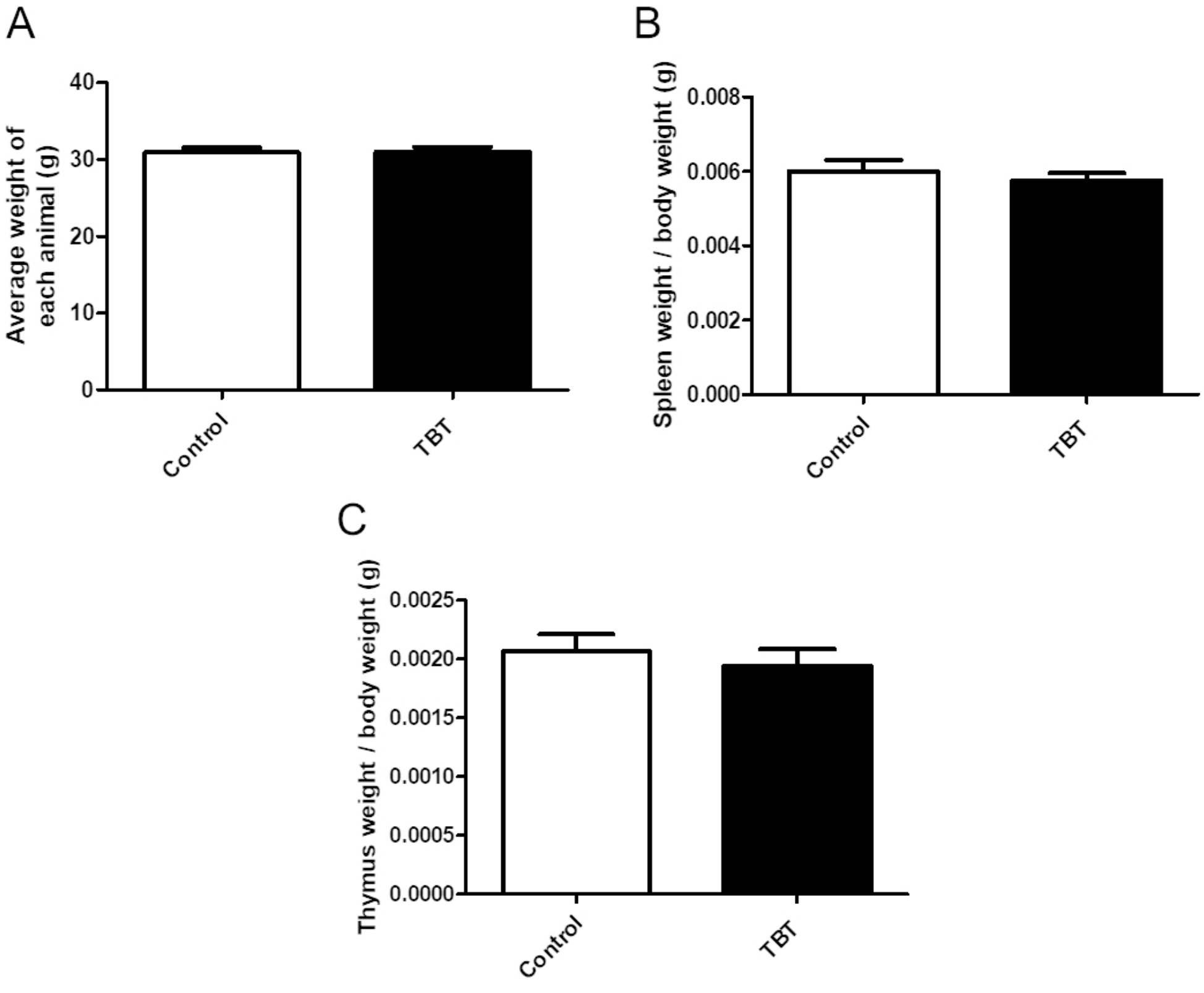
Fig. 4 Analysis of $CD4^{+}$, $CD8^{+}$, and $CD4^{+}FOXP3^{+}$ T cell populations in the spleen of male BALB/c mice. (**a**) Representative image of the gates. (**b**) Average percentage

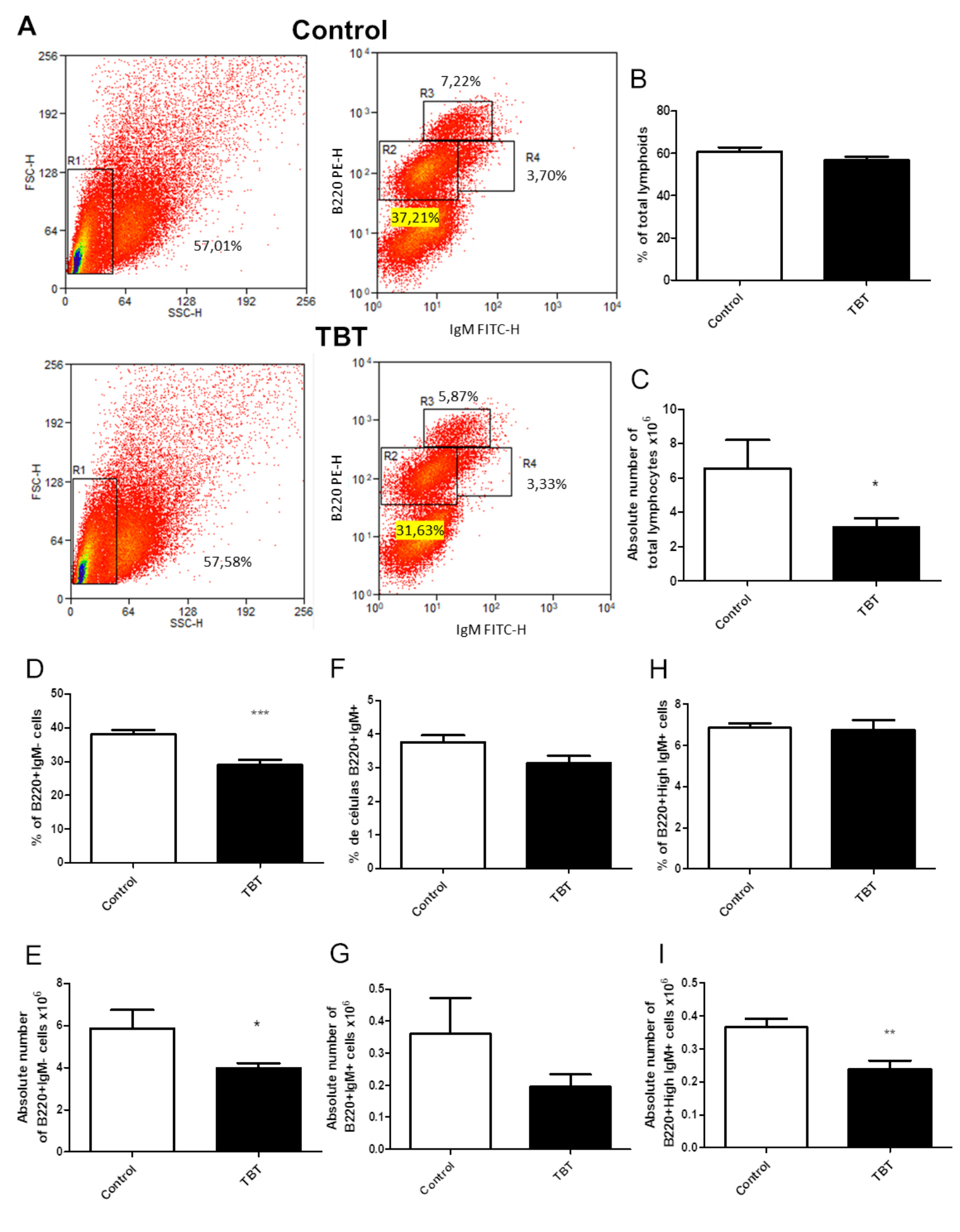
of total cells. $p = 0.0558$. (**c**) Average absolute number of total cells. $p = 0.0012$. (**d**) Average percentage of $CD4^+$ T cells. $p = 0.6218$. (**e**) Average absolute number of $CD4^+$ T cells. $p = 0.0076$. (**f**) Average percentage of $CD8^+$ T cells. $p = 0.4690$. (**g**) Average absolute number of $CD8^+$ T cells. $p = 0.0089$. (**h**) Average percentage of $CD4^+FOXP3^+$ T cells. $p = 0.6104$. (**i**) Average absolute number of $CD4^+FOXP3^+$ T cells. $p = 0.0136$. $FOXP3^+$ cells were analyzed within the $CD4^+$ T cell gate. Values are mean ± SEM of 3 independent experiments (Control: n = 8, TBT: n = 8 per experiment). Statistics: Student's t-test. $p < 0.05$, $p < 0.005$

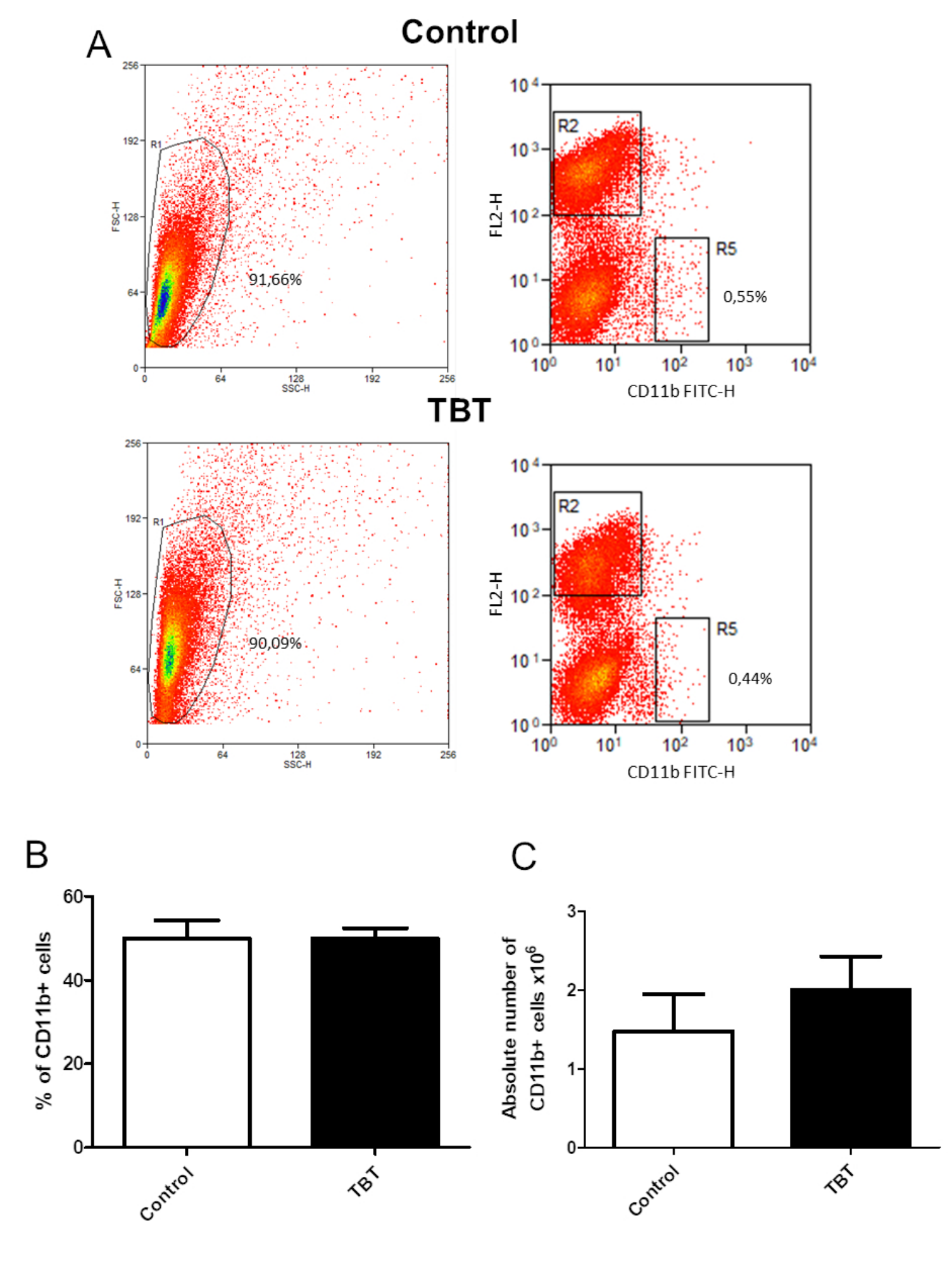
Fig. 5 Analysis of $B220^+$ and $CD11b^+$ cell populations in the spleen of male BALB/c mice. (**a**) Representative image of the gates. (**b**) Average percentage of $B220^+$ cells. $p = 0.0844$. (**c**) Average absolute number of $B220^+$ cells. $p = 0.0007$. (**d**) Average percentage of $CD11b^+$ cells. $p = 0.6525$. (**e**) Average absolute number of $CD11b^+$ cells. $p = 0.0202$. Values are mean ± SEM of 3 independent experiments (Control: n = 8, TBT: n = 8 per experiment). Statistics: Student's t-test. $p < 0.05$, $p < 0.005$, $p < 0.0005$

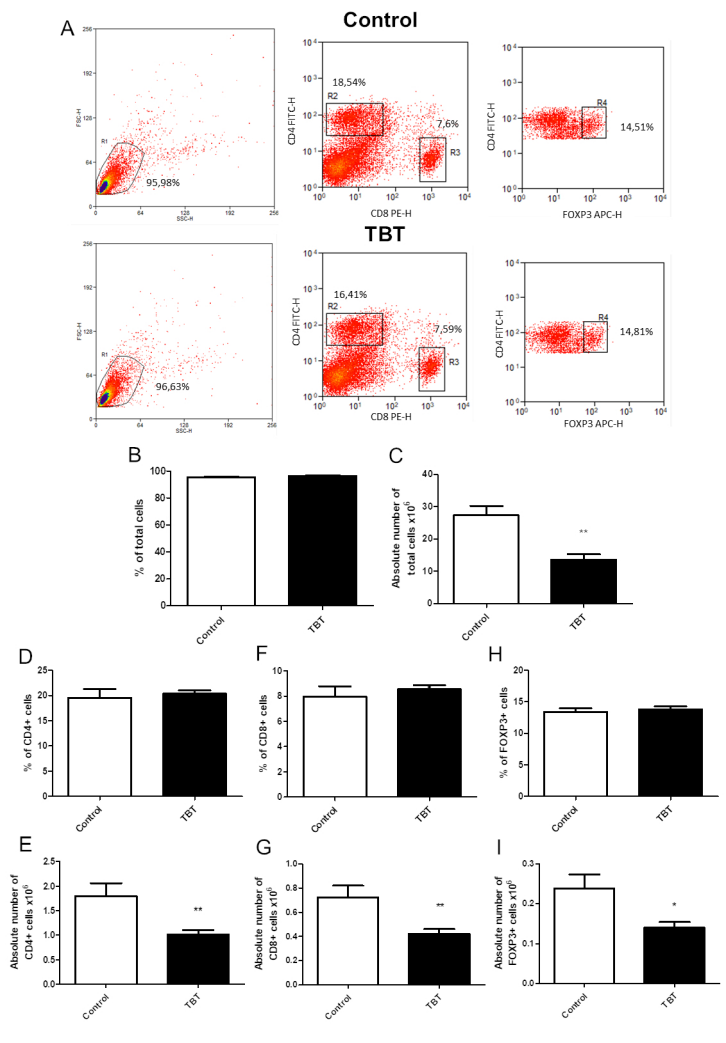
Fig. 6 Analysis of total immunoglobulin levels in the serum of BALB/c mice treated or not treated with TBT. Each bar represents mean values ± SD of serum levels of IgE (**a**), IgM (**b**), and IgG (**c**), based on duplicates for each animal. IgE: $p = 0.5796$, IgM: $p = 0.8810$, IgG: $p = 0.9423$. Values are mean ± SEM (n = 2). Statistics: Student's t-test. $p < 0.05$

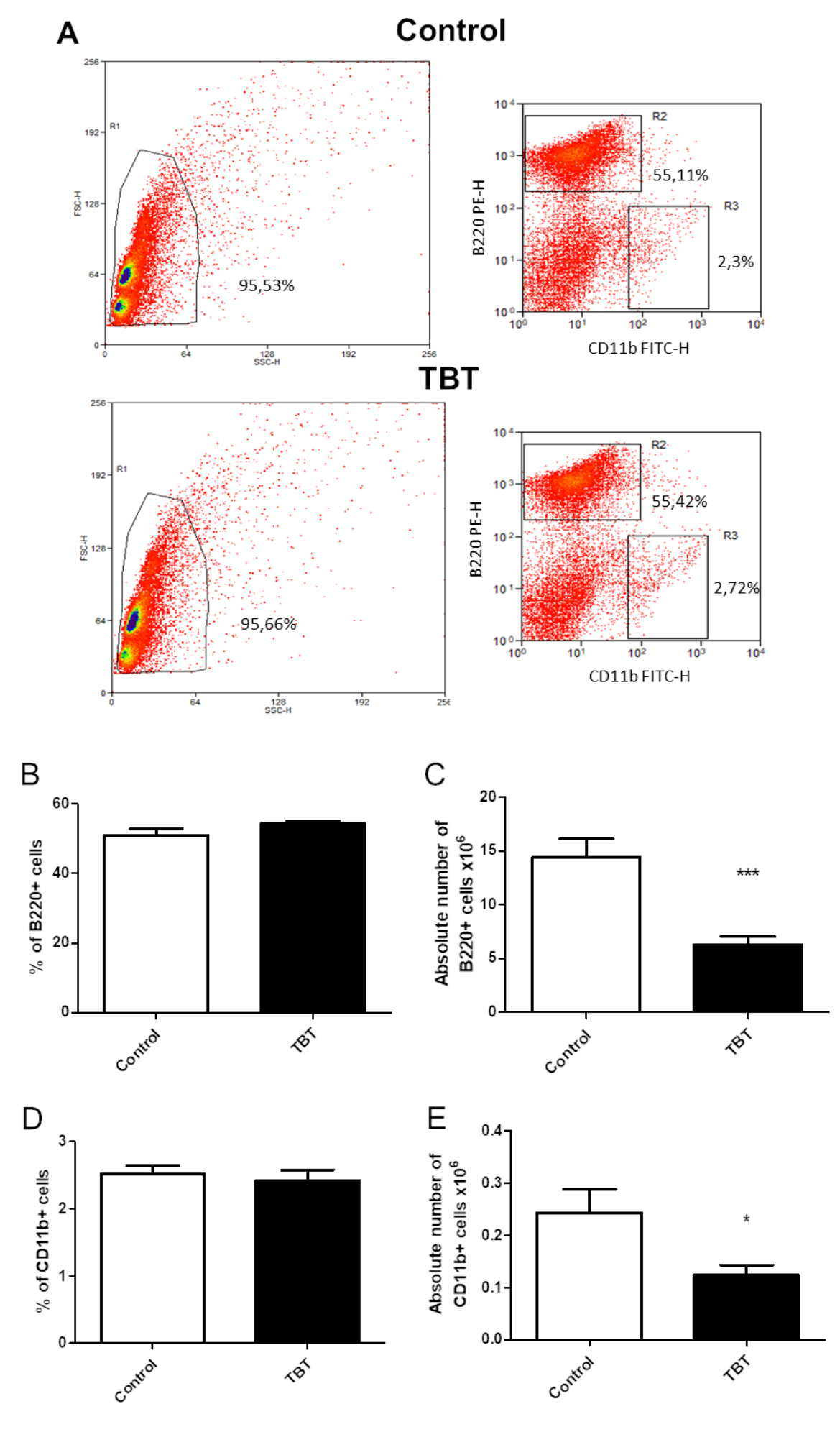
Fig. 7 Effects of TBT exposure on cytokine production in total spleen cells from BALB/c mice cultured with or without anti-CD3 stimulation. Cells isolated from untreated BALB/c mouse spleens were cultured for 72 hours in the presence or absence of 10^{-4} M TBT with or without anti-CD3. Cytokine levels (**a**) IL-6, (**b**) IL-10, and (**c**) IL-2 in culture supernatants were measured by ELISA. Data are presented as mean ± standard deviation (n = 3 independent experiments). Statistical analysis: ANOVA with Bonferroni post-test. ND = not detectable

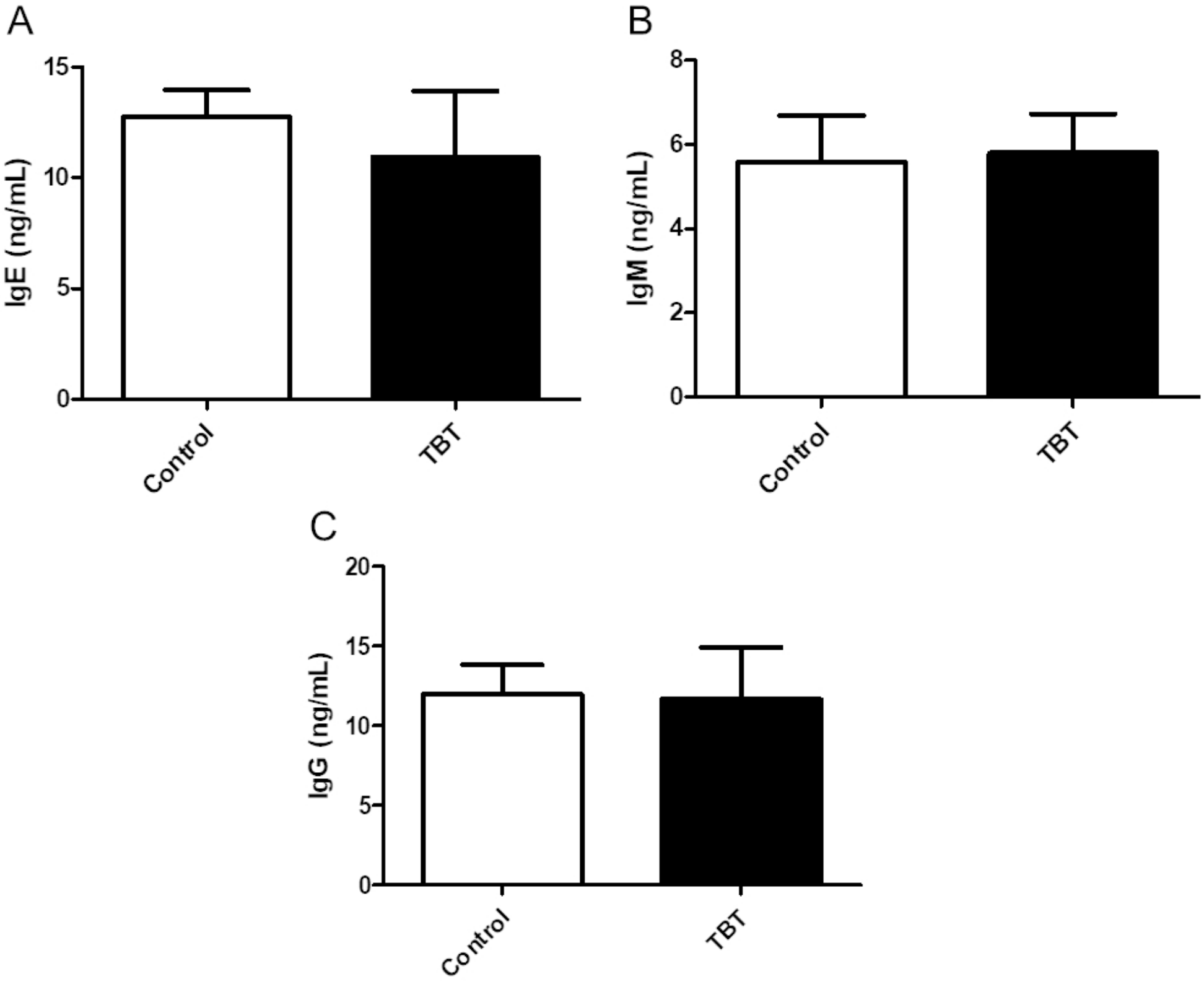

A
Average weight of each animal (g)
40
30
20
10
0
Control
TBT
B
Spleen weight / body weight (g)
0.008
0.006
0.004
0.002
0.000
Control
TBT
C
Thymus weight / body weight (g)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Control
TBT

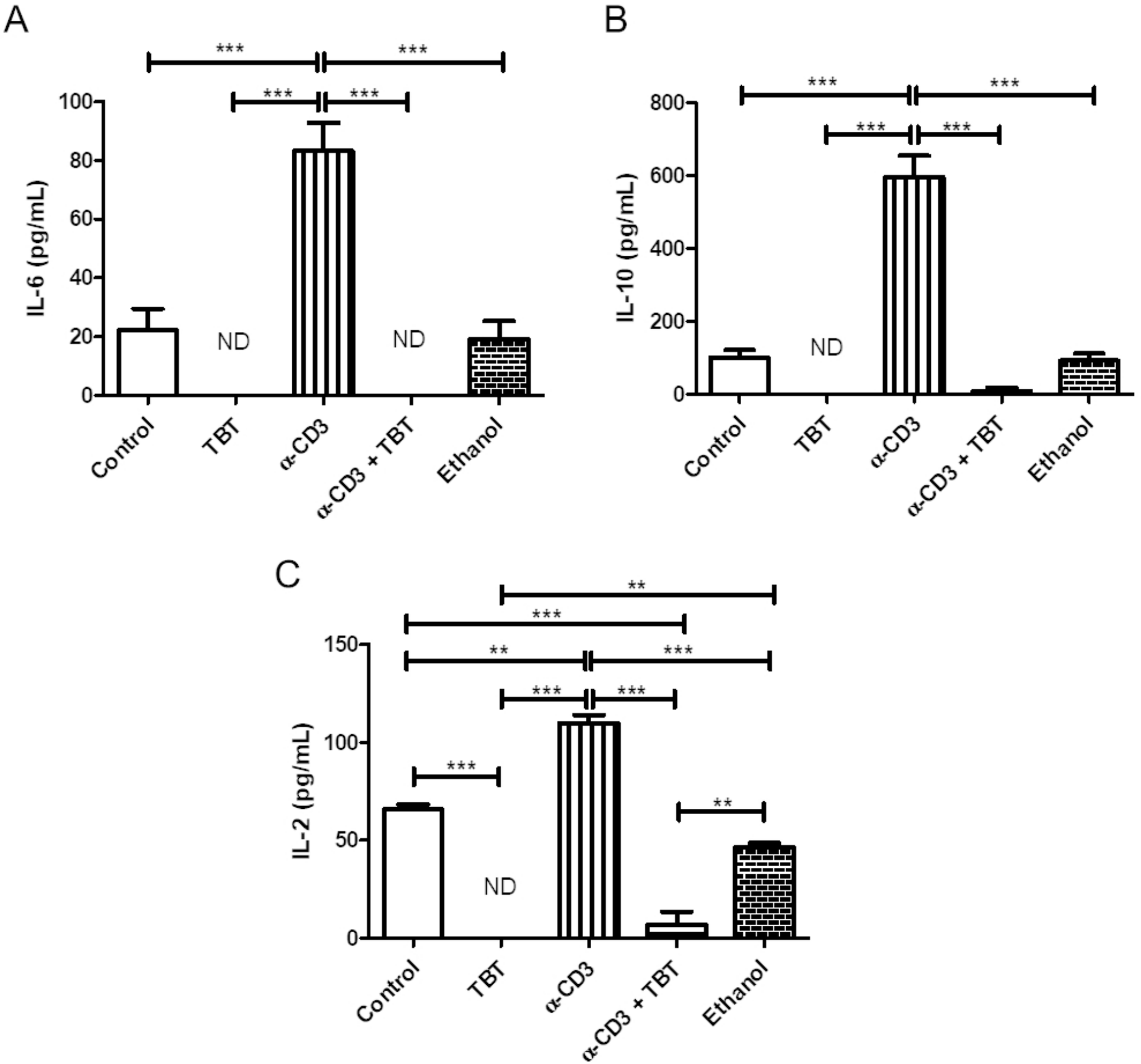

A
Control
FSC-H
SSC-H
R1
57,01%
B220 PE-H
IgM FITC-H
R2
R3
R4
7,22%
3,70%
37,21%
B
% of total lymphoids
Control
TBT
TBT
R1
57,58%
R2
R3
5,87%
R4
3,33%
31,63%
C
Absolute number of total lymphocytes x10^6
*
D
% of B220+IgM- cells
***
F
% de células B220+IgM+
H
% of B220+High IgM+ cells
E
Absolute number of B220+IgM- cells x10^6
G
Absolute number of B220+IgM+ cells x10^6
I
Absolute number of B220+High IgM+ cells x10^6
**


A
Control
R1
FSC-H
SSC-H
91,66%
R2
R5
0,55%
FL2-H
CD11b FITC-H
TBT
90,09%
0,44%
B
% of CD11b+ cells
Control
TBT
C
Absolute number of CD11b+ cells x10^6
Control
TBT


A
Control
95,98%
R1
18,54%
R2
7,6%
R3
CD4 FITC-H
CD8 PE-H
14,51%
R4
FOXP3 APC-H
TBT
96,63%
16,41%
7,59%
14,81%
B
% of total cells
Control
TBT
C
Absolute number of total cells x10^6
**
D
% of CD4+ cells
E
Absolute number of CD4+ cells x10^6
F
% of CD8+ cells
G
Absolute number of CD8+ cells x10^6
H
% of FOXP3+ cells
I
Absolute number of FOXP3+ cells x10^6
*


A
Control
R1
FSC-H
SSC-H
95,53%
R2
R3
55,11%
2,3%
B220 PE-H
CD11b FITC-H
TBT
95,66%
55,42%
2,72%
B
% of B220+ cells
Control
TBT
C
Absolute number of B220+ cells x10^6
***
D
% of CD11b+ cells
E
Absolute number of CD11b+ cells x10^6
*


A
IgE (ng/mL)
15
10
5
0
Control
TBT
B
IgM (ng/mL)
8
6
4
2
0
Control
TBT
C
IgG (ng/mL)
20
15
10
5
0
Control
TBT


A
B
C
IL-6 (pg/mL)
IL-10 (pg/mL)
IL-2 (pg/mL)
ND
***
**
Control
TBT
α-CD3
α-CD3 + TBT
Ethanol

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