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Different wild type strains of zebrafish show divergent susceptibility to TNBS-induced intestinal inflammation displaying distinct immune cell profiles

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ABSTRACT

Little is known about the diversity in immune profile of the different wild type strains of zebrafish (*Danio rerio*), despite its growing popularity as an animal model to study human diseases and drug testing. In the case of data resulting from modeling human diseases, differences in the background Danio fishes have rarely been taken into consideration when interpreting results and this is potentially problematic, as many studies not even mention the source and strain of the animals. In this study, we hypothesized that different wild type zebrafish strains could present distinct immune traits. To address the differences in immune responses between two commonly used wild type strains of zebrafish, AB and Tübingen (TU), we used an intestinal inflammation model induced by 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and characterized the susceptibility and immune profile in these two strains. Our data demonstrates significant differences in survival between AB and TU strains when exposed to TNBS, suggesting important physiological differences in how these strains respond to inflammatory challenges. We observed that the AB strain presented increased mortality, higher neutrophilic intestinal infiltration, decreased goblet cell numbers and decreased IL-10 expression when exposed to TNBS, compared to the TU strain. In summary, our study demonstrates strain-specific immunological responses in AB and TU animals. Finally, the significant variations in strain-related susceptibility to inflammation and the differences in the immune profile shown here, highlight that the background of each strain need to be considered when utilizing zebrafish to model diseases and for drug screening purposes, thus better immune characterization of the diverse wild type strains of zebrafish is imperative.

1. Introduction

The zebrafish (*Danio rerio*) has boosted in popularity over the past decade as a vertebrate research animal to model human disease, overcoming some of the murine model restrictions which require longer developmental times and have large-scale screening limitations (Lieschke and Currie, 2007; MacRae and Peterson, 2015; Adamson et al., 2018). Traditionally, zebrafish had been used in genetic and developmental studies, due to its rapid development, cost-effectiveness, genetic tractability, larval optical transparency and high degree of genetic and organ homology to humans (Novoa et al., 2006; Liu et al., 2017). Whole-genome sequencing and emerging data have demonstrated that physiology and immunity of zebrafish present specializations parallel to mammals, turning this small fish into a widely used model for human disease studies (Renshaw and Trede, 2012; Santoriello and Zon, 2012; Holtzman et al., 2016). Thus, a wide range of human

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diseases has continually and successfully been translated to zebrafish, among them tumor models, inflammatory diseases, autoimmune diseases, hematological disorders and infections (Dooley and Zon, 2000; Shelef et al., 2013; Yen et al., 2014).

In any disease, the immune system plays at least a partial role in the pathogenesis and resolution of the illness. Interindividual divergence in immune responses such as variation in serum protein concentrations, cytokine production and immune cell subsets, can be influenced by certain nonheritable factors including environment, microbiome and age, (Brodin and Davis, 2017; Tao and Reese, 2017), however a strong genetic component is estimated to account for 20–40% of immune responses (Liston et al., 2016). Likewise, some diseases are influenced by a combination of host genetics, microbiome and immunity factors that can lead to different outcomes in the severity of a disease. Inflammatory bowel diseases (IBDs), which are a group of recurrent and chronic inflammatory conditions of the intestine, portray examples of complex diseases influenced by multifactorial triggers, most common IBDs include Crohn's disease (CD) and Ulcerative Colitis (UC) (Van Limbergen et al., 2014; Hall et al., 2017). Currently, a variety of IBD models can be reproduced in animals, but often therapeutic research in animal models is criticized because of relatively low success into clinical translation; however, this is likely a result from misguided interpretation of data, flaw in experimental design and lack of communication between basic researchers and clinicians (Denayer et al., 2014). In animal research, to warrant reliable data, it is important that genetic and phenotypic nature of experimental animals is understood and in the case of inbred strains, genetic consistency is paramount, as small genetic variations may greatly skew results, a common observation when investigating immune responses (Zeldovich, 2017). It is well known that different strains of wild type (WT) mice present diversified immune profiles which make them more or less susceptible to specific diseases (Sellers, 2017). In zebrafish, although immune system complexity has shown quite high similarity to mammals, there are distinctive features that must be considered (Trede et al., 2004; Garcia-Moreno et al., 2019). A particularity of zebrafish immune system is that the innate immunity is already functional from 48 hours post-fertilization (hpf) and it was recently shown also that lymphocytes are detectable as early as 5 days-post-fertilization (dpf) (Trede et al., 2004; Coronado et al., 2019).

One important issue to consider is that the term WT zebrafish, commonly used in many research tests, actually corresponds to many different strains (e.g., AB, TU, WIK, and others) (Vignet et al., 2013). Moreover, with the growing use of zebrafish as a model organism in several research areas, the number of strains available and in use has increased in recent years (Oswald and Robison, 2008; Kalueff et al., 2014). Importantly, growing evidence of differences in behavior, morphological and genetic patterns have been reported between different zebrafish strains (Oswald and Robison, 2008; Norton et al., 2011; van den Bos et al., 2017; Mustafa et al., 2019). Strain-specific immunological profiles should always be carefully taken into consideration when modeling disease on animals; however, no study has addressed this issue regarding WT zebrafish.

Thus, we hypothesized that WT zebrafish strains display particular immune profiles that would reflect on predisposition to different disease models. AB and TU are commonly used WT strains in research, often transgenic animals are generated on either background; whereas the TU strain was chosen as reference for the genome sequencing project; however, background is not irrelevant as data have demonstrated significant behavioral differences between AB and TU (Howe et al., 2013; Vignet et al., 2013). In our study, a comparison between the immune profile of AB and TU was performed, and their susceptibility to a well-established model of IBD induced by TNBS (2,4,6-Trinitrobenzenesulfonic acid) was evaluated (Fleming et al., 2010; Oehlers et al., 2013; Brugman, 2016). We found significant differences in mortality and inflammatory responses between AB and TU, namely, AB presented increased mortality combined with higher neutrophilic intestinal infiltration, reduced number of goblet cells and decreased cytokine expression when exposed to TNBS. Our study shows that immune characteristics between AB and TU are distinct and thus researchers must not neglect strain background when reporting disease studies using zebrafish.

2. Material and methods

2.1. Animals

Embryos of the AB, TU and Tg(lysC:DsRed2)^{nz50} (Hall et al., 2007) strains were provided and maintained at the Zebrafish Animal Facility of the Institute of Biosciences of the University of São Paulo (USP) and were obtained by natural spawning, collected in Petri dishes with embryo medium E3 (5mM NaCl, 0.17mM KCl, 0.33mM CaCl2, 0.33mM MgSO4, pH 7.2) with methylene blue and kept in the incubator at 28.5 °C. Of note, the TU and AB strains available in our facility came from two different facilities (University of Chile-Chile and Pennsylvania University-USA, respectively). To analyze if a mixed cross between AB and TU would affect strain survival to TNBS, F1 spawn (named here AB/TU) were obtained from crossing AB and TU adults. The Tg(lysC:DsRed2)^{nz50} was originated from University of Auckland, were crossed with AB or TU to generate (LysC:AB) and (LysC:TU), the F1 spawns were used in the studies. Also, all the different lines of zebrafish parents were trained weeks prior to experiment execution and presented similar egg spawning, quality and retrieval of fertilized eggs. All protocols used in this study were approved by the ethics committee in animal research of the Institute of Biomedical Sciences according to project approved number 2016/101.

2.2. Analysis of the survival of AB and TU in response to TNBS

For modeling of inflammatory bowel disease, exposure of zebrafish larvae was performed as previously described (Oehlers et al., 2013, 2017; Hanyang et al., 2017), groups of 20 embryos were placed in 6 well plates containing a total volume of 7.5 ml of medium/well. Embryos were kept in E3 1X medium (controls) or TNBS (2,4,6-trinitrobenzenesulfonate) was added in increasing concentrations (60 to 100 μg/ml) at 5 days post-fertilization (dpf) and followed up until 8 dpf. The number of individual deaths was recorded daily. The experiment was performed at least 3 times with different egg batches. Each experiment was performed in experimental replicates that belonged to the same egg batch.

2.3. Gene expression analysis by RT-qPCR

At 6 dpf, a pool of 20 larvae were euthanized with an overdose of Tricaine (MS-222) (0.3 mg/mL) (Wilson et al., 2009). The RNA extraction was performed using TRIzol (Invitrogen, EUA) following the manufacturer's instructions. The RNA was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, EUA) and 2 μg of RNA was used to synthesize cDNA, using M-MLV reverse transcriptase (Promega, EUA). For PCR reactions, 4 μL of diluted cDNA 1:4, 500 nM of primers, 5 μL of Power Master Mix Syber (Thermo fisher, EUA) were used. PCR amplification and analysis were performed using the expression $2^{(-\Delta\Delta CT)}$, using *efl1a1* as reference gene, as previously described (Schmittgen and Livak, 2008). Primer's description: efl1a1 (Forward (F): AGTGTTGCCTTCGTCCCAAT, Reverse (R): TTCCATCCCTTGAACCAGCC), IL-1β (F: TGGACTTCGCAGCACAAAATG, R: GTTCACTTCACGCTCTTGGATG), TNF-α (F: TCGGGTGTATGGAGGGTGTT, R: CTGGGTCTTATGGAGCGTGA), IL-10 (F: ACTCCACAACCCCAATCG, R: CCCCTTTTCCTTCATCTTTTCA), NF-kB (F: TTTCCCCCACTGATGTCCAC, R: GCTTTGGTTCGCTACAGTCG).

2.4. Analysis of myeloid cell infiltrate by flow cytometry

At 5 dpf, groups of twenty larvae (LysC:AB and LysC:TU) were

exposed to 70 μg/mL TNBS. The next day, larvae from control and TNBS groups were euthanized with an overdose of Tricaine (MS-222 Sigma) (0.3 mg/mL) (Wilson et al., 2009), and the intestines were collected for tissue dissociation. Also, the kidneys of an AB and a Tg(LysC:DsRed2) adult animals were dissected to be used as reference for compensation and gating panels. For tissue dissociation, intestine and kidneys were placed in 750μl of 1X PBS / 2% Fetal Bovine Serum (FBS, GIBCO), homogenized in a 40 μM cell strainer with the aid of a plunger and washed with 500 μl of 1X PBS / 2% FBS. Then, the samples were centrifuged for 12 seconds and the pellet resuspended with 200 μl 1X PBS for larvae intestines and 500 μl for adult kidneys. Finally, the samples were processed in LSRFortessa X-20 cytometer and analyzed with FlowJo Software. Gating strategies are available in Supplementary data.

2.5. Analysis of neutrophilic infiltrate by microscopy

Larvae LysC:AB and LysC:TU, exposed to 70 μg/ml TNBS were analyzed at 6 dpf, anesthetized with Tricaine (0.168 mg/mL) and placed in a glass slide with 3% methylcellulose. The larvae were carefully orientated for lateral view so that the intestine was in focus. Images were captured in the ZEISS AxioZoom v16 microscope and analyzed in Zen Blue software. The analysis of the neutrophils in the intestine was performed by quantifying fluorescent cells throughout the extent of the organ, from the anterior intestine (bulb) until the anus.

2.6. Goblet cells staining

Control and TNBS-treated larvae were euthanized at 6 dpf with a tricaine overdose as described (Wilson et al., 2009). Then, larvae were fixed with 4% paraformaldehyde overnight at 4 °C. On the following day, the samples were washed in acid ethanol (1% hydrochloric acid in 70% ethanol), incubated in Alcian Blue solution (0.1% Alcian Blue in 80% ethanol and 20% glacial acetic acid) for 3 hours at room temperature and washed repeatedly with acid ethanol. Subsequently, they were washed in 1 X PBS and placed in 2% methylcellulose for imaging in the ZEISS AxioZoom v16 microscope. The goblet cells counting was done manually on the images obtained, by counting the number of alcian blue-stained cells within the intestinal tract.

2.7. Statistics

The data were shown as mean and standard deviation. The difference between experimental groups was determined with a t-student test between two groups. The survival curve was evaluated using the log-rank test (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon. The $p < 0.05$ value was considered significant for all graphs. All graphs and statistical analyses were performed using the GraphPad Prism ® 6.

3. Results

3.1. AB strain presents higher mortality to TNBS-induced inflammation

In zebrafish larvae, TNBS exposure causes enterocolitis with induction of pro-inflammatory pathways, mucosal barrier disruption and leukocytosis in intestine, hallmarks similar to human IBD (Oehlers et al., 2011, 2013; Hanyang et al., 2017). We wondered whether different wild type strains of zebrafish could present varied susceptibility and immunity to TNBS-induced intestinal inflammation.

Thus, to test this hypothesis, we first performed a survival analysis of zebrafish larvae exposed to increasing doses of TNBS. For this experiment, TNBS was added to the medium at 5 dpf and survival of larvae was registered daily until 8 dpf (Fig. 1 and Supplementary Fig. 1). Our results

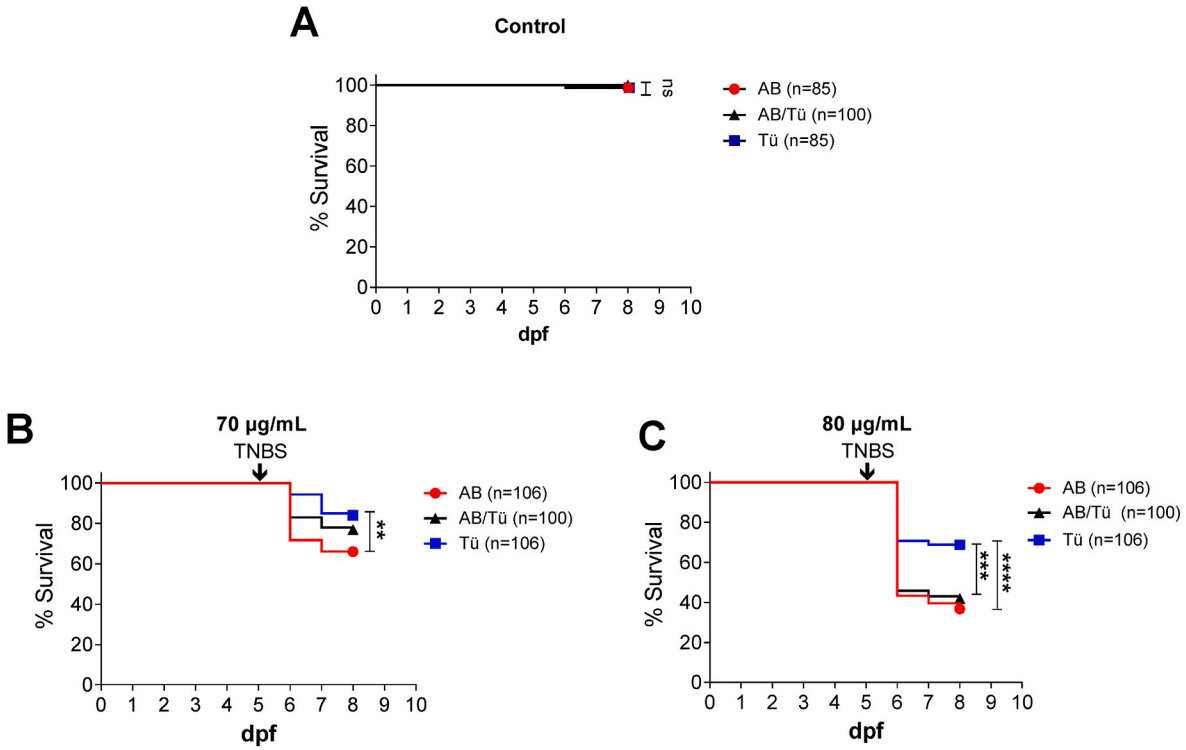

Fig. 1. Survival comparison between AB, TU and AB/TU shows that TU presents a higher survival rate. At 3 days post-fertilization (dpf), 15–20 larvae were selected per group and placed in embryonic medium, then at 5 dpf, larvae were either kept in embryonic medium or TNBS was added to a final concentration of 70 μg/mL (T70) and 80 μg/mL (T80) and survival of larvae was followed up daily from 5 to 8 dpf. **(A)** No significant mortality was observed in the control group, ns = not significant. **(B)** In T70, survival of TU was significantly higher than AB and AB/TU presented intermediate mortality to AB and TU, **p = 0.0016. **(C)** In T80, survival of TU was significantly higher than AB and AB/TU, ****p < 0.0001, ***p = 0.0001, respectively. Differences were evaluated using the log-rank test (Mantel-Cox) test. Data is representative of the sum of four independent experiments.

demonstrated that there were significant differences in survival between AB and TU strains when exposed to TNBS (Fig. 1A–B and Supplementary Fig. 1). Concentrations lower than 60 μg/mL TNBS were not toxic to AB nor TU larvae and did not result in significant mortality (Supplementary Fig. 1). However, doses of 70 μg/mL and 80 μg/mL of TNBS caused significant death in both AB and TU, with mortality being significantly higher in the AB group compared to TU (83,96% *vs* 66,03% in 70 μg/mL and 68,86% *vs* 36,79% in 80 μg/mL, respectively) (Fig. 1A–C). These data demonstrate that the TU strain has greater resistance to TNBS than AB. TNBS concentrations of 90 μg/mL or higher provoked complete loss or too low viability of larvae to detect differences in survival between AB and TU strains (Supplementary Fig. 1). Interestingly, by crossing the AB and TU backgrounds and generating hybrid AB/TU larvae, sensitivity to TNBS was altered. The AB/TU hybrid presented intermediate sensitivity to TNBS when compared to AB and TU strains alone at TNBS concentrations of 70 μg/mL (76.0% survival) and 80 μg/mL (42.0% survival) (Fig. 1A–B), however at low and high concentrations of TNBS (60, 90 μg/mL) AB/TU had an increased mortality compared to AB and TU (Supplementary Figs. 1A–C). These results corroborate our initial hypothesis that different wild type zebrafish strains have divergent sensitivity to TNBS-induced inflammation.

3.2. The AB and TU strains present different profiles of cytokine expression in inflammatory conditions

Following the observation that AB presented higher mortality than TU during TNBS exposure, we then proceed to analyze if these strains displayed divergent gene expression of inflammatory markers under physiological conditions or when exposed to TNBS. The expression of Nuclear Factor kappa B (NF-kB) transcription factor, that regulates cytokine production as well as pro-inflammatory (IL-1β, TNF-α, IL-12) or regulatory cytokines (IL-10) were measured at 6 dpf (Fig. 2). Our data showed that under physiological conditions AB and TU presented differences in expression of genes regulating inflammation. When AB and TU were exposed to a range of concentrations of TNBS, from 60 to 90 μg/mL (T60 -T90), NF-kB expression was higher in TU at the basal level and at T60 and T70, compared to AB exposed to the same conditions (Fig. 2A). Likewise, TNF-α expression tended to be higher in TU compared to AB at basal levels (CTR) and T60 (Fig. 2B). No statistically significant differences were observed in IL-12 expression between AB and TU (Fig. 2C). The pro-inflammatory cytokine IL-1β was significantly higher at T70 in TU compared to AB (Fig. 2D). However, the expression of anti-inflammatory cytokine IL-10 was also higher in TU compared to the AB strain in CTR and T70 conditions (Fig. 2E). Finally, we performed an analysis of the ratio between IL-1β and IL-10 expression (Fig. 2F), that was higher in AB under physiological conditions (CTR) and the highest

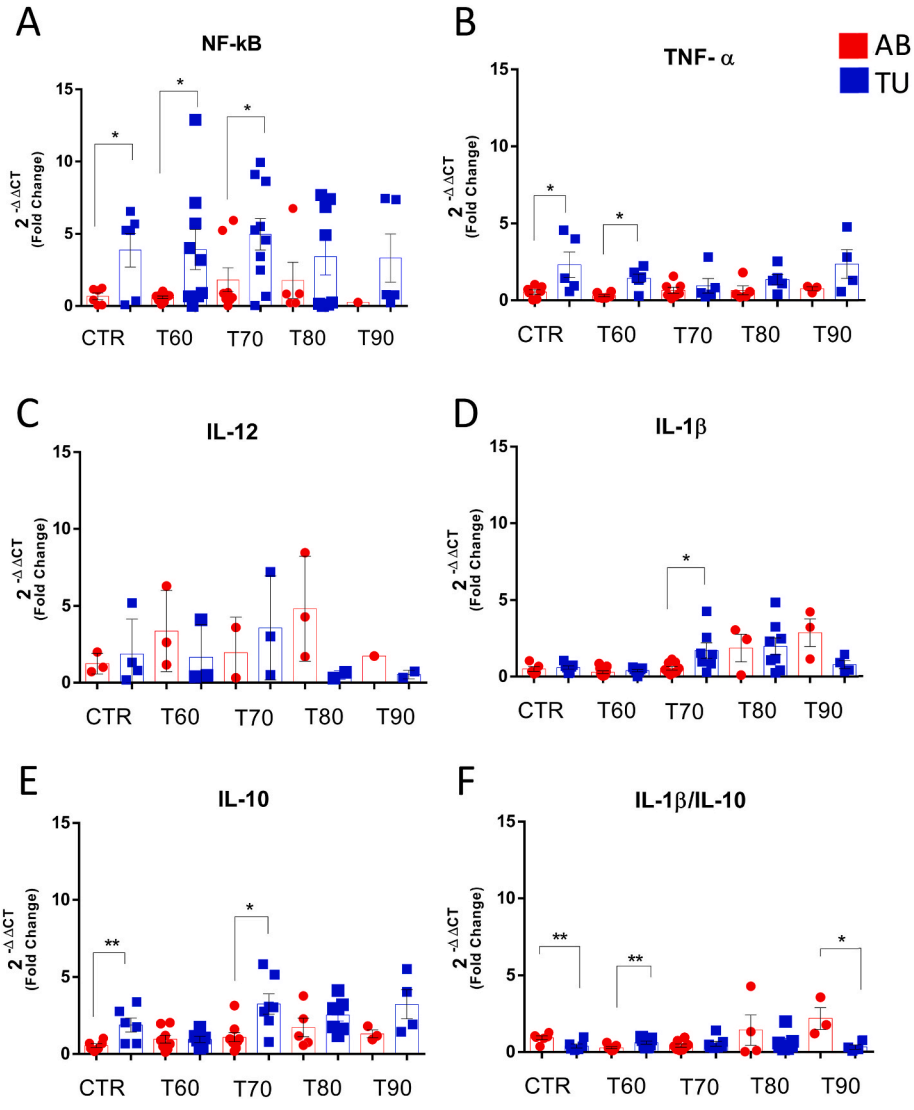

Fig. 2. TU presents higher expression of genes regulating inflammation including NF-kB and IL-10. Control or larvae exposed to increasing doses of TNBS (T60-T90) were euthanized at 6 dpf, RNA was extracted, and real time PCR was performed. Relative gene expression was calculated using the $2^{(-\Delta\Delta CT)}$ method, using the elongation factor 1 - alpha (elf1-α) as internal control. **(A)** NF-kB expression was higher in TU compared to AB in CTR, T60 and T70, AB vs. TU, *P ≤ 0.05 **(B)** TNF- α was higher in TU in CTR and T60, AB vs. Tü; *P ≤ 0.05 **(C)** IL-12 analysis showed no significant differences between AB and TU **(D)** IL-1β expression significantly increased in TU after high exposure of TNBS (T70); AB vs. TU, *P ≤ 0.05. **(E)** IL-10 expression was generally more elevated in TU compared to AB in CTR and T70, *P ≤ 0.05, **P ≤ 0.01. **(F)** IL-1β/IL-10 ratio analysis showed higher ratio of IL-1β in AB compared to TU in control and T90, AB vs TU; *P ≤ 0.05, **P ≤ 0.01; however in T60, ratio was lower compared to AB CTR, **P ≤ 0.01. Each data point corresponds to 2–9 samples, each sample was obtained from a pool of 20 larvae. Data are presented as mean and standard error of mean and differences between AB and TU, or doses were calculated by unpaired t-student test.

dose of TNBS (T90). Of note, in the AB line IL-1β/IL-10 ratio decreased in T60 compared to control.

Overall, the data indicate that the TU strain has higher expression profile of genes related to cytokine transcription in response to TNBS. In particular, TU showed a higher expression in all cytokines (NF-kB, TNF-α, IL-1β and IL-10), with a lower IL-1β/IL-10 ratio at CTR and T90 than AB. This suggests that AB and TU strains differ in their immune response but possibly the TU strain may have better immunoregulatory responses compared to AB.

3.3. TNBS induces higher infiltration of neutrophils in the gut of AB larvae

To investigate if TNBS exposure in larvae induced organ-specific inflammation conducive to an IBD phenotype in AB and TU strains, we isolated intestines from control larvae or larvae exposed to T70 at 6 dpf and analyzed the percentages of myeloid cells and neutrophils by flow cytometry (Fig. 3A–B). To compare whether differences in genetic background could lead to differential leukocyte infiltration in TNBS-induced colitis, the transgenic line Tg(LysC:DsRed2)^{nz50} with red fluorescent neutrophils were crossed with AB or TU and their offspring (LysC:AB, LysC:TU) were analyzed. The kidney is the main hematopoietic organ in adult zebrafish, equivalent to the human bone marrow. Based on this fact, we used the cells isolated from the kidney of adult zebrafish to select the myeloid gate using size and granularity, as previously described (Langenau et al., 2003; Trede et al., 2004) (Supplementary Fig. 2). We projected this kidney myeloid gate as a reference to analyze cells from larval intestines (Fig. 3A). We did not find any differences in percentage of myeloid cells between AB and TU controls nor after exposure to TNBS (Fig. 3A). After doublet exclusion, we evaluated the percentage of neutrophils by gating on LysC:DsRed2+ cells inside the myeloid population (Hall et al., 2007; Stoddard et al., 2019). No statistically significant differences were observed in the percentage of neutrophils between the groups LysC:AB, LysC:TU in controls and T70 (Fig. 3B).

Next, taking advantage of the transparency of the zebrafish larvae, which allows visual analysis of *in vivo* intestinal inflammation by the examination of fluorescent immune cells infiltrating the gut. At 6 dpf, control and T70 larvae were anesthetized and imaged for quantification of neutrophils in the intestinal region (Fig. 3C). The number of neutrophils significantly increased in LysC:AB larvae when exposed to T70 compared to control (24.69 ± 2.73 vs 39.0 ± 3.30 cells). In contrast LysC:TU displayed the opposite behavior, a slight decrease in cellular infiltrate after T70 exposure (29.10 ± 2.15 vs 24 ± 1.09) (Fig. 3D), showing that TNBS can induce inflammation in the LysC:AB line, while LysC:TU seems to be resistant. One hypothesis raised by our group is that the differences observed in these analyses could be more evident if we had used filial animals with a cleaner AB and TU genetic background, meaning descendants from at least one or two generations after the crossing of Tg(lysC:DsRed2)^{nz50} with AB or TU. However, it is important to note that survival data (Fig. 1) shows that only one crossing was sufficient to alter hybrid AB/TU resistance to TNBS compared to pure AB or TU strains.

3.4. The AB strain shows a decrease in mucus-producing goblet cells after intestinal inflammation

Mucus production is crucial to sustain intestinal epithelial barrier and is one of the first lines of defense against infections. Therefore, mucus-producing goblet cells have an important indirect role in maintaining intestinal immune homeostasis (Takiishi et al., 2017). In IBD patients, the mucosal thickness is reduced compromising the intestinal barrier integrity (Okumura and Takeda, 2018), our data shows that TNBS provokes a similar effect in AB larvae, causing a significant decline in the number of goblet cells in their intestinal tracts (Fig. 4). In contrast, TU presented lower numbers of goblet cells in physiological conditions and TNBS exposure did not alter the number of goblet cells. These data suggest that AB, in fact, has greater sensitivity to TNBS-induced colitis when compared to the TU strain.

4. Discussion

In animal models, differences in genetic backgrounds can influence the responses produced by the same stimulus. This study proposed to investigate whether there were differences in susceptibility to TNBS-induced inflammation between two zebrafish WT strains: AB and TU; and further characterize their immune profiles. Our data demonstrated significant differences in survival, cytokine profile, neutrophil intestinal infiltration and goblet cell numbers between AB and TU strains when exposed to an inflammatory agent like TNBS. We corroborated our hypothesis, that distinct WT strains of zebrafish possess specific immune responses which affect disease susceptibility. First, we demonstrated that the TU strain was more resistant to death induced by TNBS, than the AB strain. Then, analyzing the cytokine profile during TNBS exposure, we showed that the TU larvae presented a significant increase in the expression of NF-kB in both the controls and the treated groups (T60 and T70). This transcription factor is important for the regulation of genes linked to various pro-inflammatory cytokines, including TNF-α and IL-1β (Atreya et al., 2008). TNF-α is activated throughout the Myd88 pathway, as observed in mammals (Beutler et al., 1985). Bates et al. (2007), demonstrated that zebrafish recognize LPS exposure via Toll-Like Receptors (TLR), which leads to the activation of Myd88 signaling, stimulating the nuclear translocation of NF-kB, and, consequently, the expression of TNF. The triggering of TLR pathways by LPS might be seen in cases of microbiota imbalance and intestinal inflammation (Bates et al., 2007). Accordingly, microbiota disbalance and intestinal inflammation are causes of IBD (Marjoram et al., 2015). Moreover, TNF-α is released by several immune cells during infections and tissue damage (Roca et al., 2008). In that sense, zebrafish respond to intestinal inflammation in a similar manner to mammals with IBD, by presenting overexpression of the TNF locally (Bates et al., 2007). Currently, the use of TNF-α inhibitors is one of the most effective treatments for IBD, leading to mucosal healing, however, treatment with this drug can also cause adverse reactions by affecting intestinal microbiome (Pache et al., 2009; Peyrin-Biroulet, 2010; Jones-Hall and Nakatsu, 2016). Therefore, TNF-α expression is linked to intestinal inflammation in IBD cases and was evaluated in our study. We observed that in fact, the expression of TNF-α differed between TU and AB. Our data shows that when exposed to 60 μg/mL of TU presented significantly higher expression of TNF-α compared to AB. Paradoxically, the animals that had lower mortality in the survival experiment, TU, also showed greater expression of TNF-α. This could be explained by the broad effects of TNF-α, as studies have demonstrated that TNF-α regulates the growth of enterocytes and alter the permeability of the epithelial barrier, in addition to stimulating the production of matrix metalloproteinases and other factors that act in the tissue remodeling of intestinal mesenchymal cells (Bain and Mowat, 2014). In addition, TNF-α could possibly exert different types of bioactivities in fish compared to its counterparts in mammals, since they have a duplicated genome (Bradford et al., 2017). In agreement with this hypothesis, there is data showing that teleost fish, such as zebrafish, have two types of TNF-α with some distinct functions: one at a pro-inflammatory level promoting apoptosis and the second acting in signaling for tissue repair and angiogenesis, therefore the increase in TNF-α in TU could be related to the repair function and not necessarily to inflammation (Roca et al., 2008). Noteworthy, a recent study in AB larvae showed the lack of expression of TNF-α using TNBS at 50 μg/mL in different time points, corroborating the pattern of TNF-α expression seen in this strain in this study (Fénero et al., 2021).

On the other hand, IL-1β was found to be highly expressed at basal levels and in TU at 70 μg/mL TNBS, which is also observed in the context of IBD (McEntee et al., 2019). This cytokine is produced in response to PAMPs (pathogen-associated molecular patterns), DAMPs

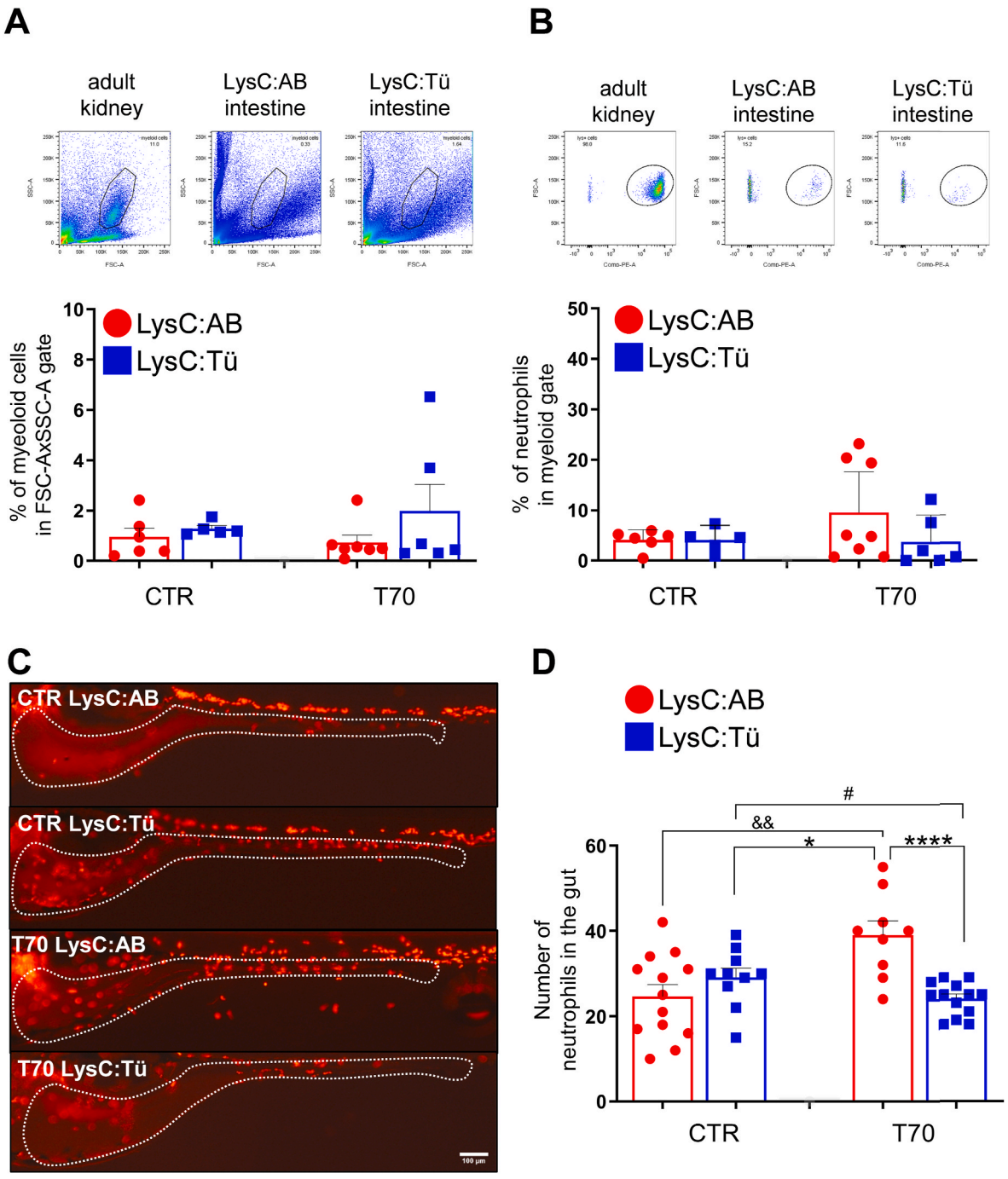

Fig. 3. Neutrophils are increased in AB but not in TU after TNBS exposure. AB and TU were crossed with LysC;Dsred + zebrafish the Dsred + progeny was selected (LysC:AB and LysC:AB) and exposed to TNBS (T70). At 6 dpf analysis of intestinal immune cell infiltrate was analyzed by flow cytometry **(A and B)** or microscopy **(C and D)**. For FACs analysis intestines of LysC:AB and LysC:AB were dissected and dissociated; **(A)** a gate on granulocytes was performed based on size and granularity and using cells isolated from the hematopoietic organ (kidney) of an adult zebrafish as reference and no significant differences between LysC:AB and LysC:TU was found; **(B)** the frequency of neutrophils (DsRed + cells) was unaltered in LysC:TU after TNBS exposure compared to control and in LysC:AB mean value increased compared to control but was not statistically significant. Each sample is a pool of 100–200 larval intestines, data is representative of three independent experiments. On another set of experiments, microscopic quantification of neutrophils in the gut of control or TNBS-exposed (T70) larvae was performed; **(C)** representative images of larvae showing delimitation of gut area (inside dotted lines) can be seen; **(D)** our data shows that in physiological conditions there are no differences between LysC:AB and LysC:TU and that TNBS exposure significantly increases the number of neutrophils in gut LysC:AB compared to control $^{\&\&}P \leq 0.01$ and vs. T70 LysC:TU, $^{**}P \leq 0.01$. In LysC:TU, TNBS caused a small but significant decrease in neutrophils compared to control $^{\#}P \leq 0.05$. Each point represents 1 animal, for each group 9–13 animals were evaluated, data is representative of two independent experiments. Data are presented as mean and standard error of mean and differences were calculated by unpaired t-student test.

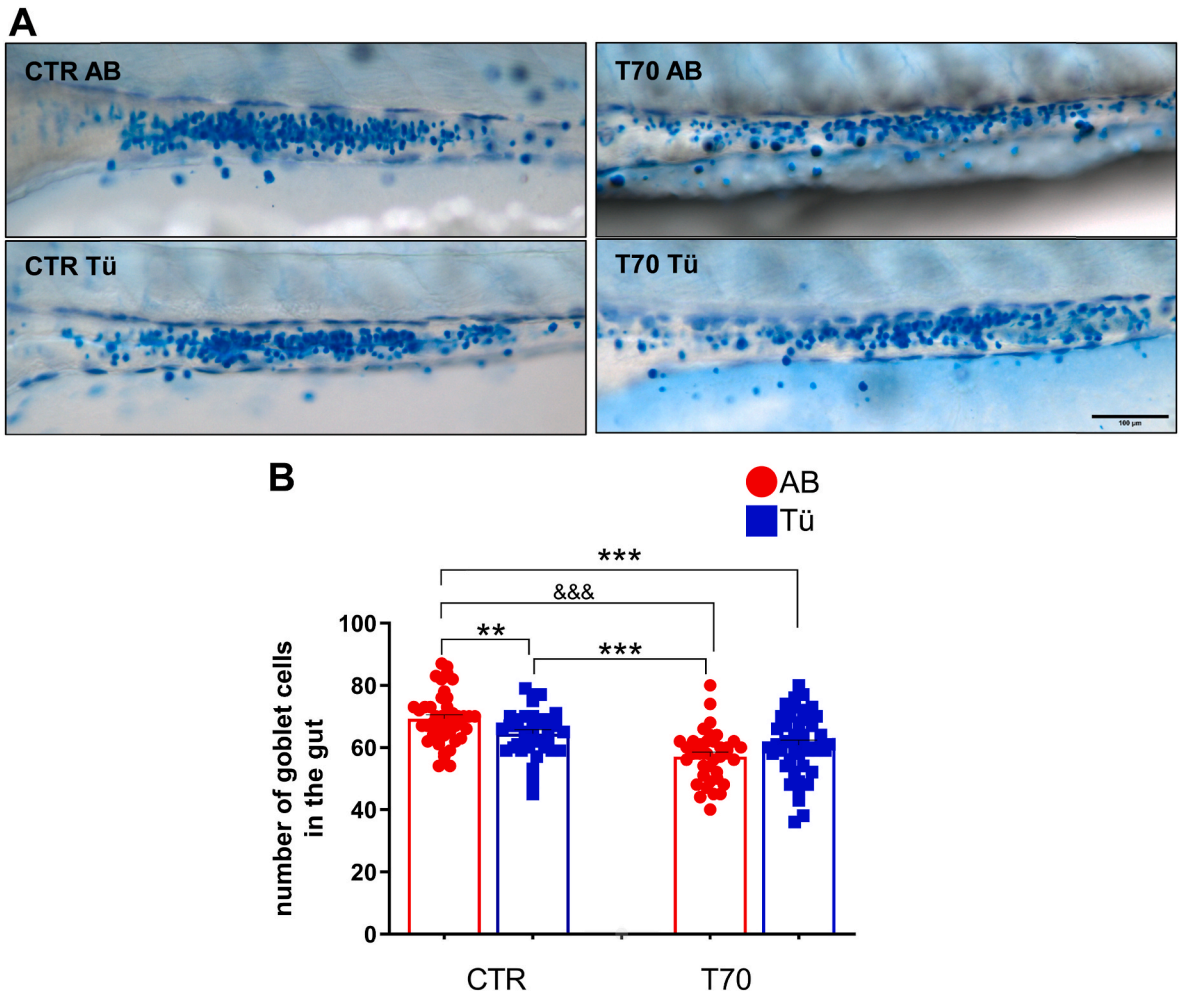

Fig. 4. TNBS significantly reduces the number of mucus-producing goblet cells in AB but not TU. At 6dpf, control or larvae exposed to 70 μg/mL of TNBS (T70) were euthanized and stained with alcian blue to quantify mucus-producing goblet cells in the intestine. **(A)** representative images of mid intestinal region of larvae showing goblet cells stained in blue **(B)** Analysis of the number of goblet cells in the intestines show that in physiological conditions, AB presents a higher number of goblet cells compared to TU (CTR TU vs CTR AB; $^{**}P \leq 0.01$), however TNBS exposure causes a significant decrease in the number of goblet cells in AB larvae (CTR AB vs T70 AB $^{\&\&\&}P \leq 0.001$), whilst in TU, TNBS does not cause alterations in the number of goblet cells. CTR AB vs. T70 TU, $^{***}P \leq 0.001$; CTR TU vs. T70 AB, $^{***}P \leq 0.001$. Data are presented as mean and standard error of mean and differences were calculated by unpaired t-student test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(damage-associated molecular patterns) and cytokines, including TNF-α (Mao et al., 2018; McEntee et al., 2019). It is noteworthy that the role played by IL-1β in colitis models is controversial. It has been reported that inflammation may improve by blocking IL-1β signaling, however, it was also observed that IL-1β-deficient animals become more susceptible to the colitis model. It was also reported that changes in bacterial groups in the intestinal microbiota may affect the release of this cytokine via the NLRP3 inflammasome (Seo et al., 2015).

In regard to IL-10, TU animals showed a higher expression upon TNBS treatment. IL-10 regulates immune responses in the gastrointestinal tract, being able to suppress aberrant type 1 and type 2 responses, critically, if IL-10 is deficient, spontaneous gut inflammatory disorders can be developed (Kühn et al., 1993; Shah et al., 2012). Moreover, it is also known that IL-10 has become a candidate for therapy, since it plays an important role in regulating inflammatory processes involved in the pathogenesis of IBD, as it has been clearly shown that IL-10 knockout animals develop colitis whereas its expression is related to the attenuation of the disease (Oehlers et al., 2013; Engelhardt and Grimbacher, 2014). Zebrafish has a conserved motif of IL-10, its expression can be detected in gills, kidney, and gut, and it can be produced by different cell types, including non-immune and immune cells (Zhang et al., 2005; Mosser and Zhang, 2008). It has been shown that IL-10 deficient zebrafish lose mucosal tissue homeostasis and a nonsense mutation in the IL-10 gene in $il10^{e46/e46}$ zebrafish, induce type 1 and type 2 responses, pro-inflammatory cytokine production (TNF-α and IL-1β) and different infection susceptibility to *Mycobacterium marinum* compared to WT (Harjula et al., 2018; Bottiglione et al., 2020). Moreover, IL-10 can modulate the immune response by inhibiting antigen presentation and the release of pro-inflammatory cytokines (Li and He, 2004). Therefore, the higher expression of IL-10, as well as lower IL-10/ IL-1β ratio in TU compared to the AB, could account for the increased resistance to TNBS found in TU.

The enterocolitis score includes the infiltration of immune cells in the gut, depletion of goblet cells and increase of pro-inflammatory cytokines (Hanyang et al., 2017). Oehlers et al. (2011) demonstrated that larvae exposed to low-dose TNBS-treatment (50 μg/mL) displayed shortening of the mid-intestine, disruption of the intestinal vasculature and leukocyte enrichment with recruitment from the caudal hematopoietic tissue to the intestine and epidermis (Oehlers et al., 2011). Accordingly, our study has indicated that TNBS-induced inflammation in LysC:AB larvae led to an increase in myeloid cells migration within the intestine in a greater proportion than in LysC:TU larvae, which is indicative that AB strain displays a higher susceptibility to TNBS compared to TU. One point to consider is that different batches of eggs might display

variations in their response, thus may not present uniform reaction to TNBS exposure, therefore it is expected to observe some differences in the survival or inflammatory response of the fishes in separate experiments, which might account for some of the higher standard deviations within our groups, particularly in the case of hybrids (e.g. LysC:AB and LysC:TU) that result from a single crossing and have less stable background. It would warrant further investigation if cleaner LysC backcrossings to AB or TU would render animals more or less susceptible to TNBS-induced inflammation.

The intestinal mucus layer acts as the first line of defense by protecting the host epithelium from the intestinal content, which includes indigestible food particles and gut bacteria. Mucus is secreted from specialized secretory cells called goblet cells that are found dispersed over the intestinal epithelium (Stange and Schroeder, 2019). Defects in the intestinal mucosal barrier are a hallmark of IBD (Lee et al., 2018). Uyttebroek et al. (2020) showed a decrease in the number of globet cells in adult zebrafish treated with TNBS, and this it was also seen in larvae (Uyttebroek et al., 2020). Accordingly, we observed that the number of mucus-producing cells was remarkably decreased in AB strain upon TNBS-induced colitis, but this was not seen in the TU strain. In addition, a study carried out in zebrafish larvae as well demonstrated that larvae treated with retinoic acid exhibit impaired mucus production resulting in an exacerbation of the experimental enterocolitis (Oehlers et al., 2012). The greater susceptibility to TNBS seen in the AB strain, which resulted on lower survival rate, can be explained by the decrease in the number of mucus-producing cells, leading to a higher infiltration of myeloid cells in the intestine which could cause more tissue damage. A hypothesis for the highest resistance TNBS-induced colitis found in TU strain is the highest expression of IL-10 expressed in these animals' cytokine could be contributing to better immunomodulation.

The data presented here suggest that the most commonly used zebrafish wild type strains AB and TU, have different basal expression of cytokines and present different responses to TNBS-induced colitis. To our knowledge, this is the first time a comparative research in immune profile is performed testing the response of zebrafish WT strains to an inflammatory insult. In mice the fact that individual strains have different basal representatives of immune cells or cytokine expression, and therefore respond differently to the same stimulus, has already been reported and is well established, however, few studies show this difference in zebrafish (Watanabe et al., 2004; Velasquez et al., 2016). Our work demonstrates that the choice of strain is fundamental to reproduce experimental models and to achieve consistent and comparative data between facilities.

5. Conclusions

The results obtained suggest that AB and TU strains present physiological differences when submitted to the experimental model of inflammatory bowel disease induced by TNBS. Our data support our hypothesis that wild-type zebrafish strains can manifest contrasting responses to the same stimulus. Based on this, the characterization of the immune response profile of these strains will help us to understand the variation in susceptibility in different experimental models of disease and thus to adequate and apply this to zebrafish studies. Moreover, we encourage the comparison of these and other zebrafish WT strains in different immunological challenges.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crimmu.2021.12.003.

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