Lithium limits trimethyltin-induced cytotoxicity and pro-inflammatory response in microglia without affecting the concurrent autophagy impairment

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ABSTRACT

Trimethyltin (TMT) is a highly toxic molecule present as an environmental contaminant causing neurodegeneration particularly of the limbic system both in humans and in rodents. We recently described the occurrence of an impairment in the late stages of autophagy in TMT-intoxicated astrocytes. Here we show that similarly to astrocytes also in microglia TMT induces the precocious block of autophagy determining the accumulation of the autophagosome marker microtubule associated protein light chain 3 (LC3). Consistently with an autophagy impairment we observe in TMT-treated microglia the accumulation of p62/SQSTM1, a protein specifically degraded through this pathway.

Lithium has been proved to be effective in general in limiting neurodegenerations and in particular in ameliorating symptoms of TMT intoxication in rodents. In our in vitro model lithium displays a pro-survival and anti-inflammatory action reducing both cell death and pro-inflammatory response of TMT-treated microglia. To note, lithium exerts these activities without affecting autophagy. In fact, the autophagic block imposed by TMT is unaffected by lithium administration. These results are of interest since defects in the execution of autophagy are frequently observed in neurodegenerative diseases and lithium is considered a promising therapeutic agent for these pathologies. Thus, it is relevant that this cation can still maintain its pro-survival and anti-inflammatory role in conditions of autophagy block.

Key words: microglia, trimethyltin, autophagy, neurodegeneration, neuroinflammation

INTRODUCTION

Trimethyltin (TMT) is a highly neurotoxic molecule belonging to the family of organotins. These compounds during the last decades have been used for a variety of industrial and agricultural applications as pesticides, fungicides and antifouling paints for ship hulls (Appel 2004). Some of them have been banned such as tributyltin chloride (TBT) in the United States during the 1980s (Fent 1996) but despite their toxicity methyltins are still heavily used as heat stabilizers in PVC production (Dobson et al., 2005). Leaching of organotins from PVC material has been proposed to be one of the principal sources of environmental contamination (Richardson and Edwards, 2009) and in fact, they can be detected as contaminants in fresh water, tap water, seawater, algae and fish (Fent, 1996; Borghi and Porte, 2002; Mundy and Freudenrich, 2006). Although the more widely used methyltin, dimethyltin (DMT), is characterized by a low direct toxicity its conversion into TMT by the addition of a methyl group has been reported to occur in vivo (Furuhashi et al., 2008).

TMT damages specific populations of neurons and in particular the limbic system, including the pyriform/enthorinal cortex, olfactory bulb, amygdala and hippocampal formation (Chang and Dyer, 1985; Kawada et al., 2008). The main symptoms of TMT intoxication comprehend aggressive behavior, disorientation, seizures, and severe memory loss (Reuhl and Cranmer, 1984; Kreyberg et al., 1992). Similarly to other neurodegenerative disorders, TMT-induced neurodegeneration is characterized by neuronal selectivity and neuroinflammation always accompanied by both astroglial and microglial activation (Geloso et al., 2011; Pompili et al., 2004; Pompili et al., 2011). In the hippocampus of TMT-intoxicated rodents, microglia activation is observed during the first post-intoxication day and then persists over a long period (Koczyk and Oderfeld-Nowak, 2000). At the same time in the hippocampus pro-inflammatory cytokines are upregulated (Bruccoleri and Harry, 2000). TMT administered in vitro to microglial cultures determines the increase in the expression of M1 markers (TNF-α, IL-1β, IL-6 and iNOS) and the decrease of the M2 markers (CD206 and arginase-1) (Kim et al., 2014). Recently, TMT was demonstrated to directly activate the BV-2 microglial cell line via the NADPH oxidase-dependent ROS generation (Kim and Kim, 2015).

A general response of cells exposed to stressful conditions is the activation of autophagy. This evolutionary conserved catabolic pathway is aimed to remove damaged organelles and misfolded proteins and generally displays a cytoprotective function (Nikoletopoulou et al., 2013). It is initiated by the formation of a double-membrane vesicle known as autophagosome which engulfs aggregated proteins or entire organelles. After fusion with lysosomes the cargo is finally degraded (for a recent review see Klionsky et al., 2016). Our previous data showed the importance of the autophagic pathway in improving the survival of neuronal cells exposed to TMT (Fabrizi et al., 2012; Fabrizi et

al., 2014). Moreover, we recently reported that in astrocytes TMT determines the impairment of the late stages of autophagy limiting autophagosome movement and thus their maturation into autophagolysosomes (Fabrizi et al., 2016).

In this paper we wanted to answer the question as whether the TMT-induced impairment of autophagy was restricted to astrocytes or instead could be a possible common feature of the reaction of glial cells to this toxic compound. Thus, we extended our previous observations to microglia which are considered the resident macrophages of the central nervous system. During neurodegenerative diseases microglial cells become activated and can produce highly toxic molecules such as radical species leading to further neuronal dysfunction and neurodegeneration.

Moreover, since lithium neuroprotective activity has been proved in many different experimental settings including TMT intoxication (Kim et al., 2013; Yoneyama et al., 2014), we studied if lithium could limit microglial response to TMT in our culture model system. Interestingly, lithium has been shown to modulate the autophagic pathway functioning both as an inducer of autophagy at low concentrations (0.5 mM) and as an inhibitor of the same pathway when used at concentrations higher than 2 mM (Sarkar and Rubinsztein, 2006; Sarkar et al., 2008). Due to its multiple molecular targets lithium mechanism of action still remains elusive (for a review see Chiu et al., 2013).

MATERIALS AND METHODS

Cell cultures and treatments

All procedures were carried out in accordance with the Italian laws and guidelines established for the care and the use of animals in research. Microglial cells were prepared from the cortex of newborn (P4) Sprague Dawley rats, as described previously (Pompili et al., 2011). Briefly, after 14 days in culture, microglial cells were separated from the underlying astrocytic monolayer by gentle agitation, and cells were cultured in DMEM (Invitrogen, Italy) supplemented with 10% fetal calf serum (Sigma, Milan, Italy) in 5% CO_2. Cultures routinely consisted of ≥95% microglial cells as determined by staining with *Griffonia simplicifolia* isolectin B4 (IL B4; Sigma, St Louis, MO).
Microglial cells ($1x10^5$ cells/cm^2) were seeded onto 96, 24 or 6-well plates depending on the experiment. After 24 hours from seeding cells were treated with TMT (10 μM; Heraeus, Karlsrube, Germany), lithium chloride (0.5, 1, 2 mM), 0.5 μM rapamycin, 10 μM indirubin-3'-monoxime, 20 nM SB-216763, 100 nM bafylomicin A1, 10 mM 3-methyladenine. All these compounds were used alone or in combination for different times ranging from 3 to 48 hours as reported in the figure legends. All reagents were from Sigma-Aldrich unless otherwise stated.

Assessment of cell viability

Cell death was evaluated by measuring the release of lactate dehydrogenase (LDH) in the culture medium by the Cytotoxicity Detection Kit (Roche, Mannheim, Germany) according to manufacturer's protocols.

TUNEL

The DNA fragmentation of apoptotic cells was detected using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) kit (In situ Cell Death Detection Kit, Roche). Cells were cultured on coverslips and at the end of the drug treatment fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (PBS) at room temperature for 15 min and then incubated with a permeabilizing solution (0.1% Triton X-100) for 10 min at 4°C. The cells were then incubated with the TUNEL reaction mixture for 60 min at 37°C and visualized by inverted fluorescence microscopy (Eclipse E600, Nikon Instruments SpA, Italy). TUNEL-positive nuclei were counted in ten nonoverlapping fields per coverslip and then converted to percentage by comparing TUNEL-positive counts with the total cell nuclei visualized by DAPI (4',6'-diamino-2-phenylindole) counterstaining.

MDC

Living cells plated on coverslips were incubated with 0.05 mM monodansylcadaverine (MDC; Sigma) (Biederbick et al., 1995) in PBS at 37°C for 10 min. Cells were washed three times in PBS and then immediately analyzed by fluorescence microscopy.

Western blotting

Treated and untreated cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2, Sigma). For estimation of p62/SQSTM1 levels only, cells were lysed directly in 100 μl 2x SDS-PAGE gel loading buffer (125 mM Tris-HCl, pH 7.4, 4% SDS, 0.04% bromophenol blue, 30 mg/ml DTT added immediately before use), sonicated 3 times for 5 sec and then boiled for 5 min (Bjørkøy et al., 2009). All samples were clarified by centrifugation at 1000 rpm for 5 min. Equivalent amount of protein (10 μg) from each sample was electrophoretically resolved on 12.5% precast SDS-polyacrylamide gels (ExcelGel, GE Healthcare Biosciences) using horizontal apparatus (Pharmacia Biotech, Uppsala, Sweden). Then, separated proteins were electro-transferred onto nitrocellulose membranes (Schleicher & Schuell) by a semi-dry system (Novablot, Pharmacia Biotech). Membranes were blocked with 3% non-fat milk in PBS and then incubated (overnight at 4°C) with the following antibodies: anti-LC3B (Sigma; 1:1000 in 3% bovine serum albumin in PBS), anti-p62/SQSTM1 (MBL, PM045; 1:1000 in 3% non-fat milk in PBS). After extensive washing with PBS containing 0.1% Tween-20 (TBST), blots were incubated with 1:2000 dilution of HRP-conjugated secondary antibody (Amersham Biosciences) for 1 hour at room temperature. Immunopositive bands were detected with a chemiluminescence detection system (GE Healthcare Biosciences). To check for equal loading of the gel, membranes were stripped and reprobed with mouse anti-β-actin or anti-GAPDH antibodies (1:20000, Sigma). Densitometric analysis was performed with the Quantity One software (BioRad Laboratories).

ELISA

The release of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) into culture supernatants was determined by standard ELISA techniques according to the manufacturer's instructions (R&D Systems, MN, USA).

Animals and immunohistochemistry

Male Sprague Dawley rats (7 week-old, weighing 250 g) (Charles River, Italy) were housed in a temperature and humidity-controlled room (21±5°C and 60% humidity) and fed *ad libitum* with

standard laboratory diet and water. All procedures were carried out in accordance with the Italian laws and guidelines established for the care and the use of animals in research. Rats were administrated a single intraperitoneal dose of TMT (8 mg/ kg body weight; Heraeus, Karlsrube, Germany) or vehicle (saline) and were sacrificed at 7 days after treatment. Brains were rapidly removed, embedded in OCT compound (Killik, Bio-Optica, Italy), and frozen on methylbutane precooled with liquid nitrogen as previously reported (Pompili et al., 2011).

Sagittal cryostatic sections (7-μm thickness) from saline and TMT-treated rats were fixed with 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 10 min. After quenching autofluorescence with 0.05 M ammonium chloride and saturation of nonspecific sites with 3% normal donkey serum (BioCell Research Laboratories, Rancho Dominguez, CA) and 0.1% Triton X-100, sections were incubated overnight at 4°C with rabbit anti-p62/SQSTM1 (1:500; MBL, PM045) and mouse anti-OX42 (1:100; Serotec, Oxford, UK). After washing, the sections were incubated with a mixture of donkey Dy-light 549 anti-rabbit IgG (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) and donkey Dy-light 488-labelled anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories). Negative controls were performed substituting specific Igs with an equivalent amount of nonspecific Igs and omitting primary antibodies. Slides were mounted with Vectashield mounting medium, containing DAPI for nuclear staining (Vector Laboratories, Burlingame, CA). Examinations and photographs were made using a fluorescence microscope (Eclipse E600; Nikon Instruments S.p.A., Firenze, Italy).

Statistics

Statistical analyses were conducted using GraphPad Prism version 4.00 software. Data are expressed as averages ± SD. Comparisons were analysed using one-way ANOVA with Bonferroni-corrected t test. All experiments were performed in triple and reproduced at least three times.

RESULTS

In the experiments presented below we used the minimum concentration of TMT (10 μM) previously demonstrated to affect microglial cell viability (Pompili et al., 2011). Consistently with those previously published data, at 24 and 48 hours of TMT treatment a progressive increment respect to control in LDH release (indicative of necrosis and late-apoptosis) was measured in the culture medium of rat microglial primary cultures (Fig. 1 A). At earlier time points a statistically significant increase respect to the untreated control in the percentage of TUNEL+ apoptotic cells could be measured at 8 hours of TMT treatment which then increased further at 24 hours (Fig. 1 B). Since we already demonstrated the appearance of autophagic vesicles in both neuronal and astrocyte cultures exposed to TMT (Fabrizi et al., 2012; Fabrizi et al., 2016), we then checked for the presence of these vesicles also in TMT-treated microglia. To this aim microglial cells were first labelled by the fluorescent dye monodansylcadaverine (MDC), a compound which marks autophagic vesicles (Vazquez and Colombo, 2009). As shown in Figure 2, TMT determined the appearance of many MDC-positive structures which were not observed if this compound was administered in combination with the autophagic inhibitor 3-methyladenine (3-MA) known to block autophagy at the sequestration stage (Stroikin et al., 2004). MDC-positive vesicles were also detected, as expected, after treatment with the autophagic inducer rapamycin. In addition to late-stage autophagosomes, MDC was also reported to label acidic endosomes and lysosomes (Munafo and Colombo, 2001). Thus, in order to confirm the increased presence of autophagosomes in TMT-treated microglia, we measured on western blot the amount of the lipidated form of LC3 (LC3-II) (Fig. 3). In fact, LC3-II binds tightly to the autophagosomal membrane and is considered a reliable autophagosome marker (Kabeya et al., 2000). When treated with TMT the amount of LC3-II raised rapidly in microglia respect to the untreated control (Fig. 3). This result not necessarily indicates an increased autophagic flux since it could be also related to an impairment in the maturation of autophagic vesicles (Klionsky et al., 2016). Thus, we analyzed LC3-II levels in microglia in the presence of bafilomycin A1. This compound is a potent inhibitor of the vacuolar-type H+ ATPase which impairs lysosomal functions blocking late stages of autophagy and consequently LC3 degradation (Yamamoto et al., 1998; Boya et al., 2005). When TMT and bafylomicin A1 were administered in combination no further increase in LC3-II/β-actin ratio was measured indicating that TMT was already blocking the autophagosome maturation and the consequent LC3-II degradation (Fig. 3). The observed impairment in autophagy induced by TMT persisted up to 48 hours of treatment (not shown).

As mentioned above, lithium is a modulator of autophagy known to limit cell death and inflammatory response in many different experimental models (Chiu et al., 2013).

When added to microglia this cation was able to significantly reduce TMT toxicity when used at 1-2 mM (Fig. 4). Lithium cytoprotective action against TMT was also confirmed by the MTT test (not shown). Concentrations of lithium above 1 mM are known to inhibit the glycogen kinase synthase-3 (GSK-3) (Sarkar et al., 2008). Other GSK-3 inhibitors, such as indirubin-3'-monoxime or SB-216763 similarly to lithium limited TMT toxicity in microglia (not shown).

In addition to its cytoprotective action, lithium was also able to reduce the TMT-induced release of TNF-α. At the same time this cation favoured the production of the anti-inflammatory cytokine IL-10 (Fig. 5).

Lithium is also known to affect the autophagic pathway. Thus, we decided to check if lithium pro-survival and anti-inflammatory actions observed in our model could be related to its ability to force the autophagic impairment induced by TMT. The analysis of LC3-I/II conversion and p62 (also known as SQTSM1) expression revealed that lithium was not able to revert the autophagic block imposed by TMT at none of the concentrations used ranging from 0.5 to 2 mM (Fig. 6 A, B). To note this is the same range of lithium concentrations shown before to protect microglia from TMT toxicity and to limit its pro-inflammatory response (Fig. 4 and 5). Figure 6 also shows the levels of expression of LC3 and p62/SQSTM1 measured in TMT-treated astrocytes which result higher respect to the ones obtained in microglia using the same experimental settings.

Moreover, when we checked for the expression of p62/SQSTM1 in brain slices of intoxicated rats most of the immunoreactivity was observed in astrocytes as previously reported (Fabrizi et al., 2016), while only few OX-42+ microglial cells were stained (Fig. 7). In control sections obtained from rats treated with saline only, p62/SQSTM1 was hardly detectable.

DISCUSSION

Our observations of TMT-treated microglial cultures showed the appearance of many MDC+ vesicles and an increased LC3-I/II conversion after exposure to this toxic compound. Both these results are indicative of autophagosome formation. Besides, the analysis of the autophagic flux during TMT treatment indicated a block of autophagy at its late stages since no further increase in LC3-II/β-actin ratio was observed after the combined treatment of this organotin with bafylomicin A1 (lysosomal inhibitor). The accumulation of p62/SQSTM1 in TMT-treated samples is in line with an impairment in autophagy since this protein is preferentially degraded through this catabolic route (Bjørkøy et al., 2005). We recently described the occurrence of an autophagic block in astrocytes intoxicated with TMT (Fabrizi et al., 2016). Although an autophagic block was observed both in astrocytes and microglia following TMT treatment, this compound appeared to induce a much higher accumulation of LC3-II and p62/SQSTM1 in the former population of glial cells respect to the latter. Similarly the expression of p62/SQSTM1 in the brain of intoxicated animals was mostly associated to astrocytes (Fabrizi et al., 2016) while less evident in microglia (present data). Although in vitro and in vivo data are in general difficult to compare, they are both indicative of a higher tendency of astrocytes to accumulate autophagosomes respect to microglia during an autophagic block which could be related to their high cytoplasmic/nuclear ratio.

The observed occurrence of an autophagic block during TMT intoxication in glial cells could be possibly due to the engulfment of autophagic vesicles with undigested materials which cells are unable to further process. In fact, only some microorganisms like bacteria (such as Pseudomonas) and phytoplankton have the ability to degrade organotin compounds under certain conditions (Hoch, 2001; Sampath et al., 2012). To note the inhibition of the autophagic flux and the p62/SQSTM1-dependent activation of the kep1-Nrf2 pathway has been reported to occur following the exposure to other toxic molecules, such as arsenic (Lau et al., 2013). Besides, the Nrf2/p62 signalling pathway is involved in the apoptosis resistance from cadmium exposure (Son et al., 2014).

During the past few decades an increased number of accidental poisoning in workers acutely exposed to methyltins was reported due to their wide industrial use (Tang et al., 2008). At the moment no effective treatment is available for these patients.

Recently, in rodents lithium was shown to ameliorate the symptoms of TMT intoxication. In fact, this cation can rescue seizures and ameliorate memory deficits in TMT-intoxicated rodents increasing at the same time the inhibitory phosphorylation of glycogen kinase synthase-3 (GSK-3)

in the hippocampus (Kim et al., 2013). Moreover, lithium enhances neuritogenesis ameliorating the depression-like behaviour observed in mice treated with TMT (Yoneyama et al., 2014).

During neurodegenerative diseases neuronal loss is always accompanied by the activation of the glia and in TMT-intoxicated brains activated microglial cells are present in affected brain areas such as the hippocampus (Koczyk and Oderfeld-Nowak, 2000). In these regions the expression of pro-inflammatory cytokines results upregulated (Bruccoleri and Harry, 2000) and chronic and exaggerated inflammatory response by microglia can be potentially detrimental for neurons further damaging brain tissue. Thus, a wide-range anti-inflammatory agents with different blood-brain barrier permeability are currently used in clinical trials for different neurodegenerative diseases in order to suppress pro-inflammatory cytokines production by chronically activated microglia (for a recent review see Perry and Holmes, 2014).

Our data are in line with previous reports showing a beneficial effect of lithium administration in TMT-intoxicated rodents (Kim et al., 2013; Yoneyama et al., 2014). In fact, we show that lithium at least in vitro limits the release of a pro-inflammatory mediator such as TNF-α potentiating at the same time the production of the anti-inflammatory cytokine IL-10.

Lithium mechanism of action is often difficult to assess for its multiple molecular targets which comprehend among others inositol monophosphatase (IMPase) and some related phosphomonoesterases, phosphoglucomutase and GSK-3 (O'Brien and Klein, 2009). In particular, lithium can inhibit GSK-3 both indirectly and directly competing with magnesium at its catalytic site; the Ki for GSK-3 inhibition at typical intracellular magnesium concentration is approximately 1.0 mM (Ryves and Harwood, 2001). GSK-3 is known to promote the inflammatory response (Martin et al., 2005) and lithium, such as other GSK-3 inhibitors, has been demonstrated to limit the production of inflammatory mediators in macrophages (Park et al., 2011), dendritic cells (Rodionova et al., 2007) and microglia (Huang et al., 2009; Yuskaitis and Jope, 2009).

Interestingly, in our microglial cultures lithium could display its pro-survival and immunomodulatory action without being able to overcome the autophagic block imposed by TMT.

This appears particularly relevant because this cation, alone or in combination with valproic acid, is considered a promising agent for treating neurodegenerative diseases (Chiu et al., 2013) and common features of these pathologies are protein misfolding, aggregation and deposition which are indicative of the possible failure of catabolic processes in general and of autophagy in particular (Vidal et al., 2014).

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FIGURE LEGENDS

Figure 1

TMT cytotoxicity in microglial primary cultures

A) LDH release in the culture medium of microglia treated with TMT for 24 and 48 hours.
B) Apoptotic cells labelled by TUNEL in microglial cultures treated with TMT or staurosporine (0.5 μM; positive control) at 3, 8 and 24 hours.

Averages ± SD of four independent experiments. ANOVA with Bonferroni's corrected t test. **p≤0.01, ***p≤0.001 versus untreated control.

Figure 2

Autophagic vesicles detected by MDC staining

Microglial cells were left untreated or treated for 24 hours with TMT alone, TMT+10 mM 3-MA or 0.5 μM rapamycin. Numerous MDC+ vesicles were detected in TMT-treated samples. These vesicles were not observed if TMT was added in combination with the autophagy inhibitor 3-MA. Results obtained using the autophagy inducer rapamycin are shown as positive control. Scale bar 20 μm.

Figure 3

LC3 turnover assay in TMT-treated microglia in the presence of a lysosomal inhibitor.

A) Western blot analysis of LC3-I/II conversion in microglia treated for 3 hours with TMT alone or in combination with 100 mM bafilomycin A1 (baf);
B) Densitometric analysis of western blot data shown in (A).

Averages ± SD of three independent experiments. ANOVA with Bonferroni's corrected t test. ***p≤0.001 versus untreated control; ns, not significant

Figure 4

Lithium protects microglial cells from TMT toxicity.

Microglia cells were treated with TMT alone or in combination with lithium (2 mM) for 48 hours. Cell viability was assessed by LDH release. Averages ± SD of four independent experiments. ANOVA with Bonferroni's corrected t test. **p≤0.01, ***p≤0.001 versus untreated control.

Figure 5

Immunomodulatory action of lithium in TMT-treated microglia.

Microglial cells were treated for 16 hours with TMT alone or in combination with lithium (0.5, 1, 2 mM) and the levels of TNF-α and IL-10 in the supernatants measured by ELISA. Averages ± SD of three independent experiments. ANOVA with Bonferroni's corrected t test. *p≤0.05, ***p≤0.001 versus TMT alone.

Figure 6

Lithium is ineffective in modifying LC3 and p62/SQSTM1 accumulation in TMT-treated microglia.

A) Western blot analysis of LC3-I/II conversion and p62/SQSTM1 expression in primary microglia treated for 24 hours with TMT alone or in combination with lithium (0.5, 1 and 2 mM). GAPDH is shown as loading reference control. The level of expression of LC3-I/II and p62/SQSTM1 in astrocytes treated for 24 hours with TMT is shown for comparison.
B) Densitometric analysis of western blot data shown in (A).

Averages ± SD of three independent experiments. ANOVA with Bonferroni's corrected t test. *p≤0.05, **p≤0.01 versus untreated control; ns, not significant versus TMT alone.

Figure 7

Detection of p62/SQSTM1 in brain slices of TMT intoxicated rats.

Immunofluorescence analysis of OX-42 and p62/SQSTM1 in the CA3/hilus hippocampal subfields from rats sacrificed 7 days after TMT intoxication or treated with saline only (ctrl). Nuclei are counterstained with DAPI. Scale bar=10 μm. A magnification of an $OX\text{-}42^{+}$ microglial cell present in TMT-intoxicated brain is shown on the right to better visualize p62/SQSTM1 localization; the p62/SQSTM1 staining is also present in many $OX\text{-}42^{-}$ cells (arrows).

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