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2024

DOI (link to publisher)
10.1016/j.tube.2024.102495

document version
Publisher’s PDF, also known as Version of record

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Marked IDO2 expression and activity related to autophagy and apoptosis in brain tissue of fatal tuberculous meningitis

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\textbf{ABSTRACT}

In about 1% of tuberculosis (TB) patients, \textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}) can disseminate to the meninges, causing tuberculous meningitis (TBM) with mortality rate up to 60%.

Chronic granulomatous inflammation (non-necrotizing and necrotizing) in the brain is the histological hallmark of TBM. The tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1) and the generated kynurenine metabolites exert major effector functions relevant to TB granuloma functioning. Here we have assessed immunohistochemically IDO1 expression and activity and its effector function and that of its isoform, IDO2, in post-mortem brain tissue of patients that demised with neurotuberculosis. We also related these findings to brain tissue of fatal/severe COVID-19. In this study, IDO1 and IDO2 were abundantly expressed and active in tuberculoid granulomas and were associated with the presence of \textit{M. tuberculosis}, as well as markers of autophagy and apoptosis. Like in fatal/severe COVID-19, IDO2 was also prominent in specific brain regions, such as the inferior olivary nucleus of medulla oblongata and cerebellum, but not associated with granulomas or with \textit{M. tuberculosis}. Spatially associated apoptosis was observed in TBM, whereas in fatal COVID-19 autophagy dominated. Together, our findings highlight IDO2 as a potentially relevant effector enzyme in TBM, which may relate to the symptomology of TBM.

\section{1. Introduction}

\textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}) causes tuberculosis (TB), which currently is the second most common fatal infectious disease after the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) \textsuperscript{[1]}. \textit{M. tuberculosis} primarily infects the lungs and can disseminate from the lungs to other organs. In the central nervous system, \textit{M. tuberculosis} can disseminate to the brain meninges causing tuberculous meningitis (TBM), which is the most lethal form of TB with a mortality rate of up to 60% \textsuperscript{[2]}. Symptoms in TBM range from a normal level of consciousness...
and no focal signs (grade I; Medical Research Council criteria) [3], lethargy or behavioral changes, meningeal irritation, or minor neurologic deficits (grade II), to stupor or coma, abnormal movements, or severe neurologic focal deficit (grade III) [4].

Recently, Zaharie et al. distinguished three types of granulomas in post-mortem TBM brains, predominantly in the leptomeninges and subarachnoid space: non-necrotizing cellular granuloma, necrotizing gummatous granuloma, and necrotizing ‘abscess’-type granuloma [2]. There were distinct differences between these types of granulomas histologically, and with respect to size and bacterial load. M. tuberculosis was found only in necrotizing granuloma, predominantly in necrotic areas. Inflammatory cells were present in granulomas but also dissociated from granulomas, i.e., both neutrophilic and lymphocytic inflammation in leptomeninges and intra-parenchymal border zones. IFN-γ was distributed intercellularly in granulomas with the highest concentration near the necrotic areas and the lowest concentration in the periphery. It was concluded that granulomatous inflammation was initiated in the leptomeninges and that the three types of granulomas were three different stages of the same pathological process. A mechanistic explanation that could reconcile the occurrence of these different types of granulomas and possibly symptomology of TBM, however, has still not been identified.

Popov et al. were the first to identify the presence of the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1) in granulomas [5] that contain intracellular bacteria like Listeria monocytogenes and M. tuberculosis. Based on earlier work by Mellor and Munn [6,7], Popov and colleagues suggested that IDO1 may contribute to the immune-privileged nature of granulomas by dampening innate and adaptive immune responses. Both the IDO1-mediated reduction of tryptophan and the generation of cytotoxic kynurenine metabolites can affect cell functioning in general and more specifically dampen immune responses [8]. IDO2 is an isoform of IDO1, but in contrast to IDO1 it is not induced by interferon and/or TNF-α, but rather by the aryl hydrocarbon receptor (AHR) [9,10]. AHR is a ligand-activated transcription factor, which surprisingly can be activated by the IDO-generated ligand kynurenine and downstream products like xanthurenic acid [23], kynurenic acid and cinnamic acid [11]. This harbors the risk of a positive feedback loop, which is prevented by the AHR repressor. Whereas IDO1 expression tends to be spatially associated with the infectious agent, i.e., where interferon is produced, IDO2 expression depends on a reduced activity of the AHR repressor and diffusion of AHR agonists. Therefore, IDO2-induced pathology may occur outside granulomas, as we have indeed observed in fatal COVID-19 [12]. Given the impact that IDO activity has on cell functioning, this led us to examine TBM brain tissue for expression of IDO1, IDO2, its metabolites, and the consequences of IDO activity, i.e., autophagy and apoptosis, in the three types of granulomas and outside granulomas. As granulomatous IDO activity has been implicated in controlling M. tuberculosis infection [13,14] (and according to our own unpublished findings), we also determined the location of the M. tuberculosis bacilli and the presence of extracellular DNA traps, a bacteria-capturing system [15].

2. Materials and methods

2.1. Ethics statement

The study was approved by the Health Research Ethics Committee of the Faculty of Health and Medical Sciences, Stellenbosch University, Cape Town, Western Cape, South Africa (ethics approval numbers S12/11/298 and N09/07/185). The post-mortem material was collected after a full autopsy was performed immediately after the patient’s demise. In South Africa, consent from relatives for a post-mortem autopsy has always been mandatory, including the period from 1975 to 2012 when the material for the present study was collected. Informed consent for the present study was waived by the Stellenbosch University Human Research Ethics Committee. The waiver of consent has been granted due to the difficulty in tracing the families of the deceased patients, as well as due to the scientific importance of the brain tissue material. Data analyses were done anonymously, using unique study numbers. The brain tissue blocks from fatal COVID-19 patients were collected and provided as part of the biobank protocol approved by the medical ethics committee of Amsterdam UMC [12].

2.2. Patient cohort

Brain tissue from seven patients from our historical TBM cohort (the total cohort consists of 84 patients; 439 brain blocks) were selected (see below). There were three adults, three children, and an additional TBM patient (who was older than 14 years of age). Whereas information on most patients was complete, for this last patient some data were missing (Table 1) [2]. Patients were selected from the overall cohort based on the following criteria: (1) ‘well formed’ granuloma; (2) medium to high bacillary load; and (3) a brain block suitable for serial sections. All patients had stage III TBM; the pediatric cases had disseminated TB with positive CSF cultures. The short time in hospital and on treatment reflected their disease severity upon admission. As described before, most TBM patients from our cohort showed three developmental stages in granuloma formation with the following types: non-necrotizing, necrotizing gummatous and necrotizing abscess-type granuloma [2]. The stains of brain sections from fatal COVID-19 patients (n = 3) in this study (Table 2), were also used previously [12,19].

2.3. Immunohistochemistry

Immunohistochemical analyses were performed on brain tissue from TBM patients. The brains of the deceased patients were fixed in formalin (10%) for a minimum of two weeks prior to dissection [2]. The antibodies used, their validation and stain procedures have been extensively described previously [12]. IDO activity was assessed by staining for quinolinic acid (QUIN) and 3-hydroxy-anthranilic acid (3OH-AA). Supplemental T1 provides an overview of the antibodies used in this study. Microphotographs were obtained using a microscope (Leica Microsystems), and scanned overviews of neighboring brain sections were digitized to obtain whole slide images using a Zeiss Axio Scan Z1 system (manufacturer) at 40× magnification, and the multiple staining pictures were processed and separated by QuPath software. Electron microscopy (EM) was performed as described previously [16].

3. Results

3.1. IDO1 and IDO2 expression in TBM brain

IDO1 was expressed pronouncedly in granulomas, in the leptomeninges (Fig. 1A–C) and mostly perivascular between the brain stem and the cerebellum (Fig. 1M). IDO1 expression in non-necrotizing granulomas (Fig. 1A–G and R; early- and mid-stage granulomas) was intense and in foci, whereas it was more dispersed in necrotizing granulomas (Fig. 1B, C and I; late-stage granulomas). IDO1 expression was not exclusively associated with granulomas and was also found in parenchyma (Fig. 1G) and, to a small extent, in the inferior olivary nuclei (Fig. 2B).

IDO2 expression paralleled that of granulomatous IDO1 expression (Fig. 1D–F, H–L). IDO2, however, was also abundantly expressed in specific areas of the brain free of granulomas, such as in the parenchyma (Fig. 1D–F), the choroid plexus (Fig. 1L), the inferior olive and the cerebellum (Fig. 2C–F). In the cerebellum, IDO2 expression was particularly high in Purkinje cells and in the granular layer (Fig. 2F).

3.2. Kynurenine metabolites in TBM brain

Both QUIN and 3OH-AA, two major products of the tryptophankynurenine pathway, were expressed abundantly in the cytoplasm of
We and others have shown that IDO1 and IDO2-expressing cells, but also in that of cells not expressing IDO. It is intriguing that only specific non-IDO-expressing cells and not neighboring cells stain for these products (see for example Figs. 1J and 2D). Furthermore, QUIN and 3OH-AA stains are found in granulomas, in neighboring blood vessels and in areas without granulomas (e.g., Fig. 1G), indicating that the effect of IDO activity may extend to various regions in the brain. Finally, QUIN and 3OH-AA stains do not necessarily overlap, but neither are they mutually exclusive.

3.3. AHR, apoptosis and autophagy in TBM brain

As in our previous report for fatal/sever COVID-19 [12], AHR localized in nuclei of IDO-2-expressing cells, indicative of its activation and its potential role in driving IDO2 expression (Fig. 3A–B). In that same study, IDO2 activity was spatially related to apoptosis (cleaved caspase-3) and autophagy (LC3B), which is shown here too for TBM and its potential role in driving IDO2 expression (Fig. 3A). AHR, apoptosis (Fig. 2G–H), whereas autophagy was more marked in the inferior olive. In contrast, autophagy as opposed to apoptosis was more present in the parenchyma (Fig. 3C–D) of TBM patients. In the inferior olive we found evidence for both autophagy and apoptosis (Fig. 2G–H), whereas autophagy was more marked in the inferior olive.

3.4. Presence of M. tuberculosis in brain tissue

Single and clustered M. tuberculosis were found in granulomas, particularly perivascular (Fig. 4A–C). In the choroid plexus (Fig. 4D), M. tuberculosis was detected covering the epithelial layer. M. tuberculosis was also observed in the parenchyma, in the superficial layers as well as deeper in the parenchyma (Fig. 4E–F). In the latter case, many aggregates of M. tuberculosis were seen. At higher magnification, M. tuberculosis was seen in clusters or dispersed within macrophages and neutrophils (Fig. 5B–D). The presence of M. tuberculosis was spatially associated with IDO1 and IDO2 expression in the early stage of granulomas as shown in consecutive sections (compare Figs. 4A and 1A and D, arrows). Based on the M. tuberculosis detection with Light Microscope (LM), a small region close to two blood vessels (Fig. 4D–I) was cut out and prepared for EM analysis. M. tuberculosis was found in the perivascular region and mostly intracerebral although a few bacteria were detected in between collagen and extracellular (Fig. 4J–O). Due to the LM fixation and chemical removal of the paraffin, the ultrastructure of the cells was lost but the M. tuberculosis bacilli retained much of their ultrastructure with the typical electron-lucent cell wall and morphology.

As M. tuberculosis clusters were present (Fig. 4L–M), sometimes with septum formation, it is likely that bacteria replicated in tissue (Fig. 4N–O) (see Fig. 6).

3.5. Extracellular DNA traps in TBM brain

As a marker of extracellular DNA traps, citrullinated histone H3 (CitrH3) was seen in non-necrotizing (Fig. 5E–F) as well as necrotizing granulomas (Fig. 5I–J). Neutrophils (MPO+) and macrophages or microglia (CD68+) were the predominant cell types associated with the extracellular DNA traps (Fig. 5E–F, I–J).

4. Discussion

TBM is characterized by mild to severe symptomology and a potential high risk for a fatal outcome. Here we have studied the expression and activity of IDO1 and IDO2. Both deplete cellular tryptophan and can lead to the generation of cyto/neurotoxic kynurenine metabolites, constituting a major effector mechanism and potentially affecting cellular functioning. We found that both IDO1 and IDO2 were expressed and active in brain tissues of fatal TBM patients. IDO1 was associated with all types of granulomas, predominantly perivascular in the leptomeninges and the subarachnoid space (Fig. 5E–F). In contrast, autophagy as opposed to apoptosis was more present in the parenchyma (Fig. 3C–D) of TBM patients. In the inferior olive we found evidence for both autophagy and apoptosis (Fig. 2G–H), whereas autophagy was more marked in the inferior olive.
particularly perivascular and less in the parenchyma, but also bound to the epithelial layer in the choroid plexus. The presence of extracellular DNA traps, which are known to trap bacteria, may contribute to the containment of bacteria in granulomas, but its presence did not appear to prevent its spread into the parenchyma.

The strength of the current qualitative immunohistochemical study lies in the carefully characterized bio-banked brain tissues, and the combination of detecting both IDO enzymes, AHR, their products, and their potential effects, which allow us to propose the likely underlying pathophysiological process. The antibodies, apart from that for IDO2, have been validated by others. Separately, we confirmed specificity of the antibody to IDO2 [12]. IDO activity is often considered in the context of immune responses only, but there is no reason to assume why the depletion of tryptophan and generation of cyto/neurotoxic

Fig. 1. Immunohistochemistry staining of IDO1, IDO2 and products of kynurenine metabolites in autopsy brain tissues from TBM patients. A-C: Double IHC staining of IDO1 (red) combined with quinolinic acid (QUIN, blue). D-F: Double IHC staining of IDO2 (red) combined with 3-hydroxy-anthranilic acid (3OH-AA, blue). A and D show early-stage granulomas (small size). B-C and E-F show the late-stage granulomas (large size). G-L show mid-stage granulomas (middle size). M and N: overview staining of the slide. Representative images of brain tissue from 6 patients with TBM. G-H: are higher magnifications from M and N. K-L: Choroid plexus co-stained with IDO1/QUIN and IDO2/3OHAA. Magnifications are indicated in the lower right corner. Arrows show different stage of granulomas. Abbreviations: B = blood vessel, BS = brainstem, CB = cerebellum, LM = leptomeninges, N = necrosis, SS = subarachnoid space.
kynurenine metabolites would not affect non-immune cells. In our study into past-acute sequela of SARS-CoV-2 infection we showed that IDO2-expressing peripheral blood mononuclear cells showed reduced levels of amino acids besides tryptophan [24]. This likely is due to kynurenine metabolites that halt uptake of various amino acids. Together this may yield a signal to cells for autophagy. In addition, depletion of tryptophan and other amino acids may attenuate the synthesis of specific hormones such as serotonin. Reduced levels of serotonin have been associated with certain neuropsychiatric symptoms like depression and anxiety, abnormal sleep patterns and neuroinflammation. In fatal/severe COVID-19, however, we did not find reduced serotonin levels in plasma and CFS despite the abundant presence of active IDO2. A possible explanation may relate to a publication by Pantouris et al. [25], showing that not tryptophan, but an adduct of tryptophan may be the preferred substrate of IDO2. IDO2 activity triggers the production of kynurenine metabolites, some of which are neurotoxic, such as quinolinic acid. Indeed, we have shown that quinolinic acid is associated with IDO2 expression and this may explain apoptosis. Although the co-localization of IDO2 expression and activity together with autophagy and apoptosis markers strongly argues for a direct effect of IDO activity on these cells, this remains to be shown. In a recent study of post-acute sequelae of SARS-CoV-2 infection, however, we showed that inhibiting IDO2 expression in peripheral blood mononuclear cells (PBMC) with an AHR antagonist in vitro, also affected autophagy, thus confirming the relation between IDO activity and autophagy [24]. Together our findings point to an earlier unrecognized IDO2-driven autophagy and apoptosis in TBM, which may affect the functioning of specific cells in the brain and relate to TBM symptomology. As IDO2 expression is likely to be induced by the AHR ligands generated by the kynurenine pathway [11] resulting from IDO1 activity, IDO2 expression may reflect a later stage of TBM. It may be worthwhile to determine whether IDO2 expression in TBM can also be visualized in PBMC, as an indicator of IDO2 expression in the brain, as suggested by our findings in patients with post-acute sequela of SARS-CoV-2 infection.

We intended to qualitatively relate the various recognized types of granulomas to immunohistochemical (IHC) markers. IDO1 is expressed in all types of granulomas, but IDO2 expression appears more abundant.
in necrotic granulomas. As argued above, and in line with the proposed developmental states of granulomas, IDO2 expression may be associated with aged granulomas. Both IDO1 and IDO2 break down tryptophan to kynurenine and consequently we found no difference regarding kynurenine metabolites between the two. Chowdhury et al. [17] have shown that NETosis, a mechanism for formation of neutrophil extracellular DNA traps (NETs), is associated with disease severity in TB-susceptible C3HeB/FeJ mice. They demonstrated that NETs are present in necrotic lung granulomas, responding poorly to antibiotic therapy due to reduced drug permeability of granulomas. Other studies showed that the NETosis pathway plays a critical role in the course of TB progression in the human lung [15,18]. In our study, NETs were found in all types of granulomas. Bacteria, however, were not associated with the NETs, even though M. tuberculosis was also found extracellularly by EM. The presence of bacteria in all granulomas, shown both by immunohistochemistry and EM, and the association of IDO with apoptosis markers were unexpected as these do not correspond with earlier findings [2]. There we reported more granulomas than in the current study and thus our findings may have been biased by a non-representative sample. We have no explanation for the difference in apoptosis.

Fig. 3. AHR, apoptosis and autophagy in granulomas of TBM brains. A-B: IDO2 (red) and AHR (blue) Immunohistochemistry double staining (arrows). C-D: IDO2 (red, arrows) and LC3b (blue, arrows) immunohistochemistry double staining. E-F: M. tuberculosis (red), MPO (blue) and Cas3 (brown, arrows) immunohistochemistry triple staining. G-H: MPO (red), CD68 (brown) and CitH3 (blue, arrows) immunohistochemistry triple staining. These photographs are representative of 4 patients, although in one patient we found more apoptosis than autophagy in the parenchyma. Abbreviations: B = blood vessel, LM = leptomeninges, N = necrosis, SB = subarachnoid.
Fig. 4. Immunohistochemical staining and transmission EM of *M. tuberculosis* in TBM brains. A: Aggregated *M. tuberculosis* in initial perivascular granuloma. B-C: Separate and aggregated *M. tuberculosis* in perivascular tissue. D: *M. tuberculosis* in choroid plexus; E-F: *M. tuberculosis* was also observed in the parenchyma, both close to border as well as deeper in the parenchyma. G-H: *M. tuberculosis* in necrotic granulomas. Arrow pointed out *M. tuberculosis* (red). Representative images of brain tissue from six patients with TBM. I: Area that was used to perform transmission EM; J-O: Transmission EM shows *M. tuberculosis* in TBM, J-K: Single *M. tuberculosis*, L-M: Clustered *M. tuberculosis*, N-O: Septum formation of replication of *M. tuberculosis* (red, arrow). Representative images of brain tissue from 2 patients with TBM. Abbreviations: B = blood vessel, LM = leptomeninges, N = necrosis.
IDO2 is expressed in both TBM and fatal/severe COVID-19 brain tissues, although it appears more pronounced in TBM than in fatal COVID-19. It is clear, however, that IDO2 expression is limited to certain areas in the brain and that these areas are similar for TBM and fatal COVID-19. As many IDO2-positive cells in brain nuclei showed evidence for autophagy, some areas in the brain were more sensitive to developing IDO2-mediated autophagy than other areas. It is as yet not clear why IDO2 expression in the brain manifests itself as it does. IDO2 expression does not co-localize with the infectious agent and so it appears to be induced solely via the AHR pathway. As virtually every viral infection leads to interferon production and trigger IDO1 expression and kynurenine production, that in itself is unlikely to result in IDO2 expression. A possible explanation is that polymorphism for the genes in the kynurenine pathway may lead to potent AHR agonists and thus IDO2 expression [20].

Our findings relate to two recent reports by van Laarhoven et al. [21] and by Ardiansyah et al. [22] in which tryptophan concentrations and its catabolism were linked to TBM pathology. Compared to non-infected controls, low tryptophan and high kynurenine CSF levels were associated with mortality in TBM. In apparent contradiction, however, in surviving TBM patients, tryptophan CSF levels were lower than in fatal TBM. Having lower levels of tryptophan may limit the production of downstream kynurenine metabolites, which we have shown to correlate spatially with autophagy and apoptosis.

There are some limitations to the current study. Most importantly, only qualitative immunohistochemical data have been provided and no quantification has been done. This limited the conclusions that could be drawn regarding the role of IDO activity on various types of granulomas and a more accurate reflection of the co-occurrence of apoptosis and autophagy. To generate quantitative data, more sections need to be studied. To follow up, we plan to set up a new study that will also assess kynurenine metabolites in plasma and CSF, and perform IDO2 stains in

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Fig. 5. Neutrophilic extracellular DNA traps and M. tuberculosis in TBM brains. Citrullinated histone H3 (CitH3) as a marker of extracellular DNA traps was seen in non-necrotizing granulomas. CitH3 was associated with MPO (neutrophil granular product) and not with CD68 (monocytes), and not with M. tuberculosis. Fig. 5 1 shows early-stage granulomas. A-B: M. tuberculosis (red arrows), C-F: Spatially correlated among M. tuberculosis (red, arrows), MPO (C-D, blue, E-F: red), CD68 (E-F and P, brown), Cas3 (C-D, brown, arrows) and E-F and O: CitH3 (blue, arrows). Fig. 5 2 shows late-stage granulomas, G-H: Triple staining of M. tub, MPO and Cas3, K and L: Singal stain of M. tub (red) and Cas3 (brown) separated from G-H by QuPath. I-J: Triple staining of MPO CitH3 and CD68, M-N: Single stian of CitH3 (blue) and CD68 separated from I-J by QuPath. These photographs are representative of six patients. Abbreviations: B = blood vessel, LM = leptomeninges, N = necrosis.
PBMCs as a potential diagnostic factor to identify advanced TBM. This approach may yield a more integrated view of IDO activity in TBM and possibly allow us to link this to symptomology. Another limitation is, that we have not been able to compare more specific areas in the brain of TBM and fatal COVID-19 cases, which would allow us to better point out which areas are affected by IDO2 activity.

In summary, we showed that IDO2 is expressed and active in granulomatous, but also non-granulomatous tissues in TBM, which appears to affect cellular metabolism and thus cell functioning. Comparison with our earlier findings in fatal COVID-19 indicates that IDO2 expression and activity occurs in specific areas only. This warrants further studies into the role of IDO activity and its metabolites and their effect on symptomology and interventions.

Funding
None.

CRediT authorship contribution statement

Lihui Guo: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Investigation, Formal analysis, Data curation, Conceptualization. Stefan-Dan Zaharie: Writing – review & editing, Writing – original draft, Validation, Resources, Conceptualization. A. Marceline van Furth: Writing – review & editing, Writing – original draft, Supervision, Resources. Nicole N. van der Wel: Writing – review & editing, Writing – original draft, Validation, Methodology. Marianna Bugiani: Validation, Resources. Mariana Kruger: Writing – review & editing, Writing – original draft, Validation, Methodology. Martijn van der Kuip: Writing – review & editing, Writing – original draft, Validation, Supervision. René Lutter: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This study was funded by the researchers involved. The authors acknowledge the generous donation of an antibody recognizing human IDO1 by Prof. Benoit van den Eynde (Ludwig Institute for Cancer Research, Université Catholique de Louvain, Brussels, Belgium). Walter Liebrich is kindly acknowledged for proofreading the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2024.102495.

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Fig. 6. Graphic abstract of the IDO-tryptophan-aryl hydrocarbon receptor axis in a granuloma and in specific areas of the parenchyma in the brain of TBM patients. CB = cerebellum; BS = brain stem.


