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Tranexamic Acid Modulates the Cellular Immune Profile after Traumatic Brain Injury in Mice without Hyperfibrinolysis

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Essentials

- We used a model of traumatic brain injury that induces local, but not systemic fibrinolysis
- Plasmin suppresses immune activation in the cervical lymph nodes after traumatic brain injury
- Tranexamic acid facilitates a more robust immune activation after traumatic brain injury
- However, tranexamic acid does not enhance self-reactivity post-traumatic brain injury
-

Abstract

Background: Traumatic brain injury (TBI) is known to promote immunosuppression, making patients more susceptible to infection, yet potentially exerting protective effects by inhibiting central nervous system (CNS) reactivity. Plasmin, the effector protease of the fibrinolytic system, is now recognised for its involvement in modulating immune function.

Objective: To evaluate the effects of plasmin and tranexamic acid (TXA) on the immune response in wild-type and plasminogen-deficient ($plg^{-/-}$) mice subjected to TBI.

Methods: Leukocyte subsets in lymph nodes and the brain in mice post-TBI were evaluated by flow cytometry and in blood with a haemocytometer. Immune responsiveness to CNS antigens was determined by ELISPOT. Fibrinolysis was determined by thromboelastography, and measuring d-dimer and plasmin-antiplasmin complex levels.

Results: $Plg^{-/-}$ mice, but not $plg^{+/+}$ mice displayed increases in both the number and activation of various antigen-presenting cells and T cells in the cLN 1 week post-TBI. Wild-type mice treated with TXA also displayed increased cellularity of the cLN 1 week post-TBI together with increases in innate and adaptive immune cells. These changes occurred despite the absence of systemic hyperfibrinolysis or coagulopathy in this model of TBI. Importantly, neither plg deficiency nor TXA treatment enhanced the auto-reactivity within the CNS.

Conclusion: In the absence of systemic hyperfibrinolysis, plasmin deficiency or blockade with TXA increases migration and proliferation of conventional DCs and various antigen-presenting cells and T cells in the draining cLN post-TBI. TXA might also be clinically

beneficial in modulating the inflammatory and immune response after TBI, but without promoting CNS auto-reactivity.

Abbreviations

CCI: controlled cortical impact, cDC: conventional dendritic cells, cLN: cervical lymph nodes, CNS: central nervous system, CRASH-2: Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage 2, i.p.: intraperitoneal, ingLN: inguinal lymph nodes, i.v.: intravenous, MDSC: myeloid-derived suppressor cells, MOG: myelin oligodendrocyte glycoprotein, MRI: magnetic resonance imaging, PAP: plasmin-antiplasmin complex, pDC: plasmacytoid dendritic cells, $plg^{+/+}$: plasminogen wild-type, $plg^{-/-}$: plasminogen-deficient, TBI: traumatic brain injury, TEG: thromboelastography, t-PA: tissue-type plasminogen activator, Treg: regulatory T cells, TXA: tranexamic acid

Introduction

TBI is the leading cause of death in young adults in the industrialised world.[1] After surviving the first insult, patients are at risk of developing life-threatening complications, including severe infection and bleeding.[2, 3] In addition to the exposure to lines, catheters and devices, the susceptibility for hospital acquired infections is further enhanced by a condition called "CNS injury-induced immune deficiency syndrome",[3] more recently regarded as an important contributor to morbidity and mortality after TBI.[4]

Plasmin, the effector protease of the fibrinolytic system, is generated in blood from its precursor, plasminogen following activation primarily by tissue-type plasminogen activator (t-PA). This occurs optimally on the fibrin surface where fibrin itself provides co-factor activity to enhance t-PA-mediated plasminogen activation over 500-fold. Since misfolded proteins provide the same co-factor activity as fibrin [5, 6], plasmin formation can readily occur in the absence of a fibrin clot and particularly in locations where cell injury and death occurs. Plasmin therefore has become recognised for its broader involvement in

physiological and pathophysiological processes that is at least in part mediated by various plasminogen receptors on leukocytes and tissue bound cells.[7] Plasmin can also modulate the immune system on several levels[8]. It has been implicated in inflammatory processes by direct interaction with pro- and anti-inflammatory mediators as well as various leukocyte subsets, by binding to cell-surface plasminogen receptors and inducing intracellular signalling.[9] In contrast, monocyte-derived dendritic cells treated with plasmin exhibit enhanced phagocytic capacity, yet a reduced ability to migrate to the draining lymph nodes and to mount an allogeneic immune response.[10]

Severe trauma can cause hyperfibrinolysis that contributes to acute traumatic coagulopathy, a condition that occurs early after injury and is associated with severe bleeding.[11, 12] Plasmin therefore, in the absence or presence of hyperfibrinolysis, may also have non-fibrinolytic consequences on the immune and inflammatory response that until now has received little attention. The antifibrinolytic agent TXA, has recently been demonstrated to improve survival in bleeding trauma patients if administered within 3h after injury.[13, 14] However, a nested trial within the ”Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage 2” (CRASH-2) trial on intracranial haemorrhage was not conclusive[15] and large randomised controlled trials are currently assessing TXA treatment in TBI [16] and in other indications. (see [17]) Recent reports have speculated that TXA treatment may have unintended consequences on the immune system in addition to its intended anti-fibrinolytic use.[7, 18, 19]. TXA has been implemented in resuscitation guidelines for major trauma around the globe,[20] yet guidelines for its use in isolated TBI are still missing. While clinical trials addressing this important issue are underway, an improved understanding of the off-target effects of TXA is necessary to better interpret clinical outcome data and to facilitate optimal patient selection for this treatment.

In this study, we investigated the role of endogenous plasmin at modulating the immune response after TBI in mice.

Materials and Methods

Animals

Sex and age matched (8-13 week) $Plg^{+/+}$ and $plg^{-/-}$ mice were derived from $plg^{+/-}$ colonies maintained at AMREP Animal Service (AS) or the Monash Animal Research Platform. Male C57/Bl6 wild-type mice (8 weeks; 20-24g) were provided by the Animal Research Council. All experiments were approved by the AMREP AS Animal Ethics Committee in accordance with the Australian code for the care and use of animals.

Controlled Cortical Impact (CCI) model of TBI

The CCI model of TBI was performed as described.[21] For sham procedure, only anaesthesia and the scalp incision, without craniotomy (given the previously identified effect of craniotomy alone on gait impairment)[22] and TBI was performed, similar to the recent report by Ritzel et al.[23]

Administration of TXA or vehicle

TXA was administered to mice twice daily over three days following TBI. 20 min after induction of TBI or sham procedure, mice were injected intravenously (i.v.) with TXA (Pfizer, USA) (100 mg/kg; 90-140 µl) or an equivalent volume of vehicle (0.9% sodium chloride). 6 h later, mice were injected intraperitoneally (i.p.) with TXA or vehicle. On days 2 and 3, mice were again injected with TXA i.p. twice daily (8 h apart). The dose used for TXA (100 mg/kg) is equivalent to that used in surgery.[24] This repeated administration was performed to ensure persistent inhibition of lysine-dependent plasmin formation over this period. Treatment commenced 20 minutes post-injury to mimic the earliest possible treatment scenario after trauma.

Thromboelastography (TEG)

TEG was performed using a TEG 5000 Thrombelastograph (Haemonetics, USA). ~600 µl blood was drawn from the inferior vena cava of C57/Bl6 mice under terminal anaesthesia without addition of pro- or anticoagulants, using a 1 ml syringe with a 21G needle. Blood was transferred into an Eppendorf tube and 360 µl of whole blood were then transferred to the plain TEG cup for analysis. Alpha-angle, maximum amplitude and LY30 were assessed at the 1 h and 3 h time points.

Blood leukocyte profiling

Differential white blood cell count was assessed using citrated blood samples with the Hemavet 950FS analyser (Drew Scientific, USA).

Preparation of lymph nodes and brain tissue for flowcytometry

Flow cytometry of cervical lymph nodes (cLN) was performed 72 h and 1 week after TBI or sham procedure. Flow cytometry of inguinal (ing) LN and the injured brain hemisphere were performed at the 1 week time point post-TBI. We were interested in identifying and phenotyping myeloid and lymphoid cell populations, with a main focus on dendritic cells and T cell subsets. Neutrophils, macrophages, monocytes, NK cells and B cells were also assessed with our staining protocol (see **Supplementary file** for details).

Plasmin-antiplasmin (PAP) complex ELISA

Citrated plasma and brain lysates (150 mg/ml) were evaluated for PAP complex levels using an ELISA [25] as described in the **Supplementary file.** Antibodies used and the gating strategy are outlined in **Suppl. Table 1 and 2** as well as **Suppl. Figure 1 and 2**.

D-dimer ELISA

Brain lysates (150mg/ml) were assessed for D-dimer levels with an ELISA (LifeSpan BioSciences, USA) using 50 µL of sample (at a 1:2 dilution. The optical density (OD; 450nm) of each sample was assessed using the FLUOstar Optima microplate reader (BMG Labtech, Germany).

Albumin ELISA

Brain lysates (150mg/ml) were evaluated for albumin levels using a mouse albumin ELISA Quantitation Set (Bethyl Laboratories, USA).

Bicinchoninic acid assay (BCA) assay

Total protein content of brain lysates was measured using the BCA™ protein assay (Pierce, USA).

Anti-MOG antibody ELISA and ELISpot assays

Methods details are provided in the **supplementary file.** As a positive control, serum samples from an experimental autoimmune encephalomyelitis (EAE) mouse model, known

for increased levels of anti-MOG antibodies[26] were used. Optimisation of the ELISpot assay was also performed using cells from mice subjected to EAE [26].

Lectin staining, Magnetic resonance imaging and Behavioural testing

Methods are provided in the **supplementary file.**

Statistics

Statistical analysis was performed with GraphPad Prism (GraphPad Software, Inc., USA). Outliers were identified using a ROUT outlier test (Q=1%) and removed from analysis. An unpaired two-tailed Student's t-test was used when 2 groups were compared. A maximum of 2 outliers were removed from any analysis. Comparisons of more than 2 groups were performed with a one-way ANOVA and Dunnett's correction test. For comparison of 4 groups differing in two factors, a two-way ANOVA and Tukey's correction test for multiple comparisons was used. Probability values < 0.05 were considered significant.

Results

The CCI-induced model of TBI does not induce coagulopathy or hyperfibrinolysis

TEG was performed on whole blood from C57/Bl6 wild-type mice 1 h or 3 h after sham procedure or TBI. There were no changes in any of the key parameters of the TEG assay including α-angle, maximum amplitude, or the LY30 (**Table 1**), suggesting that this model of TBI does not induce coagulopathy. Lysis was undetectable in both sham and TBI animals measured 30 min after reaching maximum amplitude (LY30) and still after 60 min (LY60) (not shown).

Plasminogen deficiency significantly alters the cellular immune profile 1 week post-TBI

Although the TEG assay provided no evidence for any significant increase in fibrinolysis in this model of TBI, we tested the idea that plasmin generation was indeed increased, perhaps locally, that would not be detected by the TEG approach, a situation that was recently described in a cohort of trauma patients.[27] We first evaluated the role of plasmin at modulating the systemic and local cellular immune profile 1 week post-TBI using $plg^{-/-}$ mice. Full blood evaluation revealed a significant increase in numbers of circulating neutrophils in $plg^{-/-}$ but not $plg^{+/+}$ mice 1 week after TBI, but not in lymphocytes or monocytes **(Fig.1A)**.

Profound changes were also seen in the draining cLN in $plg^{-/-}$ mice at the 1 week time point post-TBI. Total numbers of CD8+ conventional dendritic cells (cDC) **(Fig.1Bi)** including the migratory CD103+ CD8+ cDC **(Fig.1Bii)** and resident CD11b+ cDC **(Fig.1Biv)** were all significantly elevated in the cLN post-TBI in $plg^{-/-}$ mice, but not in $plg^{+/+}$ mice. CD8+ cDC also showed significantly enhanced expression of the activation marker CD80 **(Fig.1Biii)** and CD11b+ cDC of CD80 **(Fig.1Bv)** and CD86 **(Fig.1Bvi)** in $plg^{-/-}$, but not in $plg^{+/+}$ mice. Also numbers of CD4+ **(Fig.1Ci)** and CD8+ **(Fig.1Cii)** T cells as well as B cells **(Fig.1Ciii)** were significantly increased in the cLN 1 week after TBI in $plg^{-/-}$mice. No differences were observed in the frequencies of naïve, memory, or effector T cell subsets within CD4+ and CD8+ T cell.

Hence, plasminogen deficiency results in a delayed yet significant increase in migratory and activated resident cDCs and T-cells in the cLN together with an increased blood neutrophil count in mice subjected to TBI.

TBI induces an immune response against CNS antigens, but this is not influenced by plasminogen deficiency

To test the hypothesis that the increases in the immune cell profile in $plg^{-/-}$ mice after TBI resulted in enhanced immune reactivity against CNS antigens, an anti-MOG antibody ELISA and ELISpot analyses were performed. For the latter cLN cells were cultured 2 weeks after sham or TBI surgery and treated with the CNS antigen, MOG, and the number of IFN-γ producing cells determined. The 2 week time point was selected to permit time for the adaptive immune response to respond.

Serum anti-MOG antibody levels were significantly increased 2 weeks post-TBI indicating a specific immune response against MOG in our TBI model. However, no difference was observed in the extent of this increase between $plg^{+/+}$ and $plg^{-/-}$ groups ($p<0.05$) **(Suppl. figure 3A)**. Treatment of cLN cells with MOG (50 or 100 µg/ml) also resulted in an increase in IFN-γ producing cells, however, without any apparent differences between groups (sham/TBI) and treatments (vehicle/TXA) **(Suppl. figure 3B).**

TXA inhibits local plasmin formation and fibrinolysis

We next determined whether treatment of wild-type mice with TXA subjected to TBI would induce effects similar to that seen in $plg^{-/-}$ mice.

We first determined whether plasmin generation and activity in the injured brain was affected by TXA treatment. TBI resulted in increased brain levels of the plasmin-antiplasmin (PAP) complex, representing local plasmin formation **(Fig.2Ai)** at the 3h time point. Plasma PAP levels were unaffected by TBI at 3h **(Fig.2Aii)** but we note that any decrease in levels cannot be determined at this time point due to the PAP complex half life being ~12h. Accordingly, we were able to confirm in a separate experiment that TXA was still promoting an anti-fibrinolytic effect during this time frame as baseline PAP levels were significantly reduced in TXA treated mice 24h after a single intravenous administration (n=10; data not shown).

Further, at 3h post-TBI, the ratio of PAP complex levels in the brain relative to plasma was reduced in traumatised mice treated with TXA, suggesting local inhibition of plasmin formation had occurred **(Fig.2Aiii)**. This was accompanied by a significant reduction of the fibrin breakdown product D-dimer **(Fig.2B), further** indicating inhibition of fibrinolysis in the brain by TXA. Moreover, the lack of correlation of brain PAP complex or D-dimer levels with brain albumin levels (as an index of extravasation) in the TBI groups (r=0.276, p=0.239, n=20; and r=0.272, p=0.274, n=18, respectively) further confirms that the impact of TXA on PAP and D-dimer levels is due to inhibition of local plasmin formation and activity rather than the extravasation of those markers through the compromised blood-brain barrier. Furthermore, the strong correlation between PAP complex and D-dimer levels (r=0.655, p=0.003, n=18) indicates that the reduced PAP ratio described in TXA-treated TBI animals is indeed based on local inhibition of plasmin formation (preceding fibrinolysis) rather than inhibited binding of α2-antiplasmin to plasmin[28] Hence, even though systemic hyperfibrinolysis does not occur in this model of TBI (as determined by TEG; Table 1), local fibrinolysis i.e. increased plasmin generation and d-dimer levels is observed in the damaged brain.

TXA impacts on the local immune response following TBI

We next determined whether prolonged TXA treatment in wild-type mice would alter the immune response after TBI. C57/Bl6 mice were subjected to TBI and injected with TXA 20

min post-injury, and reinjected with TXA i.p. over a 3 day period. cLN cellularity was significantly enhanced in TXA-treated animals, but not vehicle-treated mice (**Fig.3Ai)**. Specifically, macrophages, monocytes, CD11b+ cDC as well as CD4+ and CD8+ T cells, and NK cells were increased after TXA treatment **(Fig.3Aii)**. Similar to our experiments on $plg^{-/-}$ mice, no differences in the frequencies of naïve, memory, or effector T cell subsets within CD4+ and CD8+ T cell were seen in mice treated with TXA. Both vehicle and TXA-treated mice showed similar increases in levels of anti-MOG antibodies ($p<0.01$) 1 week after TBI (data not shown). Interestingly, cLN cellularity was not altered at the 72 h time point post-TBI (not shown) indicating that the TXA-mediated increase in cLN cellularity occurs between 3 and 7 days post-injury.

We next evaluated whether the increase in cLN cellularity in TXA-treated mice was a consequence of active proliferation by determining expression of the intracellular marker Ki-67, a marker of recent proliferation. One week post-TBI, a significant increase in Ki-67+ macrophages was observed as well as a trend towards increased levels of Ki-67+ B cells and NK cells **(Suppl. figure 4A)**. This response was unchanged by TXA **(Suppl. figure 4B)**. Since Ki-67 expression has been reported to be a dynamic process rather than to represent a binary parameter,[29] we also looked at the expression intensity of this marker within cells. CD11b+ cDC in the cLN of TXA-treated animals showed significantly enhanced expression of Ki-67 1 week post-TBI compared to vehicle-treated mice **(Fig.3B)**. Hence it appears that the increase in lymph node cellularity due to plasmin deficiency or blockade involves increases in both cell migration and proliferation.

TXA treatment does not enhance reactivity of cLN cells to CNS antigens

Both vehicle and TXA-treated mice showed a similar increase in levels of anti-MOG antibodies 2 weeks after TBI **(Suppl. figure 5A)**. ELISpot analyses were performed as above. In all experimental groups incubation with MOG at either 50 μg/ml or 100 μg/ml resulted in a significant increase in the number of IFN-γ producing cells at the 2 week time point, with no differences in effect size between concentrations or groups **(Suppl. figure 5B)**. Also, MOG-reactivity of splenocytes was unchanged between groups (not shown). Hence, TXA

does not enhance the reactivity to the CNS antigen MOG, despite causing significant increases in cLN cellularity.

TXA influences the cellular immune profile of peripheral lymph nodes

We next determined whether the observed immune changes in the cLN also occurred in peripherally, specifically in the inguinal lymph node (ingLN). TBI did not result in a significant change in ingLN cellularity in vehicle- or TXA-treated mice **(Fig.4A).** Note that these data show cellularity relative to the sham control group. However, TXA treatment resulted in a relative increase in several myeloid (macrophages, monocytes, CD11b+ cDC) and lymphoid cells (CD4+ and CD8+ T cells, NK cells) from sham level **(Fig.4B)** 1 week post-TBI.

TBI promotes activation and migration of microglia and macrophages to the lesion – inhibitory effects of TXA

Lectin staining was performed at various time points post-TBI (n=3) to assess activation and infiltration of microglia and macrophages to the lesion. The optimal time point that discriminated lectin positivity was found to occur at 7 days and a representative image is shown **(Fig.5A)**. Lectin positive areas within the injured hemisphere were significantly enhanced between 72 h and 28 days post-TBI **(Fig.5Bi)**. The degree of lectin positivity gradually declined up to day 28 post-TBI, albeit still being significantly enhanced compared with sham. Normalised to the 72 h time point which showed the most lectin positive staining after TBI, there was a significant reduction in lectin positive cells in TXA-treated animals at the 1 week time point **(Fig.5Bii)**. Hence TXA effectively reduced the inflammatory response i.e. the extent of microglial and macrophage activation (i.e. reduced expression of CD80 and CD86) in the lesion after TBI **(Fig.5C)**, coincident with TXA promoting the innate immune response reflected by increased the proportion of dendritic cells and T cells in the cLN at this time point post-TBI **(Fig.3Ai)**.

Having identified that day 7 post-TBI was the optimal time point, we next performed flowcytometry of the injured (ipsilateral) hemisphere of the brain at this defined time point post-TBI to further characterise the effects of TXA on inflammation at the site of injury. TBI

resulted in a significant increase in macrophages and inflammatory DCs compared with sham as well as a trend towards increase of resident and inflammatory microglia and monocytes **(Suppl. Figure 6A)**. Numbers of B cells, T cells, NKT cells, CD8+ and CD11b+ cDC were not affected by TBI at the 1 week time point (not shown).

Also, despite the fact that TXA reduced microglial and macrophage activation (Fig 5C), TXA did not significantly affect the total number of these cells after TBI **(Suppl. Figure 6B)**

TXA has no effect on lesion size and behavioural outcome

To determine whether the immune changes induced by TXA could be explained by effects on neuronal injury, we performed *ex vivo* MRI volumetric analyses on the brains of traumatised mice treated with vehicle or TXA. The volume of all assessed structures (cortex, corpus callosum and hippocampus) was significantly reduced in the injured (ipsilateral) hemisphere compared with the uninjured (contralateral) hemisphere ($p<0.0001$); however lesion volume was not affected by TXA treatment 1 week post-TBI **(Fig.6A and B)**. We also evaluated behavioural changes on days 2, 4 and 6 post-TBI with an open field box test. TBI resulted in significant increases in distance travelled, mean speed and rotations during the test run on day 4 post-TBI **(Suppl. Figure 7Ai-iii)**, indicating a state of agitation. However, TXA did not further alter this response **(Suppl. Figure 7Ai-iii)**.

Discussion

Plasmin has been described as a promoter of inflammation,[9] yet recent findings suggest a more complex role in the modulation of immunity, including its ability to induce a tolerogenic phenotype to dendritic cells by reducing their capacity to induce an allogeneic immune response.[10] Curiously, studies using plasminogen-deficient mice have reported both reduced or exacerbated inflammatory responses, depending on the disease model: plasmin(ogen) deficiency can be deleterious in models of wound healing,[30, 31] yet protective in models of LPS and EAE-induced neuroinflammation[32, 33] indicating that plasminogen is needed to exert a complete inflammatory response.

Our objective was to determine the role of the fibrinolytic system at modulating the immune response in a mouse model of isolated TBI. Coagulopathy is known to occur in patients with severe TBI; the lack of TBI-induced hyperfibrinolysis in this model, indicated by

TEG is most likely a reflection of injury severity and the nature of the injury caused by the CCI model.[34] Clinically relevant hyperfibrinolysis has been defined as an LY30 of 3% in trauma patients with uncontrolled haemorrhage.[35] In our model there was no evidence of hyperfibrinolysis at 30 min and 60 min after maximum amplitude was reached in the TEG assay (LY30 and LY60, respectively). We are aware that TEG is not the most ideal method for determining clinical hyperfibrinolysis, but our data clearly shows that within the defined parameters of the assay, there was no detectable increase in systemic fibrinolysis in our mouse model. This enabled us to evaluate the influence of plasmin in the local immune response after neurotrauma in the absence of clinically defined systemic hyperfibrinolysis.

An increase in local t-PA levels early after TBI[21, 36] together with the concomitant blood brain barrier (BBB) damage[7] that can result in extravasation of blood-derived plasminogen into the brain, raises the possibility that local plasmin formation can indeed occur in isolated TBI even in the absence of systemic hyperfibrinolysis. The physical trauma with ensuing bleeding and coagulation provides a rich source of fibrin. However, misfolded proteins formed as a direct consequence of cell death and which also occurs subsequent to TBI [5, 6], also provide cofactor activity similar to fibrin to permit optimal plasminogen activation by t-PA.[37] Additionally, plasminogen receptors on various leukocytes, and other components of the vasculature can also provide a cofactor for plasmin generation by t-PA.[38]

Consistent with an immune-modulatory role of plasmin, $plg^{-/-}$ mice subjected to TBI displayed additional changes 1 week post-injury in the cellular immune status in the blood (notably neutrophils) and in the cLN that were not seen in the $plg^{+/+}$ controls. Since the cLN is part of the draining lymph node system of the brain[39] and is in close proximity to the injury we assumed that it would reflect the local immune response better than lymph nodes in other locations or the spleen. The significant increase in activated migratory and resident cDCs 1 week post-TBI only in $plg^{-/-}$ mice is consistent with the reported suppressive effects of plasmin on DC migration and activation.[10] Given the central role of plasmin in the induction of an adaptive immune response, this is likely to have significant downstream results on the subsequent immune response. The increase in antigen-presenting cells in the $plg^{-/-}$ mice was accompanied by a significant increase in helper- and cytotoxic T cells and B

cells, indicating that plasmin(ogen) deficiency promotes a heightened immune response in the cLN.

Plasmin-mediated alteration in the immune response post-TBI is an important consideration in the pharmacological treatment of TBI patients. As our findings in $plg^{-/-}$ mice suggested profound immune-modulatory properties of plasmin and previous studies suggesting immune-modulatory effects of TXA[18] we then determined whether pharmacological inhibition of plasmin generation would produce similar effects on the immune response after TBI.

TBI resulted in a profound increase in both PAP complex and D-dimer in the brain 3 h post-TBI, indicating local plasmin formation and fibrinolysis. Since the presence of brain PAP complexes can in part be explained by extravasation of circulating PAP complexes into the brain parenchyma following neurovascular damage, we assessed the ratio of brain and plasma PAP levels. This confirmed that TXA significantly inhibited PAP formation in the brain. While this might in part be explained by an inhibition of the binding of α2-antiplasmin to plasmin,[28] the concomitant reduction of D-dimer levels in the injured hemisphere strongly suggests a true inhibition of plasmin generation and activity in the brain occurred following TXA treatment. Hence, despite the lack of systemic hyperfibrinolysis in the CCI model of TBI, TXA inhibits localised fibrinolysis in the brain.

Accordingly, mice treated with TXA also displayed an increase in cellularity of the cLN 1 week post-TBI including increases in antigen-presenting cells, macrophages and lymphocytes. This effect was not due to an effect of TXA on lesion volume that was evaluated using MRI, neither was it correlated to neurological outcome within the first week after TBI. Although unlikely, it remains to be determined whether there is a longer term attenuation of neurological dysfunction after TBI. TXA therefore appears to promote the local immune response following TBI, by preventing plasmin-mediated immunosuppression. Enhanced expression of Ki-67 in CD11c+ cDC 1 week post-TBI in TXA-treated animals might represent longer-lasting proliferation of this cell subset after TBI, based on a report demonstrating that this marker slowly degrades over time in the non-proliferative phases of the cell cycle.[29] This further supports the notion of enhanced proliferation of leukocytes in the cLN as one of the reasons for the increase in cellularity with TXA. While other cell types

appeared unaffected, the peak of proliferation may have occurred earlier than the 1 week time point and further investigation is warranted.

As mentioned above, TBI compromises BBB permeability allowing influx of immune cells into the parenchyma. Since plasmin itself has been implicated in the promotion of BBB permeability[40], it would be anticipated that BBB permeability changes would also be attenuated, at least in part, when plasmin generation is inhibited by TXA. However, if TXA was altering the immune response merely by preserving the BBB, it would then be expected to see minimal immune cell infiltration into the brain, reduced migration of antigen presenting cells to the cLN, and reduced cellularity. Since the opposite was seen, the immune modulating effects of TXA are unlikely to be linked to any effect it may have on restoring BBB function.

Our studies on the impact of TXA on the immune response post-TBI in more distant inguinal lymph node (ingLN) also revealed some interesting effects. While TXA resulted in some relative increases in myeloid and lymphoid cells in the ingLN, overall ingLN cellularity after TBI was not increased by TXA treatment. Moreover, no difference was observed in leukocyte subset levels and their activation marker expression in the ingLN between $plg^{+/+}$ and $plg^{-/-}$ mice, indicating that the peripheral effects of plasmin activity or its inhibition are either of smaller scale or delayed.

TBI reportedly predisposes humans to CNS autoimmunity.[41] Therefore it was important to investigate whether TXA-mediated alleviation of early immunosuppression in the brain after injury could trigger an autoimmune-like response. Indeed, we detected significantly enhanced levels of anti-MOG antibodies, indicating a specific immune response against MOG after TBI. However, neither plasminogen deficiency nor TXA augmented this response. The low level of MOG-specific T cells (~3-10 in $1x10^6$ cells) was remarkably consistent across animals and groups, and similar to the levels reported in human volunteers,[42] and similar in magnitude to responses to some cancer associated autoantigens.[43] Results obtained for $plg^{+/+}$ and $plg^{-/-}$ mice were more variable, whereby the $plg^{-/-}$ sham group had the lowest response. Curiously, this effect was not observed when cells were incubated with 100 µg/ml MOG, suggesting that higher doses of MOG can equalise levels of $plg^{-/-}$ sham and TBI mice. This can potentially be explained by the lower MHC class II affinity of self-reactive T cells[44] that might be overridden when the MOG concentration is doubled.

The lectin staining data revealed that TXA-treated mice have temporarily accelerated clearance of microglial and macrophage activation/infiltration at least at the 1 week time point post-TBI, indicating that TXA mitigates cellular inflammation after injury. In accordance with those results obtained from lectin-stained brain sections we also observed a reduction in activation markers on macrophages and microglia at the 1 week time point in brain samples. This demonstrates an inhibitory effect of TXA on CNS tissue inflammation while simultaneously enhancing immune activation in the cLN. This is consistent with previous reports implicating plasmin in inflammation on the one hand [32, 33] and in immunosuppression on the other. [10] A summary of the findings observed in $plg^{-/-}$ mice and in wild-type mice treated with TXA is shown in Table 2.

It is important to highlight the fact that the plasminogen activation system has also been shown to modulate the immune response in other CNS paradigms, including models of multiple sclerosis.[32, 45] Curiously, an earlier study on experimental autoimmune encephalomyelitis demonstrated delayed disease onset in TXA-treated animals,[26] suggesting an unexplained yet protective effect of TXA in CNS autoimmunity.

It is particularly noteworthy that the effects of plasmin deficiency or blockade were observed in mice without a coagulopathic or hyperfibrinolytic phenotype. In the CRASH-2 trial, TXA likely improved outcome even in patients that were not coagulopathic, and our study might provide a clue how this may have occurred. On the other hand, it is well known that trauma patients with hyperfibrinolysis are at greater risk of mortality.[46] The hyperfibrinolytic state in such conditions is likely to promote immunosuppression due to the systemic overload of plasmin which may contribute to the increased infection risk in trauma[47] and hence increased mortality.

We have recently reviewed the complex implications of plasmin as a modulator of the immune response [8]. We propose that plasmin is differentially regulating the inflammatory and the innate immune response after TBI: plasmin boosts the local inflammatory response to tissue injury by enhancing leukocyte recruitment and phagocytic activity, thereby promoting the clearance of dead cell material and tissue repair. On the other hand, plasmin prevents the migration of antigen presenting cells to secondary lymphatic organs (such as the cLN) (illustrated schematically in Fig. 7).

Accordingly, after TXA treatment there is a possibility that DC-mediated antigen presentation to lymphocytes (B and T cells) would be boosted, thereby potentially resulting in self-reactivity with the risk of developing an autoimmune response. However, as no increase in T cell reactivity against the self-antigen MOG was detected in plg-deficient or TXA-treated mice, it appears that the regulatory mechanisms and immune checkpoints in place inhibit the development of a true self-reactive immune response.

Hence, we can conclude that even with prolonged TXA treatment (for 72 h), TBI patients are unlikely to develop a CNS autoimmune disease, despite the immune-activating effects of TXA. This is an interesting consideration, given that we have recently shown that TXA treatment – via its immune-activating effects – reduces post-surgical infection rates in cardiac surgery.[48] Therefore, it can be speculated that TBI patients might also potentially experience beneficial effects from TXA treatment by reducing infection rates (as recently explored in a mouse model of pneumonia [49]), in the absence of an enhanced risk of CNS autoimmune disease in this patient collective.

Randomised clinical trials are underway to address the effect of TXA in TBI and in other patient cohorts[16, 17]. While results are eagerly awaited, these trials are mostly focused on the haemostatic effects of TXA. The results of this current study implicating plasmin as an immune regulator further underscores the need to better understand the consequences of plasmin inhibition in order to correctly interpret clinical studies evaluating anti-fibrinolytic agents like TXA.

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Conflict of interest declaration

The authors declare, there are no conflict of interest with regard to the present manuscript.

Addendum: Authorship Contributions

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Optimisation and execution of experiments: all authors

Analysis, or interpretation of data: Dominik F. Draxler, Maithili Sashindranath, Magdalena Plebanski, Robert L. Medcalf

Critical revision and editing of the manuscript: all authors.

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Tables

Table 1. Thromboelastography (TEG) parameters 1 h and 3 h after sham procedure or TBI do not indicate presence of coagulopathy or hyperfibrinolysis in response to TBI

TEG parameter	sham (mean±SEM)	TBI (mean±SEM)	p value (t test)
	1h time point		
α-angle	82.2 ± 0.2082, n=3	81.83 ± 1.386, n=6	0.8620
Maximum amplitude	73.4 ± 4.065, n=3	73.03 ± 1.521, n=6	0.9188
LY30	0, n=3	0, n=6	
	3h time point		
α-angle	82.07 ± 0.5364, n=3	81.08 ± 0.675, n=8	0.4216
Maximum amplitude	75.27 ± 0.6173, n=3	75.4 ± 0.5296, n=7	0.8878
LY30	0, n=3	0, n=8	

Table 2. Summary of the effects of TXA administration to wild-type mice and in plg deficient mice subjected to TBI

	WT mice +TBI: effect of TXA	**$Plg^{-/-}$ mice + TBI compared with $plg^{+/+}$**

Blood cells	unchanged	↑ in neutrophils
Cervical Lymph Node	↑cellularity overall ↑ macrophages, monocytes ↑ CD11+cDC ↑ CD4+ and CD8+ T cells ↑ NK cells	↑ in CD11b+ cDCs ↑ in CD86 and CD80 on CD11b+ cDCs ↑ in both CD4+ and CD8+ T cells ↑ in B cells
Inguinal Lymph Node	no change in cellularity overall ↑ macrophages, monocytes ↑ CD11+cDC, ↑ NK cells ↑ CD4+ and CD8+ T cells	not tested
Brain cell Profile	↓ on resident microglia ↓ CD80 on macrophages ↓ CD86 on macrophages	not tested
autoreactivity to CNS antigens	no effect	no effect

Figures

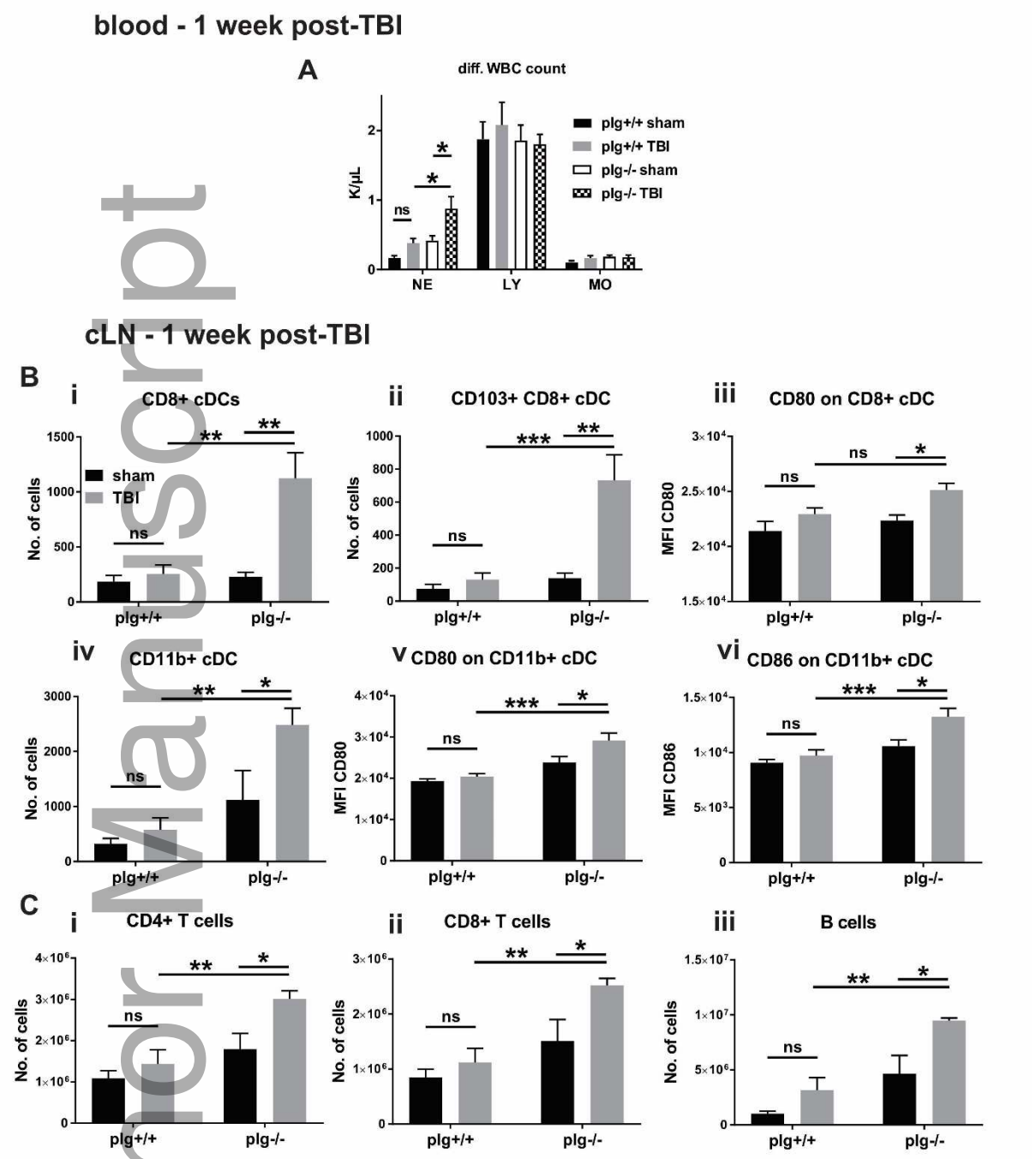

Figure 1. TBI induces profound changes in the draining cLN in plg$^{-/-}$, but not in plg$^{+/+}$ mice 1 week post-TBI

A. Neutrophils assessed in citrated whole blood with a haemocytometer were significantly elevated 1 week post-TBI in plg$^{-/-}$, but not plg$^{+/+}$ mice. No changes were observed in lymphocytes or monocytes (n=8-12). **B.** Flowcytometry was performed and exhibited increased numbers of CD8+ cDC **(i)**, CD103+ CD8+ (migratory) cDC **(ii)** as well as CD11b+ (resident) cDC **(iv)** in the cLN of plg$^{-/-}$ mice, but not plg$^{+/+}$ mice 1 week post-TBI (n=5-7). Also, the activation markers CD80 (n=8-11 for CD8+ cDC) **(iii)** (n=8-10 for CD11b+ cDC) **(v)** and

CD86 (n-8-10) **(vi)** were significantly enhanced 1 week post-TBI in CD8+ cDC (only CD80) and CD11b+ cDC only in $plg^{-/-}$ mice, as indicated by their mean fluorescence intensity (MFI). **C.** Moreover, numbers of CD4+T cells **(i)** and CD8+ **(ii)** T cells as well as B cells **(iii)** were increased in the cLN 1 week after TBI in $plg^{-/-}$, but not in $plg^{+/+}$ mice (n=5-8). Data expressed as mean±SEM; two-way ANOVA with Tukey's correction; * p<0.05, ** p<0.01; NE: neutrophils, LY: lymphocytes, MO: monocytes

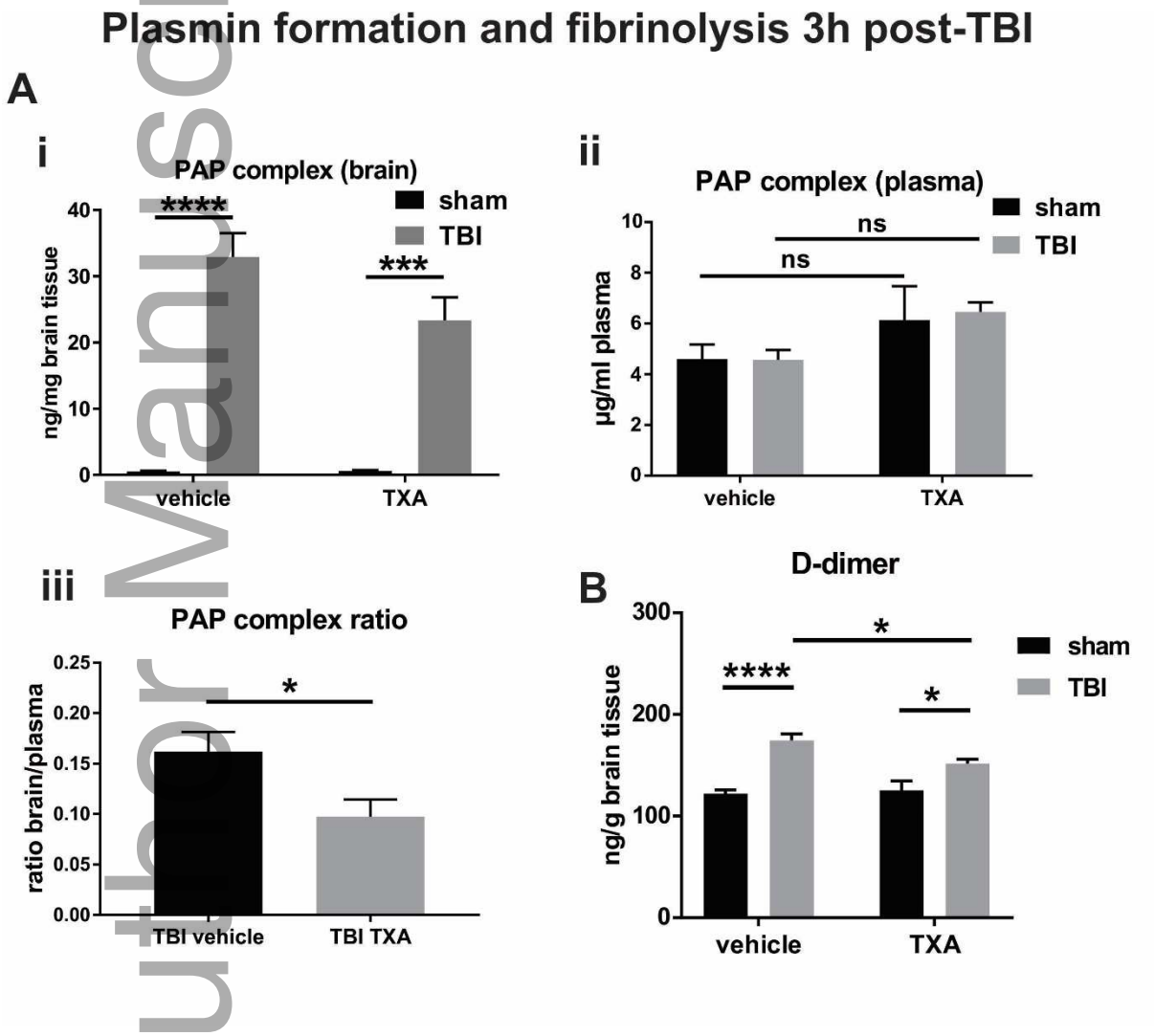

Figure 2. PAP complex formation and D-dimer levels were significantly elevated in the injured hemisphere 3h post-TBI, but this increase was inhibited by TXA

A. PAP complex levels in the perfused brain were assessed by ELISA 3 h after sham procedure and 3 h post-TBI in the injured (ipsilateral) hemisphere in both vehicle and TXA-treated animals (n=6-10) **(i)**. The PAP complex was also measured in citrated plasma at the 3

h time point (n=6-10) **(ii)** to evaluate the ratio of plasma and brain levels (n=10) **(iii)**. A significant increase in brain PAP levels was evident after TBI in both, vehicle and TXA-treated mice. Corrected for plasma levels (which were similar in all groups) a significant reduction by TXA was observed, as indicated by the PAP complex ratio. **B.** D-dimer levels were evaluated with an ELISA and showed a significant increase in the injured (ipsilateral) hemisphere 3h after TBI, suggesting presence of fibrinolysis. A significant reduction of D-dimer in mice subjected to TBI and subsequent TXA treatment indicates inhibition of local fibrinolysis.

Data expressed as mean±SEM; (Ai,Aii,B) two-way ANOVA with Tukey's correction, (Aiii) student's t-test; * p<0.05, *** p<0.001, **** p<0.0001

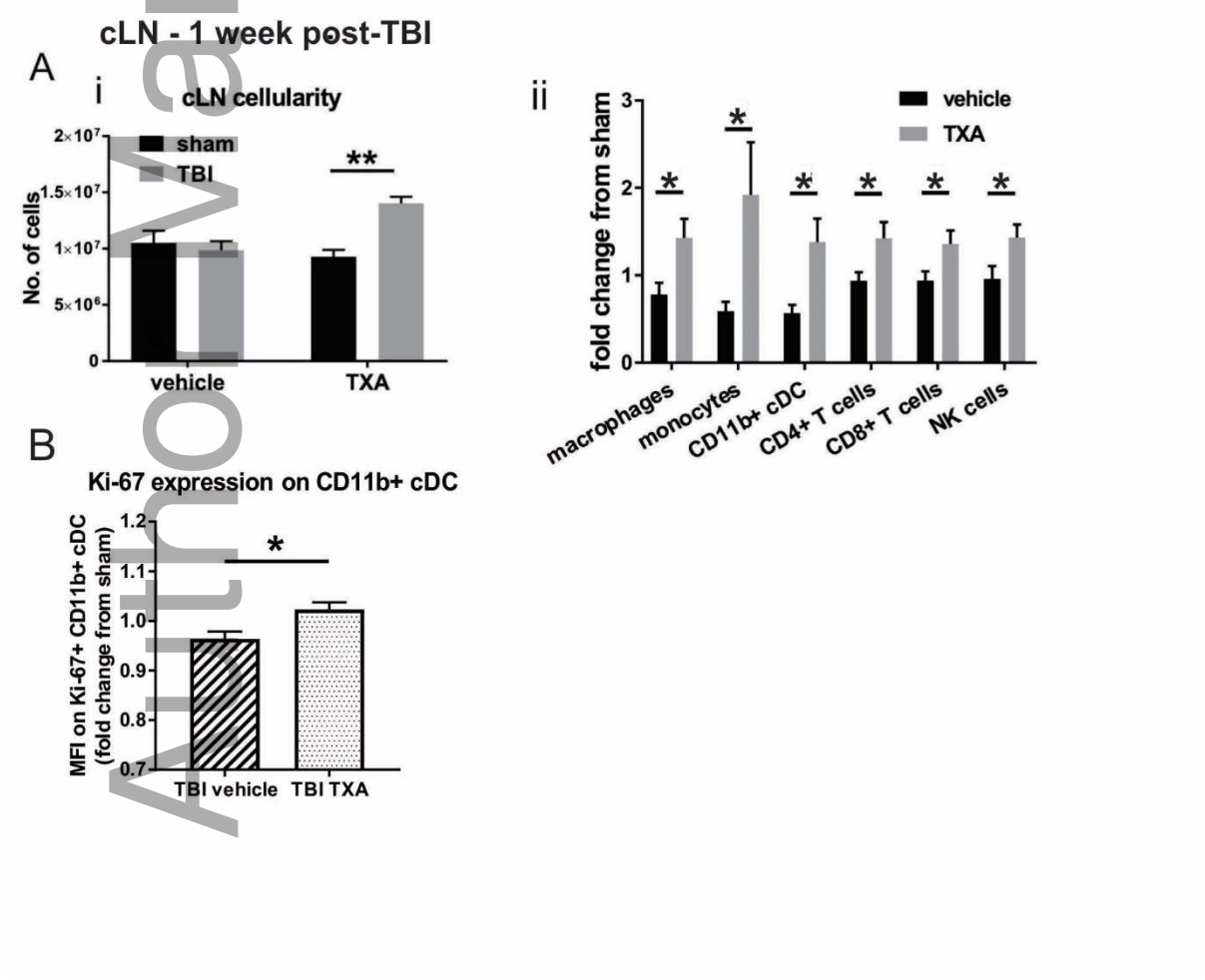

Figure 3. TXA treatment increases numbers of various immune cells in the cLN 1 week after TBI

A. 1 week post-TBI the cLN cellularity was assessed with a haemocytometer (n=9-11) **(i)** and cell subsets enumerated with flowcytometry (n=8-10). We observed increased cLN cellularity in response to TBI only in TXA-treated animals, involving an increase in macrophages, monocytes, CD11b+ cDC, CD4+ and CD8+ T cells as well as NK cells **(ii)**. **B.** The expression intensity of Ki-67 was significantly increased in response to TBI in TXA-treated animals than in vehicle-treated mice (n=9-10).

Data expressed as mean±SEM; (A): two-way ANOVA with Tukey's correction, (Aii, B) student's t-test, * p<0.05, ** p<0.01

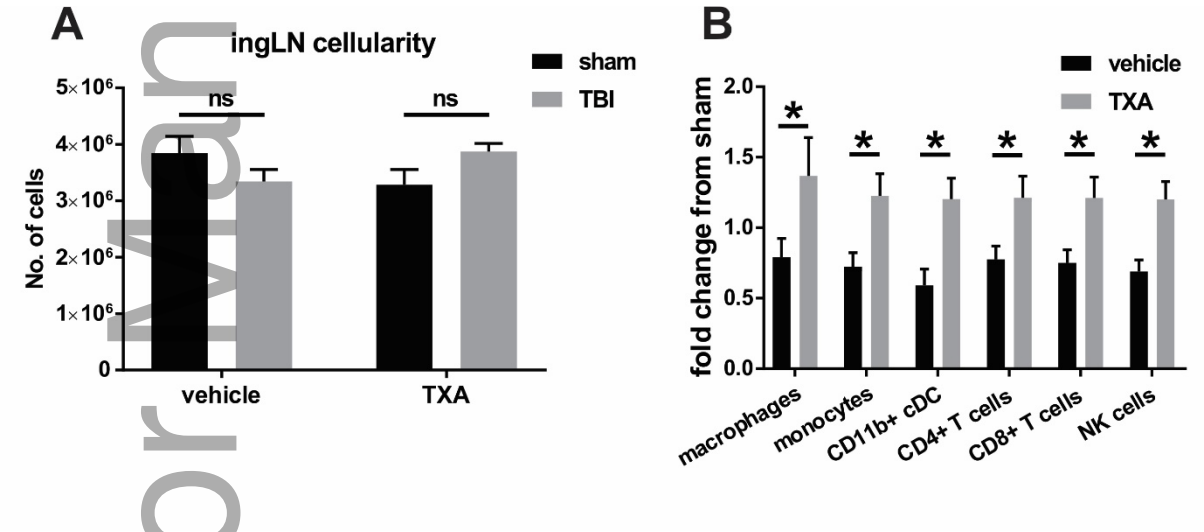

Figure 4. Effect of TXA on the inguinal lymph node immune profile 1 week post-TBI

While TBI did not result in a significant change in ingLN cellularity neither in vehicle- nor TXA-treated mice, drug treatment affected the trend of immune changes induced by the injury (p for interaction = 0.027) **(A)**. Accordingly, TXA facilitated an increase in macrophages, monocytes, CD11b+ cDC, CD4+ and CD8+ T cells and NK cells **(B)**.

Data expressed as mean±SEM; two-way ANOVA with Tukey's correction (A) ns: non-significant, student's t-test (B) * p<0.05

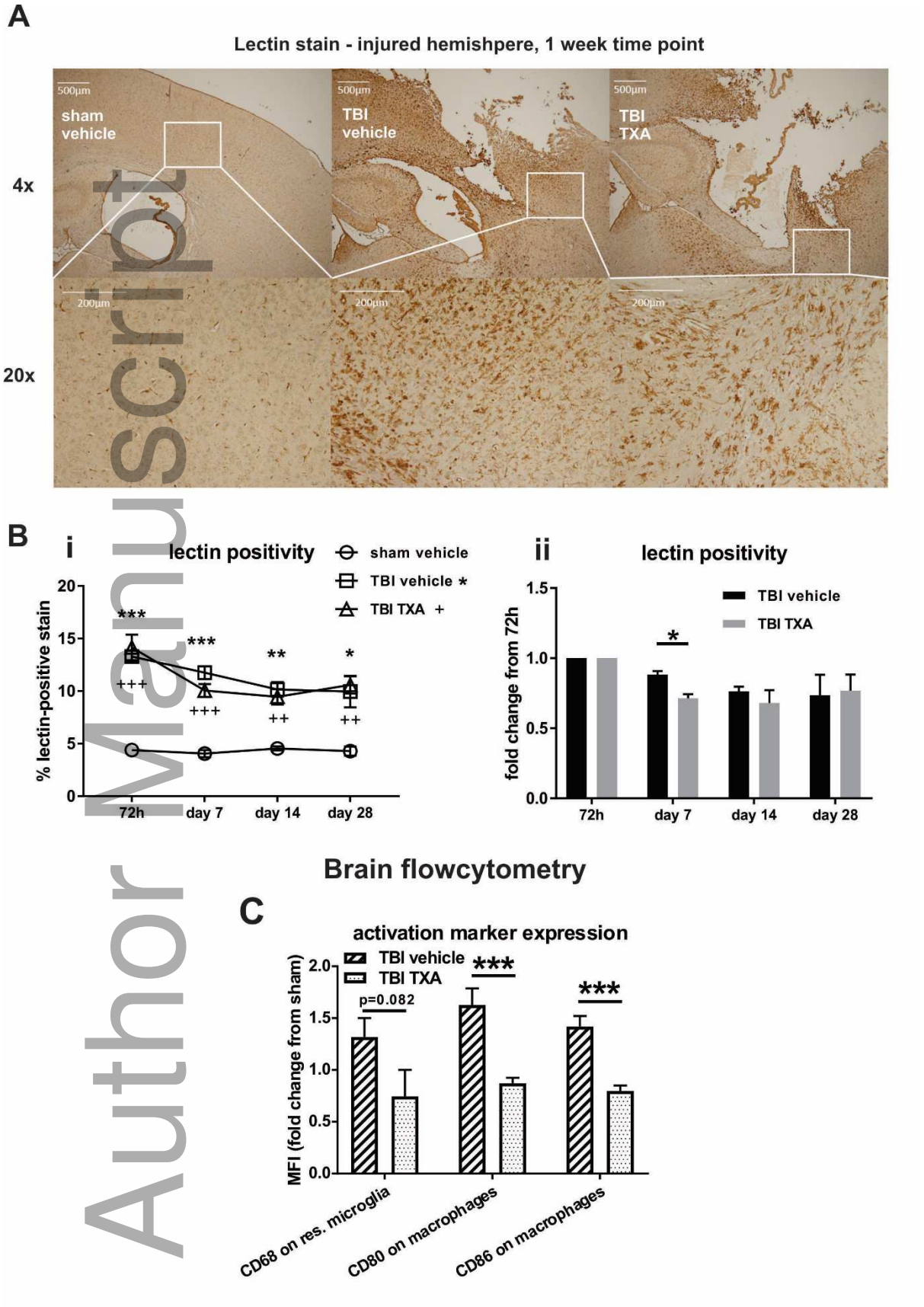

Figure 5. TBI-induced microglial and macrophage activation in the injured brain is reduced by TXA

A. Representative lectin-stained brain sections for a sham animal as well as a TBI animal treated with either vehicle or TXA at the 1 week time point. The images demonstrate the profound increase in activated microglia and macrophages in the injured hemisphere after TBI. **B.** Brain sections of sham and TBI mice treated with vehicle or TXA were stained with lectin at different time points to gauge the presence of activated microglia and macrophages illustrating the profound increase in those cells over a 28 day post-TBI period in both, vehicle and TXA-treated mice. (n=3) **(i)**. The presentation of lectin-positive areas as fold-change from 72 h levels indicates accelerated reduction in lectin signal at 1 week in TXA treated mice. (n=3) **(ii)**. **C.** TXA treatment reduced the expression of the activation markers CD80 and CD86 on macrophages.

Scale bars in **A** represent 500 µm at 4x magnification and 200 µm at 20x magnification. Data expressed as mean±SEM; (Bi) one-way ANOVA with Dunnett's correction (comparison with sham groups); (Bii,C) student's t-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

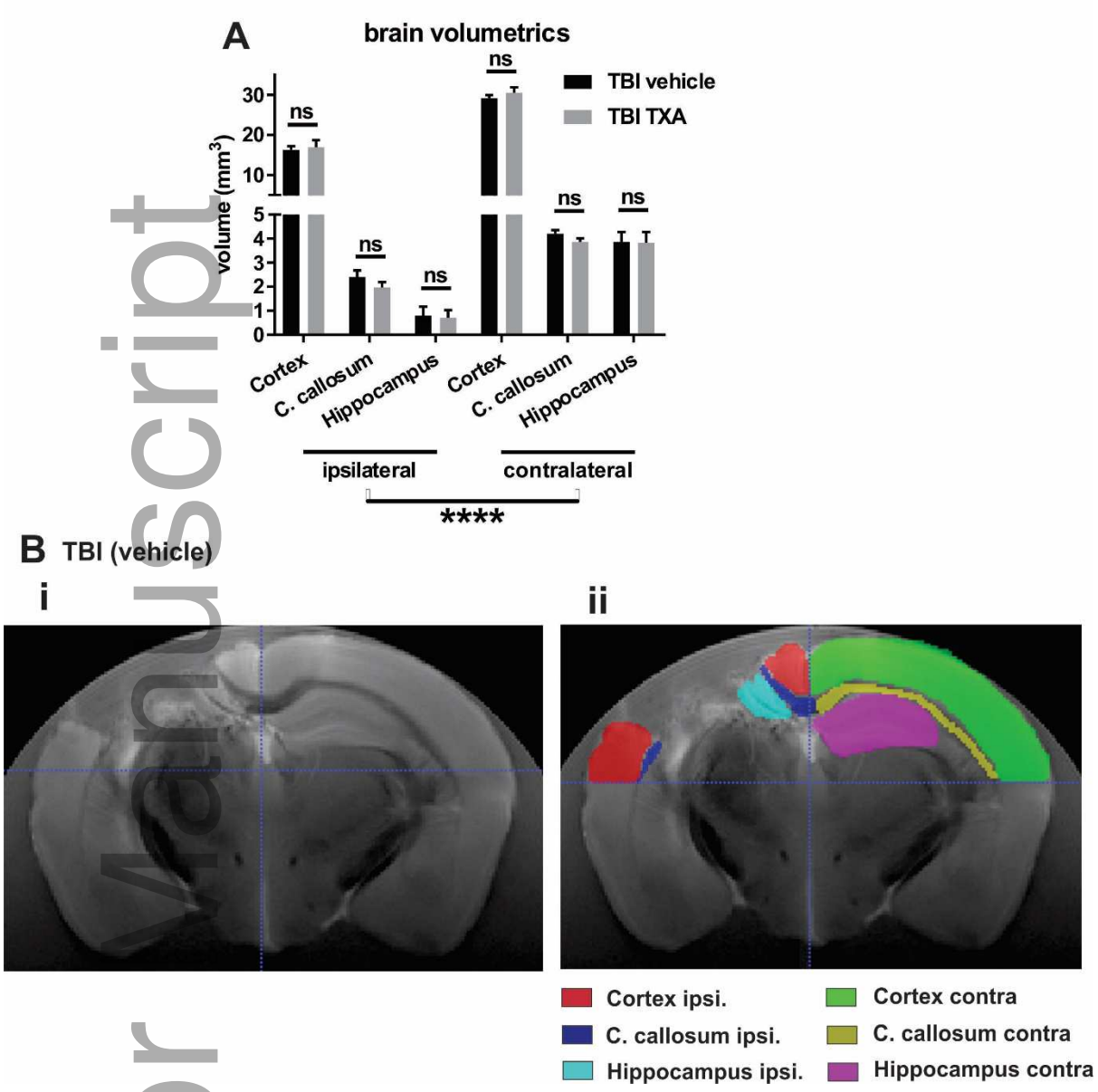

Figure 6. Brain volumetrics 1 week post-TBI

A. Ex vivo magnetic resonance imaging (MRI) was performed to quantify the volume of various brain regions (in mm^3) in the injured (ipsilateral) and uninjured (contralateral) hemisphere 1 week post-TBI (n=5-6). While a volume reduction was detected in all analysed structures (cortex, corpus callosum, hippocampus; all $p<0.0001$ analysed with a two-way ANOVA with Tukey's correction), no difference was observed between vehicle and TXA treated mice. Data expressed as mean±SEM; student's t-test; ns: non-significant, * $p<0.05$

B. Representative brain MRI image illustrating the analysis performed to quantify the volume of assessed brain structures. **(i)** shows the MRI slice without and **(ii)** with the mask generated for volumetric analysis.

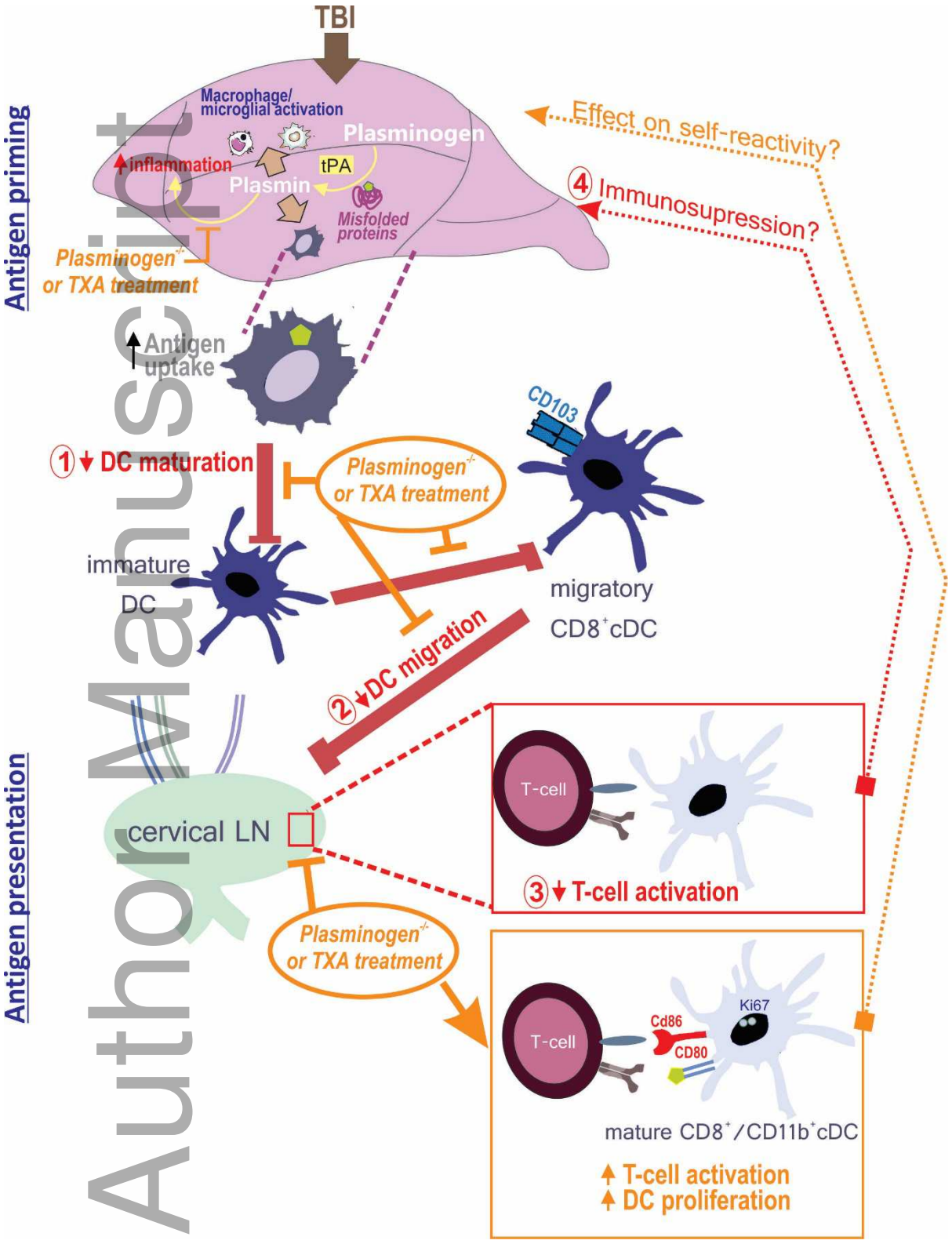

Figure 7: Schematic representation of the effect of plasmin inhibition on the cellular immune response within 7 days post-TBI.

Following TBI, t-PA levels are increased in the damaged brain. Plasminogen activation then occurs facilitated by the presence of misfolded proteins. This results in an increase in antigen uptake and macrophage/microglial cell activation and a pro-inflammatory response most likely involving engagement of plasmin(ogen) to plasminogen receptors on key leukocytes within the brain. Active plasmin reduces dendritic cell activation (1) and reduces cell migration to the cervical lymph node (2) and reduced T cell activation (3), hence promoting an immunosuppressive phenotype (4).

In contrast, inhibition of plasmin generation after TBI results in reduced inflammation, reduced antigen uptake and an increase in DC maturation and increased expression of integrin CD103 reflecting increased DC migration. In the absence of plasmin, these migratory DCs travel to the cervical lymph node (cLN) where they cross-present antigen to T-cells, evidenced by increased expression of co-stimulatory markers CD80 and CD86 and an increase in CD4 and CD8 T cells. Within the cLN, there is an increase in CD11b+ cDC proliferation (evidenced by increased Ki67).

Although not shown in the figure, the heightened immune activation does not result in an enhanced anti-MOG response at the 2 week time point post-TBI. Hence, despite the increased immune response due to plasmin blockade, it is unlikely that plasmin deficiency or inhibition will promote CNS auto-reactivity.