Toll-Like Receptor 4 Mediates Hemorrhagic Transformation After Delayed Tissue Plasminogen Activator Administration in In Situ Thromboembolic Stroke

Alicia García-Culebras, MSc*; Sara Palma-Tortosa, MSc*; Ana Moraga, PhD; Isaac García-Yébenes, PhD; Violeta Durán-Laforet, MSc; Maria I. Cuartero, PhD; Juan de la Parra, MSc; Ana L. Barrios-Muñoz, MSc; Jaime Díaz-Guzmán, MD, PhD; Jesús M. Pradillo, PhD; María A. Moro, PhD†; Ignacio Lizasoain, MD, PhD†

Background and Purpose—Hemorrhagic transformation is the main complication of revascularization therapies after stroke. Toll-like receptor 4 (TLR4) is implicated in cerebral damage and inflammation in stroke. This study was designed to determine the role of TLR4 in hemorrhagic transformation development after tissue plasminogen activator (tPA) administration.

Methods—Mice expressing ($TLR4^{+/+}$) or lacking functional TLR4 ($TLR4^{-/-}$) were subjected to middle cerebral artery occlusion using an in situ thromboembolic model by thrombin injection into the middle cerebral artery, and tPA (10 mg/kg) was administered 20 minutes or 3 hours after ischemia. Infarct size, hemorrhages, IgG extravasation, matrix metalloproteinase 9 expression, and neutrophil infiltration were assessed 24 hours after ischemia.

Results—In $TLR4^{+/+}$, early reperfusion (tPA at 20 minutes) resulted infarct volume, whereas late recanalization (tPA at 3 hours) did not modify lesion size and increased the rate of the most severe hemorrhages. In $TLR4^{-/-}$ mice, both early and late reperfusion did not modify lesion size. Importantly, late tPA administration did not result in worse hemorrhages and in an increased bleeding area as occurred in $TLR4^{+/+}$ group. In $TLR4^{-/-}$ animals, late reperfusion produced a lesser increase in matrix metalloproteinase 9 expression when compared with $TLR4^{+/+}$ animals.

Conclusions—Our results demonstrate TLR4 involvement in hemorrhagic transformation induced by delayed tPA administration, very likely by increasing matrix metalloproteinase 9 expression. **(*Stroke*. 2017;48:1695-1699. DOI: 10.1161/STROKEAHA.116.015956.)**

Key Words: blood–brain barrier ■ hemorrhage ■ inflammation ■ middle cerebral artery ■ stroke

Recanalization with tissue plasminogen activator (tPA) or endovascular thrombectomy are the only therapies available for patients with ischemic stroke.[1] Hemorrhagic transformation (HT) is the main complication of thrombolysis; to limit this problem, this drug is used only under restrictive conditions. In addition, HT is an important complication after mechanical thrombectomy, with a similar proportion of appearance.[1] Therefore, research into mechanisms of HT and potential therapies that reduce HT risk and improve prognosis of patients with acute ischemic stroke is greatly needed.

One of the major causes of HT is an increased permeability of the blood–brain barrier (BBB)[2] because of the loss of different components of the microvascular basal lamina, which is related to the increased expression of matrix metalloproteinase 9 (MMP-9) that takes place after ischemia.[3] MMP-9 is upregulated by high-mobility group box 1 via toll-like receptor 4 (TLR4),[4] a receptor implicated in cerebral damage and inflammation elicited by stroke.[5] In humans, serum levels of MMP-9 predict HT in acute stroke[6] and those of high-mobility group box 1 correlate with stroke severity.[7]

Now, our aim is to determine whether TLR4 participates in HT development using an in situ thromboembolic stroke model with early or delayed tPA administration.

Materials and Methods

Mice C57BL/10J ($TLR4^{+/+}$; mice expressing TLR4) and C57BL/10ScNJ ($TLR4^{-/-}$, lacking functional TLR4) were subjected to middle cerebral artery occlusion (MCAO) by using an in situ thromboembolic model. Recombinant tPA (10 mg/kg) was administered 20

Received November 4, 2016; final revision received February 3, 2017; accepted February 23, 2017.

From the Unidad de Investigación Neurovascular, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense and Instituto de Investigación Hospital 12 Octubre (i+12), Madrid, Spain.

*A. García-Culebras and S. Palma-Tortosa contributed equally and are joint first authors.

†Drs Moro and Lizasoain are joint last authors.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.116.015956/-/DC1.

Correspondence to Ignacio Lizasoain, MD, PhD, Departamento de Farmacología, Facultad de Medicina, Universidad Complutense Madrid, Madrid 28040, Spain. E-mail ignacio.lizasoain@med.ucm.es

minutes or 3 hours after ischemia. Infarct size, hemorrhages, IgG extravasation, MMP-9 expression and neutrophil infiltration were assessed 24 hours after ischemia as described in the online-only Data Supplement. Physiological parameters were not significantly different between groups (Table I in the online-only Data Supplement).

Results

Role of TLR4 on Infarct Volume: Effect of Early or Late tPA Administration

In TLR4$^{+/+}$ mice, early reperfusion with tPA (20 minutes) after in situ MCAO resulted in smaller infarcts than in control animals (P<0.05 versus TLR4$^{+/+}$; Figure 1B and 1C), whereas late recanalization (3 hours) did not modify the size of the lesion (P>0.05; Figure 1B and 1C). In these animals, brain TLR4 immunoreactivity was found mainly in neurons (Figure I in the online-only Data Supplement).

In TLR4$^{-/-}$ mice, MCAO produced a significantly smaller infarct volume than in TLR4$^{+/+}$ mice (P<0.05; Figure 1B and 1C), corroborating our previous results in a different model.[5] Importantly, neither early or late reperfusion of the artery modified lesion size (P>0.05 versus TLR4$^{-/-}$; Figure 1B and 1C).

Role of TLR4 in tPA-Induced HT

TLR4$^{+/+}$ mice showed mild signs of HT and a small bleeding area 24 hours after MCAO. After early reperfusion (20 minutes), both hemorrhage severity and area were similar to the MCAO control group (P>0.05; Figure 2A through 2C), whereas late recanalization (3 hours) by tPA increased the rate of the most severe hemorrhages, hemorrhagic infarction type-1, type-2 and parenchymal hemorrhage (Figure 2A through 2C). In contrast, in TLR4$^{-/-}$ mice, late tPA administration did not increase hemorrhage and bleeding area (Figure 2A through 2C). Because reduced bleeding could be a consequence of the decreased lesion size, data were normalized by dividing hemorrhage size by infarct size for each animal to avoid this bias. Importantly, after normalization, hemorrhage remained significantly reduced (P<0.05; Figure 2D).

Role of TLR4 on BBB Damage, MMP-9 Expression, and Leukocyte Infiltration After Late tPA Administration

In TLR4$^{-/-}$ animals with late reperfusion, extravasated IgG area (indicating BBB damage) was decreased when compared

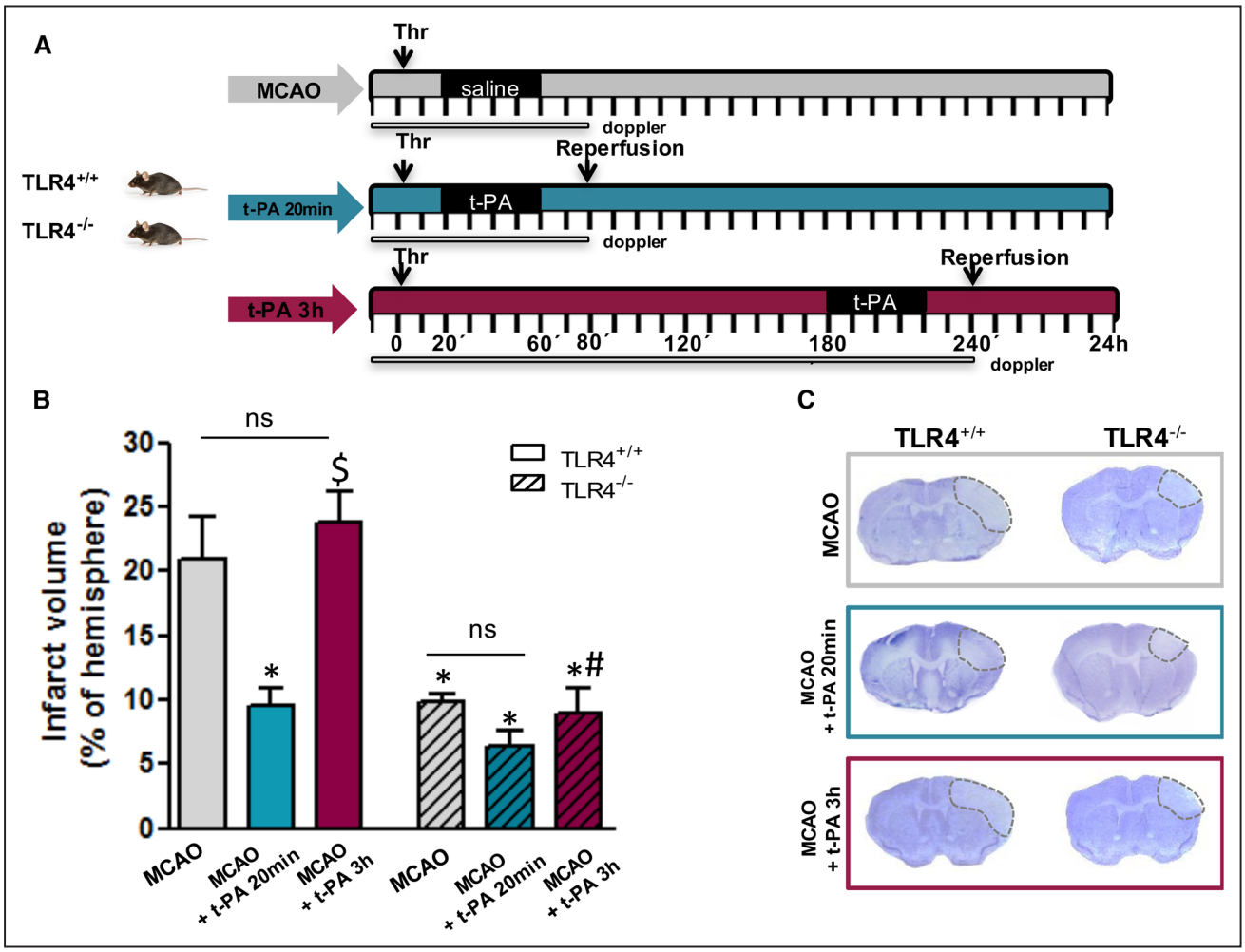

Figure 1. Effect of toll-like receptor 4 (TLR4) on infarct volume after in situ thromboembolic stroke with early or late tissue plasminogen activator (tPA) administration. **A**, Design of the study. **B**, Infarct volumes, determined 24 hours after occlusion. Data are mean±SEM. Data were compared by a nonparametric 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc testing (n=4–6; *P<0.05 vs middle cerebral artery occlusion [MCAO] TLR4$^{+/+}$; $$P$<0.05 vs MCAO tPA 20 minutes TLR4$^{+/+}$; #P<0.05 vs MCAO tPA 3 hours TLR4$^{+/+}$; for ANOVA details see Statistics in the online-only Data Supplement). **C**, Photographs of brain slices from representative experiments.

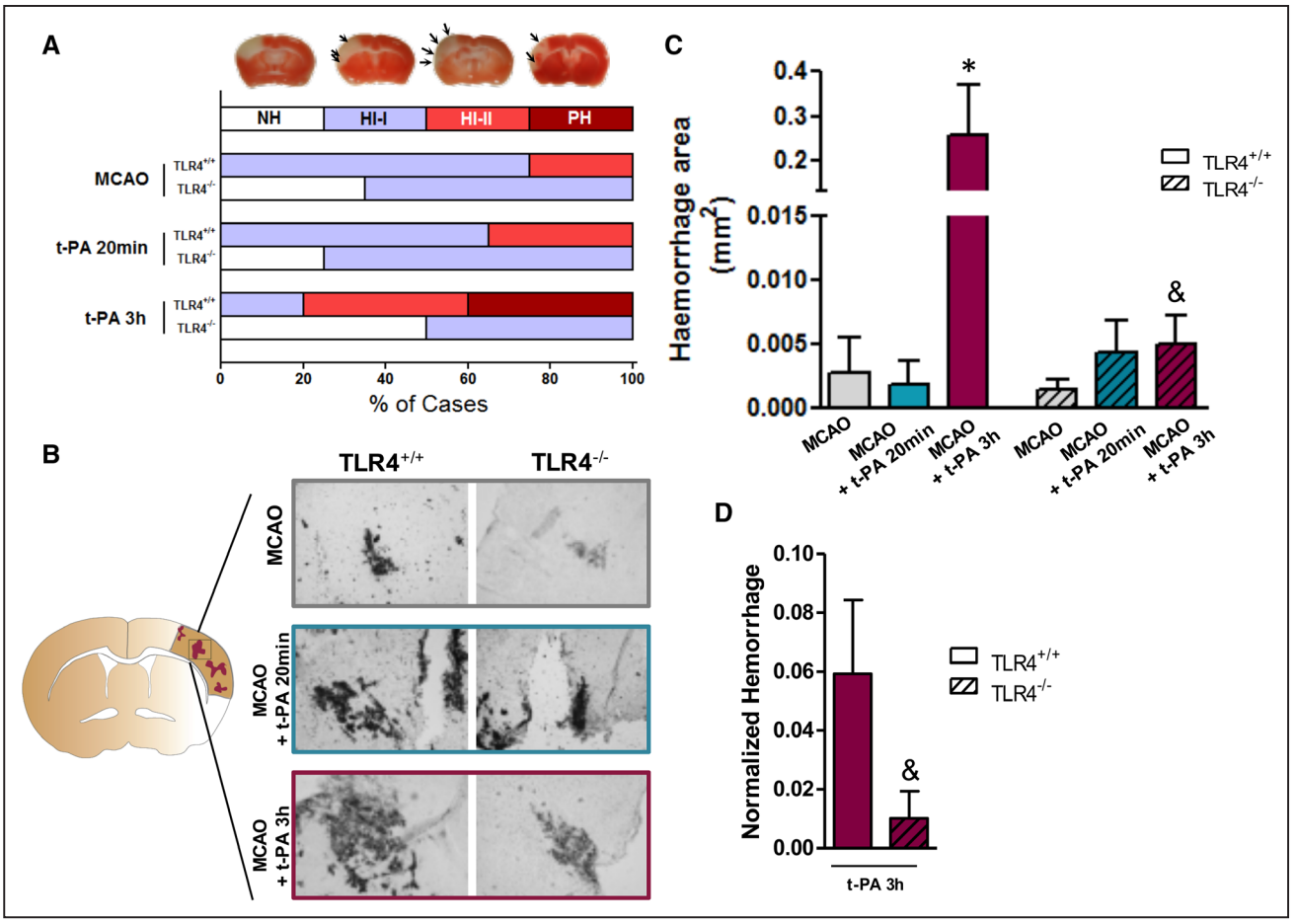

Figure 2. Effect of toll-like receptor 4 (TLR4) on hemorrhagic transformation after early or late tissue plasminogen activator (tPA) administration after in situ thromboembolic stroke. **A**, Macroscopic classification of the hemorrhage (details in the online-only Data Supplement) in no-hemorrhage (NH), hemorrhagic infarction type-1, type-2 (HI-I, HI-II), and parenchymal hemorrhage (PH). **B**, Representative diaminobenzidine staining of brain sections from all groups. **C**, Hemorrhage areas, determined 24 hours after ischemia. Data are mean±SEM. Data were compared by a nonparametric 2-way analysis of variance (ANOVA) followed by Bonferroni post hoc testing (n=4–6; *P<0.05 vs middle cerebral artery occlusion [MCAO] TLR4$^{+/+}$; &P<0.05 vs MCAO tPA 3 hours TLR4$^{+/+}$; for ANOVA details see Statistics in the online-only Data Supplement). **D**, Hemorrhage area, following normalization for infarct size, in MCAO+tPA 3 hours groups; t test &P<0.05 vs tPA 3 hours TLR4$^{+/+}$.

with TLR4$^{+/+}$ animals (P<0.05, Figure 3A). In addition, in TLR4$^{-/-}$ animals, MMP-9 expression after late tPA administration was remarkably reduced when compared with TLR4$^{+/+}$ ones (P<0.05; Figure 3B). MMP-9 expression appeared mainly vascular, as it colocalized with the endothelium marker GLUT-1, but not with neutrophil marker NIMP-R14 (Figure 3C). Importantly, there was a linear correlation between MMP-9 and hemorrhage areas, which were mostly coincident (Figure II in the online-only Data Supplement). We did not find any significant differences in neutrophil infiltration between both groups (Figure 3D).

Discussion

Recanalization therapies, either pharmacological or mechanical, are associated with an increased risk of symptomatic HT, a life-threatening complication. In preclinical studies, several compounds have been shown to decrease BBB damage and tPA-induced HT but none of them reduces the risk of HT clinically. Elucidation of mechanisms leading to HT may help to identify therapeutic targets to prevent this sequel. Our results demonstrate that TLR4 is involved in HT induced by delayed tPA administration: the presence of TLR4 (1) increases hemorrhage severity and bleeding area and (2) exacerbates BBB damage and MMP-9 expression after delayed tPA administration. To our knowledge, this study is the first to indicate that TLR4 inhibition can provide protection after ischemic stroke treated with tPA.

HT after delayed tPA infusion is mediated by TLR4, as it was significantly reduced in TLR4-deficient animals, and remained significantly reduced after normalization for infarct size. Although TLR4 implication in cerebral damage and inflammation after stroke is well known,[5] this is the first evidence that demonstrates TLR4 involvement in tPA-induced HT. Supporting our conclusion, TLR4 is upregulated after intracerebral hemorrhage[8] in neurons, as we now describe, astrocytes and microglia.[9] TLR4 signaling pathway contributes to poor outcome after intracerebral hemorrhage in animals and patients.[10] Furthermore, drugs blocking TLR4 suppress intracerebral hemorrhage–induced inflammatory injury.[9]

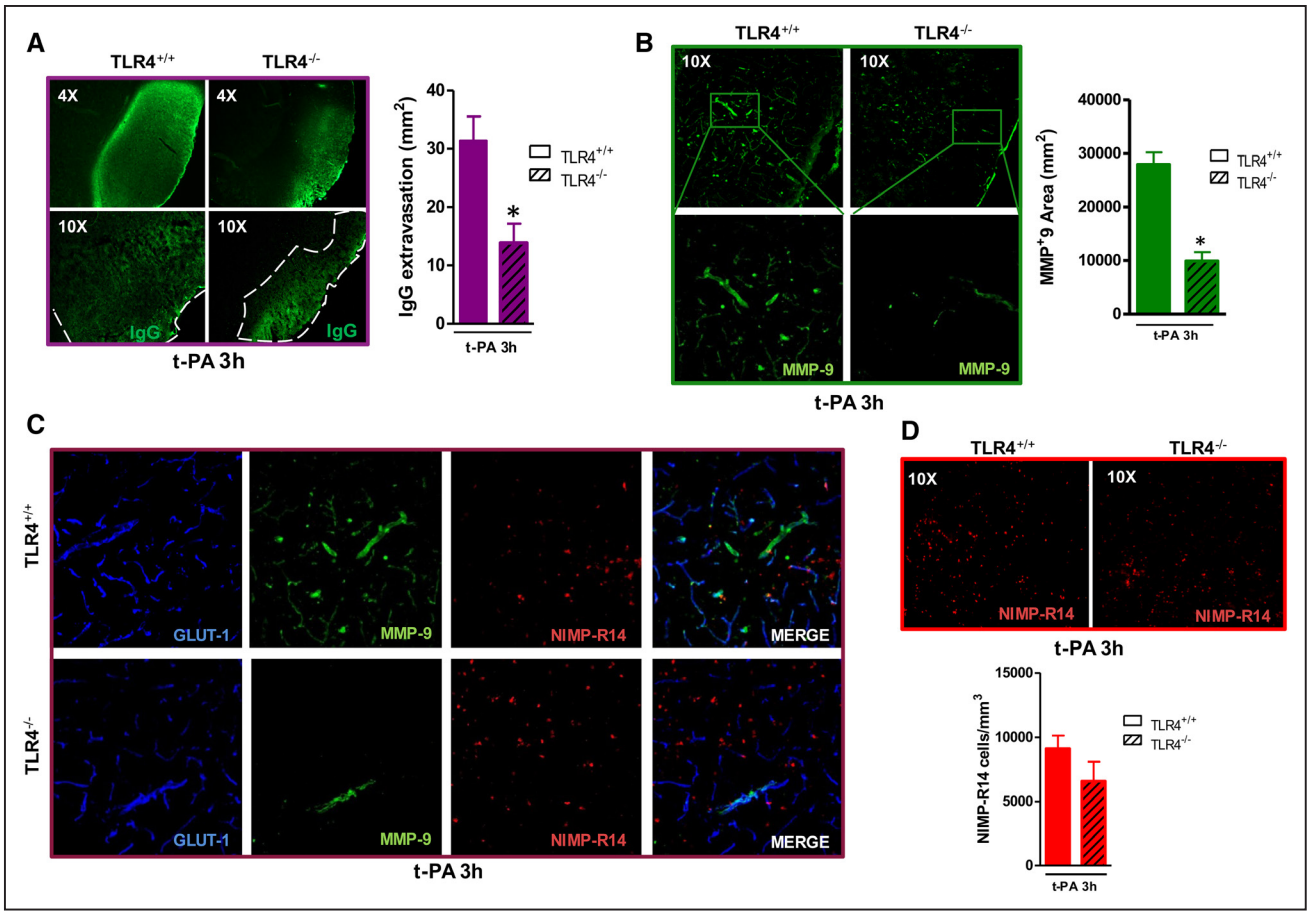

Figure 3. Effect of toll-like receptor 4 (TLR4) on IgG extravasation and MMP-9 expression after late tissue plasminogen activator (tPA) administration. **A**, IgG extravasation area in middle cerebral artery occlusion+tPA 3 hours groups. **B**, MMP-9 expression and densitometry in the ischemic hemisphere. **C**, Triple immunostaining of MMP-9, NIMP-R14 (neutrophils), and GLUT-1 (vessels). **D**, Neutrophil infiltration in the ischemic hemisphere. Data are obtained 24 hours after ischemia and are mean±SEM; n=4 to 6; *t* test **P*<0.05 vs tPA 3 hours TLR4$^{+/+}$.

Activation of proteases and infiltration of circulating white cells seem to play an important role in BBB damage and HT, particularly after tPA-induced recanalization. MMP-9 degrades basement membrane components and causes BBB disruption, an effect potentiated by tPA.[11] We previously showed increased MMP-9 immunoreactivity in the ischemic brain of wild-type mice.[5] Likewise, our data now demonstrate MMP-9 expression in animals with delayed tPA reperfusion, where HT is more frequent, supporting MMP-9 as a reliable marker of HT as clinical evidence suggests.[6] Importantly, TLR4-deficient mice present a much lower MMP-9 expression, unlikely due to the smaller infarct size because MMP-9 expression is induced early after ischemia, when infarction has not fully progressed.[4] We also show that MMP-9 immunoreactivity overlaps with the hemorrhagic area, showing a positive correlation. This association is corroborated by our results proving its vascular location. Given the expression pattern found, MMP-9 upregulation in blood vessels could be induced in a paracrine fashion by the activation of neuronal TLR4. Similarly, neuronal TLR4 signaling has been shown to induce brain endothelial activation,[12] which could lead to MMP-9 expression. This does not discard an autocrine mechanism; consistently, it has been demonstrated that high-mobility group box 1 triggers MMP-9 upregulation in neurons and astrocytes predominantly via TLR4 activation after cerebral ischemia.[4] All these data strongly support the TLR4–MMP-9 pathway as a plausible mechanism for tPA-induced HT.

Neutrophil infiltration has been involved in HT[13] but, at least in our model, its importance is diminished by the lack of differences in the groups studied after delayed tPA administration.

Our study presents some limitations: (1) the delayed administration was set at a time still safe in humans, very likely because of species differences; (2) the protective effect of TLR4 absence requires to be studied in future long-term outcome experiments and with behavioral outcomes; and (3) TLR4 involvement in thrombectomy-induced HT remains to be studied.

In conclusion, we report that TLR4 absence decreases HT after delayed tPA administration. Increased MMP-9 expression might be the mechanism underlying this effect. Our data support TLR4 inhibition as a promising therapeutic target to prevent tPA-induced HT and to increase the number of patients that benefit from this therapy.

Sources of Funding

This work was supported by grants from Spanish Ministry of Economy and Competitiveness SAF2014-52225 (Dr Lizasoain)

and SAF2015-68632-R (Dr Moro), and from Instituto de Salud Carlos III and cofinanced by Fondo Europeo de Desarrollo Regional (FEDER) "Una manera de hacer Europa" RETICS RD12/0014/0003 (Dr Lizasoain).

Disclosures

None.

References

1. Goyal M, Menon BK, van Zwam WH, Dippel DW, Mitchell PJ, Demchuk AM, et al; HERMES collaborators. Endovascular thrombectomy after large-vessel ischaemic stroke: a meta-analysis of individual patient data from five randomised trials. *Lancet*. 2016;387:1723–1731. doi: 10.1016/S0140-6736(16)00163-X.
2. Lo EH. A new penumbra: transitioning from injury into repair after stroke. *Nat Med*. 2008;14:497–500. doi: 10.1038/nm1735.
3. Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. *Stroke*. 1998;29:2189–2195.
4. Qiu J, Xu J, Zheng Y, Wei Y, Zhu X, Lo EH, et al. High-mobility group box 1 promotes metalloproteinase-9 upregulation through toll-like receptor 4 after cerebral ischemia. *Stroke*. 2010;41:2077–2082. doi: 10.1161/STROKEAHA.110.590463.
5. Caso JR, Pradillo JM, Hurtado O, Lorenzo P, Moro MA, Lizasoain I. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. *Circulation*. 2007;115:1599–1608. doi: 10.1161/CIRCULATIONAHA.106.603431.
6. Castellanos M, Leira R, Serena J, Pumar JM, Lizasoain I, Castillo J, et al. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. *Stroke*. 2003;34:40–46.
7. Yang QW, Wang JZ, Li JC, Zhou Y, Zhong Q, Lu FL, et al. High-mobility group protein box-1 and its relevance to cerebral ischemia. *J Cereb Blood Flow Metab*. 2010;30:243–254. doi: 10.1038/jcbfm.2009.202.
8. Teng W, Wang L, Xue W, Guan C. Activation of TLR4-mediated NFkappaB signaling in hemorrhagic brain in rats. *Mediators Inflamm*. 2009;2009:473276. doi: 10.1155/2009/473276.
9. Fang H, Wang PF, Zhou Y, Wang YC, Yang QW. Toll-like receptor 4 signaling in intracerebral hemorrhage-induced inflammation and injury. *J Neuroinflammation*. 2013;10:27. doi: 10.1186/1742-2094-10-27.
10. Sansing LH, Harris TH, Welsh FA, Kasner SE, Hunter CA, Kariko K. Toll-like receptor 4 contributes to poor outcome after intracerebral hemorrhage. *Ann Neurol*. 2011;70:646–656. doi: 10.1002/ana.22528.
11. Wang X, Lee SR, Arai K, Lee SR, Tsuji K, Rebeck GW, et al. Lipoprotein receptor-mediated induction of matrix metalloproteinase by tissue plasminogen activator. *Nat Med*. 2003;9:1313–1317. doi: 10.1038/nm926.
12. Leow-Dyke S, Allen C, Denes A, Nilsson O, Maysami S, Bowie AG, et al. Neuronal toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration *in vitro*. *J Neuroinflammation*. 2012;9:230. doi: 10.1186/1742-2094-9-230.
13. Rosell A, Cuadrado E, Ortega-Aznar A, Hernández-Guillamon M, Lo EH, Montaner J. MMP-9-positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. *Stroke*. 2008;39:1121–1126. doi: 10.1161/STROKEAHA.107.500868.