Identificación de la presencia de antígenos neurales en el tejido linfoide de los pacientes con ictus y análisis de su eventual implicación patogénica en la respuesta inmune adaptativa tras el daño cerebral

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Identificación de la presencia de antígenos neurales en el tejido linfoide de los pacientes con ictus y análisis de su eventual implicación patogénica en la respuesta inmune adaptativa tras el daño cerebral

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INFORME DE LOS DIRECTORES DE TESIS

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El Dr. Ángel Chamorro Sánchez, Consultor Senior del Servicio de Neurología del Hospital Clínic de Barcelona y Profesor Asociado de la Facultad de Medicina de la UB, y la Dra. Anna M. Planas Obradors, Investigador Científico del IIBB- CSIC

CERTIFICAN:

Que la tesis doctoral "Identificación de la presencia de antígenos neurales en el tejido linfoide de los pacientes con ictus y análisis de su eventual implicación patogénica en la respuesta inmune adaptativa tras el daño cerebral", presentada por Manuel Jesús Gómez-Choco Cuesta para optar al grado de Doctor por la Universidad de Barcelona se ha realizado bajo nuestra dirección y cumple todos los requisitos necesarios para ser defendida ante el Tribunal de evaluación correspondiente.

Que los artículos que conforman esta tesis y sus factores de impacto son:

- Brain-Derived Antigens in Lymphoid Tissue of Patients with Acute Stroke. The Journal of Immunology. Factor de impacto: 5.745
- Presence of heat shock protein 70 in secondary lymphoid tissue correlates with stroke prognosis. Journal of Neuroimmunology. Factor de impacto: 3.014

Que estos artículos no han sido incluidos en ninguna otra tesis doctoral ni serán incluidos en el futuro.

Dr. Ángel Chamorro
Consultor Senior

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ÍNDICE

I.-Listado de abreviaturas

BHE: Barrera hemato-encefálica.

BRAID: *Brain-associated immune deviation*.

CD: *Cluster of differentiation* (moleculas CD o grupo de diferenciación)

CPA: Célula presentadora de antígenos.

DAMPS: *Damage associated molecular patterns*.

DC: *Dendritic cell* (célula dendrítica).

EAE: Encefalomielitis alérgica experimental.

FRC*: Fibroblastic reticular cells*.

GFAP: *Glial fibrillar acidic protein* (proteína acídica fibrilar glial).

GL: Ganglio linfático.

GLC: Ganglios linfáticos cervicales.

HEV: *High endothelium venules* (venas de endotelio alto).

HLA: *Human leukocyte antigen* (antígenos leucocíticos humanos).

Hsp-70: *Heat shock protein* 70 (proteína de estrés térmico).

LCR: Líquido cefalorraquídeo.

MALT: *Mucose associated lymphoid tissue* (tejido linfoide asociado a mucosas).

MAP-2: *Microtubule associated protein* (proteína asociada a los microtúbulos).

MBP: *Myelin basic protein* (proteína básica de la mielina).

mDC: Célula dendrítica mieloide.

MHC: *Major histocompatibility complex* (complejo principal de histocompatiblidad).

NR-2A: Subunidad 2A del receptor de glutamato.

NSE: *Neural specific enolase* (enolasa neural específica).

PAMPs: *Pathogen associated molecular patterns*.

RT-PCR: *Real time polymerase chain reaction* (Reacción en cadena de la polimerasa).

pDC: Célula dendrítica plasmocitoide.

PLP: *Myelin proteolipidic protein* (proteína proteolipídica de la mielina).

MOG: *Myelin oligodendrocite protein*.

SNC: Sistema nervioso central.

TGF-β: *Transforming growth factor* (factor de crecimiento transformador β).

Th: Célula T *helper.*

TLR: *Toll like receptor*.

TLS: Tejido linfoide secundario.

TNF-α: *Tumoral necrosis factor* (factor de necrosis tumoral).

II.- Introducción

1. Necesidad de nuevas estrategias terapéuticas en el ictus.

El ictus es una de las principales causas de mortalidad y dependencia en nuestro medio, con una incidencia anual de 187 casos por 100.000 habitantes. El ictus es isquémico, debido a una oclusión arterial, es el más frecuente y representa en torno a un 80% de todos los ictus, siendo menos frecuentes la hemorragia intracerebral y otras formas menos habituales de patología vascular cerebral como la hemorragia subaracnoidea (van der Worp and van Gijn 2007; Roger, Go et al. 2011; Díaz-Guzman, Egido et al. 2012). A pesar de la mayor disponibilidad de terapias recanalizadoras para el tratamiento del ictus isquémico, sólo entre un 5 y un 7% de los pacientes ingresados por esta patología son candidatos a recibir este tipo de terapias (Abilleira, Davalos et al. 2011), mientras que aún no se dispone de ningún tratamiento específico que modifique la evolución natural de la hemorragia intracerebral (Morgenstern, Hemphill et al. 2010). Por ello, es necesario profundizar en el desarrollo de nuevas estrategias terapéuticas que puedan modificar la evolución natural de esta patología. Cada vez se dispone de más información sobre la importancia de la inmunidad en la fisiopatología del ictus agudo (Iadecola and Anrather 2011; Chamorro, Meisel et al. 2012) y por ello, modular los procesos inflamatorios e inmunológicos que tienen lugar durante la fase aguda del ictus podría aportar opciones terapéuticas en el futuro (Macrez, Ali et al. 2011).

2. El sistema inmune y el ictus.

2.1. El síndrome de inmunodepresión asociado al ictus.

Los pacientes con ictus desarrollan un síndrome de inmunodepresión asociado como consecuencia de las interacciones que se establecen entre el sistema nervioso central (SNC) y el sistema inmune a varios niveles y cuyos mecanismos más íntimos están pendientes de determinar (Urra, Obach et al. 2009). Este síndrome se caracteriza por una reducción de los linfocitos circulantes y de la actividad inflamatoria de las células mononucleares (Vogelgesang, Grunwald et al. 2007; Haeusler, Schmidt et al. 2008; Urra, Cervera et al. 2009) y la magnitud de la respuesta del sistema inmune se ha relacionado con el volumen del tejido cerebral infartado (Hug, Dalpke et al. 2009). El incremento de los niveles circulantes de corticoides y de catecolaminas como consecuencia del estrés que supone el ictus es probablemente la causa de la reducción del número de linfocitos circulantes debido a un aumento de la apoptosis linfocitaria

(Urra, Cervera et al. 2009). Esta linfopenia e incremento de la apoptosis linfocitaria también se extiende a los órganos linfoides (Liesz, Hagmann et al. 2009), ocasionando la atrofia tímica y esplénica que se observa en diversos modelos experimentales de isquemia cerebral (Prass, Meisel et al. 2003; Offner, Subramanian et al. 2006). La reducción de la actividad inflamatoria de las células mononucleares se ve reflejada en una reducción de la producción de TNF-α y de la expresión de moléculas HLA (human leukocyte antigen) por estas células (Vogelgesang, Grunwald et al. 2007; Hug, Dalpke et al. 2009; Urra, Cervera et al. 2009). Este síndrome de inmunodepresión asociado al ictus puede favorecer la aparición de complicaciones infecciosas (Haeusler, Schmidt et al. 2008; Urra, Cervera et al. 2009), pero por otra parte no es descartable que pueda constituir una respuesta adaptativa que contribuya a limitar el daño inflamatorio cerebral (Chamorro, Urra et al. 2007; Dirnagl, Klehmet et al. 2007).

2.2. La respuesta inmune innata y el ictus.

La respuesta inmune innata y la respuesta inmune adaptativa pueden desempeñar un papel relevante en la fisiopatología del ictus. La respuesta inmune innata es aquella que se establece por el reconocimiento de ciertos patrones moleculares (pathogen associated molecular patterns o PAMPs), presentes habitualmente en patógenos como virus y bacterias, por receptores presentes en macrófagos y monocitos. Este reconocimiento se lleva a cabo fundamentalmente por los receptores Toll like (toll like receptors o TLR), lo cual conlleva la activación de células inflamatorias que destruyen el patógeno o las células infectadas por él (Akira, Uematsu et al. 2006). En el ictus, el daño celular ocasionado por la isquemia cerebral libera diferentes señales de alarma denominados DAMPs (damage associated molecular patterns), en analogía a los PAMPs. Entre ellas se encuentran las proteínas de estrés térmico (heat shock proteins), HMGB1 (high mobility box group1), la proteína neuronal S100 y el ácido úrico, entre otras (Bianchi 2006; Kono and Rock 2008). Estos DAMPs pueden ser también reconocidos por receptores presentes en macrófagos y monocitos y contribuir al desarrollo de respuestas inflamatorias. A este respecto, se sabe que TLR4 puede funcionar como receptor de diversos ligandos endógenos (Marshak-Rothstein 2006) y se ha implicado en el desarrollo de la respuesta inflamatoria y en el daño tisular durante la isquemia cerebral (Caso, Pradillo et al. 2007). En los pacientes con ictus, la expresión de TL4 en

monocitos circulantes se ha correlacionado con la severidad del ictus (Yang, Li et al. 2008) y con un mal pronóstico funcional (Urra, Cervera et al. 2009).

2.3. La respuesta inmune adaptativa y el ictus.

La respuesta inmune adaptativa, que es aquella que se desarrolla de manera específica frente a antígenos presentados a las células T mediante moléculas del complejo principal de histocompatibilidad (major histocompatibility complex, MHC), también podría tener importancia en la fisiopatología del ictus (Iadecola and Anrather 2011). La presentación antigénica es llevada a cabo por células especializadas denominadas células presentadoras de antígenos (CPA). Dentro de esta respuesta adaptativa, los antígenos pueden ser presentados a través de moléculas MHC tipo I o tipo II. Las moléculas MHC-I se encargan fundamentalmente de la presentación de antígenos intracelulares, están presentes en todas las células y contribuyen a desarrollar respuestas citolíticas a través de la estimulación de células CD8. Clásicamente, intervienen en la respuesta frente a antígenos virales o tumorales. Mientras tanto, la respuesta mediada a través de moléculas MHC-II es la utilizada para la respuesta frente a antígenos externos, como pueden ser los antígenos bacterianos. Sólo las CPA expresan este tipo de moléculas y los antígenos presentados a través de estas moléculas tienden a ser presentados a células CD4 T helper (Th) (Neefjes, Jongsma et al. 2011).

Dependiendo de los estímulos recibidos por las CPA a través de sus receptores, estas transmiten diferentes tipos de señales a las células Th que se pueden traducir en diferentes tipos de respuesta inmune. Así, la activación de receptores intracelulares por ácidos nucleicos virales promueve la producción de IFN-I y el desarrollo de respuestas denominadas tipo Th1. Este tipo de respuesta se caracteriza por la producción de IFN-γ y promover respuestas citolíticas frente a las células infectadas. Mientras, el estímulo de receptores de superficie promueve el desarrollo de respuestas humorales Th2, las cuales se caracterizan por producir IL-4, y favorecer la estimulación de células B y producción de anticuerpos. En los últimos años se ha descrito el desarrollo de respuestas denominadas Th17, caracterizadas por la producción de IL-17 y el desarrollo de respuestas inflamatorias con predominio de neutrófilos (Diebold 2008).

Se sabe que en pacientes con ictus se pueden encontrar niveles elevados en el suero de proteínas de origen cerebral como por ejemplo la proteína básica de la mielina (myelin basic protein, MBP), la proteína S100 y la enolasa neuronal específica (neuronal specific enolase, NSE) (Jauch, Lindsell et al. 2006). Por ello, es argumentable que se pudiera desarrollar en el ictus algún tipo de respuesta inmune de tipo adaptativo frente a estos antígenos cerebrales. De este modo, se ha descrito un incremento de células T reactivas frente a antígenos mielínicos como MBP y PLP (myelin proteolipidic protein) en pacientes con ictus (Wang, Olsson et al. 1992), así como frente antígenos neurales como la proteína acídica fibrilar glial (glial fibrillar acidic protein, GFAP) (Becker, Kalil et al. 2011). Esta respuesta originada frente a antígenos mielínicos pudiera llegar a tener una implicación pronóstica, ya que pacientes con ictus más severos presentan una mayor respuesta Th1 frente a MBP y dicha respuesta se ha relacionado con un peor pronóstico independientemente de la severidad clínica inicial (Becker, Kalil et al. 2011). También se han observado respuestas de tipo Th2 caracterizadas por la producción de anticuerpos frente a antígenos de origen cerebral como el receptor de glutamato, el neurotransmisor más prevalente en el SNC, (Dambinova, Khounteev et al. 2003; Kalev-Zylinska, Symes et al. 2013) o neurofilamentos(Bornstein, Aronovich et al. 2001).

Sin embargo, no siempre la respuesta frente a un antígeno es efectora, sino que puede desarrollarse tolerancia o anergia frente al antígeno presentado mediante mecanismos diversos e interrelacionados. Para que se desarrolle una respuesta efectora, además de la interacción del antígeno con el receptor de célula T es necesaria la presencia de moléculas coestimuladoras. Las más relevantes son las moléculas CD80 y CD86, las cuales se encuentran presentes en las CPA e interaccionan con las moléculas CD28 y CTLA-4 presentes en los linfocitos. Del balance entre CD28 y CTLA-4 dependerá que se desarrolle una respuesta efectora o tolerancia frente a un determinado antígeno. En aquellas circunstancias en que la expresión de CTLA-4 es mayor se tiende a producir tolerancia, mientras que si predomina la interacción CD80/CD86 con CD28 se produce una respuesta efectora frente al antígeno presentado (Walker and Sansom 2011).

Además, el sistema inmune ha desarrollado respuestas celulares que controlen la respuesta frente a patógenos y evite que sea deletérea para el propio organismo. Además de las células T reguladoras naturales CD25+, existen células reguladoras antígeno especificas como las células Th3, que secretan TGF-β, y las células T1 reguladoras

(TR1) que producen gran cantidad de IL-10 (Diebold 2008; Sakaguchi, Miyara et al. 2010).

En modelos animales de isquemia cerebral se ha observado que las respuestas de tolerancia antigénica pueden tener un papel en la evolución del ictus. Se sabe que la inducción de tolerancia frente a antígenos cerebrales como la MBP o MOG (myelin oligodendrocyte glycoprotein) puede reducir el volumen de infarto y mejorar el pronóstico final (Becker, McCarron et al. 1997; Becker, Kindrcik et al. 2003; Frenkel, Huang et al. 2003; Gee, Kalil et al. 2008) o reducir respuestas inflamatorias en el SNC (Harling-Berg, M. et al. 1991). También, al igual que ocurre con otros órganos inmunoprivilegiados como el ojo, el organismo es capaz de "desviar" la respuesta inmune para limitar el daño cerebral. Este "desvío de la respuesta inmune asociado al cerebro" (brain-associated immune deviation, BRAID) es independiente de la integridad de la barrera hemato-encefálica (BHE) y de la actividad del bazo y pudiera relacionarse con la creación de células T reguladoras a nivel cervical (Wenkel and Streilein 2000).

3. El cerebro como órgano inmunoprivilegiado.

Si consideramos que la respuesta inmunes adaptativa puede tener relevancia en la fisiopatología del ictus, para su desarrollo se necesita ineludiblemente la presentación de los antígenos a las células T. Este proceso de presentación antigénica habitualmente se lleva a cabo en el tejido linfoide secundario (TLS), fundamentalmente en los ganglios linfáticos (GL) y en el tejido linfoide asociado a mucosas (mucose associated lymphoid tissue, MALT)(Itano and Jenkins 2003).

El cerebro se ha considerado clásicamente como un órgano inmunoprivilegiado, idea derivada de la observación de que células tumorales implantadas en el cerebro eran capaces de sobrevivir más que aquellas implantadas en otros tejidos. Esto se consideraba en parte debido a la ausencia de drenaje linfático en el SNC, y la presencia de la BHE. La ausencia de drenaje linfático evitaría el reconocimiento de antígenos cerebrales por las células del sistema inmune mientras que la presencia de la BHE evitaría la entrada de células inmunes en el SNC. Este "inmunoprivilegio" se encuentra reducido al parénquima cerebral, mientras que las meninges, plexos coroideos y órganos

periventriculares se comportan de forma similar al resto de los órganos debido a la ausencia de BHE (Galea, Bechmann et al. 2007).

La primera evidencia experimental de la existencia de la BHE se debe a Ehrlich, quien observó que la inyección de tinciones hidrosolubles en el sistema circulatorio teñía todos los órganos excepto el cerebro y la médula espinal (Hawkins and Davis 2005). La baja permeabilidad de la BHE se atribuye a las uniones entre las células endoteliales y la baja actividad pinocítica de las mismas. En el lado abluminal (o parte externa) de los vasos del SNC se pueden encontrar macrófagos perivasculares, los cuales pueden captar antígenos cerebrales y realizar vigilancia de las barreras del SNC (Muldoon, Alvarez et al. 2012). Situados a intervalos irregulares en la cara abluminal del endotelio se encuentran los pericitos, los cuales junto a las células endoteliales se encuentran englobados por la lámina basal, la cual se encuentra compuesta principalmente de colágeno tipo IV, laminina, fibronectina y proteoglicanos(Hawkins y Davis 2005). (Figura 1)

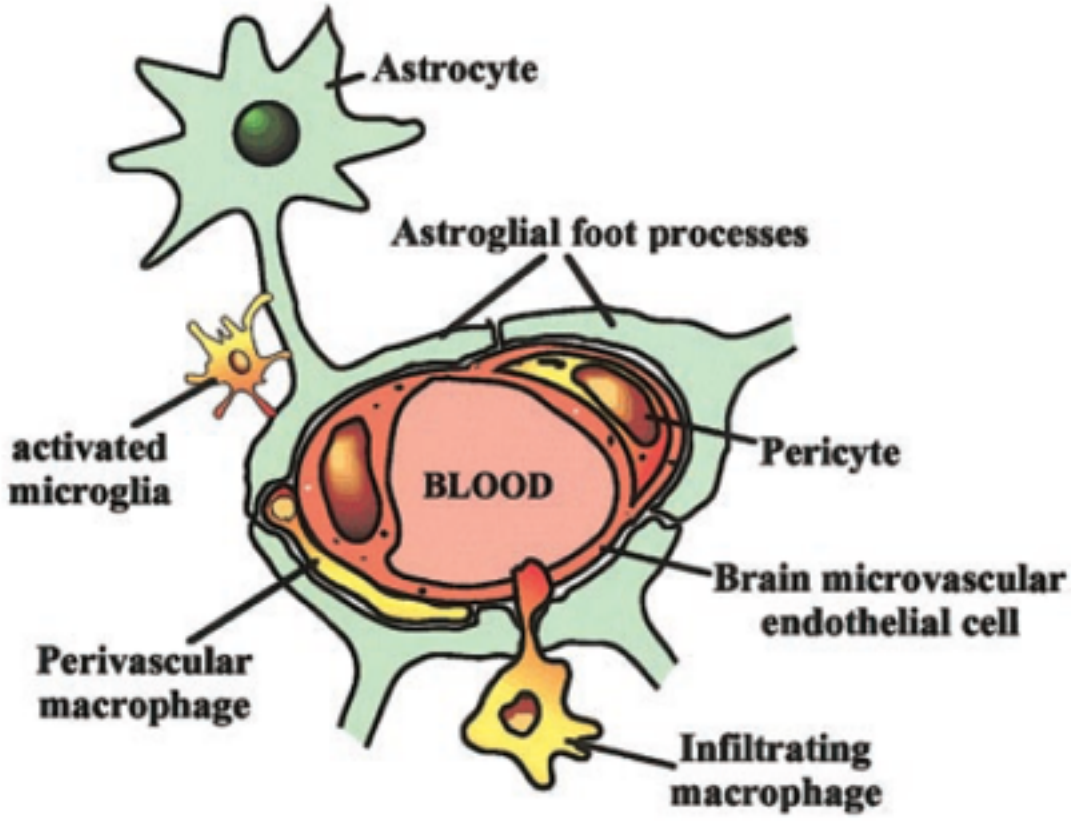

Figura 1. Esquema de la estructural BHE (Adaptado de Guillemin et al., 2003).

Sin embargo, el parénquima cerebral no se encuentra completamente aislado del sistema inmune. En condiciones fisiológicas es posible la circulación de un reducido número leucocitos a través de los diferentes compartimentos cerebrales dentro de un sistema de inmunovigilancia. La gran mayoría de los leucocitos que entran al SNC en condiciones fisiológicas son células T, y pueden pasar de la sangre al LCR a través de los plexos coroideos, directamente al espacio subaracnoideo a través de vénulas postcapilares en la superficie pial, o directamente al parénquima cerebral por medio de los espacios perivasculares (Ransohoff, Kivisäkk et al. 2003). Se sabe que la BHE es más restrictiva a nivel capilar mientras que a nivel precapilar y postcapilar es posible el tráfico celular hacia los espacios perivasculares (Bechmann, Galea et al. 2007).

Por otra parte, es posible que tanto antígenos solubles como CPA cargadas con antígenos cerebrales puedan abandonar el SNC y llegar a alcanzar el tejido linfoide, donde pueda realizarse la presentación antigénica a las células T (Karman, Ling et al. 2004; Walter, Valera et al. 2006). Este drenaje de células y/o de solutos es posible que se realice a través de tres rutas diferentes: 1) a lo largo de trayectos nerviosos, 2) espacios perivasculares, y 3) vía sanguínea.

La vía que ha captado más atención es aquella que implica el drenaje del LCR, el cual se produce principalmente en los plexos coroideos a partir de sangre arterial y en una pequeña proporción a partir del líquido intersticial del SNC. Tras circular por los ventrículos hacia el espacio subaracnoideo, el LCR es reabsorbido por las granulaciones subaracnoideas y se mezcla con el retorno venoso (Ransohoff and Engelhardt 2012). Sin embargo, una pequeña parte del LCR puede drenar como líquido intersticial a través de canales linfáticos alrededor de los nervios craneales y espinales, habiendo sido especialmente estudiado a nivel de los filetes olfatorios en la lámina cribiforme del etmoides (Johnston, Zakharov et al. 2004; Johnston, Zakharov et al. 2005; Walter, Valera et al. 2006). Por otra parte, cuando los vasos arteriales penetran el espacio subaracnoideo para nutrir el parénquima cerebral, se forman unos espacios perivasculares delimitados por piamadre y aracnoides que se mantienen conectados con el espacio subaracnoideo. Son los denominados espacios de Virchow-Robin. A través de estos espacios perivasculares es posible el drenaje de líquido intersticial del parénquima cerebral, representando una fuente de salida de antígenos solubles y de

productos del metabolismo cerebral (Harling-Berg, Park et al. 1999; Ball, Cruz et al. 2009; Ransohoff and Engelhardt 2012). El drenaje de fluido intersticial y solutos procedentes del cerebro a través de estos espacios y a lo largo de la pared vascular pudiera alcanzar los ganglios linfáticos cervicales (GLC) dada la intima relación entre estos y la arteria carótida interna (Clapham, O'Sullivan et al. 2010). Tanto el drenaje a lo largo de trayectos nerviosos como de los espacios perivasculares representan un equivalente al drenaje linfático para el parénquima cerebral, el cual se dirige a los GLC (Weller, Djuanda et al. 2008; Ransohoff and Engelhardt 2012)(Figura 2).

La funcionalidad de estas rutas se ha comprobado en trabajos como los de Hatterer y colaboradores, los cuales mostraron que cuando se injertaban células dendríticas (dendritic cells, DC) en el parénquima cerebral, estas no migraban a los GL, pero si lo hacían cuando se inyectaban en el ventrículo lateral (Hatterer, Davoust et al. 2005). Cuando estas células se inyectaban en animales con encefalomielitis alérgica experimental (EAE), tendían a localizarse en las zonas del parénquima afectadas por la inflamación, encontrándose posteriormente en espacios perivasculares y meninges, y finalmente en las zonas B de los GLC (Hatterer, Touret et al. 2008). También se ha descrito que las células T pueden ser capaces de drenar hacia los GLC a través de la lamina cribiforme del etmoides y ser identificadas en estas estructuras (Goldmann, Kwidzinski et al. 2006). Por otra parte, Carare y colaboradores sugieren que sólo los antígenos solubles de pequeño tamaño pueden abandonar el SNC a través de los espacios perivasculares pero que esta ruta no podría ser empleada por células (Carare, Bernardes-Silva et al. 2008). A nivel de los GLC, estos antígenos solubles, son capaces de desarrollar preferentemente respuestas humorales Th2 y en menor medida respuestas Th1(Harling-Berg, Park et al. 1999).

Dado su proximidad anatómica es posible que la amígala palatina, la cual forma parte del MALT, pueda recibir drenaje linfático del SNC. Las amígdalas palatinas reciben irrigación de la arteria carótida externa, están inervadas por nervios procedentes del ganglio esfenopalatino y su drenaje linfático se encuentra conectado con los GLC (Jácomo, Akamatsu et al. 2010).

Respecto a la vía sanguínea, Hochmeister y colaboradores fueron capaces de seguir el drenaje de DC inyectadas en parénquima cerebral a través del espacio perivascular,

subendotelio y el lumen de los vasos sanguíneos. Estas células se encontraron finalmente en los GL mesentéricos y en el bazo (Hochmeister, Zeitelhofer et al. 2010).

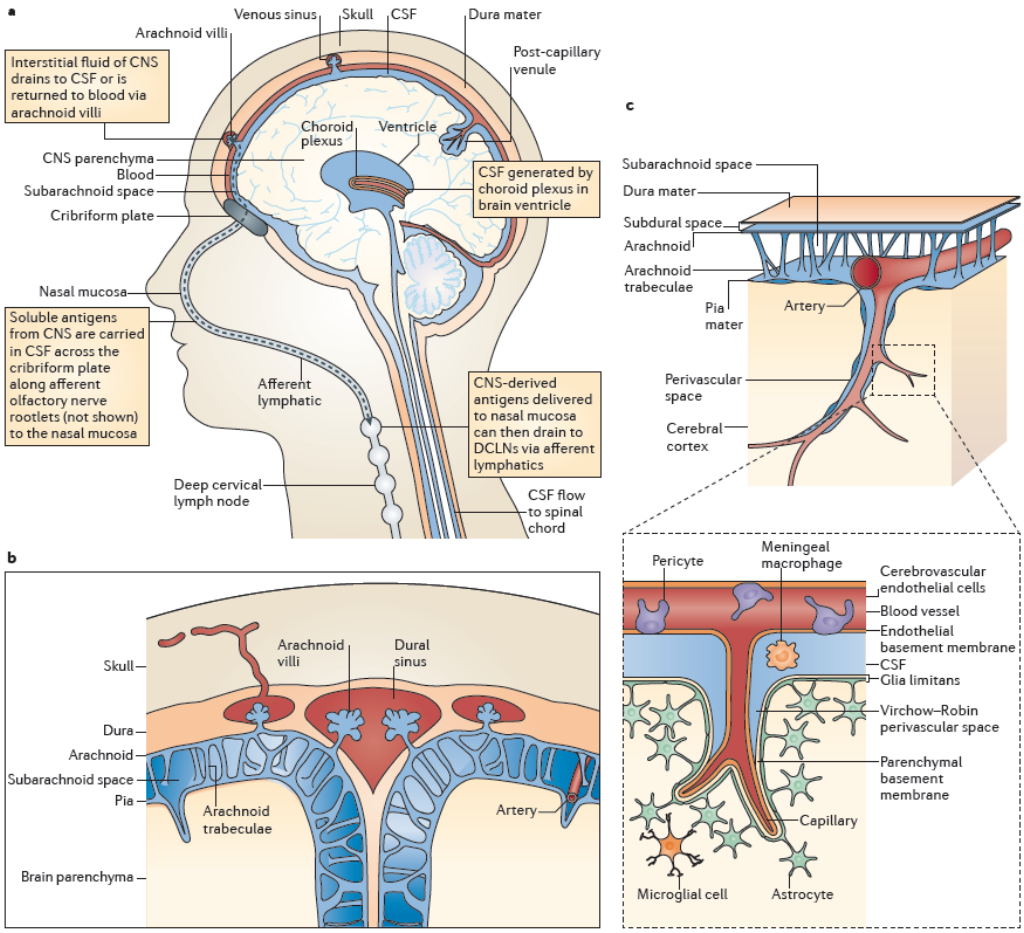

Figura 2. Esquema de vías de drenaje del LCR(adaptado de Ransohoff y Engelhardt, 2012).

A diferencia de los mecanismos de drenaje de antígenos y vigilancia inmune fisiológicos, en situaciones patológicas, la rotura de la BHE podría permitir la entrada de células inflamatorias dentro del SNC, y que antígenos cerebrales "secuestrados" dentro del SNC puedieran entrar en contacto con células inmunes (Engelhardt and Ransohoff 2005). La isquemia cerebral causa un daño directo a la unidad neurovascular ocasionando un aumento de permeabilidad a moléculas y células (Muldoon, Alvarez et al. 2012). Dentro de las células inflamatorias que infiltran el parénquima cerebral, los neutrófilos son las primeras que acceden, seguidas de macrófagos, linfocitos y DC. Los neutrófilos se pueden encontrar entre 30 minutos y unas pocas horas tras la isquemia y

alcanzan su pico entre 1 y 3 días, mientras que los linfocitos infiltran los bordes del infarto en torno al tercer día y aumentan progresivamente hasta el séptimo. Los macrófagos procedentes de la sangre pueden comenzar a infiltrar el cerebro a las 48 h y alcanzan su pico a los 7 días. (Jin, Yang et al. 2010). La máxima entrada de DC periféricas ocurre en torno las 72 h, encontrándose estas células preferente en el centro (core) del infarto, si bien se ha descrito una población de DC residentes cerebrales que tienden a acumularse en la periferia del infarto próximas a células T infiltrantes, que muestran una marcada expresión de moléculas presentadoras y coestimuladorass (Felger, Abe et al. 2010).

La presencia de antígenos cerebrales en el tejido linfoide secundario, especialmente en los GLC ha sido demostrada en diversas condiciones patológicas. Así, en animales con EAE y en pacientes con esclerosis múltiple se ha demostrado la presencia de antígenos mielínicos en los GLC (deVos, van Meurs et al. 2002; Fabriek, Zwemmer et al. 2005). También se han encontrado antígenos cerebrales como la MBP y la proteína asociada a microtúbulos (microtubule associated protein, MAP-2) en GLC de diversos modelos animales de isquemia cerebral (van Zwam, Huizinga et al. 2008). En ambos trabajos, los antígenos cerebrales se encontraban en células que expresaban marcadores característicos de CPA, como CD40 o moléculas MHC. Por otra parte, en el trabajo de van Zwam y colaboradores se observó que el perfil de citocinas expresado por las CPA variaba en función del antígeno presente (van Zwam, Huizinga et al. 2008) .

4. Presentación antigénica y estructura del tejido linfoide.

Una vez los antígenos han llegado al tejido linfoide para su presentación, describiremos la estructura y el destino de los antígenos en el GL por ser más extensamente estudiado.
El GL tiene dos zonas principales: el córtex y la médula. Dentro del córtex se puede diferenciar el paracortex o zona T, y la zona más periférica de células B formada por folículos y centros germinales (figura 3). El paracortex se organiza en cordones celulares rodeados por canales linfáticos organizados por una estructura fibrorreticular (Angel, Chen et al. 2009), diferenciándose una zona con elevada densidad de células T y zonas circundantes más difusas. El “esqueleto” fibroreticular del GL se encuentra formado por una trama fibrilar en cuya composición intervienen fundamentalmente el colágeno y otras proteínas constituyentes de la matriz extracelular como fibronectina y

laminina. Sin embargo, a diferencia de lo que ocurre en otras partes del organismo, los componentes de la matriz extracelular se encuentran rodeados por los fibroblastos que los producen (fibroblastic reticular cells, FRC)(Roozendaal, Mebius et al. 2008). De esta manera se forma una estructura que además de constituir el "esqueleto" del ganglio, establece un entramado de canales o sistema de conducción a través del cual la linfa circula desde el espacio subcapsular hacia la médula del GL. Estos canales se encuentran rodeados por CPA, que son capaces de captar y presentar los antígenos que circulan a través de ellos. De este modo la linfa, que puede transportar antígenos solubles o CPA cargadas con antígenos, entra al GL a través de vasos linfáticos aferentes y se distribuye por el espacio subcapsular, circulando posteriormente a lo largo del paracortex hasta la médula del ganglio y abandonándolo finalmente a través de un vaso linfático eferente (Roozendaal, Mebius et al. 2008; Angel, Chen et al. 2009).

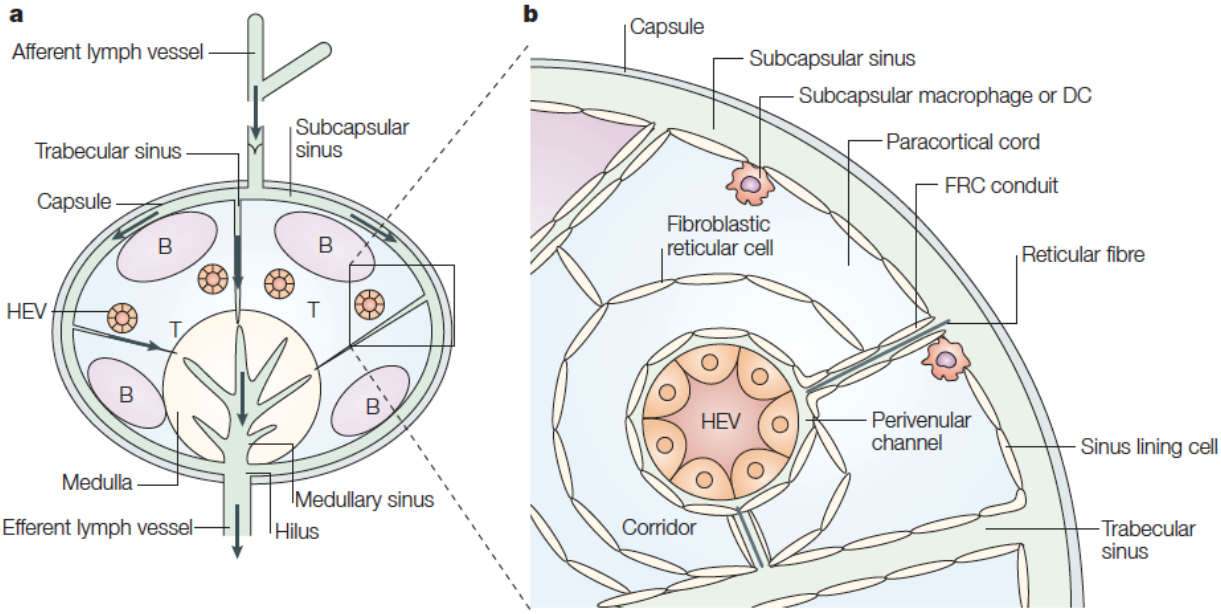

Figura 3. Esquema de la estructura del ganglio linfático (adaptado de von Andrian y Memple, 2003).

La llegada de las CPA al GL y su encuentro con las células Th se encuentra regulado por un complejo entramado de señales compuesto por citocinas y sus respectivos receptores. Así, CCR7 (un receptor de las citocinas CCL19 y CCL21) es expresado por

las CPA maduras y es necesario para la migración hacia el GL, la cual se realiza gracias a un gradiente citoquínico presente en el vaso linfático (Randolph, Angeli et al. 2005). Una vez dentro del GL, las CPA son dirigidas a la zona T donde pueden entrar en contexto con células Th y desarrollar respuestas inmunes efectoras. Los linfocitos pueden entrar en el GL a través de las vénulas de endotelio alto (high endothelium venules, HEV) o vasos linfáticos aferentes. En las HEV la entrada de los linfocitos al parénquima del GL se realiza a través de puntos determinados para posteriormente migrar a lo largo de trayectos trazados por la estructura fibroreticular del GL (Bajénoff, Egen et al. 2006).

La interacción entre CPA y los linfocitos Th se produce en el paracortex del GL, en la frontera entre la zona T y la zona B (Katakai, Hara et al. 2004). La localización tanto de las células linfoides como de las CPA en determinadas zonas del GL también viene determinado por un equilibrio de interacciones receptor-citoquina en el cual intervienen las células que constituyen la trama fibroreticular del ganglio (Katakai, Hara et al. 2004; Bajénoff, Egen et al. 2006). A este respecto, la expresión del receptor CCR7 y el reconocimiento de CCL19 favorece la localización de las células Th en la zona T, mientras el binomio receptor-citoquina CXCR5/CXCL13 favorece la movilización hacia zonas B, necesario para ayudar a las células B (Hardtke, Ohl et al. 2005).

En el proceso de presentación antigénica entre CPA y célula T se alternan diferentes fases. En momentos iniciales las células Th interaccionan de forma breve con múltiples CPA, si bien entre 8 a 12 h tras la entrada de la célula Th en el GL las interacciones CPA-célula Th son más duraderas y se forman agregados. Posteriormente estos agregados se disocian y las células Th presentan de nuevo una mayor movilidad a medida que expresan marcadores de activación y comienzan a proliferar (Mempel, Henrickson et al. 2003; Miller, Safrina et al. 2004). Si la activación de la célula Th no se produce, el linfocito sale del GL a través de los vasos sanguíneos y circula hasta otro GL (von Andrian and Memple 2003).

La activación de las células Th conlleva la expresión de diversos marcadores como CD25, CD40L (CD154) o CD69 (Caruso, Licenziati et al. 1997). Dentro de los marcadores de activación es importante la expresión de CD69, un marcador de activación temprano que se expresa de forma transitoria tras la activación linfocitaria

(Sancho, Gómez et al. 2005). CD69 contribuye a mantener a las células Th secuestradas en los órganos linfoides (Shiow, Rosen et al. 2006), interviene también en el desarrollo de linfocitos memoria Th (Shinoda, Tokoyoda et al. 2012) y su déficit favorece el desarrollo de respuestas inflamatorias (Cruz-Adalia, Jimenez-Borreguero et al. 2010; Martin, Gómez et al. 2010).

5. Las células presentadoras de antígenos.

Tres tipos celulares son capaces de expresar moléculas MHC-II y pueden actuar como CPA: las DC, los macrófagos y los linfocitos B. Si bien los macrófagos y los linfocitos B pueden actuar como CPA, estas lo hacen de manera menos eficiente que las DC, auténticas células profesionales presentadoras de antígenos. Las principales CPA en el GL son las DC, mientras que los macrófagos son más abundantes en órganos no linfoides aunque también se pueden encontrar en el ganglio linfático.

Tanto las DC, como macrófagos y monocitos forman parte del sistema monofagocítico y su capacidad fagocítica les permite la captación de antígenos externos, su procesamiento y presentación por medio de moléculas MHC-II. En este proceso encontramos una primera fase de fagocitosis en la que el antígeno acaba localizado en un compartimento delimitado por membranas denominado fagosoma. Posteriormente este fagosoma se une a lisosomas, formando el denominado fagolisosoma, donde tiene lugar el procesamiento del antígeno y los péptidos resultantes son cargados en moléculas MHC-II, para ser posteriormente presentados en superficie a células Th (Vyas, Van der Veen et al. 2008).

En humanos, todas las DC se caracterizan por expresar niveles elevados de MHC-II (HLA-DR) y carecer de marcadores de otros linajes celulares como CD3 (marcador de célula T), CD19/20(célula B) y CD56 (células natural killer). Si bien hay autores que sugieren que las DC son más un estado funcional que un linaje celular determinado (Hume 2008), se han clasificado clásicamente en DC mieloides (mDC) o plasmocitoides (pDC) atendiendo a la presencia de determinados marcadores celulares. Las mDC expresan CD11c, CD13, CD33 y CD11b, mientras que las pDC se caracterizan por la expresión de CD123, CD303 y CD304. Las mDC se dividen principalmente en una fracción CD1c+ y otra CD141+. Las mDC CD1c+ son la mayor

población de mDC en sangre, tejidos y órganos linfoides y cuando se encuentran en el tejido expresan marcadores de activación como CD86, CD80 y CD40. Estas DC expresan CD11c al igual que los monocitos, pero carecen de los marcadores CD14 y CD16, si bien se ha descrito una fracción CD14+ presente en tejidos y GL que pudieran derivar de monocitos (Collin, McGovern et al. 2013).

De acuerdo a su localización y función las DC clásicamente se ha definido como residentes o migratorias, aunque se puedan considerar algún otro tipo de DC como las presentes en sangre y las DC inflamatorias (Itano and Jenkins 2003; Collin, McGovern et al. 2013). Las DC residentes serían células "inmaduras" localizadas en el GL en íntimo contacto con su entramado trabecular, y estarían encargadas de la captación de antígenos solubles que acceden al GL a través de los vasos linfáticos aferentes. Mientras tanto, las DC migratorias son aquellas que habiendo tenido contacto con un antígeno en tejidos periféricos, migran al GL para presentarlo a células Th (Cavanagh and Weninger 2008). Estas células se localizan inicialmente en el seno subcapsular y posteriormente penetran en el parénquima del GL a través de las zonas interfoliculares, para posteriormente dirigirse hacia el límite del córtex, en la vecindad de las HEV, pero menos unidas a la estructura fibroreticular del GL que las células residentes (Cavanagh and Weninger 2008). Ambos tipos de DC podrían actuar de forma cooperativa, de manera que las DC residentes serían encargadas de seleccionar y retener en el GL las células Th específicas mientras que las DC migratorias inducirían la proliferación de las mismas (Allenspach, Lemos et al. 2008).

En un estudio realizado en GL humano para caracterizar las células presentadoras de antígenos de la piel se han descrito dos grandes poblaciones de CPA. Una población estaba compuesta por células CD209+ que expresan además diferentes combinaciones de otros marcadores: CD206, CD14 y CD68. Estas células se encontraron en el paracortex y/o en los cordones medulares de todos los ganglios estudiados y representarían fundamentalmente la población de CPA residentes. La mayoría de las células CD209+ presentes en los cordones medulares expresaban también los marcadores CD206, CD14 y CD68 perinuclear, encontrándose células similares alineadas a lo largo de la cápsula del ganglio y de las trabéculas que irradian hacia el interior del ganglio. En el paracortex, se encontraron células CD209*low* CD68+ alrededor del folículo linfoide, mientras que aquellas células que expresaban CD209 y

CD14 se encontraban en íntimo contacto con estructuras de colágeno y HEV. Estas CPA podrían corresponder tanto a DC como macrófagos dado que la expresión de alguno de estos marcadores como CD209 o CD206 puede estar presente en ambos tipos celulares (Soilleux, Morris et al. 2002; Wollenberg, Mommaas et al. 2002; Choi, Kashyap et al. 2010). CD68 ha sido utilizado clásicamente como un marcador de macrófagos, aunque puede estar presente en otros tipos celulares como fibroblastos y células endoteliales (Gottfried, Kunz-Schughart et al. 2008) y en DC en un patrón perinuclear (Pavli P, Maxwell L et al. 1996).

Otra población celular estaba compuesta por CPA que expresaban combinaciones de los marcadores CD1a, CD207 y CD208; las cuales no se encontraban presentes en todos los GL y representarían la población de CPA migratorias (Brand, Hunger et al. 1999). Estas células se encontraban limitadas al paracortex del ganglio y de la combinación de diferentes marcadores se definían 3 tipos de CPA: CD1a+ CD207- CD208+/-, CD1a+CD207+CD208+/-, y CD208+CD1a-CD207-. Parte de estos subtipos de células también expresan el marcador CD1b. Las células CD1a+ se encontraban en contacto con estructuras que expresaban fibronectina, mientras que las mayoría de las CD208+ se unían a fibras de colágeno, sin embargo raramente se observaba a ambas en la proximidad de HEV (Angel, Chen et al. 2009).

En el GL también se encuentran presentes macrófagos que pueden actuar como CPA, y que pudieran tener un papel relevante en la presentación de antígenos a las células B (Carrasco and Batista 2007; Martinez-Pomares and Gordon 2007). Clásicamente se han definido como células con elevada capacidad fagocítica presentes en todos los tejidos del organismo que internalízan y degradan partículas y patógenos y liberan mediadores que alertan a el sistema inmune (Gray and Cyster 2012). En estudios en ratón, los macrófagos se encuentran distribuidos en tres localizaciones particulares dentro del GL. De este modo, se puede diferenciar macrófagos del seno subcapsular, macrófagos de los senos medulares y macrófagos de los cordones medulares. El mejor marcador para definir a los macrófagos es la integrina Mac1, también conocida como receptor de complemento CD3, un heterodímero formado por CD11b y CD18. Otros dos marcadores importantes son CD169 o sialoadhesina, que es capaz de unir unas serie de proteínas glicosiladas, y F4/80 o EMR1, cuya función no se encuentra completamente definida.

Los macrófagos asociados a los senos subcapsulares protruyen hacia el seno capsular donde son capaces de captar partículas virales e inmunocomplejos y traslocarlos para ser presentados a células B foliculares (Carrasco and Batista 2007). Los macrófagos asociados a los senos medulares presentan una alta capacidad fagocítica y parece que también son capaces de captar antígenos, mientras que los macrófagos de los cordones medulares pudieran estar implicados en el aclarado de células plasmáticas apoptóticas y en la provisión de sustancias tróficas a las células plasmáticas. En el humano, estas diferentes características fenotípicas entre los macrófagos según su localización no se han encontrado por ahora tan claramente definidas (Gray and Cyster 2012).

6. Señales de alarma y moléculas coadyuvantes: *Heat shock protein* 70.

Como mencionamos previamente, la destrucción celular puede liberar señales de alarma o DAMPS que activan las vertientes innata y adaptativas del sistema inmune (Basu, Binder et al. 2000; Bianchi 2006; Kono and Rock 2008). Entre estas moléculas se encuentran las proteínas de estrés térmico (heat shock proteins o Hsp), un grupo de proteínas altamente conservado entre especies que son sintetizadas como respuesta celular frente a diversos estímulos nocivos, como altas temperaturas, estrés oxidativo o tóxicos (Williams and Ireland 2007). Dentro de ellas se diferencian varios subtipos dependiendo de su peso molecular, siendo Hsp-70 uno de los más estudiados.

Mientras que la expresión de Hsp-70 es prácticamente indetectable en el cerebro en condiciones fisiológicas, su producción se encuentra aumentada de manera importante en situación de isquemia (Nowak, J. et al. 1990; Kinouchi, Sharp et al. 1993; Planas, Soriano et al. 1997; Sharp, Lu et al. 2000; de la Rosa, Santalucía et al. 2013). Durante la isquemia cerebral Hsp-70 es inducida en gran cantidad por células viables y morfológicamente intactas pero no en aquellas severamente dañadas. Ello ha llevado a que la expresión de Hsp-70 se pudiera considerar el correlato molecular de la penumbra isquémica (Li, Chopp et al. 1992; Hata, Maeda et al. 2000; Hata, Maeda et al. 2000), siendo las neuronas las células más sensibles y las que antes producen Hsp-70, seguidas de las células gliales, y las células endoteliales (Li, Chopp et al. 1992; Kinouchi, Sharp et al. 1993; Planas, Soriano et al. 1997).

Abundante evidencia experimental muestra que Hsp-70 tiene un papel neuroprotector en la isquemia cerebral (Nowak and Jacewicz 1994; Yenari, Fink et al. 1998; Yenari, Giffard et al. 1999; Yenari 2002) probablemente debido a sus funciones como chaperona, ayudando en la estabilización y reparación de péptidos dañados (Yenari, Giffard et al. 1999; Hartl and Hayer-Hartl 2002), y sus propiedades antiapoptóticas (Yenari, Liu et al. 2005). De este modo, ratones *knock-out* para Hsp70.1 presentan mayor volumen de infarto tras inducción transitoria de isquemia focal (Lee, Kim et al. 2001), mientras que ratones transgénicos expresando Hsp-70 se encuentran protegidos frente al daño isquémico(Plumier, Krueger et al. 1997; Rajdev, Hara et al. 2000; Tsuchiya, Hong et al. 2003; Xu, Emery et al. 2010). Además, la administración exógena de Hsp-70 en modelos de isquemia/reperfusión ya sea a través de un vector viral o dentro de una proteína de fusión también ha mostrado efectos neuroprotectores (Badin, Lythgoe et al. 2005; Doeppner, Nagel et al. 2009; Zhan, Ander et al. 2010).

Por otra parte, debido a su papel como DAMP, Hsp-70 puede promover la secreción de óxido nítrico (NO), citocinas inflamatorias y quimiocinas por las CPA, así como la maduración de estas y su migración a los GL(Basu, Binder et al. 2000; Saito, Dai et al. 2005).

Debido a sus funciones de chaperona, Hsp-70 también puede unirse a péptidos derivados de células necróticas y así facilitar su presentación por moléculas MHC; un proceso llamado cross-presentation (Srivastava 2002; Binder and Srivastava 2005). Los complejos péptido-Hsp-70 pueden modular la respuesta inmune mostrado propiedades proinflamatorias y antiinflamatorias (Quintana and Cohen 2005). Este mecanismo de presentación antigénica se ha considerado especialmente eficaz en la inducción de respuestas CD8 y puede contribuir a desarrollar respuestas autoinmunes frente a antígenos propios (Millar, Garza et al. 2003; Binder and Srivastava 2005). Sin embargo, Hsp-70 ha mostrado que también puede reducir la estimulación de células T mediada por la acción de DC (Stocki, Wang et al. 2012), inhibir la migración de células T (Zanin-Zhorov, Tal et al. 2005) y ha sido implicada en la inducción de citocinas antiinflamatorias como IL-10 por células T (Tanaka, Kimura et al. 1999). Por tanto, además de un efecto anti-apoptótico (Yenari, Liu et al. 2005) y antiinflamatorio (Zheng, Kim et al. 2008) en la isquemia cerebral, es posible que Hsp-70 también atenúe la respuesta inmune.

En este contexto, el objetivo principal de la presente doctoral es determinar si en el tejido linfoide de los pacientes con ictus se encuentran todos los elementos necesarios para el desarrollo de una respuesta inmune adaptativa frente a los antígenos cerebrales y su posible influencia en la evolución de la enfermedad.

III.- Hipótesis

Tanto modelos experimentales como diversos procesos patológicos del SNC han mostrado que algunos antígenos cerebrales pueden alcanzar el tejido linfoide secundario. Esta tesis pretende estudiar si este fenómeno se encuentra presente y desempeña algún papel en el paciente con ictus. Para ello, planteamos las siguientes hipótesis:

1. Durante el ictus agudo se produce una llegada de antígenos cerebrales al tejido linfoide secundario.

2. Esos antígenos son capturados y presentados por células presentadoras de antígenos.

3. La presentación de antígenos neurales en el tejido linfoide secundario podría tener un papel en la fisiopatología y pronóstico del ictus.

IV.- Objetivos

1. Cuantificar diversos antígenos cerebrales (MBP, MAP-2, NR-2A) y moléculas coadyuvantes (Hsp-70) en tejido linfoide secundario (amígdala palatina y ganglios linfáticos cervicales) de pacientes con ictus y controles.

2. Describir la distribución de estos antígenos cerebrales y moléculas coadyuvantes en el tejido linfoide secundario y su relación con células presentadoras de antígenos, células linfoides y estructura fibroreticular.

3. Determinar la relación de antígenos cerebrales y moléculas coadyuvantes con la severidad clínica, volumen de lesión y pronóstico del ictus.

V.- Resultados

Trabajo número 1

Brain-Derived Antigens in Lymphoid Tissue of Patients with Acute Stroke.

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Objetivos

Describir la presencia y localización de antígenos cerebrales en la amígdala palatina y ganglios linfáticos cervicales de pacientes con ictus y en sujetos controles.

Determinar la potencial relación de la presencia de antígenos cerebrales en el tejido linfoide con el curso clínico y el pronóstico del ictus.

Resultados

Todos los antígenos cerebrales estudiados estuvieron presentes en mayor cantidad en amígdala palatina y ganglios linfáticos cervicales de pacientes con ictus que en sujetos control. Dentro del tejido linfoide, estos antígenos se encontraron preferentemente en las zonas T, localizados dentro de células presentadoras de antígenos y en la vecindad de estructuras ricas en fibronectina. Los pacientes que mostraban mayor inmunoreactividad a antígenos neuronales como MAP-2 y NR-2a presentaron infartos más pequeños y mejor pronóstico que aquellos pacientes que presentaban más inmunoreactivdad a antígenos mielínicos. Los pacientes con ictus también mostraron un mayor número de células T que expresaban el marcador de activación temprano CD69.

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Brain-Derived Antigens in Lymphoid Tissue of Patients with Acute Stroke

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In experimental animals, the presence of brain-derived constituents in cervical lymph nodes has been associated with the activation of local lymphocytes poised to minimize the inflammatory response after acute brain injury. In this study, we assessed whether this immune crosstalk also existed in stroke patients. We studied the clinical course, neuroimaging, and immunoreactivity to neuronal derived Ags (microtubule-associated protein-2 and *N*-methyl D-aspartate receptor subunit NR-2A), and myelin-derived Ags (myelin basic protein and myelin oligodendrocyte glycoprotein) in palatine tonsils and cervical lymph nodes of 28 acute stroke patients and 17 individuals free of neurologic disease. Stroke patients showed greater immunoreactivity to all brain Ags assessed compared with controls, predominantly in T cell zones. Most brain immunoreactive cells were CD68$^+$ macrophages expressing MHC class II receptors. Increased reactivity to neuronal-derived Ags was correlated with smaller infarctions and better long-term outcome, whereas greater reactivity to myelin basic protein was correlated with stroke severity on admission, larger infarctions, and worse outcome at follow-up. Patients also had more CD69$^+$ T cells than controls, indicative of T cell activation. Overall, the study showed in patients with acute stroke the presence of myelin and neuronal Ags associated with lymph node macrophages located near activated T cells. Whether the outcome of acute stroke is influenced by Ag-specific activation of immune responses mediated by CD69 lymphocytes deserves further investigation. ***The Journal of Immunology*****, 2012, 188: 000–000.**

The CNS is regarded as an immune-privileged site sequestered from the immune system by the blood-brain barrier and the lack of CNS-draining lymphatic vessels (1, 2). However, the importance of immunity in the pathobiology of acute stroke is increasingly recognized (3). In stroke patients, it was recently described a syndrome of stroke-induced immunodepression (SIID), characterized by a decline of circulating lymphocytes, and reduced inflammatory drive of mononuclear cells that increased the risk of nosocomial infections and influenced the mortality rate (4–6). Nonetheless, SIID is regarded as an adaptive response to limit local inflammation after acute brain damage (7), although the underlying cellular, molecular, and immunologic mechanisms, and functional consequences of SIID, are still debated (8).

A significant number of leukocytes may enter the CNS across the blood-CNS barriers during inflammation and encounter different Ags presented by MHC class II (MHC-II) positive APCs, such as macrophages and dendritic cells (9). APCs and soluble Ags may leave the CNS and reach the cervical lymph nodes (CLNs) via the blood stream (10). In addition, molecules can reach the CLN by bulk flow of the intracranial fluids (11, 12). Indeed, brain-derived Ags or APCs carrying these Ags can reach the CLN under experimental conditions, (13–16) and the presence of brain-derived Ags in the CLN is recognized in human anatomic studies (17, 18) and in sporadic clinical descriptions (13, 19, 20) of patients with multiple sclerosis.

Several lines of evidence support that the lymphatic drainage of the CNS might contribute to shut down neuroinflammatory responses after brain injury, in view of the findings showing that lymphocytes isolated from the CLN emphasized anti-inflammatory responses and suppressed proinflammatory signals (11, 21). Nonetheless, it is unknown whether the reactivity of lymphocytes to brain-derived Ags is nonspecific, or it is directed against specific brain Ags (22). We evaluated the presence of brain-derived Ags in lymphoid tissue of patients with acute stroke, and explored whether this could affect their clinical condition. The main results of this investigation highlight the relevance of the dialogue between the CNS and the immune system in patients with brain ischemia.

Materials and Methods

Patients

We studied 22 stroke patients who did not have ongoing infection and had not received antibiotics, steroids, or immunosuppressants within the preceding 3 mo. Neurologic function was first assessed within 12 h of clinical onset, daily until discharge, and at day 90 with the National Institutes of Health Stroke Scale (NIHSS). The volume of brain infarction was measured at mean (SD) day 7 (2) after stroke on diffusion-weighted magnetic resonance imaging (MRIcro software, Chris Roden, University of Nottingham, Nottingham, U.K.). Functional status was measured at 3 mo with the modified Rankin Scale (mRS), and defined as good outcome when the mRS was 0–2. The study was approved by the local ethics committee and

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The online version of this article contains supplemental material.

Abbreviations used in this article: CLN, cervical lymph node; MHC-II, MHC class II; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; NMDA, *N*-methyl-D-aspartate; PT, palatine tonsil; SIID, stroke-induced immunodepression.

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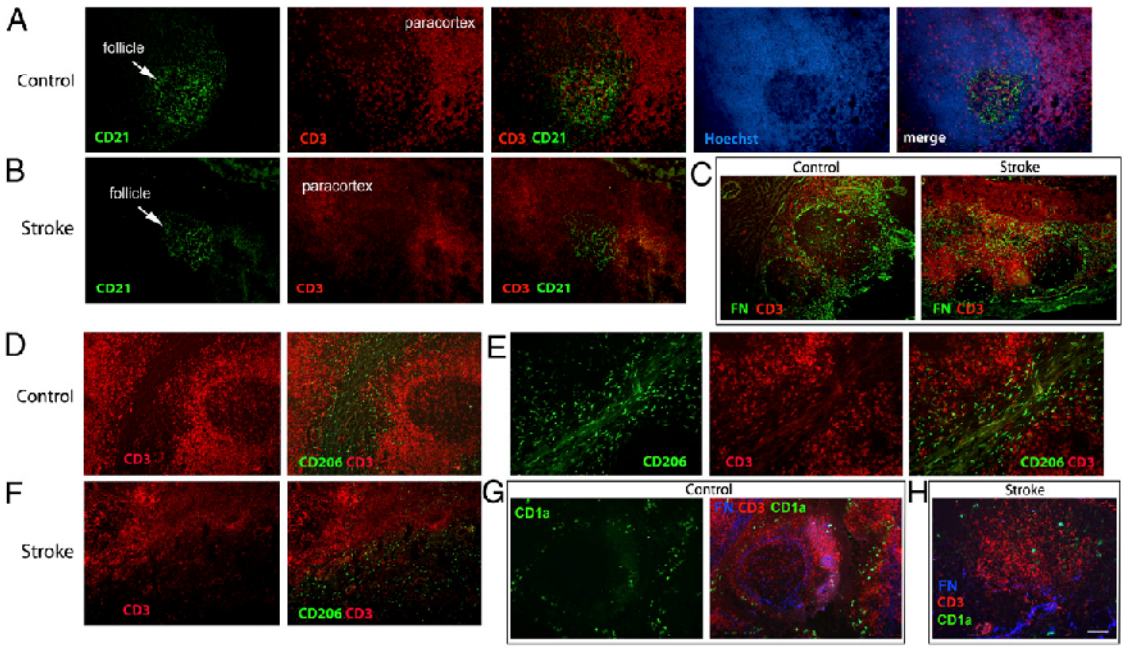

FIGURE 1. Lymphocytes and APCs in palatine tonsils of controls and stroke patients. Biopsy specimens of PTs of controls and stroke patients were studied by immunofluorescence. PTs of controls (**A**) and patients (**B**) show CD21 (green) immunostaining in the follicles, surrounded by enriched T cell areas ($CD3^+$, red) in the paracortex. Cell nuclei are stained in blue with Hoechst. (**C**) The fibroreticular mesh immunoreactive for fibronectin (FN, green) surrounds the follicles and extends through T cell-rich areas (CD3, red). (**D**–**F**) Numerous dendritic cells expressing CD206 (green) are located between T cell-rich areas ($CD3^+$, red) outside the follicular zones in controls (D, E) and patients (F). (**G** and **H**) $CD1a^+$ migratory dendritic cells (green) are observed aligned at the borders of T cell-rich areas ($CD3^+$, red), not far from the fibroreticular mesh stained with fibronectin (FN, blue). Scale bars, 200 μm (C, G), 100 μm (A, B, D, F, H), 50 μm (E).

participants or their relatives signed a written informed consent according to the Declaration of Helsinki.

In vivo and post mortem study of lymphoid tissue

We performed palatine tonsil (PT) biopsies in 22 patients and 11 controls (8 obstructive sleep apnea, and 3 healthy volunteers), at mean (SD) 76 (34) hours from stroke onset, based on the assumption that PT may receive immune signals and orchestrate an adaptive immune response (23). These lymph nodes are irrigated by branches of the external carotid artery, receive innervation from nerves derived from the sphenopalatine ganglion, and have lymphatic vessels that reach the deep CLNs (24). It is arguable that alike the CLN, the PT might also receive immunogenic signals from the CNS, via either the blood stream or the bulk flow of intracranial fluids. PT biopsy specimens were used for flow cytometry or were snap frozen in OCT and stored at −80°C, and lymphocytes were analyzed using standard surface flow cytometry by blinded investigators to clinical data. CLN and

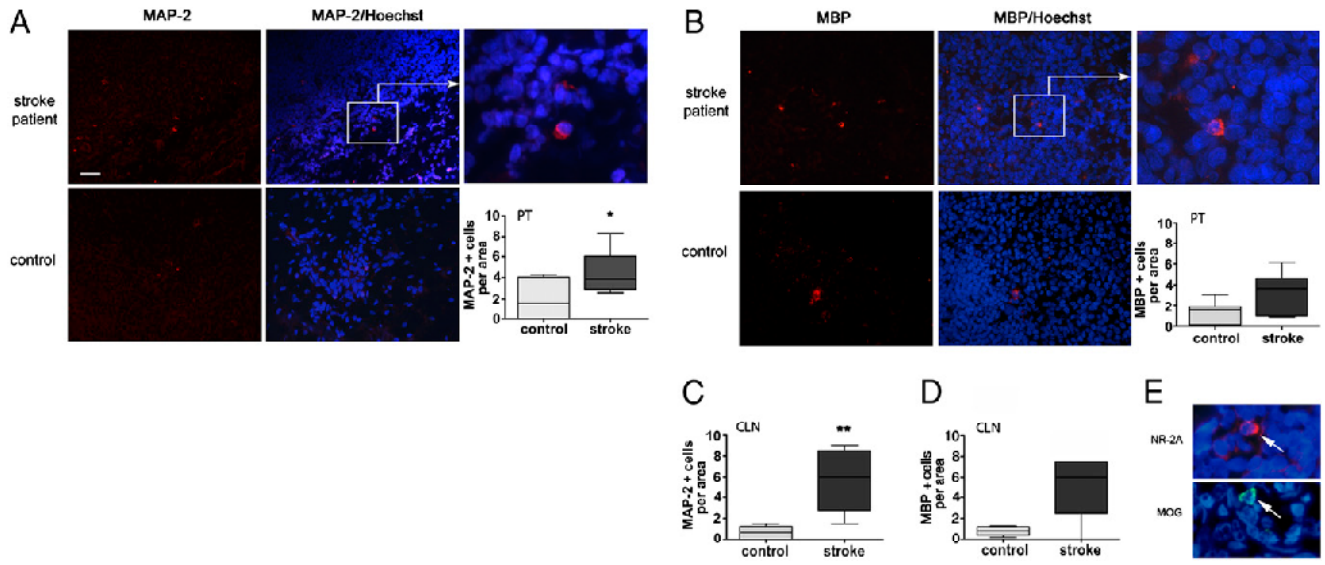

FIGURE 2. Cells immunoreactive to brain Ags are found in human lymphoid tissue samples and are more abundant in stroke patients than in controls. Biopsy specimens PTs (n = 6 stroke; n = 7 controls; **A**–**C**) and necropsy specimens of CLNs (n = 5 stroke; n = 5 controls; **D**, **E**) were used for immunofluorescence staining (red) against MAP2 (A, D) or MBP (B, C, E). MAP2 (A) and MBP (B) immunoreactive cells were observed both in patients and in controls. Patients had a greater number of $MAP2^+$ cells (A) and a trend to greater number of MBP^+ cells (B) than controls in PTs and in CLNs (C, D). E, Cells immunoreactive to the NMDA receptor NR-2A (red) and to MOG (green) in the PTs of stroke patients. Nuclei are stained in blue with Hoechst. Scale bar, 50 μm (A, B); magnified *insets* in (A) and (B) and images in (E), 15 μm. $*p = 0.05$, $**p < 0.05$.

PT were also obtained post mortem in 6 patients with malignant brain infarction, mean (SD) age 80 (7) years, and 6 controls, mean (SD) age 62 (17) years free of CNS disease at autopsy.

Flow cytometry of palatine tonsils

A cellular suspension of PT was obtained after repetitive squirting with culture medium using a fine needle, and 100 mL of suspension containing at least 2.5×10^5 cells were used for each test. Lymphocyte subsets were analyzed using standard surface flow cytometry with the following monoclonal Abs: CD25 and CD19 (FITC), CD3, CD4 and CD86 (PE), CD45, CD8 and CD4 (peridinin-chlorophyll), and CD3 (allophycocyanin; BD Biosciences). The following Ab combinations were used to identify lymphocyte subsets: $CD45^+$ for lymphocytes, $CD3^+$ for T cells, $CD3^+CD4^+$ for Th cells, $CD3^+CD8^+$ for CTLs, $CD3^-CD56^+$ for NK cells, and $CD19^+$ for B cells. Cell acquisition was performed on a FACSCalibur flow cytometer (Becton Dickinson) and CellQuest-software (Becton Dickinson) was used for analysis.

Immunofluorescence

Two-micrometer-thick frozen tissue sections were dried, washed, and fixed in ethanol 70% for 10 min. Tissue was immersed in 0.3% Triton X-100 in PBS for 10 min, blocked with 5% normal serum for 1 h, and incubated with primary Abs overnight at 4°C in the presence of 5% normal serum, followed by secondary Abs. A list of the Abs used is provided in Supplemental Table I. For double immunofluorescence, only combinations of Abs produced in different animal species were performed. Specificity of the immunoreaction was verified by omission of one of the primary Abs (Supplemental Fig. 1). Samples were counterstained with Hoechst. Observations were performed with an Olympus IX70 fluorescence microscope. Selected samples were examined with a confocal microscope (SP5, Leica). Pictures of control and stroke samples were taken using the same microscopic settings, and the samples were labeled with a code that did not reveal the group of the sample. The number of positive immunoreactive cells was counted in a blinded fashion in areas of each sample photographed under the ×40 objective. The mean value obtained in three to five areas was taken as representative of the sample value. Two or three different observers independently performed cell counting, and the mean value obtained by them was taken as the value for each sample.

Western blotting

Frozen PT biopsy tissue was homogenized in lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (Complete; Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA). Twenty micrograms of protein extracts were resolved by 8–10% SDS-PAGE, and Western blotting was performed as reported (25). Primary Abs were as follows: rabbit polyclonal Abs against the *N*-methyl-D-aspartate (NMDA) receptor subunit NR-2A (no. AB155, diluted 1:100, Chemicon; and no. G903, diluted 1:250, Sigma) and a mouse mAb against MAP2 (Sigma, diluted 1:100). Mouse monoclonal Abs against GAPDH (no. CSA-335, Stressgen) diluted 1:500 or β-tubulin (no. T4020, Sigma) diluted 1:5000, were used as protein gel loading controls. For quan-

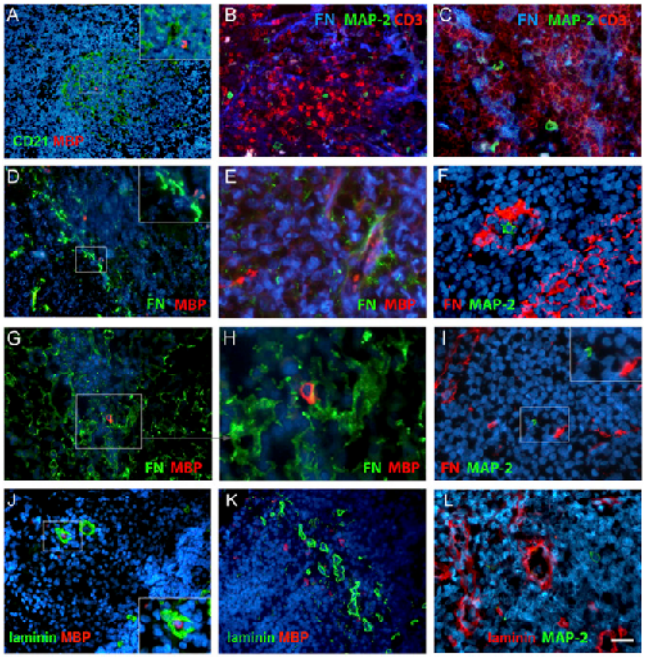

FIGURE 3. Localization of cells immunoreactive to neural Ags. (**A**–**C**) Cells immunoreactive to neural Ags do not express markers of B (CD21, green) or T (CD3, red) cells, as illustrated for MBP (red) in (A) and for MAP2 (green) in (B) and (C). Blue staining in (A) corresponds to Hoechst to visualize the nuclei, whereas blue in (B) and (C) corresponds to fibronectin (FN) to visualize the fibroreticular network. MAP2 and MBP immunoreactive cells are normally seen within T cell-rich areas (B, C), whereas these cells are rarely seen within the follicles (A). This pattern of localization is similar in patients and controls. (A) A control PT biopsy. (B and C) A stroke CLN necropsy. (**D**–**I**) MBP (red in D, E, G, H) and MAP2 (green in F and I) immunoreactive cells are seen in the proximity of the fibroreticular network immunostained with FN (green in D, E, G, and H; red in F and I). PT of controls (D–F) and patients (G–I). (**J**–**L**) MBP (J and K, red) and MAP2 (L, green) immunoreactive cells are occasionally seen nearby blood vessels immunostained with laminin in PT (green in J and K; red in L). (J and K) Necropsy specimens from a control (J) and a stroke patient (K). (L) Biopsy specimen of a control patient. Scale bars, 200 μm (A), 100 μm (J, K), 50 μm (B, D, G), 30 μm (F, I, L, *insets* in A and J), 25 μm (C, E, and *inset* in D), 20 μm (*inset* in I), 15 μm (H).

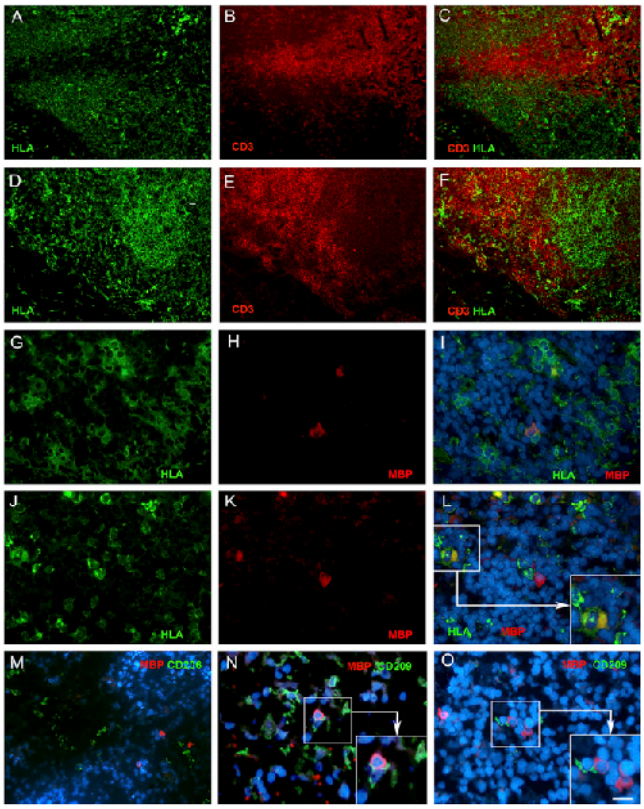

FIGURE 4. Neural Ag immunoreactive cells are APCs. APCs expressing HLA (**A**–**F**, green) are abundant in PTs. They are mainly located in the follicles and surrounding T cell-rich areas ($CD3^+$, red in A–F), and occasionally within T cell areas, both in controls (A–C) and patients (D–F). (**G**–**I**) A control PT biopsy specimen. (**J**–**O**) CLN necropsy specimens from stroke patients. (G–L) Some cells immunoreactive to MBP (red) express HLA (green) in controls (I), and more frequently in patients (L and *inset*). (M) MBP-immunoreactive cells (red) do not express CD206 (green). (N and O) Likewise, MBP-immunoreactive cells (red) do not express CD209 (DC-SIGN), but they are frequently in contact with $CD209^+$ cells (see *insets* in N and O). Nuclei (I, L–O) are stained in blue with Hoechst. Scale bars, 200 μm (A–F), 30 μm (G–L), 20 μm (*inset* in L), 50 μm (M), 25 μm (N, O), 15 μm (*insets* in N and O).

tification, the OD of selected Western blotting bands was measured and normalized by the control protein. Samples from control and stroke subjects were run in each gel. The value of each sample was expressed as the percentage of the mean value of the samples of the control subjects per gel. Rat brain tissue was used as a positive control for brain proteins.

RNA studies

Total RNA was extracted from frozen PT biopsy samples using RNeasy minikits and the RNAse-Free DNAse Set (Qiagen, Germantown, MA). The RNA quantity and quality were determined with Nanodrop and Agilent analyzers. RNA from postmortem human brain tissue was used as positive control. Eight hundred micrograms of RNA were used for cDNA synthesis using the AMV First-Strand cDNA Syhthesis Kit (no. 12328-040; Invitrogen, Carlsbad, CA). Two microliters of cDNA synthesis reaction were used in a 25-μl real-time PCR reaction with FAM-labeled Taqman Gene Expression Assays for NR-2A (NMDAR2a; Hs00168219_m1) and MAP2 (Hs01103234_g1; Applied Biosystems, Foster City, CA). β-Actin (Hs03023943_g1) was used as the reference control gene. The amplification conditions were: 10 min at 95˚C followed by 40 cycles of 15 s at 95˚C, 1 min at 60˚C. CT values were analyzed using the 2-ΔΔCT method.

Statistical analysis

Fisher's exact test, Student *t* test, or Mann–Whitney *U* test were used as appropriate. Correlations were calculated with the Pearson or Spearman Rank correlation coefficient. Linear regression models were age adjusted, and the level of statistical significance was corrected for multiple testing using the Bonferroni *t* method. Statistical analysis was performed with SPSS 17.0 statistics package (SSPS, Chicago, IL).

Results

Study population

Patients were older than controls, mean (SD) 69.5 (12.7) versus 53.0 (16.0) years ($p = 0.003$), but disclosed similar risk factors, including hypertension (61%), diabetes (27%), high cholesterol (39%), coronary artery disease (18%), and smoking (15%). Patients had large vessel atherosclerotic disease ($n = 6$), cardioembolism ($n = 7$), or stroke of unknown cause ($n = 9$). The median (interquartile range) NIHSS score on admission was 10 (4–14), and 3 (1–7) at day 90. The median (interquartile range) infarction volume at day 7 was 21 (5–56) ml. At 3 mo, 10 patients (46%) had good outcome (mRS 0-2), and none of the participants experienced any complications because of PT biopsy.

Secondary lymph node architecture and lymphocyte subsets

Patients and controls disclosed germinal centers containing mature B lymphocytes ($CD21^+$), and interfollicular areas enriched in T cells ($CD3^+$) in the paracortex of PT. The germinal centers were less prominent in the lymphoid tissue of patients than of controls (Fig. 1A, 1B). After staining for fibronectin, both groups showed a reticular meshwork surrounding the follicles, trabeculae, endothelium of blood vessels, and connective tissue underlying the epithelium (Fig. 1C), and medullary cords with APCs expressing the pattern recognition mannose receptor C type 1 CD206 (Fig. 1D–

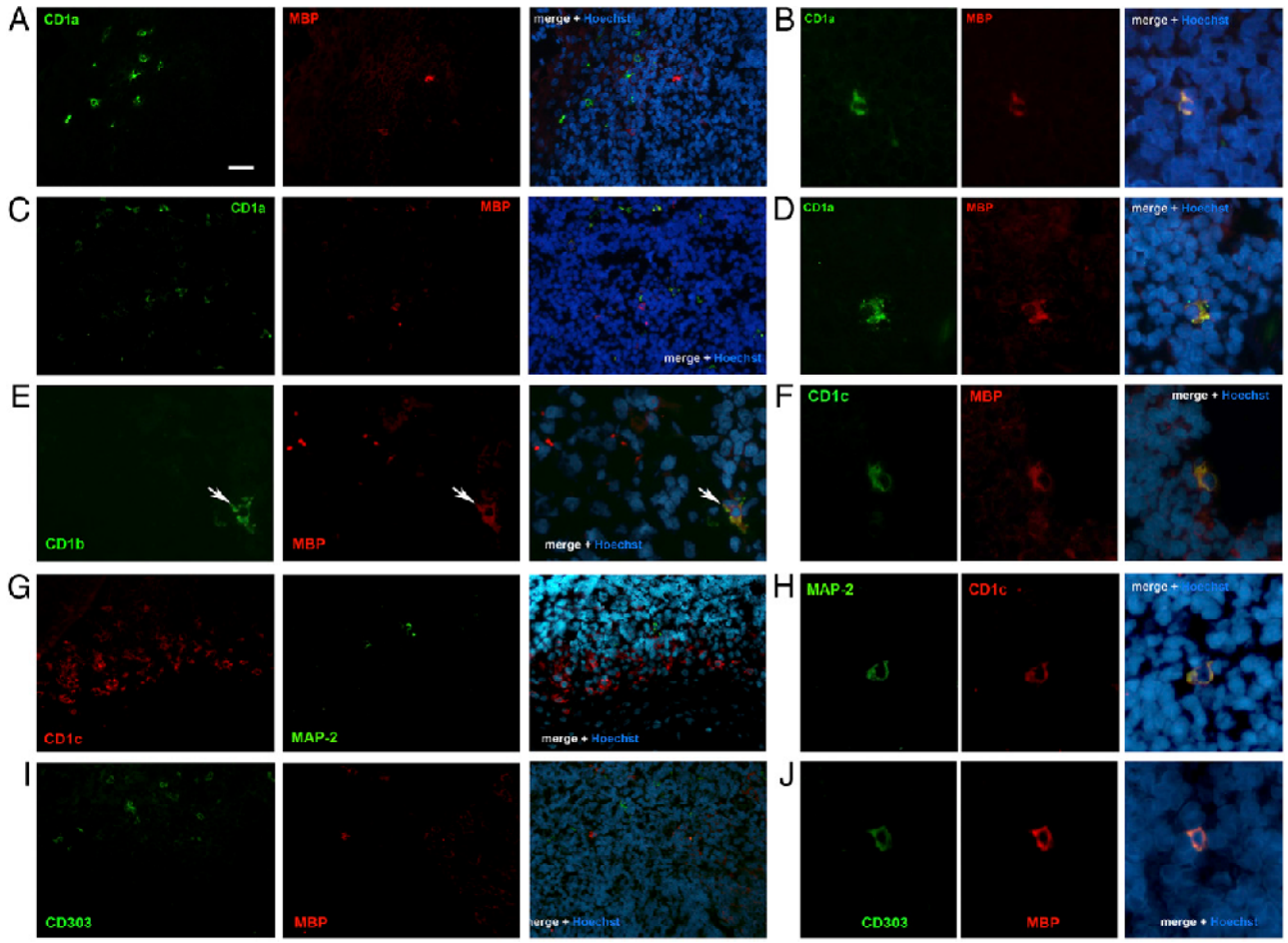

FIGURE 5. Few cells immunoreactive to neural Ags express dendritic cell markers. The following dendritic cell markers were found in lymphoid tissue of controls and patients: CD1a (**A**–**D**), CD1b (**E**), CD1c (**F**–**H**), and CD303 (**I**–**J**). Frequently, dendritic cell makers did not colocalize with immunoreactivity to neural Ags (A, C, E, G, I). However, several cells immunoreactive to MBP (red in B, D, E [arrow], F, and J) or MAP2 (green in H) coexpress one of the above dendritic cell markers in both controls (A, B, F, G–I) and patients (C, D, E). PT biopsy specimens (A, B, F, G–I) and (C–E) necropsy specimens of CLNs. Scale bars, 50 μm (A, C, G, I), 20 μm (E), 17 μm (B, D, F, H, J).

F). APCs expressing CD1a, a marker of migratory dendritic cells (26), were mainly found at the borders of the T cell areas and following the fibronectin mesh (Fig. 1G, 1H). Similar findings were observed in PT and CLN. Patients and controls disclosed similar proportions of lymphocyte subsets in the PT as assessed by flow cytometry (Supplemental Fig. 2A). The small number of macrophages in PT (Supplemental Fig. 2B) precluded a study of these cells by flow cytometry because of the limited amount of sample material.

Presence of brain Ags is increased in lymphoid tissue after acute stroke

The number of neuronal MAP2-immunopositive cells was higher ($p < 0.05$) in stroke patients compared with controls in PT (Fig. 2A) and CLN (Fig. 2C). Patients had more myelin basic protein (MBP)-immunopositive cells in PT (Fig. 2B) and CLN (Fig. 2D), but the increment was not statistically significant after age adjustment ($p = 0.60$). NMDA receptor subunit NR-2A positive cells (Fig. 2E), and myelin oligodendrocyte glycoprotein (MOG)-positive cells (Fig. 2E) were also found in patients and controls, but group comparisons were not performed for insufficient material. Cells immunoreactive to neural Ags were scarce within the follicles (Fig. 3A), prevailed in T cell-enriched zones (Fig. 3B, 3C) and nearby or in contact to fibronectin staining (Fig. 3D–I), and were rare near the blood vessels (Fig. 3J–L), both in patients and controls.

Cells immunoreactive to neural Ags express markers of APCs

of B or T lymphocytes (Fig. 3A–C) but expressed MHC-II HLA in patients and controls (Fig. 4A–F). MBP-immunopositive cells co-localized with HLA more markedly in patients than in controls (Fig. 4G–L). Cells loaded with brain-derived Ags did not coexpress C-type lectin receptors CD206 (Fig. 4M) or DC-SIGN (CD209; Fig. 4N, 4O). However, these cells were often seen adjacent to DC-SIGN$^+$ dendritic cells (Fig. 4N, 4O).

Cells immunoreactive to neural Ags are compatible with macrophages and a few are dendritic cells

In patients and controls, only few MBP or MAP2 immunopositive cells expressed the dendritic cell markers CD1a, CD1b, and CD1c (BDCA-1; Fig. 5A–H), or CD303 (BDCA-2; Fig. 5I, 5J), a molecule that can inhibit IFN-α/β synthesis (27). Quantification of the numbers of CD1a$^+$ cells showed no differences between stroke patients and controls (Supplemental Fig. 3). MBP and MAP2 were detected in (mean ± SD) 15.3 ± 23 and 18.2 ± 28% of CD1a$^+$ cells, respectively, in PTs of stroke patients. Alternatively, CD68$^+$ cells were more abundant in lymphoid tissue from patients than controls (Fig. 6), and cells double-immunoreactive to brain-derived Ags and CD68 had a morphology consistent with macrophages (Fig. 6F–L), with large cytoplasm and often patches of immunostaining for brain-derived Ags (see *inset* in Fig. 6F–I). Quantification of the numbers of CD68$^+$ cells showed more cells in samples of stroke patients than in controls, with statistically significant differences for the CLNs ($p = 0.02$; Fig. 6M) and a statistical trend ($p = 0.08$) in the PT (Fig. 6N). MAP2 immunoreactivity was found in (mean ± SD) 44.7 ± 30% of CD68$^+$ cells, whereas

Western blotting showed that MAP2 and NR-2A immunoreactivity in PT corresponded to bands at a molecular mass similar to

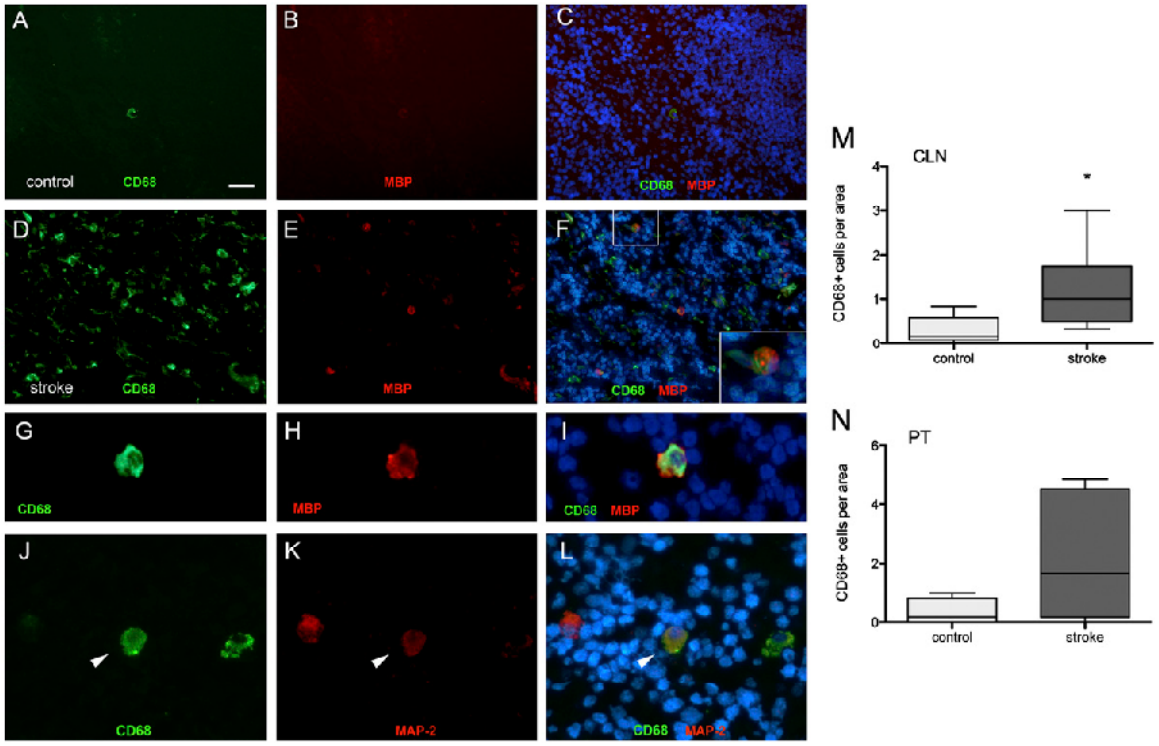

FIGURE 6. Neural Ags are often found in CD68$^+$ macrophages and more abundantly in stroke patients. CD68$^+$ macrophages were seen in lymphoid tissue of controls (A–D) and stroke patients (D–L), but were more abundant in the tissue samples of stroke patients. (**A–I**) MBP immunoreactive cells (red) are often positive for CD68 (green) (D–I), suggesting that at least some of them are macrophages. (G–I) The morphology of these cells is also consistent with macrophages. Patches of MBP immunoreactivity are observed within the cells. (**J–L**) Some of the CD68$^+$ cells (green) are immunoreactive for MAP2 (red). Nuclei are stained in blue with Hoechst. Arrowhead points to a double stained cell. (**M** and **N**) Quantification of the numbers of CD68$^+$ cells per area shows higher numbers in lymphoid tissue of stroke patients than in control tissue in the CLNs (*M*; $n = 9$ patients and $n = 5$ controls) and the PTs (*N*; $n = 9$ patients and $n = 4$ controls). The statistical significance is indicated by the p value: $p = 0.02$ for the CLN (*), and $p = 0.08$ for the PT (Mann–Whitney U test). (A–C) CLN control. (D–I) stroke PT; (J–L) stroke CLN. Scale bars, 50 μm (A–F), 15 μm (G–L, *inset* in F).

or slightly less than the molecular mass of full MAP2 (280 kDa, 70 kDa) and NR-2A (180 kDa), and fragments of lower molecular mass (Fig. 7A, 7C, 7E). Higher immunoreactivity to Abs anti-NR-2A was found in patients than in controls using a semiquantitative band intensity assessment method (Fig. 7B, 7D, 7F). For mRNA expression, real-time RT-PCR showed that both patients and controls had a very low mRNA expression of MAP2 and NR-2A in PT (Fig. 7E, 7F).

CD69 activated T-cells are found in lymphoid tissue of stroke patients

To assess whether APCs bearing brain-derived Ags could induce signals in T cells, we examined the expression of CD69 in the lymphoid tissues. While control tissue showed low or absence of immunoreactivity to $CD69^+$ (Fig. 8A), patients had $CD69^+$ cells that were often seen near MAP2 immunoreactive cells (Fig. 8B–G). Occasionally, colocalization of MAP2 with CD69 was observed in isolated cells (Fig. 8H–J), indicating that APCs carrying neural Ags can also show CD69 expression. Quantification of $CD69^+$ cells showed higher ($p < 0.05$) numbers in samples of stroke patients than in controls (Fig. 8K, 8L). The majority of the $CD69^+$ cells were $CD3^+$ T cells (Fig. 9A–F). However, $CD69^+$ cells that were not reactive to CD3 were occasionally seen (Fig. 9G–I). We can conclude that $CD69^+$ cells are mainly T cells and that this marker was sporadically seen in macrophages, because we observed colocalization of isolated $CD69^+$ cells with CD68 (Fig. 9J–L).

Infarction volume and functional outcome might be related to the immunoreactivity to specific brain Ags in the palatine tonsil

The NIHSS score at baseline was associated with the immunoreactivity to MBP ($r = 0.92$, $p = 0.02$), but not to MAP2 ($r = -0.38$, $p = 0.51$), or NR-2A ($r = -0.24$, $p = 0.36$). The volume of infarction was directly correlated with immunoreactivity to MBP ($r = 0.98$, $p = 0.005$) and inversely correlated with immunoreactivity to MAP2 ($r = -0.77$, $p = 0.07$) or NR-2A ($r = -0.65$, $p = 0.005$). Patients with good outcome had more $MAP2^+$ cells (50.0 versus 22.34 [6.34]; $p = 0.01$, ANOVA), more NR-2A immunoreactivity (367.52 [101.73 versus 156.75 {117.78}]; $p = 0.01$, ANOVA), and less MBP^+ cells (1.20 versus 5.30 [2.61]; $p = 0.25$, ANOVA) than patients with poor outcome. In models adjusted for age and initial severity of stroke (NIHSS score), MAP2 ($\beta = -0.80$, $p = 0.06$), NR-2A ($\beta = -0.52$, $p = 0.02$), and MBP ($\beta = -0.78$, $p = 0.06$) showed significant trends with functional outcome at 3 mo.

Discussion

The presence of brain-derived Ags in the CLN was previously shown in patients with multiple sclerosis (13, 15, 19) and in animals with neurologic autoimmune disease or brain ischemia (13–15). To our knowledge, this study is the first to describe increased immunoreactivity to brain-derived compounds in the CLN and PT of patients with acute stroke and that this immunologic trait was associated with the volume of infarction and with the functional outcome of patients at follow-up. Although these associations did not prove direct causality, the study showed the presence of neural Ags in the draining lymph nodes of stroke patients and suggested that transfer of neural Ags to secondary lymphoid tissue could be a mechanism of immune control after acute stroke. Our findings showed that increased immunoreactivity to neuronal MAP2 and NR-2A was associated with smaller brain infarctions at day 7, and better outcome at 3 mo. In contrast, increased immunoreactivity to myelin-derived MBP was associ-

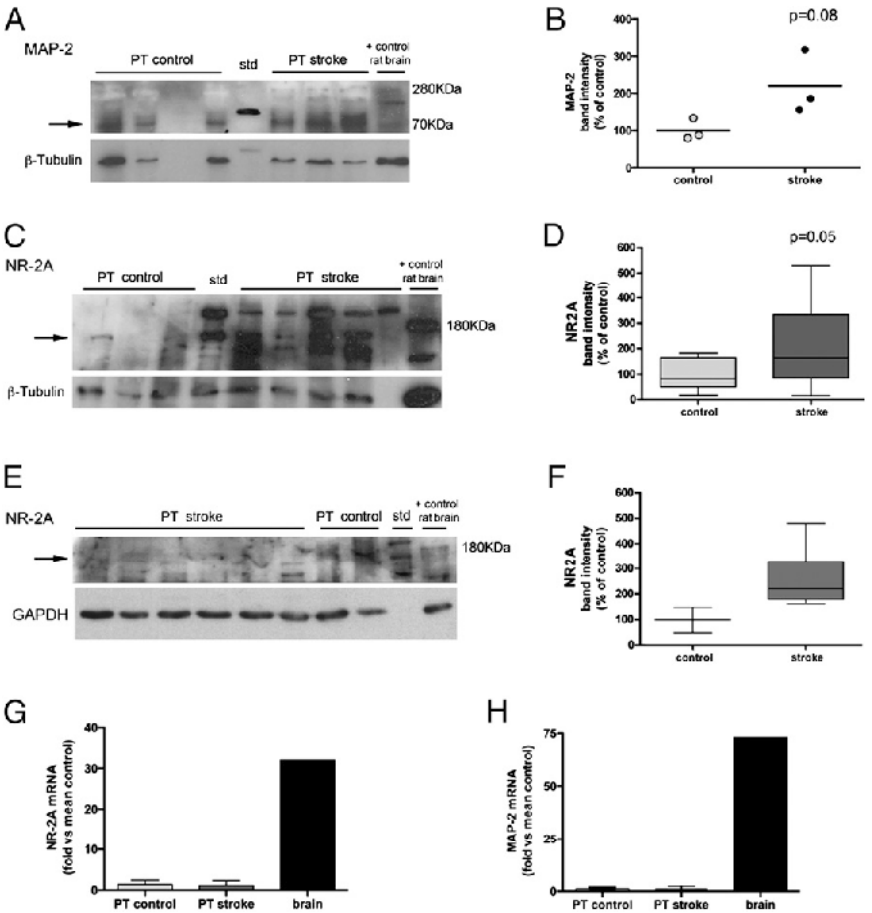

FIGURE 7. Western blotting and real-time RT-PCR of PT samples. (**A**–**F**) Western blotting of protein extracts obtained from PTs shows the presence of MAP2 (A) and NR-2A (C, E). Immunoreactive bands with molecular mass resembling that of the original proteins are found, together with fragments of lower molecular mass. This finding is compatible with phagocytosis of the proteins by macrophages. (B, D, and F) Semiquantification of band intensity (quantified bands are indicated by arrows) shows a higher presence of neuronal proteins in patients than in controls. (A) MAP2 ($n = 3$ controls and $n = 3$ strokes). (C) NR-2A immunoreactivity to the Ab no. AB155 (Chemicon; $n = 10$ controls and $n = 17$ strokes). (E) NR-2A immunoreactivity to Ab no. G903 (Sigma; $n = 2$ controls and $n = 6$ strokes). *Std* indicates molecular mass standards. Band intensity values are expressed as a percentage of mean control. β-Tubulin or GAPDH are shown as the loading controls for each gel. Samples of rat brain were used as a positive control. (**G** and **H**) Quantitative RT-PCR of RNA obtained from PT shows very low expression of MAP2 and NR-2A mRNA in both control and patients, compared with RNA of control human brain as the positive control. $n = 11$ controls; $n = 17$ stroke PT samples.

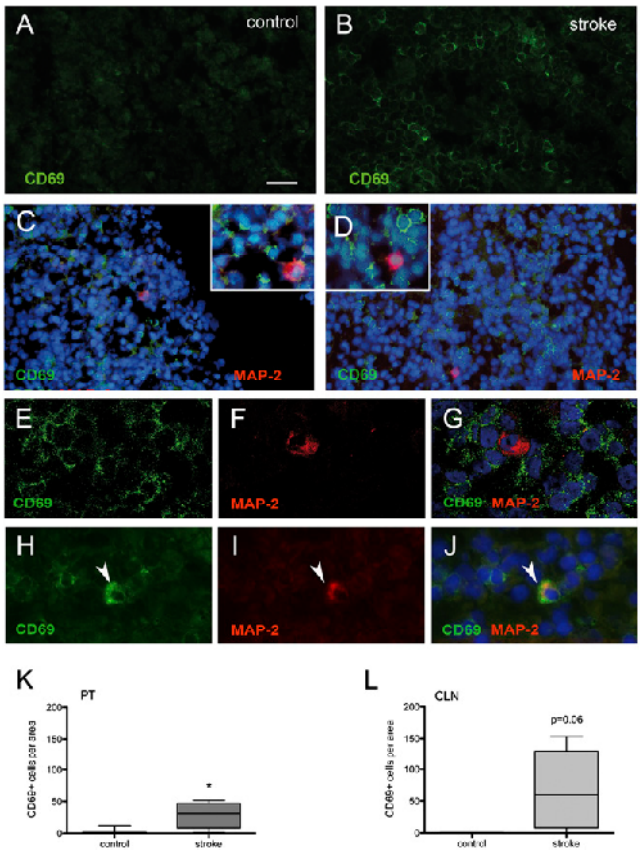

FIGURE 8. Neural Ags are often found in the vicinity of cells expressing the early activation marker CD69 after stroke. Low or absent immunoreactivity to CD69 was found in lymphoid tissue of controls (**A**), whereas in stroke patients (**B**) $CD69^+$ cells were more abundant and showed a comparatively more intense staining than in controls. (**C** and **D**) $CD69^+$ cells (green) are seen in the vicinity of MAP2 immunoreactive cells (red) in patients, indicating T cell activation. *Insets* in (C) and (D) and images in (**E**–**G**) illustrate at higher magnification the apparent contact between these cells. (**H**–**J**) Isolated $MAP2^+$ cells immunoreactive to CD69 were also seen, indicating that APCs carrying neural Ags can express CD69. (**K** and **L**) Quantification of the numbers of $CD69^+$ cells per area shows significant increases in stroke patients versus controls in PTs (K; $n = 5$ controls, $n = 8$ stroke) and a trend to significance ($p = 0.06$) in the CLNs (L; $n = 7$ controls, $n = 4$ stroke). Nuclei are stained in blue with Hoechst in C, D, G, and J. Images in (E–G) were taken with a confocal microscope. (A) CLN control. (B–J) CLN stroke. Scale bars, 30 μm (A–D), 20 μm (*insets* in C, D, H–J), 15 μm (E–G). $*p < 0.05$, nonparametric Mann–Whitney *U* test

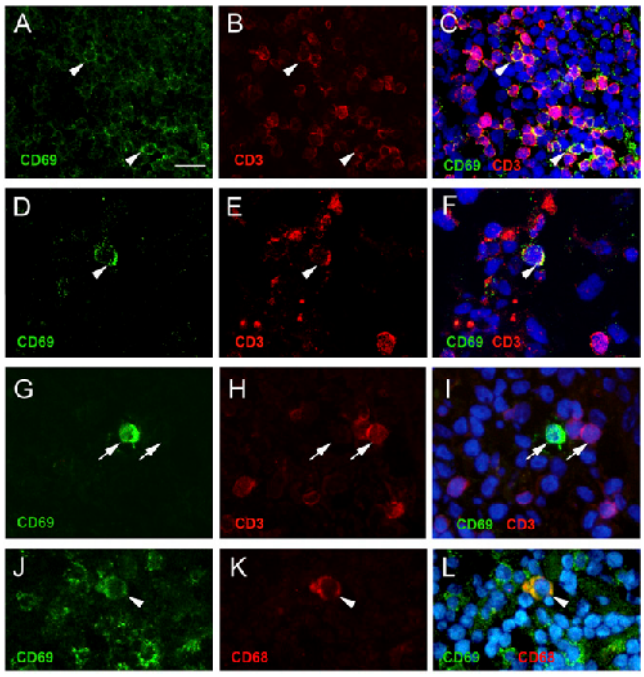

FIGURE 9. $CD69^+$ cells in stroke cases are mainly T cells. The majority of $CD69^+$ cells (green) are T cells ($CD3^+$, red; **A**–**F**) that appear in bundles (A–C), but isolated $CD69^+$ cells negative for CD3 are sporadically seen (**G**–**I**). (**J**–**L**) Double immunohistochemistry for CD69 (green) and CD68 (red) shows one $CD68^+$ macrophage that is immunoreactive to CD69 (arrowhead). Arrowheads indicate $CD69^+$ cells double stained with either CD3 (A–F) or CD68 (J–L). Arrows (G–I) indicate the same location in the tissue for the different immunofluorescence channels and evidence cells reactive to either CD69 (green) or CD3 (red). Nuclei are stained in blue with Hoechst in (C), (F), (I), and (L). Images in (D–F) were taken at the confocal microscope. Samples are from PTs (A–I) and CLNs (J–L) of stroke patients. Scale bars, 20 μm (A–C), 15 μm (D–L).

ated with severer impairment on admission, larger infarctions on brain imaging, and worse outcome at clinical follow-up. Further study will be required to validate these associations in a larger cohort.

The quantitative analysis of mRNA expression excluded a local synthesis of neural Ags in the lymph nodes, while suggesting that these Ags originated in the brain. Experimental and human studies have described anatomic and physiologic links between the CNS and the lymphoid tissue, via either the blood stream (10, 18) or the bulk flow of cerebrospinal and interstitial fluids (2, 12), that could explain our findings. Accordingly, stroke facilitated the crosstalk between the brain and the immune system, and Ags such as MBP, but not MAP2 or NR-2A, were correlated with the initial severity of stroke. The cells loaded with brain-derived Ags were rare near blood vessels, but were found in the proximity of T cells and in areas stained with fibronectin, suggesting that bulk flow might be involved in the presence of brain Ags at the lymph nodes (28). The similarities between the findings at the PT and the CLN also supported that both lymphoid tissues were part of the lymphatic drainage of the CNS.

At the lymph nodes, macrophages were the most frequent cellular type loaded with brain-derived Ags in stroke patients, and only few brain-immunoreactive dendritic cells were found. The study did not determine whether phagocytosis of brain Ags by macrophages had occurred in the injured brain or at the lymph nodes, but it showed that the cells immunoreactive to brain Ags expressed costimulatory MHC-II receptors. Because costimulatory signals are necessary for efficient activation of lymphocytes (29), our findings stressed that the patients were equipped for immune regulation (6). Furthermore, APCs immunoreactive to brain Ags were often seen near resident $DC\text{-}SIGN^+$ dendritic cells. Further study is needed to discern whether this observation underlies functional interaction between APCs, because this mechanism could facilitate Ag presentation to T cells. The presence of $CD69^+$ T cells in lymphoid tissue of stroke patients suggested activation of T cells that was not seen in controls.

The presence of brain-derived compounds was associated with impaired or improved stroke outcome, depending on the transfer of myelin- or neuronal-derived epitopes, respectively. Furthermore, $CD69^+$ T cells were found in the proximity of MAP2-immunoreactive cells in the stroke group. Previous studies showed that $CD69^+$ T cells facilitated protective immune mechanisms including self-limited immune responses (30), lymphocyte retention in lymph nodes (31), and prevention of autoimmune reactions (32). In addition, CD69 exerted a negative regulation of allergen-

induced T cell effector responses and prevented Th17 cell differentiation in allergic diseases (30). Accordingly, mice lacking CD69 had exacerbated forms of arthritis, contact dermatitis, allergic asthma, and autoimmune myocarditis (31, 32). Although our study does not demonstrate the direct activation of T cells by CD69 or its effects, we can hypothesize that the abundant expression of $CD69^+$ T cells in the proximity of neuroantigen-immunoreactive APCs resulted in a tighter regulation of harmful autoimmune responses.

Collectively, this study is, to our knowledge, the first to describe in human stroke an increased transfer to lymph nodes of brain Ags and the presence of brain Ag immunoreactive APCs equipped for effective Ag presentation, signal transmission, and activation of T cells. Further study is warranted to clarify whether CD69 mediates immune tolerance in acute stroke patients and to unveil whether peripherally activated lymphocytes recognize cognate brain epitopes at the site of stroke injury in an Ag-specific manner facilitating beneficial autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

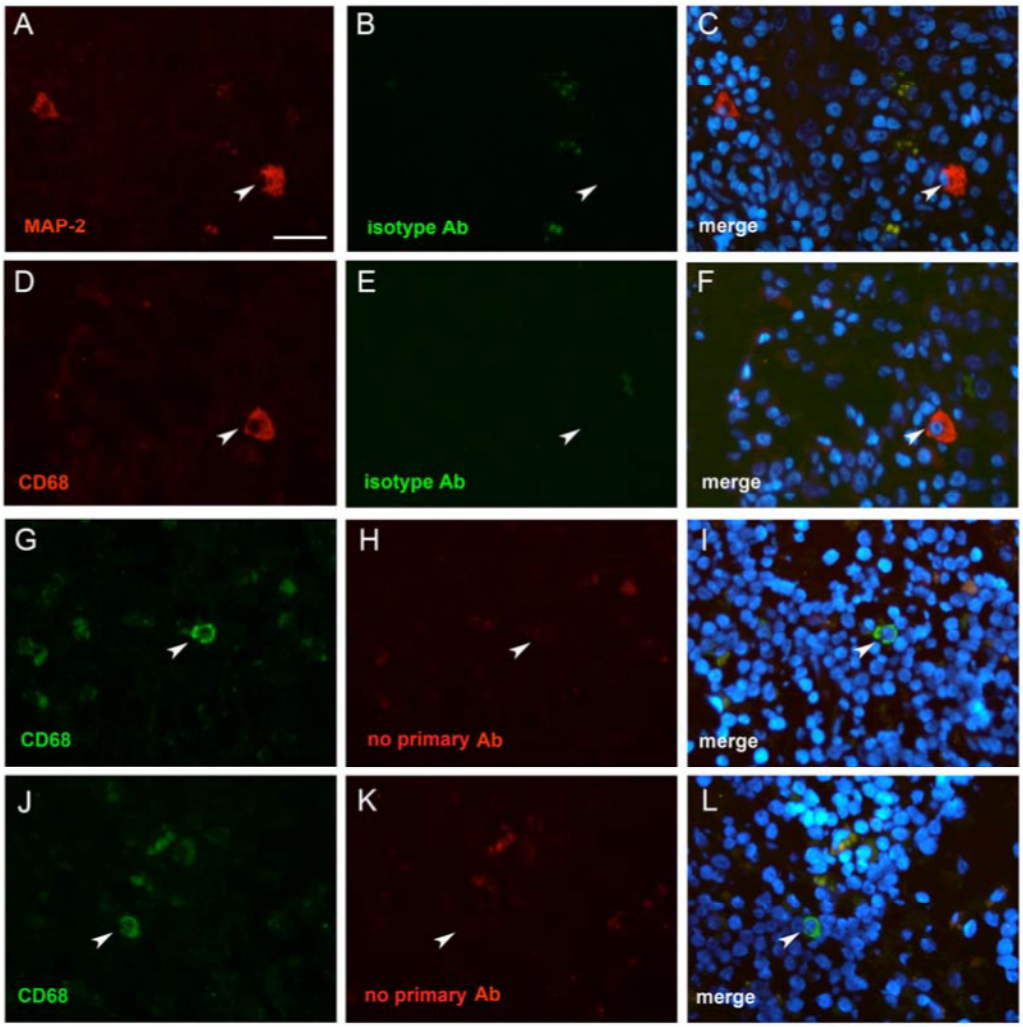
References

1. Galea, I., I. Bechmann, and V. H. Perry. 2007. What is immune privilege (not)? *Trends Immunol.* 28: 12–18.
2. Weller, R. O., E. Djuanda, H. Y. Yow, and R. O. Carare. 2009. Lymphatic drainage of the brain and the pathophysiology of neurological disease. *Acta Neuropathol.* 117: 1–14.
3. Iadecola, C., and J. Anrather. 2011. The immunology of stroke: from mechanisms to translation. *Nat. Med.* 17: 796–808.
4. Vogelgesang, A., U. Grunwald, S. Langner, R. Jack, B. M. Bröker, C. Kessler, and A. Dressel. 2008. Analysis of lymphocyte subsets in patients with stroke and their influence on infection after stroke. *Stroke* 39: 237–241.
5. Haeusler, K. G., W. U. Schmidt, F. Föhring, C. Meisel, T. Helms, G. J. Jungehulsing, C. H. Nolte, K. Schmolke, B. Wegner, A. Meisel, et al. 2008. Cellular immunodepression preceding infectious complications after acute ischemic stroke in humans. *Cerebrovasc. Dis.* 25: 50–58.
6. Urra, X., A. Cervera, N. Villamor, A. M. Planas, and A. Chamorro. 2009. Harms and benefits of lymphocyte subpopulations in patients with acute stroke. *Neuroscience* 158: 1174–1183.
7. Chamorro, A., X. Urra, and A. M. Planas. 2007. Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. *Stroke* 38: 1097–1103.
8. Urra, X., V. Obach, and A. Chamorro. 2009. Stroke induced immunodepression syndrome: from bench to bedside. *Curr. Mol. Med.* 9: 195–202.
9. Engelhardt, B., and R. M. Ransohoff. 2005. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol.* 26: 485–495.
10. Hochmeister, S., M. Zeitelhofer, J. Bauer, E. M. Nicolussi, M. T. Fischer, B. Heinke, E. Selzer, H. Lassmann, and M. Bradl. 2008. After injection into the striatum, in vitro-differentiated microglia- and bone marrow-derived dendritic cells can leave the central nervous system via the blood stream. *Am. J. Pathol.* 173: 1669–1681.
11. Harling-Berg, C. J., T. J. Park, and P. M. Knopf. 1999. Role of the cervical lymphatics in the Th2-type hierarchy of CNS immune regulation. *J. Neuroimmunol.* 101: 111–127.
12. Bradbury, M. W., H. F. Cserr, and R. J. Westrop. 1981. Drainage of cerebral interstitial fluid into deep cervical lymph of the rabbit. *Am. J. Physiol.* 240: F329–F336.
13. de Vos, A. F., M. van Meurs, H. P. Brok, L. A. Boven, R. Q. Hintzen, P. van der Valk, R. Ravid, S. Rensing, L. Boon, B. A. 't Hart, and J. D. Laman. 2002. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J. Immunol.* 169: 5415–5423.
14. Mutlu, L., C. Brandt, E. Kwidzinski, B. Sawitzki, U. Gimsa, J. Mahlo, O. Aktas, R. Nitsch, M. van Zwam, J. D. Laman, and I. Bechmann. 2007. Tolerogenic effect of fiber tract injury: reduced EAE severity following entorhinal cortex lesion. *Exp. Brain Res.* 178: 542–553.
15. van Zwam, M., R. Huizinga, M. J. Melief, A. F. Wierenga-Wolf, M. van Meurs, J. S. Voerman, K. P. Biber, H. W. Boddeke, U. E. Höpken, C. Meisel, et al. 2009. Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. *J. Mol. Med.* 87: 273–286.
16. Harling-Berg, C. J., P. M. Knopf, and H. F. Cserr. 1991. Myelin basic protein infused into cerebrospinal fluid suppresses experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 35: 45–51.
17. Johnston, M., A. Zakharov, C. Papaiconomou, G. Salmasi, and D. Armstrong. 2004. Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. *Cerebrospinal Fluid Res.* 1: 2–15.
18. Clapham, R., E. O'Sullivan, R. O. Weller, and R. O. Carare. 2010. Cervical lymph nodes are found in direct relationship with the internal carotid artery: significance for the lymphatic drainage of the brain. *Clin. Anat.* 23: 43–47.
19. Fabriek, B. O., J. N. Zwemmer, C. E. Teunissen, C. D. Dijkstra, C. H. Polman, J. D. Laman, and J. A. Castelijns. 2005. In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology. *J. Neuroimmunol.* 161: 190–194.
20. Hatterer, E., N. Davoust, M. Didier-Bazes, C. Vuaillat, C. Malcus, M. F. Belin, and S. Nataf. 2006. How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes. *Blood* 107: 806–812.
21. Wenkel, H., J. W. Streilein, and M. J. Young. 2000. Systemic immune deviation in the brain that does not depend on the integrity of the blood-brain barrier. *J. Immunol.* 164: 5125–5131.
22. Wang, W. Z., T. Olsson, V. Kostulas, B. Höjeberg, H. P. Ekre, and H. Link. 1992. Myelin antigen reactive T cells in cerebrovascular diseases. *Clin. Exp. Immunol.* 88: 157–162.
23. von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* 3: 867–878.
24. Jácomo, A. L., F. E. Akamatsu, M. Andrade, and N. F. Margarido. 2010. Pharyngeal lymphatic ring: anatomical review. *J. Morphol. Sci.* 27: 47–49.
25. Gorina, R., M. Font-Nieves, L. Márquez-Kisinousky, T. Santalucia, and A. M. Planas. 2011. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signaling, MAPK, and Jak1/Stat1 pathways. *Glia* 59: 242–255.
26. Brand, C. U., R. E. Hunger, N. Yawalkar, H. A. Gerber, T. Schaffner, and L. R. Braathen. 1999. Characterization of human skin-derived CD1a-positive lymph cells. *Arch. Dermatol. Res.* 291: 65–72.
27. Dzionek, A., Y. Sohma, J. Nagafune, M. Cella, M. Colonna, F. Facchetti, G. Günther, I. Johnston, A. Lanzavecchia, T. Nagasaka, et al. 2001. BDCA-2, a novel plasmacytoid dendritic cell-specific type II C-type lectin, mediates antigen capture and is a potent inhibitor of interferon alpha/beta induction. *J. Exp. Med.* 194: 1823–1834.
28. Angel, C. E., C. J. Chen, O. C. Horlacher, S. Winkler, T. John, J. Browning, D. MacGregor, J. Cebon, and P. R. Dunbar. 2009. Distinctive localization of antigen-presenting cells in human lymph nodes. *Blood* 113: 1257–1267.
29. Fathman, C. G., and N. B. Lineberry. 2007. Molecular mechanisms of CD4+ T-cell anergy. *Nat. Rev. Immunol.* 7: 599–609.
30. Martín, P., M. Gómez, A. Lamana, A. Matesanz Marín, J. R. Cortés, M. Ramírez-Huesca, O. Barreiro, P. López-Romero, C. Gutiérrez-Vázquez, H. de la Fuente, et al. 2010. The leukocyte activation antigen CD69 limits allergic asthma and skin contact hypersensitivity. *J. Allergy Clin. Immunol.* 126: 355–365, 365, e1–e3.
31. Cruz-Adalia, A., L. J. Jiménez-Borreguero, M. Ramírez-Huesca, I. Chico-Calero, O. Barreiro, E. López-Conesa, M. Fresno, F. Sánchez-Madrid, and P. Martín. 2010. CD69 limits the severity of cardiomyopathy after autoimmune myocarditis. *Circulation* 122: 1396–1404.
32. Sancho, D., M. Gómez, and F. Sánchez-Madrid. 2005. CD69 is an immunoregulatory molecule induced following activation. *Trends Immunol.* 26: 136–140.

Supplementary Table I. List of antibodies used for immunofluorescence studies

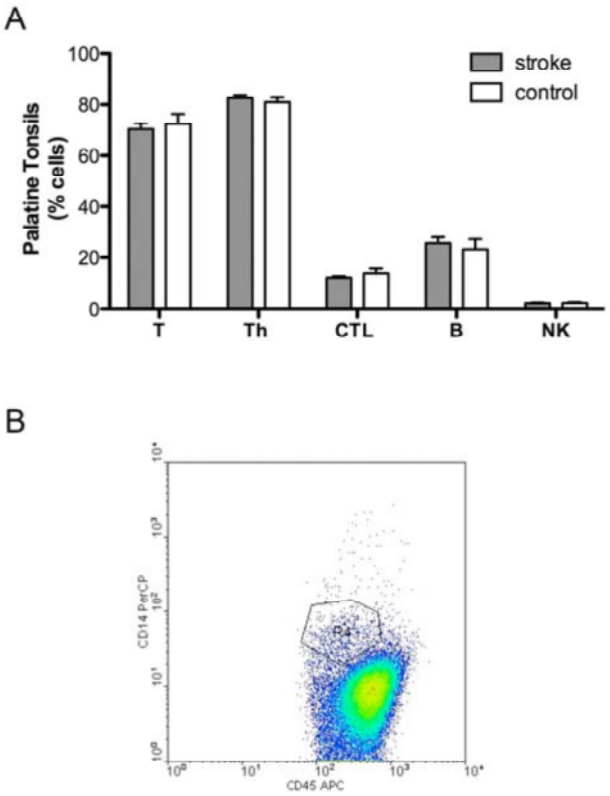
Primary antibodies	Supplier	species	type	dilution	reference
CD1a	Serotec (Kidlington, Oxford, UK)	Mouse	Monoclonal	1/100	MCA80
CD1b	Serotec	Mouse	Monoclonal	1/50	MCA1315
CD1c (BDCA-1)	MACS (Auburn, CA, USA)	Mouse	Monoclonal	1/1	130-090-695
CD3	ABCAM (Cambridge, UK)	Rabbit	Polyclonal	1/100	ab5690
CD14	Serotec	Mouse	Monoclonal	1/100	MCA596G
CD21	Serotec	Mouse	Monoclonal	1/50	MCA1195
CD68	Santa Cruz (Santa Cruz, CA, USA)	Goat	Polyclonal	1/10	SC-7083
CD69	Serotec	Mouse	Monoclonal	1/20	MCA2806
CD206	BD Bioscience (Franklin Lakes,NJ, USA)	Mouse	Monoclonal	1/100	555953
CD209 (DC-SIGN)	R&D System (Minneapolis, MN, USA)	Mouse	Monoclonal	1/100	MAB161
CD303 (BDCA-2)	MACS	Mouse	Monoclonal	1/20	130-090-690
Fibronectin	BD Bioscience	Mouse	Monoclonal	1/50	555867
HLA-DP,DQ,DR	DakoCytomation (Glostrup, Denmark)	Mouse	Monoclonal		M0775
Laminin	Dako (Carpinteria, CA, USA)	Rabbit	Polyclonal	1/100	Z0097
MAP-2	Sigma (St Louis, MI, USA)	Mouse	Monoclonal	1/100	M4403
MAP-2	Cell Signaling (Danvers, MA,USA)	Rabbit	Polyclonal	1/100	4542
MBP	ABCAM	Mouse	Monoclonal	1/200	ab66188
MBP	Dako	Rabbit	Polyclonal	1/100	A0623
MOG	ABCAM	Mouse	Monoclonal	1/100	ab24022
Secondary antibodies (Invitrogen Corp., Carlsbad, CA, USA)		**species**	**type**	**dilution**	**reference**
Alexa fluor 546 F(ab´)2 fragment of goat anti-rabbit IgG (H+L)		Rabbit	Polyclonal	1/500	A11071
Alexa fluor 546 F(ab´)2 fragment of goat anti-Mouse IgG (H+L)		Mouse	Polyclonal	1/500	A11018
Alexa fluor 488 F(ab´)2 fragment of goat anti-rabbit IgG (H+L)		Rabbit	Polyclonal	1/500	A11070
Alexa fluor 488 F(ab´)2 fragment of goat anti-mouse IgG (H+L)		Mouse	Polyclonal	1/500	A11017
Alexa fluor 488 donkey anti-goat		Goat	Polyclonal	1/500	A11055
Alexa fluor 405 goat anti-rabbit IgG (H+L)		Rabbit	Polyclonal	1/500	A31556
Alexa fluor 405 goat anti-Mouse IgG (H+L)		Mouse	Polyclonal	1/500	A31553

Supplementary Fig. 1



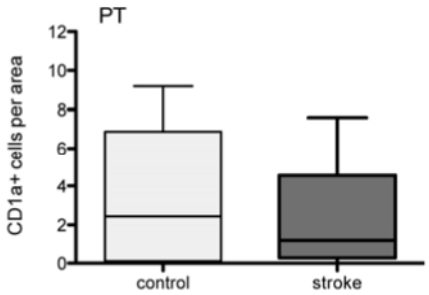
Supplementary Figure 1: Examples of controls for immunoreactions by the use of isotype control antibodies (Ab) (A-F) and by omission of one primary antibody (Ab) (G-L) Images show lack of cross-reactivity of MAP-2 (A-C) and CD68 (D-F) with an isotype control antibody, or with a red (G-I) or green (J-L) secondary antibody. Some punctuate non-specific staining is sometimes apparent outside the cells, often with a similar reaction for the green and the red fluorescence. Nuclei are stained in blue with Hoechst (C, F, I, L). Samples are from stroke patients. Bar scale= 20 μm.

Supplementary Fig. 2



Supplementary Figure 2: Flow cytometry analysis of lymphocytes in PT biopsies of stroke patients (n=22) and controls (n=11). A) No significant differences between groups are found in the proportion of lymphocyte subtypes. T (T cells), Th (T helper cells), CTL (cytotoxic T cells), B (B cells), NK (natural killers). B) In preliminary studies we verified that the population of macrophages (CD45+ $CD14^{h}$) was very small in the PT samples.

Supplementary Fig. 3



Supplementary Figure 3: Quantification of the numbers of CD1a+ cells per area in palatine tonsils of stroke patients and controls shows no significant differences (p=0.48) using the non-parametric Mann-Whitney test. Stroke: n= 10, controls: n=14.

Trabajo número 2

Presence of heat shock protein 70 in secondary lymphoid tissue correlates with stroke prognosis.

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Objetivos

Describir la presencia y distribución de Hsp-70 en el tejido linfoide secundario de pacientes con ictus frente a sujetos control.

Determinar la potencial relación entre la presencia de Hsp-70 en el tejido linfoide secundario con la evolución y pronóstico del ictus.

Resultados

Hsp-70 se encontró presente en mayor cantidad en el tejido linfoide secundario de pacientes con ictus que en controles. La distribución de Hsp-70 siguió un patrón similar a la descrita para los antígenos cerebrales y Hsp-70 se encontraba presente en células con características de célula presentadora de antígenos. Una mayor cantidad de Hsp-70 en el tejido linfoide se correlacionó con menores volúmenes de infarto y mejor pronóstico funcional independientemente de la edad y de la severidad clínica inicial.

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Presence of heat shock protein 70 in secondary lymphoid tissue correlates with stroke prognosis

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ABSTRACT

Heat shock protein 70 (Hsp-70) can act as a danger signal and activate immune responses. We studied the presence of Hsp-70 in lymphoid tissue and plasma of acute stroke patients and asymptomatic controls free of neurological disease. Immunofluorescence, Western blotting, qRT-PCR and flow cytometry studies were performed. Plasma Hsp-70 concentration at day 7 was similar in patients and controls, whereas patients disclosed stronger immunoreactivity to Hsp-70 in lymphoid tissue than controls. Most Hsp-70 + cells were antigen presenting cells located in T cell zones. Stronger immunoreactivity to Hsp-70 was associated with smaller infarctions and better functional outcome.

1. Introduction

Dead cells can behave like damage-associated molecular patterns (DAMPs) and activate the innate and adaptive arms of the immune system (Kono and Rock, 2008). Moreover, DAMPs are produced after brain ischemia and may affect the clinical course and outcome of patients with acute stroke (Iadecola and Anrather, 2011; Chamorro et al., 2012). Brain-derived antigens may leave the central nervous system (CNS) *via* the blood stream (Hochmeister et al., 2010) or *via* the bulk flow of intracranial fluids along cranial nerves and perivascular spaces of brain cortical arteries and arterioles (Bradbury et al., 1981; Weller et al., 2008). After stroke, brain-derived antigens and antigen presenting cells (APCs) can reach the brain-draining secondary lymph nodes, such as the cervical lymph nodes (CLN) (Harling-Berg et al., 1999; deVos et al., 2002; van Zwam et al., 2008) and the palatine tonsils (PT) (Planas et al., 2012). At these lymph nodes, brain derived-antigens loaded onto MHC molecules could be presented to T cells, and subsequently activate them (von Andrian and Memple, 2003). Interestingly, it has been argued that immune responses originating in the CLN have a Th2 phenotype to facilitate tolerogenic mechanisms against further inflammation and brain injury (Harling-Berg et al., 1999).

Heat shock protein 70 (Hsp-70) is a molecular chaperone that may behave as a danger signal under certain conditions (Basu et al., 2000). While Hsp-70 expression is virtually undetectable in the brain under physiological conditions, it is strongly induced in neurons following brain ischemia (Nowak et al., 1990; Kinouchi et al., 1993; Sharp et al., 1993; Planas et al., 1997; de la Rosa et al., 2013). Robust experimental evidence supports the hypothesis that Hsp-70 induction is protective in brain ischemia (Nowak and Jacewicz, 1994; Yenari et al., 1998, 1999; Yenari, 2002), and that these neuroprotective effects may be due to various mechanisms including a more efficient formation, folding and assembly of nascent proteins, a better refolding and stabilization of damaged peptides, and some anti-apoptotic effects (Yenari et al., 1999; Hartl and Hayer-Hartl, 2002; Yenari et al., 2005). Likewise, Hsp70.1 knockout mice show larger infarct volumes after transient focal ischemia (Lee et al., 2001a), while transgenic mice constitutively expressing the human (Plumier et al., 1997; Rajdev et al., 2000) or rat (Tsuchiya et al., 2003) inducible Hsp-70, and mice overexpressing Hsp-70 in astrocytes (Xu et al., 2010), are protected against ischemic brain damage. Hsp-70 overexpression, however, was not protective in certain models of permanent focal (Lee et al., 2001b) or global (Olsson et al., 2004) ischemia. In ischemia/reperfusion, virally delivered Hsp-70 (Badin et al., 2006) or intravenous administration of cell penetrating recombinant

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0165-5728/

Hsp-70 (Doeppner et al., 2009; Zhan et al., 2010) exerts neuroprotective actions mediated, at least in part, by anti-apoptotic effects. Recently, anti-inflammatory effects of TAT-Hsp70 involving downregulation of nuclear factor kappa B (NF-κB) activation have been reported after ischemia/reperfusion (Doeppner et al., 2013).

In contrast to these findings, stimulation of macrophages with Hsp-70 increased the secretion of nitric oxide, proinflammatory cytokines and chemokines, and enhanced the maturation and migration of APCs to the draining lymph nodes (Basu et al., 2000; Saito et al., 2005). Indeed, Hsp-70 has important immunoregulatory functions that include binding to peptides derived from necrotic cells to facilitate their uptake and presentation to lymphocytes, and the activation of antigen-specific T cells (Binder and Srivastava, 2005). Recently, Hsp-70 purified from a non-bacterial origin to exclude the possibility of microbial contamination, was shown to reduce the stimulatory capacity of dendritic cells and T cell responses *in vitro*, indicating that Hsp-70 moderated immune mediated inflammatory reactions (Stocki et al., 2012). Therefore, in addition to anti-apoptotic (Yenari et al., 2005) and anti-inflammatory (Zheng et al., 2008) actions on brain cells after ischemia, Hsp-70 may also attenuate immune responses.

In this study, we sought to identify the presence of Hsp-70 in brain draining lymph nodes of patients with acute stroke, as well as to assess whether the expression levels of Hsp-70 were associated with the main clinical outcomes in these patients.

2. Materials and methods

2.1. Patients

Forty-six patients with acute stroke were enrolled in this study after excluding patients with a history of infection, and an intake of antibiotics, steroids, or immunosuppressants within the preceding 3 months. Neurological impairment was assessed with the National Institutes of Health Stroke Scale (NIHSS), and functional outcome was measured at 3 months with the modified Rankin Scale (mRS). The volume of brain infarction was measured at day 7 ± 2 (mean ± SD) after stroke, using diffusion-weighted imaging (DWI) MRI (MRIcro software, Chris Roden, University of Nottingham, Nottingham, UK). Controls free of neurological symptoms (n = 16) were recruited from patients' relatives, or from obstructive sleep apnea syndrome clinics. The study was approved by the local ethics committee and participants or their relatives gave their written informed consent.

2.2. Blood and tissue samples

Blood samples were collected in 46 patients at day 7 of stroke and in 11 controls to measure the plasmatic levels of Hsp-70 protein using a commercial ELISA kit (#ADI-EKS-715, ENZO Life Sciences). PT biopsies were obtained without complications in 12 stroke patients at 85 ± 36 (mean ± SD) hours from stroke onset, and in 7 controls. Biopsy samples were divided into 1, 2 or 3 parts depending on the size of the specimens obtained; that on average were approximately $5 \times 3 \times 2$ mm^3 in size. Post mortem CLN and/or PT samples of 15 additional patients with malignant brain infarction were obtained from the Tumour Bank, Hospital Clínic-IDIBAPS Biobank at a median of 7 days after stroke onset (ranging from 1 to 21 days).

2.3. Flow cytometry

Lymphocyte subsets were analyzed in fresh tissue from PT biopsies of patients (n = 7) and controls (n = 6) using flow cytometry immediately after tissue extraction by investigators blinded to the clinical data. A cellular suspension of the PT was obtained after repetitive squirting with RPMI culture medium using a fine needle, obtaining a total of around 10 to 15×10^5 cells. A volume of 100 μL of the cell suspension, containing at least 2×10^5 cells, was used for each test, which allowed for the quantification of different lymphocyte subtypes and the use of appropriate isotype controls. The following monoclonal antibodies were used to identify T lymphocytes, T helper lymphocytes, cytotoxic T lymphocytes, natural killer cells and B lymphocytes using flow cytometry: CD25 and CD19 conjugated to fluorescein isothiocyanate, CD3, CD4 and CD56 conjugated to phycoeritrin, CD45, CD8 and CD4 conjugated to peridinin chlorophyll, and CD3 conjugated to allophycocyanin (BD Biosciences). Apoptotic lymphocytes were identified using the Annexin V FITC kit (Bender MedSystems, Vienna, Austria). Each lymphocyte subset was recognized using CD3, CD4, CD8, CD19 and CD56 antibodies conjugated to PE (all from BD Biosciences). The proportion of Annexin-V positive cells was calculated for each lymphocyte subset.

2.4. Immunofluorescence

Immunofluorescence for Hsp-70 was performed in the PT biopsies of 6 patients and 7 controls. PT biopsies and post mortem PT and CLN samples were snapped frozen in optimum cutting temperature (OCT) compound and stored at −80 °C. Cryostat sections were fixed in acetone, blocked with corresponding goat or rabbit serum and incubated overnight at 4 °C with primary antibodies followed by corresponding fluorescent secondary antibodies (Table 1). Immunofluorescence controls were carried out by omission of the primary antibody to verify the absence of non-specific staining. Samples were counterstained with Hoechst. The number of Hsp-70 positive cells in PT samples was counted under the fluorescence microscope by an investigator blinded to the clinical data. The mean value obtained in three areas per sample was taken as representative of the sample value. Double immunohistochemistry was carried out to identify the location and nature of the Hsp-70 immunoreactive cells within the lymphoid tissue. The two primary antibodies were obtained in different species to avoid cross-reactions (see Table 1).

2.5. Western blotting

Western blotting was carried out in PT biopsy tissue (weighing around 30 mg) from stroke patients (n = 9) and controls (n = 5). After extraction, tissue was immediately frozen and kept at −80 °C. Protein extraction was carried out using radioimmunoprecipitation (RIPA) buffer. Ten μg of protein was loaded in 10% polyacrilamide gels, run under denaturing conditions and proteins were transferred to a PVDC membrane. Primary antibodies used for Western blotting were a mouse monoclonal antibody against Hsp-70 (#HSP01 Millipore, diluted 1:1,000) and a rabbit polyclonal antibody against N-methyl-D-aspartate (NMDA) receptor subunit NR-2A (#AB155, Chemicon; 1:100). Mouse monoclonal Abs against GAPDH (#CSA-335, Stressgen; 1:500), or β-tubulin (#T4020, Sigma; 1:5000), were used as protein gel loading controls. Control and patient samples were run in parallel in the gels. The optical density of the bands was measured in a densitometer. After correcting for protein loading with the density of the reference band, relative band intensity was expressed as percentage of control.

2.6. RT-PCR

PT biopsy tissue from patients (n = 12) and controls (n = 5) was immediately frozen, RNA was extracted, and gene expression analysis was carried out. Quantitative real-time PCR (qRT-PCR) was performed using FAM-labeled Taqman® Gene Expression Assay probes for Hsp72 (Hs00358147_s1) and β-actin (Hs03023943_g1) (Applied Biosystems, Foster City, CA, USA). The amplification conditions were: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. CT values were analyzed using the $2^{-\Delta\Delta C_T}$ method.

2.7. Statistical analysis

The Kolmogorov–Smirnov test was used to assess the normal distribution of the data, and the Fisher's exact test, Student's *t* test, Mann–

Table 1
List of antibodies used for immunofluorescence studies.

Primary antibodies	Supplier	Species	Type	Dilution	Reference
CD1a	Serotec (Kidlington, Oxford, UK)	Mouse	Monoclonal	1/100	MCA80
CD3	ABCAM (Cambridge, UK)	Rabbit	Polyclonal	1/100	ab5690
CD68	Santa Cruz Biotech. Inc. (St. Cruz, CA, USA)	Goat	Polyclonal	1/10	SC-7083
CD21	Serotec	Mouse	Monoclonal	1/100	MCA1195
CD206	BD Bioscience (Franklin Lakes, NJ, USA)	Mouse	Monoclonal	1/100	555953
Fibronectin	BD Bioscience	Mouse	Monoclonal	1/50	555867
HLA-DR-DR	ABCAM (Cambridge, UK)	Rat	Monoclonal	1/100	ab6340
Hsp-70	Calbiochem	Mouse	Monoclonal	1/50	HSP01
Hsp-70	Calbiochem	Rabbit	Polyclonal	1/100	386035
Secondary antibodies (Invitrogen Corp., Carlsbad, CA, USA)		**Species**	**Type**	**Dilution**	**Reference**
Alexa Fluor 546 F(ab') 2 fragment of goat anti-rabbit IgG (H + L)		Rabbit	Polyclonal	1/500	A11071
Alexa Fluor 546 F(ab') 2 fragment of goat anti-Mouse IgG (H + L)		Mouse	Polyclonal	1/500	A11018
Alexa Fluor 488 F(ab') 2 fragment of goat anti-rabbit IgG (H + L)		Rabbit	Polyclonal	1/500	A11070
Alexa Fluor 488 F(ab') 2 fragment of goat anti-mouse IgG (H + L)		Mouse	Polyclonal	1/500	A11017
Alexa Fluor 488 donkey anti-goat		Goat	Polyclonal	1/500	A11055

Whitney *U* test and Kruskal–Wallis test were used as appropriate for group comparisons of continuous data. Correlations were calculated with Spearman's Rank correlation coefficient and multiple linear regression analysis was used to adjust for the effects of potential confounders on stroke outcome, such as age and stroke admission NIHSS score. Statistical analyses were performed with the SPSS 17.0 statistical package (SSPS Inc., Chicago, IL, USA).

3. Results

3.1. Study population

The mean ± SD age of the stroke patients was 72.4 ± 12.3 years, 60% were males, and the most prevalent risk factors were hypertension (68%), dyslipidemia (34%), diabetes (32%), coronary artery disease (17%), or smoking (15%). The mean ± SD age of the controls was 58.8 ± 18.6 years, 57% were males, and prevalent risk factors included hypertension (57%), dyslipidemia (19%), diabetes (10%), coronary artery disease (10%), or smoking (5%). Most stroke subtypes were classified into cardioembolic (28%) or of an undetermined cause (34%), and the median (IQR) NIHSS score on stroke admission was 11 (6–17). The median (IQR) volume of infarction at day 7 was 19 (7–47) cc, and 19% of the patients had an infection within the first 7 days of stroke. At 3 months 36% of the patients had a good outcome (mRS 0–2), and 53% had a poor outcome, including 11% of patients who had died.

3.2. Measurement of Hsp-70 in blood

Mean ± SD Hsp-70 plasma concentration at day 7 was 0.40 ± 0.45 ng/mL in patients (n = 46) and 0.38 ± 0.41 ng/mL in controls (n = 11), Mann–Whitney *U* test, p = 0.87. Hsp-70 plasma levels were associated neither with the demographic factors, vascular risk factors, stroke subtypes nor infections (data not shown).

3.3. Hsp-70 in lymphoid tissue

Western blotting of Hsp-70 protein in PT biopsy samples (relative optical density expressed as the percentage of one control sample; Fig. 1A) showed higher amounts of Hsp-70 in stroke patients (1072.02 ± 1211.77%) than in controls (85.67 ± 39.09%) (Mann–Whitney *U* test, p = 0.04). Hsp-70 relative optical density in the PT was also higher in patients with good outcome than in patients with a poor outcome at 3 months, or controls (Kruskal–Wallis test, p = 0.01, Fig. 1B). However, Hsp-70 mRNA expression in the PT was similar in patients than in controls (Fig. 1C), suggesting that Hsp-70 protein in the PT of stroke patients was not locally produced at the lymph nodes but had reached the lymphoid tissue from external sources.

A previous study from our group identified the presence of the neuronal antigen NMDA glutamate receptor subunit NR-2A in the PT of patients with acute stroke and reported that it was associated with a better outcome (Planas et al., 2012). Therefore, in the present study, we further assessed whether the levels of Hsp-70 and NR-2A were correlated

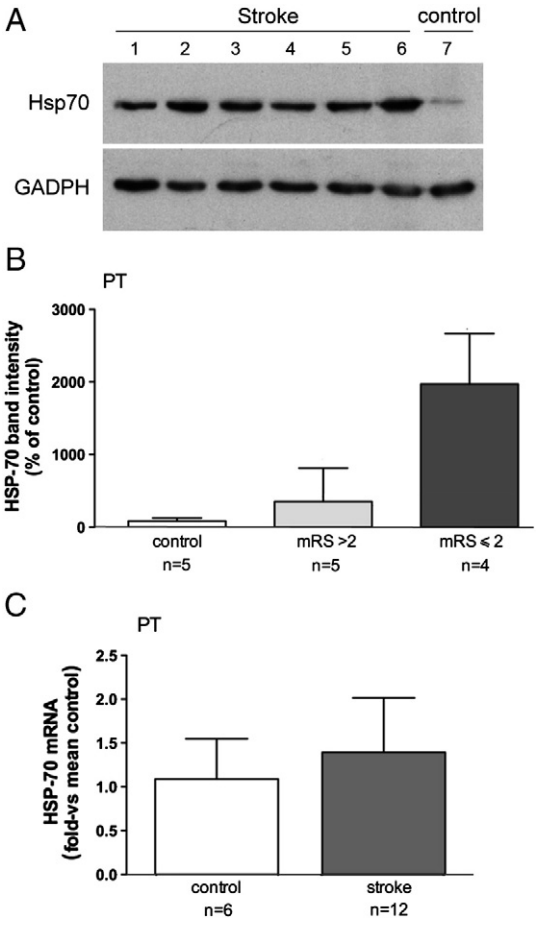

Fig. 1. Hsp-70 expression in lymphoid tissue. A) Western blotting showing Hsp-70 expression in the PT of stroke patients (lanes 1–6), and a control subject (lane 7). GAPDH illustrates the amount of protein loaded in each lane. B) Quantification of Hsp-70 revealed a significantly higher band intensity from the blots in stroke patients with a good outcome (mRS 0 to 2, n = 4) than in patients with a poor outcome (mRS > 2, n = 5), or controls (n = 5) (Kruskal–Wallis test, p = 0.01). The differences were also significant when comparing good outcome patients with poor outcome patients (Mann–Whitney *U* test, p = 0.02). C) Hsp-70 mRNA expression (qRT-PCR) was not significantly different in patients (n = 12) *versus* controls (n = 5) (ANOVA test, p = 0.48). Values are mean ± SD.

in the PT. Thus, we confirmed a positive correlation between the expression of Hsp-70 and the neuronal-derived antigen NR-2A in the PT, as patients with a greater expression of the chaperone protein also had a greater expression of the neuronal derived antigen [Spearman's correlation $r = 0.764$, $p = 0.002$] (Fig. 2A).

The reactivity to Hsp-70 in the PT of the patients was highly inversely correlated with the severity of stroke on admission (NIHSS score). Thus, patients with a more severe neurological impairment on admission had a lower reactivity to Hsp-70 in the PT (Fig. 2B). In correspondence with this finding, the volume of brain infarction at day 7 was also inversely correlated with the reactivity to Hsp-70, since we confirmed that the patients with the larger brain infarctions had lower reactivity to Hsp-70 in the PT [Spearman's correlation $r = -0.683$, $p = 0.04$] (Fig. 2C). The reactivity to Hsp-70 in the PT was also inversely correlated with the mRS score at day 90 [Spearman's correlation $r = -0.653$, $p = 0.05$] (Fig. 2D). Since the mRS is a functional scale that increases the score according to the severity of the residual symptoms, this result highlighted a better functional outcome at follow up in patients with greater reactivity to Hsp-70 in the PT. To minimize the effect of major factors that influence the outcome of patients with stroke we confirmed the validity of the correlation between Hsp-70 expression and functional outcome in multivariate models adjusted for the confounding effect of age and clinical severity. Accordingly, we confirmed that a greater reactivity to Hsp-70 at the PT was independently correlated with a better mRS score at day 90 [$\beta = -0.531$, $p = 0.01$].

There were no differences between patients and controls regarding the proportions of T lymphocytes, T helper lymphocytes, cytotoxic T lymphocytes, natural killer cells and B lymphocytes in the PT assessed by flow cytometry, nor in the proportions of apoptotic cells in any of the lymphocyte subtypes. The reactivity to Hsp-70 in the PT shown above was not correlated with the lymphocyte subpopulations or with the proportion of apoptotic cells in any of the lymphocyte subtypes (data not shown).

3.4. Cellular localization of Hsp-70 in lymphoid tissue

Immunofluorescence was carried out to study the lymphoid tissue from biopsies and necropsies. The PT contains lymph nodules with follicles rich in B cells, a surrounding diffuse lymphoid tissue rich in T cells, and reticular fibers (Fig. 3A–B). Hsp-70 immunoreactive cells were detected in the PT (Fig. 3C). The number (mean ± SD) of Hsp-70 immunoreactive cells in PT biopsies was significantly higher (Mann–Whitney *U* test, $p = 0.02$) in stroke patients (10.52 ± 7.20 cell number per area; $n = 6$) than in controls (3.74 ± 2.68 cell number per area; $n = 7$) (Fig. 3C).

Hsp-70 immunoreactive cells were localized in T cell rich zones of lymphoid tissue (CD3+), but Hsp-70 did not co-localize with CD3+ cells (Fig. 3 D–F and G–H), confirming that the Hsp-70+ cells were not T cells. Interestingly, Hsp-70+ cells were not observed in CD21+ B cell areas (Fig. 3I); however, they were nearby the fibroreticular network that was stained with fibronectin (Fig. 3J–K). Hsp-70 immunoreactivity was not observed in zones rich in resident dendritic cells expressing the pattern recognition mannose receptor C type 1 CD206 (Fig. 3L), although, Hsp-70 often co-localized, but not always, with APCs expressing the MHC class II cell surface receptor HLA-DR (Fig. 4). Furthermore, co-localization with the APC marker of migratory dendritic cells CD1a was observed (Fig. 5A–C and D–F), and isolated Hsp-70+

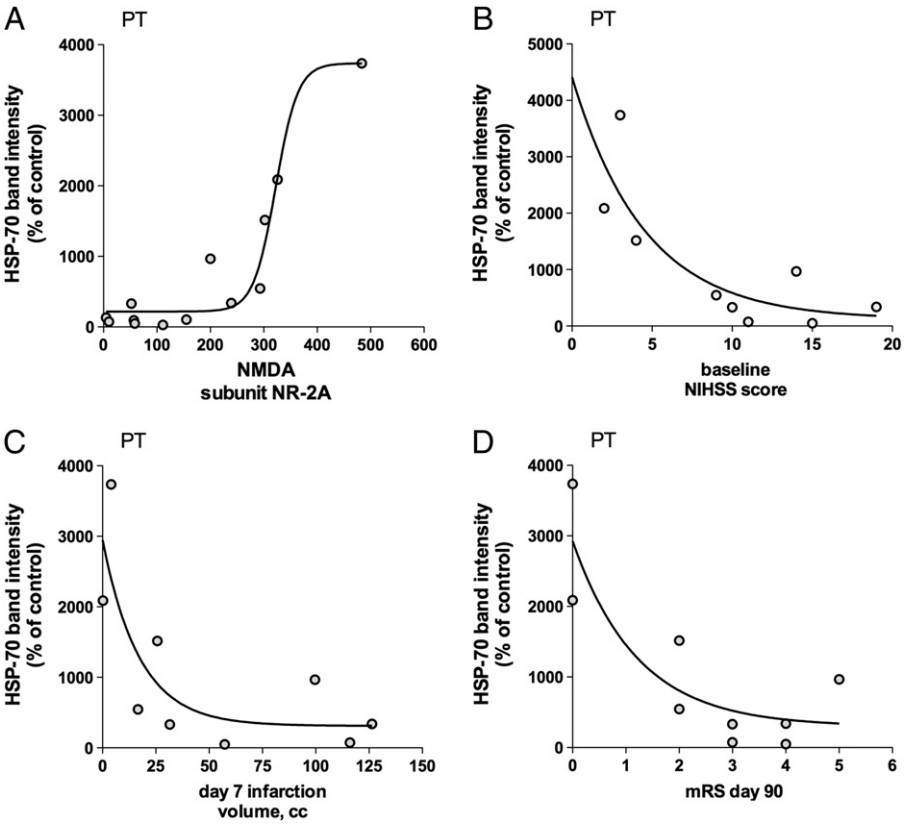

Fig. 2. Correlations between the degree of reactivity to Hsp-70 (Western blotting) in the PT and other parameters. (A) Expression of neuronal-derived antigen NR-2A (Western blotting) in the PT was higher in the PT of patients with greater reactivity to Hsp-70. (B) Baseline NIHSS score (ranging from 0 to 42, with higher scores indicating a more severe neurologic deficit) was inversely correlated to Hsp-70 immunoreactivity. The figure illustrates that patients with a worse neurological deficit at baseline had lower reactivity to Hsp-70 in the PT. (C) Patients with large infarct volumes at day 7 had lower reactivity to Hsp-70 compared with patients with small infarct volumes. (D) A greater reactivity to Hsp-70 was also found in the PT of patients with lower modified Rankin Score at 3 months. Since the mRS ranges from 0, indicating no residual symptoms, to 6, indicating death, this finding indicated that reactivity to Hsp-70 was increased in the PT of patients with better functional outcome. For graphical representation data were fit to a curve with non-linear regression using GraphPad software.

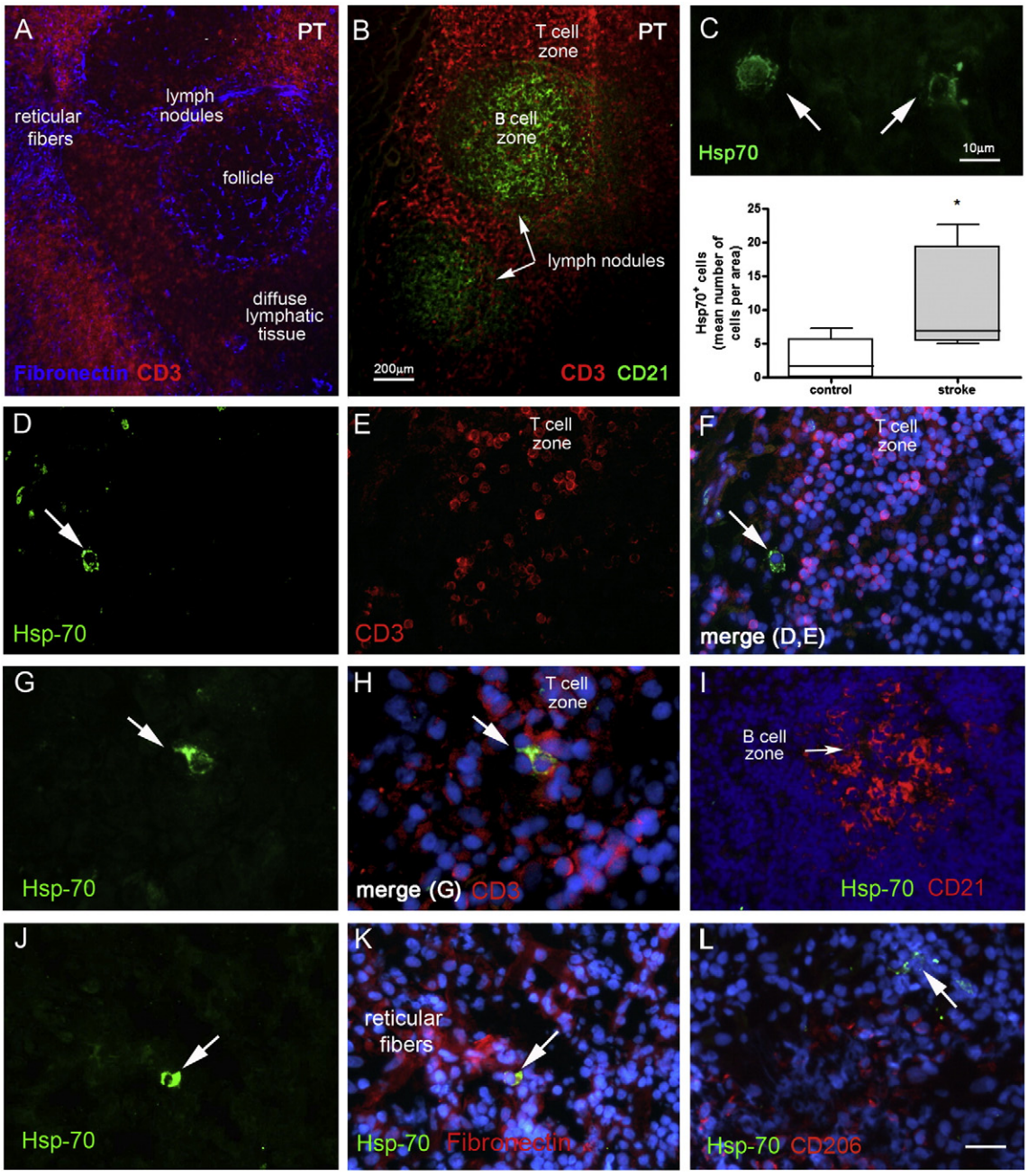

Fig. 3. Localization of Hsp-70 immunoreactivity. A) The structure of the palatine tonsil (PT) biopsies is illustrated showing lymph nodules surrounded by CD3+ (red) diffuse lymphoid tissue, and fibronectin+ (blue) reticular fibers of the fibroreticular network. B) CD3+ (red) T cell zones surround CD21+ (green) B cell zones. C) Higher numbers of Hsp-70 immunoreactive cells (green) are present in the PT biopsies of stroke patients (n = 6) *versus* controls (n = 7) (Mann–Whitney test, $^{*}p < 0.05$). D–F) Hsp-70+ cells (green, D) are seen within T-cell rich zones (CD3+, red, E) as shown in the merged image (F) where nuclei are stained with Hoechst (blue). G–H) Higher magnification illustration of the location of Hsp-70 (green, G) in CD3 (red) zones, as shown in the merged image (H) (blue is Hoechst). I) Hsp-70 (green) is not seen in B-cell rich areas stained for CD21 (red) (blue is Hoechst). J–K) Hsp-70 (green, J) is seen within the fibroreticular network (fibronectin, red) in the merged image (K) (blue is Hoechst). L) Hsp-70 (green) does not co-localize with the marker of resident dendritic cells (CD206, red) (blue is Hoechst). Lymphoid tissue (A–C) was obtained from PT biopsies of stroke patients and (D–L) from necropsies of patients with fatal stroke (CLN). Arrows point to Hsp-70 immunoreactive cells. Images were obtained with a fluorescence microscope. Bar scale: A–B = 200 μm, C = 10 μm. D–F = 50 μm, G–H = 15 μm; I = 200 μm, J–L = 30 μm.

cells were found to co-localize with CD68+ macrophages (Fig. 5G–I, J–L and M–O).

4. Discussion

We report the first study identifying an increased presence of Hsp-70 in brain draining lymph nodes of patients with acute stroke compared with control subjects free of symptomatic neurological disease, and show that Hsp-70 was not locally generated in the PT but was derived from non-lymphoid cellular sources. Previously, it was known that expression of Hsp-70 was induced in the brain after ischemia (Nowak and Jacewicz, 1994; Planas et al., 1997), particularly in penumbral neurons as a marker of cell viability (Li et al., 1992; Planas et al., 1997; Hata et al., 2000a,b; Yenari, 2002). We further illustrate that the amount of Hsp-70 at the brain-draining lymph nodes was correlated in crude and adjusted analyses with a better outcome and smaller infarctions at follow up in the stroke patients, but not with the plasma levels of the chaperone protein. While these results do not prove a causal relationship between the presence of Hsp-70 at the brain draining lymph nodes and the outcome of the patients we cannot exclude the

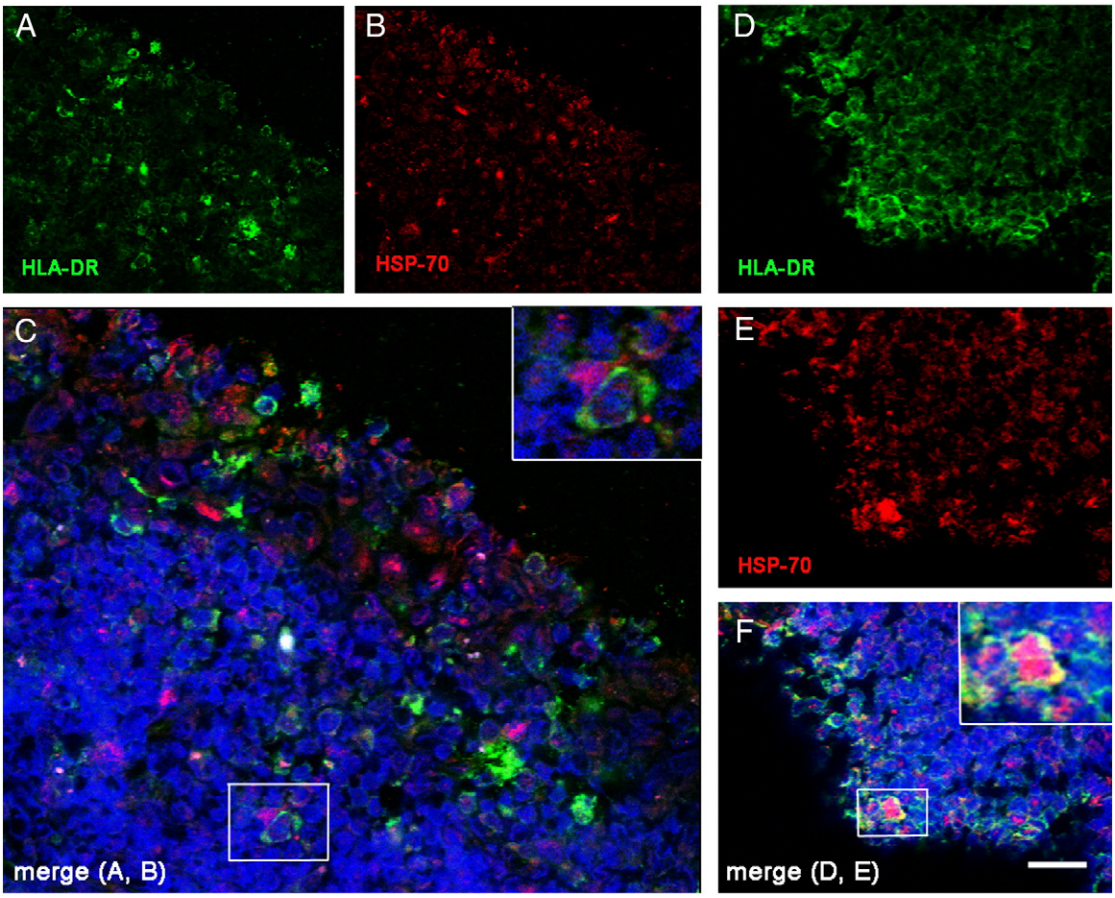

Fig. 4. Hsp-70 was found in antigen presenting cells immunoreactive for HLA-DR. Hsp-70 immunoreactive cells (red) are seen in areas immunoreactive for the MHCII HLA-DR (green). A–C) HLA-DR is shown in the green channel (A), corresponding Hsp-70 in the same field is shown in the red channel (B), and the merged image (C) illustrates both immunostainings together with cell nuclei stained with Hoechst (blue). D–F) HLA-DR is illustrated in the green channel (D), Hsp70 (E) in the red channel, and the merged image (F) illustrates the nuclei after Hoechst staining (blue). Insets in C and F correspond to higher magnifications of the areas marked with squares. Inset in C highlights an Hsp-70 negative HLA-DR positive cell, while inset in F highlights two HLA-DR positive cells that are immunoreactive for Hsp-70 as well. Samples were CLN necropsies of fatal stroke cases and images were obtained in a confocal microscope. Bar scale: A–B = 40 μm; C–F = 20 μm; insets in C and F = 10 μm.

possibility that Hsp-70 conveyed some clinical benefits in relation with its immunoregulatory properties (Quintana and Cohen, 2005).

Clinical (Planas et al., 2012) and experimental (Harling-Berg et al., 1999; deVos et al., 2002; van Zwam et al., 2008) studies previously showed an increased presence of brain-derived antigens in brain-draining lymph nodes of subjects with several neurological conditions including acute stroke. In the present study, we found a positive correlation between the presence of Hsp-70 at the PT and an increased amount of the NMDA glutamate receptor subunit NR-2A, a neuronal derived brain antigen that in previous studies by our group was shown to be associated with a better prognosis in human stroke (Planas et al., 2012). Collectively, these results highlighted that an increased transfer of specific brain antigens and chaperones to the draining lymph nodes of stroke patients might be of clinical significance although the causality of this association awaits confirmation in further studies and larger stroke cohorts.

Brain components present in the extracellular milieu might reach the draining lymphoid tissue either through direct fluid drainage by bulk flow (Harling-Berg et al., 1999; deVos et al., 2002; van Zwam et al., 2008), or after their capture by migrating cells. Previous studies showed that neuronal and myelin-derived brain antigens increased in lymphoid tissue located within the draining territory of the CNS of stroked mice and humans (van Zwam et al., 2008; Planas et al., 2012), although it remains unclear as to whether this process is antigen specific. In this study, we extended these observations and described that Hsp-70 was found in cells in contact with the fibroreticular network of the lymph node, in an anatomical distribution similar to that previously described for the presence of brain-derived antigens (Planas et al., 2012). We also showed that Hsp-70+ cells were equipped to induce T cell activation since these cells co-localized with APC expressing MHC class II molecules and prevailed in T-cell rich areas. Overall, these findings suggest that the transfer of Hsp-70 to brain-draining lymph nodes may represent a previously unrecognized mechanism linking innate and adaptive responses in human stroke.

The presence of Hsp-70 at the lymphoid tissue might facilitate the presentation of brain antigens to T cells (Binder and Srivastava, 2005). Indeed, previous studies showed that Hsp-70 reduced the amount of antigen necessary to trigger an adaptive immune response (Binder and Srivastava, 2005), and Hsp-70 was implicated in the development of tolerogenic responses against self-antigens (Millar et al., 2003; Schildknecht et al., 2009; Stocki et al., 2012). Furthermore, Hsp-70 may activate regulatory T cells that control pathogenic T cells specific for self-antigens, and anti-inflammatory activities in human T cells (Zanin-Zhorov et al., 2005). Our findings may suggest that Hsp-70 facilitates the identification of self-brain antigens, such as the NMDA glutamate receptor subunit NR-2A, and this facilitation might promote tolerogenic immune responses or tone down inflammatory responses, as previously described in certain autoimmune disorders, such as type 1 diabetes, and some forms of arthritis (Quintana and Cohen, 2005). Quantification of Hsp-70+ cells in lymph nodes that do not drain the

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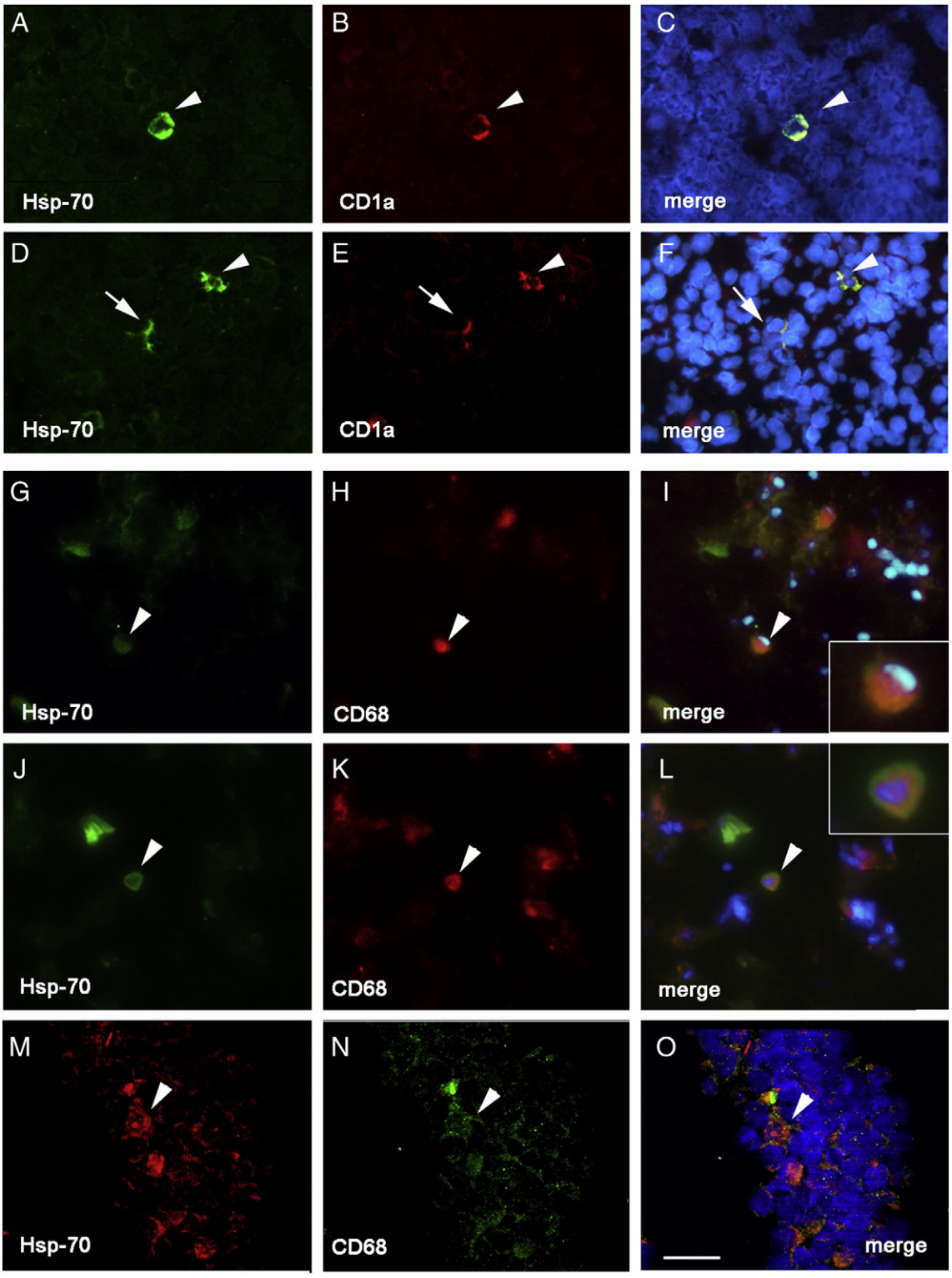

Fig. 5. Co-localization of Hsp-70 immunoreactivity with migrating dendritic cells and CD68 positive macrophages in cervical ganglion of stroke patients. A–C) Hsp-70 immunoreactivity is shown in the green channel (A); CD1a, a marker of migratory dendritic cells, is shown in the red channel (B); and co-localization is illustrated in the merged image (C) where nuclei are stained with Hoechst (blue). D–F) Hsp-70 (green, D) and CD1a (red, E) co-localize in the same cells as illustrated in the merged image (F). G–O) Illustration of Hsp-70 immunoreactivity (green in G, J, and red in M) that occasionally co-localizes with the macrophage marker CD68 (red in H and K, and green in N). I, L, O) Merged images of the corresponding Hsp-70 (G, J, M) and CD68 (H, K, N) immunoreactions counterstained with Hoechst (blue) to show the cell nuclei. Insets in I and L are magnifications of the cells pointed with arrowheads. Images were obtained from necropsies of patients with fatal stroke (CLN). Images were obtained with either a fluorescence microscope (A–L) or a confocal microscope (M–O). Bar scale: A–C = 30 μm, D–F = 10 μm; G–I = 30 μm; J–L = 20 μm; insets in I, L: 10 μm.

intracranial fluids of the brain might also help to clarify the specificity of the interaction between the central nervous system and the lymphoid system in subjects with acute stroke.

In summary, the current results highlight the presence of Hsp-70 in brain draining lymph nodes in human stroke and stress the rich interplay between the CNS and the immune system in this condition

(Chamorro et al., 2012). While Hsp-70 is recognized as a marker of brain tissue viability after ischemia (Li et al., 1992; Planas et al., 1997), the immunoregulatory and anti-inflammatory effects of Hsp-70 are also being increasingly recognized, and the current clinical findings may indicate that Hsp-70 contributes to keeping autoimmunity more effectively at bay after acute stroke. Altogether, the findings of this study contribute to make this molecule an attractive target for future study in acute stroke.

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References

Badin, R.A., Lythgoe, M.F., van der Weerd, L., Thomas, D.L., Gadian, D.G., Latchman, D.S., 2006. Neuroprotective effects of virally delivered HSPs in experimental stroke. J. Cereb. Blood Flow Metab. 26, 371–381.

Basu, S., Binder, R.J., Suto, R., Anderson, K.M., Srivastava, P.K., 2000. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maduration signal to dendritic cells and activate the NF-kappa B pathway. Int. Immunol. 12, 1539–1546.

Binder, R.J., Srivastava, P.K., 2005. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8 + T cells. Nat. Immunol. 6, 593–599.

Bradbury, M.W., Cserr, H.F., Westrop, R.J., 1981. Drainage of cerebral interstitial fluid into deep cervical lymph of the rabbit. Am. J. Physiol. 240, F329–F336.

Chamorro, A., Meisel, A., Planas, A.M., Urra, X., van de Beek, D., Veltkamp, R., 2012. The immunology of acute stroke. Nat. Rev. Neurol. 8, 401–410.

de la Rosa, X., Santalucía, T., Fortin, P.Y., Purroy, J., Calvo, M., Salas-Perdomo, A., Justicia, C., Couillaud, F., Planas, A.M., 2013. *In vivo* imaging of induction of heat-shock protein-70 gene expression with fluorescence reflectance imaging and intravital confocal microscopy following brain ischaemia in reporter mice. Eur. J. Nucl. Med. Mol. Imaging 40, 426–438.

deVos, A., van Meurs, M., Brok, H.P., Boven, L.A., Hintzen, R.Q., van der Valk, P., Ravid, R., Rensing, S., Boon, L., Hart, B.A., Laman, J.D., 2002. Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. J. Immunol. 169, 5415–5423.

Doeppner, T.R., Kaltwasser, B., Fengyan, J., Hermann, D.M., Bähr, M., 2013. TAT-Hsp70 induces neuroprotection against stroke *via* anti-inflammatory actions providing appropriate cellular microenvironment for transplantation of neural precursor cells. J. Cereb. Blood Flow Metab. http://dx.doi.org/10.1038/jcbfm.2013.126 (2013 Jul 24).

Doeppner, T.R., Nagel, F., Dietz, G.P., Weise, J., Tonges, L., Schwarting, S., Bähr, M., 2009. TAT-Hsp70-mediated neuroprotection and increased survival of neuronal precursor cells after focal cerebral ischemia in mice. J. Cereb. Blood Flow Metab. 29, 1187–1196.

Harling-Berg, C., Park, J.T., Knopf, P.M., 1999. Role of the cervical lymphatics in the Th2-type hierarchy of CNS immune regulation. J. Neuroimmunol. 101, 111–127.

Hartl, F.U., Hayer-Hartl, M., 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295, 1852–1858.

Hata, R., Maeda, K., Hermann, D., Mies, G.N., Hossmann, K.-A., 2000a. Dynamics of regional brain metabolism and gene expression after middle cerebral artery occlusion in mice. J. Cereb. Blood Flow Metab. 20, 306–315.

Hata, R., Maeda, K., Hermann, D., Mies, G.N., Hossmann, K.-A., 2000b. Evolution of brain infarction after transient focal cerebral ischemia in mice. J. Cereb. Blood Flow Metab. 20, 937–946.

Hochmeister, S., Zeitelhofer, M., Bauer, J., Nicolussi, E.-M., Fischer, M.-T., Heinke, B., Selzer, E., Lassmann, H., Bradl, M., 2010. After injection into the striatum, *in vitro*-differentiated microglia- and bone marrow-derived dendritic cells can leave the central nervous system *via* the blood stream. Am. J. Pathol. 173, 1669–1681.

Iadecola, C., Anrather, J., 2011. The immunology of stroke: from mechanisms to translation. Nat. Med. 17, 796–808.

Kinouchi, H., Sharp, F.R., Hill, M.P., Koistinaho, J., Sagar, S.M., Chan, P.H., 1993. Induction of 70-kDa heat shock protein and hsp70 mRNA following transient focal cerebral ischemia in the rat. J. Cereb. Blood Flow Metab. 13, 105–115.

Kono, H., Rock, K.L., 2008. How dying cells alert the immune system to danger. Nat. Rev. Immunol. 8, 279–289.

Lee, S.H., Kim, M., Yoon, B.W., Kim, Y.J., Ma, S.J., Roh, J.K., Lee, J.S., Seo, J.S., 2001a. Targeted hsp70.1 disruption increases infarction volume after focal cerebral ischemia in mice. Stroke 32, 2905–2912.

Lee, J.E., Yenari, M.A., Sun, G.H., Xu, L., Emond, M.R., Cheng, D., Steinberg, G.K., Giffard, R.G., 2001b. Differential neuroprotection from human heat shock protein 70 overexpression in *in vitro* and *in vivo* models of ischemia and ischemia-like conditions. Exp. Neurol. 170, 129–139.

Li, Y., Chopp, M., Garcia, J.H., Yoshida, Y., Zhang, Z.G., Levine, S.R., 1992. Distribution of the 72-kd heat-shock protein as a function of transient focal cerebral ischemia in rats. Stroke 23, 1292–1298.

Millar, D.G., Garza, K.M., Odermatt, B., Elford, A., Ono, N., Li, Z., Ohashi, P.S., 2003. Hsp70 promotes antigen presenting cell function and converts T-cell tolerance to autoimmunity *in vivo*. Nat. Med. 9, 1469–1476.

Nowak Jr., T.S., Ikeda, J., Nakajima, T., 1990. 70-kDa heat shock protein and c-fos gene expression after transient ischemia. Stroke 21 (11 Suppl.), III107–III111.

Nowak Jr., T.S., Jacewicz, M., 1994. The heat shock/stress response in focal cerebral ischemia. Brain Pathol. 4, 67–76.

Olsson, T., Hansson, O., Nylandsted, J., Jäättelä, M., Smith, M.L., Wieloch, T., 2004. Lack of neuroprotection by heat shock protein 70 overexpression in a mouse model of global cerebral ischemia. Exp. Brain Res. 154, 442–449.

Planas, A.M., Gomez-Choco, M., Urra, X., Gorina, R., Caballero, M., Chamorro, A., 2012. Brain-derived antigens in lymphoid tissue of patients with acute stroke. J. Immunol. 188, 2156–2163.

Planas, A.M., Soriano, M.A., Estrada, A., Sanz, O., Martin, F., Ferrer, I., 1997. The heat shock stress response after brain lesions-induction of 72 kDa heat shock protein (cell types involved, axonal transport, transcriptional regulation) and protein synthesis inhibition. Prog. Neurobiol. 51, 607–636.

Plumier, J.C., Krueger, A.M., Currie, R.W., Kontoyiannis, D., Kollias, G., Pagoulatos, G.N., 1997. Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury. Cell Stress Chaperones 2, 162–167.

Quintana, F.J., Cohen, I.R., 2005. Heat shock proteins as endogenous adjuvants in sterile and septic inflammation. J. Immunol. 175, 2777–2782.

Rajdev, S., Hara, K., Kokubo, Y., Mestril, R., Dillmann, W., Weinstein, P.R., Sharp, F.R., 2000. Mice overexpressing rat heat shock protein 70 are protected against cerebral infarction. Ann. Neurol. 47, 782–791.

Saito, K., Dai, Y., Ohtsuka, K., 2005. Enhanced expression of heat shock proteins in gradually dying cells and their release from necrotically dead cells. Exp. Cell Res. 310, 229–236.

Schildknecht, A., Probst, H.C., McCoy, K.D., Miescher, I., Brenner, C., Leone, D.P., Suter, U., Ohashi, P.S., van den Broek, M., 2009. Antigens expressed by myelinating glia cells induce peripheral cross-tolerance of endogenous CD8 + T cells. Eur. J. Immunol. 39, 1505–1515.

Sharp, F.R., Kinouchi, H., Koistinaho, J., Chan, P.H., Sagar, S.M., 1993. HSP70 heat shock gene regulation during ischemia. Stroke 24 (12 Suppl.), I72–I75.

Stocki, P., Wang, X.N., Dickinson, A.M., 2012. Inducible heat shock protein 70 reduces T cell responses and stimulatory capacity of monocyte-derived dendritic cells. J. Biol. Chem. 287, 12387–12394.

Tsuchiya, D., Hong, S., Matsumori, Y., Shiina, H., Kayama, T., Swanson, R.A., Dillman, W.H., Liu, J., Panter, S.S., Weinstein, P.R., 2003. Overexpression of rat heat shock protein 70 is associated with reduction of early mitochondrial cytochrome *C* release and subsequent DNA fragmentation after permanent focal ischemia. J. Cereb. Blood Flow Metab. 23, 718–727.

van Zwam, M., Huizinga, R., Melief, M.-J., Wierenga-Wolf, A.F., Meurs, M., Voerman, J.S., Biber, K.P.H., Boddeke, H.W.G.M., Höpken, U.E., Meisel, C., Meisel, A., Bechmann, I., Hintzen, R.Q., 't Hart, B.A., Amor, S., Laman, J.D., Boven, L.A., 2008. Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE. J. Mol. Med. 87, 273–286.

von Andrian, U.H., Memple, T.R., 2003. Homing and cellular traffic in lymph nodes. Nat. Rev. Immunol. 3, 867–878.

Weller, R.O., Djuanda, E., Yow, H.-Y., Carare, R.O., 2008. Lymphatic drainage of the brain and the pathophysiology of neurological disease. Acta Neuropathol. 117, 1–14.

Xu, L., Emery, J.F., Ouyang, Y.B., Voloboueva, L.A., Giffard, R.G., 2010. Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia. Glia 58, 1042–1049.

Yenari, M.A., 2002. Heat shock proteins and neuroprotection. Adv. Exp. Med. Biol. 513, 281–599.

Yenari, M.A., Fink, S.L., Sun, G.H., Chang, L.K., Patel, M.K., Kunis, D.M., Onley, D., Ho, D.Y., Sapolsky, R.M., Steinberg, G.K., 1998. Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy. Ann. Neurol. 44, 584–591.

Yenari, M., Giffard, R.G., Sapolsky, R.M., Steinberg, G.K., 1999. The neuroprotective potential of heat shock protein 70 (HSP70). Mol. Med. Today 5, 525–531.

Yenari, M.A., Liu, J., Zheng, Z., Vexler, Z.S., Lee, J.E., Giffard, R.G., 2005. Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. Ann. N. Y. Acad. Sci. 1053, 74–83.

Zanin-Zhorov, A., Tal, G., Shivtiel, S., Cohen, M., Lapidot, T., Nussbaum, G., Margalit, R., Cohen, I.R., Lider, O., 2005. Heat shock protein 60 activates cytokine-associated negative regulator suppressor of cytokine signaling 3 in T cells: effects on signaling, chemotaxis, and inflammation. J. Immunol. 175, 276–285.

Zhan, X., Ander, B.P., Liao, I.H., Hansen, J.E., Kim, C., Clements, D., Weisbart, R.H., Nishimura, R.N., Sharp, F.R., 2010. Recombinant Fv-Hsp70 protein mediates neuroprotection after focal cerebral ischemia in rats. Stroke 41, 538–543.

Zheng, Z., Kim, J.Y., Ma, H., Lee, J.E., Yenari, M.A., 2008. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. J. Cereb. Blood Flow Metab. 28, 53–63.

VI.- Síntesis de resultados y discusión

En conjunto, los trabajos presentados muestran que en el tejido linfoide del paciente con ictus se encuentran los elementos necesarios para desarrollar una respuesta específica frente a antígenos cerebrales, lo cual podría influir en la fisiopatología y evolución de la enfermedad.

Estructura del tejido linfoide en el paciente con ictus.

El primer trabajo ofrece por primera vez una descripción del TLS en pacientes con ictus agudo. En estos pacientes el TLS presentó su habitual estructura, consistente en centros germinales con linfocitos B maduros (CD21+), rodeados un área interfolicular rica en linfocitos T (CD3+). El marcaje para fibronectina mostró que ambos tipos de células se encontraban englobados por una estructura reticular dispuesta alrededor de los folículos linfoides, en los espacios interfoliculares, y rodeando al endotelio de los vasos sanguíneos. Se observó también la presencia de unas células CD206+, localizadas preferentemente en los espacios interfoliculares ricos en células T y unas células CD1a+ localizadas cerca de estructuras ricas en fibronectina en la periferia de la zona T. Estos datos son compatibles con la distribución de CPA en ganglios linfáticos humanos descrita por Angel y colaboradores, correspondiendo las células CD206+ a la subpoblación de CPA residentes y las CD1a+ a las células migratorias(Angel, Chen et al. 2009).

Presencia de antígenos cerebrales en el tejido linfoide de pacientes con ictus.

Tanto los antígenos mielínicos (MBP y MOG) como neuronales (MAP-2,NR-2A) estaban presentes en el TLS de pacientes con ictus y sujetos control. Los primeros mostraron más células inmunoreactivas frente a MAP-2 y MBP que los controles tanto en la amígdala palatina como en el GL, si bien la diferencia no fue estadísticamente significativa tras ajustar por la edad. El antígeno neuronal NR-2A estaba significativamente incrementado respecto a controles cuando fue determinado de forma semicuantitativa mediante Western blot. Si bien en modelos animales de isquemia o EAE, y en pacientes con esclerosis múltiple ya se había descrito la presencia de antígenos cerebrales en el TLS (deVos, van Meurs et al. 2002; van Zwam, Huizinga et al. 2008), ésta es la primera vez que se describe en pacientes con ictus.

La expresión de RNA de los antígenos MAP-2 y NR-2A mediante RT-PCR cuantitativa fue mínima tanto para los ictus como para los controles. Este dato, junto al incremento observado para ciertos antígenos en pacientes con ictus respecto a controles, creemos que nos permite sugerir que el origen de los antígenos encontrados en el TLS no se debe a una síntesis local y posiblemente provengan del SNC.

Las células inmunoreactivas para antígenos cerebrales se localizaron preferentemente en zonas T, próximas a estructuras teñidas para fibronectina correspondientes a la estructura fibroreticular del GL. La mayoría de estas células fueron identificadas principalmente como macrófagos, dada su morfología y la expresión del marcador CD68. En menor medida se trató de DC migratorias, dada su expresión de otros marcadores como CD1a, CD1b, CD1c o CD303, pero no de células CD206+ o CD209+, correspondientes a DC residentes. Sin embargo, muchas de las células inmunoreactivas para antígenos cerebrales se encontraban en la vecindad de células CD209+. Al estudiar de forma cuantitativa las células portadoras de antígenos, sólo los macrófagos fueron significativamente más abundantes en pacientes con ictus que en controles, mientras las células dendríticas estuvieron presentes en cantidades similares en ambos grupos. Cuando se realizó Western blot para los antígenos neuronales MAP-2 y NR-2A a partir de extractos proteicos de amígdala palatina se observaron bandas con un peso molecular similar al de las proteínas nativas, pero también fragmentos de menor peso molecular.

En conjunto, estos datos muestran que algunos antígenos cerebrales pueden alcanzar el TLS tanto en pacientes con ictus como en controles y probablemente ser procesados por células con capacidad de presentar antígenos. Se ha descrito que tanto moléculas como células pueden dejar el SNC y alcanzar el TLS a través del torrente sanguíneo (Hochmeister, Zeitelhofer et al. 2010) o del drenaje de linfa y líquidos intersticiales (Bradbury MW, Cserr HF et al. 1981). Aunque en nuestro trabajo no podemos determinar la vía de llegada de los antígenos cerebrales encontrados, la localización de las células cargadas con antígenos cerebrales en proximidad de estructuras ricas en fibronectina apoyaría que el drenaje de líquidos intersticiales tendría un papel importante en la llegada de los antígenos. No podemos determinar si la fagocitosis de los antígenos tuvo lugar en el parénquima cerebral lesionado o en el GL si bien las células portadoras de estos antígenos presentan características de CPA. Por otra parte, la presencia de fragmentos de menor peso molecular que la proteínas nativas al realizar los

estudios de Western blot es compatible con la fagocitosis y procesamiento de estas proteínas por las CPA previo a su presentación a través de moléculas MHC-II (Vyas, Van der Veen et al. 2008).

Presencia de moléculas coadyuvantes (Hsp-70) en el tejido linfoide de pacientes con ictus.

En el segundo trabajo mostramos que la proteína Hsp-70 se encuentra también presente en mayor cantidad en el TLS de pacientes con ictus. Hsp-70 tiene funciones de chaperona, encargándose del ensamblaje de péptidos nacientes y de la estabilización de péptidos dañados, y debido a estas propiedades se le ha atribuido un papel neuroprotector en la isquemia cerebral. Pero Hsp-70 también puede funcional como una señal de alarma o DAMP (Bianchi 2006; Kono and Rock 2008) y favorecer la presentación antigénica (Binder and Srivastava 2005).

En nuestro trabajo observamos que Hsp-70 se encontraba en mayores cantidades en el TLS de pacientes con ictus que en controles tanto en los estudios de inmunofluorescencia como en el estudio semicuantitativo mediante Western blot. Sin embargo, no se observaron diferencias significativas entre ictus y controles cuando Hsp-70 fue determinado en plasma. Por otra parte, la expresión de RNA para Hsp-70 en TLS de ictus y controles determinada mediante RT-PCR fue prácticamente insignificante. Ello nos sugiere que la mayor cantidad de proteína Hsp-70 presente en el TLS de pacientes con ictus no parece deberse a una síntesis local en el tejido linfoide.

Hsp-70 se encontró localizada en el TLS siguiendo un patrón similar al descrito previamente para los antígenos cerebrales. Gran parte de las células inmunoreactivas para Hsp-70 expresaron también el marcador CD1a+, pero ninguna expresó CD206+. Como mencionamos previamente las células CD1a+ se consideran DC migratorias mientras que las CD206+ se corresponderían principalmente con las DC residentes. Además, gran parte de las células Hsp-70+ se encontraron en zonas T y en la vecindad de estructuras ricas en fibronectina. En nuestra opinión estos datos sugieren en su conjunto que probablemente la proteína Hsp-70 detectada en el TLS de pacientes con ictus pudo haber sido producida en el SNC durante la situación de isquemia y llegar al TLS a través de las vías descritas previamente. Hsp-70 es inducida en gran cantidad

durante la isquemia cerebral (Nowak and Jacewicz 1994; Planas, Soriano et al. 1997) y es por tanto factible que se encuentre en mayores cantidades en pacientes que en controles. Por otra parte, Hsp-70 presentó una estrecha correlación con el antígeno neural NR-2ª (subunidad 2A del receptor de glutamato) determinado mediante Western blot. Ello apoya un posible origen neural para la presencia de Hsp-70 en el TLS.

Presentación de los antígenos cerebrales.

Hasta ahora hemos mostrado que algunos antígenos cerebrales pueden encontrarse en CPA presentes en el TLS, en el cual disponemos también de la presencia de la molécula coadyuvante Hsp-70 y que tanto antígenos como Hsp-70 se encuentran en mayor cantidad en los pacientes con ictus, pero desconocemos si ello tiene un papel en la fisiopatología del ictus o se trata de un mero epifenómeno.

Hemos observado que las células inmunoreactivas para antígenos cerebrales y para Hsp-70 expresaron con más frecuencia moléculas HLA-DR del MHC en pacientes con ictus que en controles, lo cual sugiere que estas células tienen capacidad para presentar antígenos. Por otra parte, las células inmunoreactivas para antígenos cerebrales se encontraban habitualmente en la proximidad de células residentes DC-SIGN positivas(CD209+), lo cual podría sugerir mecanismos de cooperación en la presentación antigénica por ambos tipos de células como ha sido descrito para las células migratorias y residentes (Allenspach, Lemos et al. 2008).

Cuando estudiamos el marcador de activación linfocitaria CD69, pudimos ver que en los pacientes con ictus se encontraron con más frecuencia células CD69+ en contacto o próximas a células MAP-2+ que en controles. En escasas ocasiones una misma célula fue simultáneamente MAP-2+ y CD69+. La mayoría de las células CD69+ fueron linfocitos T(CD3+) y ocasionalmente se trató de macrófagos (CD68+). Aunque no pudimos determinar otras medidas de activación linfocitaria, creemos que la presencia de CPA reactivas para antígenos neurales puede ejercer algún tipo de activación sobre las células T adyacentes.

Potenciales implicaciones clínicas.

Respecto a las potenciales implicaciones clínicas de la llegada de antígenos cerebrales al TLS, se observó que la gravedad del ictus, el volumen de infarto y el pronóstico final de los pacientes estaban en relación con la presencia de antígenos neuronales o mielínicos. De este modo, la gravedad inicial del ictus y el volumen de infarto se correlacionaron positivamente con la inmunoreactividad frente a MBP, mientras que la inmunoreactividad frente a MAP-2 y NR-2A se correlacionó inversamente al volumen del infarto pero no con la gravedad clínica inicial. Los pacientes con buen pronóstico funcional presentaron más inmunoreactividad frente a los antígenos neuronales MAP-2 y NR-2A y menos células MBP+ que los pacientes con peor pronóstico.

CD69 contribuye a mantener a las células Th secuestradas en los órganos linfoides (Shiow, Rosen et al. 2006) y su déficit favorece el desarrollo de respuestas inflamatorias (Cruz-Adalia, Jimenez-Borreguero et al. 2010; Martin, Gómez et al. 2010). La íntima relación espacial entre las células MAP-2+ y CD69+, y el efecto inmunomodulador relacionado con la expresión de este marcador podría ser unos de los potenciales mecanismos que explicaran el mejor pronóstico de aquellos pacientes que presentaban en mayor cantidad células inmunoreactivas para antígenos neurales.

Por otra parte, en nuestro segundo trabajo describimos como la cantidad de Hsp-70 en TLS se encontró inversamente relacionada con la gravedad inicial del ictus, la extensión de la lesión y el pronóstico a los 3 meses. Dado que Hsp-70 se sintetiza en las zonas de penumbra isquémica (Hata, Maeda et al. 2000; Hata, Maeda et al. 2000), se podría especular que aquellos pacientes con más tejido potencialmente “en riesgo” o “salvable” serían aquellos con mayor capacidad para producir Hsp-70 y de ello se derivaría la relación encontrada. Sin embargo la relación de HSP70 con un mejor pronóstico funcional a los 3 meses se mantuvo tras ajustar por la severidad clínica inicial, lo cual podría sugerir algún otro papel fisiopatológico para Hsp-70.

Dado que Hsp-70 puede actuar como molécula coadyuvante y se encuentra preferentemente localizada en células presentadoras CD1a+ en próximo contacto con células T, se podría especular que Hsp-70 podría facilitar la presentación de antígenos

neurales como NR-2A y favorecer el desarrollo de respuestas inmunomoduladoras (Millar, Garza et al. 2003; Schildknecht, Probst et al. 2009; Stocki, Wang et al. 2012).

VII.-Conclusiones

1. En el tejido linfoide secundario del paciente con ictus se encuentran antígenos cerebrales y la molécula coestimuladora Hsp-70 en mayor cantidad que en sujetos controles. Ello pudiera deberse a su mayor llegada a través de las vías de drenaje del líquido intersticial del SNC.

2. Los antígenos cerebrales y la Hsp-70 se localizan preferentemente en la zona T del tejido linfoide, en células que presentan características de célula presentadora de antígeno.

3. La mayoría de las células portadoras de antígenos cerebrales tienen características de macrófagos y en menor medida de células dendríticas. Estas células se encuentran adyacentes al entramado fibroreticular del tejido linfoide a través del cual circula la linfa.

4. La presencia de antígenos cerebrales en células presentadoras de antígenos puede derivar en algún tipo de activación linfocitaria, dada la expresión del marcador de activación temprano CD69 en células T próximas a células portadoras del antígeno MAP-2.

5. El pronóstico del paciente con ictus pudiera estar determinado por la respuesta frente a determinados antígenos cerebrales y el desarrollo de respuestas inmunomoduladoras, presentado mejor pronóstico aquellos pacientes con una mayor inmunoreactividad frente a los antígenos neurales MAP-2 y NR-2a, y una mayor inmunoreactividad frente a Hsp-70.

VIII.- Bibliografía

Abilleira, S., A. Davalos, et al. (2011). "Outcomes of Intravenous Thrombolysis After Dissemination of the Stroke Code and Designation of New Referral Hospitals in Catalonia: The Catalan Stroke Code and Thrombolysis (Cat-SCT) Monitored Study." Stroke 42: 2001-2006.

Akira, S., S. Uematsu, et al. (2006). "Pathogen Recognition and Innate Immunity." Cell 124: 783-801.

Allenspach, E. J., M. P. Lemos, et al. (2008). "Migratory and Lymphoid-Resident Dendritic Cells Cooperate to Efficiently Prime Naive CD4 T cells." Immunity 29: 795-806.

Angel, C. E., C. J. J. Chen, et al. (2009). "Distinctive localization of antigen-presenting cells in human lymph nodes." Blood 113: 1257-1267.

Badin, R. A., M. F. Lythgoe, et al. (2005). "Neuroprotective effects of virally delivered HSPs in experimental stroke." Journal of Cerebral Blood Flow & Metabolism 26: 371-381.

Bajénoff, M., J. G. Egen, et al. (2006). "Stromal Cell Networks Regulate Lymphocyte Entry, Migration, and Territoriality in Lymph Nodes." Immunity 25: 989-1001.

Ball, K. K., N. F. Cruz, et al. (2009). "Trafficking of glucose, lactate, and amyloid-β from the inferior colliculus through perivascular routes." Journal of Cerebral Blood Flow & Metabolism 30: 162-176.

Basu, S., R. J. Binder, et al. (2000). "Necrotic but not apoptotic cell death realeases heat shock proteins, which deliver a partial maduration signal to dendritic cells and activate the NF-KB pathway." International Immunology 12: 1539-1546.

Bechmann, I., I. Galea, et al. (2007). "What is the blood-brain barrier (not)?" Trends in Immunology 28: 5-11.

Becker, K., K. Kindrcik, et al. (2003). "Adoptive Transfer of Myelin Basic Protein-Tolerized Splenocytes to Naive Animals Reduces Infarct Size: A Role for Lymphocytes in Ischemic Brain Injury?" Stroke 34: 1809-1815.

Becker, K. J., A. J. Kalil, et al. (2011). "Autoimmune Responses to the Brain After Stroke Are Associated With Worse Outcome." Stroke 42: 2763-2769.

Becker, K. J., R. M. McCarron, et al. (1997). "Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia." Proceedings of the National Academy of Sciences 94: 10873-10878.

Bianchi, M. E. (2006). "DAMPs, PAMPs and alarmins: all we need to know about danger." Journal of Leukocyte Biology 81: 1-5.

Binder, R. J. and P. K. Srivastava (2005). "Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells." Nature Immunology 6: 593-599.

Bornstein, N. M., B. Aronovich, et al. (2001). "Antibodies to brain antigens following stroke." Neurology 56: 529-530.

Bradbury MW, Cserr HF, et al. (1981). "Drainage of cerebral interstitial fluid into deep cervical lymph of the rabbit." American Journal of Physiology 240: F329-F336.

Brand, C. U., R. E. Hunger, et al. (1999). "Characterization of human skin-derived CD1a-positive lymph cells." Archives of Dermatological Research 291: 65-72.

Carare, R. O., M. Bernardes-Silva, et al. (2008). "Solutes, but not cells, drain from the brain parenchyma along basement membranes of capillaries and arteries: significance for cerebral amyloid angiopathy and neuroimmunology." Neuropathology and Applied Neurobiology 34: 131-144.

Carrasco, Y. R. and F. D. Batista (2007). "B Cells Acquire Particulate Antigen in†a Macrophage-Rich Area at the Boundary between the Follicle and†the Subcapsular Sinus of the Lymph Node." Immunity 27: 160-171.

Caruso, A., S. Licenziati, et al. (1997). "Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation." Cytometry 27: 71-76.

Caso, J. R., J. M. Pradillo, et al. (2007). "Toll-Like Receptor 4 Is Involved in Brain Damage and Inflammation After Experimental Stroke." Circulation 115: 1599-1608.

Cavanagh, L. L. and W. Weninger (2008). "Dendritic cell behaviour in vivo: lessons learned from intravital two-photon microscopy." Immunology and Cell Biology 86: 428-438.

Chamorro, A., A. Meisel, et al. (2012). "The immunology of acute stroke." Nature Reviews Neurology 8: 401-410.

Chamorro, A., X. Urra, et al. (2007). "Infection After Acute Ischemic Stroke: A Manifestation of Brain-Induced Immunodepression." Stroke 38: 1097-1103.

Choi, K. M., P. C. Kashyap, et al. (2010). "CD206-Positive M2 Macrophages That Express Heme Oxygenase-1 Protect Against Diabetic Gastroparesis in Mice." Gastroenterology 138: 2399-2409.e2391.

Clapham, R., E. O'Sullivan, et al. (2010). "Cervical lymph nodes are found in direct relationship with the internal carotid artery: Significance for the lymphatic drainage of the brain." Clinical Anatomy 23: 43-47.

Collin, M., N. McGovern, et al. (2013). "Human dendritic cell subsets." Immunology 140: 22-30.

Cruz-Adalia, A., L. J. Jimenez-Borreguero, et al. (2010). "CD69 Limits the Severity of Cardiomyopathy After Autoimmune Myocarditis." Circulation 122: 1396-1404.

Dambinova, S. A., G. A. Khounteev, et al. (2003). "Blood Test Detecting Autoantibodies to N-Methyl-D-aspartate Neuroreceptors for Evaluation of Patients with Transient Ischemic Attack and Stroke." Clinical Chemistry 49: 1752-1762.

de la Rosa, X., T. Santalucía, et al. (2013). "In vivo imaging of induction of heat-shock protein-70 gene expression with fluorescence reflectance imaging and intravital confocal microscopy following brain ischaemia in reporter mice." European Journal of Nuclear Medicine and Molecular Imaging 40: 426-438.

deVos, A., M. van Meurs, et al. (2002). "Transfer of Central Nervous System Autoantigens and Presentation in Secondary Lymphoid Organs." Journal of Immunology 169: 5415-5423.

Díaz-Guzman, J., J. A. Egido, et al. (2012). "Stroke and transient ischemic attack incidence rate in Spain: the IBERICTUS study." Cerebrovascular Diseases 34: 272-281.

Diebold, S. S. (2008). "Determination of T-cell fate by dendritic cells." Immunology and Cell Biology 86: 389-397.

Dirnagl, U., J. Klehmet, et al. (2007). "Stroke-Induced Immunodepression: Experimental Evidence and Clinical Relevance." Stroke 38: 770-773.

Doeppner, T. R., F. Nagel, et al. (2009). "TAT-Hsp70-mediated neuroprotection and increased survival of neuronal precursor cells after focal cerebral ischemia in mice." Journal of Cerebral Blood Flow & Metabolism 29: 1187-1196.

Engelhardt, B. and R. M. Ransohoff (2005). "The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms." Trends in Immunology 26: 485-495.

Fabriek, B. O., J. N. P. Zwemmer, et al. (2005). "In vivo detection of myelin proteins in cervical lymph nodes of MS patients using ultrasound-guided fine-needle aspiration cytology." Journal of Neuroimmunology 161: 190-194.

Felger, J. C., T. Abe, et al. (2010). "Brain dendritic cells in ischemic stroke: Time course, activation state, and origin." Brain, Behavior, and Immunity 24: 724-737.

Frenkel, D., Z. Huang, et al. (2003). "Nasal Vaccination with Myelin Oligodendrocyte Glycoprotein Reduces Stroke Size by Inducing IL-10-Producing CD4+T Cells." Journal of Immunology 172: 6549-6555.

Galea, I., I. Bechmann, et al. (2007). "What is immune privilege (not)?" Trends in immunology 28: 12-18.

Gee, J. M., A. Kalil, et al. (2008). "Induction of Immunologic Tolerance to Myelin Basic Protein Prevents Central Nervous System Autoimmunity and Improves Outcome After Stroke." Stroke 39: 1575-1582.

Goldmann, J., E. Kwidzinski, et al. (2006). "T cells traffic from brain to cervical lymph nodes via the cribroid plate and the nasal mucosa." Journal of Leukocyte Biology 80: 797-801.

Gottfried, E., L. A. Kunz-Schughart, et al. (2008). "Expression of CD68 in Non-Myeloid Cell Types." Scandinavian Journal of Immunology 67: 453-463.

Gray, E. E. and J. G. Cyster (2012). "Lymph Node Macrophages." Journal of Innate Immunity 4: 424-436.

Haeusler, K. G., W. U. H. Schmidt, et al. (2008). "Cellular Immunodepression Preceding Infectious Complications after Acute Ischemic Stroke in Humans." Cerebrovascular Diseases 25: 50-58.

Hardtke, S., L. Ohl, et al. (2005). "Balanced expression of CXCR5 and CCR7 on follicular T helper cells determines their transient positioning to lymph node follicles and is essential for efficient B-cell help." Blood 106: 1924-1931.

Harling-Berg, C., J. T. Park, et al. (1999). "Role of the cervical lymphatics in the Th2-type hierarchy of CNS immune regulation." Journal of Neuroimmunology 101: 111-127.

Harling-Berg, C. J., K. P. M., et al. (1991). "Myelin basic protein infused into cerebrospinal fluid suppresses experimental autoimmune encephalomyelitis." Journal of Neuroimmunology 35: 45-51.

Hartl, F. and M. Hayer-Hartl (2002). "Molecular chaperones in the cytosol: from nascent chain to folded protein." Science 295: 1852-1858.

Hata, R., K. Maeda, et al. (2000). "Dynamics of Regional Brain Metabolism and Gene Expression After Middle Cerebral Artery Occlusion in Mice." Journal Of Cerebral Blood Flow & Metabolism 20: 306-315.

Hata, R., K. Maeda, et al. (2000). "Evolution of Brain Infarction After Transient Focal Cerebral Ischemia in Mice." Journal Of Cerebral Blood Flow & Metabolism 20: 937-946.

Hatterer, E., N. Davoust, et al. (2005). "How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes." Blood 107: 806-812.

Hatterer, E., M. Touret, et al. (2008). "Cerebrospinal Fluid Dendritic Cells Infiltrate the Brain Parenchyma and Target the Cervical Lymph Nodes under Neuroinflammatory Conditions." PLoS ONE 3: e3321.

Hawkins, B. T. and T. P. Davis (2005). "The Blood-Brain Barrier/Neurovascular Unit in Health and Disease." Pharmacological Reviews 57: 173-185.

Hochmeister, S., M. Zeitelhofer, et al. (2010). "After Injection into the Striatum, in Vitro-Differentiated Microglia- and Bone Marrow-Derived Dendritic Cells Can Leave the Central Nervous System via the Blood Stream." The American Journal of Pathology 173: 1669-1681.

Hug, A., A. Dalpke, et al. (2009). "Infarct Volume is a Major Determiner of Post-Stroke Immune Cell Function and Susceptibility to Infection." Stroke 40: 3226-3232.

Hume, D. A. (2008). "Macrophages as APC and the Dendritic Cell Myth." The Journal of Immunology 181: 5829-5835.

Iadecola, C. and J. Anrather (2011). "The immunology of stroke: from mechanisms to translation." Nature Medicine 17: 796-808.

Itano, A. A. and M. K. Jenkins (2003). "Antigen presentation to naive CD4 T cells in the lymph node." Nature Immunology 4: 733-739.

Jácomo, A. L., F. E. Akamatsu, et al. (2010). "Pharyngeal lymphatic ring: anatomical review." Journal of Morphological Science 27: 47-49.

Jauch, E. C., C. Lindsell, et al. (2006). "Association of Serial Biochemical Markers With Acute Ischemic Stroke: The National Institute of Neurological Disorders and Stroke Recombinant Tissue Plasminogen Activator Stroke Study." Stroke 37: 2508-2513.

Jin, R., G. Yang, et al. (2010). "Inflammatory mechanisms in ischemic stroke: role of inflammatory cells." Journal of Leukocyte Biology 87: 779-789.

Johnston, M., A. Zakharov, et al. (2005). "Subarachnoid injection of Microfil reveals connections between cerebrospinal fluid and nasal lymphatics in the non-human primate." Neuropathology and Applied Neurobiology 31: 632-640.

Johnston, M., A. Zakharov, et al. (2004). "Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species." Cerebrospinal Fluid Research 1: 2.

Kalev-Zylinska, M. L., W. Symes, et al. (2013). "Stroke Patients Develop Antibodies That React With Components of N-Methyl-d-Aspartate Receptor Subunit 1 in Proportion to Lesion Size." Stroke 44: 2212-2219.

Karman, J., C. Ling, et al. (2004). "Initiation of Immune Responses in Brain Is Promoted by Local Dendritic Cells." Journal of Immunology 173: 2353-2361.

Katakai, T., T. Hara, et al. (2004). "A novel reticular stromal structure in lymph node cortex: an immuno-platform for interactions among dendritic cells, T cells and B cells." International Immunology 16: 1133-1142.

Katakai, T., T. Hara, et al. (2004). "Lymph Node Fibroblastic Reticular Cells Construct the Stromal Reticulum via Contact with Lymphocytes." The Journal of Experimental Medicine 200: 783-795.

Kinouchi, H., F. R. Sharp, et al. (1993). "Induction of 70-kDa Heat Shock Protein and hsp70 mRNA Following Transient Focal Cerebral Ischemia in the Rat." Journal Of Cerebral Blood Flow & Metabolism 13: 105-115.

Kono, H. and K. L. Rock (2008). "How dying cells alert the immune system to danger." Nature Reviews Immunology 8: 279-289.

Lee, S.-H., M. Kim, et al. (2001). "Targeted hsp70.1 Disruption Increases Infarction Volume After Focal Cerebral Ischemia in Mice." Stroke 32: 2905-2912.

Li, Y., M. Chopp, et al. (1992). "Distribution of the 72-kd Heat-Shock Protein as a Function of Transient Focal Cerebral Ischemia in Rats." Stroke 23: 1292-1298.

Liesz, A., S. Hagmann, et al. (2009). "The Spectrum of Systemic Immune Alterations After Murine Focal Ischemia: Immunodepression Versus Immunomodulation." Stroke 40: 2849-2858.

Macrez, R., C. Ali, et al. (2011). "Stroke and the immune system: from pathophysiology to new therapeutic strategies." The Lancet Neurology 10: 471-480.

Marshak-Rothstein, A. (2006). "Toll-like receptors in systemic autoimmune disease." Nature Reviews Immunology 6: 823-835.

Martin, P., M. Gómez, et al. (2010). "The leukocyte activation antigen CD69 limits allergic asthma and skin contact hypersensitivity." Journal of Allergy and Clinical Immunology 126: 355-365.e353.

Martinez-Pomares, L. and S. Gordon (2007). "Antigen Presentation the Macrophage Way." Cell 131: 641-643.

Mempel, T. R., S. E. Henrickson, et al. (2003). "T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases." Nature 427: 154-159.

Millar, D. G., K. M. Garza, et al. (2003). "Hsp70 promotes antigen presenting cell function and converts T-cell tolerance to autoimmunity in vivo." Nature Medicine 9: 1469-1476.

Miller, M. J., O. Safrina, et al. (2004). "Imaging the Single Cell Dynamics of CD4+ T Cell Activation by Dendritic Cells in Lymph Nodes." Journal of Experimental Medicine 200: 847-856.

Morgenstern, L. B., J. C. Hemphill, et al. (2010). "Guidelines for the Management of Spontaneous Intracerebral Hemorrhage: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association." Stroke 41: 2108-2129.

Muldoon, L. L., J. I. Alvarez, et al. (2012). "Immunologic privilege in the central nervous system and the blood-brain barrier." Journal of Cerebral Blood Flow & Metabolism 33: 13-21.

Neefjes, J., M. L. M. Jongsma, et al. (2011). "Towards a systems understanding of MHC class I and MHC class II antigen presentation." Nature Reviews Immunology 11: 823-836.

Nowak, T. S., Jr., I. J., et al. (1990). "70-kDa heat shock protein and c-fos gene expression after transient ischemia." Stroke 21 (11 Suppl.) III107-111.

Nowak, T. S., Jr. and M. Jacewicz (1994). "The heat shock/stress response in focal cerebral ischemia." Brain Pathology 4: 67-76.

Offner, H., S. Subramanian, et al. (2006). "Splenic Atrophy in Experimental Stroke Is Accompanied by Increased Regulatory T Cells and Circulating Macrophages." Journal of Immunology 176: 6523-6531.

Pavli P, Maxwell L, et al. (1996). "Distribution of human colonic dendritic cells and macrophages." Clinical & Experimental Immunology 104: 124-132.

Planas, A. M., M. A. Soriano, et al. (1997). "The heat shock stress response after brain lesions-induction of 72 Kda heat shock protein(cell types involved, axonal transport, transcriptional regulation) and protein synthesis inhibition." Progress in Neurobiology 51: 607-636.

Plumier, J., A. Krueger, et al. (1997). "Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury." Cell Stress Chaperones: 162-167.

Prass, K., C. Meisel, et al. (2003). "Stroke-induced Immunodeficiency Promotes Spontaneous Bacterial Infections and Is Mediated by Sympathetic Activation Reversal by Poststroke T Helper Cell Type 1-like Immunostimulation." Journal of Experimental Medicine 198: 725-736.

Quintana, F. J. and I. R. Cohen (2005). "Heat Shock Proteins as Endogenous Adjuvants in Sterile and Septic Inflammation." The Journal of Immunology 175: 2777-2782.

Rajdev, S., K. Hara, et al. (2000). "Mice overexpressing rat heat shock protein 70 are protected against cerebral infarction." Annals of Neurology 47: 782-791.

Randolph, G. J., V. Angeli, et al. (2005). "Dendritic-cell trafficking to lymph nodes through lymphatic vessels." Nature Reviews Immunology 5: 617-628.

Ransohoff, R. M. and B. Engelhardt (2012). "The anatomical and cellular basis of immune surveillance in the central nervous system." Nature Reviews Immunology 12: 623-635.

Ransohoff, R. M., P. Kivisäkk, et al. (2003). "Three or more routes for leukocyte migration into the central nervous system." Nature Reviews Immunology 3: 569-581.

Roger, V. L., A. S. Go, et al. (2011). "Heart Disease and Stroke Statistics--2011 Update: A Report From the American Heart Association." Circulation 123: e18-e209.

Roozendaal, R., R. E. Mebius, et al. (2008). "The conduit system of the lymph node." International Immunology 20: 1483-1487.

Saito, K., Y. Dai, et al. (2005). "Enhanced expression of heat shock proteins in gradually dying cells and their release from necrotically dead cells." Experimental Cell Research 310: 229-236.

Sakaguchi, S., M. Miyara, et al. (2010). "FOXP3+ regulatory T cells in the human immune system." Nature Reviews Immunology 10: 490-500.

Sancho, D., M. Gómez, et al. (2005). "CD69 is an immunoregulatory molecule induced following activation." Trends in Immunology 26: 136-140.

Schildknecht, A., H. C. Probst, et al. (2009). "Antigens expressed by myelinating glia cells induce peripheral cross-tolerance of endogenous CD8+ T cells." European Journal of Immunology 39: 1505-1515.

Sharp, F., A. Lu, et al. (2000). "Multiple Molecular Penumbras After Focal Cerebral Ischemia." Journal Of Cerebral Blood Flow & Metabolism 20: 1011-1032.

Shinoda, K., K. Tokoyoda, et al. (2012). "Type II membrane protein CD69 regulates the formation of resting T-helper memory." Proceedings of the National Academy of Sciences 109: 7409-7414.

Shiow, L. R., D. B. Rosen, et al. (2006). "CD69 acts downstream of interferon-alfa/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs." Nature 440: 540-544.

Soilleux, E. J., L. S. Morris, et al. (2002). "Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro." Journal of Leukocyte Biology 71: 445-457.

Srivastava, P. (2002). "Roles of Heat-Shock Proteins in Innate and Adaptative Immunity." Nature Reviews Immunology 2: 185-194.

Stocki, P., X. N. Wang, et al. (2012). "Inducible Heat Shock Protein 70 Reduces T Cell Responses and Stimulatory Capacity of Monocyte-derived Dendritic Cells." Journal of Biological Chemistry 287: 12387-12394.

Tanaka, S., Y. Kimura, et al. (1999). "Activation of T Cells Recognizing an Epitope of Heat-Shock Protein 70 Can Protect Against Rat Adjuvant Arthritis." Journal of Immunology 163: 5560-5565.

Tsuchiya, D., S. Hong, et al. (2003). "Overexpression of Rat Heat Shock Protein 70 Is Associated With Reduction of Early Mitochondrial Cytochrome c Release and Subsequent DNA Fragmentation After Permanent Focal Ischemia." Journal of Cerebral Blood Flow & Metabolism 23: 718-727.

Urra, X., A. Cervera, et al. (2009). "Monocytes Are Major Players in the Prognosis and Risk of Infection After Acute Stroke." Stroke 40: 1262-1268.

Urra, X., A. Cervera, et al. (2009). "Harms and benefits of lymphocyte subpopulations in patients with acute stroke." Neuroscience 158: 1174-1183.

Urra, X., V. Obach, et al. (2009). "Stroke induced immunodepression syndrome: from bench to bedside." Current Molecular Medicine 9: 195-202.

van der Worp, H. B. and J. van Gijn (2007). "Acute Ischemic Stroke." New England Journal of Medicine 357: 572-579.

van Zwam, M., R. Huizinga, et al. (2008). "Brain antigens in functionally distinct antigen-presenting cell populations in cervical lymph nodes in MS and EAE." Journal of Molecular Medicine 87: 273-286.

Vogelgesang, A., U. Grunwald, et al. (2007). "Analysis of Lymphocyte Subsets in Patients With Stroke and Their Influence on Infection After Stroke." Stroke 39: 237-241.

von Andrian, U. H. and T. R. Memple (2003). "Homing and cellular traffic in lymph nodes." Nature Reviews Immunology 3: 867-878.

Vyas, J. M., A. G. Van der Veen, et al. (2008). "The known unknowns of antigen processing and presentation." Nature Reviews Immunology 8: 607-618.

Walker, L. S. K. and D. M. Sansom (2011). "The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses." Nature Reviews Immunology 11: 852-863.

Walter, B. A., V. A. Valera, et al. (2006). "The olfactory route for cerebrospinal fluid drainage into the peripheral lymphatic system." Neuropathology and Applied Neurobiology 32: 388-396.

Wang, W. Z., T. Olsson, et al. (1992). "Myelin antigen reactive T cells in cerebrovascular diseases." Clinical & Experimental Immunology 88: 157-162.

Weller, R. O., E. Djuanda, et al. (2008). "Lymphatic drainage of the brain and the pathophysiology of neurological disease." Acta Neuropathologica 117: 1-14.

Wenkel, H. and J. W. Streilein (2000). "Systemic Immune Deviation in the Brain That Does Not Depend on the Integrity of the Blood-Brain Barrier." The Journal of Immunology 164: 5125-5131.

Williams, J. H. H. and H. E. Ireland (2007). "Sensing danger--Hsp72 and HMGB1 as candidate signals." Journal of Leukocyte Biology 83: 489-492.

Wollenberg, A., M. Mommaas, et al. (2002). "Expression and Function of the Mannose Receptor CD206 on Epidermal Dendritic Cells in Inflammatory Skin Diseases." Journal of Investigative Dermatology 118: 327-334.

Xu, L., J. F. Emery, et al. (2010). "Astrocyte targeted overexpression of Hsp72 or SOD2 reduces neuronal vulnerability to forebrain ischemia." Glia 58: 1042-1049.

Yang, Q.-w., J.-c. Li, et al. (2008). "Upregulated expression of toll-like receptor 4 in monocytes correlates with severity of acute cerebral infarction." Journal of Cerebral Blood Flow & Metabolism 28: 1588-1596.

Yenari, M., R. G. Giffard, et al. (1999). "The neuroprotective potential of heat shock protein 70 (HSP70)." Molecular Medicina Today 5: 525-531.

Yenari, M. A. (2002). "Heat shock proteins and neuroprotection." Advances in Experimental Medicine and Biology 513: 281-299.

Yenari, M. A., S. L. Fink, et al. (1998). "Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy." Annals of Neurology 44: 584-591.

Yenari, M. A., J. Liu, et al. (2005). "Antiapoptotic and Anti-inflammatory Mechanisms of Heat-Shock Protein Protection." Annals of the New York Academy of Sciences 1053: 74-83.

Zanin-Zhorov, A., G. Tal, et al. (2005). "Heat Shock Protein 60 Activates Cytokine-Associated Negative Regulator Suppressor of Cytokine Signaling 3 in T Cells: Effects on Signaling, Chemotaxis, and Inflammation." The Journal of Immunology 175: 276-285.

Zhan, X., B. P. Ander, et al. (2010). "Recombinant Fv-Hsp70 Protein Mediates Neuroprotection After Focal Cerebral Ischemia in Rats." Stroke 41: 538-543.

Zheng, Z., J. Y. Kim, et al. (2008). "Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke." Journal of Cerebral Blood Flow & Metabolism 28: 53-63.