UNIVERSIDAD AUTÓNOMA DE BARCELONA
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PAPEL DE LA MICROGLÍA EN LA REGULACIÓN DE LA RESPUESTA INMUNITARIA ADQUIRIDA

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Memoria para optar al grado de Doctor en Ciencias que presenta

BEATRIZ ALMOLDA ARDID

Realizada en la Unidad de Histología de la Facultad de Medicina bajo la dirección del Dr. Bernardo Castellano y Dra. Berta González

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CERTIFICAN:

Que la presente tesis doctoral titulada: "Papel de la microglía en la regulación de la respuesta inmunitaria adquirida", ha sido realizada bajo nuestra dirección por Beatriz Almolda Ardid, en la Unidad de Histología del Departamento de Biología Celular, Fisiología e Inmunología, estimando que se encuentra concluida y en condiciones de ser presentada y defendida públicamente para optar al grado de Doctor.

Y para que así conste firman la presente en Bellaterra a 20 de Mayo de 2010.

Dr. Bernardo Castellano

Dr. Berta González

A mi familia,

A Pol,

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Esta tesis te la dedico a ti Pol, por ser como eres, porque te quiero y porque lo eres todo para mí.

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ABREVIATURAS

AP-1	Activador de proteínas 1
CD	Célula dendrítica
CD14	Cluster de diferenciación 14
CD28	Cluster de diferenciación 28
CD4	Cluster de diferenciación 4
CD45	Cluster de diferenciación 45
CD8	Cluster de diferenciación 8
CPA	Célula presentadora de antígenos
CR	Receptor del complemento
CTLA-4	Antigeno de linfocitos T citotóxicos 4
dpi	Dias post-inducción
EAE	Encefalopatía autoinmune experimental
FasL	Ligando de Fas
IFN-γ	Interferón-gamma
IL4, 6, 10, 17A, 17F, 21, 22	Interleucinas 4, 6, 10, 17A, 17F, 21, 22
IRF	Factor regulador de interferones
iT-reg	T-reguladores inducibles
LPS	Lipopolisacárido bacteriano
LT	Lectina de tomate
MBP	Proteína básica de la mielina
MHC	Complejo mayor de histocompatibilidad
MS	Esclerosis múltiple
NF-kappaB	Factor nuclear kappa B
NK	Células asesinas naturales
nT-reg	T-reguladores naturales
PAMPs	Patrones moleculares asociados a patógenos
SNC	Sistema nervioso central
SR	Receptor basurero
TCR	Receptor de linfocitos T
TGF-β	Factor de crecimiento tumoral-betta
Th	T-cooperador
TLR	Receptor "toll-like"
TNF-α	Factor de necrosis tumoral-alpha
T-reg	T-reguladores
VIH	Virus de la inmunodeficiencia humana

RESUMEN

Numerosos estudios han demostrado a lo largo de los años, el papel fundamental que juegan las células de microglía en el funcionamiento del SNC. No sólo en condiciones normales, donde controlan la correcta homeóstasis del tejido, sino también en todas aquellas situaciones que, como consecuencia de alteraciones y procesos patológicos diversos, conllevan a una pérdida de esta homeóstasis. En respuesta a todas estas situaciones, las células de microglía son capaces de detectar rápidamente el daño y actuar de una manera específica en función del tipo de perturbación que se produzca en su entorno. Esta respuesta microglial ha sido ampliamente estudiada en daños agudos, en los que la microglía actúa como parte del sistema inmune innato, sin embargo los procesos que subyacen a la reactividad microglial ante una situación de inmunidad adquirida, así como la comunicación que se establece entre estas células microgliales y las células inmunes periféricas infiltradas permanecen en muchos aspectos sin esclarecer. En el presente trabajo hemos caracterizado el patrón de reactividad microglial y su relación con las diferentes poblaciones de linfocitos infiltrados a lo largo de las diferentes fases de la evolución que acontecen en un modelo agudo de encefalopatía autoinmune experimental (EAE).

Nuestros estudios demuestran que las células de microglía se activan en respuesta a la inducción de la EAE y presentan un patrón de activación específico en cada una de las fases. Durante la fase de inducción y pico del proceso patológico, en estrecha relación con el aumento de la sintomatología clínica que se manifiesta por un progresivo deterioro de las funciones motoras, estas células microgliales experimentan cambios morfológicos y en su distribución acumulándose alrededor de los vasos sanguíneos. Además de microglía reactiva y posiblemente de macrófagos de origen sanguíneo, se observa también un gran número de linfocitos infiltrados, mayoritariamente del tipo T-cooperador y subtipo Th1 (pro-inflamatorio), si bien también se observan linfocitos T-citotóxicos y T-$\gamma\delta$. En estas fases, la activación microglial se caracteriza a nivel fenotípico por el aumento en la expresión de moléculas del complejo mayor de histocompatibilidad clase I y clase II (MHC-clase I y MHC-clase II) sin expresión concomitante de moléculas co-estimuladoras B7.1 o B7.2, es decir las células de microglía activadas presentan en estas circunstancias un fenotipo característico de células dendríticas inmaduras; hecho que hemos corroborado con la demostración de la expresión del marcador CD1. En este contexto, la señal que inducen estas células de microglía a los linfocitos infiltrados, podría estar implicada en la modulación del proceso inflamatorio induciendo la apoptosis o anergia linfocitaria.

Durante la fase de recuperación, a pesar de que los animales experimentan una progresiva mejora sintomatológica, las células de microglía siguen mostrando una morfología, distribución y patrón de expresión de moléculas característicos de células reactivas. Si bien, en general, las células de microglía siguen mostrando el mismo patrón fenotípico (CD1+, MHC-I+, MHC-II+, B7.1- y B7.2-), aquellas localizadas en el entorno de algunos vasos sanguíneos expresan diferencialmente la molécula B7.2. Durante esta fase, además, el número total de linfocitos T-cooperadores, T-citotóxicos y T-$\gamma\delta$ se mantiene muy elevado con valores similares a los observados en las fases anteriores. Es interesante señalar que ya no encontramos linfocitos Th1 y la población de T-cooperadores está constituida por los subtipos Th17 y T-regs. Los linfocitos que se acumulan en las inmediaciones de los vasos sanguíneos expresan CTLA-4, uno de los co-receptores de B7.2. En este nuevo contexto, la interacción de las células de microglía B7.2+ con estos linfocitos CTLA-4+ podría ser la responsable de la resolución de la respuesta immunitaria y la inducción de la tolerancia.

En su conjunto los resultados obtenidos evidencian que la microglía juega un papel clave en la evolución de la respuesta inmune adquirida modulando la activación e inactivación de las diferentes poblaciones linfocitarias implicadas tanto en la inducción del proceso inmune/inflamatorio como en su posterior resolución.

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ABSTRACT

Over the years, numerous studies have demonstrated the fundamental role played by microglial cells in the CNS, not only in normal conditions where they control the correct tissue homeostasis, but also in all those situations in which, as a result of alterations and pathologies, homeostasis may be disturbed. Thus, when the integrity of the nervous tissue is disrupted, microglial cells are activated, showing specific activation patterns which fully depend on changes in the particular micro-environment. The microglial response has been widely studied in acute injuries in which microglia act as an intrinsic element of the innate immune system. However, the processes underlying microglial reactivity in situations of acquired immunity, as well as the relationship established between these microglial cells and infiltrating peripheral immune cells, remain poorly understood. In this study we have characterized the pattern of microglial reactivity and their relationship with the different populations of infiltrated lymphocytes, along the evolution of an acute model of experimental autoimmune encephalopathy (EAE) induced in Lewis rat.

Our studies have demonstrated that microglial cells became activated in response to EAE induction, showing a specific activation pattern in each phase along disease evolution. During the inductive and peak phases, microglial cells showed changes in morphology and distribution, in close association with the increase of clinical symptoms, manifested by a progressive deterioration of motor function. These microglial cells progressively shorten their ramifications leading to amoeboid morphologies. Microglia and blood-borne macrophages increased in number and accumulated around blood vessels. In addition, a large number of infiltrated lymphocytes were also detected during the inductive and peak phases. These lymphocytes belong mostly to the T-helper phenotype (pro-inflammatory Th1 cells), although T-cytotoxic and $\gamma\delta$ T-cells were also observed. Activated microglial cells displayed an immature dendritic cell phenotype characterized by expression of CD1 and major histocompatibility complexes class I and class II (MHC-class I and MHC-class II) without concomitant expression of B7.1 or B7.2 co-stimulatory molecules. In addition, these activated microglia expressed CD1, a marker of immature dendritic cells. In this context, the signal that these immature dendritic cell-like microglial cells induced to infiltrated lymphocytes may provoke lymphocyte apoptosis or anergy.

During the recovery phase, animals experienced a gradual improvement in symptomatology, although microglial cells in this phase still showed a morphology, distribution and phenotype characteristics of reactive cells. In general, these microglial cells displayed the same immature dendritic cell phenotype (CD1+, MHC-I+, MHC-II+, B7.1- and B7.2-) observed during earlier phases. Noticeably, microglial cells located around blood vessels express B7.2. The total number of T-helper, T-cytotoxic and $\gamma\delta$ T-cells remained very high with values close to those observed during the inductive and peak phases. Interestingly, we do not find Th1 lymphocytes during the recovery phase, and the population of T-helper cells mainly consists of Th17 and T-regs subtypes. Lymphocytes accumulated in the vicinity of blood vessels expressed CTLA-4, one of the B7.2 co-receptors. In this new context, the interaction between B7.2+ microglial cells and CTLA-4+ lymphocytes could be responsible for the immune response resolution and the induction of subsequent tolerance.

Altogether, these results show that microglia play a key role in the evolution of the acquired immune response, modulating the activation and inactivation of the different lymphocyte populations involved in both the induction of the immune/inflammatory process and its subsequent resolution.

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INTRODUCCIÓN

El sistema nervioso central (SNC) ha sido considerado durante mucho tiempo como un órgano inmunológicamente aislado del sistema inmune periférico, debido a la presencia de una barrera hematoencefálica y la ausencia de vasos linfáticos (129) y, por otra parte, al hecho de que injertos de piel, inoculación de virus o bacterias y antígenos introducidos directamente en el parénquima nervioso no inducen una respuesta inmune (12,108,117,165). Sin embargo, a lo largo de los últimos años, numerosos estudios han demostrado que el SNC no sólo es inmune-competente sino que además interacciona activamente con células del sistema inmune periférico (6,14,164), que pueden ser reclutadas al interior del parénquima nervioso (42,43,143). Hoy en día, esta visión de aislamiento ha cambiado drásticamente hacia un escenario mucho más activo, en el cual la tolerancia inmune se mantiene continuamente dentro del SNC. A esta tolerancia contribuyen la expresión constitutiva de FasL, un receptor involucrado en la muerte de células inmunes infiltradas (16,46) y la producción local de mediadores anti-inflamatorios como indolamina 2,3-dioxigenasa, en respuesta a la interacción con linfocitos pro-inflamatorios (84). Además, se ha demostrado la presencia de macrófagos y células dendríticas, células clave en el inicio de respuestas inmunitarias, en lugares estratégicos como las meninges, los plexos coroideos (115,116) o en el espacio perivascular (176). Por todo esto, actualmente se considera al SNC como un sistema inmune-privilegiado, no inmune-aislado (29,49). En este contexto, se ha sugerido que una de las células con un papel clave tanto en el mantenimiento de esta tolerancia inmune como en el control de las respuestas inmunitarias que tienen lugar en el SNC son las células de microglía (5,27,55).

Reactividad microglial

Las células de microglía son consideradas como las células inmunitarias residentes del SNC (5,27,83). Hoy en día se considera que existen al menos dos tipos de microglía en el SNC adulto sano: la microglía quiescente ("resting microglia") que se encuentra distribuida por todo el parénquima nervioso, tanto en áreas de sustancia blanca como de sustancia gris; y las células de microglía perivascular (también denominadas macrófagos perivasculares) que constituyen una población minoritaria de células, dentro del SNC, localizadas tanto en el espacio perivascular como en el de Virchow-Robins, espacios situados por debajo de la membrana basal de las células endoteliales (176). La microglía quiescente representa una población de células estable con una baja tasa de recambio (88), morfología ramificada y con expresión basal muy baja de CD45 y ausencia de MHC-clase II (153). Las células de microglía/macrófagos perivasculares, sin embargo, tienen una tasa de recambio más elevada (15), no presentan las prolongaciones características de la microglía quiescente y expresan CD45, MHC-clase II y ED2 de manera constitutiva (39). Debida a esta expresión y a su localización estratégica, estas células perivasculares parecen jugar un papel importante durante la respuesta a procesos inmunológicos.

En condiciones basales, las células de microglía supervisan de manera constante el parénquima nervioso (37,122) y son capaces de reaccionar rápidamente cuando la homeostasis se ve perturbada como consecuencia de una gran variedad de situaciones incluyendo lesiones, neurotoxicidad, infecciones, etc (38,52,55,58). Este proceso de activación microglial conlleva un cambio fenotípico y de activación génica caracterizado por cambios morfológicos, por el aumento, disminución o expresión *de novo* de numerosas moléculas de superficie y por la secreción de un amplio número de sustancias como citocinas, quimiocinas y factores tróficos (35,38,58,141,144). Esta gran variedad de cambios, unido al hecho de que hoy en día se sugiere que existen diferentes subpoblaciones de microglía dentro del SNC (28), hace que la reactividad microglial no pueda considerarse como un evento uniforme, sino más bien como un proceso heterogéneo que, dependiendo del tipo de señal activadora y del ambiente en el cuál se produce esta activación, presenta diferentes evoluciones.

Aunque la función que tiene la microglía tanto en el cerebro normal como después de una lesión aguda ha sido ampliamente estudiada, el papel específico que juegan estas células en la respuesta inmunitaria dentro del SNC, así como la comunicación que se establece entre ellas y las células involucradas en esta respuesta, son aspectos que suscitan todavía muchos interrogantes.

Microglía en la respuesta inmune innata

Tal y como se puede leer en cualquier texto básico de Inmunología, la respuesta inmune innata se basa en la existencia de una serie de elementos, tanto barreras físicas como células específicas (neutrófilos, macrófagos, células asesinas naturales ó NK, etc.) cuya principal función es proteger al individuo frente a la presencia de patógenos invasores o células dañadas (1). Esta respuesta es antígeno-independiente y muy rápida, y se induce por la activación de una serie de receptores, presentes en la membrana de estas células inmunitarias, que reconocen una gran variedad de motivos estructurales altamente conservados en los microorganismos, comúnmente denominados PAMPS (pathogen-associated molecular patterns) o presentes en células dañadas denominados

ACAMPS (apoptotic cell-associated molecular patterns) (2). Algunos de los receptores más conocidos actualmente son:

1) Receptores "toll-like" (TLRs), un grupo de receptores transmembrana localizados en la membrana celular o en la membrana del endosoma. Tras la unión con su ligando, activan cascadas de señalización intracelular específicas que resultan en la activación de una serie de factores de transcripción como NF-kappaB, AP1 e IRFs, los cuales en último término promueven la secreción de diferentes moléculas pro-inflamatorias.
2) Receptores del complemento, como CR3 y CR4. Estos receptores se encuentran en la membrana de varios tipos celulares, incluyendo neutrófilos y macrófagos. La unión de estos receptores con sus ligandos induce la fagocitosis de elementos opsonizados por moléculas del complemento, principalmente C3bi.
3) Receptor de la manosa, un tipo de lectina que se localiza en la membrana celular y reconoce polisacáridos complejos expresados frecuentemente por patógenos. Se encuentra en células dendríticas, macrófagos y algunos tipos de células endoteliales (95,97) y su función, igual que en el caso de los receptores del complemento, está relacionada con la fagocitosis (96).
4) Receptores basureros ("scavenger"): SR-A, SR-BI, CD36. Consiste en un grupo de receptores expresados en macrófagos, cuyas funciones están relacionadas con la adhesión celular, endocitosis y señalización celular.
5) Receptores Fc, que reconocen el fragmento constante de las inmunoglobulinas y median la fagocitosis de productos opsonizados.

Las células de microglía, en condiciones normales, expresan niveles bajos/moderados de CR3 (3), receptores Fc (125) y TLRs (126), pero no muestran expresión del receptor de manosa (97) o receptores "scavenger" (19), los cuales están restringidos a poblaciones específicas de macrófagos situados en los plexos coroideos de los ventrículos cerebrales y las meninges (19,50). Tras su activación, sin embargo, las células de microglía, pueden aumentar o expresar *de novo* una gran variedad de estos receptores (5,26,65,90). En algunas ocasiones, las variaciones en estos receptores se manifiestan ante la presencia de algún tipo de patógeno: por ejemplo, la administración de lipopolisacárido bacteriano (LPS) induce un aumento en la expresión del receptor CD14 (120) y de TLR-4 (91); la infección con el virus del herpes simple tipo 1 de TLR-2 (9); la infección con el virus del Este del Nilo induce TLR-3 (34) y la presencia de abscesos cerebrales mediados por *S. aureus* induce expresión de TLR-2 (75). La inducción de estos receptores se produce también en situaciones de neurodegeneración en las que no hay evidencias de la presencia de ningún tipo de microorganismo o patógeno. De esta manera, se ha demostrado en células de microglía el aumento en la expresión de TLR-2 después de una lesión en la vía perforante de la corteza entorrinal (11) y tras hipoxia-isquémia (92) así como expresión de CD14 (44), diferentes TLRs y CD36 (30,41) en la enfermedad de Alzheimer. Además se ha descrito un aumento en la expresión de TLR-2 y CD14 en modelos de autoinmunidad, como la encefalopatía autoinmune experimental (EAE) (178).

La microglia también participa en estos tipos de respuesta innata mediante la secreción de un amplio número de mediadores inflamatorios tales como citocinas, pro-inflamatorias y anti-inflamatorias, quimiocinas y prostanoides (5). La secreción de estos mediadores induce la migración y activación del resto de células de la respuesta inmune tanto innata (neutrófilos, células NK, macrófagos) como adquirida (linfocitos T y B).

Microglía en la respuesta inmune adquirida

En comparación con la respuesta inmune innata, la respuesta inmune adquirida, es una respuesta más tardía y antígeno-dependiente. Los linfocitos, constituyen el principal tipo celular asociado a este tipo de respuestas. Existen dos tipos principales de linfocitos, los linfocitos B que reconocen el antígeno soluble y son productores de anticuerpos; y los linfocitos T encargados de la respuesta celular. Estos linfocitos T, a su vez, se dividen en dos tipos: los linfocitos T citotóxicos (CD8+) cuya función consiste en destruir directamente, mediante la secreción de moléculas como perforina y granzima, a células infectadas o dañadas; y los linfocitos T cooperadores ("T-helper") (CD4+) encargados de regular la activación de otros tipos de células inmunes, principalmente macrófagos y linfocitos B, a través de la secreción de una gran variedad de citocinas. Dado que estos linfocitos T son incapaces de reconocer los antígenos de manera soluble, necesitan de la ayuda de un tipo de células especializadas, las células presentadoras de antígenos (CPA) que, mediante el proceso denominado de presentación antigénica, captan, procesan y presentan en su superficie antígenos de patógenos, virus u otras estructuras extrañas permitiendo su reconocimiento por parte de estos linfocitos T. Este proceso, consiste en dos señales, la primera deriva de la unión entre el receptor de linfocitos T (TCR) y las moléculas del complejo mayor de histocompatibilidad (MHC) que se encuentran en la membrana de las CPAs y llevan acomplejado el antígeno: MHC clase I en el caso de linfocitos T citotóxicos y MHC de clase II en el caso de linfocitos T cooperadores. La segunda señal, la señal de co-estimulación se produce tras la unión de

receptores como B7.1 o B7.2 en la membrana de la CPA y sus co-receptores en linfocitos (86). Las células dendríticas (CDs) constituyen el grupo de CPA profesionales en la periferia. Sin embargo, en el SNC la presencia de estas células está restringida a lugares específicos como son los plexos coroideos y las meninges (115,116).

Las células de microglía, en este sentido, son consideradas las principales CPA dentro del SNC (5,141). Aunque en situaciones basales, en la mayoría de cepas de animales de laboratorio, estas células de microglía no muestran expresión de MHC-clase I ni MHC-clase II (83,129), tras su activación, pueden aumentar rápidamente la expresión de estas moléculas en todo tipo de situaciones (83,129). Sin embargo, no todos estos estudios han caracterizado la expresión de moléculas co-estimuladoras en estas células microgliales. En este sentido, se ha descrito la expresión *de novo* de B7.2, pero no B7.1 en microglía tras lesión de la corteza entorrinal (17,85), lesión de nervio periférico (149), axotomía del nervio facial (24) o desmielinización inducida por cuprizona (147); y aumento de ambas moléculas en modelos de autoinmunidad en ratón, como la EAE y la infección con el virus de Theiler (67,100,141).

Por otro lado, la infiltración de linfocitos, principalmente linfocitos T, en el SNC ha sido descrita en diferentes tipos de patologías (148) tales como infecciones víricas (virus de la inmunodeficiencia humana ó VIH (131), virus del Este del Nilo (53)); enfermedades neurodegenerativas (Parkinson (25), esclerosis lateral amiotrófica (62)); lesiones agudas (axotomía del nervio facial (142), lesión de la corteza entorrinal (10)) o procesos autoinmunes como EAE (40). Esta infiltración linfocitaria se ha relacionado en algunas ocasiones con una función protectora, como en el caso de la axotomía del nervio facial (161), la infección con el virus del Este del Nilo (53) o la esclerosis lateral amiotrófica (18), mientras que en otras situaciones esta infiltración contribuye al aumento de la patología. Este es el caso de la enfermedad de Parkinson (25), la infección con el virus VIH (131) o modelos de autoinmunidad (40).

Tal y como se ha descrito anteriormente, existen dos tipos de linfocitos T, los citotóxicos y los cooperadores. Estos últimos a su vez de subdividen en diferentes subtipos. Hasta hace unos años, esta subdivisión de linfocitos cooperadores era dicotómica: por un lado se describían los linfocitos Th1, capaces de secretar citocinas pro-inflamatorias como interferon-γ (IFN-γ) o factor de necrosis tumoral-α (TNF-α) y por otro lado los linfocitos Th2 encargados de producir citocinas anti-inflamatorias como interleucina-4 (IL-4) e IL-10. Sin embargo, en los últimos años, numerosos estudios han demostrado que además de Th1 y Th2, existen otros subtipos de linfocitos T cooperadores con funciones y patrones de secreción de citocinas muy variados (40,146,169). Uno de estos subtipos son los linfocitos Th17 (60). Los linfocitos Th17 secretan, generalmente, citocinas pro-inflamatorias como IL17A e IL17F y han sido descritos especialmente en relación a procesos de autoinmunidad. No obstante, recientemente también se ha descrito su capacidad, en determinadas circunstancias, de producir citocinas anti-inflamatorias como IL10 e IL21 (8,21,59,113,173). La polarización y diferenciación de linfocitos naïve hacia estos linfocitos Th17 depende de la presencia en el ambiente de dos citocinas específicas: factor de crecimiento transformante-β (TGF-β) e IL6 (20,103). Es interesante señalar que TGF-β es también una de las citocinas clave en la diferenciación de otro subtipo de linfocitos T, los T-reguladores (T-regs) (104). Los linfocitos T-regs son una subpoblación especializada de linfocitos T que actúa suprimiendo la activación del sistema inmune. Su principal función es el mantenimiento de la homeostasis inmunológica y de la tolerancia frente a autoantígenos (23). Existen dos subtipos de linfocitos T-regs: T-regs naturales (nT-reg) y T-regs inducibles (iT-reg) (33,63). Los nT-reg se generan en el timo durante el proceso de maduración de linfocitos T y son los encargados del mantenimiento de la homeostasis del sistema inmune. Por el contrario, los iT-regs, se producen en órganos linfoides periféricos tras la estimulación de linfocitos CD4+ con la citocina TGF-β. Dada su capacidad para suprimir la respuesta inmune, la participación de estos linfocitos T-regs en la evolución de las respuestas inmunes adquiridas en el SNC, sobretodo aquellas relacionadas con la autoinmunidad, ha generado un gran interés en los últimos años. En este sentido se ha demostrado su acumulación en gliomas cerebrales (56) y en algunos modelos de EAE (80,81,114). Sin embargo, muchos aspectos relacionados con la función y el papel que juegan estas células en la regulación de la respuesta inmune adquirida dentro del SNC, así como su regulación por parte de las células residentes del SNC, especialmente la microglía, están todavía por determinar.

El descubrimiento de todos estos subtipos de linfocitos ha hecho que el escenario de la respuesta neuroimmune se haya complicado en los últimos años. Además, a esto hay que añadir el hecho de que recientemente ha sido demostrada la presencia de algunos subtipos de células dendríticas infiltradas en el parénquima nervioso en algunos modelos experimentales de isquemia y de autoinmunidad (82,153,154). En conjunto, todos estos datos sugieren que el resultado final neto de la respuesta inmune adquirida dentro del SNC, no sólo dependerá de la mayor o menor presencia de linfocitos y CPAs, sino que estará también directamente relacionado, con el tipo específico de linfocitos y el fenotipo particular de la CPA en cada situación. Por lo tanto, en este contexto, es clave establecer el papel que juega la microglía activada en el control de la

respuesta inmune adquirida para poder entender los mecanismos subyacentes a la conexión que se establece entre dos sistemas tan complejos como son el SNC y el sistema inmunitario.

Uno de los modelos experimentales más utilizados en neuroinmunología para el estudio de las respuestas celulares y moleculares asociadas a los procesos de inmunidad adquirida, es el de la encefalopatía autoinmune experimental (EAE), un modelo prototipo de autoimmunidad mediada por células T.

La encefalopatía autoinmune experimental

La EAE se puede inducir en animales susceptibles, principalmente roedores, mediante dos tipos de inmunización: 1) inmunización activa, que consiste en la inyección subcutánea de una emulsión que contiene o bien tejido nervioso o bien algún péptido de proteínas de la mielina, mezclados con un coadyuvante o 2) inmunización pasiva, que se basa en la inyección intravenosa de linfocitos T específicos contra alguna de las proteínas de la mielina. La EAE se caracteriza por una dificultad progresiva en la movilidad, que tiene su origen en el tren posterior del animal y va evolucionando de manera postero-anterior hasta producir paraplejia o tetraplejia. Asociada a esta sintomatología clínica se produce una pérdida significativa del peso corporal del animal. La EAE afecta principalmente a la sustancia blanca de la médula espinal, aunque hoy en día cada vez son más los trabajos que demuestran afectación también a nivel de sustancia gris y en otras áreas del SNC como el cerebelo y el tronco cerebral (36,101). A nivel tisular, la EAE está caracterizada por una reactividad glial, principalmente de células de microglía, y por la infiltración de una gran cantidad de células del sistema inmune que invaden el parénquima nervioso a través del torrente sanguíneo (27,43,141). Hay modelos animales, además, donde se produce desmielinización, normalmente asociada con estadios más avanzados de la evolución de la enfermedad.

Existen varios modelos de EAE con características diferenciales tanto a nivel sintomatológico como a nivel tisular y que simulan distintas fases dentro de la evolución de la esclerosis múltiple (MS) humana (170,174). Estas diferencias entre modelos dependen de múltiples factores, como la especie animal, la cepa, el sexo, la edad y el péptido usado en la inmunización.

Frente a otros modelos en los que se induce una sintomatología crónica o remitente-recurrente, el modelo de EAE agudo en rata Lewis se caracteriza por poseer un único pico de sintomatología que empieza alrededor de los 10 días post-inducción (dpi), con una pérdida parcial del tono de la cola. La sintomatología evoluciona de manera progresiva, causando la parálisis completa de la cola seguida de paraparesia del tren posterior, hasta llegar a los 12-14 dpi cuando los animales manifiestan paraplejia, máxima expresión de la enfermedad. Después de dos o tres días, da comienzo una espontánea y progresiva recuperación que culmina, alrededor de los 21-23 dpi, cuando los animales ya no muestran ningún signo clínico de EAE. Este patrón de inducción/recuperación podría simular un brote inicial de la MS humana. En este modelo de EAE agudo, los animales, una vez recuperados, generan tolerancia a posteriores inducciones con el mismo antígeno (102,121). Todas estas características hacen que el modelo de inducción aguda de EAE en rata Lewis sea de gran utilidad para el estudio de los procesos que tienen lugar no sólo durante la fase de inducción o establecimiento de la enfermedad, sino también para el estudio de todos aquellos cambios, tanto a nivel celular como molecular relacionados con la recuperación espontánea y posterior tolerancia. Finalmente, es importante destacar que este es un modelo de encefalopatía en el que no se produce desmielinización, lo que permite estudiar con mayor eficacia y simplicidad los mecanismos asociados al proceso inflamatorio “per se” evitando las posibles interferencias derivadas de una desmielinización adicional.

HIPÓTESIS

La hipótesis que nos planteamos al iniciar este estudio es que en una respuesta inmune adquirida, las células de microglía activadas adquieren la capacidad de actuar como células presentadoras de antígenos regulando la activación/inactivación de las poblaciones linfocitarias infiltradas.

OBJETIVOS

El objetivo general de esta tesis ha sido determinar el patrón fenotípico de las células de microglía/macrófagos y su relación con la población linfocitaria infiltrada a lo largo de las diferentes fases de un modelo agudo de encefalopatía autoinmune experimental (EAE).

Los objetivos concretos que nos planteamos fueron:

1) Analizar los cambios morfológicos y de distribución que sufren las células de microglía/macrófagos durante las fases de inducción, pico y recuperación en la EAE inducida en rata Lewis mediante la inyección de proteína básica de la mielina (MBP) y correlacionar estos cambios con la sintomatología clínica.

2) Caracterizar el patrón de infiltración de las diferentes poblaciones de linfocitos T inflamatorios y reguladores a lo largo de la evolución de la EAE.

3) Caracterizar, tanto en microglía/macrófagos como en linfocitos, el patrón de expresión de moléculas relacionadas con los mecanismos de presentación antigénica, incluyendo el complejo mayor de histocompatibilidad y moléculas co-estimuladoras.

4) Determinar si las células de microglía activada expresan marcadores de células dendríticas maduras e inmaduras.

RESUMEN DE RESULTADOS Y DISCUSIÓN

Con la realización de esta tesis doctoral hemos caracterizado el patrón de activación y reactividad de las células de microglía y su implicación en la regulación de la respuesta linfocitaria en un modelo de inmunidad adquirida, concretamente la inducción de EAE aguda en rata Lewis. Los resultados obtenidos muestran que las células de microglía adquieren un fenotipo de célula dendrítica inmadura y son capaces de resolver la respuesta inmunitaria mediante la modulación de la activación/inactivación de poblaciones linfocitarias.

Fenotipo de las células de microglía en los animales control

Los diversos marcadores empleados, NDPasa, lectina de tomate (LT) e Iba1 nos han permitido corroborar que las células de microglía en los animales control mostraron en todo momento la morfología ramificada característica. Asimismo, en concordancia con estudios anteriores (83,129), nuestros resultados demuestran que estas células microgliales no expresan MHC-clase II, ni las moléculas co-estimuladoras B7.1 o B7.2. Sin embargo, hemos detectado una expresión basal de MHC-clase I en estas células. Aunque estudios anteriores describen ausencia de MHCs en situaciones normales (83), la presencia de esta molécula en nuestro estudio puede atribuirse a características específicas de la cepa de animales utilizada. De esta misma manera, se ha descrito una expresión basal de MHC-clase II en células de microglía derivadas de ratas Brown-Norway (158) y expresión constitutiva de MHC-clase II y moléculas co-estimuladoras B7.1 y B7.2 en ratones de la cepa C57BL/6 (179).

Los mismos marcadores NDPasa, LT e Iba1 nos han permitido observar la presencia de células con morfología fusiforme ubicadas en el espacio perivascular. Estas células se caracterizaron fenotípicamente como macrófagos perivasculares, dada su expresión constitutiva de ED2 (39,133), MHC-clase II (47,87) y CD4 (130).

Inducción de EAE: Sintomatología clínica

La inyección de MBP provoca la aparición de los primeros síntomas clínicos de la EAE hacia los 10 días post-inducción (dpi) manifestándose en primera instancia por la pérdida del tono de la cola (*score* clínico 0.5). Esta sintomatología aumenta rápidamente produciendo parálisis de la cola (*score* 1), dificultades en la movilidad de las extremidades posteriores (*score* 2) y por último, hacía los 12dpi, paraplegia completa del tren posterior (*score* 3). La paraplegia se mantiene durante 1 o 2 días y a partir de ese momento, los animales empiezan a recuperar la movilidad de forma progresiva empezando por las patas posteriores (*score* 2R) hasta mostrar únicamente parálisis de la cola (*score* 1R). Alrededor de los 21dpi, se observa una recuperación total de los animales (*score* 0R) los cuales no vuelven a mostrar signos externos de la enfermedad. En este estudio, la confección de los grupos experimentales y el ulterior análisis se han proyectado teniendo en cuenta únicamente el *score* clínico y no los dpi. Hemos considerado pues: a) una fase de inducción, que comprende los *scores* 0.5, 1 y 2; b) una fase de pico de la enfermedad correspondiente al *score* 3; c) una fase de recuperación, correspondiente a los *scores* 2R, 1R y 0R; y d) una fase de post-recuperación, en la que se incluyeron aquellos animales sin sintomatología clínica que fueron mantenidos tras la recuperación y estudiados a varios tiempos de supervivencia entre 28-90dpi correspondientes a los *scores* 0R-28dpi, 0R-32dpi, 0R-40dpi y 0R-90dpi.

Nuestros resultados nos han permitido establecer una clara correspondencia entre la sintomatología clínica observada durante la fase de inducción de la EAE y los cambios progresivos en la reactividad de la población microglial/macrofágica por una parte, y con la infiltración linfocitaria por otra.

Durante el pico de la enfermedad, momento en que los animales se ven más afectados clínicamente, es cuando la reactividad microglial es máxima y el número de linfocitos infiltrados alcanza su valor más alto.

Una de las observaciones más interesantes de nuestro estudio es que durante la fase de recuperación, a pesar de que los síntomas clínicos remiten de manera progresiva, persiste una gran reactividad de células de microglía/macrófagos y la población de linfocitos infiltrados se mantiene. Incluso en aquellos animales que se han recuperado totalmente (*score* 0R) se observa aún reactividad glial y linfocitos infiltrados varias semanas después de que no presenten alteración clínica.

Activación de las células de microglía y entrada de monocitos sanguíneos

Durante la fase de inducción y en concordancia con el progresivo aumento de la sintomatología clínica, hemos observado una reactividad de las células de microglía monitorizada por un incremento progresivo en la expresión de NDPasa, LT e Iba1. A nivel morfológico las células reactivas muestran un engrosamiento y retracción de las prologanciones celulares, hasta dar lugar a morfologías ameboides y macrofágicas durante el pico de la enfermedad. El análisis minucioso al microscopio nos ha permitido revelar la presencia, des del *score* 1, de prolongaciones de células de microglía activada que rodean somas neuronales. Esta observación podría sugerir un mecanismo que ha sido descrito anteriormente en

otros modelos de lesión, conocido como "synaptic stripping", y que consiste en el desplazamiento de los contactos sinápticos de las neuronas para aislarlas del ambiente tóxico generado por la lesión (22,54). Un mecanismo como este podría ser el responsable en nuestro modelo de la alteración de los circuitos neuronales, que conducirían a un progresivo deterioro de la capacidad de movilidad y explicaría la parálisis observada en estos animales.

Los cambios morfológicos vienen acompañados de un aumento progresivo en el número de células de microglía reactiva tanto en sustancia gris como en sustancia blanca. Este incremento podría deberse a una proliferación de las células de microglía o bien a una entrada de monocitos sanguíneos desde la periféria. Mediante el uso de marcadores de proliferación como la fosfo-histona 3, en combinación con marcadores de microglía, algunos de nuestros resultados aún no publicados, revelan la existencia de células CD11b+ en proliferación, mayoritariamente durante los *scores* 2 y 3. A pesar de que estas células muestran morfologías macrofágicas, el hecho de que no existan marcadores específicos para diferenciar entre un tipo celular y otro, no nos permite afirmar si esta población de células en proliferación corresponde a células de microglía o bien si son monocitos infiltrados los que se dividen. Las células de microglía/ macrófago activada se encuentran acumuladas alrededor de vasos sanguíneos, lo cual sugiere la posible implicación de la microglía en el control de la infiltración de células inmunitarias periféricas o la interacción con ellas. En este sentido, diversos autores muestran que la microglía es capaz de secretar una gran variedad de sustancias quimio-atrayentes, involucradas en la entrada de leucocitos sanguíneos (5,7).

En correspondencia con algunos estudios que describen la entrada de monocitos sanguíneos en modelos de EAE (45,66,183), nuestras observaciones también revelan la presencia de una población de células pequeñas con morfología redonda, cuya presencia se inicia en las primeras fases de la inducción y aumenta de manera progresiva a lo largo de esta fase hasta el pico y expresan MHC-clase I+, MHC-clase II+, B7.2+ y CD4+. Estas células podrían corresponder a monocitos infiltrados que podrían contribuir a la población de células con aspecto macrofágico que se encuentran a lo largo de la fase de inducción y pico de la enfermedad.

Infiltración de linfocitos durante la fase de inducción y pico

Durante la fase de inducción y en paralelo a la activación microglial nuestras observaciones ponen de manifiesto también la presencia de un gran número de linfocitos infiltrados. Estas observaciones correlacionan con estudios anteriores donde ya se ha descrito la presencia de linfocitos tras la inducción de la EAE (163,177). Nuestro estudio detallado, sin embargo, muestra el patrón temporal específico de infiltración de diferentes tipos de linfocitos: 1) linfocitos T-cooperadores (CD3+CD4+), linfocitos T-citotóxicos (CD3+CD8+) y linfocitos T-$\gamma\delta$ (CD3+ V65+). Todos estos tipos de linfocitos infiltrados los hemos detectado desde el inicio de la sintomatología, manteniéndose su número constante durante toda la fase de inducción y el pico. Proporcionalmente son los linfocitos T-cooperadores los que representan la mayor parte de la población, concretamente hemos podido determinar que durante estas fases pertenecen al subtipo de linfocitos pro-inflamatorios Th1 (Tbet+).

Presentación antigénica: inducción de anergia y apoptosis linfocitaria durante las fases de inducción y pico

En paralelo a los cambios morfológicos, las células de microglía reactiva muestran un aumento progresivo en la expresión de MHC-clase I y MHC-clase II a lo largo de la fase de inducción y pico, en directa correlación con la sintomatología clínica. Estas moléculas de MHC-clase I y MHC-clase II constituyen la primera señal del proceso de presentación antigénica, proceso necesario para la activación de linfocitos T. La expresión de las moléculas de MHC determina la especificidad de la respuesta linfocitaria. El aumento de expresión de estas moléculas en microglía, ha sido ampliamente descrito tras su activación ante diferentes tipos de daño en el SNC (83,129), incluyendo EAE (31,72,94,105,106,110,111,139,140). Sin embargo, es importante tener en cuenta que los trabajos anteriormente citados no realizan un estudio exhausto de los cambios en la expresión de estas moléculas en las diferentes fases de la EAE, como los que se describen en la presente tesis.

La expresión de moléculas del MHC por sí no es suficiente para la activación de los linfocitos T, siendo necesaria una segunda señal denominada de co-estimulación. La expresión de MHC sin la expresión de co-estimulación induce la anérgia clonal (incapacidad de activación) o/y la muerte por apoptosis de los linfocitos (76). Esta señal de co-estimulación, es antígeno-independiente y se produce por la unión entre diferentes receptores presentes en la superficie de los linfocitos T con sus respectivos ligandos localizados en las CPAs (86). De entre las diferentes moléculas co-estimuladoras descritas hoy en día, la pareja que juega un papel más relevante en la activación de linfocitos T, es la formada por las moléculas B7.1 y B7.2 en las CPAs y sus co-receptores CD28 y CTLA-4 presentes en linfocitos (93,151,162). La unión de B7.1 o B7.2 con CD28 promueve la activación de los linfocitos T, induciendo su proliferación, la expresión de proteínas anti-apoptóticas y la secreción de diferentes tipos de citocinas. En cambio, la unión de

estos mismos receptores B7.1 y B7.2 con el co-receptor CTLA-4 genera una señal inhibidora en los linfocitos que dejan de proliferar y de secretar citocinas, induciendo la finalización de la respuesta inmunitaria (73,152) (consultar Figura 1 para ver un resumen del resultado de estas interacciones). En nuestro estudio no hemos detectado en ningún caso expresión de B7.1 ni de B7.2 en células de microglía reactiva durante las fases de inducción y pico de la EAE. Este fenotipo celular, caracterizado por la expresión de moléculas de MHC y ausencia de moléculas co-estimuladoras, se asocia comúnmente con el fenotipo que muestran las células dendríticas inmaduras en órganos periféricos (1). Esto nos lleva a proponer que en estas fases la microglía está actuando como una célula dendrítica inmadura. Apoya esta idea el hecho de que hemos demostrado la expresión de CD1, un marcador de célula dendrítica inmadura (160), en microglía reactiva localizada tanto a nivel perivascular como en el parénquima. También cabe añadir que las células de microglía a pesar de expresar CD1, nunca llegan a expresar fascina, un marcador de células dendríticas maduras (4), indicando que no llegan nunca a actuar como una célula dendrítica madura.

En lo que respecta a la expresión de moléculas co-estimuladoras en linfocitos, hemos detectado que durante la fase de inducción el número de células CD28+ aumenta de manera progresiva hasta alcanzar su máximo durante el pico de la enfermedad. Sin embargo, tan solo se observa una pequeña población de células CTLA-4+ durante estas fases.

En conjunto todos estos resultados, nos llevan a proponer que durante la inducción y pico de la enfermedad, la microglía reactiva mediante su fenotipo de célula dendrítica inmadura (MHC-clase I+, MHC-clase II+, B7.1-, B7.2-, CD1+), puede estar promoviendo la apoptosis o anérgia de los linfocitos pro-inflamatorios CD28+ infiltrados. La promoción de esta apoptosis podría explicar la no-cronificación de la enfecefalopatía e incluso su inminente resolución. Apoyando esta hipótesis nuestras observaciones muestran la presencia de numerosos linfocitos T-cooperadores (CD4+) con núcleo apoptótico durante el pico de la EAE, algunos de los cuales se observan en estrecha relación con células de microglía reactiva.

Las células de microglía se mantienen activadas durante la remisión de la sintomatología clínica al mismo tiempo que aparecen nuevos subtipos de linfocitos

Coincidiendo con el momento en que empieza la recuperación a nivel sintomatológico (*score* 2R), la población de microglía/macrófagos experimenta ciertos cambios morfológicos, emitiendo nuevamente prolongaciones citoplasmáticas más o menos desarrolladas, que a partir del *score* 1R, aunque vuelven a recordar la microglía ramificada, muestran todavía signos evidentes de reactividad con fuerte expresión de NDPasa, LT e Iba1. Estas células de microglía siguen manteniendo una estrecha relación con los vasos sanguíneos, observándose acúmulos perivasculares similares a los observados durante la fase de inducción y pico.

La apoptosis de linfocitos T es uno de los fenómenos que se ha relacionado con la recuperación de la EAE (112,127,128,155). Este mecanismo, que como hemos descrito anteriormente puede estar inducido por las propias células de microglía, podría explicar la drástica disminución del número de linfocitos Th1 observada a partir del *score* 2R, momento en que empieza la fase de recuperación. Cabe señalar sin embargo, que el número y proporción total de linfocitos T-cooperadores se mantiene constante durante toda esta fase, lo cual indica que posiblemente la infiltración de linfocitos al interior del parénquima nervioso se mantiene durante toda la evolución de la EAE. En este sentido, durante la etapa de recuperación hemos observado un progresivo incremento en el número y proporción de otras dos subpoblaciones de linfocitos T, los Th17 y los T-reg. Nuestros estudios no nos permiten determinar si estos linfocitos Th17 y T-reg son nuevos linfocitos infiltrados, o bien si derivan de la transformación de algunos linfocitos infiltrados durante la fase de inducción y pico. En este sentido estudios recientes apuntan hacía una plasticidad entre las diferentes poblaciones de linfocitos que podría determinar el cambio de fenotipo de linfocito efector dependiendo de factores como las citocinas presentes (124,182). Lo que sí podemos afirmar es que estas poblaciones no son el resultado de una proliferación de linfocitos dentro del parénquima, ya que mediante el uso de marcadores específicos no hemos detectado expresión de moléculas de proliferación en la población de linfocitos, en ninguna de las fases de la EAE.

Los linfocitos Th17 se definen de forma general como un subtipo de linfocitos con función patogénica en la EAE. Estas afirmaciones derivan del hecho de que estos linfocitos se han observado dentro del SNC durante el pico de la EAE (119) y son capaces de inducir EAE cuando se inyectan en ratones susceptibles (68,123). Sin embargo, algunos autores defienden la posibilidad de que los linfocitos Th17 puedan jugar también un papel beneficioso (77). En este sentido, se ha descrito que estos linfocitos además de producir citocinas pro-inflamatorias como la IL17, son capaces de secretar algunas citocinas anti-inflamatorias como IL10 (113) e IL22 (169). Además, el papel que juegan algunas de las citocinas secretadas por linfocitos Th17, como la IL21 en la evolución de la EAE, ha generado mucha controversia. Por un lado, se ha demostrado que la

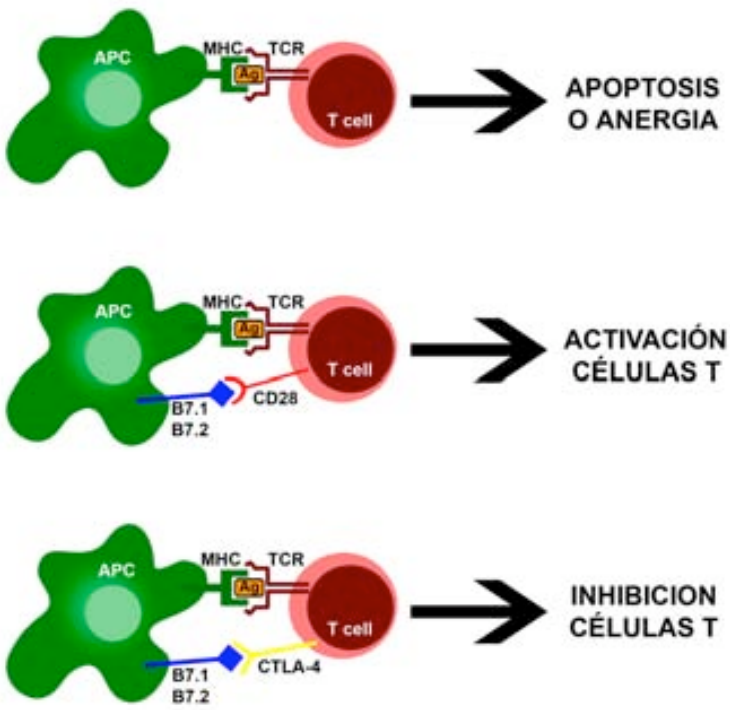

Figura 1: Posibles efectos derivados de la interacción entre las células presentadoras de antígeno (APC) y los linfocitos T.

presencia de IL21 antes del inicio de la EAE agrava la patología (171), mientras que por otra parte inhibir su acción igualmente induce un empeoramiento de la sintomatología (99,132).

El papel de los linfocitos T-reg en la patogénia de la EAE genera menos discrepancia, ya que su presencia ha sido siempre descrita en etapas de remisión en modelos de ratón (79,81,114), y su efecto protector ha sido ampliamente demostrado: no sólo se ha probado que la inyección de estas células induce una disminución de la severidad de la EAE (79,114), sino que se ha visto que su ausencia vuelve a los animales más susceptibles a desarrollar autoinmunidad (180). El hecho de que en nuestro estudio la mayor presencia de T-reg coincida con la fase de recuperación nos lleva a pensar que estas células están jugando un papel clave en la resolución de la EAE y que podrían estar directamente relacionadas con la recuperación espontánea característica de este modelo.

Además de linfocitos T-cooperadores, también hemos observado que las proporciones de los linfocitos T-citotóxicos y T-γδ se mantienen estables a lo largo de toda la fase de recuperación. La infiltración de ambos tipos de linfocitos ha sido préviamente demostrada en otros modelos de EAE (51,138,163,177), si bien todavía no se conoce el papel que juegan estas poblaciones linfocitarias en la evolución de la enfermedad. Por un lado se apunta hacia un posible papel patogénico, ya que se ha demostrado la presencia de linfocitos T-γδ durante el pico de la EAE (51,177) y se ha descrito la inducción de EAE mediante la inmunización pasiva de linfocitos T citotóxicos (64,167). Por otro lado, sin embargo, se ha demostrado que la eliminación de estos dos tipos de linfocitos produce un empeoramiento de la sintomatología (48,71,78,98,137,138), lo cual sugiere su posible papel regulador. Dado que no hemos observado diferencias a lo largo de la evolución de la EAE, nuestros resultados no nos permiten definir a estas poblaciones como patogénicas o reguladoras, pero el hecho de que se mantengan presentes a lo largo de todas las fases de la EAE nos indica que podrían estar relacionadas de alguna manera con la evolución de la patología.

Las células de microglía alrededor de los vasos expresan moléculas co-estimuladoras durante la recuperación

A lo largo de toda la fase de recuperación, las células de microglía reactiva mantienen una alta expresión de MHC-clase I y muestran una disminución progresiva en la expresión de MHC-clase II. A pesar de esto, los niveles de expresión de ambas moléculas, incluso en animales analizados en ausencia de sintomatología (*score* 0R), se mantienen muy por encima de los observados en animales control, indicando que todavía juegan un

papel activo. Hemos de destacar que durante esta fase de recuperación, y por primera vez, se observa la expresión de la molécula co-estimuladora B7.2 en una subpoblación de células de microglía reactiva localizada alrededor de algunos vasos sanguíneos. Estos resultados sugieren que las células de microglía ubicadas en los alrededores de los vasos sanguíneos adquieren una funcionalidad específica. Algunos autores ya han sugerido que la población microglial puede expresar fenotipos diferentes en función de su localización o microambiente en el cual se encuentran (28). Por otro lado, el hecho de que la co-expresión de MHC-clase I o MHC-clase II junto con B7.2 se observe específicamente alrededor de algunos vasos, nos indica que en esta localización específica las células de microglía interaccionan con la población linfocitaria de una forma particular. Tenemos que resaltar en este contexto que justo alrededor de los vasos sanguíneos es donde hemos observado un progresivo aumento en la expresión de CTLA-4, uno de los co-receptores de B7.2. La unión de estas moléculas, tal y como hemos descrito anteriormente, induce la inhibición de las vías de señalización que activan a los linfocitos, llevando en último término a la finalización de su respuesta (89). Sin embargo, hoy en día aún no está bien definida la función que ejerce CTLA-4 en los linfocitos T-reg, los cuales expresan esta molécula de manera constitutiva (168). Estudios recientes han demostrado que la señal a través de CTLA-4 es necesaria para la inducción de Foxp3 y para la activación de estas células T-reg (69,145,181). Esto nos lleva a sugerir que los linfocitos T-reg que hemos observado durante la fase de recuperación pueden estar recibiendo una señal de la microglía perivascular que promueve su activación.

Por otro lado, la microglía parenquimática mantiene la expresión de MHC-clase I y MHC-clase II pero no expresa moléculas co-estimuladoras. De la misma manera que ocurre durante la fase de inducción y pico, estas células, podrían estar induciendo una señal de anergia y/o apoptosis a los linfocitos infiltrados en el parenquima.

En conjunto, estos resultados sugieren que la expresión diferencial de moléculas co-estimuladoras durante la fase de recuperación, tanto en linfocitos como en células de microglía, participa en la resolución de la EAE y probablemente como discutiremos más tarde en la inducción de tolerancia.

Para concluir este apartado merece la pena mencionar que a diferencia de lo que ocurre en otros modelos de EAE (67), en este modelo agudo, la microglía no muestra expresión de B7.1 en ninguna de las fases analizadas. La expresión conjunta de MHC-clase II y B7.2 pero no de B7.1 en microglía reactiva se ha descrito en situaciones de lesión aguda al SNC; como la axotomía del nervio facial (24) o de la vía perforante de la corteza entorrinal (17,85), en las que la destrucción de la mielina no conlleva posteriormente a la inducción de auto-inmunidad. Este hecho nos hace sugerir que el fenotipo microglial observado en nuestro estudio presentaría las mismas características, ejerciendo un papel neuroinmunomodulador que evitaría el desarrollo de un ulterior ataque inmunitario. En línea con esta hipótesis, se ha asociado la expresión de B7.1 en modelos de EAE remitente-recurrentes con la generación del fenómeno de expansión de epítopo ("epitope spreading"), que se postula como uno de los mecanismos responsables de las recaídas en estos modelos de autoinmunidad (118).

Una subpoblación de células de microglía expresa CD4 exclusivamente durante la fase de recuperación

Mediante análisis inmuno-histoquímicos y de citometría de flujo, hemos podido observar la expresión de la molécula CD4 en una subpoblación de microglía reactiva tan solo durante la fase de recuperación. Esta molécula, expresada constitutiva-mente en monocitos y macrófagos en la periferia (32,70), se ha descrito en macrófagos perivasculares y meníngeos dentro del SNC normal (130). Respecto a su expresión en microglía, se ha asociado *in vitro* (cultivos de microglía) a la presencia de moléculas como el factor neurotrófico ciliar (57) y citocinas como IL4 e IL6 (172); e *in vivo*, a microglía ameboide en etapas post-natales (130). También ha sido descrita la expresión de CD4 en microglía reactiva asociada a algunos tipos de daño agudo como lesión por aspiración (130), isquemia focal (156), axotomía del nervio facial (166) y neurodegeneración inducida por serotonina (175), aunque en ninguno de estos estudios se describe el posible papel que juega CD4 en estas células de microglía. En nuestro estudio no hemos determinado el papel de CD4 en células de microglía, sin embargo, el hecho de que se exprese especí-ficamente durante la fase de recuperación, nos lleva a sugerir que CD4 podría ser una molécula involucrada en la resolución de la respuesta inflamatoria/inmune, asociada a este modelo de EAE.

¿Qué papel juegan los macrófagos perivasculares?

Tal y como hemos descrito en el primer apartado, ya en los animales control se detecta una población de células ED2+ que tras la inducción de la EAE muestran cambios asociados con la sintomatología. Por un lado se ha observado un incremento en el número de células ED2+ y por otro un cambio morfológico progresivo hacia formas macrofágicas, en clara correlación con el aumento de la sintomatología clínica. Hay que destacar que durante el pico de la EAE, muchas de estas células se observan no sólo en el espacio perivascular sino también infiltradas en el parénquima cerebral. Debido a su localización estratégica y a su expresión

constitutiva de MHC-clase II, se ha propuesto a estos macrófagos perivasculares como una de las células clave en el inicio de las respuestas inmunes adquiridas en el SNC. Estas células podrían participar en la activación de los linfocitos, hecho necesario para la posterior infiltración en el parénquima (13,74). No obstante, muchas de las evidencias experimentales basadas en la eliminación específica de estas células (134) muestran resultados contradictorios (135,136), por lo que su papel en la evolución de la EAE no está todavía bien definido.

El número de macrófagos perivasculares disminuye durante la fase de recuperación, aunque sigue siendo mayor al observado en animales control. Asimismo, la morfología progresivamente adopta las formas fusiformes detectadas en animales control.

Infiltración de células con fenotipo de dendríticas maduras

Tal y como hemos comentado anteriormente, las células de microglía activada o macrófagos, identificados con los marcadores habituales, nunca presentan expresión de fascina en este modelo. Sin embargo, nuestras observaciones ponen de manifiesto la presencia de una población de células dendríticas maduras fascina+ con morfología fusiforme, que se localizan inicialmente en la pared de los vasos sanguíneos. Posteriormente, durante el pico de la enfermedad, hemos podido identificar estas células fascina+ en el interior del parénquima. Aunque no hemos determinado la naturaleza exacta de estas células, el hecho de que no muestren marcadores de células microgliales ni de macrófagos, nos lleva a especular que en este modelo agudo de EAE podría producirse un reclutamiento de células dendríticas periféricas, que indudablemente jugarían un papel importante en la evolución de la EAE. Algunos autores han descrito la infiltración de células que identifican como dendríticas en modelos de EAE (107,159), no obstante hay que destacar que estos autores no identifican estas células como dendríticas maduras o inmaduras, y éste es el primer estudio que demuestra la entrada de células fascina+ en algún modelo de EAE. Obviamente se precisan más estudios enfocados a desvelar el papel que juegan estas células.

Durante la fase de recuperación el número de células dendríticas maduras (fascina+) disminuye progresivamente. A pesar de ello, su número sigue siendo mayor al que muestran los animales control, incluso en aquellos animales estudiados en ausencia de sintomatología (*score* 0R). Durante esta fase de recuperación además, estas células se encuentran localizadas exclusivamente en el interior del espacio perivascular, y no dentro del parénquima. No es hasta la fase de post-recuperación cuando ya no se observan células dendríticas maduras (fascina+).

El papel que juegan estas células en este modelo de EAE es totalmente desconocido. De hecho nunca antes se ha descrito la presencia de células fascina+ en este u otros modelos de EAE. Cabría especular que estas células se infiltran en el tejido nervioso a la espera de jugar un papel activo en la presentación de antígenos y activación de linfocitos pro-inflamatorios, pero ante la acción inhibidora de la respuesta inmune ejercida por las células de microglía, no pueden cumplir con su función y finalmente desaparecen.

Posible implicación de la microglía en los mecanismos de generación de tolerancia

Como hemos mencionado anteriormente nuestros resultados revelan que la reactividad microglial e infiltración linfocitaria no concluyen tras la recuperación funcional de los animales afectados por la EAE, sino que por el contrario se mantienen durante varias semanas en el período que hemos denominado de post-recuperación. Así pues, entre las 4-6 semanas tras la recuperación funcional, hemos observado focos de células de microglía reactivas con elevada expresión de MHC-clase I y MHC-clase II, tanto en el parénquima como alrededor de algunos vasos. Algunas células microgliales asociadas a los vasos sanguíneos siguen mostrando expresión de B7.2, si bien ya no podemos detectar expresión de CD1. Asimismo, hemos observado que a los 28dpi, la proporción de Th17 y T-reg se mantiene elevada. Sin embargo, a partir de los 40dpi, se observa una disminución en la proporción de estos linfocitos T-cooperadores.

Cabe destacar que a los 90dpi las células de microglía adquieren ya una morfología ramificada que recuerda a la que se observa en animales control, sin embargo en una pequeña subpoblación, aún se observa una expresión mayor de MHC-clase I, MHC-clase II y B7.2 que la observada en animales control. A los 90dpi no se ha podido detectar la presencia de linfocitos T-cooperadores en el interior del parénquima cerebral.

Estas observaciones sugieren que la reactividad persistente de la población microglial y la presencia de poblaciones linfocitarias pueden estar en conjunto implicadas en la generación del fenómeno de tolerancia. La tolerancia se define como la ausencia de respuesta ante un antígeno, ya sea propio o extraño, que se genera tras exposiciones previas al mismo antígeno (1). Existen dos tipos principales de tolerancia: la tolerancia central y la periférica. La tolerancia central ocurre durante la etapa de maduración de los linfocitos T en el timo y es la encargada de asegurar que no se producen linfocitos contra autoantígenos (61,109). Durante esta etapa de maduración, aquellos linfocitos específicos que reconocen antígenos propios con gran afinidad son eliminados por apoptosis. Además de esta eliminación, hoy en día se sabe que algunos de los

linfocitos que reconocen antígenos propios en el timo no son eliminados sino que se transforman en los denominados linfocitos T-reg. Los mecanismos que determinan que un linfocito muera o se transforme en T-reg no son conocidos. La tolerancia periférica por otro lado es el mecanismo por el cual linfocitos T que reconocen antígenos en órganos periféricos se vuelven incapaces de responder nuevamente a estos antígenos. Esta tolerancia puede ser inducida por varios mecanismos: por anergia clonal, por apoptosis o por supresión mediada por los T-reg (150,157). Es importante destacar que, en situaciones normales, el mecanismo de tolerancia tiene una gran importancia en el control de la respuesta contra antígenos propios. En este mecanismo juegan un papel primordial las células dendríticas. Concretamente, en el modelo agudo de EAE en rata Lewis se ha descrito inducción de tolerancia a nuevas inmunizaciones con el mismo antígeno MBP (102,121), de tal forma que una segunda inmunización con MBP no genera ninguna respuesta inmune. Nuestras observaciones aún sin publicar, demuestran sin embargo que puede volverse a inducir EAE en estos animales inmunizando una segunda vez mediante la utilización de otros antígenos de la mielina diferentes.

A modo de resumen se han elaborado las Figuras 2 y 3 que recogen las principales observaciones del estudio y la posible interpretación de las mismas.

CONCLUSIONES

Los resultados alcanzados con la realización de este trabajo indican que las células de microglía juegan un papel clave en la regulación de la respuesta inmune adquirida asociada a un modelo agudo de EAE modulando la activación/inactivación de las diferentes poblaciones de linfocitos infiltrados a lo largo de las diferentes fases de la evolución de la enfermedad. Concretamente:

- La inducción de EAE aguda en rata Lewis provoca la manifestación de una sintomatología clínica que correlaciona con la activación de la población microglial/macrofágica y una fuerte infiltración linfocitaria pro-inflamatoria durante la fase de inducción y la fase de máxima afectación (pico) de la enfermedad. Esta correlación no es evidente durante la fase de recuperación en la que, a pesar de que los animales llegan a recuperarse completamente, se aprecia todavía una importante activación de las células de microglía y una gran infiltración linfocitaria.

- La infiltración masiva de linfocitos tiene lugar a lo largo de la fase de inducción. El número de linfocitos se mantiene elevado durante la fase de recuperación. La mayor parte de la población linfocitaria son Th1 pro-inflamatorios durante la fase de inducción y pico, mientras que en las siguientes fases predominan los Th17 y T-reguladores.

- A lo largo de la fase de inducción y pico de la enfermedad, pero también durante la fase de remisión, las células de microglía adoptan un fenotipo de células dendríticas inmaduras el cual puede ser responsable de la modulación de la respuesta inmunitaria. La expresión microglial de moléculas del complejo mayor de histocompatibilidad de tipo I y II en ausencia de la expresión de moléculas co-estimuladoras puede ser el mecanismo responsable de la apoptosis o anergia de los linfocitos inflamatorios.

- Únicamente durante la fase de recuperación hemos detectado una subpoblación de microglía con expresión de moléculas co-estimuladoras en áreas circundantes a los vasos sanguíneos, coincidiendo con la presencia de linfocitos Th17 y T-reg. La interacción de las células de microglía con estos subtipos de linfocitos podría estar estrechamente relacionada con los mecanismos de inducción de tolerancia.

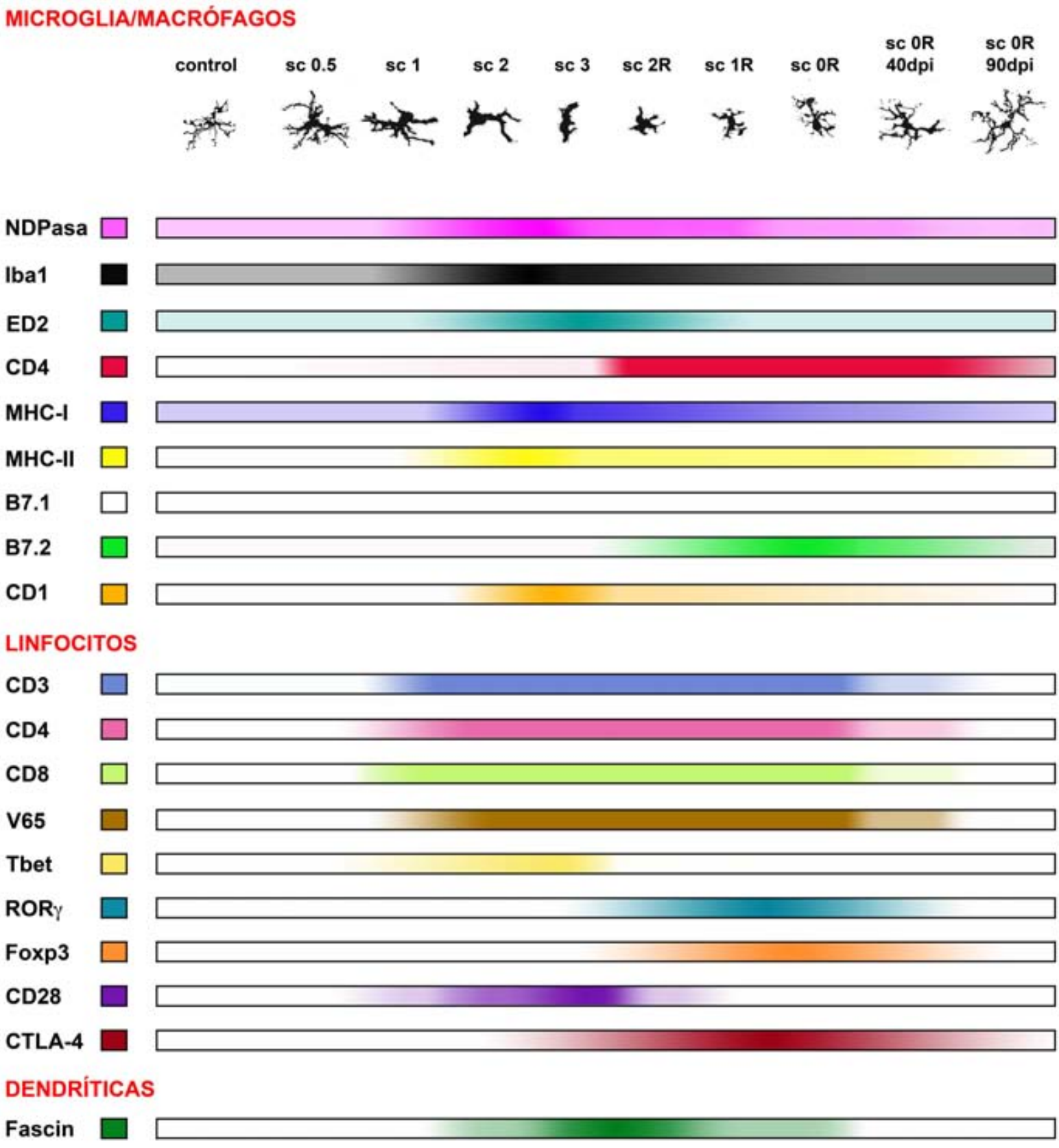

Figura 2. Patrón de expresión de las moléculas analizadas a lo largo de las diferentes fases de la evolución de la EAE

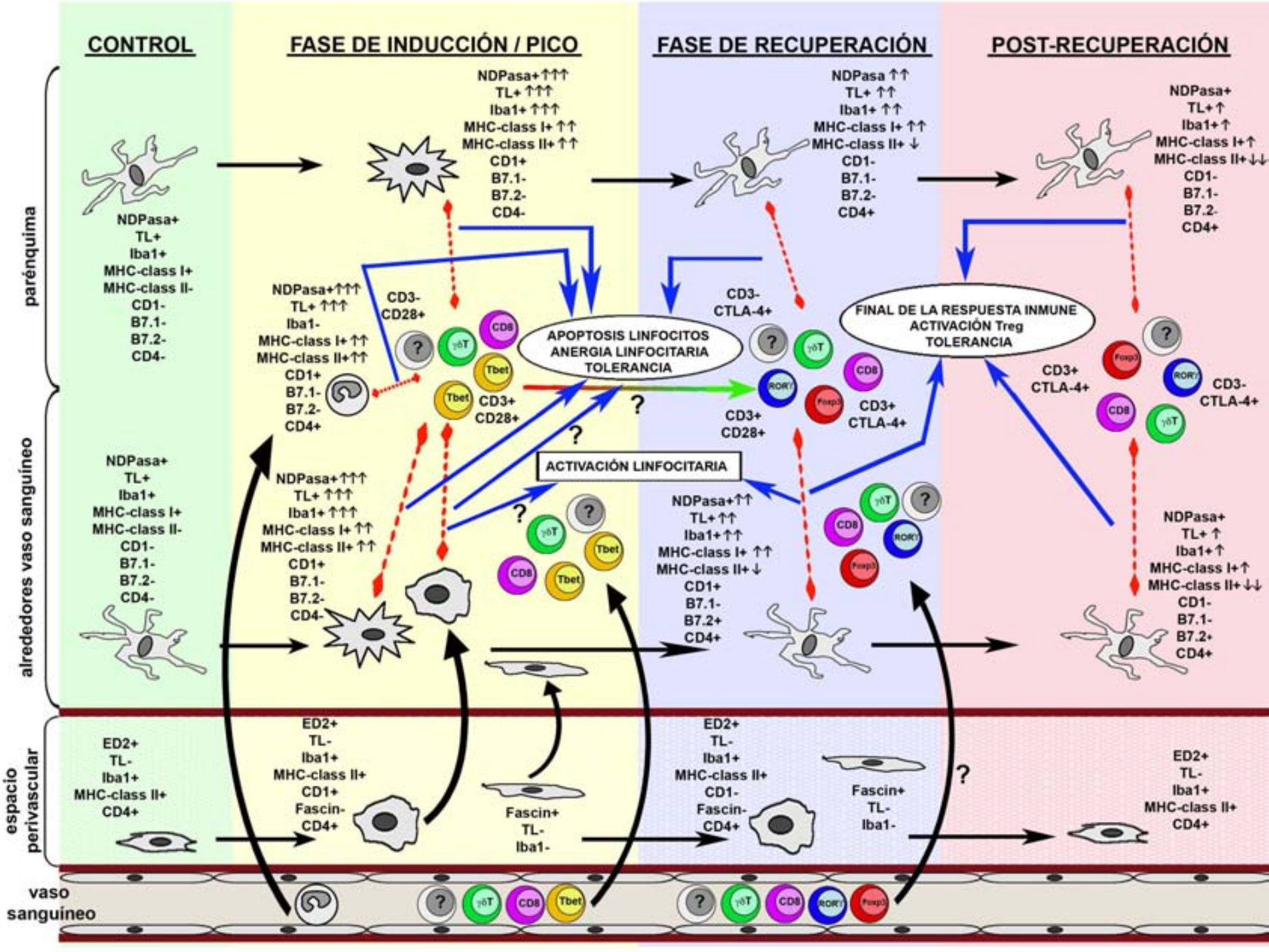

Figura 3. Representación esquemática del proceso de presentación antigénica e infiltración linfocitaria propuestos

interacción entre células; efecto derivado de la interacción; plasticidad linfocitaria

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ANEXO 1

Artículos originales

1. CD4 microglial expression correlates with spontaneous clinical improvement in the acute Lewis rat EAE model. **Almolda, B**.; Costa, M.; Montoya, M.; González, B.; Castellano, B. *J. Neuroimmunol. 2009, Apr 30; 209(1-2):65-80*

2. Activated microglial cells acquire an immature dendritic cell phenotype and may terminate the immune response in an acute model of EAE. **Almolda, B.**; González, B.; Castellano, B. *J. Neuroimmunol. 2010, Jun; 223(1-2):39-54.*

3. Quick decrease in Th1 lymphocytes and high increase in Th17 and T-reg populations may explain the spontaneous recovery of acute EAE. **Almolda, B.**; Costa, M.; Montoya, M.; González, B.; Castellano, B. *J. Neuroimmunol. (enviado).*

Capítulos de libro

1. Antigen presentation mechanisms in EAE: role of microglia, macrophages and dendritic cells. **Almolda, B.**; González, B.; Castellano, B. *In: Frontiers in Biosciences: Microglia and brain macrophages in health and disease. Kaur Charanjit (Ed.) (enviado).*

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CD4 microglial expression correlates with spontaneous clinical improvement in the acute Lewis rat EAE model

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ABSTRACT

CD4 is a molecule commonly expressed on the surface of T-helper lymphocytes with a recognized critical role in the antigen presentation process that has also been reported in monocytes and macrophages, although its role in these cells remains unknown. The objective of the present study was to analyze whether experimental conditions involving a potent acquired immune component, as occurs in experimental autoimmune encephalomyelitis (EAE), are able to induce CD4 expression in the population of microglia/macrophages. Myelin Basic Protein (MBP) immunized female Lewis rats, were examined at different phases during the course of EAE according to their clinical score. Spinal cords were analyzed by flow cytometry for CD11b, CD4 and CD45, by histochemistry for NDPase and by immunohistochemistry for ED2, Iba1, CD45 and CD4. Flow cytometry analysis showed that EAE induced CD4 expression in macrophages (CD11b+/CD45^{high}) and microglia (in both CD11b+/CD45^{intermediate} and CD11b+/CD45^{low} phenotypes). Noticeably, microglial CD4 expression was found during the recovery phase and was maintained until 40 days post-induction. In agreement, immunolabelled sections revealed CD4 expression in microglial cells with ramified morphology during the recovery and post-recovery phases. In conclusion, our results indicate that, in this EAE model, perivascular cells, microglia and macrophages showed different dynamics during the course of the disease in close relation with symptomatology and that microglial cells expressed CD4 interestingly during the recovery phase, suggesting a role of microglial CD4 expression in the resolution of the immune response.

1. Introduction

CD4 is a glycoprotein expressed on the surface of some T lymphocytes including T helper and regulatory T cells (Reinherz et al., 1979; Sakaguchi, 2000). CD4 has four immunoglobulin domains (D1 to D4) that are exposed to the extracellular space, a transmembrane domain and a cytoplasmic tail that interacts with the leukocyte-specific protein tyrosine kinase (Lck) that is essential for activating the intracellular signalling pathway of activated T lymphocytes (Shaw et al., 1990). On T cells, CD4 is a coreceptor of the T-cell receptor (TCR). CD4 uses its D1 domain to interact specifically with MHC-II molecules expressed on the surface of professional antigen presenting cells (APCs) ensuring the specificity of the T cell response (Fleury et al., 1991). In addition to lymphocytes, expression of CD4 has been also described in human monocytes and rat macrophages (Crocker et al., 1987; Jefferies et al., 1985) although its role in these cells remains unclear.

It is well established that microglia, the resident macrophagic population within the central nervous system (CNS), becomes rapidly activated when the integrity of the CNS is disturbed as consequence of lesions, neurotoxicity, infections and also during autoimmune processes (Garden and Moller, 2006; Hanisch and Kettenmann, 2007; Kreutzberg, 1996). This activation is a complicated and gradual phenomenon characterized by a broad range of alterations including morphological changes and different modifications at biochemical and functional levels. In this context, activated microglia produce a variety of different cytokines, chemokines and trophic factors and may exhibit an important upregulation or de novo expression of a large array of molecules on their surface including sugar residues, adhesion molecules, enzymes, complement and immunogoblulin receptors and molecules related to T-cell-mediated immune functions such as CD45, MHCs and costimulatory molecules (Aloisi, 2001; Ambrosini and Aloisi, 2004; Garden and Moller, 2006; Raivich and Banati, 2004). All these activation changes are often related to the nature of the injury and influenced by the environment in which the cells are immersed (Hanisch and Kettenmann, 2007; Raivich et al., 1999).

In agreement with the monocytic origin of microglia, CD4 expression has been described in amoeboid microglial cells in the postnatal rat brain (Perry and Gordon, 1987; Wang et al., 1996). In the

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adult normal rat brain, however, CD4 expression has only been reported in the population of resident perivascular macrophages, but not in parenchymal microglia (Perry and Gordon, 1987) with the exception of microglial cells located in areas that exhibit an incomplete blood brain barrier (BBB) such as the area postrema, the pineal gland and the subfornical organ (Pedersen et al., 1997).

While it is accepted that microglia may acquire functions closer to macrophages and some in vitro studies reported an increase in microglial CD4 expression in certain circumstances (Wang et al., 2002), only a few studies have reported CD4 expression in reactive microglia in vivo. These studies showed that after acute injuries such as aspiration lesion (Perry and Gordon, 1987), focal ischemia (Schroeter et al., 1999), facial nerve axotomy (Streit and Graeber, 1993) and HT5 induced neurodegeneration (Wilson and Molliver, 1994), which involved an important glial reaction in the context of an innate immune response, there is a population of CD4+ cells that can be identified as microglial cells. Nevertheless, in these studies, the significance of microglial CD4 expression has not been discussed.

As CD4 has a well-established function in the immune response and resident microglia/macrophages have been widely accepted as cells with immunoregulatory properties in the CNS (Aloisi, 2001) it is not unexpected to hypothesize that CD4 expression in these cells may be involved in control of the immune response. In this regard the objective of the present work was to analyze whether experimental conditions involving a potent acquired immune component, as occurs in experimental autoimmune encephalomyelitis (EAE), are able to induce CD4 expression in the population of microglia/macrophages.

The induction of EAE in Lewis rats by Myelin Basic Protein (MBP) immunization has been chosen in this study as an experimental model due to its acute monophasic evolution characterized by a single episode of paralysis with a spontaneous recovery, allowing the study of microglial/macrophage reaction and CD4 expression not only during the inductive phase, but also during the resolution of the inflammatory process (recovery phase).

For this study we have performed a detailed analysis of microglial and macrophage reaction as well as CD4 expression in relation to clinical symptomatology by using histological and flow cytometry methods.

2. Materials and methods

2.1. Animals and EAE induction

A total of 113 female Lewis rats (180/200 g) susceptible to develop experimental autoimmune encephalomyelitis (EAE), were purchased from Charles River (IFFA Credo Belgique). Rats were maintained with food and water *ad libitum* in a 12 h light/dark cycle.

EAE was induced by the injection, in each hindlimb, of an emulsion containing 100 µg MBP (M2295; Sigma, St Louis, USA), Complete Freud's Adjuvant (CFA) (Ref. 0638; Difco, USA) and 0.2 mg of *Mycobacterium tuberculosis* H37 Ra (Ref. 3114; Difco, USA) in saline solution. Animals injected with vehicle solution were used as control.

All animals were evaluated daily for the presence of clinical symptoms, using the following clinical score test: 0, absence of symptoms; 0.5, partial loss of tail tonus; 1, paralysis of tail; 2, paraparesis of hindlimb; 3, paraplegia; 4, tetraparesis; 5, tetraplegia and 6, death.

All experimental animal work was conducted according to Spanish regulations in agreement with European Union directives and was approved by the ethical commission of the Autonomous University of Barcelona.

2.2. Experimental groups

MBP-injected rats ($n=100$) were sacrificed at different phases of the EAE course according to their clinical score and then distributed for both flow cytometry and histological studies (Fig. 1). In addition, a number of control animals were used for flow cytometry ($n=4$) and histological analysis ($n=9$).

2.3. Flow cytometry analysis

Rats processed for flow cytometry were anesthetized and intracardially perfused with phosphate buffer solution (PBS). The entire spinal cord was quickly removed and dissociated through 140 µm and 70 µm meshes in order to obtain a cell suspension for each rat. Subsequently, each individual cellular suspension was centrifuged

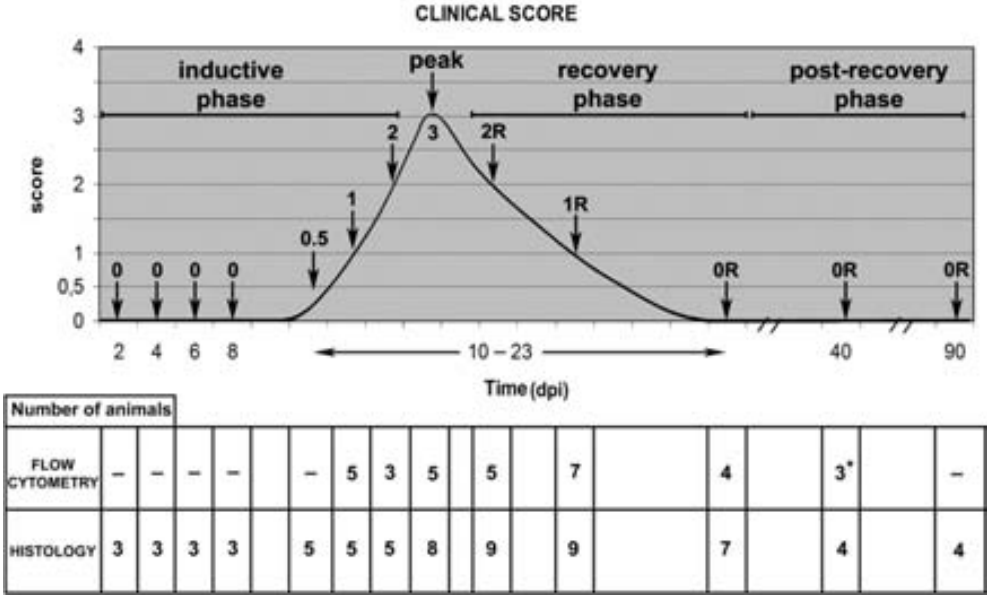


Fig. 1. Clinical score. The different phases of EAE evolution were schematized in this figure. Clinical symptoms of EAE appeared around 10 dpi with a partial loss of tail tone (score 0.5). During the inductive phase, the symptoms increased during the time peaking at around 12–13 dpi when the animals showed paralysis of the hindlimbs (score 3). During the recovery phase (R), animals progressively recovered the movement since 15–16 dpi and around 21 dpi were completely recovered (score 0R). Arrows corresponded to the experimental groups used in this study. Note that from days 10 to 23, animals were sacrificed according to their clinical score. Two groups of animals were maintained after the recovery and sacrificed at 32 and 90 dpi for flow cytometry; and 40 and 90 dpi for histology.

at 2400 rpm for 20 min in a discontinuous density Percoll gradient (17-0891-02, Amersham-Pharmacia) between 1.08 g/ml and 1.03 g/ml. Myelin layer at the top of the tube was removed. Cells in the interphase and the clear upper phase were collected, washed in PBS and labelled during 30 min at 4 °C with the following surface antibodies: anti-CD11b-FITC (1:400; 554982; BD Pharmingen, San Diego, CA), anti-CD45-PE (1:400; 554878; BD Pharmingen, San Diego, CA) and anti-CD4-PE-Cy5 (1:400; 554839; BD Pharmingen, San Diego, CA). Isotype-matched control antibodies (BD Pharmingen) were used as negative control. Finally the cells were acquired using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) and the results analyzed using the FlowJo® software.

2.4. Tissue processing for histological analysis

Animals used for histochemistry and immunohistochemistry, under deep anesthesia, were perfused intracardially for 20 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) + 5% sucrose. Spinal cords (cervical and dorsal part) were removed and immersed in the same fixative for 4 h at 4 °C. Samples were cut using a Leica VT 1000S vibratome and eight parallel longitudinal 40 µm thick sections were obtained. One of these series was processed for histoenzymatic demonstration of nucleoside diphosphatase (NDPase), a specific microglial marker (Murabe and Sano, 1981), as described below, and the others were stored at −20 °C in Olmos antifreeze solution and used for immunohistochemistry.

2.5. Hystoenzymatic demonstration of NDPase

NDPase technique was performed by collecting vibratome sections in 0.1 M cacodylate buffer (pH 7.4) (C0250; Sigma, St Louis, USA) with 5% sucrose as described earlier (Castellano et al., 1991). Briefly, sections were incubated for 20 min at 37 °C in a medium containing 7 ml distilled water, 10 ml 0.2 M Trizma-maleate (pH 7.4) (T3128; Sigma, St. Louis, USA), 5 ml 0.5% $MnCl_2$, 3 ml 1% $Pb(NO_3)_2$ and 25 mg sodium salt of 5′-inosine diphosphate (I4375; Sigma, St Louis, USA) as substrate. After several washes in distilled water, visualization of the reaction product was achieved by treating the sections with 2% ammonium sulfide followed by distilled water washes and by $AgNO_3$ treatment. Some sections were counterstained using toluidine blue. Finally, sections were mounted, dehydrated in graded alcohols and after xylene treatment coverslipped in DPX.

2.6. Single and double immunohistochemistry

Parallel free-floating vibratome sections were processed for either Iba1, CD4, ED2 (perivascular cells) or CD45 (LCA, leukocytic common antigen). After endogenous peroxidase blocking (2% H_2O_2 in 70% methanol for 10 min), sections were incubated for 1 h in Blocking Buffer solution (BB) containing 0.05 M Tris-buffered saline (TBS) pH 7.4, 10% fetal calf serum, 3% bovine serum albumin and 1% Triton X-100. Afterwards, sections were incubated overnight at 4 °C with either one of the following antibodies: 1) rabbit anti-Iba-1 (1:5000; 019-19741; Wako), 2) mouse anti-CD4 (1:1000; MCA55G; AbD Serotec), 3) mouse anti-ED2 (1:1000; MCA342R; AbD Serotec), and 4) mouse anti-CD45 (1:2000; MCA43GA; AbD Serotec) diluted in BB. As negative controls, some sections were incubated in media lacking the primary antibody. After washes with TBS+ 1% Triton, sections were incubated at room temperature for 1 h with either biotinylated anti-mouse (1:200; BA-2001) or biotinylated anti-rabbit (1:200; BA-1000) secondary antibodies (from Vector Laboratories, Inc, Burlingame, CA). Sections were then rinsed in TBS+ 1% Triton and incubated with horseradish peroxidase streptavidin (1:400; SA-5004; Vector Laboratories, Inc, Burlingame, CA). The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.5 mg/ml 3, 3′-diaminobenzidine (DAB) and 0.33 µl/ml H_2O_2. Finally, sections were mounted, counterstained with toluidine blue, dehydrated in graded alcohols and after xylene treatment coverslipped in DPX.

Double-immunolabelling for CD4 and either Iba1 (microglia/macrophages) or CD3 (T cells) was carried out by processing the sections for CD4 immunolabelling as described above but using AlexaFluor® 555-conjugated anti-mouse (1:1000, A31570; Molecular Probes) as secondary antibody. After several washes, sections were incubated overnight at 4 °C with either rabbit anti-Iba-1 (1:5000; 019-19741; Wako) or rabbit anti-CD3 (1:500; A0452; Dakopatts, Denmark) followed by AlexaFluor® 488-conjugated anti-rabbit (1:1000; A21206; Molecular Probes). Finally, sections were mounted on slides, dehydrated in graded alcohols and coverslipped in DPX.

Double labelling for ED2 and laminin was achieved by processing the sections for ED2 immunolabelling as described above but using AlexaFluor® 555-conjugated anti-mouse (1:1000, A31570; Molecular Probes) as secondary antibody. After several washes, sections were incubated overnight at 4 °C with rabbit anti-laminin (1:5000; L-9393; Sigma, St. Louis, USA) followed by AlexaFluor® 488-conjugated anti-rabbit (1:1000; A21206; Molecular Probes). Sections were mounted on slides and coverslipped as detailed above.

2.7. Morphometric analysis

Morphometric analysis was performed on sections immunoreacted for Iba1. To accomplish that, a total of 60 Iba1+ cells per animal were randomly chosen from 6 different photographs containing either grey or white matter, taken at 20× using a Nikon digital camera DXM 1200F join to a Nikon Eclipse 80i microscope. Three animals per score plus three controls were used. By means of analySIS® software, individual cells were isolated and different parameters including shape factor, elongation, mean diameter and area were recorded for each cell. Data were analyzed individually for grey and white matters. The statistic analysis was performed using the ANOVA test.

3. Results

Our observations showed that experimental conditions involving a potent acquired immune component were able to induce CD4 expression not only in T cells but also in the population of microglia/macrophages and that this expression was in close relationship with the clinical symptoms. Thus, during the inductive phase, populations of perivascular cells and microglia became widely activated and displayed important changes in their number, distribution and morphology. Only perivascular cells and macrophages but not reactive microglia showed increased or de novo expression of CD4. In the recovery phase, in parallel with a gradual extinction of clinical symptoms, our findings demonstrated that the macrophage population strongly decreased whereas reactive microglial cells progressively returned to their ramified state and some of them, noticeably, displayed de novo expression of CD4. During the post-recovery phase, in the absence of any symptomatology, there was still a persistent CD4 expression related to ramified microglial cells.

3.1. Clinical symptoms

Compared with control animals which never showed symptoms of disability, MBP-injected animals exhibited the first symptoms of EAE, characterized by weight loss and lightly loss of tail tonus (score 0.5), around 10 days post-immunization (dpi). In these animals, the disability increased progressively from tail paralysis (score 1) around 11 dpi, to hindlimb paraparesis (score 2) around 12 dpi and posterior hindlimb paralysis (score 3) around 14 dpi. During the recovery phase, animals, in addition to start to gain weight, progressively improved the movement of their hindlimbs, showing paraparesis (score 2) around 17 dpi, and tail paralysis around 19 dpi (score 1). Around 21 dpi animals did not show signs of disability (score 0). After their recovery, EAE animals which were monitored until either 40 or 90 dpi

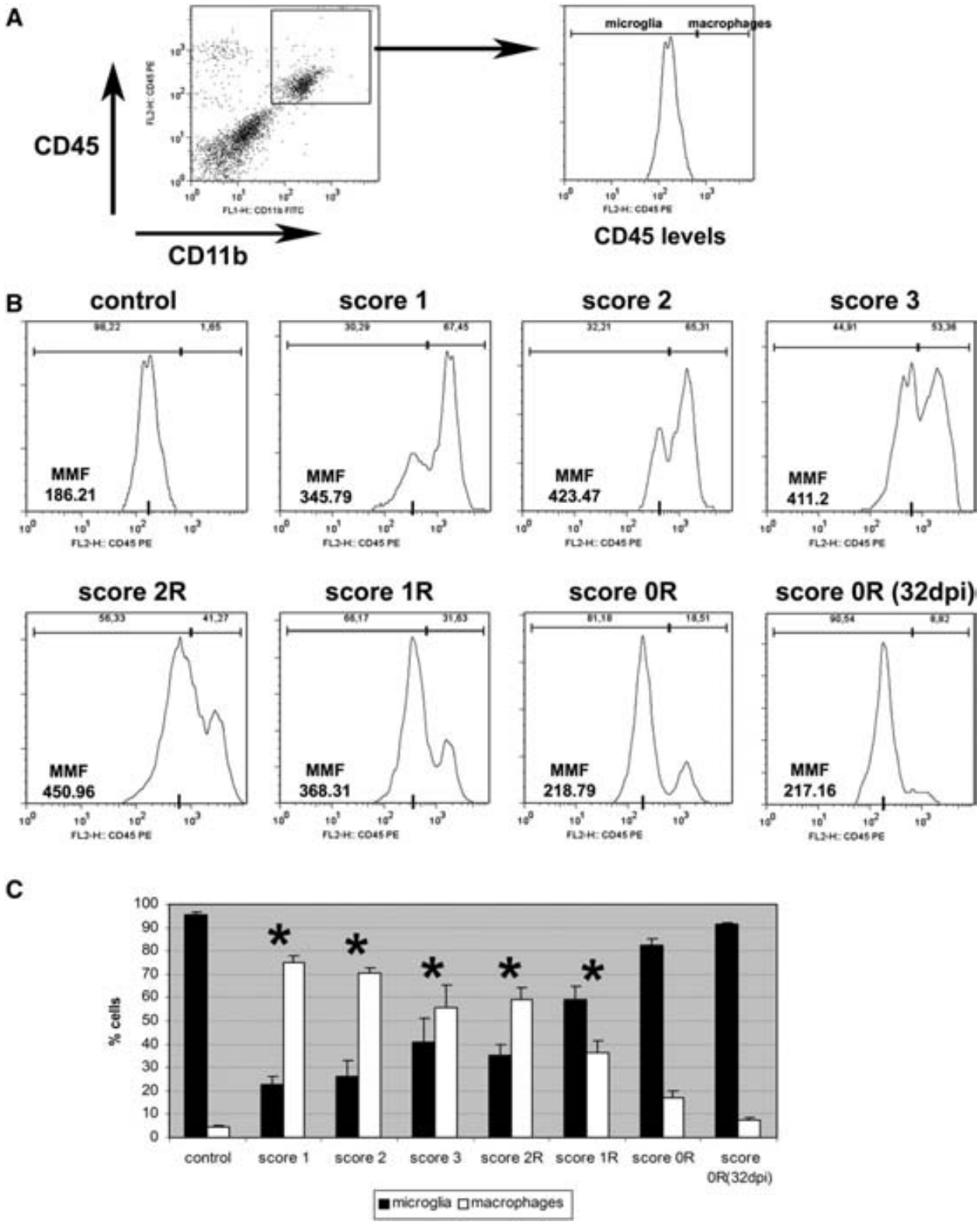


Fig. 2. Flow cytometry analysis of microglia and macrophage populations during the evolution of EAE. A) On the left side, representative dot-plot of CD11b/CD45 expression in cells obtained from spinal cord homogenates of control animals. The square delimits the CD11b+/CD45+ population of cells used in this study. On the right side, representative histogram where populations of CD11b+/CD45^{low} cells (microglia) and CD11b+/CD45^{high} cells (macrophages) were defined. B) Representative histogram-plot showing the dynamics of microglia and macrophage populations at the inductive phase (score 1 and 2), peak (score 3) and recovery phase (score 2R to 0R) during EAE evolution. Note that in comparison with control animals, EAE animals presented an important increase in the ratio of macrophages that is more apparent in the inductive phase and the peak. In addition, histograms showed (MMF: microglial mean fluorescence) that in EAE animals displaying clinical symptoms, microglial population had higher levels of CD45 than in controls. C) The histogram shows the mean values of microglia and macrophage population ratios during the course of EAE evolution (ANOVA test, $*p \leq 0.01$ relative to control).

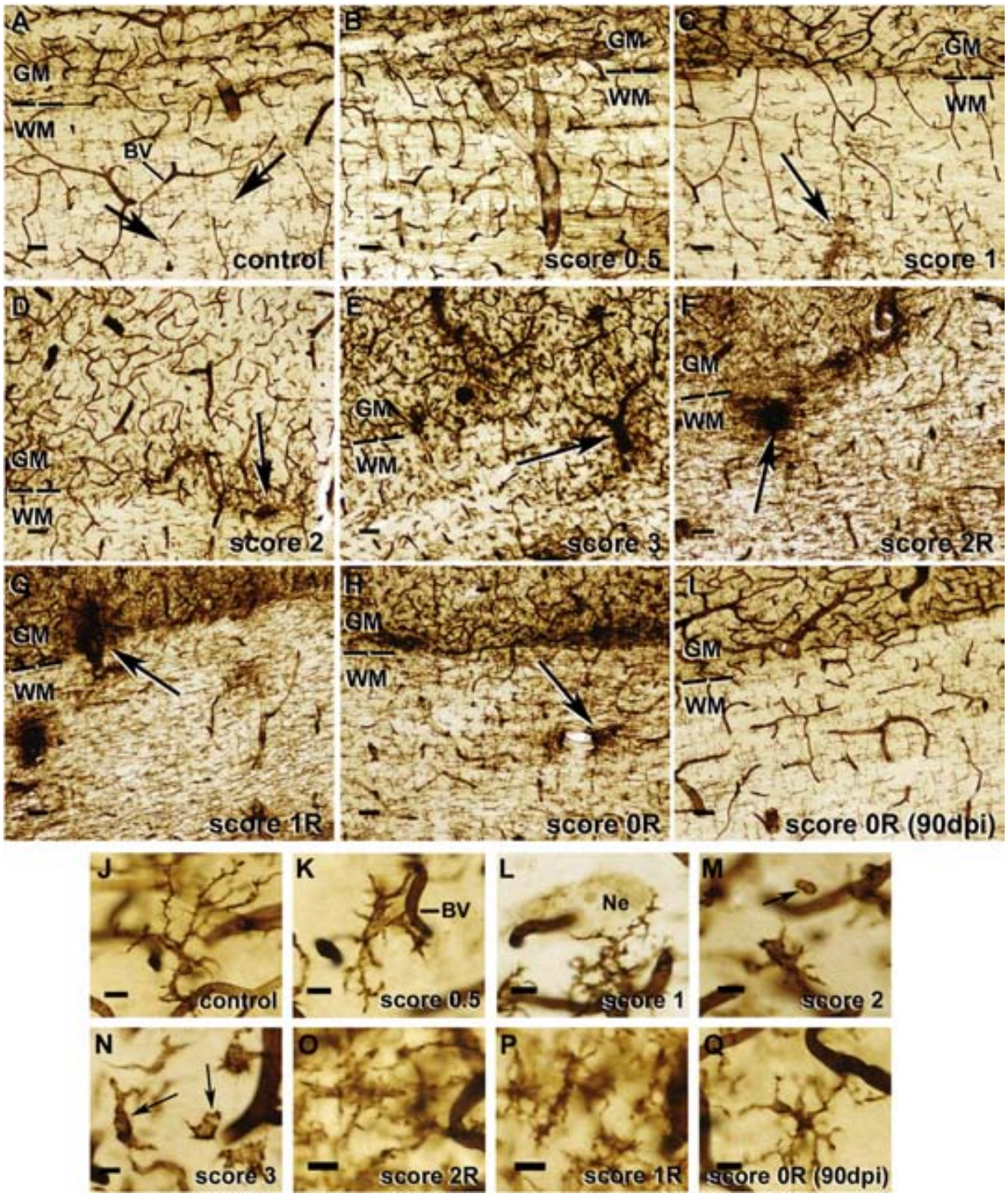


Fig. 3. NDPase histochemistry in control and EAE animals. NDPase histochemistry in the spinal cord of control animals (A) revealed the presence of ramified microglial cells (arrows) and blood vessels (BV) in both grey (GM) and white matters (WM). In MBP-injected animals (B–I), by comparing with their control counterparts, distribution and number of NDPase+ cells change during the different scores. Note that NDPase expression increases during the inductive phase (B–D), peaking at score 3 (E) and remains higher during the recovery phase (F, G) and even at score 0R (H). At long survival times (I), NDPase levels are similar to control animals. From score 1 to 0R, NDPase+ cells accumulate near blood vessels (arrows in C–H). High magnification (J–Q), shows that NDPase+ cells changed their morphology from the characteristic ramified cells found in control animals (J) to amoeboid shapes seen at score 3 (arrows in N), gradually reverting to ramified morphologies during the recovery (O, P) and post-recovery phase (Q). Note that during the inductive phase, reactive ramified NDPase+ cells approached to blood vessels (K, L) and their processes often wrapped neuronal somas (Ne in L). In addition, a distinct population of round NDPase+ cells was observed at scores 2 and 3 (arrow in M). Scale bar (A–I) = 50 μm; (J–Q) = 10 μm.

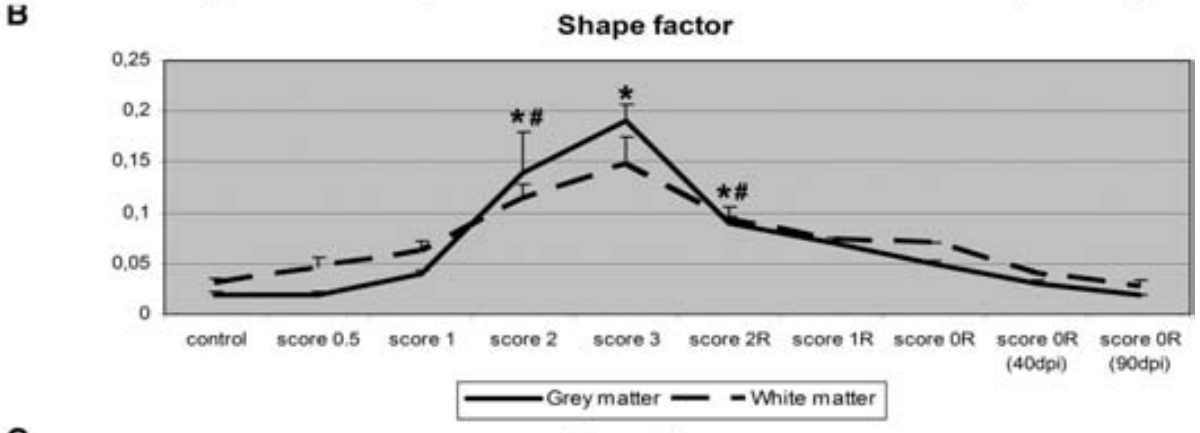
did not exhibit any relapse, in accordance to the known acute monophasic character of this model.

In this study, in order to appropriately identify animal groups and avoid confusion, we used "score 0", "score 1" and "score 2" in the inductive phase and "score 1R", "score 2R" and "score 0R" in the recovery phase (Fig. 1).

3.2. Microglia and macrophages during the evolution of EAE

The dynamics of the microglia and macrophage populations during the evolution of EAE was assessed by determining the levels of CD45 in the population of CD11b+ cells by flow cytometry and by evaluating, at histological level, their distribution and morphological


A
GM
WM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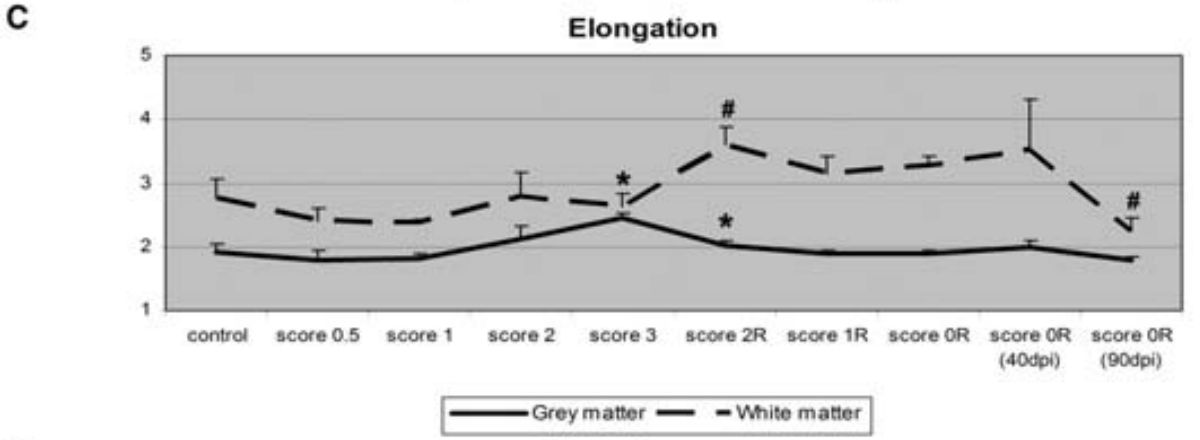
0,2
0,15
0,1
0,05
0
control
score 0.5
score 1
score 2
score 3
score 2R
score 1R
score 0R
score 0R (40dpi)
score 0R (90dpi)
Grey matter
White matter


C
Elongation
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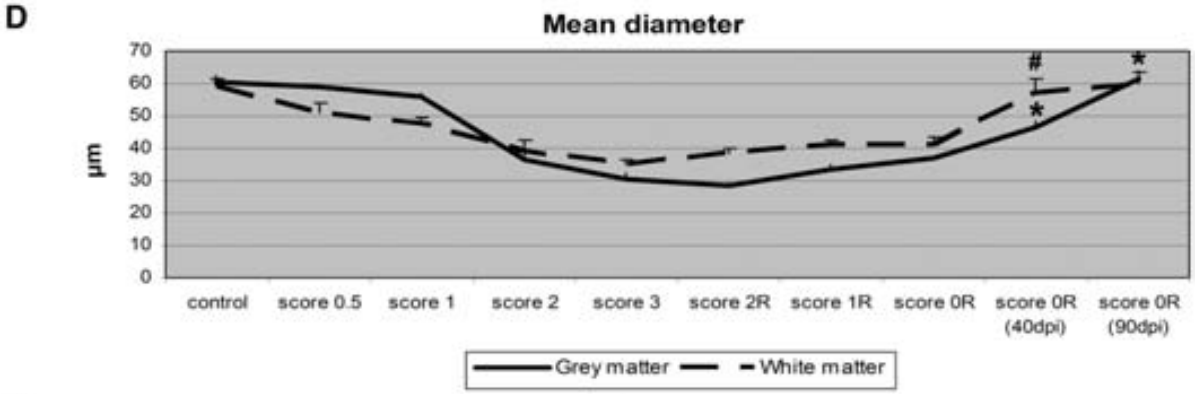
score 0R (90dpi)
Grey matter
White matter


D
Mean diameter
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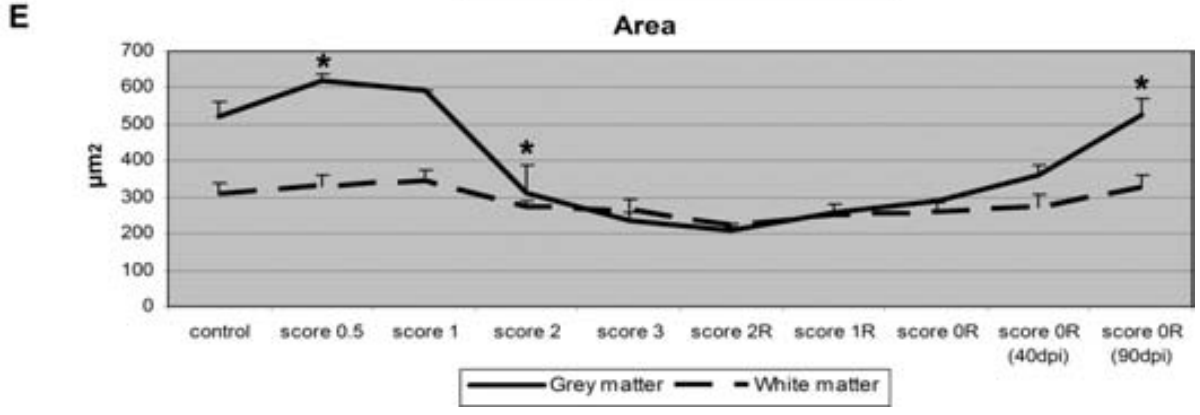
score 0R (40dpi)
score 0R (90dpi)
Grey matter
White matter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score 2R
score 1R
score 0R
score 0R (40dpi)
score 0R (90dpi)
Grey matter
White matter

changes by means of NDPase histochemistry and Iba1, ED2, CD45 immunohistochemistry.

3.2.1. Dynamics of CD11b+CD45+ cell populations

As shown in Fig. 2 A, in control animals there was a population of CD11b+/CD45+ cells identified as microglial cells according to their low CD45 expression (CD11b+/CD45^{low} cells). In these animals, there was a very low ratio of macrophages, which were identified on the basis of their high expression of CD45 (CD11b+/CD45^{high} cells).

In comparison with controls, MBP-injected animals showed variations in the relative proportions of both populations during the evolution of EAE in close relationship to clinical symptoms (Fig. 2 B and C). During the inductive phase, from score 1, there was a large increase in the percentage of CD11b+/CD45^{high} cell population. This ratio of CD11b+/CD45^{high} cells remained high during the peak of clinical symptoms (score 3) and even at score 2R in the recovery phase. From score 2R to score 0R, and in parallel with the improvement of symptomatology, the ratio of CD11b+/CD45^{high} cells decreased gradually but without reaching the values found in control animals.

In addition, flow cytometry analysis revealed that, in EAE animals, microglial cells exhibited increased levels of CD45 displaying a CD11b+/CD45^{intermediate} phenotype, which suggested an activated state of these cells. CD11b+/CD45^{intermediate} activated microglia were found from score 1 in the inductive phase to score 1R in the recovery phase. At score 0R, microglia displayed again a CD11b+/CD45^{low} phenotype.

3.2.2. NDPase histochemistry

Microglial cells were visualized using the histochemical demonstration of nucleoside diphosphatase (NDPase), a purine-related enzyme located in the microglial plasma membrane, widely accepted as a marker of resting and reactive microglia (Castellano et al., 1991; Murabe and Sano, 1981). In addition, NDPase histochemistry stained the blood vessels, which allowed us to evaluate putative changes in the distribution of microglia in relation to the vasculature.

In control animals (Fig. 3 A and J), microglia were identified as ramified cells distributed in both the grey and the white matters without any particular relationship to neurons or vasculature except that some of their processes were in contact with blood vessels. During the study, no changes in distribution and morphology of microglia were seen in control animals.

In EAE animals, in correspondence with the existence of clinical signs, the microglial cell distribution changed, exhibiting a close relationship with some blood vessels in both grey and white matters. In addition, microglial cells increased NDPase expression and showed marked variations in their morphology adopting reactive morphologies sometimes indistinguishable from other NDPase+ monocyte/macrophage-like cells found in these animals.

First signs of microglial alterations were evident at score 0.5 when the cells increased NDPase expression, enlarged their cell body, presented coarse ramifications and some of them approached to blood vessels (Fig. 3 B and K). At score 1, microglial cells started to accumulate around some blood vessels (Fig. 3 C). At this score, noticeably, reactive microglia extended their processes wrapping some neuronal bodies (Fig. 3 L). At score 2, microglial processes became shorter and thicker. Moreover, round NDPase+ cells were found in both grey and white matters, often near blood vessels (Fig. 3 M). At score 3, the number of NDPase+ cells increased substantially and the main part of these cells displayed amoeboid-like morphologies (Fig. 3 N). At scores 2R and 1R, in both the grey and white matters, reactive NDPase+ cells adopted a more ramified morphology and no amoeboid-like or round cells were observed in either case (Fig. 3 O and P). An important number of these NDPase+ cells remained adjacent to blood vessels (Fig. 3 F and G). At score 0R, in the absence of clinical signs, microglial cells still showed a reactive ramified morphology similar to those found at scores 2R and 1R, although the number of microglial cells associated with blood vessels decreased considerably (Fig. 3 H). It was not until 90 dpi that distribution and morphology of microglial population were comparable to control animals (Fig. 3 I and Q).

3.2.3. Iba1 immunohistochemistry and morphometric analysis

Iba1 is a macrophage/microglia-specific calcium-binding protein that is involved in Rac-GTPase-dependent membrane ruffling and phagocytosis (Ito et al., 1998; Kanazawa et al., 2002). Sections stained with Iba1 allowed us the study of microglial distribution and morphology without the interference of blood vessels. Our study using this marker (Fig. 10) corroborated NDPase observations. In addition, taking advantage of the clear identification of individual cells provided by Iba1 immunohistochemistry, we performed a detailed morphometrical analysis of the changes observed in microglia in both grey and white matters during the course of EAE (Fig. 4). The parameters analyzed were the shape factor, the elongation factor, the mean diameter and the area of Iba1+ cells. Control animals presented low shape factor values (Fig. 4 B) and low elongation values (Fig. 4 C). In EAE animals, these values increased during the inductive phase, peaking at score 3, indicating that Iba1+ cells lost their ramifications and became more elongated. In the recovery phase, from score 2R, both the shape and the elongation factors experimented a significant decrease until score 0R (90 dpi) when the cells presented values comparable to controls. Elongation factor in the white matter did not change during the inductive phase but increased during the recovery phase and returned to normality at score 0R (90 dpi). In comparison to controls, the mean diameter of Iba1+ cells in EAE animals decreased from score 2 during the inductive phase and remained low during all the recovery phase (Fig. 4 D). At score 0R (40 dpi) the mean diameter of Iba1+ cells increased, without reaching control values until score 0R (90 dpi). Finally, we observed that, compared to controls, EAE animals exhibited an increase in the area of Iba1+ cells in the grey matter at score 0.5 (Fig. 4 E). After that, the area decreased drastically at score 2 and was not until score 0R (90 dpi) when the area of Iba1+ cells reached values similar to controls. No changes in white matter were detected regarding this parameter.

3.2.4. ED2 immunohistochemistry

In control animals, ED2 immunoreactivity stained a population of cells located in the wall of blood vessels (Fig. 5 A, J, P). These cells displayed a spindle-shaped morphology following the blood vessel axis. Whereas the distribution and morphology of ED2+ cells in controls did not change throughout the study, in EAE animals, these cells exhibited variations during the course of the disease in parallel with the clinical scores.

During the inductive phase, from score 0.5, the number of ED2+ cells increased progressively until the peak of symptomatology, at score 3, when the maximum presence of ED2+ cells was observed (Fig. 5 E). Simultaneously, these perivascular ED2+ cells changed their appearance shortening their processes and displaying macrophage-like morphologies (Fig. 5 M). In addition, at score 3, as shown by double immunolabelling with laminin, rounded scattered ED2+ cells were detected within the parenchyma, outside the basal lamina of blood vessels, in both grey and white matters (Fig. 5 Q).

During the recovery phase, from score 2R to 0R, the number of ED2+ cells declined progressively (Fig. 5 F–H). ED2+ cells were seen only at

Fig. 4. Morphometric analysis of Iba1+ cells. A) Representative morphologies displayed by Iba1+ cells in control and EAE animals in grey (GM) and white matters (WM). B) Histogram corresponding to shape factor. High values are indicative of round shape, whereas low values indicate ramified morphology. C) Histogram corresponding to elongation factor. Value equal to 1 indicates round morphology and high values correspond to increased elongation. D) Histogram corresponding to the mean diameter. E) Histogram corresponding to the area. (* in grey matter and # in white matter indicates significance, $p \leq 0.05$, in relation to precedent value).

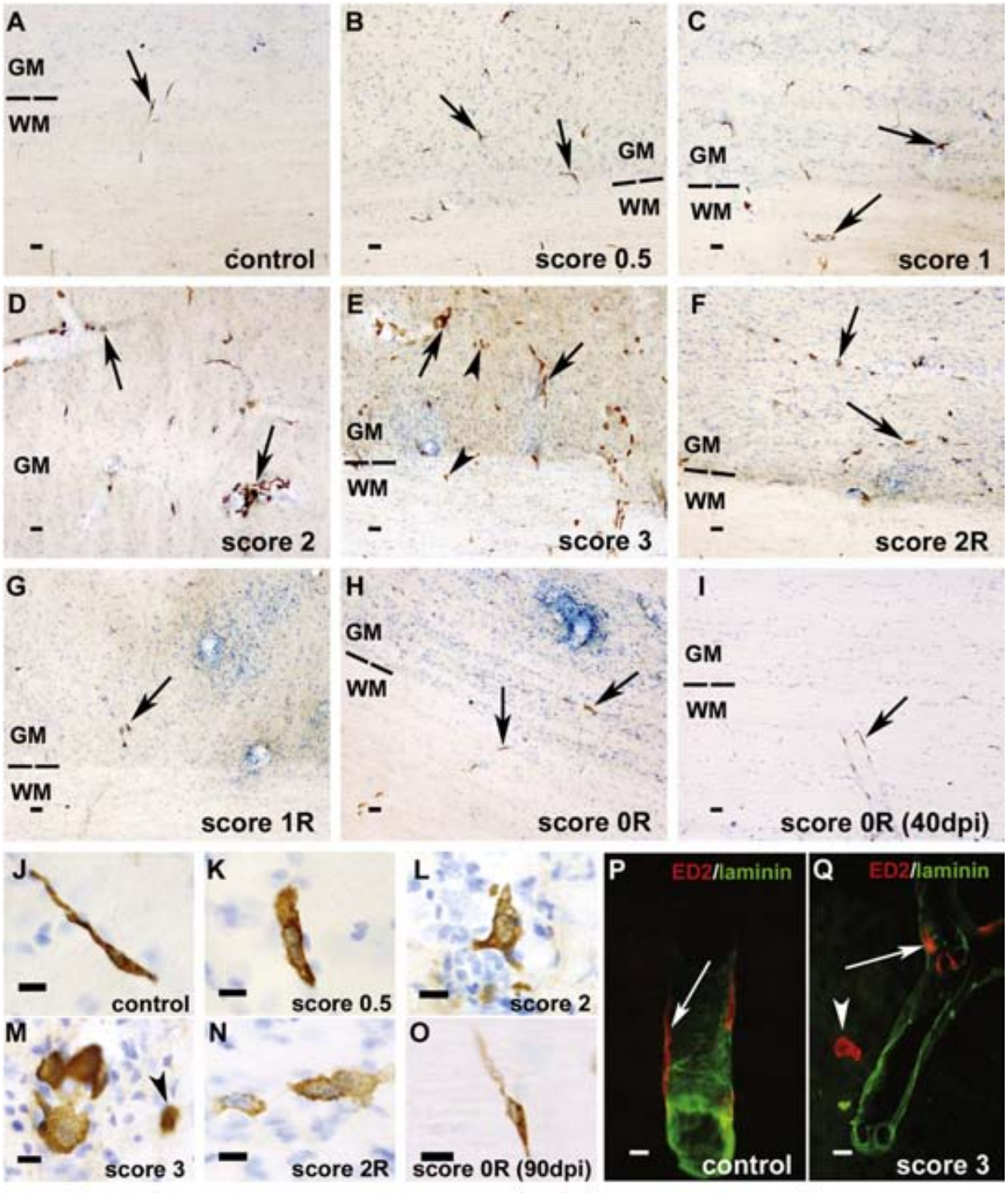

Fig. 5. ED2 immunohistochemistry in control and EAE animals. In comparison to controls (A), EAE animals (B–I) presented changes in distribution and morphology of ED2+ cells. In control animals, ED2+ cells were always located in the perivascular space (arrow in P), following the axis of blood vessels in the grey (GM) and the white matters (WM) (arrow in A) displaying a characteristic spindle-shaped morphology (J). During the inductive phase and peak, perivascular ED2+ cells increased in number (arrows in B–E). From score 0.5, perivascular ED2+ cells showed morphological changes shortening their prolongations (K) and adopting macrophage-like morphology at scores 2 and 3 (L, M). Note the presence of some rounded ED2+ cells in the parenchyma at score 3 (arrowheads in E and M). Double labelling with laminin (Q) allowed the unequivocal visualization of ED2+ cells in the perivascular space (arrow) and in the parenchyma (arrowhead). During the recovery phase from score 2R, the number of ED2+ cells decreased progressively (arrows in F–H) and started to elongate (N) although it was not until score 0R (40 dpi) when the number, distribution and morphology of ED2+ cells approached to those seen in controls (arrow in I, O). Sections were counterstained with toluidine blue. Scale bar (A–I) = 30 μm; (J–Q) = 10 μm.

perivascular position showing macrophage-like morphologies and no round parenquimal ED2+ cells were detected. It was not until 40 dpi when ED2+ cells showed a distribution and morphology similar to those observed in control animals (Fig. 5 I and O).

3.2.5. CD45 immunohistochemistry

The study of sections immunoreacted for CD45 demonstrated, in control animals, the presence of CD45+ cells in both the grey and the white matters displaying ramified microglia-like morphology (Fig. 6 A).

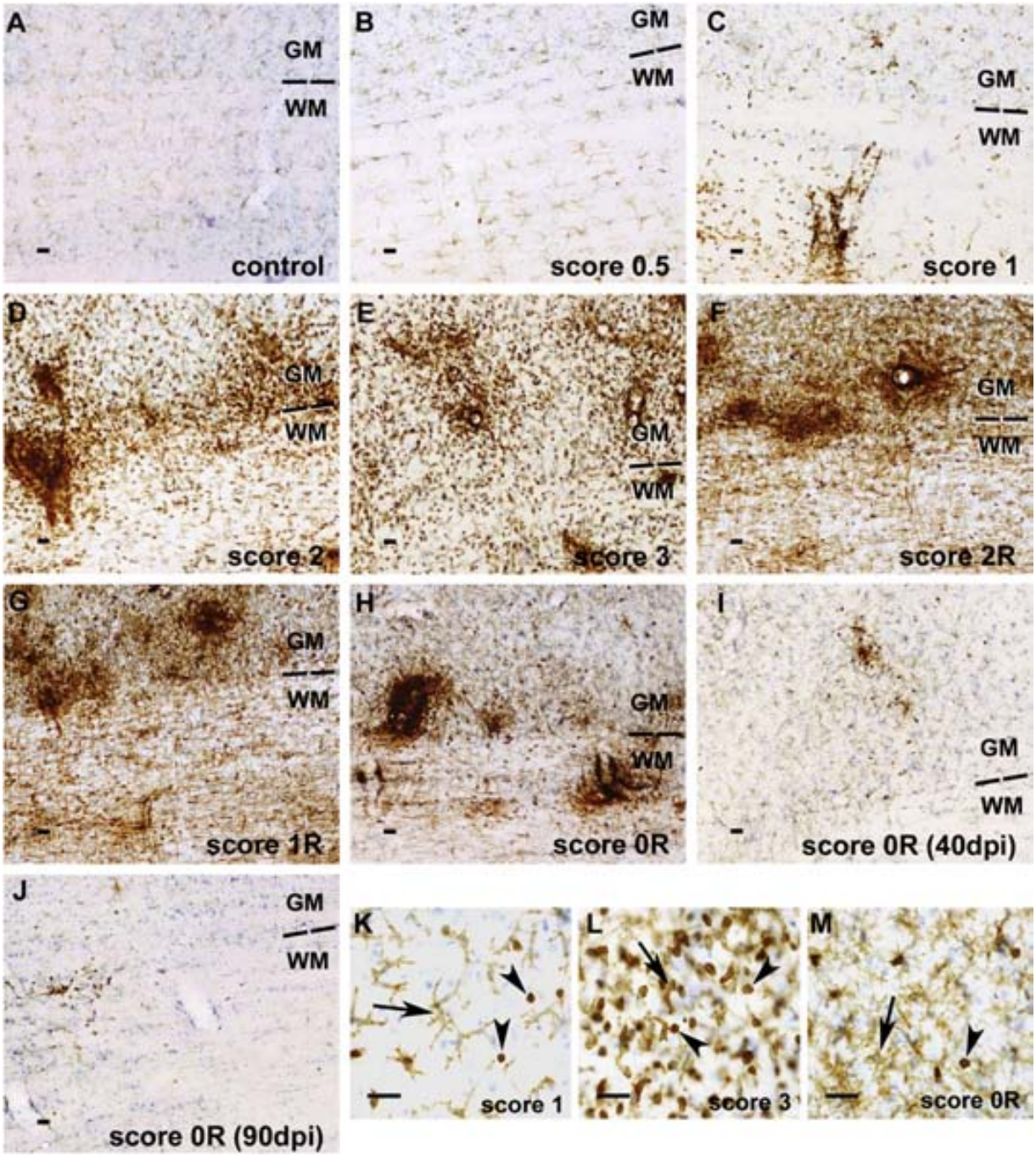

Fig. 6. CD45 immunohistochemistry in control and EAE animals. In control animals (A), ramified microglia-like CD45+ cells were seen distributed in both grey (GM) and white matters (WM). In EAE animals (B–M) strong changes in distribution and morphology of CD45+ cells were found during the evolution of the disease. During the inductive phase (B–D) from score 0.5, but particularly at score 2, an important increase in the number of immunoreactive cells and levels of CD45 expression was observed. These changes persisted during the peak and the recovery phase until score 0R (E–H). Observe the high accumulations of CD45+ cells around blood vessels (C–H). In the post-recovery phase, at score 0R (40 dpi) and score 0R (90 dpi), a remarkable decrease in the number of positive cells and CD45 expression was detected although some CD45+ cell accumulations remain around some blood vessels (I, J). In addition to modifications in number, distribution and level of immunoreactivity, CD45+ cells experimented important changes in morphology. From score 0.5 to 3, ramified microglia-like CD45+ cells experimented a gradual transformation into amoeboid cells (arrows in K and L) and subsequently started to ramify during the recovery phase (arrow in M). In addition, a population of small round CD45+ cells was evident during the inductive, peak, and recovery phases (arrowheads in K–M). All the sections were counterstained with toluidine blue. Scale bar = 30 μm.

In addition some few spindle-shaped CD45+ cells were seen in association with blood vessels. In EAE animals, these cells regulate their CD45 expression and exhibited progressive changes in distribution and morphology that correlated with the presence of clinical symptoms.

During the inductive phase, at score 0.5, a small increase in CD45 expression was observed and, in addition to the presence of positive ramified microglia-like cells, some scattered small round CD45+ cells were found in relation to vasculature in both grey and white matters (Fig. 6 B). At score 1, ramified microglia-like CD45+ cells shortened and widened their processes whereas round CD45+ cells increase in number and started to accumulate around some blood vessels (Fig. 6 C and K). At scores 2 and 3, CD45 immunoreactivity becomes more intense and the number of CD45+ cells increased considerably, and often crowded around blood vessels. These cells had heterogeneous morphologies ranging from round to amoeboid shapes, but no ramified CD45+ cells were seen (Fig. 6 D, E and L).

During the recovery phase, CD45 immunoreactivity remained high and many cells accumulated around blood vessels. From score 2R to 0R, round and amoeboid CD45+ cells progressively diminished in number whereas CD45+ ramified microglia-like cells rapidly increase (Fig. 6 F–H and M).

During the post-recovery phase, when clinical symptoms disappeared, CD45 expression diminished considerably at score 0R (40 dpi) although was higher than those observed in control animals (Fig. 6 I). CD45+ cells mainly displayed ramified microglia-like morphology, but round CD45+ cells remained present frequently in relation to blood vessels. It was not until score 0R (90 dpi) when CD45 immunoreactivity was comparable to control animals, although, in addition to ramified microglia-like shapes, there were still a small number of round CD45+ cells (Fig. 6 J).

3.3. CD4 expression during the evolution of EAE

The dynamics of CD4 expression as well as the nature of cells expressing this antigen during the progression of EAE was determined by flow cytometry and immunohistochemistry using single and double labelling.

3.3.1. Dynamics of CD4 expression

Flow cytometry showed that in control animals, the population of CD11b+ cells was mainly negative for CD4. In EAE animals, during the inductive phase, from score 1, a new and large population of CD4+ cells was evident. The major part of these CD4+ cells corresponded to CD11b− cells (lymphocytes). From the peak, at score 3, and during the recovery phase, in addition to CD11b−/CD4+ cells, a significant

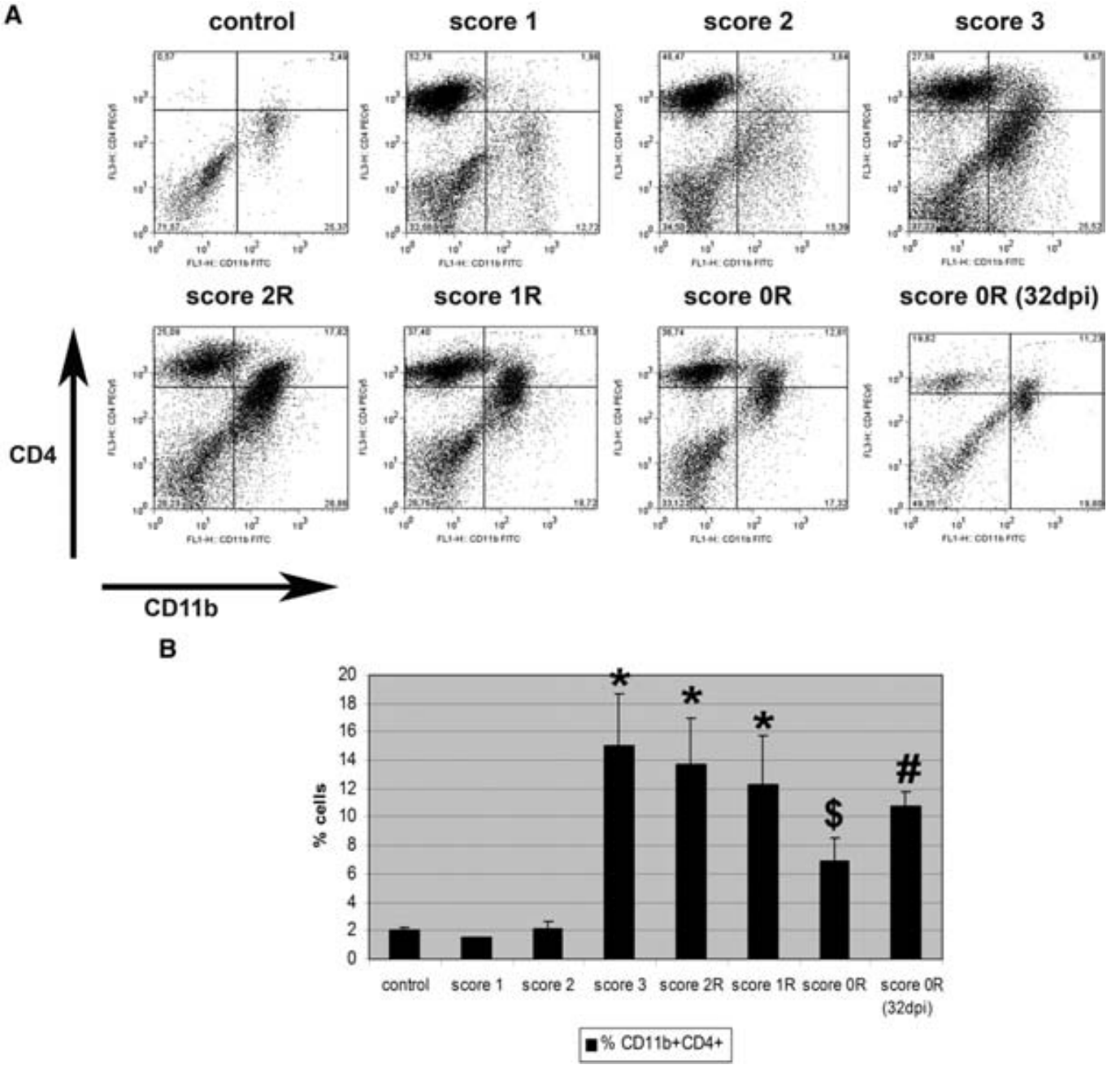

Fig. 7. Flow cytometry analysis of CD11b/CD4 populations during EAE. A) Representative dot-plots corresponding to dynamics of CD4 and CD11b expression during the EAE evolution. Note that whereas no CD4+ cells were found in control animals, in EAE animals, from score 1 and during peak and recovery phase, there were two populations of CD4+ cells corresponding to CD11b− lymphocytes and CD11b+ microglia/macrophages. Those CD4+ populations remained during the post-recovery phase (score 0R, 32 dpi). B) The histogram shows the mean values of CD11b+/CD4+ population ratios during the course of EAE. (ANOVA test $*p \leq 0.01$, $\#p \leq 0.05$, and $\$p \leq 0.2$ relative to control).

subpopulation of CD4+ cells that were CD11b+ (microglia/macrophages) was also observed. From score 1R, CD4+ cells started to decrease, although CD11b−/CD4+ and CD11b+/CD4+ cells were still present in the post-recovery phase (Fig. 7).

After gating for the CD11b+ population and analyzing the CD45 and CD4 expression (Fig. 8), our analysis showed that in control animals the population of $CD45^{low}$ cells (microglia) did not express CD4. In EAE animals, since score 1 in the inductive phase, only a part of the CD11b+/$CD45^{high}$ cell population (macrophages) expressed CD4 whereas CD11b+/$CD45^{low}$ cells (microglia) remained CD4−. The ratio of CD11b+/$CD45^{high}$ cells expressing CD4 peaked at score 3 and diminished progressively during the recovery phase. At score 2R a subpopulation of the CD11b+/$CD45^{intermediate}$ cells (activated microglia) expressed CD4. Remarkably, expression of CD4 remained in those cells at score 1R and in CD11b+/$CD45^{low}$ cells at both score 0R and score 0R (32 dpi).

3.3.2. CD4 immunohistochemistry

In control animals, a small population of CD4+ cells displaying spindle-shaped morphology was found in perivascular location in the grey and white matters (Fig. 9 A). In EAE animals, a large increase in the number of CD4+ cells was detected. In addition to perivascular spindle-shaped cells, other round and ramified CD4+ cells were observed during the evolution of EAE.

During the inductive phase, at score 0.5 when the clinical symptoms started to manifest, only perivascular CD4+ cells were found (Fig. 9 B). However, from score 1, a population of small round CD4+ cells appeared in the vicinity of blood vessels (Fig. 9 C). This population exhibited a gradual increase in number until score 3 and was located not only in the proximity of blood vessels but also disseminated in the parenchyma in both the grey and white matters (Fig. 9 E). During the recovery phase, the number of small round CD4+

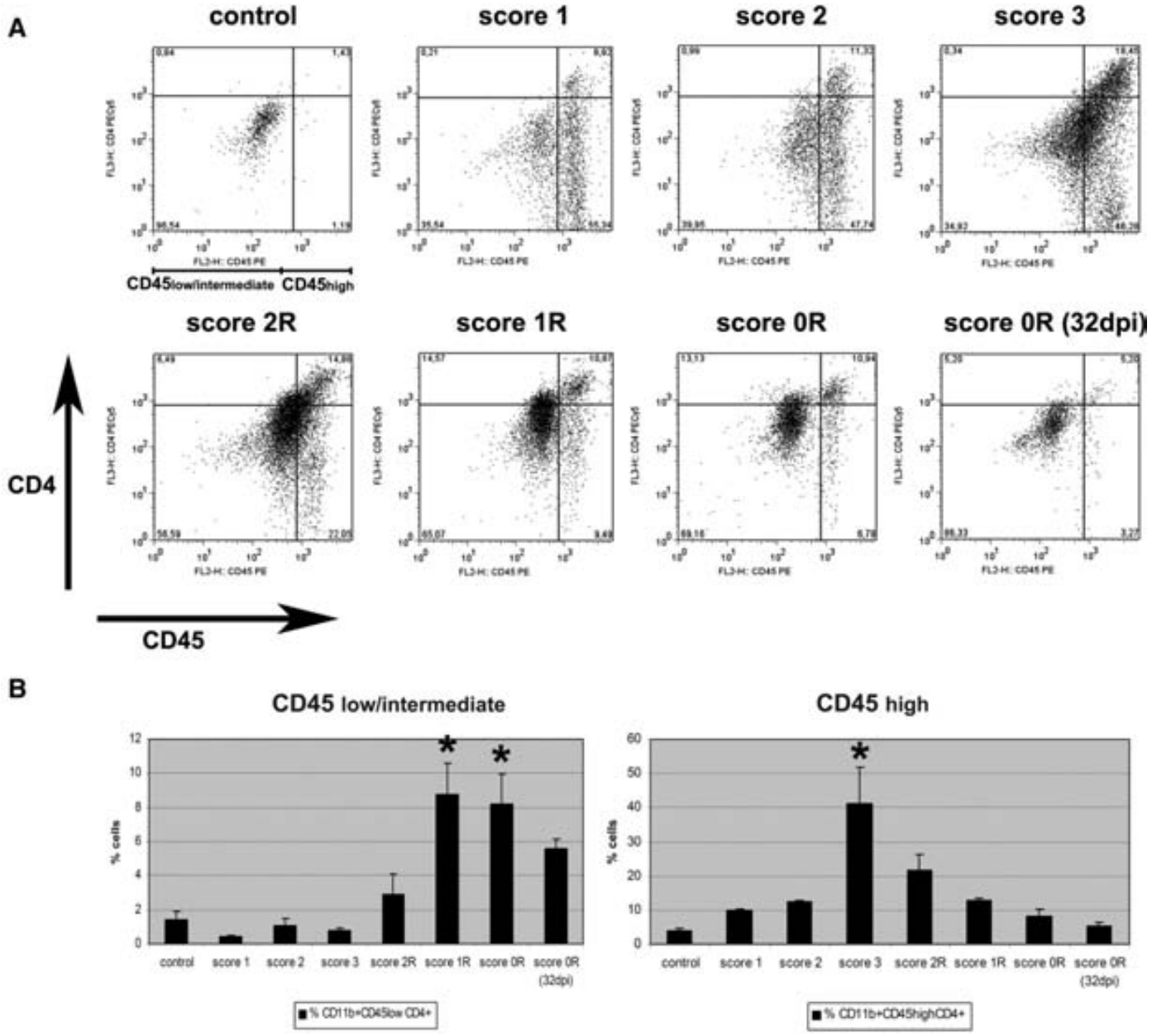

Fig. 8. Flow cytometry analysis of CD45/CD4 populations during EAE. A) Representative dot-plots of the dynamics of CD45 and CD4 expression in the gated CD11b+ cells. Whereas in control animals all CD45+ cells were CD4−, after EAE induction some CD45+ cells corresponding to microglia and macrophages expressed CD4. $CD45^{high}$/CD4+ cells were found from score 1 in the inductive phase, peaked at score 3 and declined in the recovery phase. Moreover, during the recovery phase, at scores 2R and 1R, a subpopulation of $CD45^{intermediate}$ cells expressed CD4. Note that at score 0R and score 0R (32 dpi) there still was an important ratio of $CD45^{low}$/CD4+cells. B) The histograms show on the left the mean values of $CD45^{low/intermediate}$/CD4+ cells and on the right the mean values of $CD45^{high}$/CD4+ cells during the course of EAE. (ANOVA test $*p \leq 0.01$ relative to control).

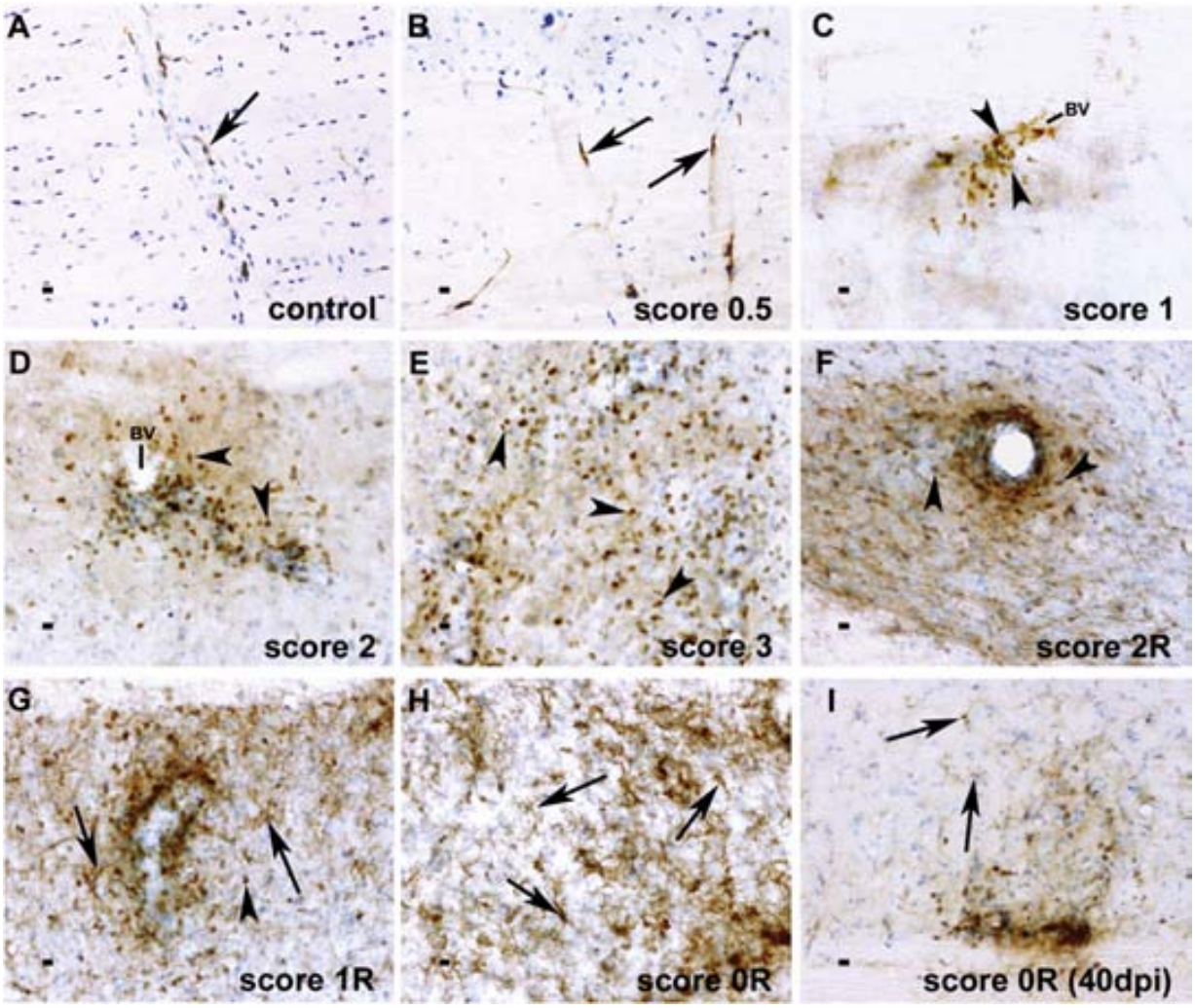

Fig. 9. CD4 immunohistochemistry in control and EAE animals. In control animals (A), CD4+ perivascular cells with spindle-shaped morphology were detected (arrow). In EAE animals, during the inductive phase (B–D), in addition to CD4+ perivascular spindle-shaped cells (arrows in B), from score 1, a population of small round CD4+ cells was observed (arrowheads in C and D) near blood vessels (BV). At score 3, small round CD4+ cells were also seen in the parenchyma (arrowheads in E). Note that during the recovery phase, in addition to small round CD4+ cells (arrowheads in F and G), a population of CD4+ ramified cells was evident (arrows in G and H). At score 0R (40 dpi), the presence of ramified CD4+ cells was still observed (arrows in I). All the sections were counterstained with toluidine blue. Scale bar = 10 μm.

cells declined gradually from score 2R to 0R (Fig. 9 F–H). In addition to these small round CD4+ cells, during the recovery phase, a population of CD4+ cells with ramified morphology became apparent from score 2R. Ramified CD4+ cells increased progressively, mainly in the grey matter, during the recovery phase peaking at score 0R. At score 0R (40 dpi), a significant reduction in the number of CD4+ cells was observed, although some patches with ramified and small round CD4+ cells were still detected (Fig. 9 I).

3.3.3. CD4/CD3 and CD4/Iba1 double-immunohistochemistry

In order to analyze the nature of CD4+ cells, double immunolabelling combining CD4 with either CD3 or Iba1 was carried out (Fig. 10). Our analysis showed that, in control animals, CD4 expression co-localized with spindle-shaped Iba1+ cells located in the blood vessel walls (Fig. 10 B). In EAE animals, the small round CD4+ cells observed during the inductive, the peak and the recovery phases, co-localized with CD3 (Fig. 10 A) but not with Iba1 (Fig. 10 C). In contrast, the population of CD4+ ramified cells, observed during the recovery phase and at score 0R (40 dpi), always co-localized with Iba1 (Fig. 10 D–F). It is important to emphasize that these ramified CD4+/Iba1+ cells represented only a fraction of the Iba1+ population.

4. Discussion

In the last decade, the interest in the study of the "immune function" of microglia and its role in the modulation of the immune response in the CNS has increased substantially (Aloisi, 2001; Raivich and Banati, 2004) although the full role played by microglial cells and molecular mechanisms involved in these processes remain to be established. The lack of good markers to clearly distinguish between perivascular cells, reactive microglia and macrophages has contributed to some confusion in the results obtained with different experimental models and techniques.

In spite of microglial reaction that has been largely studied in different kinds of experimental injuries and diseases (Dheen et al., 2007; Garden and Moller, 2006), studies on microglia reaction in the context of an acquired immune component, as occurs in EAE are restricted to specific time points during the inductive phase or peak of the disease without analysis of the temporal course of microglial activation and deactivation in the recovery phase. Among the different available models of EAE, we have chosen the acute EAE model induced in Lewis rat as it is an inflammatory model in which the animals spontaneously recovered and become tolerant to another immunization (MacPhee and Mason, 1990; Namikawa et al., 1986). These

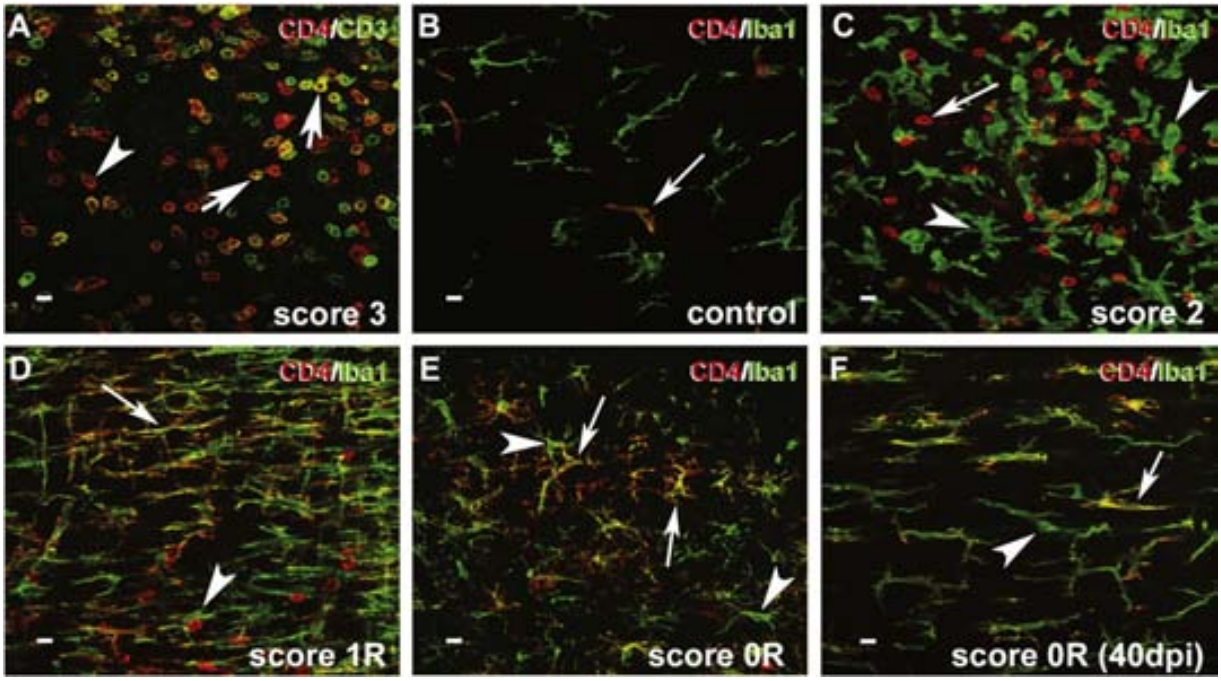

Fig. 10. CD4/CD3 and CD4/Iba1 double-immunohistochemistry. Double labelled CD4+/CD3+ cells at score 3 (arrows in A). Note that CD4+/CD3+ is only a portion of CD3+ cells and that there are some CD4+ cells that are not labelled with CD3 antibody (arrowhead in A). In control animals (B), Iba1+ cells were usually negative for CD4 immunolabelling, with the exception of some spindle-shape cells observed in perivascular location (arrow). In EAE animals (C–F), no CD4+/Iba1+ cells were observed during the inductive phase (C). Note the amoeboid morphology of CD4−/Iba1+ cells (arrowhead in C) and the presence of small round CD4+/Iba1− cells (arrow in C). During the recovery and post-recovery phases, in addition to the small round CD4+/Iba1− cells, a noteworthy population of CD4+/Iba1+ cells displaying ramified morphology was evident (arrows in D–F). Arrowheads in D–F point to CD4−/Iba1+ cells. CD4 in red, CD3 and Iba1 in green, co-localization in yellow. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

features make this model very useful for the study of the contribution of perivascular cells, microglia and macrophages to the inflammatory component (without the interference of the demyelinating component) during the inductive phase and peak of the disease but in addition allowed us to extend our study to the recovery phase where mechanisms associated with this spontaneous resolution took place.

The detailed study we have performed during the different phases of the disease, combining flow cytometry analysis and immunohistochemistry on histological sections has not been performed formerly and has allowed us to study the dynamics of perivascular cell, microglia and macrophage populations and the unequivocal and specific demonstration of CD4 expression in these populations.

CD4 is a molecule commonly associated with different subsets of lymphocytes, particularly T-helper (Reinherz et al., 1979) and some regulatory T cells (Sakaguchi, 2000). In agreement with these observations, in our study a large population of CD3+/CD4+ lymphocytes, displaying small round morphology, appeared during the inductive phase of EAE, but interestingly remained present during all the recovery phase.

In addition, in agreement with the fact that CD4 expression was also described in human monocytes and rat macrophages (Crocker et al., 1987; Jefferies et al., 1985) in this study we also found a population of CD3−/CD4+ cells that does not correspond to lymphocytes and was identified as perivascular, macrophages and microglial cells. Interestingly, CD4 expression in these populations presented significant differences between the inductive, peak and recovery phases: macrophages showed CD4 expression during all the phases but mainly in the inductive phase and peak; perivascular cells displayed CD4 during EAE evolution; and microglial cells only during the recovery phase even at long survival times.

4.1. Macrophages and CD4 expression

Flow cytometry analysis demonstrated the presence of a large population of CD11b+/CD45^{high} cells in EAE animals, that exhibited a rapid increase in number in close relationship with worsening of clinical symptoms and gradually declined during the recovery phase. CD11b+/CD45^{high} cells are usually identified as macrophages although it should be noted that also monocytes, pericytes and dendritic cells were described as CD11b+/CD45^{high} populations (Ford et al., 1995; Sedgwick et al., 1991). Immunohistochemical observations confirmed the presence of round and amoeboid CD45+ cells around blood vessels. Small round CD45+ cells may correspond to infiltrated lymphocytes whereas other rounded or amoeboid cells may correspond to blood borne monocytes, macrophages or reactive microglia. Entry of both lymphocytes and monocytes has been extensively reported in EAE models (Floris et al., 2004; Lafaille, 1998; Swanborg, 2001).

T cell infiltration into the CNS is mediated by different signalling mechanisms including production of chemokines that guide T cell traffic, and adhesion molecules and matrix metalloproteinases (MMPs) that facilitate cell migration through blood vessel walls (Engelhardt and Ransohoff, 2005). Macrophages and lymphocytes produce MMPs in EAE (Toft-Hansen et al., 2004) and expression of adhesion molecules by cells involved in blood brain barrier is regulated by inflammatory cytokines (Engelhardt and Ransohoff, 2005). The fine mechanism whereby monocyte/macrophages modulate the entry of T cells is not completely established, albeit TNFalpha seems to be involved by directing chemokine production in glial cells in EAE (Murphy et al., 2002).

Expression of CD4 in the CD11b+CD45^{high} population was found during the inductive phase of EAE but mainly during the peak of clinical symptoms, gradually diminishing during the recovery phase. Surprisingly, in the histological analysis, we did not observed CD4+/Iba1+ cells in the inductive phase of EAE. A possible explanation may be that CD11b and Iba1 antibodies do not stain the same cell populations (Matsumoto et al., 2007). Although some investigators have observed CD4-mediated signalling in monocytes (Graziani-Bowering et al., 2002), the role played by CD4 expression in monocyte/macrophages remains uncertain and in spite of its well established

role in lymphocytes in antigen presentation mechanisms, probably here may play an antigen presentation independent function. In addition, controversial results are found in the literature in regards to the intracellular components involved in CD4 signalling in these cells. Whereas some studies reported that in monocytes and macrophages CD4 signalling was not associated with Lck, as reported in lymphocytes (Shaw et al., 1990), nor any other kinase (Pelchen-Matthews et al., 1991), recently the presence of the tyrosine kinase Hck has been reported specifically in monocytes (Lynch et al., 2006). Further studies are necessary to reveal the role of CD4 in these cells.

4.2. Perivascular cells and CD4 expression

The so-called perivascular cells are located in the basal lamina of capillaries (Lawson et al., 1990) and were often referred as perivascular macrophages although some authors have considered these cells as a subpopulation of microglia. In comparison to resting microglia, which is a permanent population with a very low turnover (Lawson et al., 1992) expressing low levels of CD45 (Ford et al., 1995) but not detectable levels of ED2 or MHC-II (Aloisi, 2001; Kreutzberg, 1996), perivascular cells are periodically replaced (Bechmann et al., 2001) and express high levels of CD45, MHC-II and ED2 antigen. ED2 expression has been commonly related to different populations of mature macrophages (Polfliet et al., 2006) although in the CNS it was restricted to perivascular macrophages in rats (Dijkstra et al., 1994) and humans (Fabriek et al., 2005). In agreement with these previous reports, our observations in control animals demonstrated the presence of spindle-shaped ED2+ cells only in perivascular locations. However, in the inductive phase of EAE these perivascular ED2+ cells not only increased their number but also changed their appearance shortening their shape and acquiring a rounded macrophage-like phenotype. Subsequently, in close correspondence with the peak of symptomatology, a significant number of ED2+ cells were found in the brain parenchyma, outside the basal lamina of blood vessels, suggesting their transformation into active parenchymal macrophages, although we cannot rule out that those cells are a subpopulation of monocyte-derived macrophages. In fact, this marker has also been described in other cells including rat CNS pericytes in culture (Balabanov et al., 1996) and human blood monocytes and some dendritic cell populations (Maniecki et al., 2006). Due to their strategically important location, perivascular cells are ideal candidates to have a central role in diseases as EAE in which the infiltration of lymphocytes and their restimulation in the parenchyma play a crucial role (Lafaille, 1998). Experimental evidence indicated that selective depletion of perivascular and meningeal macrophages (Polfliet et al., 2001a) may induce gross changes in the evolution of immune response. Thus, after bacterial meningitis, the absence of perivascular macrophages produced a worsening of the disease (Polfliet et al., 2001b), whereas induction of EAE in depleted animals resulted in a decreased symptomatology (Polfliet et al., 2002).

Noticeably, CD4 is expressed constitutively in perivascular cells and remains throughout EAE evolution. As discussed previously for macrophages, the role played by CD4 expression in perivascular cells is still unknown.

4.3. Microglial reaction and CD4 expression

Our observations showed that in EAE animals the first morphological signs of microglial activation coincided with the appearance of the first signs of disability at score 0.5, when microglial cells increased NDPase, Iba1 and CD45 expression. Other studies on EAE have also reported first signs of microglial activation in close relationship to the onset of clinical signs (Gehrmann et al., 1993; Matsumoto et al., 1992), and even before the appearance of clinical symptoms (Brown and Sawchenko, 2007; Ponomarev et al., 2005).

Interestingly, as we have shown in our study, together with first signs of activation, microglial cells align more closely with blood vessels suggesting a putative role of these cells in the events modulating the entry of blood-borne cells. It has been extensively reported that activated microglia may release different molecules such as cytokines, chemokines, etc. that can act as potent chemoattractants for neutrophils, monocytes and lymphocytes (Aloisi, 2001; Babcock et al., 2003; Raivich and Banati, 2004).

Therefore, microglial activation during inductive phase of EAE seems to be associated with potent activation of immune reaction. In this way microglial reaction in those processes where acquired immunity takes place has often perceived as a detrimental factor. Giving support to this hypothesis, it has been reported that when microglial activation is blocked, EAE development was repressed (Guo et al., 2007; Heppner et al., 2005). Furthermore, a recent work (Bhasin et al., 2007) showed that MIF, a microglial inhibitor, produced a substantive reduction in EAE severity in mice when administered seven days after EAE induction, although there were no effects if administered just one day before induction. Conversely, when these authors administered tufsin, a microglial activator, either one day before or 7 days after induction, EAE symptoms were drastically reduced.

Another important point to take into account in our study is the fact that during the inductive phase, microglial cell processes wrapped some neuronal bodies. This finding was extensively reported in the facial nucleus after facial nerve axotomy (Blinzinger and Kreutzberg, 1968; Graeber et al., 1993; Moran and Graeber, 2004) and has been suggested to be a neuroprotective mechanism where activated microglia protect neurons by physically removing synaptic input (synaptic stripping) providing the suitable environment for regeneration (Blinzinger and Kreutzberg, 1968; Graeber et al., 1993). The same phenomenon has been observed in other CNS injury models, such as ischemia (Neumann et al., 2006) and focal cortical inflammation induced by BCG bacteria (Trapp et al., 2007). Although physical association between microglia and neurons has been reported in multiple sclerosis (MS) (Peterson et al., 2001), this observation has so far escaped notice in MS animal models, except for a work reporting microglial ensheathment of motoneurons after passive EAE (MBP-T cell injection) (Gehrmann et al., 1993). To our knowledge, the present work is the first study describing this phenomenon in acute EAE. The meaning of neuronal wrapping by microglia in EAE animals is unknown but if synaptic stripping is taking place, that could be one of the factors contributing to disrupt neuronal circuitry and consequently to promote hindlimb paralysis. On another hand, we cannot rule out that neuronal ensheathment by microglia may represent a mechanism of isolation to prevent the exposition of neurons to inflammatory products released by infiltrated lymphocytes and monocyte-derived-macrophages.

Interestingly, our observations (flow cytometry and immunohistochemical analysis) showed that reactive microglial cells did not display CD4 expression during the inductive phase when ramified cells gradually transformed into amoeboid shaped cells. In contrast, as shown by flow cytometry, a subpopulation of microglial cells displaying activated ($CD45^{intermedium}$) or resting phenotype ($CD45^{low}$) during the recovery and post-recovery phases respectively, was CD4+. In agreement, our immunohistochemical observations corroborated the presence of a subpopulation of CD4+ ramified microglial cells during the recovery and post-recovery phases. The fact that only a part of the microglial population expressed CD4 together with the observation of remaining reactive microglial cells at long survival times, supported the idea that different subpopulations of microglial cells with different phenotypes may co-exist within the CNS exerting different functions as already suggested by other authors (Carson et al., 2007).

Different stimuli have been associated with an increase in CD4 levels in microglia in vitro including ciliary neurotrophic factor (Hagg et al., 1993) and some cytokines such as IL-4 and IL-6 (Wang et al., 2002). It should be emphasized that in different EAE models, including the model used in this study, IL-4 has been reported to increase,

peaking during the recovery phase (McCombe et al., 1998). In addition to engagement with MHC-II, it is known that CD4 acts as a receptor for IL-16 (Center et al., 2000). IL-16 is an immunomodulatory cytokine produced by a variety of cells including microglia, which is involved in CD4+ cell recruitment (Cruikshank et al., 1998; Cruikshank et al., 2000). In the acute EAE MBP-induced in Lewis rat, a subpopulation of microglial cells has been reported to express IL-16 during the recovery phase (Guo et al., 2004). Altogether these observations indicate that microglial cells during the recovery phase of EAE may attract and or activate specific populations of lymphocytes, which by means of release of anti-inflammatory signals may contribute to revert the immune response. Microglial cells can interact with regulatory T cells (Tregs) (Kipnis et al., 2004), which are a subtype of CD4+ lymphocytes involved in the resolution of EAE (Kohm et al., 2002; McGeachy et al., 2005). Once activated, Tregs secrete the anti-inflammatory cytokines TGF-β and IL-10 (Zhang et al., 2004) which may play a role in the downregulation of the inflammatory/immune response.

5. Conclusions

In conclusion our study demonstrated that in the EAE model induced by MBP injection in the Lewis rat, perivascular cells, microglia and macrophages showed different dynamics during the course of the disease in close relationship with worsening and amelioration of clinical symptoms. The specific pattern of CD4 expression in these populations during the course of EAE suggests a specific role of this molecule in the modulation of inflammatory/immune response. Concretely, the temporal expression of CD4 in some microglial cells during the recovery and post-recovery phases strongly indicates that microglia may have a crucial and active participation in the resolution of the immune response during EAE and that CD4 may be one of the molecules involved in this process. Further studies are however necessary to clarify the exact function played by CD4 in microglia in the context of EAE.

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Activated microglial cells acquire an immature dendritic cell phenotype and may terminate the immune response in an acute model of EAE

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ABSTRACT

Antigen presentation, a key mechanism in immune responses, involves two main signals: the first is provided by the engagement of a major histocompatibility complex (MHC), class I or class II, with their TCR receptor in lymphocytes, whereas the second demands the participation of different co-stimulatory molecules, such as CD28, CTLA-4 and their receptors B7.1 and B7.2. Specific T-cell activation and deactivation are achieved through this signalling. The aim of our study is to characterise, in the acute experimental autoimmune encephalomyelitis (EAE) model in Lewis rat, the temporal expression pattern of these molecules as well as the cells responsible for their expression. To accomplish that, MBP-immunised female Lewis rats were daily examined for the presence of clinical symptoms and sacrificed, according to their clinical score, at different phases during EAE. Spinal cords were cut with a cryostat and processed for immunohistochemistry: MHC-class I and MHC-class II, co-stimulatory molecules (B7.1, B7.2, CD28, CTLA-4) and markers of dendritic cells (CD1 for immature cells and fascin for mature cells). Our results show that microglial cells are activated in the inductive phase and, during this phase and peak, they are able to express MHC-class I, MHC-class II and CD1, but not B7.1 and B7.2. This microglial phenotype may induce the apoptosis or anergy of infiltrated CD28+ lymphocytes observed around blood vessels and in the parenchyma. During the recovery phase, microglial cells express high MHC-class I and class II and, those located in the surroundings of blood vessels, displayed the B7.2 co-stimulatory molecule. These cells are competent to interact with CTLA-4+ cells, which indicate an active role of microglial cells in modulating the ending of the immune response by inducing lymphocyte activity inhibition and Treg activation. Once clinical symptomatology disappeared, some foci of activated microglial cells (MHC-class II+/B7.2+) were still present in concomitance with CTLA-4+ cells, suggesting a prolonged involvement of microglia in lymphocyte inhibition and tolerance promotion. In addition to microglia, during the inductive and recovery phases, we also found perivascular ED2+ cells and fascin+ cells which are able to migrate to the parenchyma and may play a role in lymphocytic regulation. Further studies to understand the specific function played by these cells are warranted.

1. Introduction

Antigen presentation is a crucial process in T-cell activation and modulation of immune responses. Two main signals are involved in this process. The first, provided by the engagement of either the MHC-class I or MHC-class II on antigen-presenting cells (APCs) with the T-cell receptor (TCR) on T lymphocytes, controls the specificity of the immune response, as MHC-class I is recognised by CD8+ T cells whereas MHC-class II interacts with CD4+ T cells (Janeway, 1992). The second signal, the co-stimulatory signal, is antigen non-specific, involves the interaction of different T-cell surface receptors with their respective ligands on APCs (Lanzavecchia, 1997; Lenschow et al., 1996) and is essential for the full T-cell activation, as TCR-MHC binding in the absence of co-stimulation can lead to T-cell apoptosis or anergy (Kishimoto and Sprent, 1999). Different combinations of co-stimulatory molecules and receptors providing stimulatory or inhibitory signals have been described (Nurieva et al., 2009), however the signal provided by the B7 molecules, B7.1 and B7.2 on APCs with their receptors CD28 and CTLA-4 in lymphocytes appear to be the predominant molecular interactions for T-cell activation (Salomon and Bluestone, 2001; Sharpe and Freeman, 2002). Binding of B7.1 or B7.2 with CD28 provides a potent stimulatory signal in T cells, whereas binding of the related but higher-affinity CTLA-4 receptor, delivers an inhibitory signal (Karandikar et al., 1996; Sansom, 2000).

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Therefore, the delicate balance established between these positive and negative signals may provide different outcomes of the immune response.

Evidence for the involvement of antigen presentation in experimental autoimmune encephalomyelitis (EAE) comes from the observations that microglial cells express MHC-class II in different EAE models in both rats (Craggs and Webster, 1985; Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; McCombe et al., 1994; McCombe et al., 1992) and mice (Juedes and Ruddle, 2001; Lindsey and Steinman, 1993; Ponomarev et al., 2005; Pope et al., 1998), and from studies that reported the expression of co-stimulatory molecules and the beneficial or detrimental effects derived from the lack of these molecules in EAE (Chang et al., 1999; Chang et al., 2003; Girvin et al., 2000; Hurwitz et al., 1997; Karandikar et al., 1998a; Miller et al., 1995; Perrin et al., 1999). It should be emphasised, however, that all the aforementioned studies have been focused only on the peak of the disease and did not address the co-expression of MHCs and B7.1/B7.2-CD28/CTLA-4 molecules in the same context. Thus, it remains to be determined, first, if MHCs expression matches with the presence of these co-stimulatory molecules and if this is the case, whether different combinations of B7.1/B7.2-CD28/CTLA-4 molecules differ during the different phases of EAE and may be playing a role in the initiation and resolution of the immune response. A second question that remains is which are the cells driving the antigen-presenting mechanism within the CNS in this acute EAE model, as in spite of the task of microglia as APCs, some recent studies have reported that a specific population of dendritic cells (DCs), the main APCs in the periphery, infiltrate the CNS in EAE rats (Matyszak and Perry, 1996; Serafini et al., 2000) and MS (Serafini et al., 2006) and play a central role in antigen presentation in murine EAE models (Bailey et al., 2007; Greter et al., 2005), opening the possibility that, in addition to microglia, DCs can be involved in the mechanism of antigen presentation also in this acute model.

Among the different EAE models in rodents, the acute EAE model induced in Lewis rat is of special interest, as it is characterised by a single peak of paralysis after which animals recover spontaneously (Swanborg, 2001; Tsunoda and Fujinami, 1996). Taking advantage of this model, which gave us the opportunity to investigate the mechanisms involved in the induction, peak and resolution of the inflammatory–immune response, the present study provides a detailed description of the temporal expression pattern of MHC/co-stimulatory molecules, and the phenotype of cells responsible for their expression.

2. Materials and methods

2.1. EAE induction

A total of 77 female Lewis rats (180–200g) susceptible to develop experimental autoimmune encephalomyelitis (EAE) were used in this study. Animals were purchased from Charles River (IFFA Credo; Belgique) and maintained with food and water *ad libitum* in a 12 h light/dark cycle.

Rats were induced to develop EAE by a subcutaneous injection, in each hindfoot, of an emulsion containing 100 µg of MBP (M2295; Sigma; St Louis, USA), Complete Freud's Adjuvant (CFA) (Ref. 0638; Difco; USA) and 0.2 mg of *Mycobacterium tuberculosis* H37 Ra (Ref. 3114; Difco; USA) in saline solution.

Animals were evaluated daily for the presence of clinical symptoms, using the following clinical score test: 0, absence of symptoms; 0.5, partial loss of tail tonus; 1, tail paralysis; 2, paraparesis of hindlimb; 3, paraplegia; 4, tetraparesis; 5, tetraplegia and 6, death.

All experimental animal work was conducted according to Spanish regulations in agreement with European Union directives and was approved by the Ethics Committee of the Autonomous University of Barcelona.

2.2. Experimental groups

EAE-induced animals ($n=68$) were analysed, at the different phases along the EAE course, according to their clinical score, that is to say, inductive, peak, recovery and post-recovery phases (See Fig. 1) following the method previously described by Almolda et al. (2009). It is important to emphasise that those animals that did not reach the score of 3 were excluded from the study.

In addition to EAE-induced animals, a group of rats ($n=9$) was injected with vehicle solution and used as control.

2.3. Tissue processing for histological analysis

For immunohistochemistry, anaesthetised animals were perfused intracardially for 20 min with 4% paraformaldehyde in 0.1M PBS (pH

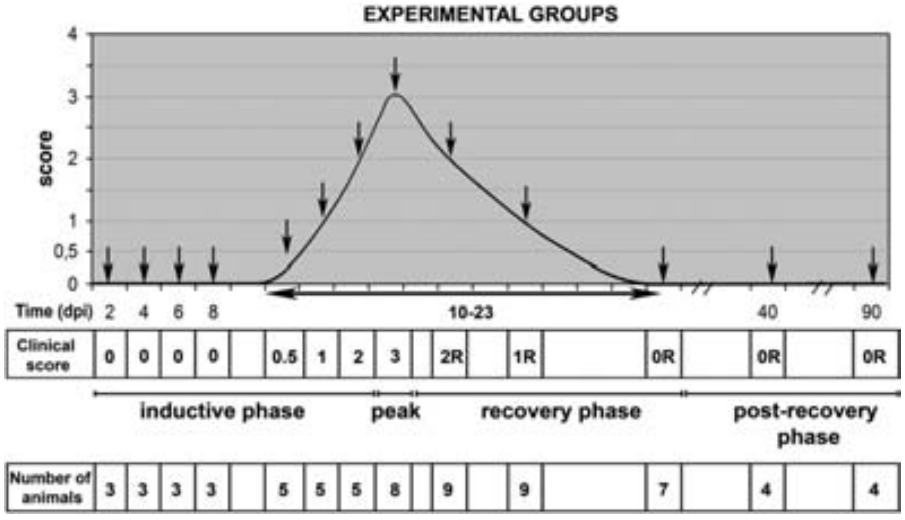


Fig. 1. Experimental groups. Schematic representation of clinical score evolution in the acute Lewis rat EAE model. In this work we do not sacrifice animals taking into account the post-immunisation time, but animals were analysed at different points according to the clinical score (represented by arrows). In order to avoid confusion, different nomenclature was used in the different experimental groups. Thus, score 0, score 0.5, score 1 and score 2 were used for animals sacrificed during the inductive phase whereas score 1R, score 2R and score 0R were used for those sacrificed during the recovery and post-recovery phase. The number of EAE animals used in each score is indicated in the table. In addition to those animals, a total of nine rats injected with vehicle were used as controls.

7.4) + 5% sucrose. Spinal cords (cervical and dorsal parts) were removed and post-fixed in the same fixative for 4 h at 4 °C. Samples were cut using a Leica VT 1000S vibratome and parallel longitudinal 40-μm-thick sections were obtained and stored at −20 °C in Olmos antifreeze solution until used.

2.4. Single immunohistochemistry

Parallel free-floating vibratome sections were processed for MHC-I, MHC-II, the co-stimulatory molecules B7.1, B7.2, CD28 and CTLA-4 and two DC-specific markers: CD1 (immature DCs; (Serafini et al., 2006)) and fascin (mature DCs; (Al-Alwan et al., 2001)). After blocking the endogenous peroxidase (2% H_2O_2 in 70% methanol for 10 min), sections were incubated for 1 h in Blocking Buffer solution (BB) containing 0.05 M Tris-buffered saline (TBS) pH 7.4 + 1% Triton X-100, 10% fetal calf serum and 3% bovine serum albumin. Sections were then incubated overnight at 4 °C with one of the following antibodies: 1) mouse anti-rat MHC-class I (RT1A; 1:1000; MCA51G; AbD Serotec), 2) mouse anti-rat MHC-class II (RT1B; 1:1000; MCA46G; AbD Serotec), 3) mouse anti-rat CD86 (B7.2; 1:1000; 555016; BD Pharmingen), 4) mouse anti-rat CD152 (CTLA-4; 1:1000; MCA2092; AbD Serotec) or 48h at 4 °C with 5) mouse anti-rat CD28 (1:300; MCA1331; AbD Serotec) and 6) rabbit anti-mouse CD1 (1:250; M276; Santa Cruz Biotechnology) diluted in BB. In parallel, some sections were incubated in media lacking the primary antibody and used as negative controls; sections from the spleen were used as a positive control. Subsequently, after washes with TBS+ 1% Triton, sections were incubated at room temperature for 1 h with biotinylated anti-mouse secondary antibody (1:500; BA-2001; Vector Laboratories, Inc; Burlingame, CA) or biotinylated anti-rabbit secondary antibody (1:500; BA-1000; Vector Laboratories, Inc; Burlingame, CA) in the case of CD1, rinsed in TBS+ 1% Triton and incubated for 1 h with streptavidin–peroxidase (1:500; SA-5004; Vector Laboratories, Inc; Burlingame, CA). The peroxidase reaction was visualized by incubating the sections in 3,3′-diaminobenzidine and hydrogen peroxide using the DAB kit (SK-4100; Vector Laboratories, Inc; Burlingame, CA). Finally, sections were mounted, counterstained with toluidine blue, dehydrated in alcohol and, after xylene treatment, coverslipped in DPX. Sections were analysed and photographed with a DXM 1200F Nikon digital camera joined to a Nikon Eclipse 80i microscope.

2.5. Double immunohistochemistry

Double-immunolabelling was carried out by firstly processing the sections with MHC-I, MHC-II, B7.2, CD28, CTLA-4, Iba1, CD1 or fascin, mouse anti-human fascin (1:1000; M3567; Dakopatts; Denmark) immunolabelling as described above, but using AlexaFluor® 555-conjugated anti-mouse (1:1000, A31570; Molecular Probes) or AlexaFluor® 488-conjugated anti-rabbit (1:1000; A21206; Molecular Probes) in the case of Iba1 and CD1 as secondary antibody. After several washes, these same sections were incubated overnight at 4 °C with either rabbit anti-Iba1 (1:3000; 019-19741; Wako), rabbit anti-bovine GFAP (1:1800; Z0334; Dakopatts; Denmark), rabbit anti-human CD3 (1:300; A0452; Dakopatts; Denmark), rabbit anti-mouse laminin (1:5000; L-9393; Sigma; St. Louis, USA), mouse anti-rat CD4 (1:1000; MCA55G; AbD Serotec), mouse anti-rat ED2 (1:1000; MCA342R; AbD Serotec) or tomato lectin (TL) (1:150; L-0651; Sigma Aldrich) followed by AlexaFluor® 488-conjugated anti-rabbit (1:1000; A21206; Molecular Probes), AlexaFluor® 555-conjugated anti-mouse (1:1000, A31570; Molecular Probes) or Streptavidin-Cy2 (1:1000; PA-42001; Amersham) in the case of TL. Finally, sections were mounted on slides, dehydrated in graded alcohol and coverslipped in DPX. Sections were analysed using a Nikon DXM 1200 digital camera join to a Nikon Eclipse E600 microscope of fluorescence and a Leica DMIRE 2 confocal microscope.

3. Results

MBP immunisation in Lewis rat produces an acute monophasic disease characterised by a progressive clinical motor impairment, starting around 9–11 days post-immunisation (dpi) reaching complete hindlimb paralysis (12–14 dpi) after which progressive–spontaneous recovery takes place (15–23 dpi). After 23 dpi, clinical symptoms were absent. In this work, in contrast to the major part of studies where experimental groups are determined on the basis of the days post-immunisation, the animals were analysed at the different clinical scores along the EAE course, as already reported (Almolda et al., 2009). Therefore, as outlined in Fig. 1, four different phases have been identified: a) the inductive phase, which included the score 0.5 (slight loss of tail tonus), score 1 (tail paralysis) and score 2 (hindlimb paraparesis); b) the peak or score 3, in which animals showed complete hindlimb paralysis; c) the recovery phase, which included score 2R (hindlimb paraparesis), score 1R (tail paralysis) and score 0R (absence of clinical symptoms); d) the post-recovery phase, which included animals with score 0R at long-time survival times (0R-40dpi and 0R-90dpi).

Our results demonstrate that no significant differences are observed between EAE animals with score 0 at the inductive phase and controls. Expression of MHC-class I and MHC-class II are not only found during the inductive phase (since signs started to be apparent) and the peak but also remained during the recovery and post-recovery phases. In addition, co-stimulatory molecules are expressed following specific expression patterns along EAE evolution. The main cell responsible for antigen presentation was microglia, which expressed immature DC markers. A transient population of mature dendritic cells, different from microglia, was found during the inductive, peak and recovery phases. It is important to mention that no variability was observed between animals sacrificed in the same clinical score in any molecule analysed.

3.1. MHC-class I and MHC-class II expression along the evolution of EAE

The dynamics as well as the nature of cells responsible for MHC-class I and MHC-class II expression were analysed in the different phases of EAE evolution by the use of single and double immunohistochemistry. Our observations in EAE animals demonstrate progressive changes in the expression, distribution, number and morphology of MHC-class I+ and MHC-class II+ cells along the evolution of the disease.

3.1.1. Dynamics of MHC-class I expression

In control animals, the study of sections immunostained for MHC-class I revealed the presence of low MHC-class I expression restricted to cells displaying ramified microglial-like morphology (Fig. 2A, J). In EAE animals, during the inductive phase, at score 0.5, a slight increase in MHC-class I immunolabelling was observed in ramified microglial-like cells (Fig. 2B). A few little-round MHC-class I+ cells were occasionally observed around some blood vessels (Fig. 2K). At score 1, MHC-class I+ cells showed a coarser cell body and shorter ramifications; some of them were accumulated in close relationship to blood vessels (Fig. 2C). In addition to those ramified cells, little-round MHC-class I+ cells were also detected around blood vessels. MHC-class I immunolabelling became more intense at scores 2 and 3, and perivascular accumulations of MHC-class I+ cells increased considerably in both the white and the grey matter (Fig. 2D, E). At score 2, MHC-class I+ cells were constituted mainly by little-round and ramified morphologies, although some amoeboid cells (elongated cells lacking or with few wide processes) were occasionally found (Fig. 2L). At score 3, the major part of the cells presented little-round or amoeboid shapes and no ramified cells were observed (Fig. 2M). Additionally, a population of MHC-class I+ cells displaying big-round morphology and mitotic nuclei was observed around blood vessels and in the parenchyma at both scores, but more often at score 3.

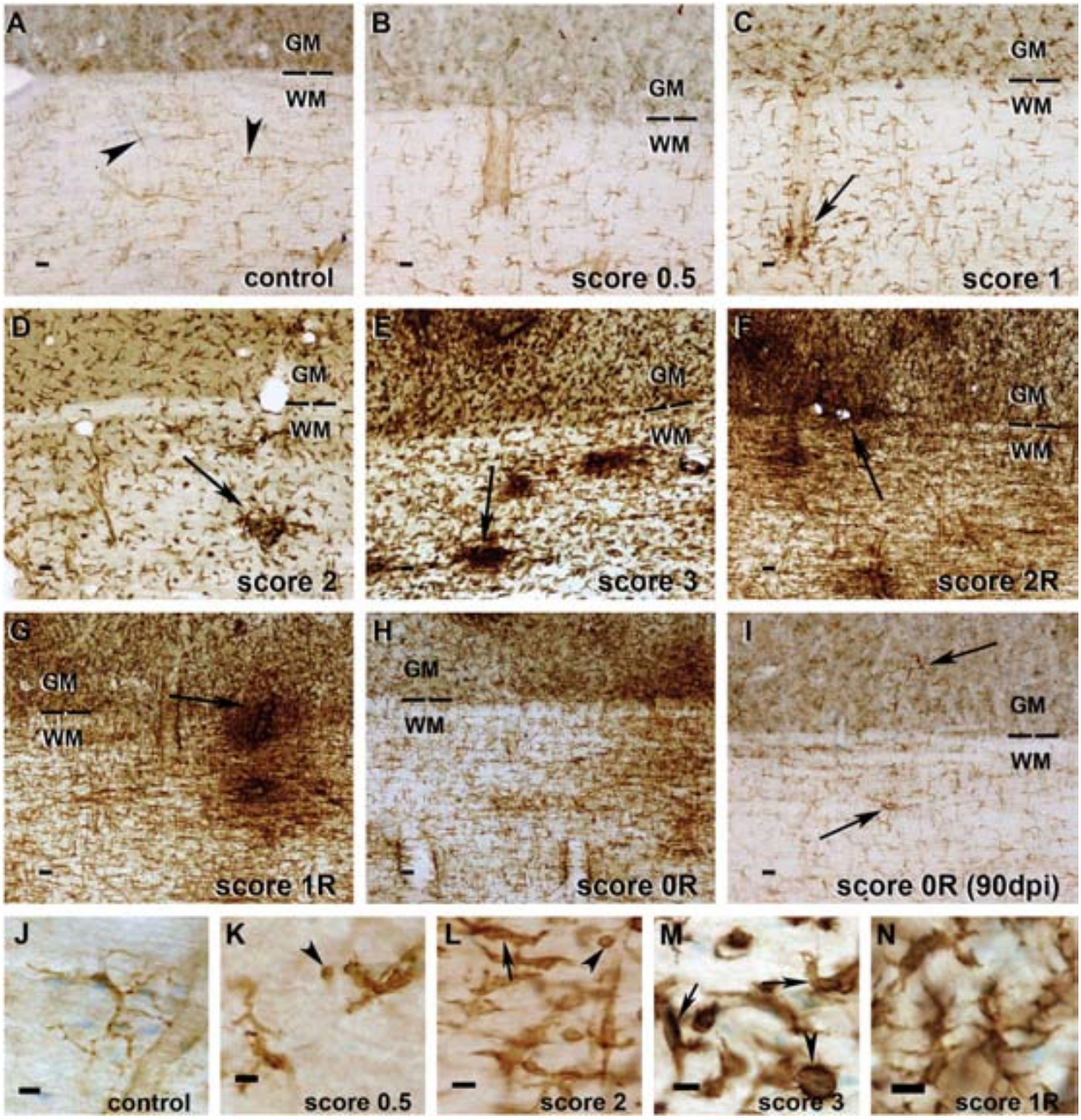
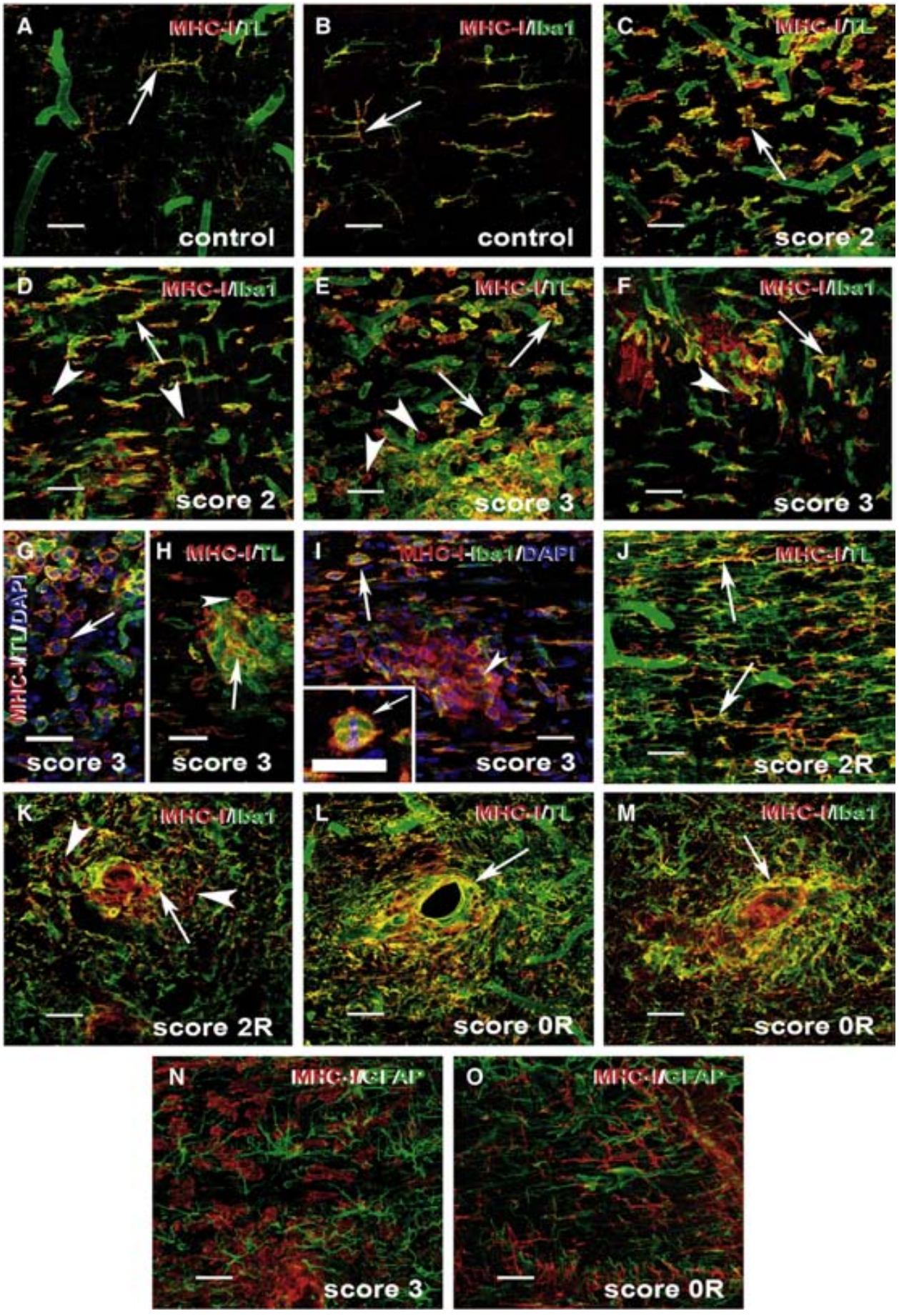


Fig. 2. Temporal expression pattern of MHC-class I. (A and J) MHC-class I+ cells in the parenchyma of control animals in both white (WM) and grey matter (GM) of the spinal cord; arrowheads point characteristic ramified microglial-like cells observed in these animals. (B–I) Representative pictures of different phases along EAE induction are shown; (B–D) inductive phase, (E) peak, (F–H) recovery phase and (I) post-recovery phase. Arrows indicate accumulation of MHC-class I+ cells around blood vessels. (J–N) High-magnification images in controls (J) and in EAE animals (K–N) showing the different morphologies of MHC-class I+ cells; ramified microglial-like (J) in controls and little-round (arrowheads in K and L), amoeboid (arrows in L and M) big round (arrowheads in M) and ramified (N) along different phases of EAE. Bar scale A–I = 30 μm; J–N = 10 μm.

During the recovery phase, MHC-class I expression remained high and repeated accumulations of MHC-class I+ cells were observed around blood vessels (Fig. 2F–H). From score 2R to score 0R, the major part of MHC-class I+ cells displayed ramified morphologies (Fig. 2N), whereas the proportion of little-round and big-round positive cells progressively decreased. No amoeboid MHC-class I+ cells were found.

During the post-recovery phase, at both score 0R-40dpi and score 0R-90dpi, a considerable reduction in the immunolabelling and the number of MHC-class I+ cells was observed, but still remained higher

Fig. 3. Identification of MHC-class I+ cells. Double-immunolabelling combining MHC-class I with TL (A, C, E, G, H, J and L), Iba1 (B, D, F, I, K and M) and GFAP (N and O) in both controls (A, B) and EAE animals during the inductive (C, D), peak (E–I and N) and recovery phases (J–M and O). Double MHC-class I+/TL+ cells (arrows in A, C, E, G, H, J and L) and MHC-class I+/Iba1+ (arrows in B, D, F, I, K and M) were observed. Arrowheads in D, E and F point to MHC-class I+/TL− (E) and MHC-class I+/Iba1− cells (D and F). Big-round MHC-class I+ cells showing co-localisation with Iba1 (arrowhead in I) but not with TL (arrowhead in H) are shown. Arrows in G and I indicate MHC-class I+/TL+/Iba1+ cells with mitotic nuclei. Inset image in I corresponds to a high-magnification image of one MHC-class I+/Iba1+ cells presenting nuclei in metaphase. No double-labelling with GFAP was observed (N and O). Bar scale = 30 μm.


A
MHC-I/TL
control
B
MHC-I/Iba1
control
C
MHC-I/TL
score 2
D
MHC-I/Iba1
score 2
E
MHC-I/TL
score 3
F
MHC-I/Iba1
score 3
G
MHC-I/TL/DAPI
score 3
H
MHC-I/TL
score 3
I
MHC-I/Iba1/DAPI
score 3
J
MHC-I/TL
score 2R
K
MHC-I/Iba1
score 2R
L
MHC-I/TL
score 0R
M
MHC-I/Iba1
score 0R
N
MHC-I/GFAP
score 3
O
MHC-I/GFAP
score 0R

than in control animals (Fig. 2I). Only ramified microglial-like morphologies were seen during this post-recovery phase.

3.1.2. Nature of MHC-class I+ cells

Double-immunolabelled sections for MHC-class I with TL, Iba1 or GFAP demonstrated that ramified MHC-class I+ cells found in control animals (Fig. 3A and B) co-localised with TL and Iba1, but not with GFAP.

Similarly, in EAE animals, all ramified and amoeboid MHC-class I+ cells observed during the inductive phase, the peak, the recovery and the post-recovery phase co-localised with TL and Iba1 (Fig. 3C–M). Noticeably, some little-round MHC-class I+ cells observed along the different phases of EAE did not co-localise with TL or Iba1 (Fig. 3, arrowheads in D, E, F and K). Moreover, whereas all of the big-round MHC-class I+ cells with mitotic nuclei located in the parenchyma always co-localised with both TL and Iba1 (Fig. 3G, I), those cells found around blood vessels only showed co-localisation with Iba1 but never with TL (Fig. 3, arrowhead in H). In not any case, we have observed co-localisation of MHC-class I with GFAP (Fig. 3N, O).

3.1.3. Dynamics of MHC-class II expression

In control animals, analysis of immunolabelled sections for MHC-class II revealed the presence of a scarce number of immunoreactive cells, located mainly around blood vessels (Figs. 4A and 5A and B), which displayed poorly ramified morphology (Fig. 4K).

In EAE animals, during the inductive phase, at score 0.5, a progressive increase in the number of MHC-class II+ cells was

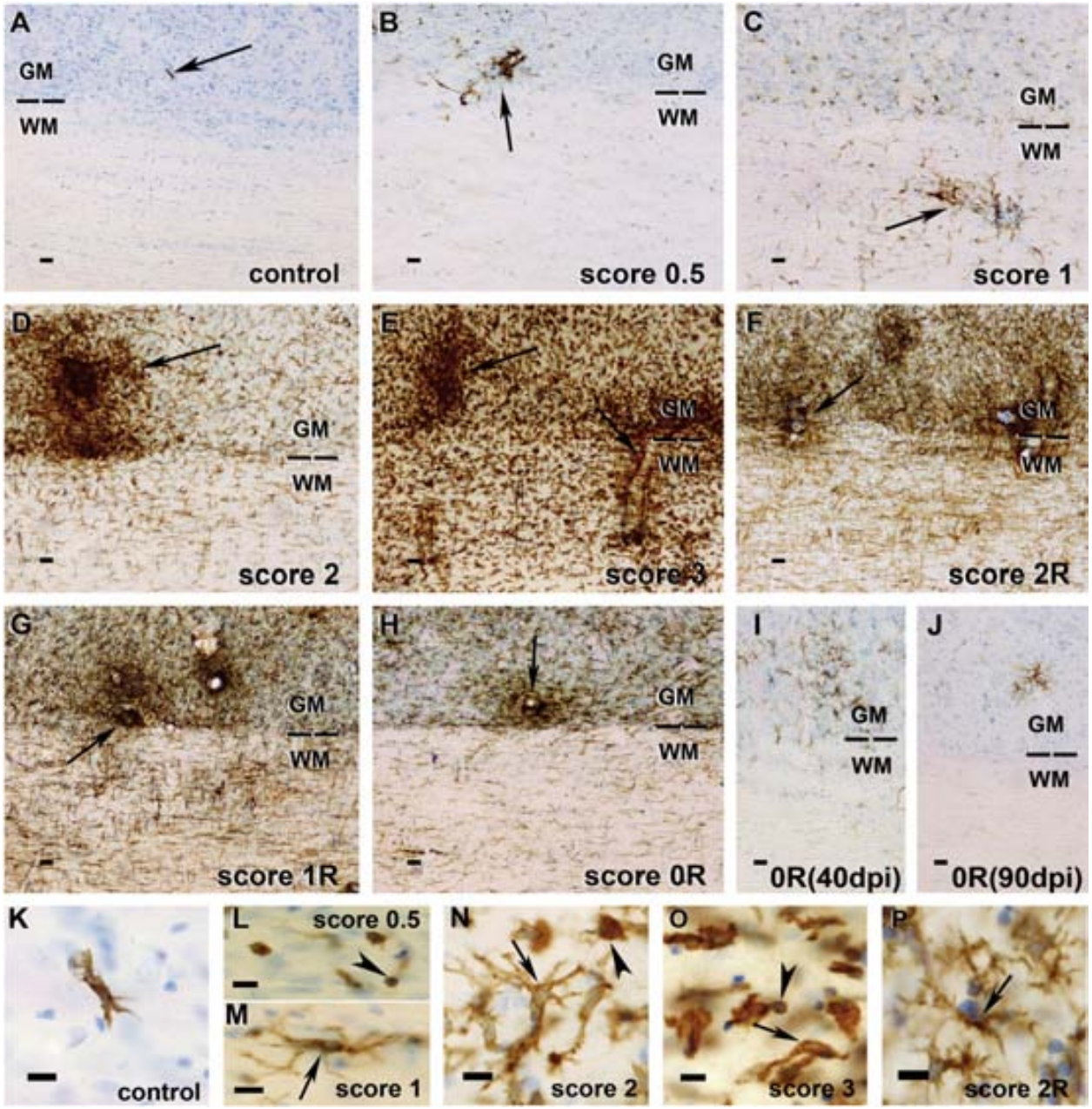


Fig. 4. Temporal expression pattern of MHC-class II. MHC-class II+ cells in controls (A) and in EAE animals (B–J) in both the grey (GM) and the white matter (WM). Different phases along EAE evolution are shown: inductive (B–D), peak (E), recovery (F–H) and post-recovery (I and J). Perivascular accumulations of MHC-class II+ cells are indicated by arrows in B–H. High-magnification micrographs show MHC-class II+ cells with poorly ramified morphology in controls (K) and little-round (arrowheads in L and O), ramified (arrows in M, N and P), amoeboid (arrow in O) and big-round (arrowhead in N) in EAE animals. Bar scale A–J = 30 μm; K–P = 10 μm.

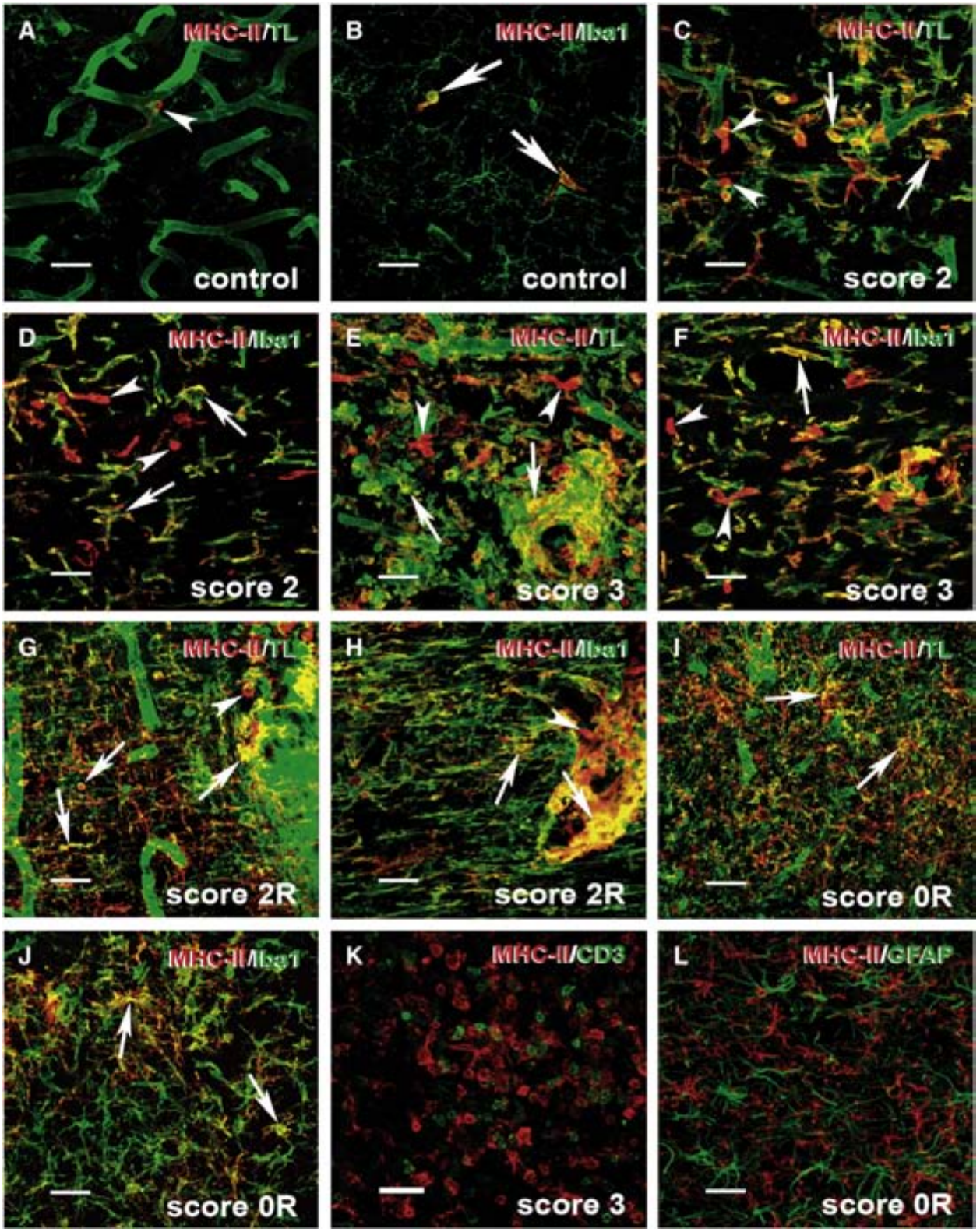


Fig. 5. Identification of MHC-class II+ cells. Double-immunolabelling combining MHC-class II with TL (A, C, E, G, I), Iba1 (B, D, F, H, J), CD3 (K) and GFAP (L) in both controls (A and B) and in different phases along EAE evolution (C–L). Arrows point to double-labelled cells with either TL (C, E, G and I) or Iba1 (B, D, F, H and J), whereas arrowheads indicated MHC-class II+/TL− (A, C, E, G) and MHC-class II+/Iba1− cells (D, F, H). No co-localisation between MHC-class II with either CD3 (K) or GFAP (L) was found. Bar scale = 30 μm.

detected (Fig. 4B). At score 1, although the major accumulation of positive cells was observed around blood vessels, MHC-class II+ cells were also found scattered in the parenchyma (Fig. 4C). At these scores, in addition to poorly ramified cells, some perivascular accumulations of MHC-class II+ cells displaying either little-round or ramified morphologies were observed (Fig. 4L, M). At score 2, the number of MHC-class II+ cells increased in both the grey and the white matter (Fig. 4D) reaching maximum levels at the peak of the

disease, at score 3 (Fig. 4E). At scores 2 and 3, the MHC-class II+ cell population was constituted by different morphologies (Fig. 4N, O). At score 2, mainly ramified morphologies were found, although some poorly ramified, amoeboid, little-round and big-round MHC-class II+ cells were also seen. At score 3, however, amoeboid cells predominated over big- and little-round morphologies, whereas no ramified or poorly ramified MHC-class II+ cells were detected.

During the recovery phase, from score 2R to 0R, a progressive decrease in MHC-class II immunoreactivity was found, although still an important number of perivascular accumulations of immunolabelled cells was found (Fig. 4F–H). The major part of these MHC-class II+ cells showed a ramified morphology (Fig. 4P). The proportion of little-round cells decreased starting from score 2R, but the number of big-round MHC-class II+ cells around blood vessels remained very high until score 1R, suffering an important reduction at score 0R. In addition, at score 0R some poorly ramified MHC-class II+ cells were observed again. No amoeboid morphologies were seen during the recovery phase.

During the post-recovery phase, at scores 0R-40dpi and 0R-90dpi, some foci of ramified and poorly ramified MHC-class II+ cells were still observed, often around blood vessels (Fig. 4I, J) and mainly at score 0R-40 dpi.

3.1.4. Nature of MHC-class II+ cells

The analysis of double-immunolabelled sections revealed that poorly ramified MHC-class II+ cells observed in control animals, at scores 0.5, 1 and 2 of the inductive phase, at score 0R of the recovery phase and during the post-recovery phase (score 0R-40dpi and score 0R-90dpi) always co-localised with Iba1 but not with TL or GFAP (Fig. 5A, B). Interestingly, while ramified MHC-class II+ cells were TL+ and Iba1+, only some amoeboid MHC-class II+ cells showed co-localisation with TL and Iba1 (Fig. 5C–J). Parenchymal big-round MHC-class II+ cells observed at scores 2 and 3 were TL+ and Iba1+, whereas some of those big-round cells observed in the surroundings of blood vessels co-localised with Iba1 but not with TL. Little-round MHC-class II+ cells did not usually show co-localisation with either TL or Iba1, although at scores 2 and 3 some of these cells displayed TL staining (Fig. 5C).

No co-localisation of MHC-class II with CD3 or GFAP was observed in any case (Fig. 5K, L).

3.2. Expression of co-stimulatory molecules

In contrast to control animals where expression of co-stimulatory molecules was absent (Figs. 6A, 7A and 8A), in EAE animals expression

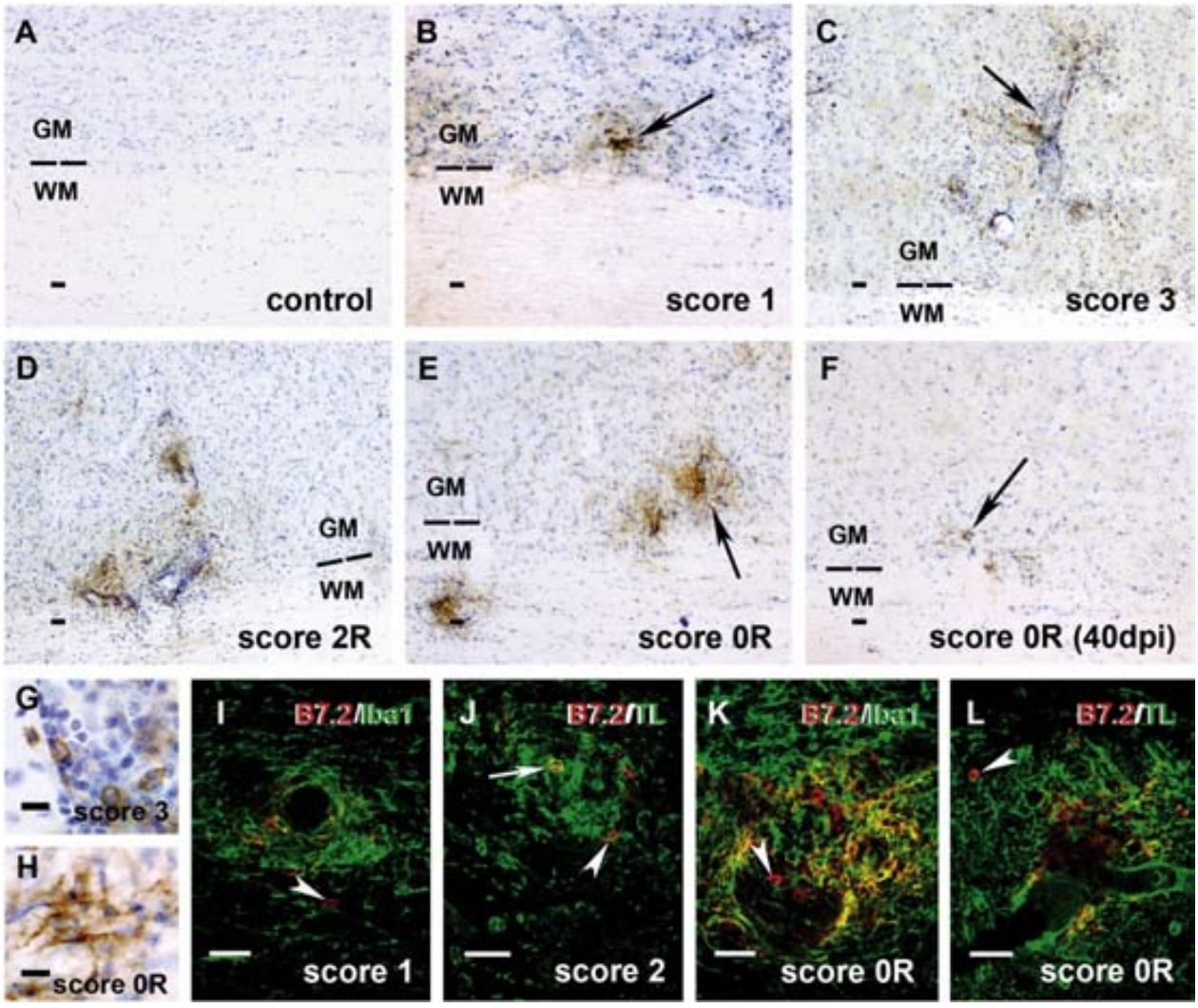


Fig. 6. Expression of B7.2. (A–H) Single immunolabelled sections of the spinal cord of control (A) and EAE animals (B–H). Arrows in B, C, E and F point to B7.2+ accumulations around blood vessels. Characteristic morphologies exhibited by B7.2+ cells are shown in higher magnification photographs in G and H. (I–L) Double-immunolabelling combining B7.2 with Iba1 (I, K) and TL (J, L). Yellow colour indicates co-localisation. Arrow in J points to a characteristic little-round B7.2+/TL+ cell. Arrowheads in I and K point to B7.2+/Iba1− cells; in J and L arrowheads point to little-round B7.2+/TL− cells. Bar scale A–F and I–L = 30 μm; G and H = 10 μm.

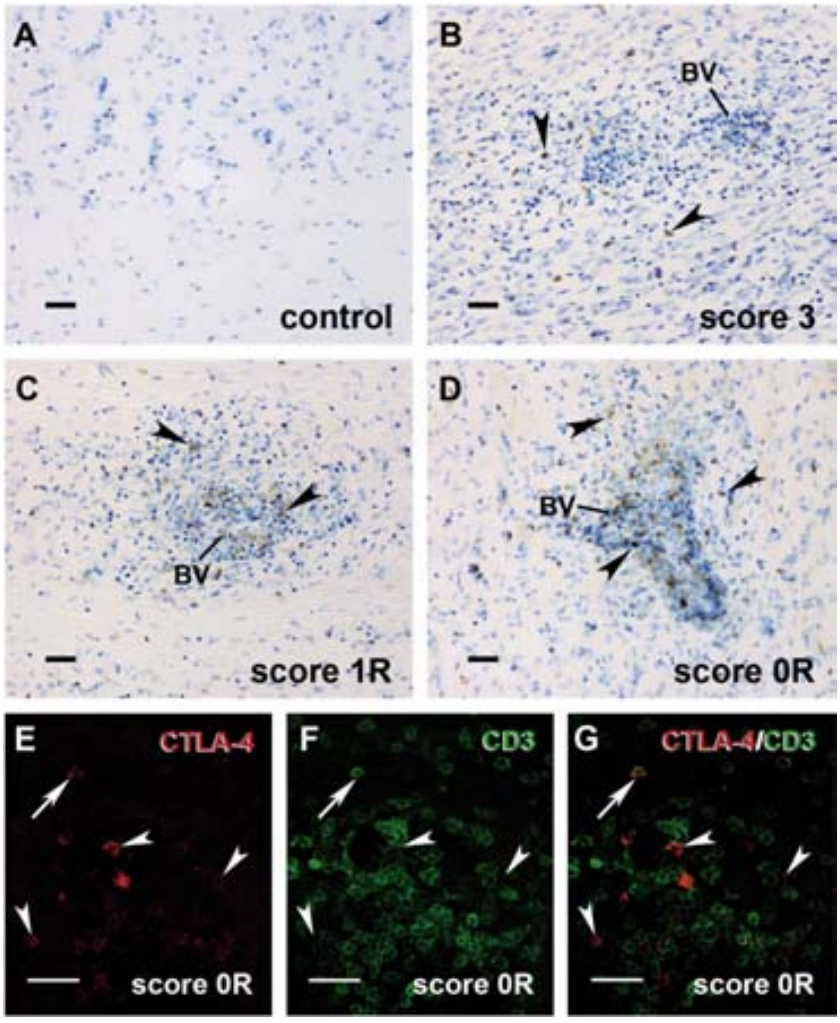


Fig. 7. Expression of CTLA-4. Single labelled sections of controls (A) and MBP-injected animals in different phases of EAE (B–D). Arrowheads in B, C and D point to CTLA-4+ cells around blood vessels (BV). (E–G) Double immunohistochemistry showing CTLA-4+/CD3+ cells (arrows) and CTLA-4+/CD3− cells (arrowheads). Bar scale = 30 μm.

of B7.2, CD28 and CTLA-4 was found along the different phases of the disease, as will be detailed below.

3.2.1. B7.1 and B7.2 expression

Whereas expression of B7.1 was not detected in EAE animals (data not shown), B7.2 expression was found in different phases along EAE. During the inductive phase, from score 1, the presence of B7.2+ cells displaying little-round morphology was detected around some blood vessels (Fig. 6B). The number of little-round B7.2+ cells increased progressively until score 3 and were always seen around blood vessels (Fig. 6C, G).

During the recovery phase, from score 2R to 0R, a reduction in the number of little-round B7.2+ cells was found in parallel to a progressive increase in ramified B7.2+ cells (Fig. 6D, E, H). Ramified B7.2+ cells were detected not only accumulated around blood vessels, but also some of them were found scattered in the parenchyma.

During the post-recovery phase, scores 0R-40dpi and 0R-90dpi, a dramatic decrease in the expression of B7.2 was detected, but some scattered ramified B7.2+ cells were still found in the parenchyma (Fig. 6F).

Double-immunolabelled sections for B7.2 with either TL or Iba1 showed that little-round B7.2+ cells observed during the inductive, the peak and the recovery phases of EAE, did not co-localise with Iba1 but a few of them co-localised with TL (Fig. 6I, J). In contrast, those B7.2+ cells displaying ramified morphology that were detected during the recovery and post-recovery phases always co-localised with Iba1 and TL (Fig. 6K, L).

3.2.2. CTLA-4 expression

In EAE animals, at score 2 during the inductive phase, and also at score 3, some CTLA-4+ cells displaying little-round morphology were detected around some blood vessels (Fig. 7B).

During the recovery phase, from score 2R to 0R, a progressive increase in the number of little-round CTLA-4+ cells was found which were highly accumulated in the vicinity of some blood vessels (Fig. 7C, D). The number of CTLA-4+ cells substantially decreased during the post-recovery phase, and at score 0R-90dpi no CTLA-4+ cells were detected (not shown).

Double immunohistochemistry combining CTLA-4 with CD3 showed that although a high density of CD3+ cells was found around blood vessels, only a few of them co-localised with CTLA-4 (Fig. 7E–G).

3.2.3. CD28 expression

In EAE, during the inductive phase, since score 1 some CD28+ cells displaying little-round morphology were found in the vicinity of blood vessels, their density progressively increasing until score 3 (Fig. 8B, C). In addition, at score 3, several little-round CD28+ cells were observed dispersed in the parenchyma, in both the grey and white matter (Fig. 8C).

During the recovery phase, from score 2R to 0R, a gradual decrease in the number of little-round CD28+ cells was observed. The major

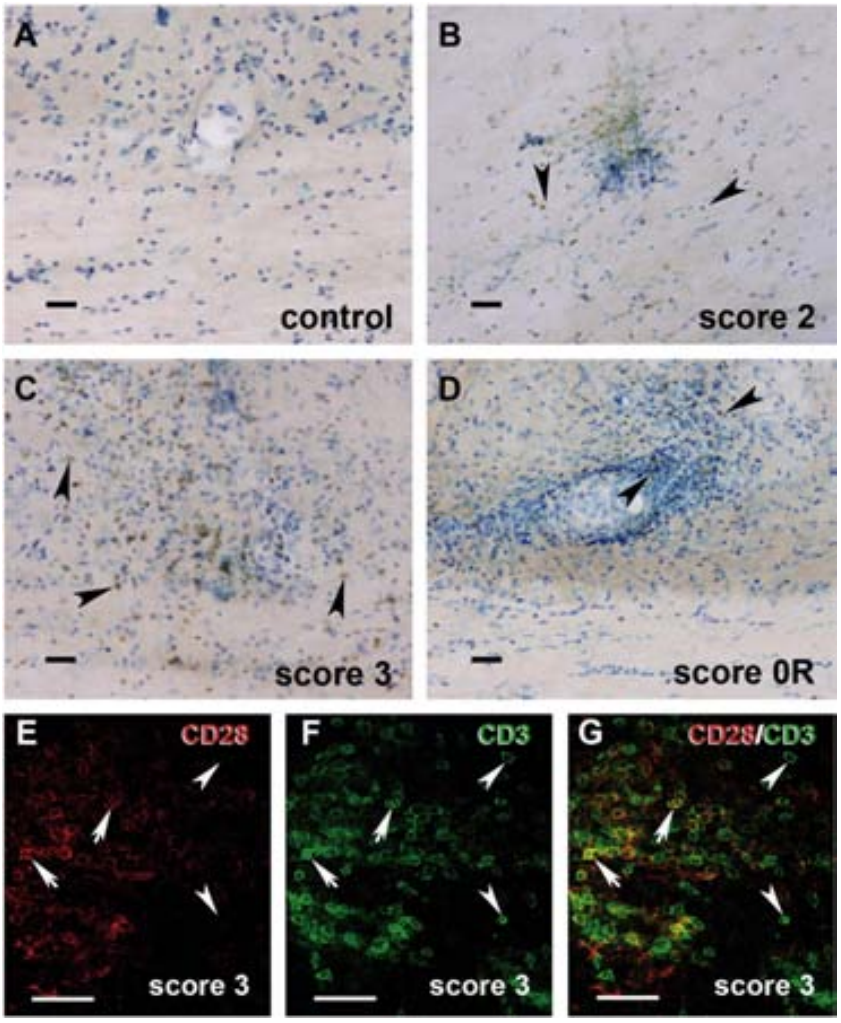


Fig. 8. Expression of CD28. Single immunolabelled sections of controls (A) and EAE animals (B–D); arrowheads point to CD28+ cells. (E–G) Double-immunolabelled sections showing CD28+ cells that co-localised with CD3 (arrows in E–G). Arrowheads in E–G point to CD28−/CD3+ cells. Bar scale = 30 μm.

part of these remaining CD28+ cells was found mainly around blood vessels (Fig. 8D). No CD28+ cells were found along the post-recovery phase.

Sections double-labelled for CD28 and CD3 showed that the major part of little-round CD28+ cells co-localised with CD3, although some CD28+CD3− and CD28−CD3+ cells were also detected (Fig. 8E–G).

3.3. Expression of dendritic cell markers

3.3.1. CD1 expression

In control animals, no expression of CD1 was detected (Fig. 9A). In EAE animals, during the inductive phase at scores 0.5 and 1, CD1+ cells showing little-round morphology were observed around some blood vessels, mostly in the white matter (Fig. 9B, C and J). At score 2, the number of CD1+ cells greatly increased not only in the vicinity of blood vessels but also in the parenchyma in both the grey and white matter (Fig. 9D). At this score, in addition to little-round positive cells, some amoeboid and big-round CD1+ cells were found (Fig. 9K). Parenchymal, big-round CD1+ cells had mitotic nuclei, as revealed by the toluidine blue counterstaining (Fig. 9, arrowheads in K, L). The maximum density of both perivascular and parenchymal CD1+ cells were observed at score 3 (Fig. 9E).

During the recovery phase, from score 2R to 0R, an important decrease in CD1 immunolabelling was observed in the parenchyma, remaining high around blood vessels (Fig. 9F–H). Starting from score 2R, in parallel to a large reduction in the number of little-round and big-round CD1+ cells, a population of ramified CD1+ cells emerged in the surroundings of blood vessels (Fig. 9M). This population of ramified CD1+ cells remained until score 1R. At score 0R, perivascular CD1+ cells consisted only of cells with a little-round morphology (Fig. 9N). No expression of CD1 was found during the post-recovery phase (Fig. 9I).

Double-immunohistochemistry analysis combining CD1 with CD11b revealed that some CD11b+ cells displaying little-round, amoeboid or big-round morphologies during the inductive phase and ramified morphology during the recovery expressed CD1; moreover, in both phases, some CD1+CD11b− cells were also found (Fig. 10A, B). Similar results were obtained combining CD1 with TL (Fig. 10C, D).

Double-immunolabelling combining CD1 with MHC-class I (Fig. 10E, F) or MHC-class II (Fig. 10G, H) showed that little-round CD1+ cells observed during the inductive and recovery phases always displayed co-localisation with MHC-class I, but only some of them were MHC-class II+. Only at score 3, all little-round CD1+ cells were MHC-class I+ and MHC-class II+. Amoeboid and parenchymal big-round CD1+ cells observed at scores 2 and 3 always co-localised with both MHC-class I and MHC-class II. In contrast, only some of the big-round CD1+ cells observed around blood vessels showed co-localisation with MHC-class I and MHC-class II.

Analysis of sections double-labelled combining CD1 with ED2 revealed that only those big-round CD1+ cells seen around blood vessels from score 2 to 1R always co-localised with ED2 (Fig. 10I). It

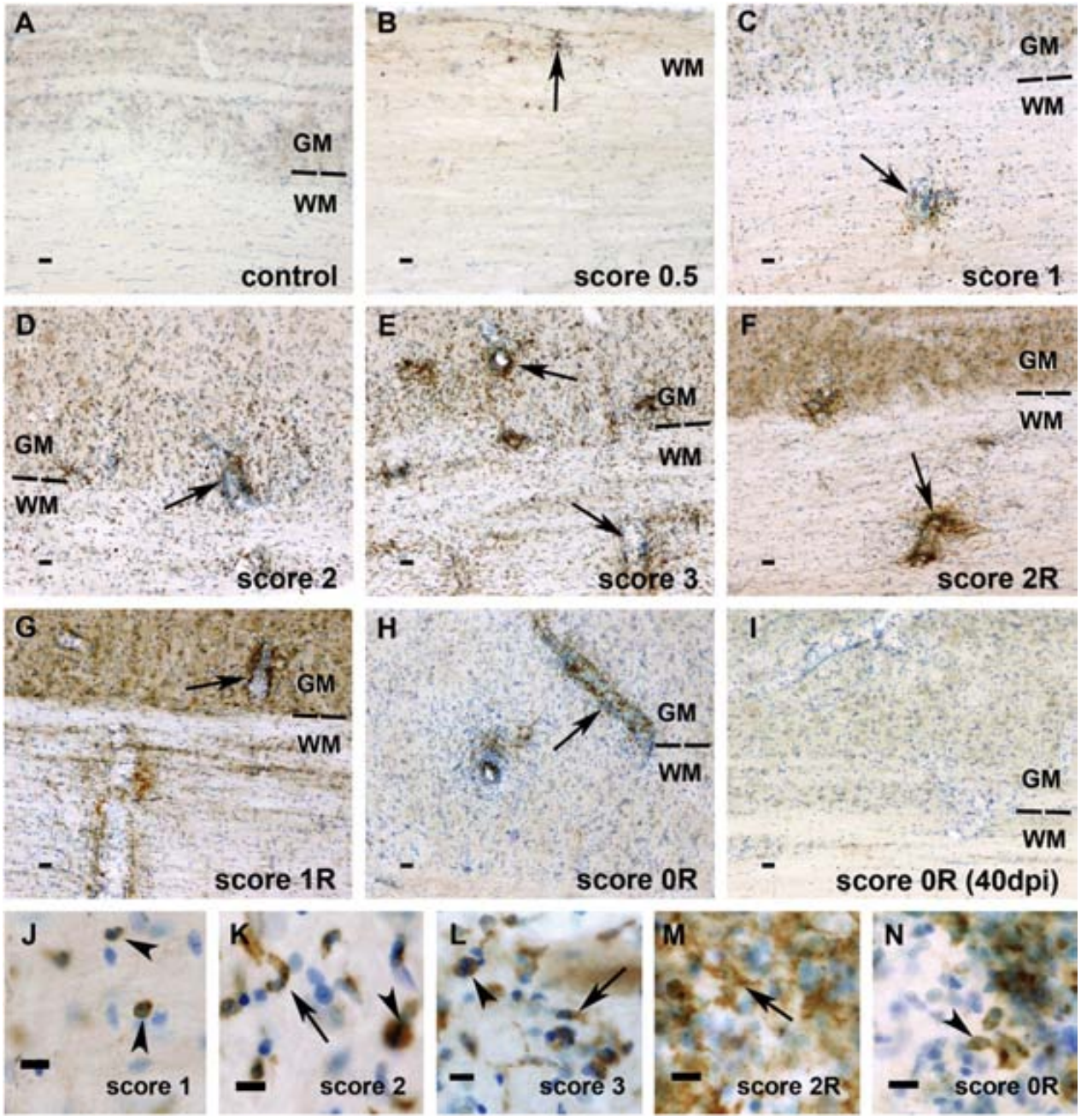


Fig. 9. CD1 expression. Expression of CD1 in control (A) and in different phases along EAE evolution: inductive (B–D), peak (E), recovery (F–H) and post-recovery phases (I). Note accumulations around blood vessels (arrows in B–H) in both the grey (GM) and the white matter (WM). (J–N) High-magnification micrographs showing the different morphologies of CD1+ cells in EAE animals: little-round (arrowheads in J and N), amoeboid (arrows in K and L), big-round with mitotic nuclei (arrowheads in K and L) and ramified (arrow in M). Bar scale A–I = 30 μm; J–N = 10 μm.

should be noted that during this period, in addition to ED2+CD1+ cells, we also found some big-round ED2+CD1− cells.

Sections immunolabelled combining CD1 with fascin or CD4 did not show co-localisation of these markers in any case (Fig. 10J–M).

3.3.2. Fascin expression

In contrast to controls (Fig. 11A), where no expression of fascin was seen, in EAE animals beginning from score 2, a population of fascin+ cells displaying a big-round morphology was observed in close relationship with blood vessels (Fig. 11B). The density of perivascular fascin+ cells increased at score 3. At this score, in addition to big-round positive cells, amoeboid fascin+ cells were also found around blood vessels and scattered in the parenchyma (Fig. 11C and I).

During the recovery phase, from score 2R to 0R, the density of amoeboid fascin+ cells remained without changes, whereas big-round fascin+ cells progressively decreased. At these scores, fascin+ cells were always detected around blood vessels, but never in the parenchyma (Fig. 11D–F). During the post-recovery phase, scores 0R-40dpi and 0R-90dpi, no fascin+ cells were found.

Fascin+ cells never showed co-localisation with CD1 (Fig. 10J and K), Iba1 (Fig. 11G) or TL (Fig. 11H) in any phase.

4. Discussion

In this study a detailed analysis of the temporal pattern of expression and cellular distribution of the different molecules (MHCs and co-stimulatory) related to the antigen-presenting mechanism along the

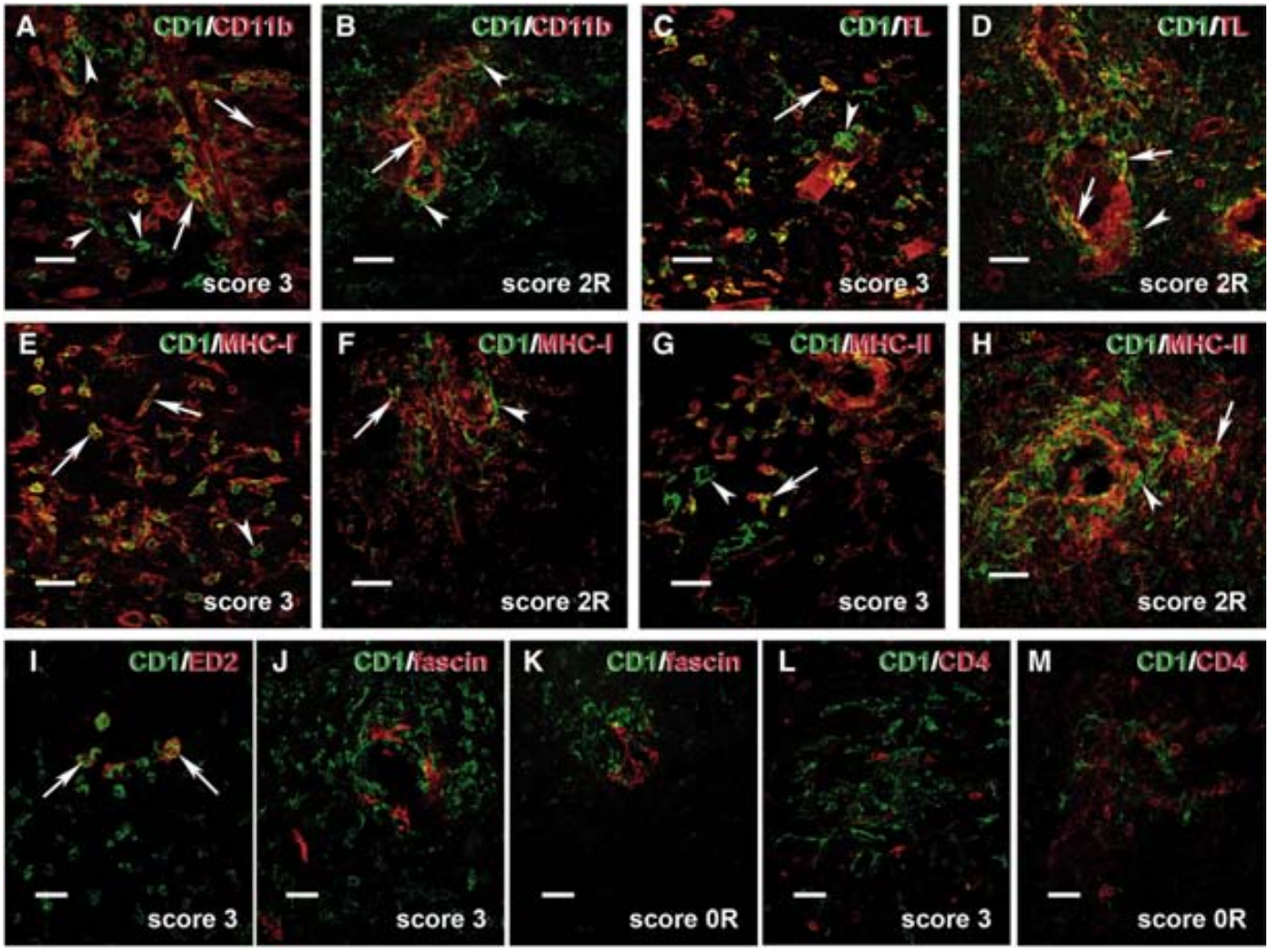


Fig. 10. Identification of CD1+ cells. Double-immunolabelled sections combining CD1 with either CD11b (A and B), TL (C and D), MHC-class I (E and F), MHC-class II (G and H), ED2 (I), fascin (J and K) or CD4 (L and M). Arrows in A–I point to double-immunolabelled cells, whereas arrowheads in A–H indicate CD1+ cells that did not show double-immunolabelling with the corresponding marker. There was no co-localisation between CD1 and fascin (J and K) or CD4 (L and M). Bar scale = 30 μm.

different phases of EAE evolution was performed. Firstly, it is important to highlight the fact that no variability was observed in animals analysed in each specific clinical score, indicating that histopathological changes within the CNS are more associated with clinical symptomatology than with time post-immunisation, providing a more accurate way to address the study of processes associated with this acute EAE model. An increase in MHC expression in cells morphologically identified as microglia during the peak of EAE has been reported in previous studies (Craggs and Webster, 1985; Juedes and Ruddle, 2001; Lindsey and Steinman, 1993; Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; McCombe et al., 1994; McCombe et al., 1992; Ponomarev et al., 2005; Pope et al., 1998). Our study not only confirm these earlier reports but, additionally, clearly demonstrate that expression of both MHC-class I and MHC-class II molecules are in a strong, close relationship with the evolution of EAE symptomatology. This expression increases during the inductive phase, reaching maximum strength at the peak, decreasing progressively during the recovery phase although, interestingly, in animals analysed during the post-recovery phase, at both 40 dpi and 90 dpi, expression levels of MHC-class I and MHC-class II remained higher than in control animals. These data suggest that, prolonged expression of these molecules may play a role in the resolution of the inflammatory/immune response. Controversial studies are found in the literature regarding the role played by MHC-class I and class II in the CNS. Some studies have pointed towards a detrimental role of MHC-class I and MHC-class II expression due to the fact that some treatments that decreased EAE severity and exerted beneficial effects in EAE evolution, such as minocycline (Nikodemova et al., 2007; Popovic et al., 2002) and nerve growth factor (NGF) (Stampachiacchiere and Aloe, 2005), correlated with a decrease in MHC expression. However, other studies have suggested that expression of both MHC-class I and class II may play a beneficial role. For example, MHC-class I-KO mice present aggravation of EAE symptoms (Linker et al., 2005a; Linker et al., 2005b), and animals with a stronger MHC-class I response exhibited better recovery after nerve transection (Sabha et al., 2008). Moreover, an impairment of remyelination has been reported in MHC-class II-KO mice after both cuprizone-demyelination (Arnett et al., 2003) and Theiler's murine encephalomyelitis (Njenga et al., 1999), and high levels of MHC-class II were found in microglial cells of EAE-resistant rat strains (Klyushnenkova and Vanguri, 1997; Sedgwick et al., 1993).

It is important to take into account that, as has been extensively demonstrated, the signal provided by MHC molecules is not sufficient, and a second co-stimulatory signal is crucial for the complete T-cell activation. However, the major part of the aforementioned studies has only been focused in MHCs expression and less studies about co-expression of these MHCs with co-stimulatory molecules are found in the literature. Therefore, we analysed whether the presence of B7.1/B7.2-CD28/CTLA-4, the most potent pair of co-stimulatory molecules, correlated with MHC expression and EAE evolution. Interestingly,

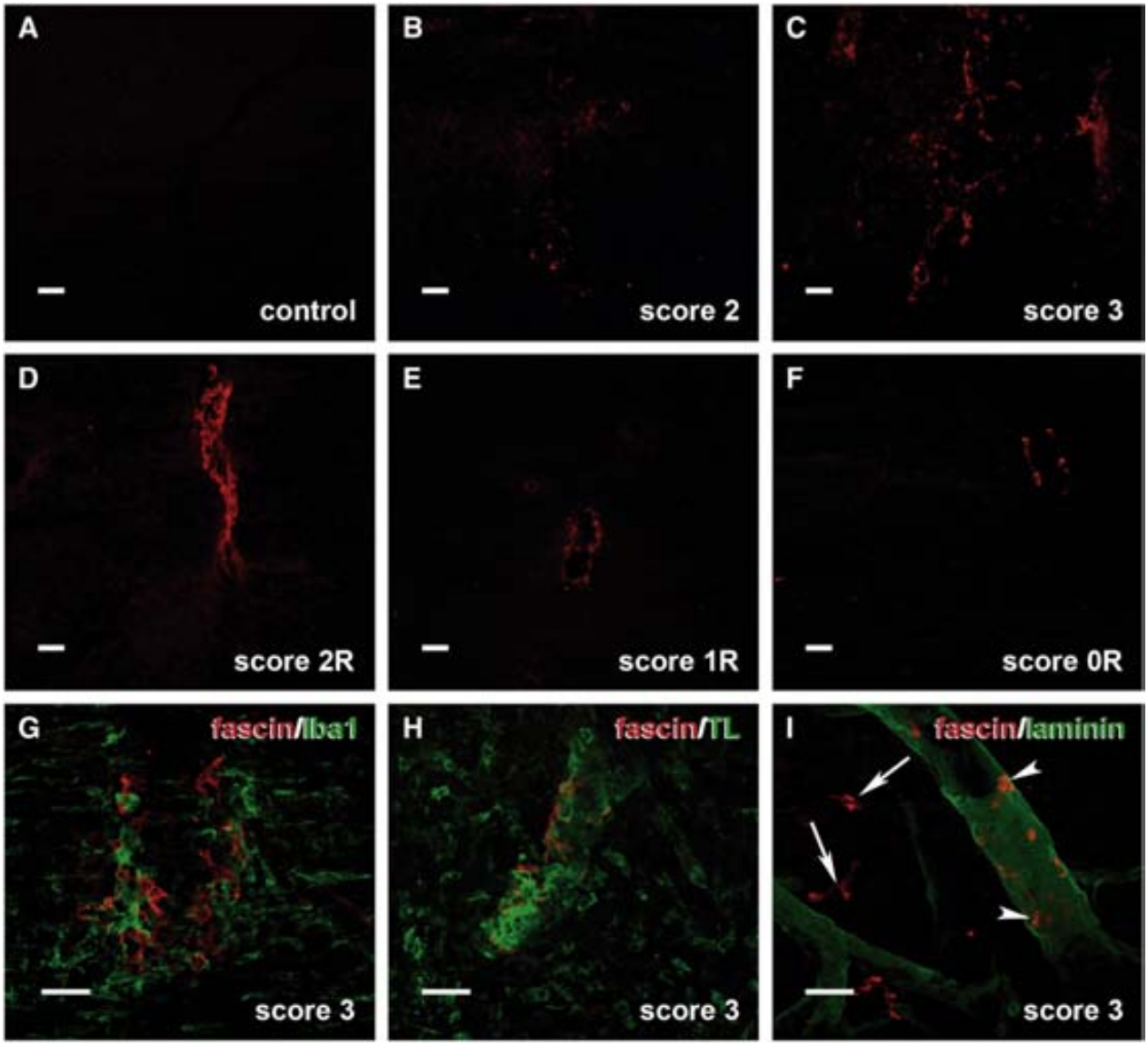


Fig. 11. Fascin expression. Immunostaining for fascin in controls (A) and in EAE animals (B–F). Double immunohistochemistry combining fascin with Iba1 (G), TL (H) and laminin (I) showing no co-localisation between these markers. Arrows in I point to fascin+ cells located in the parenchyma whereas arrowheads in I show fascin+ cells in close relationship with blood vessels. Bar scale = 30 μm.

although we found expression of MHCs along all the phases of the disease, the expression and distribution of B7.1, B7.2, CD28 and CTLA-4 molecules, exhibited a specific temporal pattern that was related with the different phases of EAE analysed (Fig. 12). Firstly, it is important to mention that, in contrast to other studies in murine EAE models (Issazadeh et al., 1998; Karandikar et al., 1998b), no expression of B7.1 in any phase of EAE in our EAE Lewis rat model was detected. To our knowledge, there are no reported studies of B7.1 temporal pattern expression in rat EAE. It should be emphasised that B7.1 expression in murine EAE models was directly related to the mechanism involved in epitope spreading and clinical relapses (Issazadeh et al., 1998; Karandikar et al., 1998b; Miller et al., 1995). If B7.1 plays the same role in the rat, this would explain why this molecule is not expressed in the EAE Lewis rat model since it is characterised by a single peak of disability without relapsing episodes. Regarding CD28 expression, our observations demonstrate that although this molecule is highly expressed during the inductive and peak phases, by T lymphocytes in the parenchyma and around blood vessels, B7.2 expression was restricted to perivascular locations, suggesting that T-cell activation takes place only at these specific sites. In contrast, CD28+ lymphocytes in the parenchyma, received the signal of antigen presentation in the context of lack of co-stimulation, a phenomenon that has been associated with apoptosis or anergy of T cells (Kishimoto and Sprent, 1999; Sprent and Tough, 2001). In fact, both apoptosis and anergy of lymphocytes are mechanisms which have been associated with the control of EAE evolution (Gold et al., 1997; Gordon et al., 2001; McCombe et al., 1996; Schmied et al., 1993) (Fig. 12).

Interestingly, during the recovery phase, B7.2 expression did not decrease, but rather its expression was maintained in the vicinity of blood vessels associated with TL+/Iba1+ ramified microglia. In the same perivascular location we found a high density of CTLA-4+ cells, some of which have been identified as CD3+ T cells, suggesting that inhibitory lymphocytic signalling mediated by microglia may occur. In addition to CTLA-4+ lymphocytes, a high proportion of CTLA-4+/CD3− cells was found whose identity was uncertain. There is a report showing that microglia can express CTLA-4 in culture (Dimayuga et al., 2005). Another study has suggested that B cells are also able to express CTLA-4 when co-cultured with T cells (Quandt et al., 2007).

Strikingly, in our study we have demonstrated that MHC and B7.2 expression did not end when clinical symptoms disappeared but,

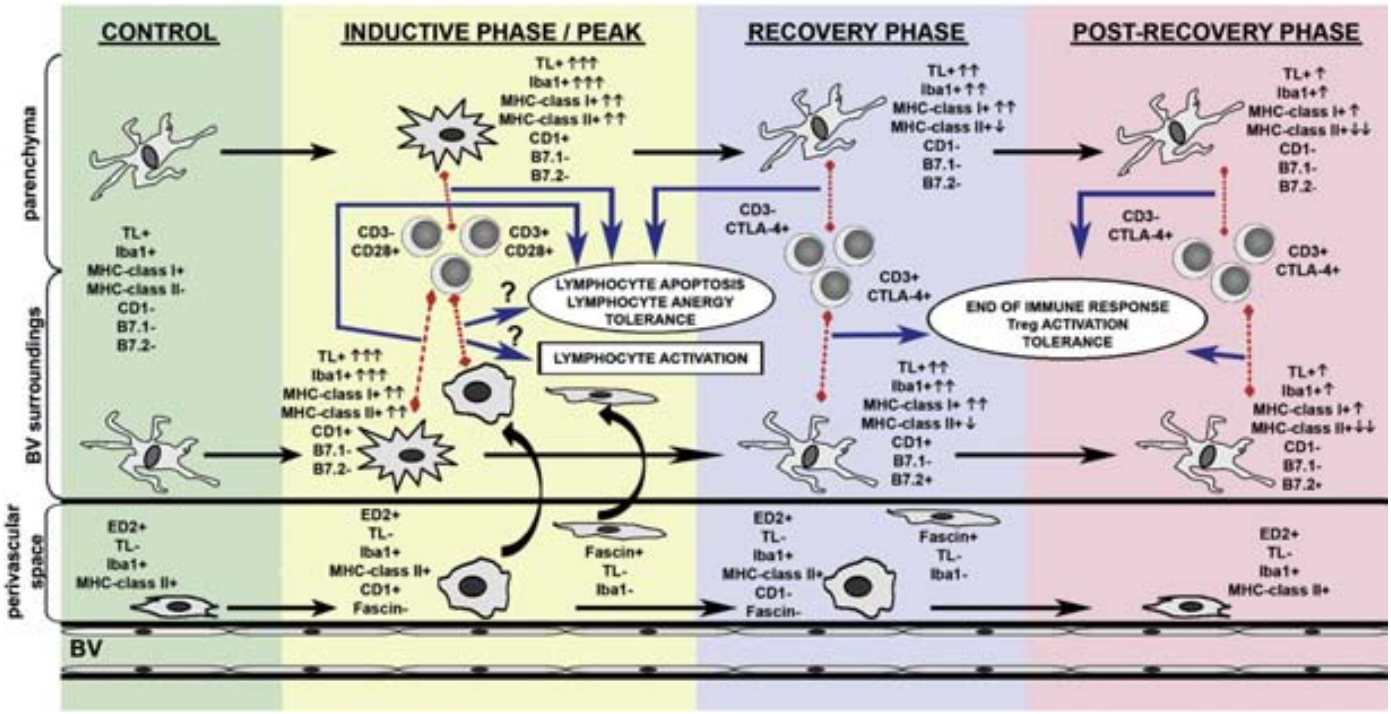


Fig. 12. Modulation of the immune response along the different phases of acute EAE Lewis rat model. Principal findings observed in this study and proposed interactions between different cell types as stated in the Results and Discussion sections were outlined in this drawing. During the inductive and peak phases of EAE, microglial cells become activated (morphological changes from ramified to reactive shapes, and up-regulation or *de novo* expression of some molecules) and interact with CD28+ lymphocytes in the context of MHC expression without co-stimulatory signals. This interaction may involve the apoptosis or anergy of infiltrated lymphocytes. During the recovery phase, microglial cells, although reverting to ramified forms, remain activated (high expression of different markers). Expression of B7.2 was only found in microglial cells located in the surroundings of blood vessels, in parallel to CTLA-4 expression in infiltrated lymphocytes. The signal derived from CTLA-4/B7.2 binding is inhibitory in effector lymphocytes but can also stimulate the activity of Tregs. Noticeably, these microglial cells near the blood vessels displayed CD1, a marker for immature DCs. During the post-recovery phase, foci of activated microglia MHC-class I+ and class II+, remain near the blood vessels and in the parenchyma, where some CTLA-4+ cells are still present. The interaction of those microglial cells (B7.2− in the parenchyma and B7.2+ around blood vessels) with lymphocytes may induce the end of immune response and tolerance by Treg activation or T-effector cell inhibition. A population of ED2+ perivascular cells was seen infiltrating the parenchyma during the peak. These cells display MHC-class II and CD1, suggesting that they also may play a role in T-cell regulation. Finally, fascin+ cells (mature DCs), found in the parenchyma during the peak, may be recruited from the periphery. The role played by these cells is uncertain.

instead, their expression continued longer, during the post-recovery phase, suggesting that they still play a role in the modulation of the immune response. In this same way, it should be noted that CTLA-4 expression was also observed during this post-recovery phase, suggesting that the mechanisms involved in lymphocytic inhibition extended into this phase. As will be discussed later, this process may be involved in peripheral tolerance and T-regulatory cells (Treg) activation, which may explain the absence of clinical relapses in this model (Fig. 12).

4.1. Microglia as dendritic cells

Microglial cells have been proposed as a population of resident, immature dendritic cells (Carson et al., 1998) that, when activated under some circumstances, may acquire a mature dendritic cell phenotype (Fischer and Reichmann, 2001; Santambrogio et al., 2001). Recent studies have shown that a subpopulation of microglial cells constitutively displayed CD11c, a marker of dendritic cells (Bulloch et al., 2008). In the present study, we have analysed the expression of characteristic dendritic cell markers, such as MHCs, co-stimulatory factors and dendritic cell maturation molecules in microglial cells.

Previous studies in different EAE models (Matsumoto and Fujiwara, 1986; Matsumoto et al., 1986; McCombe et al., 1992) suggested that microglia were the principal cell-type responsible for MHC-class I and class II expression. However, these statements were based on morphological features, as no specific co-localisation analysis was used in these studies. Here, we clearly demonstrated that along the different phases of EAE both MHC-class I and MHC-class II molecules co-localised with two different markers of microglial cells, Iba1 (Ito et al., 1998) and TL (Acarin et al., 1994). Interestingly, although the major part of microglial cells expressed MHC-class I and class II molecules, only a small proportion of microglia expressed B7.2 around blood vessels, mainly during recovery. As already discussed above, microglial co-expression of MHC and co-stimulatory factors around blood vessels suggest that during the inductive and peak phases, MHC-class I+/class II+/B7.2+ microglial cells interact with CD28+/CD3+ lymphocytes inducing their activation, whereas during the recovery phase these MHC-class I+/class II+/B7.2+ cells interact with CTLA-4+/CD3+ lymphocytes inducing their inhibition. The fact that there was a population of microglial cells expressing MHC-class I and class II without B7.2 co-expression may indicate that microglial cells are promoting the apoptosis or anergy of T lymphocytes invading the parenchyma. Nevertheless we cannot discard the possibility that this microglial expression of MHC-class I and MHC-class II is not directly related to the antigen-presentation mechanism, playing other, not well established roles, or that its expression is associated with expression of other co-stimulatory molecules not analysed in this study.

Our results show that CD1, an immature dendritic cell marker (Serafini et al., 2006), is expressed by a set of CD11b+ or TL+ cells displaying little-round, amoeboid and ramified morphologies that might correspond to a monocyte/macrophage/microglia population. In agreement, a flow cytometry study showed an increase in CD1 expression in CD11b+CD45^{low} microglial cell and CD11b+CD45^{high} macrophage populations during the peak of EAE in mice (Busshoff et al., 2001). In our model, CD1 expression closely correlated with the clinical symptomatology, increasing during the inductive phase, peaking at score 3 and decreasing during the recovery phase. Moreover, we found

CD1 expression mainly in the surroundings of blood vessels, coinciding with our observations of MHCs and B7.2 expression in microglial cells in the same perivascular locations, where CD3+ lymphocytes were accumulated. All together, these findings, suggest that microglia located in the surroundings of blood vessels acquire a specific phenotype of immature dendritic cells (CD1+/MHC-class I+/MHC-class II+/B7.2+) and participate in the modulation of T-cell responses (Fig. 12). However, it should be noted that these microglial cells did not express fascin, an actin-bundling protein whose expression strongly correlated with maturation of DCs and their ability to activate T cells (Al-Alwan et al., 2001; Mosialos et al., 1996; Ross et al., 2000). Little is known about fascin expression in the brain, as only one study in which the nature of fascin+ cells was not determined, has reported fascin expression in association with inflamed blood vessels of chronic-active human MS lesions (Serafini et al., 2006). The fact that, in our model, microglial cells displayed CD1 expression without reaching a mature fascin+ phenotype may indicate a specific role in the control of the immune response. In this sense, some studies have proposed that, in humans, immature DCs would be involved in the peripheral-tolerance maintenance by inducing the differentiation of a subset of Tregs (Dhodapkar et al., 2001; Jonuleit et al., 2000; Roncarolo et al., 2001). The fact that Tregs constitutively expressed CTLA-4 (Birebent et al., 2004) and their presence has been associated with the recovery of EAE (McGeachy et al., 2005), together with our findings showing CTLA-4 expression during the recovery and post-recovery phases, reinforce the idea that microglial cells, acting as immature DCs, modulate T-cell response and direct the termination of the immune/inflammatory process.

Our observations reveal that, in addition to CD1+ microglia, there was an important population of CD1+/CD11b−/TL− cells displaying different morphologies mainly at the peak of EAE, but also during the recovery phase. These cells are mainly located around blood vessels and usually expressed MHC-class I and class II molecules. We have not determined the nature of these little-round and amoeboid CD1+ cells, although we have identified those CD1+ cells exhibiting big-round morphology as perivascular cells due to their ED2 expression (Dijkstra et al., 1994). We have previously reported that, in acute Lewis rat EAE model, ED2+ cells undergo changes in density, distribution and phenotype that strongly correlate with the clinical symptomatology (Almolda et al., 2009). Moreover, in our previous study, we observed that ED2+ cells invaded the parenchyma particularly at the peak of the disease. In the present study, additionally, we demonstrated that some of these ED2+ cells acquire an immature DC phenotype as expressed CD1 antigen, suggesting that they are also contributing to the modulation of the inflammatory/immune response during this period (Fig. 12).

Finally, it is important to mention that we observed a population of fascin+/Iba1−/TL− cells, with big-round and amoeboid morphologies, whose density increased in correlation to the clinical score. These cells were frequently located in the vicinity of blood vessels, but also within the parenchyma at the peak of the disease. Therefore, we can speculate that, in addition to a population of resident microglial cells displaying an immature DC phenotype (CD1+), there is a recruitment of a specific population of DCs from the periphery, as already suggested by other authors (Matyszak and Perry, 1996; Serafini et al., 2000; Serafini et al., 2006). We cannot discard, however, the possibility that these fascin+ cells correspond to mature DC forms coming from immature CD1+ microglia or perivascular ED2+ cells. More studies are necessary in order to clarify the phenotype, origin and function of these specialised populations of DCs.

5. Conclusion

In conclusion, our study has shown that in this acute model of EAE, antigen-presenting mechanisms are not restricted to the inductive and peak phases of the disease, but rather they also play an important role during the recovery and post-recovery phases. Our results clearly indicate that during the inductive and the peak phases, antigen presentation to parenchymal CD28+ lymphocytes might take place in the context of MHC molecules without co-stimulatory B7.1/B7.2 signalling, thus may be inducing the apoptosis or anergy of these infiltrating CD28+ lymphocytes. Furthermore, during the recovery phase, B7.2 expression around blood vessels in concomitance with an increase in CTLA-4 expression, the molecule that triggers inhibitory signals to lymphocytes, suggests that this mechanism is powering the end of the inflammatory/immune response. Microglial cells seem to be the principal cell population involved in these processes by expressing antigen characteristics of immature DCs such as MHCs, B7.2 and CD1. In addition, a specific population of DCs displaying CD1 or fascin markers mainly located in the vicinity of blood vessels has been detected, and further studies are necessary in order to identify their nature and function.

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Quick decrease in Th1 lymphocytes and high increase in Th17 and T-reg populations may explain the spontaneous recovery of acute EAE

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE), a well-established model of multiple sclerosis, is characterised by microglial activation and lymphocyte infiltration. Induction of EAE in Lewis rats produces an acute monophasic disease characterised by a single peak of disabilitiy followed by spontaneous recovery and subsequent tolerance to further immunisations. The aim of the present study is to perform a detailed analysis of the dynamics of different lymphocyte populations along the inductive, peak, recovery and post-recovery phases of this model. To accomplish that, MBP-injected rats were examined daily for the presence of clinical symptoms and sacrificed at different phases of EAE, attending to their clinical score. Spinal cords were removed and, by the use of flow cytometry and immunohistochemistry, different populations of lymphocytes were analysed: CD3+ T-cells; CD4+ T-helper cells, T-helper subsets Th1, Th17 and T-regulatory (T-reg); CD8+ T-cytotoxic cells and γδ T-lymphocytes. Our results revealed that the number of CD3+ and CD4+ cells increased progressively during the inductive and peak phases, in parallel to an increase in symptomatology. During the inductive and peak phases, lymphocytes displayed a Th1 phenotype. In contrast, during the recovery phase, although clinical signs progressively decreased, the number and proportion of these CD3+ and CD4+ populations remained very high without significant changes. Interestingly, an abrupt decrease of Th1 and an increase in Th17 and T-reg cells took place. Moreover, an important population of Th17 and T-reg cells was observed during the post-recovery phase when clinical symptoms were absent. In addition, our findings showed that two populations of CD8+ and γδ + lymphocytes were observed without changes along all the phases analysed. The putative role played by microglia in regulating the dynamics of lymphocyte subpopulations is discussed.

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a useful animal model for the study of multiple sclerosis (MS) that is produced by the immunisation of susceptible animals with myelin proteins. Among the different EAE models, immunisation of susceptible Lewis rats with Myelin Basic Protein (MBP) induced an acute monophasic disease characterised by weight loss and hindlimb paralysis, followed by a spontaneous recovery, after which animals became resistant to further immunisations with MBP [1, 2]. This EAE model is of special interest because it allows for the study of not only the cellular and molecular mechanisms leading to the induction of the disease, but also those involved in the recovery and subsequent tolerance.

Although a wide number of studies are available about EAE in Lewis rats, the major part of these studies are focused on specific time-points, mainly at the peak of the disease, whereas only less-detailed reports at molecular and cellular levels during the induction and recovery phases are available. To address this lack of information and gain insights into the mechanisms involved in the and resolution of the pathological process, we have performed a detailed study [3, 4] of microglial reactivity including not only changes in morphology, but also in the expression of molecules involved in the antigen presentation process. Our observations have demonstrated that, along EAE evolution, microglial cells display a specific phenotype in each phase. During the inductive and peak phases, microglial cells exhibited a phenotype of an immature dendritic cell, characterised by MHC-class I and class-II expression, no co-stimulatory molecules and CD1 expression, a marker of immature dendritic cells, whereas during the recovery and even post-recovery phases, microglia maintained MHC expression and a subpopulation of cells expressed B7.2 [4] and/or CD4 [3].

In addition to microglial reactivity, our observations have also demonstrated an important infiltration of CD3+ lymphocytes along the different phases of EAE [4]. It is well established that T-cell infiltration is a crucial feature of EAE [5]. These findings, together with the fact that microglia are able to express different patterns of MHC and co-stimulatory molecules [4], led us to speculate that these microglial cells may be involved in the regulation of lymphocyte activation/deactivation during the different phases of EAE. Although an important number of reports are found in the literature about infiltration of T-cells in the different models of EAE [6-10], the dynamics of lymphocyte subtypes along the course of the disease are poorly understood. It is widely accepted that CD4+ Th1 cells are key players in leading to the immune response associated with EAE [11]. However, upon the emergence of new subsets of CD4+ T-cells with different cytokine profiles and functions [12-14], this classical assumption has been reconsidered and nowadays it is suggested that, in addition to Th1, other subtypes of CD4+ T-cells may also be involved in EAE pathogenesis. Thus, it has recently been demonstrated that Th17 lymphocytes, a new characterised subset of CD4+ T-cells [15, 16], are able to induce EAE when passively injected in mice [17]. Moreover, also in mice, accumulation of T-regulatory Foxp3+ cells (T-regs) was reported within the CNS concurrent with EAE recovery [18-20]. Furthermore, other types of T-lymphocytes such as cytotoxic CD8+ cells and γδ T-cells are putative candidates to play a role in EAE evolution [21, 22] as suggested by reports demonstrating that EAE in mice can be induced by passive immunisation of pathogenic CD8+ T-cells [23-25] and other studies showing the infiltration of γδ T-cells during the onset and peak of the disease [26].

Taking advantage of our knowledge on glial reactivity and the expression of antigen-presentation molecules in the model of acute EAE in Lewis rat, the aim of the present study is to perform a careful

analysis of CD4+ T-cell subsets, CD8+ and γδ T-cell dynamics along the inductive, peak, recovery and post-recovery phases.

2. Material and methods

2.1 Animals and EAE induction

A total of 140 female Lewis rats (180g/200g) succeptible to developing experimental autoimmune encephalomyelitis (EAE) were purchased from Charles River (France) and maintained with food and water *ad libitum* in a 12h light/dark cycle.

EAE was induced by the injection in both hindlimbs of an emulsion containing 100 µg myelin basic protein (MBP) (Sigma, USA, Ref. M2295) in Complete Freud's Adjuvant (CFA) (Ref. 0638; Difco; USA) and 0.2 mg of *Mycobacterium tuberculosis* (*M. tuberculosis*) H37 Ra (Ref. 3114; Difco; USA). Animals injected with vehicle solution were used as controls.

The presence of clinical signs was evaluated daily in all animals, using the following clinical score test: 0, no clinical signs; 0.5, partial loss of tail tonus; 1, tail paralysis; 2, paraparesis of hindlimb; 3, paraplegia; 4, tetraparesis; 5, tetraplegia and 6, death. Paralysed animals were afforded easier access to food and water.

All experimental animal work was conducted according to Spanish regulations in agreement with European Union directives and was approved by the Ethical Committee of the Autonomous University of Barcelona.

2.2 Experimental groups

As in previous studies [3, 4], EAE-induced animals were sacrificed according to their clinical score, at different phases along the EAE course, as detailed: 1) before the appearance of symptomatology (2, 4, 6 and 8 days post-immunisation); 2) during the inductive phase: at score 0.5, score 1 and score 2; 3) at the peak of the disease (score 3); 4) during the recovery phase: at score 2 of recovery (score 2R), score 1 of recovery (score 1R) and 0 of recovery (score 0R) and 5) during the post-recovery phase, at 28, 32, 40 and 90 days post-immunisation (referred to as score 0R-28dpi, score 0R-32dpi, score 0R-40dpi and score 0R-90dpi). A total of 56 EAE-induced rats and 7 controls injected with vehicle were used for flow cytometry studies. For immunohistochemistry, 68 EAE-induced rats and 9 controls were processed.

2.3 Flow cytometry analysis

For flow cytometry, animals were anesthetised and intracardially perfused with phosphate buffer solution (PBS). Quickly, the entire spinal cord was dissected out and the meninges were carefully removed. In order to obtain cell suspensions, tissue was dissociated through meshes of 140 µm and 70 µm and digested with a mixture of DNase I (28U/ml; 10 104 159 001; Roche) and collagenase (0.2mg/ml; LS004194, Worthington). Subsequently, each cellular suspension was centrifuged for 20 min at 600g at 4ºC in a discontinuous-density Percoll gradient (17-0891-02; Amersham-Pharmacia) between 1.08 g/ml and 1.03 g/ml. Myelin in the upper layer was removed. Cells in the interphase and in the upper-phase were collected, washed in PBS + 2% serum and labelled during 30 min at 4ºC with different combinations of the following surface markers: anti-CD4-PECy5 (1:400; 554839; BD Pharmingen; San Diego, CA), anti-CD4-APC.Cy7 (1:400; 201518; Biolegend), anti-CD3-FITC (1:400; 557354; BD Pharmingen, San Diego, CA), anti-CD8α-PerCP (1:400; 558824; BD Pharmingen, San Diego, CA), anti-CD45RC-PE (1:400; 554888; BD Pharmingen, San Diego, CA) and anti-V65-PE (1:400; 551802; BD Pharmingen, San Diego, CA). Subsequently, for the detection of intracellular markers, samples were permeabilised for 40 min using the Foxp3 staining buffer set (00-5523-00; eBiosciences; San Diego, CA) and labelled for 30 min at 4ºC with anti-Tbet-PerCP.Cy5.5 (1:400; 45-5825; eBiosciences; San Diego, CA), anti-GATA3-PE (1:400; 560074; BD Pharmingen; San Diego, CA), anti-RORgamma-APC (1:400; IMG-6275G; IMGENEX; San Diego, CA) and anti-Foxp3-PE.Cy7 (1:400; 25-5773; eBiosciences; San Diego, CA) following the instructions specified in the manufacturer's protocol. In parallel, isotype-matched control antibodies for the different fluorochromes were used as negative controls and spleen samples as positive control. In order to perform the quantification of the total number of cells, a known volume of fluorescence beads (CytoCountTM, S2366, DakoCytomation) was added and mixed with each sample. Finally, cells were acquired using a FACScalibur or FACsCanto flow cytometer (Becton Dickinson; San Jose, CA), and the results were analysed using FlowJo® software. Quantification of total number of cells was performed following the methodology specified in the manufacturer's data-sheet (CytoCountTM, S2366, DakoCytomation).

2.4 Tissue processing for histological analysis

Animals processed for immunohistochemistry were sacrificed under deep anaesthesia and perfused intracardially with 4% paraformaldehyde in 0.1M PBS (pH 7.4) + 5% sucrose. Spinal cords (cervical and dorsal part) were dissected out immediately, postfixed for 4h at 4ºC in the same fixative, and then eight series of parallel longitudinal sections (40 µm thick) were obtained using a Leica VT 1000S vibratome. Series were stored at -20ºC in the Olmos antifreeze solution until their later use.

2.5 Single immunohistochemistry

Some parallel free-floating vibratome sections were processed for the visualisation of different subtypes of lymphocytes: CD3 for all T-cell populations, CD4 for T-helper and CD8 for T-cytotoxic. After endogenous peroxidase blocking with 2% H_2O_2 in 70% methanol for 10 min, sections were blocked in 0.05 M Tris-buffered saline (TBS), pH 7.4, containing 10% foetal calf serum, 3% bovine serum albumine (BSA) and 1% Triton X-100 for 1h. Afterwards, sections were incubated overnight at 4ºC with one of the following antibodies: 1) anti-CD3 (1:500; A0452; Dakopatts, Denmark), 2) anti-CD4 (1:1000; MCA55G; AbD Serotec) or 3) anti-CD8α (1:1500; MCA48R; AbD Serotec), diluted in the same blocking solution. Sections incubated in media lacking the primary antibody were used as negative controls, and spleen sections as positive control. After washes with TBS + 1% Triton, sections were incubated at room temperature for 1h with either biotinylated anti-mouse secondary antibody (1:500; BA-2001; Vector Laboratories, Inc; Burlingame, CA) or biotinylated anti-rabbit secondary antibody (1:500; BA-1000; Vector Laboratories, Inc; Burlingame, CA). After 1h in streptavidin-peroxidase (1:500; SA-5004; Vector Laboratories, Inc; Burlingame, CA), the reaction was visualised by incubating the sections in a DAB kit (SK-4100; Vector Laboratories, Inc; Burlingame, CA) following the manufacturer's instructions. Finally, sections were mounted on slides, some of them counterstained with toluidine blue, dehydrated in alchohol and after xylene treatment, coverslipped in DPX. Sections were analysed and photographed with a Nikon Eclipse 80i microscope joined to a Nikon digital camera DXM 1200F.

2.6 Double immunohistochemistry

Double-immunolabelling was carried out by firstly processing the sections with either CD3, CD4 or CD8 immunolabelling as described above, but using, as secondary antibodies, AlexaFluor® 488-conjugated anti-rabbit in the case of CD3, or AlexaFluor® 555-conjugated anti-mouse (1:1000, A31570; Molecular Probes) in the cases of CD4 and CD8. After several washes, these sections were incubated overnight at 4ºC with either rabbit anti-Iba1 (1:3000; 019-19741; Wako), mouse anti-CD4 (1:1000; MCA55G; AbD Serotec), mouse anti-CD8α (1:1500; MCA48R; AbD Serotec), mouse anti-TCRgamma+TCRdelta (V65) (1:250; ab23902; AbCam), rabbit anti-Tbet for demonstration of Th1 cells (1:1000; sc-21003; Santa Cruz Biotechnology), rabbit-anti-Foxp3 for demonstration of T-reg cells (1:2000; sc-28705; Santa Cruz Biotechnology) or rabbit anti-RORγ for demonstration of Th17 cells (1:2000; ab78007; AbCam) followed, in the cases of CD4 and CD8, by AlexaFluor® 555-conjugated anti-mouse (1:1000; A31570; Molecular Probes) and by AlexaFluor® 488-conjugated anti-rabbit for Iba1. In the cases of V65, Tbet, Foxp3 and ROR-γ after the primary antibody, sections were incubated with either biotinylated anti-rabbit (1:500; BA-1000; Vector Laboratories, Inc; Burlingame, CA) or biotinylated anti-mouse (1:500; BA-2001; Vector Laboratories, Inc; Burlingame, CA) followed by Streptavidin-Cy3 (1:1000; PA-43001; Amersham). Finally, sections were mounted on slides, dehydrated in graded alchohol

and coverslipped in DPX. Sections were analysed using a fluorescence Nikon Eclipse E600 microscope and photographed with a Leica DMIRE 2 confocal camera.

2.7 CD3+ cell quantification

For CD3+ cell quantification, a total of three EAE-induced animals per clinical score and three controls were used. The analysis was performed by using one entire longitudinal section of the cervical spinal cord per animal. Each section was photographed at 4x using a Nikon Eclipse 80i microscope joined to a DXM 1200F Nikon digital camera, and pictures taken were merged using Adobe Photoshop software. The total number of CD3+ cells per section was counted by the use of analySIS® software.

2.8 Statistical analysis

All statistics along the study were performed using the Graphpad Prism 4 software. One-way ANOVA and Tukey's post-hoc test were used to determine statistically significant differences.

3. Results

MBP-induced Lewis rat developed the first signs of EAE around 10 days post-immunisation (dpi), displaying loss of tail tonus (score 0.5). Afterwards, during the inductive phase, animals presented tail paralysis (score 1) followed by hindlimb paraparesis (score 2). Clinical symptomatology reached the maximum at score 3 (approximately around 12-14 dpi) when animals showed complete hindlimb paralysis. Animals stayed at score 3 for 1-2 days and afterwards spontaneously started to recover. During the recovery phase, animals progressively improved their mobility achieving scores 2R and 1R. Around 21dpi, animals were completely recovered (score 0R) and no clinical signs were observed. During the post-recovery phase (28dpi, 32dpi, 40dpi and 90dpi), the animals did not show any visible sign of disease.

It is important to highlight here that, as we have done in previous works [3, 4], animals to be analysed for flow cytometry and immunohistochemistry were selected not based on the days post-immunisation, but rather exclusively according to their clinical score, during the inductive, peak and recovery phases. This criterion has demonstrated itself to be very useful because, as already stated in our previous studies, the variability among animals in the same score, if any, is very low.

3.1 CD3+ cells

The study of sections immunolabelled for CD3 revealed that there was no presence of T-lymphocytes in the spinal cord of control animals (Fig.1 A). In contrast, in EAE animals, from score 0.5, few little round CD3+ cells were observed in the parenchyma (Fig. 1 B). The number of CD3+ cells increased progressively during the inductive phase, mainly accumulating around blood vessels (Fig. 1 C, D and J), reaching their maximum number at score 3, when they were also extensively distributed throughout the parenchyma (Fig. 1E).

During the recovery phase, from score 2R to 1R, although the number of CD3+ cells remained without significant changes (Fig. 1 J), the density of cells around blood vessels increased, whereas a progressive decrease in the number of CD3+ cells within the parenchyma was observed (Fig. 1 F and G). The major part of positive cells at score 0R was mainly accumulated in the surroundings of blood vessels (Fig.1 H). Quantitative analysis showed that at this score 0R, the number of CD3+ cells apparently decreased, although the value did not reach statistical significance.

During the post-recovery phase, at score 0R-40dpi, a high reduction of CD3+ cells was observed (Fig.1 J) although some perivascular CD3+ cells were still detected (Fig.1 I). At score 0R-90dpi, CD3+ cells completely disappeared (Fig.1 J).

3.2 CD3+CD4+ cells

The number and proportion of CD4+ cells within the gated CD3+ cell population was analysed by flow cytometry along the different phases of EAE evolution (Fig. 2 A). In contrast to control animals where no presence of CD3+CD4+ cells was found, in EAE animals, from score 1 in the inductive phase, a high number of CD4+T-helper lymphocytes was observed (Fig. 2B). The number of CD3+CD4+ cells remained without significant changes along the inductive and peak phases and score 2R, and only decreased from score 1R. During the post-recovery phase, at score 0R-32-40dpi, an important and marked decrease in the number of these cells was found (Fig. 2B and C). It should be noted that CD4+ T-cells represented around 70% of the CD3+ lymphocytes (Fig. 2D). This high percentage of CD4+ T-cells was maintained without significant changes at the different scores analysed during the inductive, peak and recovery phases. Only during the post-recovery phase, at score 0R-32dpi, was a slight decrease in the proportion of CD4+ cells observed although without reaching statistical significance (Fig. 2 B and D).

In all phases analysed, around 90% of CD3+CD4+ cells showed a CD45RC- phenotype, characteristic of effector/memory cells, and only few CD45RC+ naïve lymphocytes were detected (Fig. 2 E).

It is important to highlight that, the study of double immunolabelled sections for CD4 and Iba1 revealed the presence of a high number of CD4+ cells showing apoptotic nuclei, mainly at the score 2 in the inductive phase and the peak, in close relationship with activated Iba1+ microglial cells (Fig. 3).

3.3 Subtypes of CD4+ lymphocytes

The different subpopulations of CD4+ T-helper lymphocytes were determined by flow cytometry by using specific antibodies against lineage-specific transcription factors. After gating in the CD3+ cell population, combinations of CD4 with Tbet (for Th1 cells), RORγ (for Th17 cells) and Foxp3 (for T-regulatory cells) were analysed.

3.3.1 Th1 cells

As shown in Fig. 4, in EAE animals, a progressive increase in the number of CD4+Tbet+ cells in the gated CD3+ cell population was detected from score 1, reaching the maximum value at scores 2 and 3 (Fig. 4 A and B). From score 2R, an abrupt decrease in the number of CD4+Tbet+ cell population was observed (Fig. 4 A and B). This population of CD4+Tbet+ cells represented, at scores 2 and 3, around 7% of CD3+ lymphocytes (Fig. 4 C). During the post-recovery phase (score 0R-28dpi and score 0R-40dpi), Th1 cells were absent (Fig. 4 A and B).

3.3.2 Th17 cells

In EAE animals, some CD4+RORγ+ cells were observed during the inductive phase, the peak and at score 2R in the recovery phase (Fig. 5 A and B). A high increase in the number of CD4+ RORγ+ cells was found from score 1R in the recovery phase, reaching the maximum value at score 0R (Fig. 5 B). During the post-recovery phase, an important decrease in the number of CD4+RORγ+ cells was detected (Fig. 5 B). It should be noted that, whereas during the inductive and peak phases the subpopulation of CD4+RORγ+ lymphocytes represented around 7% of CD3+ T-cells, from score 1R in the recovery phase the percentage of CD4+RORγ+ lymphocytes increased until a value of around 50% at score 0R (Fig. 5 C). During the post-recovery phase, a decrease in the percentage of these cells was found although levels remained higher than those observed during the inductive and peak phases (Fig. 5 C).

In addition to CD4+RORγ+ cells, our analysis also demonstrated the presence of a population of CD4-RORγ+ cells (less than 10%) (Fig. 5 A).

3.3.3 T-regulatory cells

As shown in Fig. 6, during the inductive phase, the peak, and at score 2R in the recovery phase, only few CD4+Foxp3+ cells within the gated CD3+ population were observed (Fig. 6 A and B). From score 1R during the recovery phase, a substantial increase in the number of CD4+Foxp3+ cells was observed until score 0R, when the maximum quantity of these cells was found (Fig. 6 A and B). The number of these CD4+Foxp3+ cells significantly decreased during the post-recovery phase from score 0R-28dpi to score 0R-40dpi (Fig. 6 B). This population of CD4+Foxp3+ cells represented less

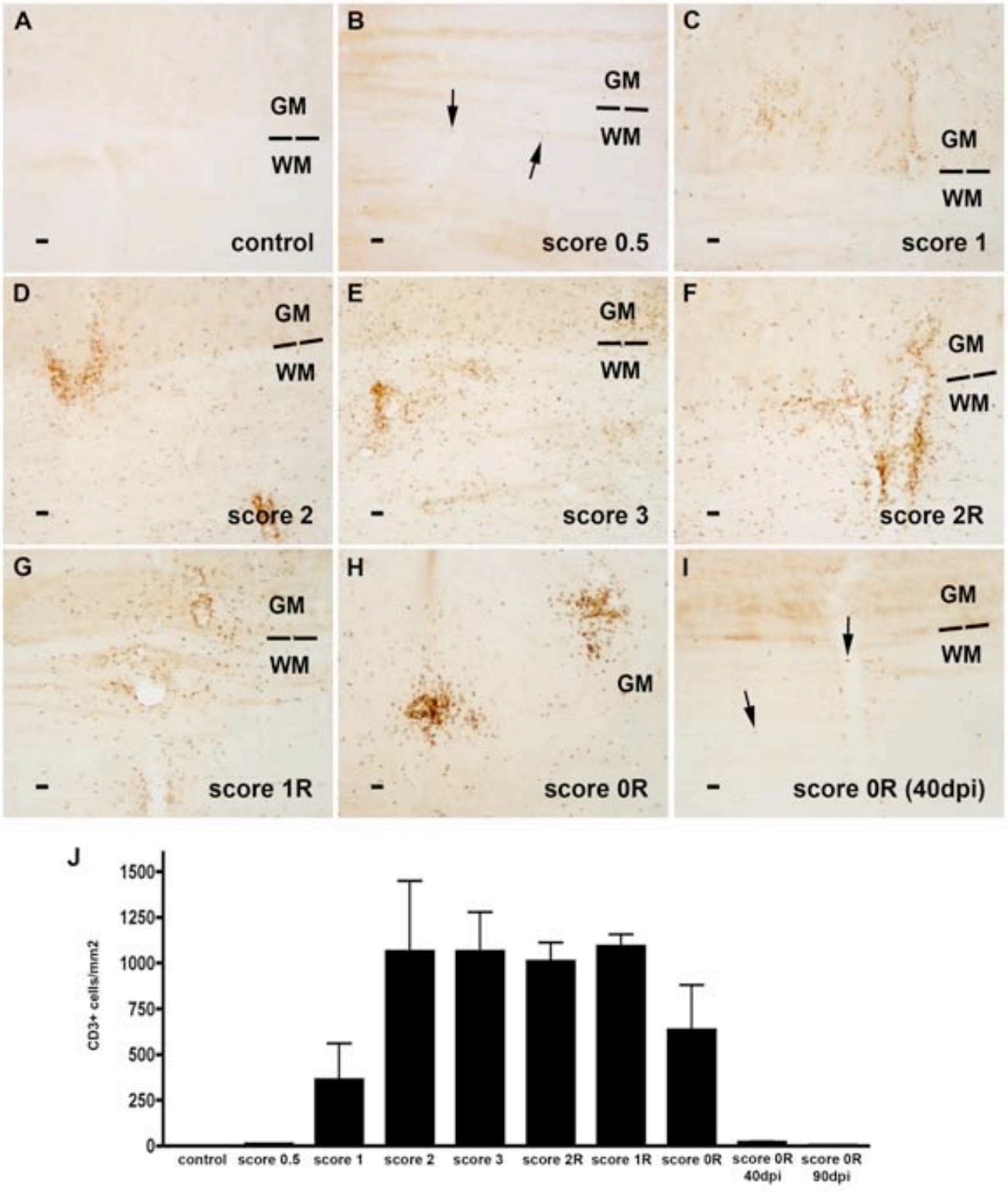

Figure 1. Dynamics of CD3+ cells. Immunohistochemistry for CD3 in control animals (A) and in EAE animals during the inductive (B-D), peak (E), recovery (F-H) and post-recovery phases (I). Arrows in B and I point to the few CD3+ cells observed in these scores. Note the progressive increase in the number of CD3+ cells in both the grey (GM) and white matter (WM) during the inductive and peak phases and the maintenance of these cells during both the recovery and post-recovery phases. Bar scale = 30μm. J) Histogramme showing analysis of CD3+ cell density observed in the different clinical scores.

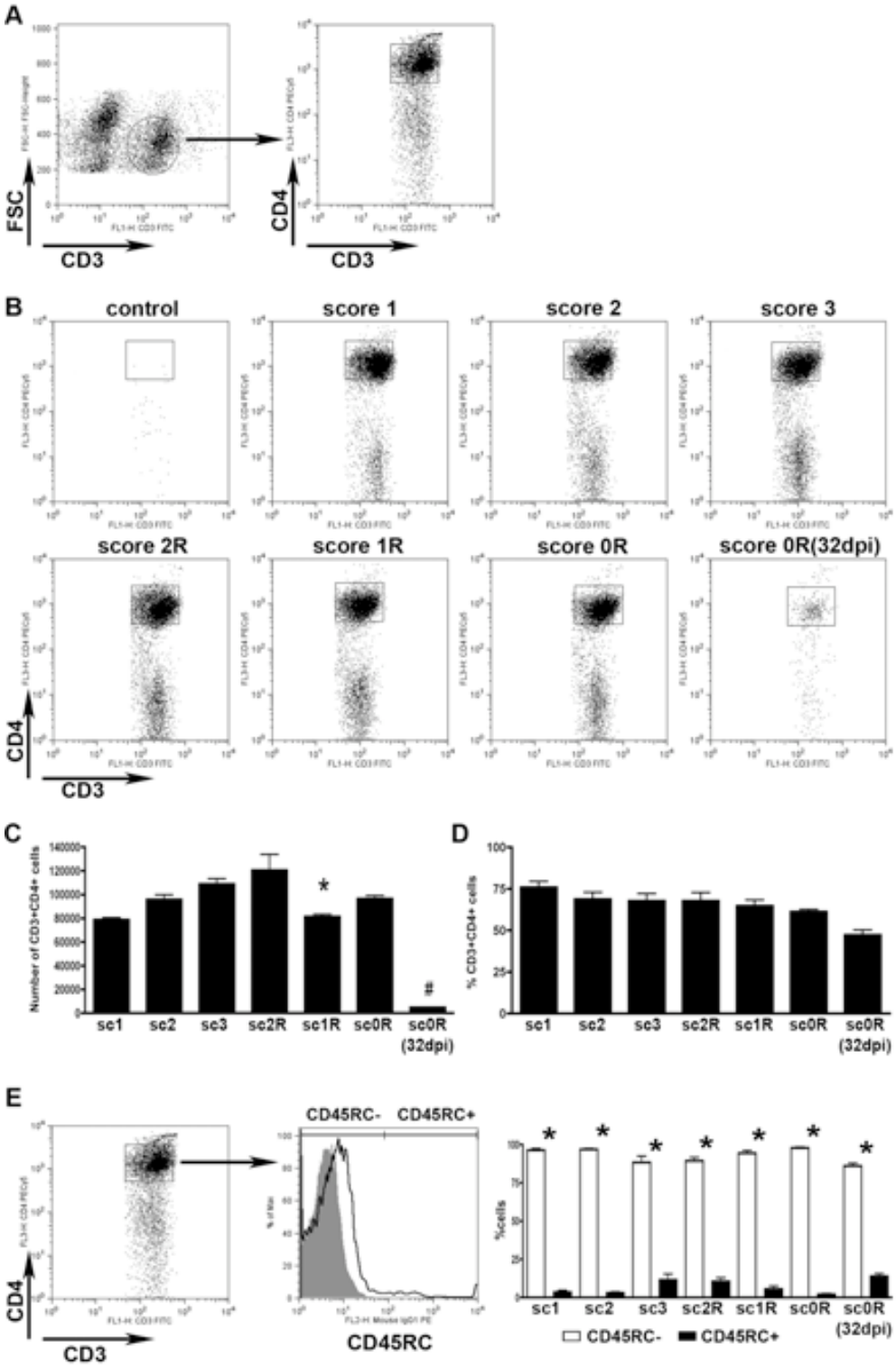

Figure 2. CD4 T-helper cells. A) Dot-plots exemplifying how the analysis of CD3+CD4+ cells was performed. CD3+ T-cells were gated (circle in left dot-plot) and the percentage of CD3+CD4+ cells was analysed in this gated population (square in right dot-plot). B) Representative dot-plots showing the dynamics of the CD3+CD4+ cell population (square) in both control and EAE animals during the inductive phase (score 1 and 2), peak (score 3), recovery phase (score 2R, 1R and 0R) and post-recovery phase (score 0R-32dpi). Note that in relation to controls, there is an important and persistent population of CD3+CD4+ cells in EAE animals. C and D) Histogrammes showing the values corresponding to CD3+CD4+ cell number and relative percentage along EAE evolution. E) On the left side, representative dot-plot of CD3+CD4+ cells of EAE animals. In the middle, representative histogramme where populations of CD45RC- cells (activated/effector lymphocytes) and CD45RC+ cells (naïve lymphocytes) were defined. Isotype control is represented in grey. On the right, histogramme showing the values of the percentages of CD45RC- (white columns) and CD45RC+ cells (black columns) in the different phases along EAE evolution (T-student, * p≤0.0001).

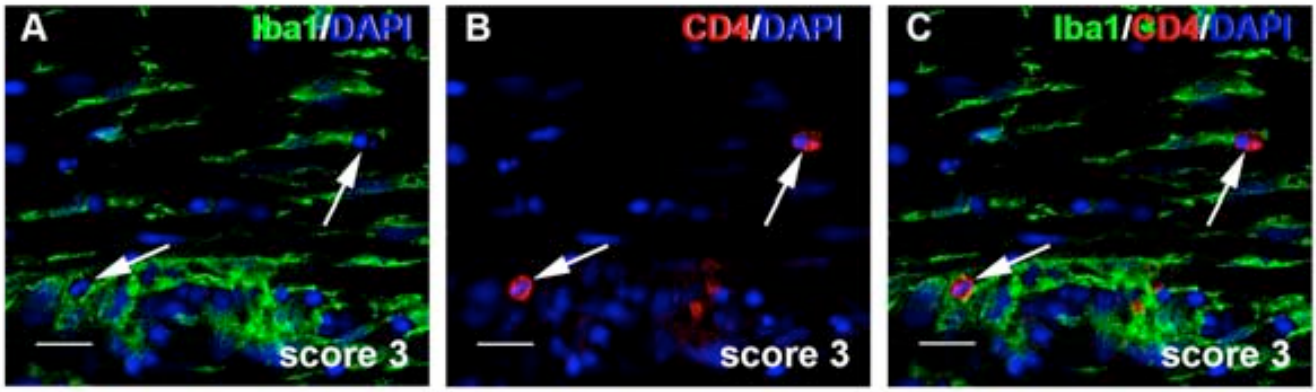

Figure 3. Double immunolabelling combining Iba1 and CD4 at the peak of the disease and counterstained with DAPI. A) Iba1+ cells (green) were observed closely with cells i displaying apoptotic nuclei (arrows). These apoptotic cells corresponded to CD4+ lymphocytes (arrows in B). C) Merged figure showing the close-relationship established between apoptotic lymphocytes and microglial cells. Bar scale = 20μm

than 10% of CD3+ T-cells at score 1R, and approximately 15% at score 0R (Fig. 6 C). Remarkably, at score 0R-28dpi, the proportion of these cells remained unchanged though the total number of CD4+Foxp3+ cells decreased considerably (Fig. 6 C). It was not until score 0R-40dpi when the proportion of this population underwent a substantial decline (Fig. 6 C). No significant Foxp3 expression in the population of CD4- cells was detected at the different scores analysed (Fig. 6 A).

In addition to flow cytometry analysis, the localisation of the different subtypes of CD4+ T-helper lymphocytes was studied by the use of double immunohistochemistry. These studies corroborated the temporal pattern observed by flow cytometry study. The presence of CD4+Tbet+, CD4+RORγ+ and CD4+Foxp3+ cells along the evolution of EAE was mostly accumulated in the vicinity of blood vessels (Figs. 4 D-F, 5 C-F, 6 C-F).

3.4 CD3+CD8+ cells

The percentage of CD8+ cells in the gated CD3+ cell population was analysed by flow cytometry (Fig. 7 A). In contrast to controls, in EAE animals, from score 1 in the inductive phase, around 6% of CD3+ cells corresponded to CD8+ cells (Fig. 7 B and C). The proportion of this population increased until about 10% at score 2 and then was maintained at similar levels during the peak and recovery phases. In the post-recovery phase, at score 0R-32dpi, a slight increase in the proportion of CD8+ cells within the CD3+ population was found, although without reaching significant levels.

The study of CD45RC expression in the gated CD3+CD8+ cell population demonstrated that during the inductive phase, the peak and scores 2R and 1R of the recovery phase, the major proportion of CD3+CD8+ cells corresponded to CD45RC+ naïve cells (Fig. 7 D). At scores 0R and OR-32dpi, no differences between the percentages of CD45RC+ versus CD45RC- CD8+ lymphocytes were detected.

Using immunohistochemistry the morphology and distribution of CD8+ cells in the spinal cord of control and EAE animals were analyzed (Fig. 8). In contrast to control animals in which no CD8+ cells were observed (Fig. 8 A), little round CD8+ cells were found in EAE animals, from score 1 (Fig. 8 B). At this score, CD8+ cells were located mainly in the vicinity of blood vessels in both the grey and white matter. At score 2, in agreement with flow cytometry data, CD8+ cell numbers increased considerably, mostly being located around blood vessels, although some positive cells were also found within the parenchyma (Fig. 8 C). At the peak, score 3, CD8+ cells were densely distributed in all of the parenchyma (Fig. 8 D).

During the recovery phase, from score 2R, a reduction in the number of CD8+ cells was found principally in the parenchyma (Fig 8 E). During this recovery phase, in addition to little round CD8+ cells, a sparse population of CD8+ cells exhibiting ramified morphology was detected in the parenchyma. The number of these ramified CD8+ cells increased progressively from score 2R to 0R, in both the grey and white matter. During the post-recovery phase, at score 0R-40dpi, no little round CD8+ cells were found, but some ramified CD8+ cells were still detected. No CD8+ cells were observed at score 0R-90dpi.

Double-immunofluorescence labelling for CD3 and CD8 demonstrated that little round CD8+ cells observed along the different phases of EAE corresponded to CD3+ lymphocytes (Fig. 8 I). CD8+ cells displaying a ramified morphology found during the recovery and post-recovery phases were not identified as being microglia or astrocytes, as they did not show co-localisation with either Iba1 (Fig. 8 J) or GFAP (Fig. 8 K).

3.5 γδT-cells

Flow cytometry analysis revealed that in EAE animals from score 1 in the inductive phase, a small population of cells within the gated CD3+ cell population expressed V65, a marker for gd T-cells (Fig. 9 B). The percentage of this population increased progressively during the inductive and peak phases, and slightly decreased during the recovery phase (Fig. 9 C). At score 0R-32dpi, a high increase in the proportion of V65+ cells within the CD3+ population was found at the same time that CD3+ cell numbers decreased.

Double-immunostained cells CD3+V65+ were found in all phases analysed, mainly being located in the surroundings of blood vessels, and some of them co-localised with CD3 (Fig. 9 C-E). In addition, CD3+V65- and CD3-V65+ cells were also found

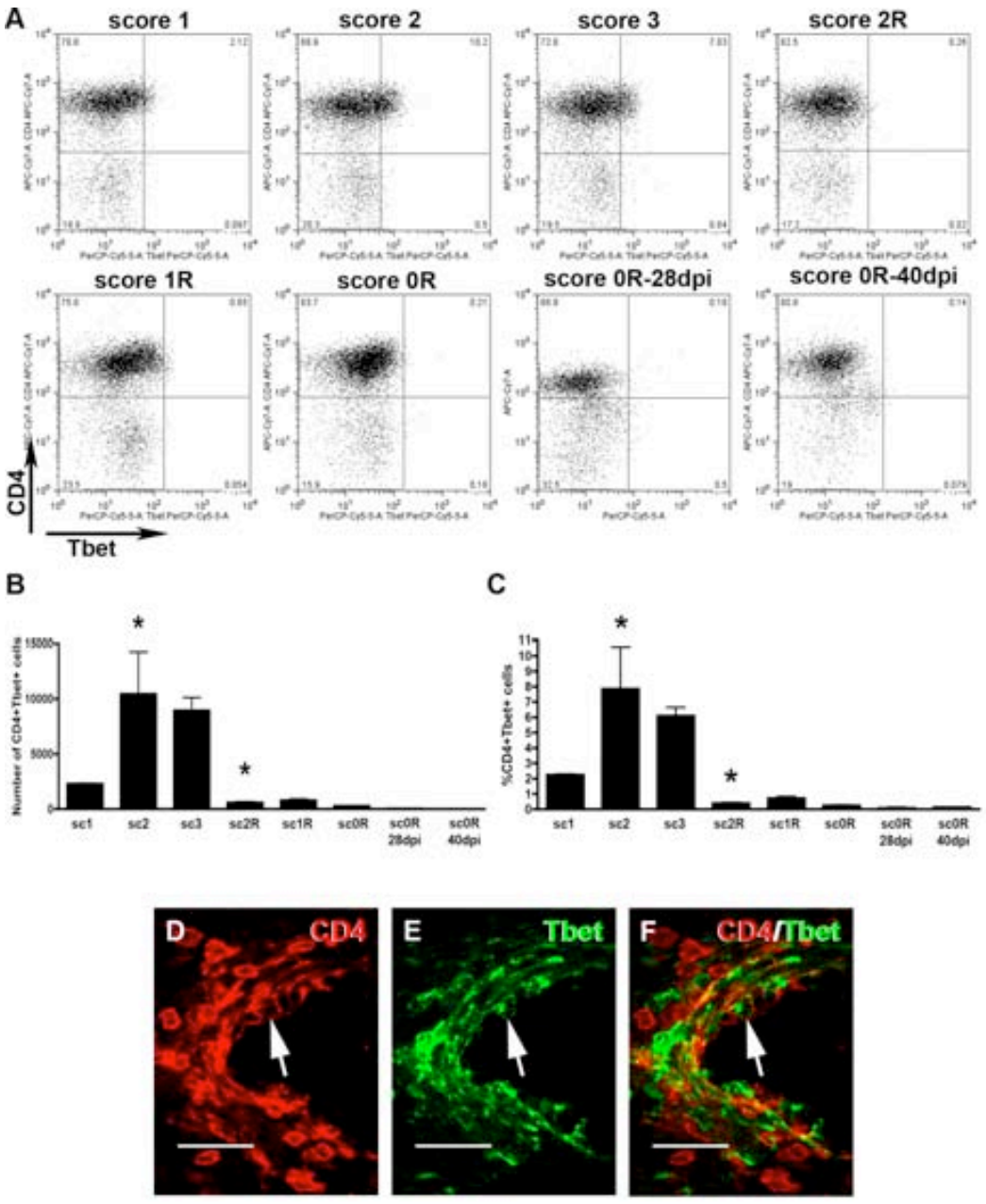

Figure 4. Dynamics of Th1 cell population. A) Representative dot-plots of the population of CD4+Tbet+ cells of EAE animals. Dot-plots were obtained by previously gating in the CD3+ T cell population. Different quadrants were defined by application of the appropriate isotype control. B and C) Histogrammes showing the values corresponding to the total number and the percentage, respectively, of CD4+Tbet+ cell population along EAE. Note that CD4+Tbet+ lymphocytes are found during the inductive and peak phases and abruptly decreased at score 2R of the recovery phase (ANOVA and Tukey's post-hoc test, *$p \leq 0.05$, with respect to the previous score). D-E) Double immunohistochemistry photographs showing a representative CD4+Tbet+ cell found around blood vessels. Arrows point to double-immunolabelled cell. Bar scale = 30μm.

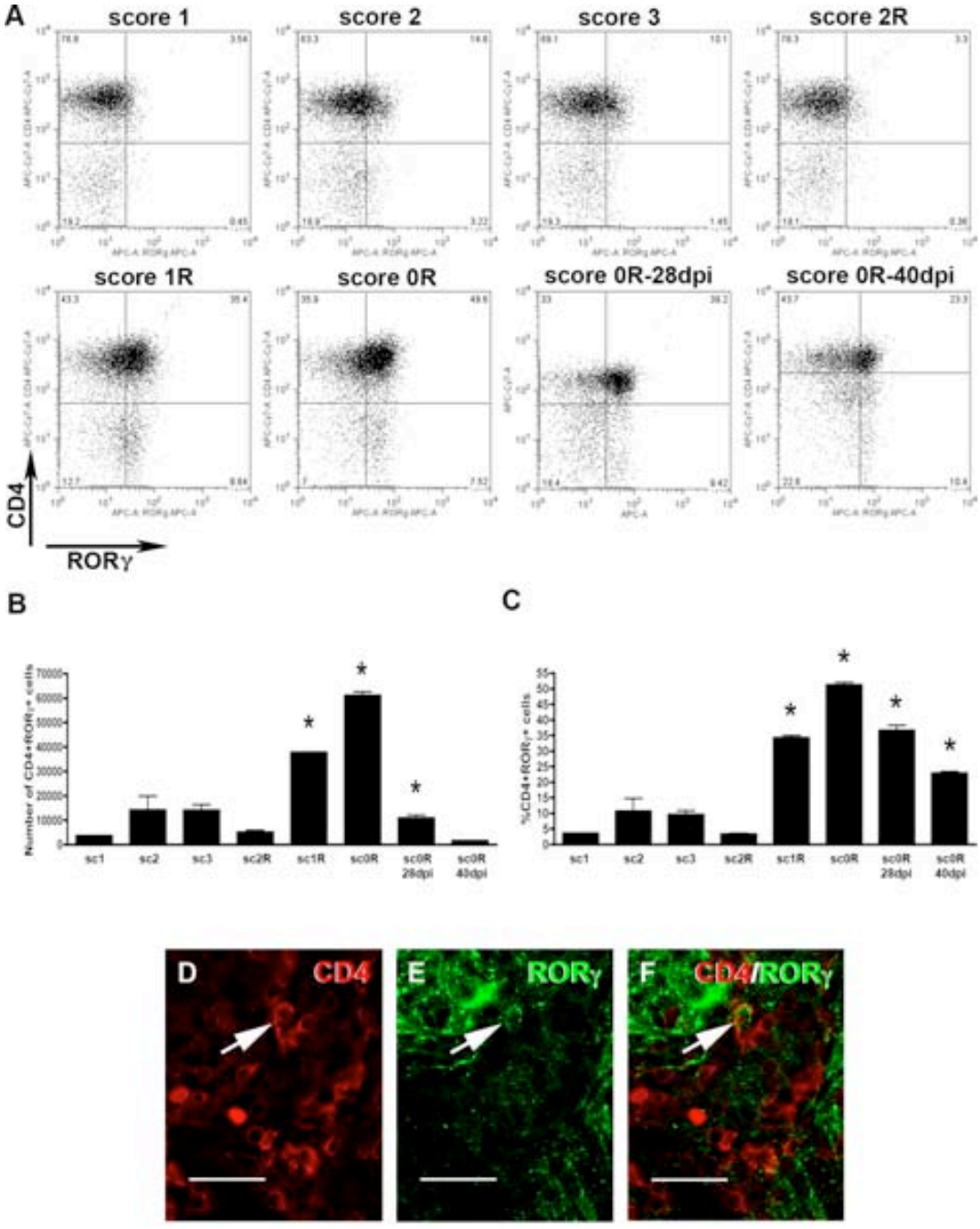

Figure 5. Dynamics of Th17 cells. A) Representative dot-plots of CD4+RORγ+ cells in the different phases along EAE evolution. Dot-plots were obtained after gating in the population of CD3+ T-cells. Different quadrants were defined by application of the appropriate isotype control. The total number and percentage of CD4+RORγ+ cells along the different phases of EAE are represented in histogrammes B and C, respectively. Note that during the recovery and the post-recovery phases, from score 1R, a great increase in the subpopulation of CD4+RORγ+ cells was observed (ANOVA and Tukey's post-hoc test, *$p \leq 0.001$, with respect to the previous score). D-E) Double immunohistochemistry photographs showing a representative CD4+RORγ+ cell observed around blood vessels (arrow). Bar scale = 30μm.

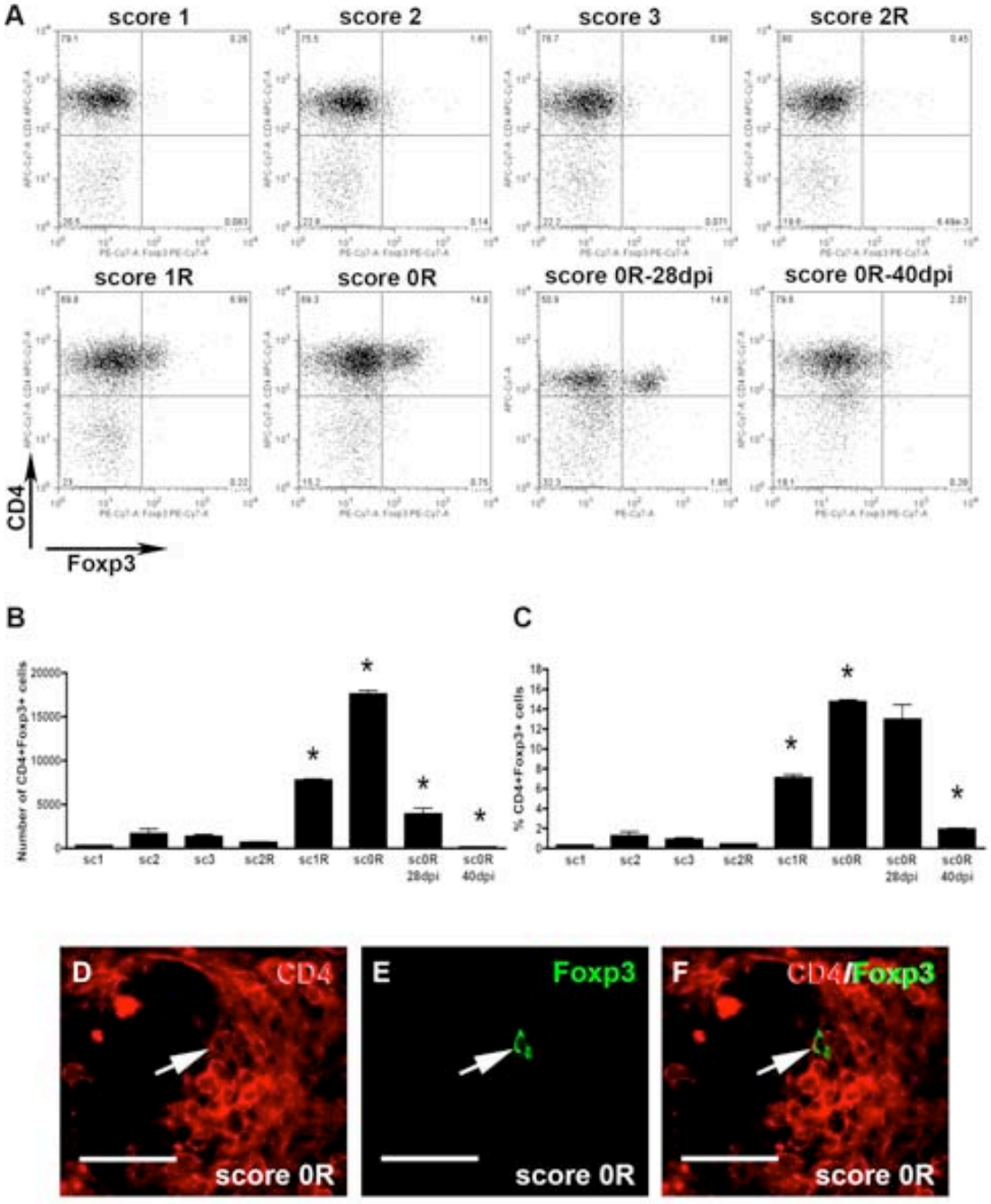

Figure 6. Dynamics of T-regulatory cells. A) Representative dot-plots of the CD4+Foxp3+ cell population along the different phases of EAE evolution. Dot-plots were obtained after gating in the population of CD3+ T-cells. Quadrants were defined by application of the appropriate isotype control. B and C) Histogrammes showing the total number and percentages of CD4+Foxp3+ cells, respectively, along the different phases of EAE. Note that although the number of CD4+Foxp3+ cells decreased at 0R-28dpi, their percentage remained high until score 0R-40dpi (ANOVA and Tukey's post-hoc test, *$p \leq 0.001$, with respect to the previous score). D-F) Double immunohistochemistry photographs showing a representative CD4+Foxp3+ cell found around blood vessels. Arrows point to double-immunolabelled cell. Bar scale = 30μm.

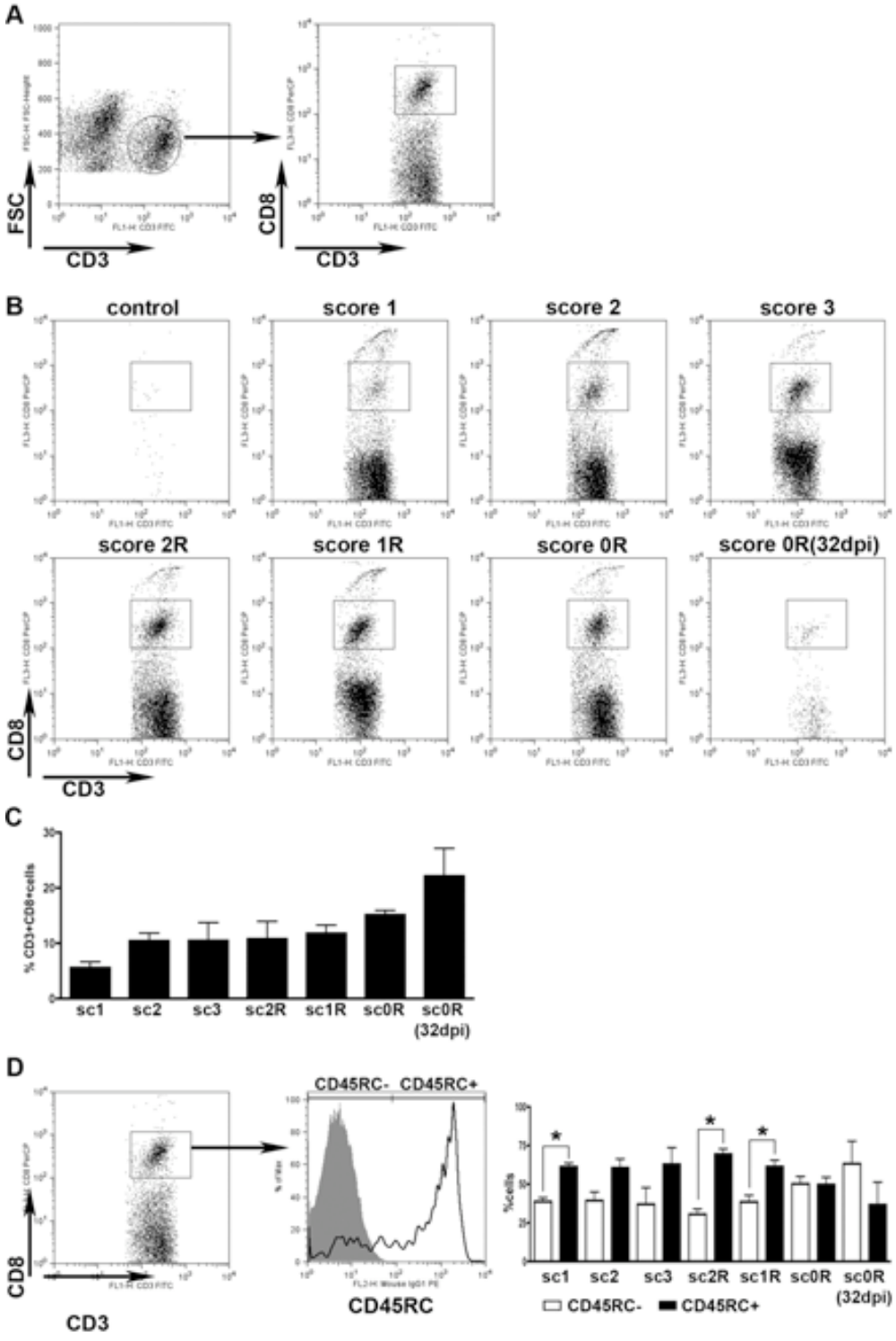

Figure 7. CD8+ T-cytotoxic lymphocytes. A) Dot-plots exemplifying how the analysis of CD3+CD8+ cells was performed. CD3+ T-cells were gated (left dot-plot) and the percentage of CD3+CD8+ cells was analysed in this gated population (right dot-plot). B) Representative dot-plots of CD3+CD8+ cell population (square) in both control animals and at the different phases along EAE evolution. C) The histogramme shows the values of the percentage of CD3+CD8+ cells at the different clinical scores along EAE. Note that the relative proportion of CD8+ lymphocytes was maintained with similar levels along all of the phases analysed, and even showed a tendency to increase in the post-recovery phase. D) On the left side, representative dot plot of CD3+CD8+ cells gated for the study of CD45RC expression (square). In the middle, representative histogramme where populations of CD45RC- cells (activated/effector lymphocytes) and CD45RC+ cells (naïve lymphocytes) were defined. Isotype control was represented in grey. On the right, the histogramme shows the values of the percentage of CD45RC- (white columns) and CD45RC+ cells (black columns) at the different phases along EAE evolution (T-student, * $p \leq 0.005$).

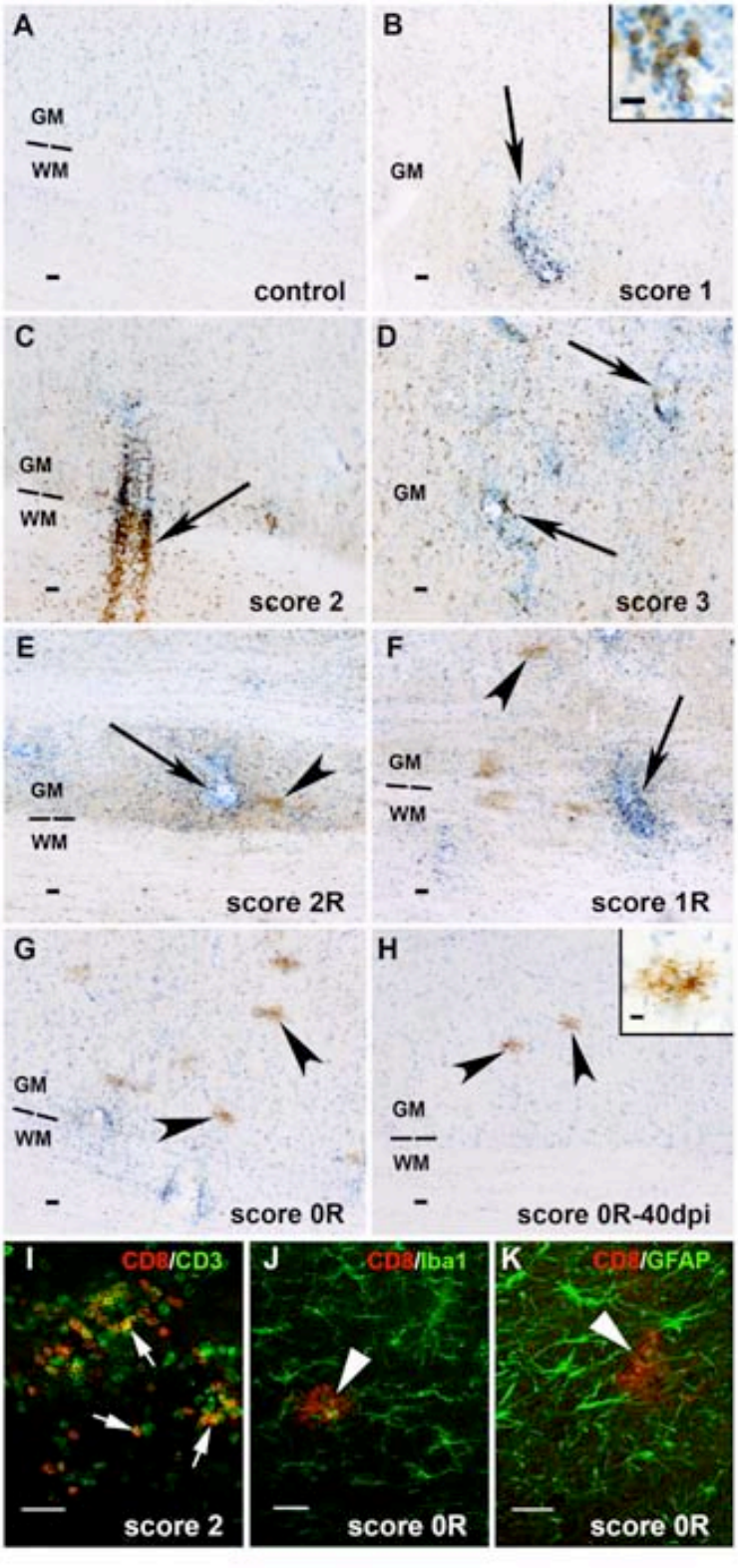

Figure 8. CD8 immunohistochemistry. In contrast to control animals (A) where no CD8+ cells were observed, in EAE animals, from score 1 (B), a progressive increase in the number of CD8+ cells was observed during the inductive and peak phases (B-D) in both the grey (GM) and white matter (WM). Note that at scores 1 and 2 cells were more concentrated around blood vessels (arrows in B-C), whereas at score 3 they were more scattered through the parenchyma (arrow in D). During the recovery phase, from score 2R to 0R, the number of positive cells progressively decreased, and again they were more frequent in the vicinity of blood vessels (arrows in E-G). The major part of CD8+ cells displayed little round morphology, as was evident in the insert at high magnification in Picture B, and were identified as T-cells by co-localisation with CD3 (arrows in I). During the recovery phase, in addition to little round CD8+ cells, some positive cells with a ramified morphology were scarcely found (arrowheads in E-G) (see insert in H). These ramified CD8+ cells did not show co-localisation with either Iba1 (J) or GFAP (K). During the post-recovery phase, at score 0R-40dpi, no little round CD8+ cells were observed, although some CD8+ cells exhibiting a ramified morphology were still found (arrowheads in H). Bar scale = 30μm.

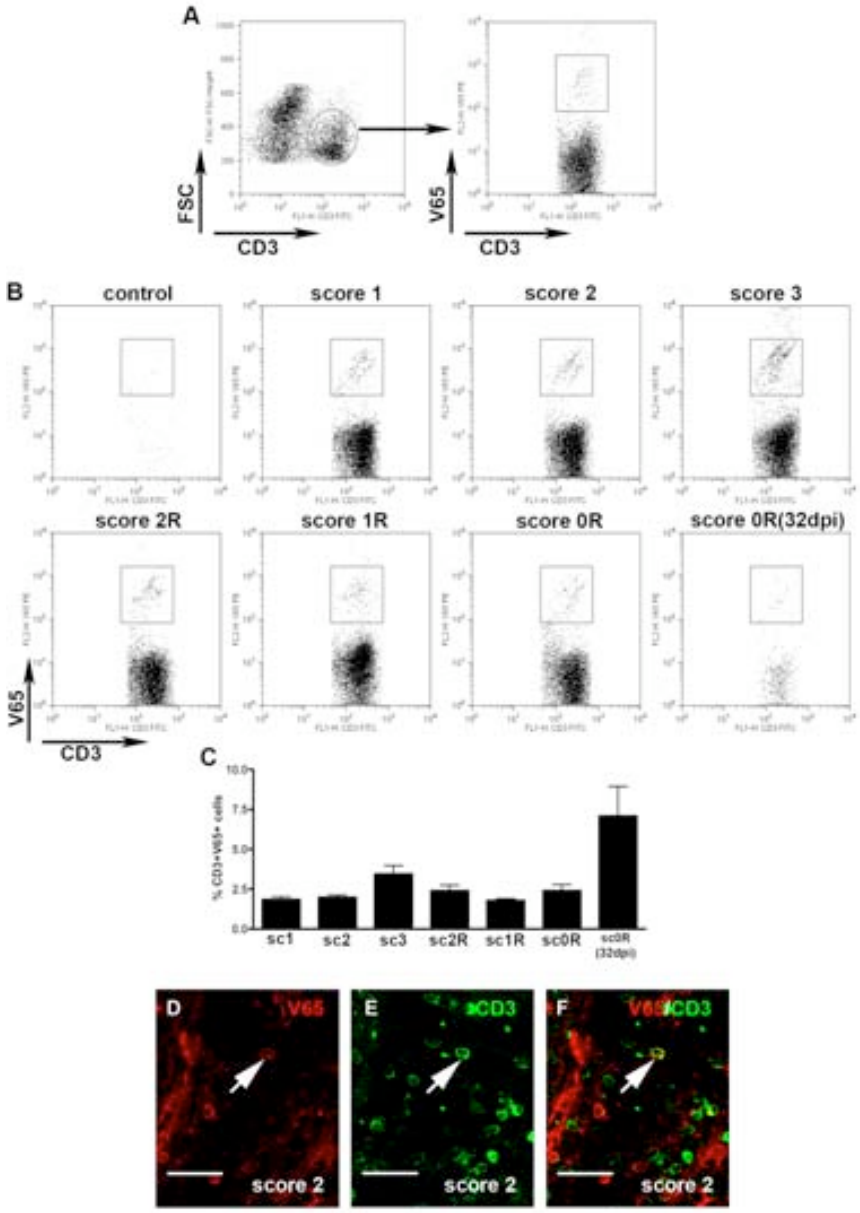

Figure 9. Dynamics of γδ T-cells. A) Dot-plots exemplifying how the analysis of CD3+V65+ cells (γδ T-cells) was performed. CD3+ T-cells were gated (left dot-plot) and the percentage of CD3+V65+ cells was analysed in this gated population (right dot-plot). B) Representative dot-plots showing the dynamics of the CD3+V65+ cell population (square) along the different phases of EAE. C) The histogramme shows the quantification of the percentage of the CD3+V65+ cell population in the different clinical scores along EAE evolution. D-F) Double-immunolabelling photographs showing a representative γδ T-cell displaying co-localisation of CD3 (green) and V65 (red) (arrows). Note that in addition to double-positive cells, some CD3+V65- cells were also found. Bar scale = 30μm.

4. Discussion

In the present study we have performed a detailed analysis of the different subtypes of lymphocytes that infiltrated the CNS along the different phases of the acute EAE model in Lewis rat. Our results show that during the inductive and the peak phases, the number of CD3+ cells increased in close relationship to disease severity. Nevertheless, during the recovery phase, although clinical signs decreased, the number of CD3+ lymphocytes remained very high without significant variations. Even during the post-recovery phase, CD3+ cells were still found within the parenchyma of the spinal cord, although their number decreased considerably. These findings do not agree with other works reporting that some treatments, such as an inhibitor of α4 integrins [27] or sRAGE [28], induced an improvement of clinical symptomatology of EAE in correlation with a decrease in lymphocyte infiltration. However, it is important to take into account that, although T-cells have been commonly recognised as pathogenic cells in EAE, nowadays it is clearly demonstrated that some subsets of lymphocytes, such as T-regs and Tr1 cells, may play a crucial role in the resolution of immune responses associated with autoimmunity [29] which may explain why the numbers of CD3+ T-cells remained high during both recovery and post-recovery phases. Noticeably, our current findings correlate with our previous observations showing that microglial cells in this EAE model remained activated [3, 4], pointing towards a cross-talk between both populations along the evolution of the disease, not only during the inductive and peak, but also during the recovery phase.

Similarly to CD3+ T-cells, we found that the proportions of CD4+ T-helper cells remained unchanged along the evolution of EAE, displaying a phenotype of activated/effector cells (CD45RC- cells)

throughout all phases analysed. Traditionally, CD4+ T-cells were considered as being the pathogenic lymphocytes in EAE, partly due to their capacity to induce the disease when passively injected into susceptible animals [30] and to studies showing the presence of CD4+ cells at the peak of the disease in different models of EAE [6, 8, 9]. Nevertheless, it is important to take into account that nowadays it is clearly demonstrated that CD4+ T-cells comprise a wide range of subpopulations with both pathogenic and regulatory/suppressive functions [12-14]. In this sense, by using lineage-specific transcription factors, we performed an accurate study of different subsets of CD4+ T-helper lymphocytes along EAE evolution, determining the specific temporal pattern of infiltration of Th1 (Tbet+), Th17 (RORγ+) and T-reg (Foxp3+) cells. Our findings revealed that these different subsets of T-helper lymphocytes are present in the spinal cord in specific phases along the course of the disease (Fig. 10). It was clearly demonstrated that the number of Th1 lymphocytes, CD3+CD4+Tbet+ cells, correlated directly with disease evolution, increasing progressively during the inductive phase, reaching the maximum at the peak and decreasing thereafter during the recovery phase.

In parallel to a decrease in Th1 cells, a high increase in Th17 and T-reg cell populations during the recovery phase was also found. Interestingly, although we found few Th17 cells within the parenchyma during the inductive and the peak phases of EAE, they greatly increased at the end of the recovery phase (at score 1R), peaking at score 0R. Th17 cells are commonly considered as a pathogenic population of lymphocytes, as they have been detected at the onset of EAE in mice [31]. Other studies have also shown that when injected into mice, they are able to induce EAE [17, 29]. In addition, recently published data indicate that Th17 cells infiltrated the CNS and induce passive EAE in mice only after an initial influx of Th1 lymphocytes [29]. Nonetheless, the pathogenic role of Th17 is controversial and is still under discussion [32] because it has been demonstrated that in addition to produce pro-inflammatory cytokines such as IL-17, they are also able to secrete anti-inflammatory cytokines such as IL-10 [33] and IL-22 [14]. Production of IL-10 by Th17 lymphocytes was correlated with increased RORγt expression [33]. This IL-10 production was induced by exposure of lymphocytes to TGF-β plus IL-6 [33], cytokines usually involved in the polarisation of the Th17 phenotype [34]. Moreover, the role of other cytokines produced by Th17 in EAE, such as IL-21, is not well established. Thus, it has been shown that the addition of IL-21 before the onset of EAE symptoms aggravates the disease [35], but blocking the IL-21/IL-21R pathway induced an enhancement of EAE severity [36, 37]. Furthermore, it has also been shown that DCs grown in the presence of IL-21 displayed an immature phenotype [38] and cannot induce T-cell responses [39, 40]. In this regard it is interesting to highlight that we have previously reported that parenchymal microglial cells acquire an immature DC phenotype during the recovery phase characterised by the expression of CD1 (an immature marker of dendritic cells) and MHCs but not co-stimulatory molecules [4]. Accumulation of Th17 lymphocytes in our study coincided with an abrupt decrease in Th1 cells. Although speculative, a plausible explanation for this delay in Th17 cell influx is that during the inductive and peak phases, Th1 cells inhibit Th17 differentiation. In agreement with this hypothesis, it has been recognised that the Th1-related cytokine IFN-γ inhibits Th17 differentiation [41]. As will be discussed later, the quick disappearance of Th1 cells may have been due to an extensive process of apoptosis mediated by microglia.

In addition to the Th17 lymphocyte population, during the recovery phase we also found an important increase in the number of Foxp3+ T-reg cells. Accumulation of Foxp3+ T-regs in the CNS has already been reported during recovery in mice EAE models [19, 20, 42], albeit to our knowledge this is the first study demonstrating accumulation of Foxp3+ cells within the spinal cord of EAE-induced rats. Several studies have demonstrated the beneficial role played by these cells in EAE pathogenesis. As such, injection of Foxp3+ T-reg cells, derived from EAE-recovered mice or *in vitro*-expanded, ameliorates EAE when injected into MOG-induced mice [20, 43]. In the same way, an induced decrease of Foxp3+ cell numbers *in vivo*, by means of anti-CD25 antibody treatment, renders these animals more vulnerable to EAE [44]. Since we found the major proportion of these cells during the recovery phase of EAE, we can speculate that Foxp3+ cells in this model play a putative role in the resolution of the immune response.

Noticeably, during the post-recovery phase, although the number of both Th17 and T-reg cells declined, the proportion of these cell populations remained high, mostly at score 0R-28dpi. This long-time permanence suggests that these cells still play an active role during this period even after the animals have fully recovered and do not show any clinical symptomatology. The presence of Th17 and T-reg cells during this period may be involved in the tolerance to further immunisations reached by Lewis rats after EAE induction [1].

Several studies have demonstrated that recovery from acute EAE is commonly associated with apoptotic elimination of pathogenic lymphocytes [45-48]. As our findings showed that when the recovery phase started the number of Th1 cells abruptly decrease, we can thus hypothesise that this decrease may be due to induced apoptotic elimination of these lymphocytes. This fits well with our previous findings [4] showing that during the inductive and peak phases, microglial cells displayed an immature dendritic-cell phenotype (MHC-class I and II+/CD1+/B7.1-/B7.2-) which may provide an anergic or apoptotic signal to the Th1-infiltrated lymphocytes. Nevertheless, we cannot discard the possibility that the different T-cell populations observed and their dynamics are the result of a phenomenon of lymphocytic plasticity, bearing the interconversion between T-cell subtypes, as has recently been postulated by some authors [49, 50].

In addition to CD4+ T-helper cells, our findings have also revealed the presence of a population of CD8+ T-lymphocytes and a population of γδ T-cells in EAE animals along all of the phases of EAE. The infiltration of CD8+ and γδ T-cells has been reported in mice models of EAE [8, 26, 51-54] but never in rat EAE models. The role of CD8+ T-cells in EAE is controversial and has been commonly associated with a regulatory, rather than disease-inducing, role. An exacerbation in EAE symptomatology has been reported in both CD8-deficient [52, 55, 56] and β-2 microglobulin KO mice [57]. However, it has also been shown that passive immunisation of encephalitogenic CD8+ T-cells can induce a severe and chronic EAE [23, 24]. Also, controversial findings have been found regarding the role played by γδ T-cells in EAE. Some studies have suggested that these cells play a pathogenic role, as they are found within the CNS around the peak phase of the disease and decrease during remission periods [26, 54]. Nevertheless, other authors have demonstrated that mice deficient in γδ T-cells could not recover from EAE [53, 58], suggesting a regulatory role of these cells. Our study does not show changes in the percentage of these two T-lymphocyte populations which remained constant along all phases of EAE. This fact is intriguing and does not allow us to correlate their presence with a protective or detrimental role in this acute EAE model. More studies in this sense are warranted in order to investigate the specific role of these cells in this model.

Conclusion:

In conclusion, we clearly demonstrate in this study that, although the number of T-lymphocytes inside of the spinal cord parenchyma remains constant along the three main phases of EAE (inductive, peak and recovery), there are specific lymphocyte-phenotype populations along these different phases. During the inductive and peak phases, lymphocytes exhibited a phenotype of Th1 cells, whereas during the recovery and post-recovery phases T-lymphocytes displayed a phenotype of Th17 or T-reg cells. In addition, two populations of T-cells (CD8+ and γδ) were found without any change in their proportions along the three different phases of the disease. Based on these findings, together with our previous studies focused on microglial cell reaction, [3, 4], we suggest that microglial cells regulate the immune response associated with EAE by controlling the apoptosis and/or differentiation of different subpopulations of T-lymphocytes.

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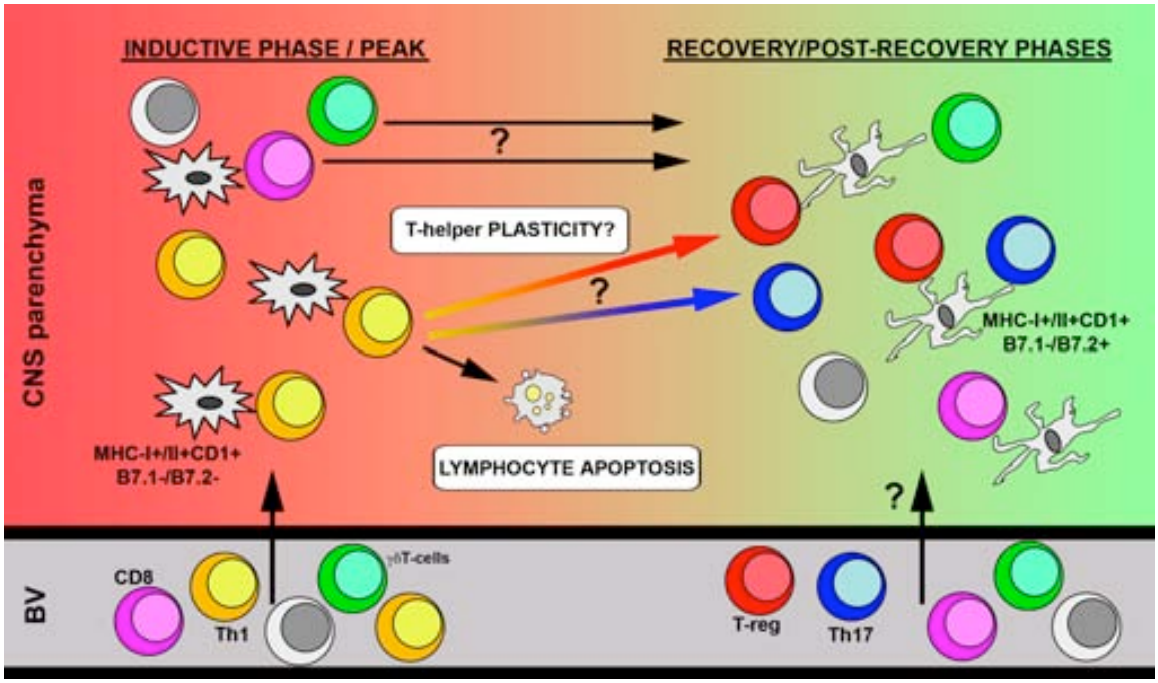

Figure 10. Schematic representation of the dynamics of specific lymphocyte populations along EAE evolution. The draft summarised the principal findings of the present study and proposed a mechanism of microglial modulation of lymphocyte subpopulations. During the inductive and peak phases of EAE, CD4+ lymphocytes infiltrate the spinal cord parenchyma. These cells display activated/effector cells showing, some of them, a Th1 cell phenotype. Microglial cells, previously reported displaying an immature dendritic cell-like phenotype (as reported in [4]) may interact with infiltrated lymphocytes and, as a result of this interaction, induce their anergy or apoptosis, which can be responsible for the abrupt decrease in Th1 cell population observed during the recovery phase. Our results do not allow us to discard the possibility that this decline in Th1 lymphocytes is the result of a phenomenon of lymphocytic plasticity, where Th1 cells differentiate into the populations of Th17 and T-reg cells observed exclusively in the recovery phase. We hypothesise that activated microglia together with these Th17 and T-reg cells, coming from Th1 differentiation or the infiltration of new cells recruited from blood vessels, may participate in the resolution of the inflammatory/immune response by means of secretion of anti-inflammatory mediators. In addition, two populations of lymphocytes (CD8+ and γδ cells) were found along the different phases of EAE.

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[Frontiers in Biosciences]

Antigen presenting mechanisms in EAE: role of microglia, macrophages and dendritic cells

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE), a well-established model of multiple sclerosis, is characterised by microglial activation and lymphocytic infiltration. Lymphocytic activation through the antigen presentation process involves three main signals, the first provided by the engagement of major histocompatibility complex molecules (MHC) with the receptor of T-cells (TCR), the second by the binding of co-stimulatory molecules and the third by the secretion or expression of T-cell polarising molecules in specific populations of antigen presenting cells (APC). Microglial cells are considered to be the main APC population in the central nervous system (CNS) because they are able to provide the three signals under specific situations. Specifically in EAE an increase in MHCs, co-stimulatory molecules and different T-cell polarising factors have been reported in microglial cells. However, a growing number of evidences suggest that dendritic cells (DCs), the main APC population in the peripheral immune system, may also participate in the regulation of T-cell responses taking place within the CNS. The origin of these CNS DCs is still not well known but in addition to their possible recruitment and infiltration from the periphery, one of the theories points towards a putative microglial origin. In this review we summarize the principal knowledge regarding microglial/macrophage function in EAE and their role in T-cell modulation, as well as the participation of DCs in the immune response associated to this disease.

Introduction

Microglial cells are considered to be the innate immune cell population in the central nervous system (CNS) (1-3). These cells originate from highly proliferative blood-borne myeloid cells infiltrating the brain parenchyma during foetal and early post-natal development to become amoeboid cells that, later, transform into the ramified microglial cells observed in adult animals (4-10). At least two subsets of microglial cells are nowadays recognised in adult CNS: 1) the so-called “resting” microglia, which are ramified cells distributed in all grey and white matter areas of the parenchyma, and 2) the so-called perivascular microglia (also called perivascular macrophages), which represent a minority population specifically located in the perivascular space of blood vessels (11). Ramified microglia are a permanent population of cells with a low turnover (12), with a very low CD45 expression and no expression of MHC-class II (13). In contrast, perivascular microglia/macrophages are periodically replaced (14), do not present the characteristic prolongations of microglia and express high levels of CD45, MHC-class II (15) and ED2 (16, 17). Due to their strategic location, these perivascular cells seem to play a key role in the initiation of immune responses in the CNS (11).

Over the years, numerous studies have demonstrated the fundamental role played by microglial cells in the CNS, not only in normal conditions where they control tissue homeostasis (18, 19), but also in all of those situations in which the integrity of the tissue is disturbed, as a result of a wide variety of situations including lesions, neurotoxicity or infections (20-23). In these circumstances, microglial cells are activated, showing specific reactivity patterns that fully depend on changes that take place in the specific micro-environment where they are located as well as the magnitude and type of injury. The process of microglial reactivity involves changes in their gene activation and phenotype, manifested in morphological modifications, increase/decrease or *de novo* expression of surface molecules and secretion of a wide range of substances such as cytokines, chemokines and trophic factors (21, 22, 24-26). Nowadays, this great variety of changes, coupled with increasing

evidence suggesting that different microglial subpopulations can co-exist within the CNS (27), indicates that microglial reactivity cannot be considered as homogeneous, but rather as a heterogeneous process that may have different outcomes according to where, how and what population of cells are activated.

Microglial cells not only interact with resident CNS cells as neurons or other glial cells, but are also able to establish a cross-talk with cells of the immune system that can be recruited to the CNS parenchyma under inflammatory conditions, through the production and secretion of the different molecules mentioned above. In particular, accumulating evidence *in vitro* and *in vivo* suggests that microglial cells may act as antigen presenting cells playing a role in the modulation of lymphocyte activation.

Antigen presentation mechanism

Antigen processing and presentation is a crucial mechanism in the modulation of the immune response, by which foreign molecules or intracellular antigens are processed and presented for recognition by T cells, thereby inducing their activation. In general, intracellular antigens, such as those produced by viruses, defective self-molecules or tumour-associated antigens, are presented by the major histocompatibility complex class I (MHC-class I). In contrast, antigens from extracellular pathogens, such as bacteria, parasites and toxins, are presented in the context of MHC-class II molecules. Whereas all nucleated cells express MHC-class I, only specialised cells, the antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells, in addition to expressing MHC-class I have the appropriate machinery for the processing and presentation of extracellular antigens through the MHC-class II molecules.

It has been shown that two main signals are involved in the mechanism of antigen presentation. The first signal is provided by the binding between MHC molecules bearing the antigen, with the receptor of T-cells (TCR) present on the surface of T-lymphocytes. This signal confers the specificity of the mechanism, as MHC-class I is specifically recognised by CD8+ T-lymphocytes, whereas MHC-class II exclusively binds CD4+ T-cells. The second signal, the so-called co-stimulatory signal, is antigen-independent and is produced by the engagement of different receptors and their respective co-receptors expressed on the surface of both APCs and lymphocytes (28). The presence of these co-stimulatory signals has been demonstrated to be essential for the full activation of T-cells, as binding of the TCR with the MHCs in the absence of co-stimulation can lead to apoptosis or anergy of T-cells (29). Different combinations of co-stimulatory molecules providing stimulatory or inhibitory signals have been described (30). For example, the binding of ICOS, CD154 or OX40 with their corresponding ligands B7h, CD40 and OX40L delivers a stimulatory signal in lymphocytes, inducing their activation and proliferation, whereas the engagement of other co-stimulatory molecules like PD-1, with its receptor PD-L1, triggers an inhibitory signal causing the apoptosis of lymphocytes. Among the different co-stimulatory molecules currently described, the pair that plays a more relevant role in T-cell activation is the one formed by B7 molecules (B7.1 and B7.2, also known as CD80 and CD86), present in the APC surface and their receptors CD28 and CTLA-4 expressed in lymphocytes (31-34). Binding of B7.1 or B7.2 with CD28 generates a stimulatory signal in T-cells inducing their proliferation, expression of anti-apoptotic molecules and secretion of different cytokines. In contrast, the binding of B7.1 and B7.2 with CTLA-4 results in an inhibitory signal that mediates the stoppage in proliferation and cytokine secretion, leading to the end of the immune response (32, 35). Therefore, the delicate balance between positive and negative signals conducted by different co-stimulatory molecules may provide different outcomes of the immune response (See Figure 1 for a summary).

In addition to these two well-known signals, a growing body of evidence indicates the existence of a third type of signal by which APCs, particularly dendritic cells (DCs), may also regulate the immune response. This third signal consists of the expression of specific sets of T-cell polarising molecules either soluble or membrane-bound (see Fig. 2). In general, Type 2 IFNs, IL12, IL23 and ICAM-1 are considered to be Th1 polarising molecules; IL4, MCP-1 and OX40L are Th2 polarising molecules; and IL10, PD-L1 and ILT3/4 are viewed as regulatory T-cell polarising molecules (36).

The selective expression profile of all of these polarising molecules is fully dependent on the signals that the DCs receive during their maturation process (Fig. 2). The binding of pathogens to selective “pattern recognition receptors” or factors produced by tissue cells in response to these pathogens induce different signalling in immature DCs that promotes the specific expression and production of these polarising factors. Basically, DCs exposed to viruses or intracellular bacteria promote Th1 responses, some helminths induce Th2, and the presence of regulatory factors or specific pathogens such as *Schistosoma mansoni* support the development of T-regulatory cells (For a more detailed review, see 37).

Antigen presenting cells

In general terms, it is assumed that those cell populations that have the capacity to process and present antigens and display them to T-cells are APCs. DCs are considered to be the most specialised APC type in the periphery. They are generated in the bone marrow and migrate as precursor cells to sites considered to be potential points of entry for pathogens. Thus, there are subsets of resident DCs in the skin, in the gastrointestinal and respiratory tracts and in lymphoid organs which can be distinguished by the expression of different cell-surface markers that are not only dependent on the specific organ but also on the animal species (38-40). In steady-state situations, DCs display an immature phenotype (41, 42) characterised by: 1) expression of specific receptors and molecules such as Fc- and mannose-receptors whose presence confers to them a high capacity to phagocytose or endocytose antigens, and 2) high expression of MHC molecules, but low or absent expression of co-stimulatory or other molecules involved in T-cell activation. This phenotype renders these immature DCs able to process antigens but not to activate T-cells. Immature DCs are, however, not inactive cells but they rather continuously circulate through tissues and into lymph nodes capturing self-antigens as well as innocuous environmental proteins, playing an active role in the maintenance of tolerance (43). Under inflammatory conditions, immature DCs become activated and differentiate into mature DCs. This maturation process involves the down-regulation of molecules related to the antigen capture and processing mechanism, and the up-regulation of the antigen presenting machinery, including an increase or *de novo* expression of co-stimulatory molecules (41). Moreover, mature DCs experiment changes in their expression pattern of chemokine receptors that allow them to migrate to the lymph nodes where they activate T-cells. For instance, DCs decrease molecules related to their tissue homing such as CCR5 and on the other hand, increase CCR7 expression, a molecule involved in their migration to the nearest lymph node.

In addition to DCs, macrophages and B-lymphocytes can also act as APCs (44). They are considered non-professional APCs because, in contrast to the professional DCs, they cannot activate naïve T-cells and they do not express MHCs constitutively. Nevertheless, they have the necessary machinery to capture and process antigens, and under inflammatory conditions they increase MHC expression, becoming able to activate T-cells.

For many years it has been assumed that there were not DCs in the CNS. This, together with the presence of the blood brain barrier, the lack of lymphatic vessels and the fact that skin grafts, viruses, bacterias or antigens directly inoculated in the parenchyma do not induce an immune response (45-48), contributes to the initial views of the CNS as an immune-isolated site. In this context, microglial cells were considered to be incompetent immune cells. However, during the last decade, the capacity of microglial cells to interact with immune cells recruited into the CNS parenchyma under several situations has been widely reported (3, 23, 49, 50), suggesting that microglia may be considered as being APCs in the CNS and under certain circumstances they differentiate into DC-like cells. Furthermore, as shall be discussed in detail later, in addition to DCs of a microglial origin, an increasing number of evidence suggests that specific subpopulations of bone marrow-derived DCs may reach the CNS parenchyma under certain inflammatory conditions, such as focal cortical ischemia (51), stroke (52), excitotoxic lesion (53), delayed type-hypersensitivity lesions (54) or experimental autoimmune encephalomyelitis (15, 54).

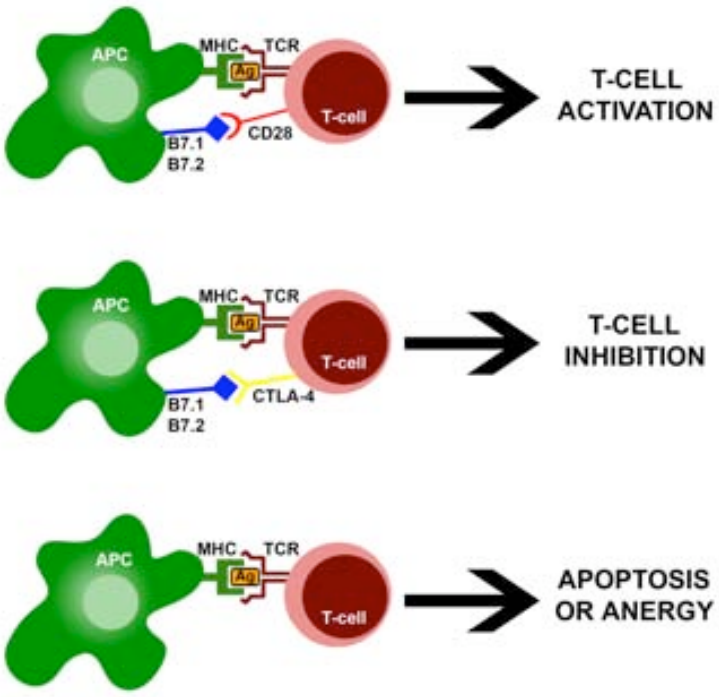

Fig. 1 Putative effects derived from the interaction between antigen presenting cells (APC) and T-cells

Experimental autoimmune encephalomyelitis

One of the most useful animal models for the study of the interactions established between CNS resident and peripheral immune cells is experimental autoimmune encephalomyelitis (EAE), which is considered to be a model of human multiple sclerosis (MS). EAE is characterised by a progressive difficulty in mobility that originates in the tail and hindlimbs and gradually progresses to cause paraplegia or even tetraplegia. Associated with this clinical symptomatology, animals induced with EAE experiment a progressive and significant loss of weight, mimicking what also happens in MS. There are several models of EAE with particular symptomatological and histopathological features that reproduce different forms of human MS (55, 56). Thus there are models, such as that induced by myelin oligodendrocyte protein (MOG) immunisation in C57BL/6 mice, which resemble a form of chronic MS; others, such as that induced in SJL mice by proteolipid protein (PLP) injection, which reproduce relapsing-remitting MS, which alternate stages of paralysis with stages of recovery, and also some models like that caused in Lewis rats by myelin basic protein (MBP) immunisation, which mimic an acute phase of MS characterised by a single peak of disability followed by a full and spontaneous recovery. Multiple factors including the species, strain, sex and age of animals used, as well as the peptide and the protocol utilised in the immunization process, seem to be crucial to yield the specific type of EAE. Susceptible animals, mainly rodents, can be induced by: 1) active immunisation, which consists of the subcutaneous injection of an emulsion containing nervous tissue or any peptide of myelin proteins together with a coadjuvant, or 2) passive immunisation, based on the intravenous injection of T-lymphocytes activated against myelin proteins.

EAE primarily affects the white matter of the spinal cord although today it has also been reported that the spinal cord grey matter and other CNS areas such as the cerebellum and the brainstem are affected (57, 58). As occurs in human MS, the main histopathological features of EAE are glial reactivity, mainly microglial activation, and a large infiltration of peripheral immune cells, principally T-cells (3, 24, 59). Due to the autoimmune nature of EAE and MS, it was widely accepted that CD4+T cells with a Th1 pro-inflammatory phenotype were the principal lymphocytes involved in the disease (60). Over the last few years, however, the identification of different subsets of lymphocytes with varied functions and cytokine profiles (61-63), have led to the re-evaluation of this initial assumption. It has been demonstrated that Th17 lymphocytes, a subtype of CD4+ T-cells (64, 65), are able to induce EAE when injected into mice (66). It has also been shown that Th17 cells are present in the spinal cord at the onset of EAE in mice (67), suggesting a pathogenic role of these lymphocytes. On the other hand, subtypes of regulatory cells, such as the extensively studied Foxp3 T-regulatory cells (Treg), are also reported in EAE. Treg cells have been detected during recovery in mice (68-70), and their beneficial role in EAE has been clearly demonstrated (69, 71, 72). Furthermore, in addition to CD4+ T-cells, some authors have also suggested the participation of other types of lymphocytes, such as CD8+ T-cells and $\gamma\delta$ T-cells (73-76).

Microglia, macrophages and monocytes in EAE

Microglial reactivity has been reported in different models of EAE (77-83). The major part of these studies has focused on the peak of the disease, where maximal levels of microglial activation were found. However, only few reports have analysed the temporal pattern of microglial reactivity along the course of EAE. Specifically in acute EAE, microglial reactivity has been detected in close correspondence with the increase in clinical symptomatology along the inductive and peak phases of the disease (83). Despite the large amount of literature describing microglial reactivity in EAE and human MS, the exact function played by these cells in these diseases still remains to be established. On the one hand, the observations of maximal microglial reactivity at the peak of the disease, together with the fact that the decrease in microglial reactivity, induced by treatments such as macrophage inhibitor factor (MIF) (84) and MW01-5-188WH (85) or observed in microglia-depleted animals (86), has been associated with beneficial effects in

EAE, led to the perception of microglia as detrimental cells in EAE pathogenesis. Moreover, it has been reported that activated microglia can release a wide range of molecules such as cytokines, chemokines and nitric oxide which may contribute to the recruitment of immune cells and the spread of the inflammatory response in the CNS (2, 24).

On the other hand, nevertheless, it has also been reported that during EAE remissions, despite the gradual recovery of animals, microglial cells remained very reactive in both mice (82) and rat EAE models (83), suggesting a putative beneficial role of these microglial cells. Microglial reactivity has also been described in EAE-resistant rats (87) and in normal-appearing white matter in human MS (88). Furthermore, earlier recovery from EAE symptoms has been reported in mice administered with tufsin, a microglial/macrophage activator (84). In this sense, the capacity of microglial cells to also produce protective factors such as anti-inflammatory cytokines, prostanoids and trophic factors which may contribute to the termination of the inflammatory response in the CNS, has been extensively demonstrated (2, 24).

In addition to morphological changes, an increase in the number of reactive microglia/macrophage cells in both chronic (79) and acute EAE (83) has been reported. This increase could be due to resident microglial cell proliferation or it could be the result of the recruitment of blood-borne monocytes, a population whose infiltration along the course of EAE has been described (89-91). The lack of good markers to distinguish between macrophages coming from the monocytic lineage and reactive macrophage-like microglial cells makes the analysis of the contribution of infiltrated monocytes to the final number of microglial/macrophage cells difficult.

Some evidence indicates that besides microglia, the perivascular macrophage population may also play a role in the pathogenesis of EAE. They are commonly distinguished from microglial cells by their specific expression of ED2 in rats (16) and its counterpart CD163 in humans (17). Data in the acute EAE model induced in Lewis rat has been reported an increase in the number of ED2+ cells before the appearance of clinical signs (92), which is in close relationship with the progressive increase in EAE symptomatology (83). Moreover, these ED2+ cells infiltrate the CNS parenchyma specifically at the peak of the disease (83). Due to their strategic location and their constitutive expression of MHC-class II (15), perivascular macrophages seem to be involved in the initiation of the immune response associated with EAE, perhaps contributing to T-cell activation, a step required for the subsequent infiltration of T-cells into the parenchyma (93, 94). Indeed, experimental evidence based on the selective elimination of perivascular macrophages by the use of chlorodate lyposomes (95) revealed that their depletion produced a decrease in the symptomatology (92), reinforcing the idea of an active participation of these cells in EAE evolution.

Microglia regulate the lymphocyte response

Infiltration and retention of T-cells in the CNS requires their interaction with local APCs (93, 94). As microglial cells are currently considered to be the principal APC within the CNS parenchyma (2, 24), it is assumed that, in the CNS, they may play a key role in modulating the lymphocyte response including their proliferation, differentiation and apoptosis through the antigen presenting mechanism. Although in basal conditions, microglia do not express MHC-class I and MHC-class II, it has been shown that once activated they can rapidly express these molecules in a variety of situations (1, 96).

Specifically in EAE, it has been widely reported that when microglial cells become activated, they increase the expression of MHC molecules in both mouse (79, 97-99) and rat models (100-104). The role played by these MHC molecules in EAE showed controversial results. On the one hand, some studies pointed

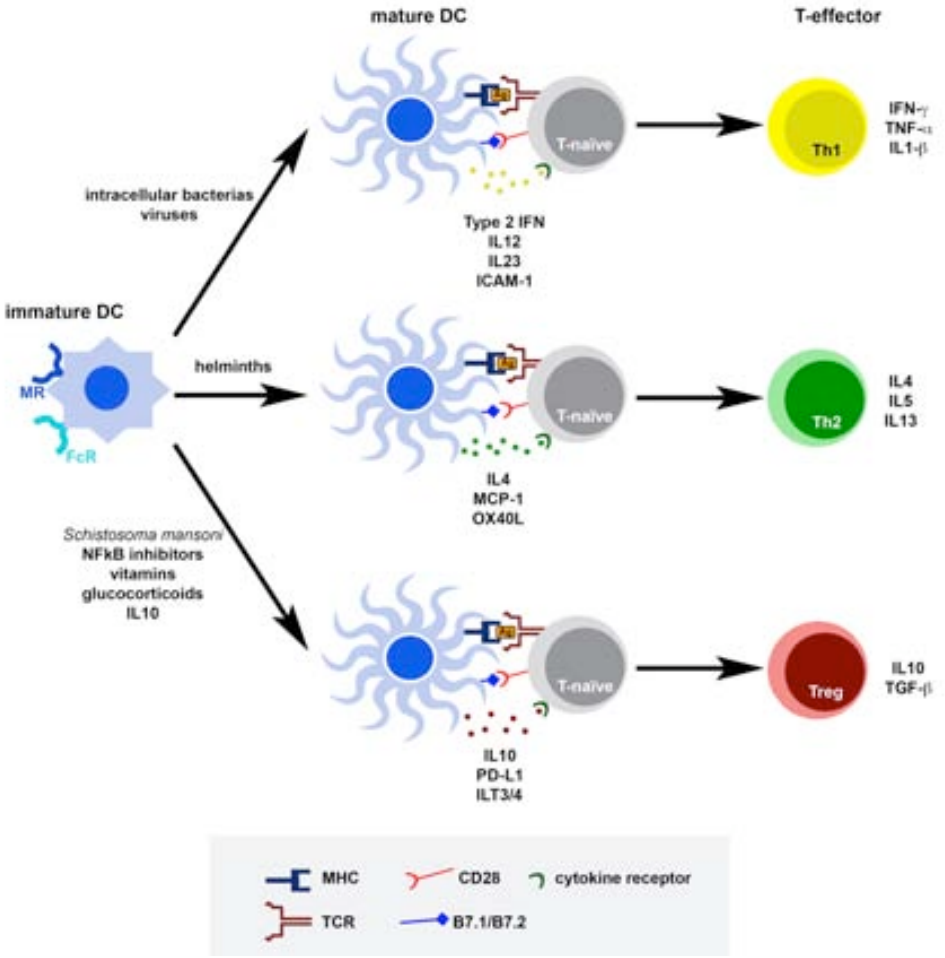

Fig. 2 Summary of signals leading to the maturation of immature dendritic cells (DCs) into different subtypes of mature DCs. These mature DCs secrete different T-cell polarising molecules that regulate the transformation of T-naïve cells into distinct T-effector cells

towards a detrimental role, as it has been reported that some treatments with a positive effect in EAE were associated with a decrease in MHC-class II expression (105, 106). However, on the other hand, a growing number of evidence indicated a putative beneficial effect of these molecules. It has been shown that MHC-class I KO mice present aggravation of EAE symptoms (107, 108) and MHC-class II KO showed impairment of remyelination after Theiler's murine encephalomyelitis (109). Interestingly, in the acute EAE model induced in Lewis rat, the expression of both MHC-class I and class II molecules increased progressively during the inductive and peak phases correlating with the increase in clinical symptomatology, but levels of MHC expression remained also high during the spontaneous recovery phase, even in animals that completely recovered and do not show any symptomatology (15).

At this point, it is important to highlight that MHC expression *per se* is not sufficient for antigen presentation, and co-stimulatory signals are necessary for the complete activation of T-cells (28, 31). The involvement of co-stimulatory molecules in EAE evolution comes principally from studies showing the effects of the absence of these co-stimulatory molecules (For a review, see 110). Thus, a reduction of EAE severity has been correlated with the blockage of CD28 (111, 112) or B7 (113, 114), whereas the blockage of CTLA-4, the co-stimulatory signal that drives the ending of the immune responses, has been shown to produce a worsening of the disease (115).

The question of whether a specific pattern of MHC and co-stimulatory molecule expression governs the evolution of EAE remains unresolved, and so far it is not clearly known which are the cells that express these molecules. Differences in the expression of B7.1 and B7.2 have been detected among different models of EAE. Whereas in mouse models B7.1 has been commonly described during the relapses, being nominated as one of those responsible for the epitope-spreading phenomenon and the induction of clinical relapses (113, 116-118), no B7.1 expression has been detected at any phase of acute EAE in rats (15). In fact, in this acute EAE model, despite the high expression of MCH molecules in microglia and the infiltration of CD28+ T-cells, neither B7.1 nor B7.2 expression was found during the inductive and peak phases (15), leading the authors to suggest that this expression of MHCs without a concomitant co-stimulatory signal can mediate either the anergy or the apoptosis of infiltrating T-cells, as described in the peripheral immune system (29). Induction of T-cell anergy or apoptosis may, therefore, be the cause of the immune response resolution leading to the spontaneous clinical recovery (15). In fact, apoptosis of encephalitogenic T-lymphocytes has been widely described in EAE models (119-122). In contrast, during the recovery phase the aforementioned study (15) revealed the expression of B7.2 in a subpopulation of microglial cells located in the vicinity of blood vessels. This pattern of expression of MHCs with B7.2, in the absence of B7.1, has been reported in reactive microglia in acute CNS injuries, such as facial-nerve axotomy (123), enthorinal-cortex lesion (124, 125) and cuprizone-induced demyelination (126), situations in which myelin destruction does not lead to autoimmunity. Furthermore, B7.2 expression has been described in concomitance with an accumulation of cells expressing CTLA-4 (15), which is the B7.2 co-receptor that inhibits T-cell activation (127). CTLA-4 was found constitutively expressed in T-regulatory cells (128), a specialised subpopulation of T-lymphocytes that suppressed the activation of the immune system, whose principal function is the maintenance of immunological homeostasis and tolerance to autoantigens (For detailed reviews, see (129-131). The role played by CTLA-4 in T-reg activation is still not well determined. However, it has emerged that the signal through this co-stimulatory molecule is necessary for the induction of Foxp3, the master transcription factor of Treg (132, 133), and for the activation of this population of lymphocytes (133-135).

Finally, it is also important to mention that an increasing number of reports currently suggests the involvement of other co-stimulatory molecules in EAE evolution (136). CD40/CD40L molecules have been described in MS plaques in humans (137) and during relapses in mice with EAE (116). It has been shown that CD40/CD40L recognition exerts detrimental effects in EAE evolution, as their absence, either by anti-CD40L treatment or as observed in CD40KO mice, prevents EAE induction (137, 138). Other molecules whose expression has been involved in EAE are PD1/PDL1,2 and ICOS/ICOS-L. The involvement of PD1 and its receptors PDL1 and PDL2, in EAE, came from studies showing the upregulation of these molecules at the peak of the disease (139, 140). Some years later, the use of KO mice demonstrated that a deficiency in either PD1 or PDL1, but not PDL2, induces an increase in EAE severity (141), suggesting a putative beneficial role of this signalling in EAE evolution. Moreover, recently it has been shown that EAE can be induced in PD1-deficient mice without the use of pertussis toxin (PTX), whereas in wild-type animals, PTX administration is essential for EAE development (142). Only few studies have addressed the role of signalling through ICOS and ICOS-L. These molecules may exert a beneficial role, as induction of EAE in ICOS-deficient mice results in severe disease (143). Nonetheless, treatment with anti-ICOS antibody suggested a dual role of ICOS in EAE: treatment during the first days post-induction (1-10 days post-induction) exacerbated EAE severity, whereas administration during the effector phase (9-20 days post-induction) promotes a delay in onset and severity (144). Despite these studies, no reports about ICOS expression in microglia or any other CNS resident cells are currently available.

Dendritic cells in EAE: recruited from the periphery or derived from microglia?

Different subtypes of DCs showing specific expression of surface molecules are currently identified in both human and mouse (39, 145). They are continuously produced from stem cells located in the bone marrow and they migrate as DC-precursor cells to peripheral organs where they differentiate into specific populations of resident DCs, by the action of different factors such as GM-CSF (granulocyte-macrophage colony stimulating factor), Flt-3 and c-Kit, in an antigen-independent manner (146).

The presence of DCs within the CNS has been a point of debate for many years. Nowadays the presence of these cells in restricted areas, such as the meninges and the choroids plexus, in the healthy CNS is accepted (147, 148). Studies in recent years have reported the existence of specific populations of DCs within the brain parenchyma under inflammatory conditions (For a review, see 149).

The presence of DCs within the CNS parenchyma in different models of EAE in rat (15, 54) and mouse (150-152) has been reported. The role played by DCs in the evolution of EAE is still controversial. Some authors point towards a detrimental role showing that intracerebral injection of DCs, pulsed *in vitro* with MOG, produces an exacerbation of EAE severity (153). In addition, it has been reported that DCs are responsible for the immune infiltration in EAE (154) and promote the polarisation of lymphocytes towards Th17-pathogenic cells (152). However, some studies have reported that if DCs were previously cultured with TNF-α, a tolerogenic phenotype would be induced in these cells which, when injected, produced a decrease in EAE symptomatology (153, 155), suggesting a beneficial role of DCs and indicating that further research is needed to clarify the exact role of DCs in CNS (For a detailed review, see 156). Additionally, the emergence of new subsets of DCs with regulatory cues, such as the tolerogenic DCs, opens new perspectives in the study of CNS DCs. Tolerogenic DCs are considered as an incomplete or modulated form of DC maturation, involved in peripheral tolerance via induction of anergy, apoptosis or Treg activation (157, 158). *In vitro* studies have demonstrated that certain cytokines such as IL10 and TFG-β, some vitamins (1,25-dihydroxy vitamin D3, vitamin C), immunosuppressive treatments using glucocorticoids or NFkB inhibitors can generate a tolerogenic phenotype in DCs (For a review, see 159). In contrast to immature DCs, also considered as inductors of tolerance, tolerogenic DCs display a stable phenotype, being resistant to further maturation. Characteristic features of these tolerogenic DCs have been described including the secretion of the anti-inflammatory enzyme indoleamine 2,3-dioxygenase (IDO), expression of MHC molecules but low levels of co-stimulatory molecules and expression of surface molecules involved in T-cell inhibition such as PDL1 or CD95L (Reviewed in 160). All of these molecules contribute to the inhibition of T-cell proliferation and induction of apoptosis. Despite the large amount of information in the peripheral immune system on the phenotype, role and modulation of these tolerogenic DCs, little is known regarding the presence and participation of these cells in the CNS during EAE.

Some evidence, nevertheless, raises the possibility that tolerogenic DCs can be involved in EAE evolution. The expression of IDO in EAE has been described in mouse, coinciding with the remission phase (161). IL10 and TGF-β expression have also been detected during the remission stage (162, 163). Moreover, low levels of co-stimulatory molecules have been detected in acute EAE (15).

Another issue that remains to be elucidated is what the origin and the differentiation cues of CNS DCs are. Two different theories can be considered to explain the possible sources of these cells: differentiation from resident microglial cells or recruitment from the periphery. Regarding the putative microglial origin, *in vitro* studies have demonstrated the capacity of microglial cells to differentiate into cells with a DC phenotype after exposure to GM-CSF (13, 151). In addition, recent studies have shown the existence of a subpopulation of microglial cells that constitutively displayed CD11c in the healthy brain (164). In accordance with these observations, the expression of CD1, a marker commonly used for the identification of immature DCs (165), has been demonstrated in a subpopulation of microglial cells located in the vicinity of blood vessels during the course of EAE (15). In agreement with this idea, an increase in CD1 expression in CD11b+CD45low microglial cells and CD11b+CD45high macrophages has been reported during the peak of EAE in mouse by flow cytometry (166). If this theory of the microglial origin of DCs were true, microglial cells should be considered as a population of immature DCs residing in the CNS parenchyma, as already suggested by some authors (167).

Alternatively, it has also been proposed that CNS DCs can be recruited from the periphery. Although monocytes were initially described as the precursors of all of the subpopulations of tissue macrophages, it is now recognised that they can also differentiate into DCs. In steady-state conditions, monocytes are the precursor of tissue-resident DCs such as the Langerhans cells in the skin, but under inflammatory situations the differentiation of monocytes into monocyte-derived DCs has recently been described (Reviewed in 168). The choice of monocytes to differentiate into DCs or macrophages is likely determined by the cytokine milieu and the signals that these cells received from the environment (169). The presence of Ly6C+ monocytes, the putative source of monocyte-DCs, has been recently reported in EAE in mouse (90, 170). Reinforcing the idea of a putative peripheral origin of the CNS DC population, recent findings have revealed the presence of a specific population of mature DCs, which does not correspond to microglia, within the CNS parenchyma (15). These mature DCs were characterised by the expression of fascin, an actin-bundling protein whose expression has been linked to DC maturation (171-173).

Taking into consideration all of these observations, the origin and function of DCs in CNS remain to be clarified and further research is warranted.

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ANEXO 2

Presentaciones del trabajo en congresos nacionales e internacionales

CONGRESOS NACIONALES

Almolda, B.; Acarin, L.; Castellano, B. and Gonzalez, B
PRESENTACIÓ D'ANTÍGENS EN L'ENCEFALOMIELITIS AUTOIMMUNE EXPERIMENTAL (EAE), UN MODEL D'ESCLEROSI MÚLTIPLE
POSTER
VI SIMPOSI DE NEUROBIOLOGIA EXPERIMENTAL
BARCELONA, NOVIEMBRE 2006

Almolda, B.; Acarin, L.; Castellano, B. and Gonzalez, B
REGULATION OF ANTIGEN PRESENTATION MECHANISMS IN THE ACUTE EAE LEWIS RAT MODEL
POSTER
2nd IBEROAMERICAN CONGRESS OF NEUROIMMUNOMODULATION
MADRID, ABRIL 2007

Almolda, B.; Gonzalez, B and Castellano, B.
LAS CELULAS DE MICROGLIA JUEGAN UN PAPEL DECISIVO EN LA FINALIZACIÓN DE LA RESPUESTA INMUNE EN UN MODELO DE ESCLEROSIS MÚLTIPLE
POSTER
XIII CONGRESO DE LA SOCIEDAD ESPAÑOLA DE NEUROCIENCIAS
TARRAGONA (ESPAÑA), SEPTIEMBRE 2009

CONGRESOS INTERNACIONALES

Almolda, B.; Acarin, L.; Castellano, B. and Gonzalez, B
TIME COURSE OF ANTIGEN PRESENTING MOLECULES EXPRESSION DURING THE EVOLUTION OF EAE IN LEWIS RATS
POSTER
7th IBRO WORLD CONGRESS OF NEUROSCIENCE
MELBOURNE(AUSTRALIA), JULIO 2007

Almolda, B.; Montoya, M.; Costa, M.; Acarin, L.; Castellano, B. and Gonzalez, B
CD4 EXPRESSION ON MICROGLIA DURING THE RECOVERY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS, A MODEL OF MULTIPLE SCLEROSIS
POSTER
33nd ANNUAL LA JOYA IMMUNOLOGY CONFERENCE
CALIFORNIA (USA), OCTUBRE 2007

Almolda, B.; Montoya, M.; Costa, M..; Castellano, B. and Gonzalez, B
MICROGLIA EXPRES CD4 ALONG THE COURSE OF ACUTE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS, A MODEL OF MULTIPLE SCLEROSIS

ORAL
INTERNATIONAL WORKSHOP. VIRAL TRIGGERS OF AUTOIMMUNITY: FOCUS ON EPSTEIN-BARR VIRUS AND MULTIPLE SCLEROSIS
ROMA (ITALIA), MAYO 2008

Almolda, B.; Montoya, M.; Costa, M..; Gonzalez, B and Castellano, B.
EXPRESSION OF IMMUNOMOLECULES BY MICROGLIAL CELLS DURING THE RECOVERY OF ACUTE EAE
POSTER
I CONGRESO IBRO/LARC DE NEUROCIENCIAS DE AMERICA LATINA, CARIBE Y PENINSULA IBÉRICA
BUZIOS (BRASIL), SEPTIEMBRE 2008

Almolda, B.; Costa, M..; Montoya, M.; Gonzalez, B and Castellano, B.
CD8 EXPRESSION DURING THE RECOVERY PHASE IN THE EAE LEWIS RAT MODEL
POSTER
INTERNATIONAL WORKSHOP: CD8 T CELLS IN CENTRAL NERVOUS SYSTEM INFLAMMATION
ROMA (ITALIA), MARZO 2009

Almolda, B.; Gonzalez, B and Castellano, B.
MICROGLIAL EXPRESSION OF MOLECULES RELATED TO ANTIGEN PRESENTING MECHANISM DURING THE RECOVERY PHASE OF ACUTE EAE
POSTER
9th EUROPEAN MEETING ON GLIAL CELLS IN HEALTH AND DISEASE
PARIS (FRANCIA), SEPTIEMBRE 2009

Almolda, B.; Gonzalez, B and Castellano, B.
MICROGLIAL INVOLVEMENT IN THE RESOLUTION OF INFLAMMATION IN ACUTE EAE
ORAL
COST NEUROINFNET MEETING: INFLAMMATION IN BRAIN DISEASE
ODENSE (DINAMARCA), MAYO 2010

Almolda, B.; Gonzalez, B and Castellano, B.
MICROGLIAL CELLS REGULATE THE ADAPTATIVE IMMUNE RESPONSE BY MODULATING THE LYMPHOCYTIC FUNCTION IN EAE
ORAL
CONGRESO BICENTENARIO DE NEUROCIENCIAS. VI REUNIÓN ANUAL DE LA SOCIEDAD CHILENA DE NEUROCIENCIAS
VALDIVIA (CHILE), SEPTIEMBRE 2010

Almolda, B.; Costa, M.; Montoya, M.; Gonzalez, B and Castellano, B.
MICROGLIAL CELLS IN ACUTE EAE MAY PLAY A KEY ROLE IN THE REGULATION OF LYMPHOCYTE DYNAMICS
ORAL
10th INTERNATIONAL CONGRESS OF NEUROIMMUNOLOGY
SITGES (BARCELONA), OCTUBRE 2010