GLIA

GLIA 59:841–856 (2011)

The Majority of Infiltrating CD8 T Lymphocytes in Multiple Sclerosis Lesions is Insensitive to Enhanced PD-L1 Levels on CNS Cells

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KEY WORDS

immunoregulation; astrocytes; microglia; oligodendrocytes; neurons

ABSTRACT

Central nervous system (CNS) cells locally modulate immune responses using numerous molecules that are not fully elucidated. Engagement of programmed death-1 (PD-1), expressed on activated T cells, by its ligands (PD-L1 or PD-L2) suppresses T-cell responses. Enhanced CNS PD-1 and PD-L1 expression has been documented in inflammatory murine models; however, human CNS data are still incomplete. We determined that human primary cultures of astrocytes, microglia, oligodendrocytes, or neurons expressed low or undetectable PD-L1 under basal conditions, but inflammatory cytokines significantly induced such expression, especially on astrocytes and microglia. Blocking PD-L1 expression in astrocytes using specific siRNA led to significantly increased CD8 T-cell responses (proliferation, cytokines, lytic enzyme). Thus, our results establish that inflamed human glial cells can express sufficient and functional PD-L1 to inhibit CD8 T cell responses. Extensive immunohistochemical analysis of postmortem brain tissues demonstrated a significantly greater PD-L1 expression in multiple sclerosis (MS) lesions compared with control tissues, which colocalized with astrocyte or microglia/macrophage cell markers. However, more than half of infiltrating CD8 T lymphocytes in MS lesions did not express PD-1, the cognate receptor. Thus, our results demonstrate that inflamed human CNS cells such as in MS lesions express significantly elevated PD-L1, providing a means to reduce CD8 T cell responses, but most of these infiltrating immune cells are devoid of PD-1 and thus insensitive to PD-L1/L2. Strategies aimed at inducing PD-1 on deleterious activated human CD8 T cells that are devoid of this receptor could provide therapeutic benefits since PD-L1 is already increased in the target organ.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disorder of the central nervous system (CNS) pathologically characterized by focal demyelination, neuronal damage, and astrocyte and microglia activation (Sospedra and Martin, 2005). Although CD4 T lymphocytes have been established as important players in MS pathogenesis, CD8 T lymphocytes are increasingly recognized as potential contributors to tissue damage (Friese and Fugger, 2009; Mars et al., 2010). CD8 T lymphocytes are detected in MS lesions, preferentially in the parenchyma and in greater amount than their CD4 counterparts (Lassmann, 2004; Neumann et al., 2002). Detection of oligoclonal expansion of CD8 T lymphocytes in the CNS of MS patients (Babbe et al., 2000; Jacobsen et al., 2002; Junker et al., 2007; Skulina et al., 2004) suggests their local activation. CD8 T lymphocytes with polarized cytolytic granules are observed in close apposition to oligodendrocytes and demyelinated axons (Neumann et al., 2002). Finally, up-regulated expression of major histocompatibility complex Class I (MHC-I) on microglia/macrophages, astrocytes, oligodendrocytes, and neurons in MS lesions supports the concept that CNS cells could be recognized and targeted by CD8 T cells (Hoftberger et al., 2004).

T cell responses are triggered by the T-cell receptor (TCR) recognition of MHC-peptide complexes and modulated by an array of co-activating or co-inhibiting molecules. The B7-CD28 family members play key roles in T-cell responses by contributing both co-stimulatory and co-inhibitory signals. Programmed death-1 (PD-1), also called CD279, belongs to this family and is a crucial co-inhibitory receptor expressed by activated immune cells (Keir et al., 2008). Whilst only small numbers of naïve or resting human T lymphocytes express detectable PD-1, a proportion of these cells up-regulate this receptor upon activation (Kinter et al., 2008); however, the mechanisms dictating PD-1 acquisition by a fraction of activated T lymphocytes are still poorly understood. PD-1 participates in immune dysfunction and anergy of

N.A. holds a Donald Paty Career Development Award from the Multiple Sclerosis Society of Canada (MSSC) and a Chercheur-Boursier from the Fonds de la Recherche en Santé du Québec. C.P. holds a studentship from the MSSC.

Grant sponsors: Canadian Institute of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada; Grant number: 355722-2008; Grant sponsor: CIHR-Neuroinflammation training Program (to C.P.).

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Received 20 July 2010; Accepted 19 January 2011

DOI 10.1002/glia.21158

Published online 28 February 2011 in Wiley Online Library (wileyonlinelibrary.com).

human CD8 T lymphocytes during chronic viral infections (Day et al., 2006; Trautmann et al., 2006; Urbani et al., 2006). The interaction between PD-1 and its ligands PD-L1 or PD-L2 inhibits the TCR-mediated antigen receptor signaling including T-cell proliferation, cytokine production, and cytotoxicity (Carter et al., 2002; Freeman et al., 2000; Rodig et al., 2003). Moreover, this interaction provides essential inhibitory signals to the fine balance between appropriate *versus* detrimental T-cell activation as illustrated by the various autoimmune diseases spontaneously developed by PD-1-deficient mice (Nishimura et al. 1999, 2001). PD-L1 (also known as B7-H1 or CD274) is widely expressed on activated T and B lymphocytes, macrophages, dendritic cells, and non-immune cells. In contrast, PD-L2 (also called B7-DC or CD273) expression is restricted to macrophages, dendritic cells, and mast cells (Keir et al., 2008).

The role and expression of PD-1 and its ligands in CNS diseases has been studied in numerous animal models including MS models. Blockade of the PD-1-PD-L1/2 pathway increased the susceptibility to or disease severity of experimental autoimmune encephalomyelitis (EAE), an MS model, in a strain-specific manner (Zhu et al., 2006). Conversely, blocking PD-1 led to a more rapid and severe disease characterized by a boosted number of CNS immune infiltrating cells, especially CD8 T cells (Kroner et al., 2009a,b; Salama et al., 2003; Wang et al., 2010). In EAE mice, up-regulated PD-1 expression has been observed on infiltrating cells, while PD-L1 was detected on microglia (Magnus et al., 2005; Salama et al., 2003) and astrocytes (Salama et al., 2003). Other murine CNS inflammatory models have illustrated the capacity of CNS cells to express PD-L1 in response to a variety of insults (Lafon et al., 2008; Lipp et al., 2007; Phares et al., 2009).

Enhanced PD-L1 immunodetection in MS brain sections compared with controls and detection on microglia/macrophage-like cells have been reported (Ortler et al., 2008). However, which CNS cell types express this molecule has not been completely resolved and whether these levels are sufficient to modulate human T-cell responses has not been addressed.

In this study, we investigated the expression of PD-L1 and PD-L2 by human CNS cells. We assessed the impact of such expression specifically on human CD8 T-cell functions since all CNS cell types in MS lesions could potentially interact with infiltrating CD8 T cells given their MHC-I expression (Hoftberger et al., 2004). We demonstrate that human CNS cells express low PD-L1 and PD-L2 levels under basal conditions, but inflammatory stimuli up-regulate PD-L1 on these cells. We establish that glial cells can locally modulate CD8 T cell responses via PD-L1 expression. Finally, we show that PD-L1 is highly expressed in MS lesions but that only small numbers of infiltrating CD8 T lymphocytes express PD-1. Our data suggest that during MS pathogenesis the inflamed CNS attempts to protect itself against active T lymphocytes via the expression of PD-L1, but fails because most infiltrating CD8 T lymphocytes lack PD-1.

MATERIALS AND METHODS

Isolation of Human Foetal Astrocytes and Neurons

Human foetal CNS tissue from 14- to 20-week-old embryos was provided by the Albert Einstein College of Medicine (Bronx, NY). Their and McGill University' ethical review boards approved the studies. Astrocytes and neurons were isolated as previously described (Saikali et al., 2007). Cells were treated for 24 (qPCR) or 48 hours (FACS/immunocytochemistry) with interferon-γ (IFN-γ, 200 U/ml; Thermo Scientific, Rockford, IL), tumour necrosis factor (TNF, 200 U/ml; Invitrogen, Camarillo, CA), interleukin-1β (IL-1β, 10 ng/ml, Invitrogen), or combinations of these cytokines.

Isolation of Adult Human Oligodendrocytes and Microglia

Adult oligodendrocytes and microglia were obtained from surgical resections performed for the treatment of nontumour-related intractable epilepsy as previously described (Saikali et al., 2007) and in accordance with guidelines of the ethical board of McGill University. Cells were treated with cytokines as described above.

Isolation of Human CD8 T Lymphocytes

These studies were approved by the ethical board of the Centre Hospitalier de l'Université de Montréal (CHUM) and informed consent was obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient. CD8 T lymphocytes were isolated using CD8 beads (MACS, Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions; purity assessed by FACS was >95%.

RNA Isolation, Reverse Transcription, and qPCR

Total RNA was isolated using TRIzol (Invitrogen) and subsequently the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions and as previously described (Saikali et al., 2007). RNA samples were transcribed into cDNA using Quantitect Reverse Transcription kit according to the manufacturer's instruction (Qiagen). PD-L1 and PD-L2 gene expression was determined by quantitative real-time PCR (qPCR) using primers and Taqman probes obtained from Applied Biosystems (Foster City, CA). Amplification of 18S was used as an endogenous control; results are presented as fold increase compared with untreated cells.

Fluorescence Activated Cell Sorting (FACS)

Brain cells were detached using PBS-EDTA and then labelled for surface (PD-L1 or PD-L2, HLA-A, B, C,

TABLE 1. List of Reagents Used to Detect Human Proteins

Reagent	Source
Biotinylated mouse α-PD-L1 (flow cytometry and immunocytochemistry)	eBioscience
Biotinylated mouse α-PD-L2 (flow cytometry and immunocytochemistry)	eBioscience
Mouse α-PD-L1 (immunohistochemistry)	Biolegend
Allophycocyanin-conjugated mouse α-granzyme B	Invitrogen
Alexa Fluor® 700-conjugated mouse α-IFN-γ	BD Biosciences
Pacific Blue-conjugated mouse α-CD8	BD Biosciences
Phycoerythrin-conjugated mouse α-TNF	BD Biosciences
Allophycocyanin-conjugated streptavidin	BD Biosciences
Pacific Blue-conjugated mouse α-HLA-A, B, C	Biolegend
V450-conjugated mouse CD11c	BD Biosciences
Alexa Fluor® 488-conjugated mouse α-GFAP	Invitrogen
Alexa Fluor® 488-conjugated mouse α-CD68	Biolegend
Rabbit α-Nogo A (flow cytometry and immunocytochemistry)	Millipore
Rabbit α-Iba-1	Wako
Rabbit α-CD8	Vector
Rat α-CD4	BD Biosciences
Mouse α-PD-1	Abcam
Biotinylated goat α-mouse	Dako
Cy™3-conjugated Streptavidin	Jackson Immuno
Alexa Fluor® 488-conjugated goat α-rabbit	Invitrogen

CD11c, Nogo-A) and intracellular (glial fibrillary acidic protein, GFAP) human molecules as previously described (Saikali et al., 2007) (see list of reagents Table 1). Appropriate isotype controls were used for all stainings. All results were acquired on a LSRII (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, OR). ΔMedian fluorescence intensity (MFI) was calculated by subtracting the fluorescence of the isotype from that of the stain.

Immunocytochemistry

CNS cells were fixed with either cold acetone or paraformaldehyde and blocked in HBSS containing goat and horse sera, FBS, HEPES buffer, and sodium azide. Cells were incubated overnight with antibodies specific for either PD-L1 or PD-L2 (see list of reagents in Table 1) and then for 2 hours with secondary reagents (Table 1; biotinylated goat-α-mouse and Cy™3-conjugated streptavidin) in combination with antibodies targeting cell-specific markers. To identify CNS cell types, astrocytes were costained for GFAP, oligodendrocytes for Nogo-A, and microglia for CD68. Cells were finally incubated with Hoechst 33258 nuclear stain (Invitrogen), and mounted in Gelvatol. Slides were observed using a Leica fluorescent microscope.

siRNA

Astrocytes were plated (2×10^5 cells/well) in a 24-well plate for 48 hours and then were washed with Opti-MEM (Invitrogen). PD-L1 specific (catalog number 4392420 from Applied Biosystems) or control siRNA (catalog number 4390844) were introduced into cells at an optimal concentration (7.5 pmol) with the Lipofectamine™ 2000 (Invitrogen) diluted in Opti-MEM. Cells were kept 24 hours with the siRNA, and then washed. Proinflammatory cytokine IFN-γ (200 U/ml) was then added to the culture for 24 hours. Freshly isolated alloreactive CFSE-labeled CD8 T cells (2×10^5 cells/well) were added to washed astrocytes in the presence of anti-CD3 (0.18 μg/ml, clone OKT3) and anti-CD28 (1 μg/ml, BD Biosciences) antibodies. Inhibition of PD-L1 expression was confirmed by FACS on astrocytes the same day that CD8 T cells were added. After a 6-day co-culture, cell supernatants were harvested and frozen for future CXCL10 ELISA assessment (OptEIA set; BD Bioscience) and CD8 T cells were collected and stained for CD8 in the presence of Live/dead fixable Aqua Dead Cell Stain kit (Invitrogen) to exclude dead cells and intracellularly stained for IFN-γ, TNF, and granzyme B (see reagents in Table 1).

Immunohistochemistry

Postmortem brain sections from tissue donors without CNS disease and patients diagnosed clinically and confirmed by neuropathological examination as having MS were obtained from the NeuroResource tissue bank, UCL Institute of Neurology, London, UK. Tissues were donated to the tissue bank with informed consent following ethical review by the London Research Ethics Committee, UK. This study was approved by the CHUM Ethical Committee. Snap-frozen coded sections were cut from blocks of normal control and MS brain tissues. Sections cut before and immediately after the ones used for the immunofluorescence studies were stained with oil red O and hematoxylin, and scored as previously described (Li et al., 1996) (Table 2). Sections were air-dried, fixed in cold acetone, and blocked in HBSS containing horse serum, FBS, HEPES buffer and sodium azide and then blocked using the Blocking Kit (Vector Laboratories). Primary antibodies targeting PD-L1 (see Table 1 for all staining reagents) or PD-1 were incubated overnight. Sections were then washed with PBS and incubated for 2 hours with biotinylated goat-anti-mouse and subsequently with Cy3-conjugated streptavidin concomitantly with antibodies targeting cell specific marker for either astrocytes (GFAP), microglia (Iba-1), oligodendrocytes (Nogo-A), CD8, or CD4 T cells followed by a 2-hour incubation with appropriate secondary reagent. Finally, sections were treated with RNase and then incubated with a nuclear stain TO-PRO®-3 iodide (Invitrogen). Sections were treated with 1% Sudan black in 70% ethanol to quench tissue autofluorescence. Controls were concurrently carried out on adjacent sections using appropriate primary isotype controls at the same concentrations. Slides were observed using a SP5 Leica confocal microscope and confocal images acquired simultaneously in different channels throughout 4 to 8 μm z-stack every 0.2 to 0.5 μm.

Statistics

Data were analyzed using GraphPad Prism software. Results are shown as mean ± SEM and statistical

TABLE 2. Description of Postmortem Brain Sections

Block	M/F	Age (yr)	DD (yr)	Cause of death	DFT (h)	Sample type	ORO, hematoxylin score	Summary observations on ORO-stained sections
1	F	68	–	Colorectal metastatic tumour	23	NC W, OV, R	0, 0	Normal white matter and cortical grey matter
2	M	49	–	Myocardial infarction and coronary artery thrombosis	11	NC W, PV, R	0, 0	Normal white matter and grey matter
3	F	47	20	Bronchopneumonia	9	MS AQ, FSv, L	4, 3	White matter and grey matter surrounding active plaque. ORO+ cells in blood vessel walls and parenchyma
4	F	47	20	Bronchopneumonia	9	MS AQ, PSv, L	5, 4	Large plaque with active and some subacute and chronic areas. Large perivascular cuffs. White and grey matter
5	F	71	32	Bronchopneumonia	19	MS SAQ, O pole Sv, L	2, 0	Hypocellular plaque surrounded by patchy abnormal-appearing white matter
6	M	53	–	Cardiac arrest	19	NC W, OSv, R	0, 0	Normal white matter.
7	F	37	10	Bronchopneumonia	24	MS AQ, basal ganglia, L	3, 3	Large subacute plaque with perivascular cuffing; areas of grey matter
8	F	60	34	Renal failure	24	MS SAQ, TV, L	2, 4	Large subacute plaque with many large and small perivascular cuffs
9	F	49	11	Bronchopneumonia	16	MS CQ, F pole V, R	0, 2	Large chronic plaque surrounded by pale abnormal white matter
10	F	29	8	Bronchopneumonia	11	MS SAQ, cerebellum, R	1, 1	Large subacute plaque with hypercellular areas

ORO and cuffing: Scored on a scale of 0 to 5 for ORO and haematoxylin staining; 0 is what would be expected in normal control white matter. Data is averaged from duplicate sections cut immediately before and after the serial sections cut.
DD: disease duration; DFT: time between death and sample freezing; NC: normal control; W: white matter; MS: multiple sclerosis; AQ: active plaque; SAQ: subacute plaque; V: ventricular; Sv: subventricular; F: frontal; P: parietal; T: temporal; O: occipital; R: right; L: left.

analyses included paired and unpaired Student's *t*-test. P values less than 0.05 were considered significant.

RESULTS

Proinflammatory Cytokines Increase PD-L1 Expression on Human Astrocytes

We elected to evaluate whether different human CNS cell types express PD-L1 or PD-L2 under basal levels or upon inflammatory conditions. Primary cultures of human astrocytes were either untreated (NIL) or activated with different proinflammatory cytokines: IFN-γ, TNF, IL-1β, IFN-γ + TNF, TNF + IL-1β, or IFN-γ + IL-1β to mimic the proinflammatory environment found in the CNS of MS patients. We first conducted qPCR analysis to assess PD-L1 and PD-L2 mRNA levels and found that astrocytes expressed only low levels of both PD-L1 and PD-L2 under basal conditions (Fig. 1A,B). However, following single cytokine treatment we detected a significant increase in PD-L1 levels compared with untreated cells (IFN-γ or TNF: $*P < 0.05$ and IL-1β: $**P < 0.01$ *vs*. NIL). PD-L1 levels were even greater when cytokine combinations which included IFN-γ were applied (IFN-γ + TNF or IFN-γ + IL-1β: $**P < 0.01$; TNF + IL-1β: $*P < 0.05$ *vs*. NIL). In contrast, cytokines or cytokine combinations had only a small impact on PD-L2 mRNA levels.

We next investigated whether astrocytes expressed detectable protein levels of these two ligands using FACS. In agreement with our qPCR results, low protein levels of PD-L1 and PD-L2 were detected on astrocytes under basal conditions (Fig. 1C–F). Adding one proinflammatory cytokine elevated PD-L1 expression and IFN-γ + TNF or IFN-γ + IL-1β combinations caused even a greater increase of PD-L1 on all astrocytes (Fig. 1C). These enhanced protein levels compared with untreated samples were observed on all samples tested reaching almost significance (ξ: $0.05 > P \leq 0.1$; TNF, IL-1β, IFN-γ + TNF, IFN-γ + IL-1β, or TNF + IL-1β *vs*. NIL). These treatments had only a small impact on PD-L2 expression (Fig. 1D,F). To document the activation status of astrocytes, we assessed their MHC-I expression and secretion of CXCL10, both known to be robustly enhanced following their activation (Jack et al., 2005). MHC-I levels were increased in response to each treatment tested (Fig. 1G) similarly to what we observed for PD-L1 expression. Moreover, CXCL10 secretion augmented in response to proinflammatory treatments, especially with IFN-γ + TNF or IFN-γ + IL-1β combinations (Fig. 1H). Addition of IL-17 or IL-22, two cytokines produced by Th17 cells, did not alter PD-L1,

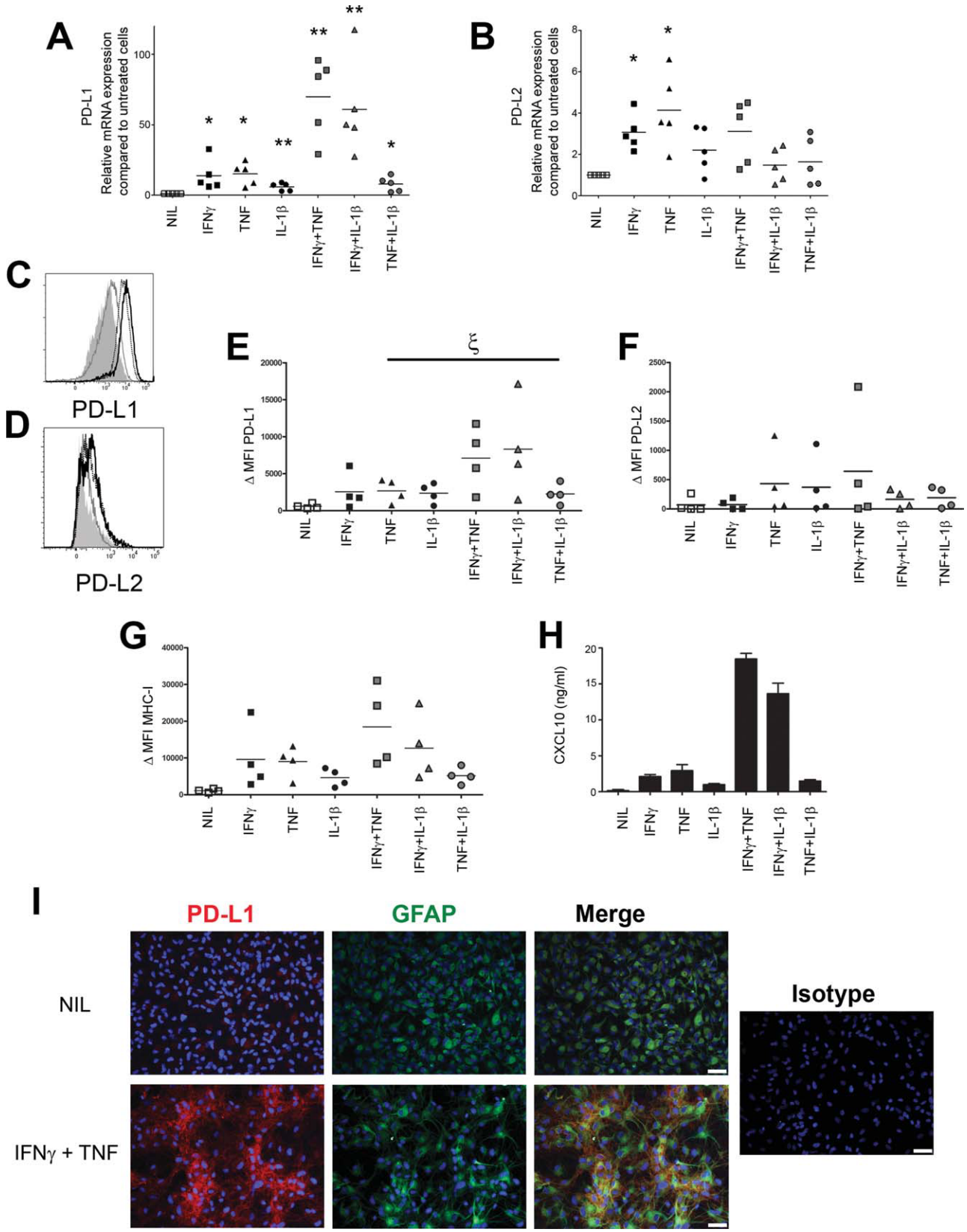

Fig. 1. Human astrocytes up-regulate PD-L1 expression following proinflammatory cytokine treatments. Human astrocytes (n = 4 or 5 donors) were either untreated (NIL) or stimulated with cytokines and PD-L1 and PD-L2 expression was determined by qPCR, FACS, or immunocytochemistry. PD-L1 (**A**) and PD-L2 (**B**) mRNA expression by astrocytes. (**C** and **D**) Representative FACS profiles gated on GFAP+ cells illustrating PD-L1 (C) and PD-L2 (D) protein expression. Filled gray histogram: isotype control; gray line: untreated cells; dotted line: IFN-γ; solid black line: IFN-γ + TNF. Quantified FACS detection (ΔMFI) of PD-L1 (**E**), PD-L2 (**F**), and MHC-I (**G**) on astrocytes. (**H**) CXCL10 production by astrocytes quantified by ELISA. (**I**) Representative micrographs of either untreated (NIL) or IFN-γ + TNF activated astrocytes stained for PD-L1 (red), GFAP (green), and nuclei (blue) with the corresponding isotype. Scale bar = 50 μm. Paired Student's t-test comparing nil *versus* indicated cytokine treatment $^{*}P < 0.05$; $^{**}P < 0.01$; ξ: $0.05 > P < 0.1$.

PD-L2, or MHC-I expression on astrocytes (data not shown).

PD-L1 was undetectable or barely detectable by immunocytochemistry on untreated astrocytes, but we observed an increased PD-L1 expression after IFN-γ + TNF treatment (Fig. 1I). IFN-γ and TNF alone also induced an increase of PD-L1 expression, but to a lesser extent than IFN-γ + TNF (data not shown). The small PD-L2 increased expression observed by qPCR and FACS following proinflammatory treatment was below detection levels by immunocytochemistry (data not shown).

Human Oligodendrocytes and Microglia Express PD-L1 in Response to Proinflammatory Cytokines

We evaluated whether other CNS cell types express PD-L1 or PD-L2 in response to proinflammatory cytokines. As experiments performed on astrocytes showed similar results between mRNA levels and protein levels, PD-L1 and PD-L2 expression were assessed at the protein level by immunocytochemistry and FACS. Adult CNS cells enriched for oligodendrocytes or microglia were either untreated or cytokine stimulated before PD-L1 and PD-L2 detection concomitantly with either CD68 or CD11c (for microglia) or Nogo-A (for oligodendrocytes) labeling. One representative example out of four tested donors is illustrated in Fig. 2. Untreated microglia and oligodendrocytes had very low or undetectable levels of PD-L1 (Fig. 2A–C,H–J,O–P) and PD-L2 (data not shown). However, PD-L1 expression was increased on microglia (Fig. 2D–F,O) and oligodendrocytes (Fig. 2K–M,P) following IFN-γ + TNF treatment; the ΔMFI for PD-L1 levels reached on average 1,213 for microglia and 403 for oligodendrocytes. IFN-γ alone had also the capacity to induce a detectable increase of PD-L1 on microglia and oligodendrocytes (Fig. 2P and data not shown). These cytokine treatments did not induce detectable expression of PD-L2 (data not shown). These results illustrate that oligodendrocytes and microglia have the capacity to express enhanced levels of PD-L1 in an inflammatory milieu.

Neurons Slightly Increase PD-L1 Expression Upon Proinflammation

We also assessed the expression of PD-L1 and PD-L2 on primary cultures of human neurons using FACS and excluded GFAP+ cells (astrocytes) from our analysis. PD-L1 and PD-L2 levels did not differ from the isotype control under basal conditions (Fig. 3A,B). However, stimulation with IFN-γ or IFN-γ + TNF induced a significant increase of PD-L1 on the surface of neurons ($n = 5$); (IFN-γ or IFN-γ + TNF *vs*. NIL: $^{*}P < 0.01$), while TNF had no effect on PD-L1 levels (Fig. 3C). The increase (ΔMFI) observed on neurons (Fig. 3C, NIL: 0 *vs*. IFN-γ + TNF: 83 ± 20 or IFN-γ: 113 ± 18) remained; however, much lower than what we detected on astrocytes similarly treated (Fig. 1E, NIL: 581 ± 163 *vs*. IFN-γ + TNF: 7098 ± 2148). PD-L2 was not detectable on neurons upon any treatment (Fig. 3B).

Human Astrocytes Modulate CD8 T Cell Responses via PD-L1

Although previous studies have shown the capacity of PD-L1 expressing murine microglia (Magnus et al., 2005) or oligodendrocytes (Phares et al., 2009) to inhibit T-cell responses, whether PD-L1 levels expressed by human CNS cells, and especially astrocytes, have a consequential impact on immune cell responses has not been previously tested. As a proof of concept we investigated whether PD-L1 provided by human astrocytes influences CD8 T-cell functions. We elected to use this CNS-immune cell interaction paradigm since PD-1 engagement by PD-L1 inhibits TCR signaling and that CD8 T lymphocytes recognize, via their TCR, MHC Class I-peptide complexes, which are enhanced on CNS cells during MS pathogenesis (Hoftberger et al., 2004). We specifically knocked down PD-L1 expression by astrocytes using a siRNA approach. Astrocytes treated with PD-L1 specific siRNA (siPDL1) typically reduced by 60% to 70% their PD-L1 mRNA levels (Fig. 4A; left panel: qPCR results) and by 70% to 77% their PD-L1 protein levels compared with control siRNA (siCTL) (Fig. 4A; right panel: FACS data; representative of four astrocyte donors). However, MHC-I expression (Fig. 4A; right panel) by these astrocytes was not affected by such treatment.

CFSE-labeled alloreactive human CD8 T cells were co-cultured with astrocytes treated with either siCTL or siPDL1 and proliferation and production of IFN-γ, TNF, and granzyme B were assessed. For comparison, PD-1 expression acquired by CD8 T cells cultured in the presence of anti-CD3+anti-CD28 is illustrated (Fig. 4B; upper panel one representative donor, lower panel three donors). Typical FACS profiles of CD8 T cells following co-culture with astrocytes are illustrated (Fig. 4C); 40% (23.6% + 16.4%) of CD8 T cells co-cultured with siPDL1-transfected astrocytes proliferated compared with only 29.1% (17.1% + 12.0%) for those co-cultured with siCTL-transfected astrocytes. Moreover, 23.6% of CD8 T cells proliferated and produced IFN-γ when co-cultured on siPDL1-transfected astrocytes whereas only 17.3% did for those co-cultured with siCTL-transfected astrocytes. We observed a similar pattern for granzyme B production as the proportion of CD8 T cells that proliferated and produced granzyme B reached 10.0% when co-cultured on siPDL1-astrocytes but only 6.9% when co-cultured with siCTL-astrocytes. We observed a significant ($^{**}P < 0.01$) increase in the proliferation and production of IFN-γ, granzyme B, and TNF by CD8 T cells co-cultured on siPDL1-astrocytes compared to siCTL-astrocytes; results obtained from five CD8 T cell donors tested on three astrocyte donors are shown in Fig. 4D. Our results demonstrate that PD-L1 expressed by astrocytes was sufficient to significantly diminish the proliferation and effector functions (cytokines and lytic enzyme) of human CD8 T cells.

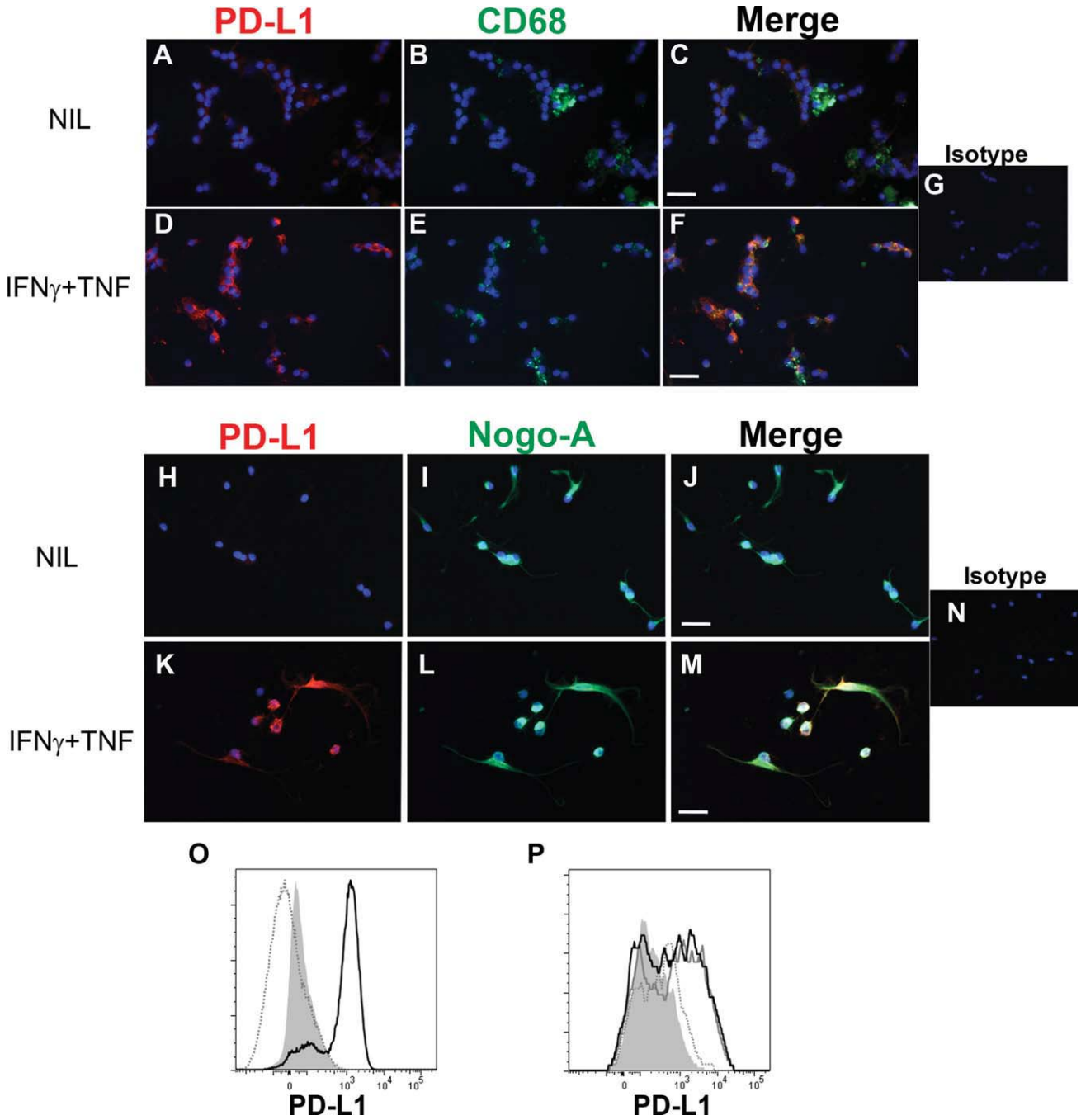

Fig. 2. Human microglia and oligodendrocytes up-regulate PD-L1 expression following proinflammatory cytokine treatments. Human microglia and oligodendrocytes were either untreated (NIL) or treated with IFN-γ + TNF before immunocytochemistry or FACS detection of PD-L1 concomitantly with a microglia (**A–G**, **O**) or oligodendrocyte cell specific marker (**H–N**, **P**). (A–G) Representative micrographs of untreated (A–C) or treated (D–F) cells stained for PD-L1 (red), CD68 (green), and nuclei (blue) and corresponding isotype control (G). (H–N) Representative micrographs of untreated (H–J) or treated (K–M) cells stained for PD-L1 (red), Nogo-A (green) and nuclei (blue) and corresponding isotype control (*N*). Scale bar = 50 μm. Results shown are representative of four donors tested. (O and P) Representative FACS histograms for PD-L1 expression on microglia (O) (gated on CD11c+ cells) and oligodendrocytes (P) (gated on Nogo-A+ cells). Filled gray histogram: isotype control; dotted gray line: untreated cells; gray line: IFN-γ; solid black line: IFN-γ + TNF.

PD-L1 is Highly Expressed in MS Brain Lesions but not in Control Tissues

To assess whether human CNS cells can provide PD-L1 *in vivo*, we performed immunohistochemistry on postmortem brain tissues obtained from normal controls and MS patients, see description in Table 2. MS lesions were characterized using the ORO and hematoxylin scoring as being acute (AQ), containing numerous phagocytic macrophages that have recently engulfed lipid

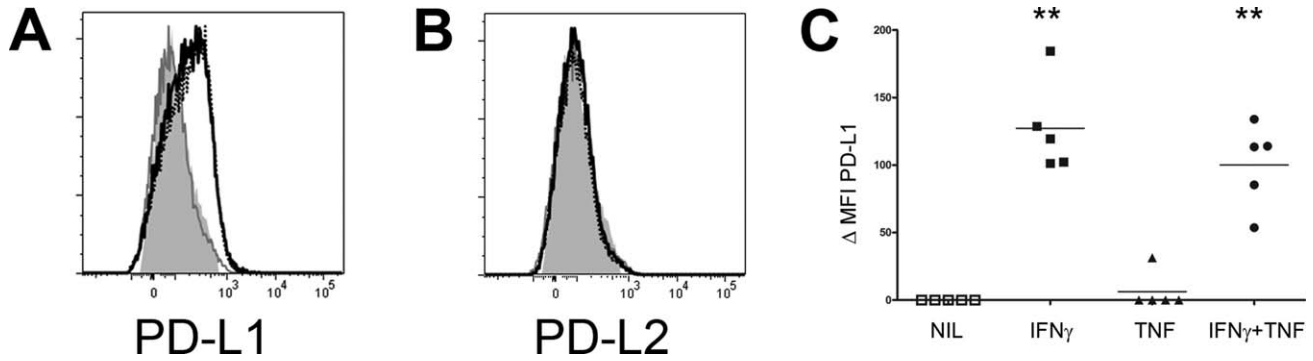

Fig. 3. Human neurons express low PD-L1 levels following cytokine treatment. Human neurons were either untreated (NIL) or treated with cytokines as indicated and analyzed for PD-L1 and PD-L2 expression. (**A** and **B**) Representative FACS histograms for PD-L1 (A) and PD-L2 (B) expression on neurons (gated on GFAP-negative cells). Filled gray histogram: isotype control; gray line: untreated cells; dotted line: IFN-γ; solid black line: IFN-γ + TNF. (**C**) Quantified flow cytometric detection (ΔMFI) of PD-L1 on neurons ($n = 5$). Paired Student's t-test comparing nil *versus* indicated cytokine treatment $^{**}P < 0.01$.

debris, or subacute (SAQ), containing demyelinated areas but demonstrating less recent myelin destruction. Brain sections were stained for PD-L1 and GFAP or appropriate isotype controls. Six to 10 fields (at 630×, each field covering 0.0625 mm^2) per section (three sections from controls and seven sections from MS patients) containing GFAP-positive cells were thoroughly analyzed to determine the percentage of cells positive for PD-L1 and representative fields are illustrated (Fig. 5A–O). Although we observed PD-L1-positive cells in all sections analyzed, the proportion of PD-L1-expressing cells as well as the PD-L1 signal intensity were greatly enhanced in MS lesions compared with control sections (Fig. 5A,F,K). The proportion of GFAP+ cells per surface area varied throughout sections. However, the majority of astrocytes expressed PD-L1 in MS lesions (Fig. 5H,M,P) while few were positive in control tissues (Fig. 5C,P). We also used the Sudan black staining to identify demyelinated *versus* non-demyelinated area and observed that most PD-L1+ cells were localized in demyelinated or juxtaposed to demyelinated areas. Finally, our quantitative analysis revealed that AQ and SAQ MS sections bore significantly more PD-L1-expressing astrocytes per mm^2 than control sections (Fig. 5N) (SAQ or AQ *vs*. NC: $^{*}P < 0.05$).

We observed PD-L1 positive cells that were negative for GFAP in MS sections (Fig. 5H, pink arrows). To identify these cells, we stained adjacent sections for Iba-1 (see Fig. 6), a marker for macrophage/microglia, or for Nogo-A (see Fig. 7), a marker for oligodendrocytes. In control sections, only few microglia expressed PD-L1 (0%–5%) (Fig. 6C,P). However, the percentage of macrophages/microglia expressing PD-L1 was statistically increased in MS lesions compared with controls (Fig. 6H,M,P) as we measured an increase of up to 60% in MS lesions (Fig. 6P). The number of PD-L1 positive microglia/macrophages per surface area was also significantly elevated in AQ and SAQ MS lesions compared with controls (Fig. 6Q; NC *vs*. SAQ $^{**}P < 0.01$; and NC *vs*. AQ $^{*}P < 0.05$). Thus, both astrocytes and microglia/macrophages displayed significantly boosted PD-L1 expression in MS lesions compared with controls.

In contrast, oligodendrocytes (Nogo-A+) did not express detectable PD-L1 in controls (Fig. 7A–C) or MS tissues either within or outside lesions (Fig. 7E–G,I–K,M–O). Nogo-A+ oligodendrocytes observed in MS sections (Fig. 7E–G,I–K) were mainly localized outside lesions (Fig. 7M–O).

The Majority of CNS-Infiltrating CD8 T Cells do not Express PD-1

Our *in vitro* functional assays demonstrated that CNS cells, in particular inflamed astrocytes, express sufficient levels of PD-L1 to modulate CD8 T cell responses. We next evaluated whether human infiltrating CD8 T cells found in MS lesions express detectable levels of PD-1, which would dictate their susceptibility to immunomodulation by PD-L1 and PD-L2 on CNS cells. We stained for CD8 and PD-1 (see Fig. 8) on the same postmortem brain tissues previously used (see Table 2) for PD-L1 detection and performed a similar analysis. We employed the same monoclonal clone (NAT) than others have successfully used to detect PD-1 in human tissues (Roncador et al., 2007; Wu et al., 2009). As expected, only rare and isolated CD8 T cells were found in the parenchyma of control patients, and few were located perivascularly (Fig. 8B). In contrast, we detected substantially more CD8 T cells per surface area in both AQ and SAQ MS lesions (Fig. 8G,L,Q) (SAQ *vs*. NC $P = 0.14$; AQ *vs*. NC $^{**}P < 0.01$). The vast majority of CD8 T cells observed in controls was positive for PD-1, regardless of localization (Fig. 8A–D). However, in MS lesions only a few CD8 T cells expressed PD-1 (Fig. 8F–I,K–N). We quantified more specifically CD8 T cells located in the parenchyma, where these cells could more likely interact with PD-L1 expressing astrocytes and microglia/macrophages, and determine their proportion expressing PD-1. Most of the very few CD8 T cells present in the parenchyma of controls were positive for PD-1 (between 50% and 100%). In lesions the percentage of CD8 T cells expressing this inhibitory receptor went down to 0%–65%, (NC *vs*. SAQ $^{*}P < 0.05$ and NC *vs*. AQ *vs*. ξ

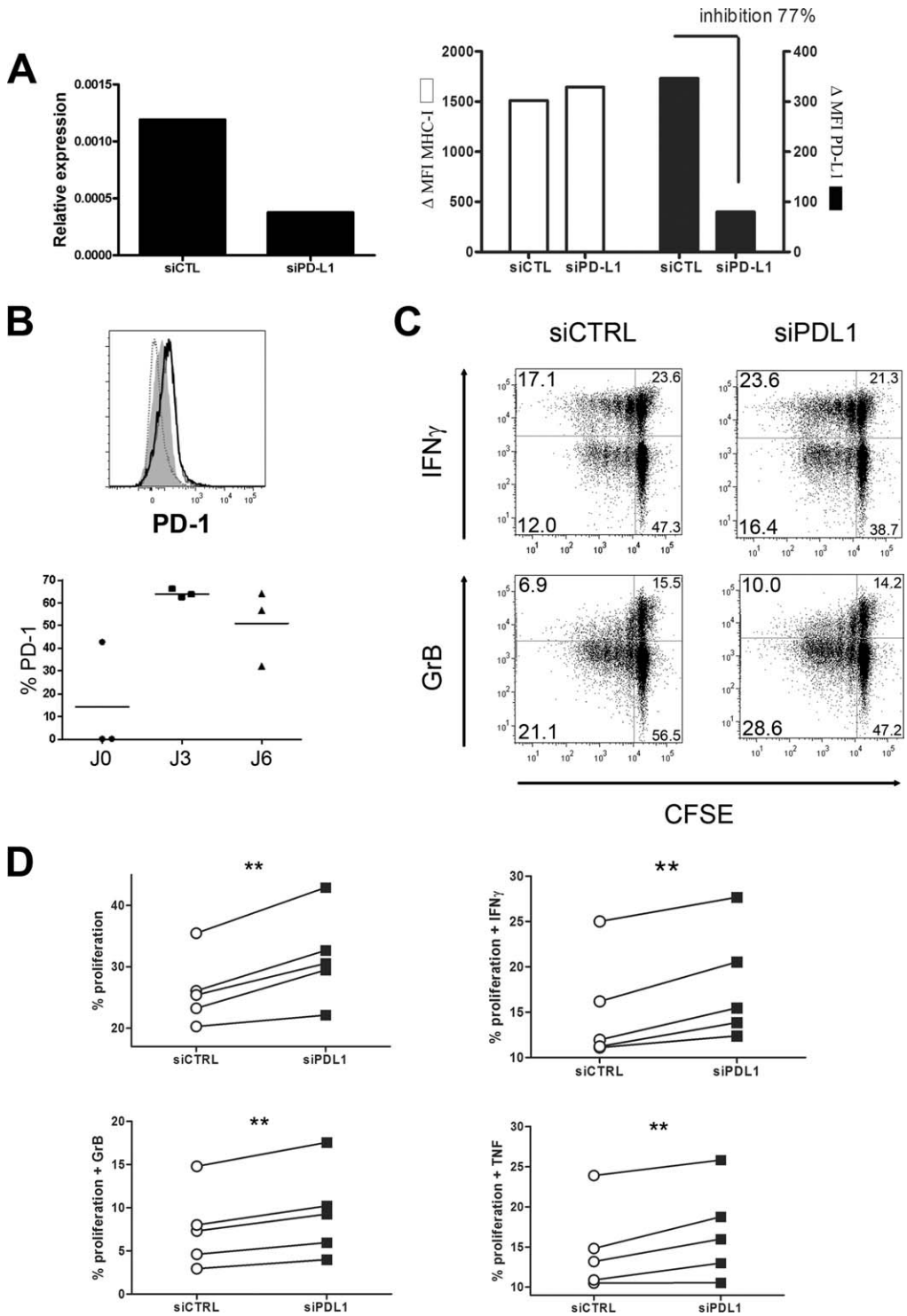

Fig. 4. Human astrocytes via PD-L1 expression decrease the activation of human CD8 T cells. Human astrocytes treated either with a control (siCTL) or a PD-L1 specific siRNA (siPDL1) were stimulated with cytokines and then co-cultured with human CD8 T cells; CD8 T cells were subsequently analyzed for proliferation and cytokine production by FACS. (**A**) One representative out of four astrocyte donors treated with siRNA analyzed for PD-L1 mRNA (left panel) and PD-L1 and MHC class I protein (right panel) expression. (**B**) Upper panel: representative FACS histogram for PD-1 expression on CD8 T cells *ex vivo* (dotted gray line) or stimulated with anti-CD3 and anti-CD28 for 3 days (solid black line) compared with isotype control (filled gray). Lower panel: pooled data of PD-1 percentage on CD8 T cells *ex vivo* or stimulated with anti-CD3 and anti-CD28 for 3 and 6 days ($n = 3$ donors). (**C**) Representative dot plots of immune responses observed for CD8 T cells (gated on living CD8 T cells) co-cultured with astrocytes treated with either siCTRL or siPDL1. Proliferation assessed by CFSE dilution (*x*-axis) *versus* IFN-γ (top panel) or granzyme B (bottom panel) production (*y*-axis). (**D**) Data obtained from five donors of CD8 T cells co-cultured with astrocytes treated with either siCTRL or siPDL assessing proliferation (top left), IFN-γ (top right), granzyme B (bottom left), and TNF production (bottom right). Paired Student's *t*-test comparing siCTRL *versus* siPDL $^{**}P < 0.01$.

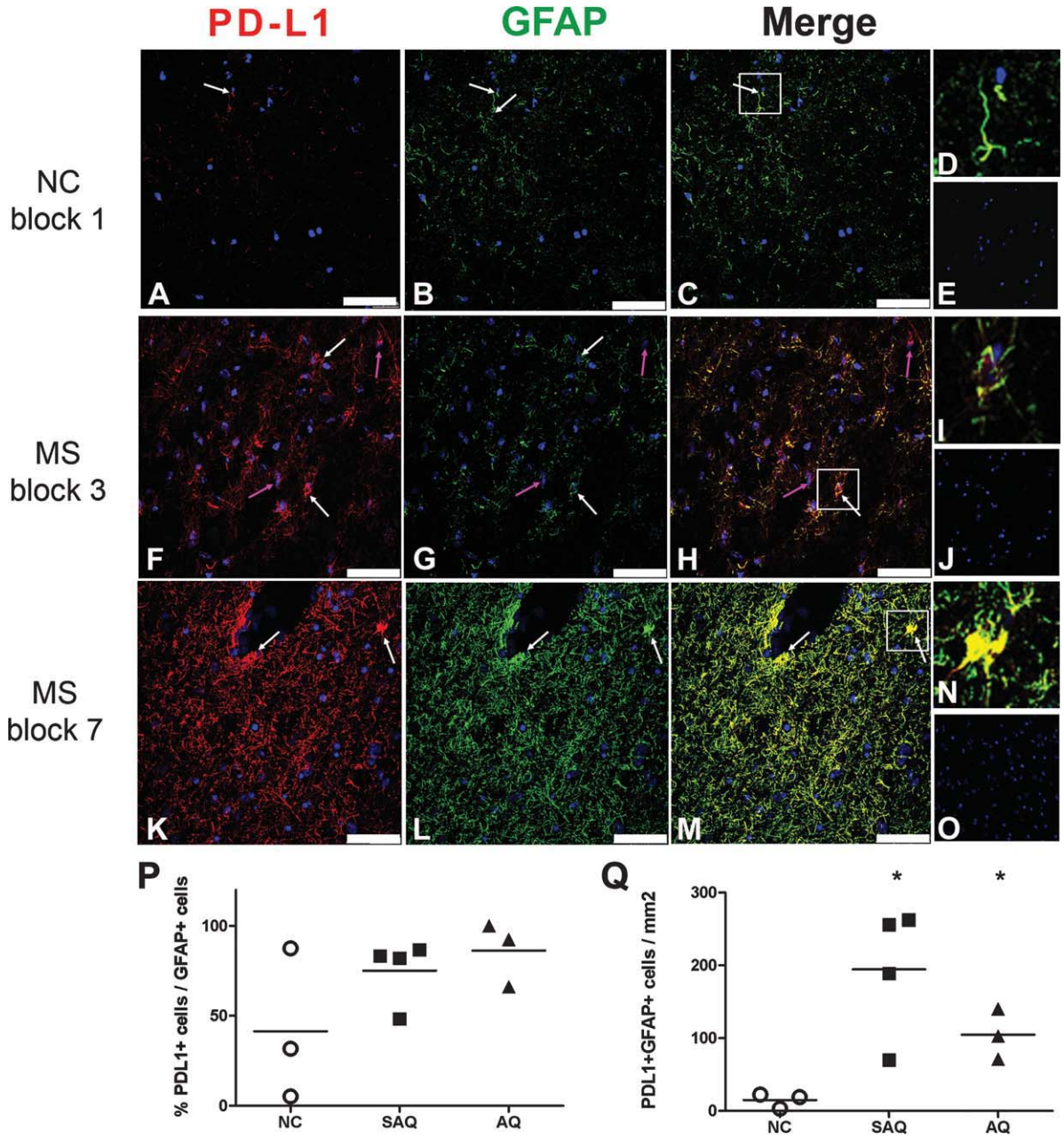

Fig. 5. PD-L1 expression by astrocytes is strongly enhanced in MS brain lesions compared with normal controls. (**A–O**) Micrographs showing brain sections stained for PD-L1 (red), GFAP (green), and nuclei (blue) of one representative normal control (A–D), and two representative MS donors (F–J and K–N). Corresponding isotypes are shown in E, J, and O. Scale bar = 50 μm. White arrows indicate examples of GFAP+ PD-L1+ cells and pink arrows indicate examples of GFAP- PD-L1+. (C-H-M) Cells in white boxes shown enlarged in D-I-N. (**P–Q**) Quantification of astrocytes expressing PD-L1 in human brain sections (at least five fields analyzed per section) from normal controls (NC), subacute (SAQ), and acute MS lesions (AQ). Each dot represents one distinct section. (P) Percentage of astrocytes positive for PD-L1 in brain sections; (Q) number of astrocytes positive for PD-L1 per mm^2 for all donors. Student's *t*-test comparing normal controls *versus* MS lesions $^*P < 0.05$.

$P < 0.09$) (Fig. 8Q). Cells displaying a leukocyte morphology and devoid of CD8 expressed detectable PD-1 in the CNS parenchyma and around blood vessels of MS lesions (Fig. 8H,M; pink arrows); an average of 86 PD-1+ cells/mm^2 in MS lesions were not CD8 T lymphocytes. To identify these cells we performed co-staining for PD-1 and CD4 and observed that most infiltrating CD4 T cells in MS lesions expressed PD-1 (Fig. 8R–V).

DISCUSSION

Our study demonstrates that exposure to proinflammatory stimuli increases the expression of PD-L1, an inhibitory ligand, on most human CNS cells. Inflamed astrocytes express sufficient levels of PD-L1 to modulate CD8 T-cell immune responses. In addition, we showed that PD-L1 is significantly more expressed in MS brain

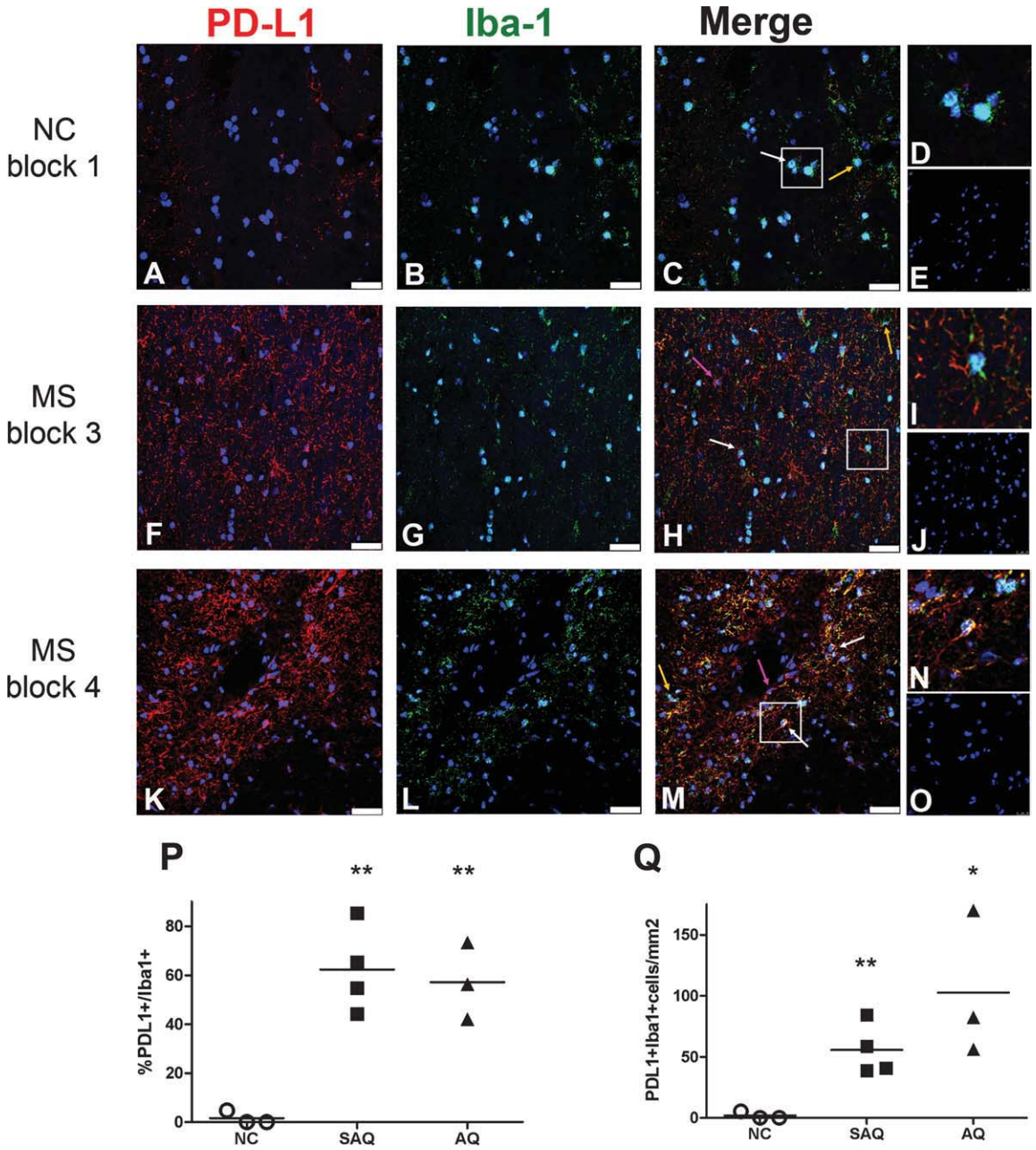

Fig. 6. PD-L1 is expressed by microglia/macrophages in MS brain lesions, but not in normal controls. (**A**–**O**) Micrographs showing brain sections stained for PD-L1 (red), Iba-1 (green), and nuclei (blue) of one representative normal control (A–D), and two representative MS donors (F–I and K–N). Corresponding isotypes are shown in E, J, and O. Scale bar = 25 μm. White arrows indicate examples of Iba-1+ PD-L1+ cells; pink arrows indicate examples of Iba-1- PD-L1+; orange arrows indicate examples of Iba-1+ PD-L1−. (C, H, M) Cells in white boxes shown enlarged in D, I, N. (**P** and **Q**) Quantification of microglia/macrophages in human brain sections (at least five fields analyzed per section) expressing PD-L1 in human brains from normal controls (NC), subacute (SAQ), and acute MS lesions (AQ). Each dot represents one distinct section. (P) Percentage of microglia/macrophages positive for PD-L1; (Q) number of microglia/macrophages positive for PD-L1 per mm^2. Student's *t*-test comparing normal controls *versus* MS lesions $^*P < 0.05$; $^{**}P < 0.01$.

lesions than in control tissues and that astrocytes and microglia/macrophages are significant and abundant sources of this ligand in MS lesions. In contrast, most infiltrating CD8 T lymphocytes found in MS lesions especially in the parenchyma are devoid of PD-1. Our observations suggest that although PD-L1 is abundantly expressed in MS lesions by astrocytes and microglia/macrophages, infiltrating CD8 T cells would not be susceptible to this local PD-L1-mediated inhibition.

We performed an extensive study of primary human CNS cells and treated astrocytes, microglia, oligodendrocytes, and neurons with cytokines known to be abundantly present in MS lesions (Sospedra and Martin, 2005). We observed low basal levels of PD-L1 and PD-

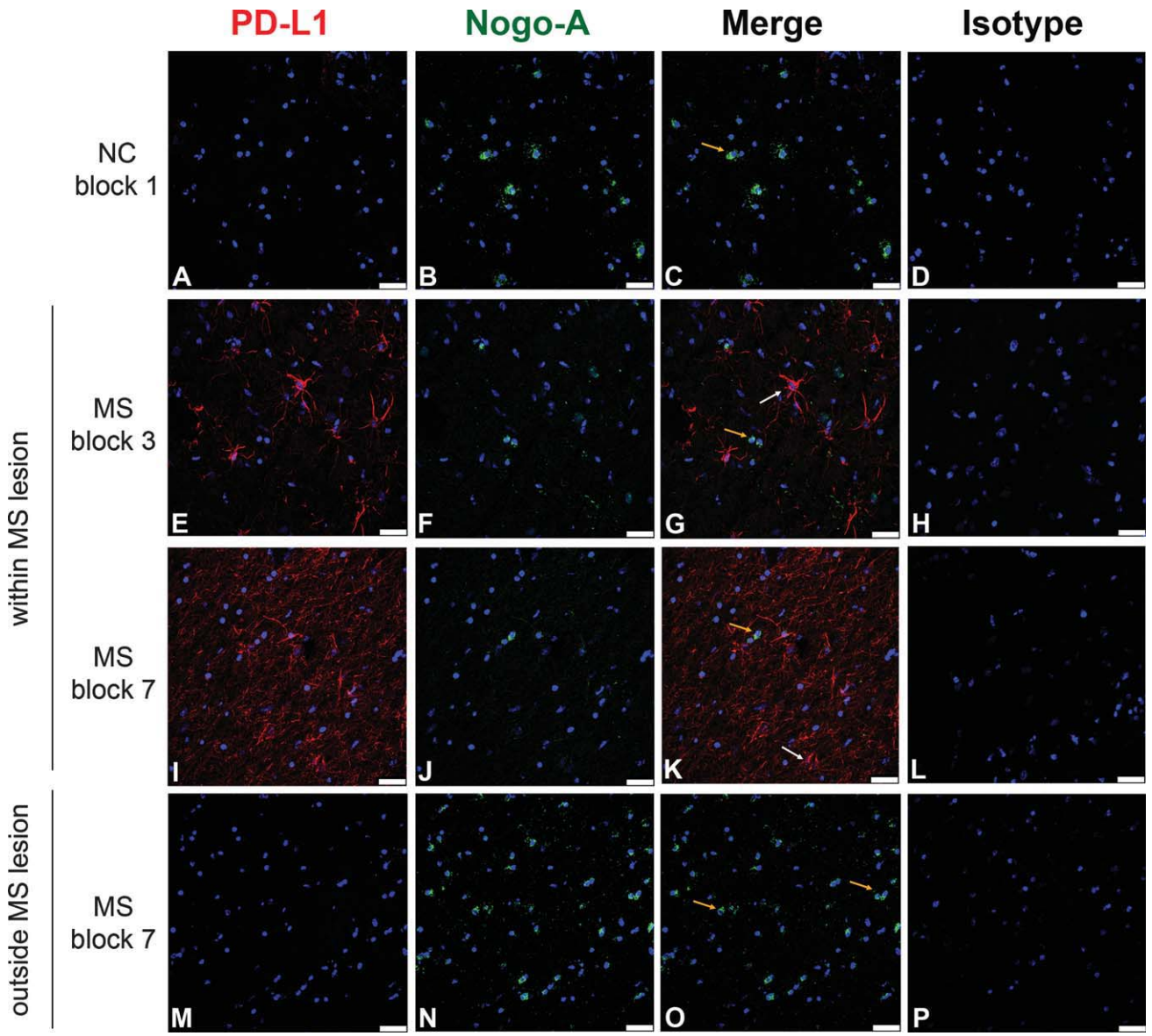

Fig. 7. Oligodendrocytes do not express detectable PD-L1 in human brain sections. (**A**–**P**) Micrographs showing brain sections stained for PD-L1 (red), Nogo-A (green), and nuclei (blue) of one representative normal control (A-C), and two representative MS donors either within or outside lesions as indicated (E–G, I–K, and M-O). Corresponding isotype controls are shown in D, H, L and P. Scale bar = 25 μm. White arrows indicate examples of Nogo-A- PD-L1+; orange arrows indicate example of Nogo-A+ PD-L1−.

L2, but IFN-γ treatment up-regulated PD-L1 and the combination of IFN-γ and TNF led to even greater levels in astrocytes but not neurons. This was unexpected as TNF receptors are constitutively expressed by neurons (Tobinick, 2009) and TNF triggers neuronal responses (Kraft et al., 2009; Rainey-Smith et al., 2010). However, PD-L1 levels on IFN-γ−treated neurons never reached those observed on other CNS cells, especially astrocytes. In contrast, cytokines had no or only a small impact on PD-L2 levels (Figs. 1–3). Our results substantiate that PD-L2 is more restricted than PD-L1 in the CNS similarly to other organs (Keir et al., 2008).

Murine *in vivo* CNS models have shown that strategies systemically inhibiting the PD-L1/2-PD-1 interaction either through peripheral blocking antibody injection or use of globally knockout mice enhanced disease severity, T cell and macrophage infiltration, and T-cell responses (Kroner et al., 2009a; Ortler et al., 2008; Phares et al., 2009; Salama et al., 2003). However, these reports did not assess the impact of local CNS PD-L1 or PD-L2 expression on T-cell functions. Since T lymphocytes can also express PD-L1, especially following their activation (Kinter et al., 2008), it was essential to specifically block the expression of this ligand in CNS cells. Our siRNA approach allowed us to specifically abolish PD-L1 expression on astrocytes which would not have been possible with antibody. Our results demonstrate that CNS cells, especially astrocytes, express functional

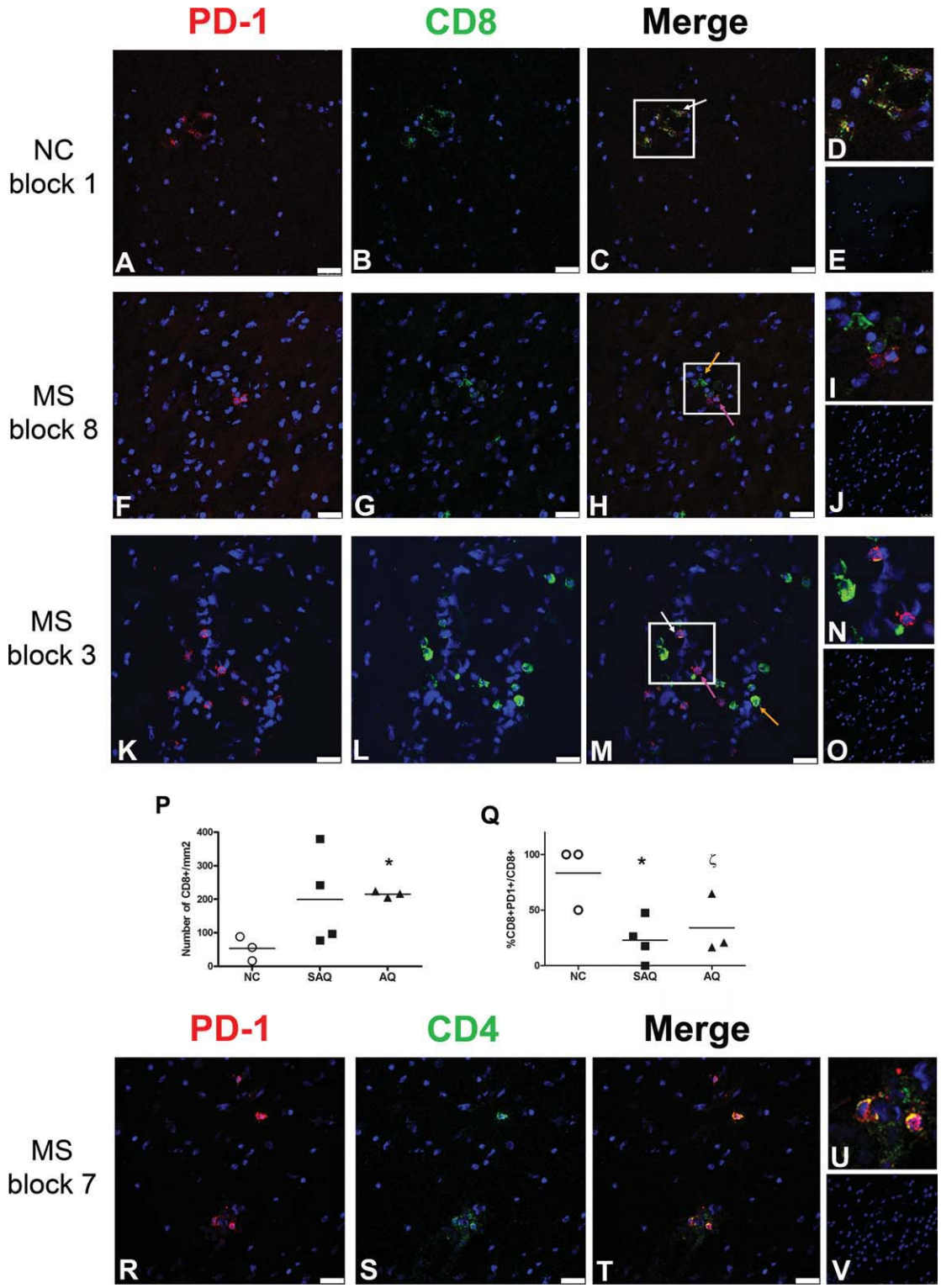

Fig. 8. The majority of CNS-infiltrating CD8 T cells in MS brain lesions do not express PD-1. (**A**–**O**) Micrographs showing brain sections stained for PD-1 (red), CD8 (green), and nuclei (blue) of one representative normal control (A–D), and two representative MS donors (F–I and K–N). Corresponding isotype controls are shown in E, J, and O. Scale bar = 25 μm. White arrows indicate examples of CD8+ PD-1+; pink arrows indicate examples of CD8- PD-1+; orange arrows indicate examples of CD8+ PD-1−. (C–H–M) Cells in white boxes shown enlarged in D-I-N. (**P** and **Q**) Quantification of CD8 T cells (at least five fields analyzed per section) and co-localization for PD-1 in human brains from normal controls (NC), subacute (SAQ), and acute MS lesions (AQ). Each dot represents one distinct section. (P) Number of CD8 T cells observed per mm^2. (Q) Percentage of CD8 T cells positive for PD-1. Student's *t*-test comparing normal controls *versus* MS lesions $^*P < 0.05$, ξ $P < 0.09$. (**R**–**V**) Micrographs showing brain sections stained for PD-1 (red), CD4 (green), and nuclei (blue) of one representative MS donor (R–U). Corresponding isotype control is shown in V. Scale bar = 25 μm. (T) Cells in white box shown enlarged in U.

and sufficient levels of PD-L1 to locally dampen CD8 T cell responses (proliferation, IFN-γ, TNF, granzyme B). The boosted immune responses of CD8 T cells we observed upon co-culture on PD-L1 knockdown astrocytes were partial as only a proportion of *ex vivo* or activated human CD8 T cells bear PD-1, the necessary receptor for PD-L1-mediated inhibition (Bengsch et al., 2010; Day et al., 2006; Kinter et al., 2008). Moreover, inflamed astrocytes express a plethora of activating mediators (boosted MHC expression, secreted cytokine, etc) thus, their final impact on T-cell functions will be dictated by the addition of these numerous factors and PD-L1 is one of them.

Murine CNS PD-L1 expression has been shown to be up-regulated upon various stimuli including inflammation and viral infections. In EAE, PD-L1 was detected on microglia (Magnus et al., 2005) and astrocytes (Salama et al., 2003); but PD-L2 was absent (Salama et al., 2003). PD-L1 expression was increased on astrocytes, but not on active microglia or neurons in mice suffering from entorhinal cortex lesions (Lipp et al., 2007). PD-L1 was detected at high and sustained levels on oligodendrocytes but more transiently on microglia in mice infected with a neurotropic coronavirus, JHM strain of mouse hepatitis virus (Phares et al., 2009). In contrast, rabies virus infection up-regulated PD-L1 in infected murine neurons and immortalized human neurons (Lafon et al., 2008). Therefore, different insults could trigger PD-L1 expression by distinct CNS cell subsets.

Few studies have addressed the PD-L1 expression in the human CNS. One group did not detect PD-L1 in brain tissue sections from normal controls (Wintterle et al., 2003); in contrast, others observed few scattered cells bearing a microglial like morphology expressing PD-L1 in nonpathological CNS sections (Ortler et al., 2008). Similarly, we also observed that up to 5% of microglia in control CNS samples display PD-L1 staining. Thus, a small subset of microglia bear PD-L1 in non-CNS disease patients suggesting that these donors had small and localized CNS insults. We also observed in controls few astrocytes co-stained for PD-L1, except for one section obtained from a donor who died from cardiac arrest (see Table 2, block 6) which had the highest number of astrocytes positive for PD-L1 of the control samples (see Fig. 5). We could speculate that a CNS insult triggered such expression in this particular donor. PD-L1 has been detected in MS patient brain especially in AQ lesions on cells bearing a microglia/macrophage like morphology (Ortler et al., 2008) but quantification or co-staining was not performed in this study. Our quantitative immunohistochemistry observations demonstrate that significantly more microglia/macrophages expressed PD-L1 in MS lesions compared with normal control tissues (see Fig. 6). Moreover, we detected a greater number of PD-L1-expressing astrocytes per surface area in MS lesions compared with controls (see Fig. 5). We could not observe detectable PD-L1 on oligodendrocytes in control and MS tissues (see Fig. 7). It is still possible that human oligodendrocytes could express PD-L1 in human brain in response to stimuli other than those present in MS lesions, as reported in mice infected with a coronavirus (Phares et al., 2009).

The susceptibility of T lymphocytes to PD-L1 or PD-L2-mediated inhibition relies on their expression of PD-1, the inhibitory receptor. Less CD8 T cells in the parenchyma of MS lesions expressed detectable PD-1 compared with normal controls (see Fig. 8). In contrast, we detected PD-1+ cells that were not positive for CD8 in MS lesions illustrating the capacity of other PD-1+ cells, some being CD4 T lymphocytes (see Fig. 8) to infiltrate the CNS of MS patients. Activated CD4 and CD8 T cells have been shown to cross the blood–brain barrier under both pathological and normal conditions (Vrethem et al., 1998) *albeit* in much lower number in normal controls. Thus, the few and scattered CD8 T cells we detected in normal controls were most likely activated T cells expressing PD-1. PD-1 levels on CD4 and CD8 T lymphocytes were lower from relapsing compared to remitting MS patients in blood and cerebrospinal fluid samples obtained from relapsing-remitting MS (RRMS) patients (Trabattoni et al., 2009). These results suggest that during active MS, when an important number of T lymphocytes infiltrates the CNS due to an impaired blood-brain barrier (Sospedra and Martin, 2005), a smaller proportion of these cells expresses PD-1. We can also speculate that CNS infiltrating CD8 T lymphocytes devoid of PD-1 have a functional advantage over their PD-1+ counterparts and can locally proliferate since their TCR signalling is not inhibited by local PD-L1. Alternatively, PD-1+ T cells could have undergone apoptosis in the MS tissues and thus be less abundant than their PD-1 negative counterparts. Indeed, CD8 T-cell clones have been shown to persist over many years in the CNS of MS patients supporting the notion that these cells have locally encountered their cognate antigen and proliferated (Skulina et al., 2004).

PD-1 expression by infiltrating immune cells was evaluated in murine models of CNS disorders. Although in EAE, PD-1 was detected in brain (Liang et al., 2003) and spinal cord (Salama et al., 2003) no quantification assessment was provided. PD-1 knockout mice demonstrated a greater susceptibility to EAE than their wild-type counterparts (Wang et al., 2010). Moreover, in the absence of PD-1, an increased expansion of T lymphocytes in the CNS and an augmented disease severity were observed in transgenic mice overexpressing a myelin gene (Kroner et al., 2009a,b). These mouse models support the hypothesis that PD-1-negative T cells have an elevated capacity to proliferate in the CNS during an ongoing demyelinating disease.

Several lines of evidence support the notion that PD-1+ CD8 T lymphocytes are not intrinsically incompetent at migrating to the CNS or to other organ attacked by deleterious autoimmune responses. A greater number of HIV-specific CD8 T cells were found to express PD-1 in the cerebrospinal fluid compared with their peripheral blood counterparts in HIV patients (Sadagopal et al., 2010). Analysis of most tissues from chronically infected mice with lymphochoriomeningitis virus illustrated that the brain contained the highest levels of PD-1 compared

with other organs (Blackburn et al., 2010). Similarly, analysis of mice infected with a neurotropic virus (strain JHM of mouse hepatitis virus) established that virus specific CD8 T cells in the CNS bore greater PD-1 levels compared with their peripheral counterparts (Phares et al., 2009). Raptopoulou et al. observed that T lymphocytes that have infiltrated the target organ (synovial tissue and fluid) of rheumatoid arthritis patients were enriched in PD-1+ cells compared with healthy tissues (Raptopoulou et al., 2010). Thus, PD-1+ T lymphocytes could be detected in human CNS and other organs targeted by autoimmune diseases supporting the notion that our results demonstrating the absence of PD-1 on most infiltrating CD8 T lymphocytes in MS lesions is novel and unique.

In summary, our novel observations revealed the presence of PD-L1 on CNS cells in an inflamed environment *in vitro* and in MS lesions. We also demonstrate that this molecule has the capacity to modulate CD8 T-cell responses by dampening proliferation and cytokine production. The local expression of PD-L1 by tissue cells has been shown to play a crucial role in inhibiting T-cell responses in different mouse models. Whilst PD-L1 expression by islet cells protects from detrimental autoimmune CD4 T cell attack in a diabetes model (Keir et al., 2006), PD-L1 expression by nonhematogenous cells diminished viral clearance and organ immunopathology mediated by CD8 T lymphocytes in a chronic infection model (Mueller et al., 2010). Thus, human CNS cells can achieve the same capacity of abrogating T-cell responses. Finally, the lack of PD-1 on numerous infiltrating CD8 T cells suggests that these cells will not receive an inhibitory signal from the PD-L1 expressing CNS cells, astrocytes, or microglia/macrophages. Strategies aimed at boosting PD-1 levels on activated autoreactive human T cells could be a new promising therapeutic approach since PD-L1 is already increased in the target organ.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Diane Beauseigle and Manon Blain. The authors acknowledge the contribution of Drs. André Oliver and Jeffrey Hall for providing adult human CNS tissues for the *in vitro* studies. The authors are grateful to Dr. Alexandre Prat for access to the Leica confocal microscope and the LSRII flow cytometer.

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