Neuroimmunoendocrine Regulation of the Prion Protein in Neutrophils*

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Background: Prion protein (PrPc) modulates inflammation, and prion diseases affect neutrophil numbers and functions, but the regulation of PrPc in neutrophils is unknown.

Results: Inflammation and stress massively up-regulated PrPc in neutrophils via glucocorticoids and TGF-β.

Conclusion: We show a novel pathway of regulation of PrPc, with functional consequences for neutrophils.

Significance: Systemic control of the expression and function of PrPc broadly modulates cellular physiology and pathology.

The prion protein (PrPC) is a cell surface protein expressed mainly in the nervous system. In addition to the role of its abnormal conformer in transmissible spongiform encephalopathies, normal PrPC may be implicated in other degenerative conditions often associated with inflammation. PrPC is also present in cells of hematopoietic origin, including T cells, dendritic cells, and macrophages, and it has been shown to modulate their functions. Here, we investigated the impact of inflammation and stress on the expression and function of PrPC in neutrophils, a cell type critically involved in both acute and chronic inflammation. We found that systemic injection of LPS induced transcription and translation of PrPC in mouse neutrophils. Up-regulation of PrPC was dependent on the serum content of TGF-β and glucocorticoids (GC), which, in turn, are contingent on the activation of the hypothalamic-pituitary-adrenal axis in response to systemic inflammation. GC and TGF-β, either alone or in combination, directly up-regulated PrPC in neutrophils, and accordingly, the blockade of GC receptors in vivo curtailed the LPS-induced increase in the content of PrPC. Moreover, GC also mediated up-regulation of PrPC in neutrophils following noninflammatory restraint stress. Finally, neutrophils with up-regulated PrPC presented enhanced peroxide-dependent cytotoxicity to endothelial cells. The data demonstrate a novel interplay of the nervous, endocrine, and immune systems upon both the expression and function of PrPC in neutrophils, which may have a broad impact upon the physiology and pathology of various organs and systems.

The prion protein (PrPc) is a highly conserved glycosylphosphatidylinositol-anchored cell surface protein, expressed mainly in the nervous and immune systems (1). Abnormal conformers of PrPc, often referred to as scrapie or PrPSc, are associated with the neurodegenerative disorders called transmissible spongiform encephalopathies, or prion diseases (2, 3). PrPc has also recently been implicated in mouse models of Alzheimer disease (4–8), and data have been reported of a modest, context-dependent influence of PrPc upon rodent models of other neurodegenerations (9). Recent work even highlights the novel hypothesis that other proteins and peptides involved in neurodegeneration may misbehave similar to PrPc in the context of disease (10).

Although physiological functions of the prion protein remain controversial (9), its cellular content affects not only neural activity and integrity (11) but also innate and acquired immunity (reviewed in Linden et al. 1 and Isaacs et al. 12). Peripheral inflammation induced by ligands of either Toll-like receptors or Fas, as well as phagocytosis by macrophages, were affected in PrPc-null mice (1, 13), whereas the content of PrPc in monocytes is modulated by IFN-γ (14). These results suggest that PrPc is involved in inflammation. Neutrophils play critical roles in both acute and chronic inflammatory responses (15, 16), but nothing is known of physiological roles of PrPc in this cell type (1). Notwithstanding, among early work on the pathogenesis of transmissible spongiform encephalopathies, changes were reported on the neutrophil numbers and functions both in transmissible spongiform encephalopathy patients and in animals infected with scrapie (17–19). Similarly, neutrophil functions were reportedly affected in both Alzheimer and Parkinson disease patients (20, 21). The functional impact of such changes has yet to be assessed, but the data suggest that neutrophils are

The abbreviations used are: PrPc, prion protein; GC, glucocorticoid; DEX, dexamethasone; PMA, phorbol 12-myristate 13-acetate; BMC, bone marrow cell; CM, conditioned medium; bAEC, bovine aortic endothelial cell; nAb, neutralizing antibody; rh, recombinant human.
affected by neurodegenerative diseases, which are in turn modulated by peripheral inflammation (22).

Physiological properties of the prion protein, often associated with those diseases, appear to be particularly associated with the processing of either systemic or cellular stress (1), arguing for a possible regulatory loop involving PrP(Sc) in inflammatory responses. Here, we examined the expression and role of the prion protein in neutrophils using the well established model system of acute systemic inflammation induced by LPS, as well as a behavioral restraint model of inflammation-independent stress.

**EXPERIMENTAL PROCEDURES**

**Reagents**—RPMI 1640 culture medium and sodium thioglycolate were from Invitrogen. LPS from *Salmonella enterica*, RU486, phorbol 12-myristate 13-acetate (PMA), catalase, neutral red, propidium iodide, and recombinant human HMG-1 were from Sigma. Hydrogen peroxide was from Merck. Percoll® and first strand cDNA synthesis kit were from GE Healthcare. Glycoprotein deglycosylation kit was from Calbiochem. Monoclonal antibodies anti-mouse Ly-6G (Gr-1), CD16/32, CD3, CD4, CD8, CD115, CD45R (B220), NK1.1, TER-119, TNF-α nAb and IL-1β nAb, as well as recombinant mouse TNF-α, IL-6, and IL-1β were from eBioscience. rhIL-4, IL-10, and IL-13 were from PeproTech. rhTGF-β1, TO-PRO-3 iodide, and TRIZol® reagent were from Invitrogen. recombinant mouse GM-CSF and polyclonal anti-TGF-β nAb were from R&D Systems. Dexamethasone (Decadron) was from Aché Laboratórios Farmacêuticos. Recombinant human macrophage migration inhibitory factor was kindly provided by Dr. Christine Metz (The Feinstein Institute for Medical Research, New York). Monoclonal SAF32 anti-PrP(Sc) antibody was from Cayman Chemical. Polyclonal anti-PrP(Sc) antisera was produced against recombinant mouse PrP(Sc) in PrP(Sc)-null mice (23). Serum from nonimmunized animals was used as negative control. Secondary antibodies for either flow cytometry or for immunofluorescence and Western blots were from Jackson ImmunoResearch and Santa Cruz Biotechnology, respectively. Coat-A-Count corticosterone radioimmunoassay kit was from Siemens, and ECL Plus kit was from Amersham Biosciences.

**Mice**—C57BL/6 mice were from either the Fluminense Federal University or the State University of Campinas (Brazil). Tumor necrosis factor receptor p55 knock-out mice (*TNFR1−/−*) (24) were a gift of Dr. Leda Quercia (Federal University of Minas Gerais, Brazil). C57BL/10 and PrP(Sc) knock-out mice (*Npu Prnp−/−*) (25), backcrossed to C57BL/10 mice for at least 10 generations, were kindly provided by Bruce Chesebro and Richard Race (Rocky Mountain Laboratories, NIAID, National Institutes of Health). Heterozygous animals were mated, and homozygous F1 littermates were crossed to generate *Npu Prnp−/−* or *Npu Prnp+/+* embryos. Animals were housed in plastic boxes with food and water *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 a.m.). Experiments were done with male mice between 8 and 12 weeks old, weighing 20–25 g, in accordance with current guidelines for the care and use of laboratory animals, as described by the National Institutes of Health and approved by the Committee for the Use of Experimental Animals from the Center of Health Sciences, Federal University of Rio de Janeiro.

**In Vivo LPS or DEX Challenges and Restraint Stress**—Mice were treated by intraperitoneal (i.p.) injections with saline, LPS, or DEX for 24 h or physically restrained in well ventilated 50-ml conical polypropylene tubes for 15 h (6:00 p.m. to 9:00 a.m.) as described previously (26). Pretreatment of mice with RU486 (500 μg/animal) by oral gavage was done at 2 h before LPS treatment or restraint. Animals were euthanized, and bone marrow cells (BMC) were collected from their femurs. Red blood cell lysis was performed with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2).

**Enrichment of Neutrophils**—BMC were collected; red blood cells were lysed, and Fc receptors were blocked with 5% normal mouse serum. Labeling was carried out by incubating cells with biotin-conjugated antibodies to Ter119, CD3, B220, CD4, CD8, NK1.1, and CD115. After washing, cells were incubated with streptavidin-conjugated Dynabeads®, and negative selection of neutrophils was performed in DynaMag™-15 magnets according to manufacturer’s instructions (Invitrogen). Alternatively, cells were layered on a two-layer Percoll® gradient (62 and 81%) after red blood cell lysis. Neutrophils were harvested from the 62/81% interface after centrifugation for 20 min at 700 × g. More than 95% (negative selection) or 85% (Percoll) of the collected cells were neutrophils as judged by both flow cytometry and May–Grünewald–Giemsa staining.

**Data Acquisition and Analysis by Flow Cytometry**—Flow cytometric analyses were done in a FACSCalibur (BD Biosciences). Cells were washed, and Fc receptors were blocked as above. Antibodies coupled to fluorochromes or polyclonal anti-PrP(Sc) were added for 20 min at 4°C. Polyclonal anti-PrP(Sc) was developed with a phycoerythrin-conjugated anti-mouse (Fab’). Propidium iodide was added to samples at 1 μg/ml immediately before acquisition, to exclude dead cells from analysis. Acquisition from at least 10,000 cells was done with CellQuest (BD Biosciences), and FlowJo (Tree Star) was used for further analysis. PrP(Sc) fluorescence intensity was measured as the difference between the median values of cells labeled with polyclonal anti-PrP(Sc) and those stained with a nonimmune serum and expressed as percentage of control.

**Immunofluorescence Microscopy**—Neutrophils were fixed with 4% paraformaldehyde for 1 h at room temperature, seeded onto poly-l-lysine-coated slides, and permeabilized with 0.5% Triton X-100 in 1% BSA for 30 min. PrP(Sc) was detected using an anti-PrP(Sc) antibody followed by a secondary DyLight 488 antibody, and nucleus was stained with TO-PRO-3 iodide. Images were taken with a Zeiss LSM 510 META confocal microscope and processed using Adobe Photoshop CS5.

**Western Blots**—Neutrophils, bone marrow, or brain samples were homogenized in extraction buffer (50 mM Tris⋅HCl, pH 7.4, 4°C, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, containing 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). After 15 min, samples were centrifuged at 10,000 × g for 20 min at 4°C, and the supernatant was collected. Protein concentration was estimated by the Lowry method using bovine serum albumin as standard. Lysates were resolved by SDS-12% PAGE and transferred to a nitrocellulose membrane, which was then blocked
with 5% nonfat dry milk in TBS-T (40 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, probed with the primary antibodies overnight at 4 °C, and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Protein levels were detected with ECL. Plus according to the manufacturer’s directions. Membranes were stripped and reprobed for Erk2 as a loading control. Protein deglycosylation with N-glycosidase F was done as per manufacturer’s protocol, except that the enzyme incubation time was increased from 3 to 16 h, as described (27).

Real Time RT-PCR—We performed the RNA extraction, cDNA synthesis, and real time RT-PCR analysis as published previously (28). Cells were isolated as described and kept on ice. RNA was extracted using TRIzol® reagent according to the manufacturer’s instructions and reverse-transcribed using the First Strand cDNA synthesis kit. Real time RT-PCR primers and probes for mouse Prion (Prnp) and GAPDH (Gadp) were designed using Primer Express software (ABI) (Reporter-FAM, Quencher-BHQ, nonfluorescent). Table 1 shows the sequences of these primers and probes. The cDNA amplifications were performed in 20-μl reactions containing 200 nM of primers, 200 nM of probe, and TaqMan Universal PCR Mastermix on an ABI 7500 System (Applied Biosystems). PCR cycle parameters were 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 75 °C for 15 s and 60 °C for 1 min.

Measurement of Serum Corticosterone—Serum samples were obtained from blood collected by cardiac puncture. Corticosterone levels from serum were measured with the Coat-A-Count corticosterone radioimmunoassay kit. Briefly, 50 μl of standard or serum and 1 ml of 125I-corticosterone were incubated for 2 h in corticosterone Ab-coated tubes. Tubes were decanted, and each radiolabeled tube was read using a gamma counter for 1 min. Corticosterone concentration was expressed in nanograms/ml.

In Vitro Cell Treatment—BMC were plated in 24-well plates and were either treated or not with recombinant cytokines. Alternatively, cells were treated with LPS alone, DEX, macrophage conditioned media, or blood serum from animals previously subjected to either restraint stress or LPS treatment. Inhibition was tested with neutralizing antibodies to TNF-α, IL-1β, or TGF-β or with RU486 added 30 min before other treatments.

Cytotoxicity Assay—Neutrophil cytotoxicity was studied following a described procedure (29). Briefly, CM were obtained from neutrophils cultured for 30 min with 1 μg/ml PMA. In some cases, catalase (1 μg/ml) was added to CM 15 min before treatment of cells. Bovine aortic endothelial cells (bAEC; Lonza Walkersville, Inc.) were incubated for 6 h with CM; the medium was removed, and cells were incubated for another 2 h with RPMI medium containing 40 μg/ml neutral red. Subsequently, cells were washed and incubated with a destaining solution (50% ethanol, 49% deionized water, and 1% glacial acetic acid) to release the dye incorporated by viable cells. Measurement of the OD of the neutral red extract was done at 540 nm in a SpectraMax microplate reader (Molecular Devices), and data were expressed as percentage of control.

Statistical Analysis—Results are expressed as means ± S.E., and the data were analyzed by unpaired t test, one-way or two-way analysis of variance with Dunnett or Bonferroni post tests, as appropriate, using GraphPad Prism software version 5.03. p < 0.05 was considered significant. Sample sizes are indicated in the figure legends.

RESULTS

Systemic Administration of LPS Regulates PrP<sup>C</sup> in Bone Marrow Neutrophils—We first analyzed the effect of systemic inflammation upon PrP<sup>C</sup> in neutrophils. Mice were subject to intraperitoneal injections of LPS, and bone marrow cells were collected at various intervals for analysis through flow cytometry. LPS induced a dose-dependent (Fig. 1, A and B) and time-dependent (Fig. 1C) up-regulation of PrP<sup>C</sup> in Gr-1<sup>+</sup> cells, a fraction highly enriched in neutrophils. Surface content of PrP<sup>C</sup> in neutrophils was confirmed by laser scanning confocal microscopy (Fig. 1D). PrP<sup>C</sup> content was also verified by Western blots; protein extracts from Gr-1<sup>+</sup> cells, but not from brain tissue, obtained from mice treated with LPS, had a higher PrP<sup>C</sup> content as compared with untreated animals, as shown with a polyclonal anti-PrP<sup>C</sup> antibody (Fig. 1F). Other populations of bone marrow cells, mostly lymphocytes and monocytes, express little or no prion protein, and its content was not changed in those cells after LPS treatment (data not shown).

We tested for a direct effect of LPS upon neutrophils in bone marrow cell cultures. Following treatment for 24 h, LPS alone had no effect on PrP<sup>C</sup> content in neutrophils, whereas cells treated with serum collected from animals previously injected with LPS showed a robust increase in surface immunoreactivity to PrP<sup>C</sup> (Fig. 1E). This was also confirmed by Western blots (Fig. 1G) and indicates that effects of LPS depend on one or more secondary mediators present in the serum. Production of such mediators is not dependent on PrP<sup>C</sup>, because serum from PrP<sup>C</sup>-null mice treated with LPS also increased PrP<sup>C</sup> content in wild-type neutrophils in vitro (data not shown).

Major Pro-inflammatory Mediators Are Not Required for LPS-dependent Up-regulation of PrP<sup>C</sup> in Neutrophils in Vivo—Next, we tested whether up-regulation of PrP<sup>C</sup> by endotoxemia was dependent on the main proinflammatory cytokines. Treatment of bone marrow cells in vitro with CM derived from LPS-activated peritoneal macrophages, a rich source of proinflammatory cytokines, resulted in up-regulation of PrP<sup>C</sup> by neutrophils, dependent on TNF-α but not IL-1β (Fig. 2, A and B). Also, recombinant TNF-α alone, but not IL-1β, IL-6, GM-CSF, macrophage migration inhibitory factor, or HMG-1, induced up-regulation of PrP<sup>C</sup> in neutrophils (Fig. 2C).

### Table 1

| Gene | 5′-Primer | Probe | 3′-Primer |
|------|-----------|-------|-----------|
| Prnp | 5′-TCTGTGTCCCCCATAGCTCAA-3′ | 5′-CCCCTGGCAGCTATGGGCC-3′ | 5′-AACAAAGACCAACTGCTCTACTCTT-3′ |
| Gadp | 5′-CTCCACTCTAGGCGAAATTCA-3′ | 5′-AGGCCGAAGATGGAAAGTGTTGTCAT-3′ | 5′-TAATGTTAGAAGGCTGCTGC-3′ |
We therefore tested whether TNF was required for the upregulation of PrP<sup>C</sup> induced by the serum collected from animals previously treated with 50 μg of LPS for 12 h (from now on referred to as “LPS-serum”). Surprisingly, treatment of bone marrow cells with an anti-TNF-α-neutralizing antibody did not prevent up-regulation of neutrophil PrP<sup>C</sup> by the LPS-serum (Fig. 2D), although the effect of recombinant TNF-α was completely blocked (data not shown). To verify that TNF-α was not required in the regulation of PrP<sup>C</sup> in vivo following injections of LPS, we treated mice that lack TNFRp55 (TNFR<sup>-/-</sup>) with LPS-serum from wild-type mice, neutrophils from bone marrow of animals either treated or not with 50 μg of LPS for 24 h were fixed, permeabilized, labeled with anti-PrP<sup>C</sup>, counterstained with TO-PRO-3, and examined by confocal microscopy. Scale bar, 5 μm.

**FIGURE 1.** Systemic administration of LPS regulates PrP<sup>C</sup> in bone marrow neutrophils. A, mice were injected intraperitoneally with 50 μg of LPS, and 24 h later BMC were collected, and PrP<sup>C</sup> content at the surface of Gr-1<sup>+</sup> cells was analyzed by flow cytometry. Control animals (CTR) were injected with sterile saline alone. NIS, nonimmune serum. B, mice were injected intraperitoneally with varying doses of LPS, and BMC were analyzed after 24 h. C, animals were injected intraperitoneally with 50 μg of LPS, and BMC were collected at various time points after injection, and PrP<sup>C</sup> content at the surface of Gr-1<sup>+</sup> cells was analyzed. D, neutrophils purified from the bone marrow of animals either treated or not with 50 μg of LPS for 24 h were fixed, permeabilized, labeled with anti-PrP<sup>C</sup>, counterstained with TO-PRO-3, and examined by confocal microscopy. Scale bar, 5 μm. E, BMC from untreated animals were cultured in vitro for 24 h either with varying doses of LPS (left panel) or with 10% serum from animals previously treated at various intervals with 50 μg of LPS (LPS-serum; right panel). The relative increase of surface PrP<sup>C</sup> in bone marrow neutrophils was estimated by flow cytometry. F, immunoblots showing PrP<sup>C</sup> content of lysates from cerebral cortex or bone marrow neutrophils from animals either treated (+) or not (−) with 50 μg of LPS for 24 h. Erk2 was used as a loading control. Molecular mass standards are indicated at the left in kDa. G, BMC were cultured in vitro for 24 h with either 10% control or 10% LPS-serum obtained 12 h after administration of PBS or LPS (50 μg). Immunoblots of the respective lysates were stained for PrP<sup>C</sup>. *, p < 0.01 (B); **, p < 0.05, and ***, p < 0.01 (C and E) compared with untreated groups; n ≥ 3.

**TGF-β and Glucocorticoids Concur to Regulate PrP<sup>C</sup>**—Both the establishment and resolution of systemic inflammation induced by LPS depend on a complex network of mediators and feedback that restores homeostasis. The main proinflammatory cytokines were not essential for the up-regulation of PrP<sup>C</sup>, which occurs relatively late in our experimental model of acute inflammation. We therefore focused on molecules with immunoregulatory and anti-inflammatory actions, which normally arise in the late phase of the reaction, consistent with the time course of the effect observed with LPS-serum (Fig. 1C). Accordingly, bone marrow cells were treated in vitro for 24 h with the cytokines IL-4, IL-10, IL-13, or TGF-β. Among these, treatment with TGF-β alone dose-dependently up-regulated PrP<sup>C</sup> in neutrophils (Fig. 3A).

In addition to cytokine-mediated regulation, resolution of LPS-induced inflammation involves additional mediators. Notably, treatment with LPS induces the activation of the hypothalamic-pituitary-adrenal axis and the release of glucocorticoids (GC), which in turn both help inhibit the production of proinflammatory mediators, and induce anti-in-
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FIGURE 2. LPS-induced PrP<sup>C</sup> regulation is not dependent on major proinflammatory cytokines. A, BMC were cultured in vitro for 24 h with CM prepared from thioglycolate-elicited peritoneal macrophages stimulated for 24 or 48 h with 0.1 or 1 μg/ml LPS. The relative increase of surface PrP<sup>C</sup> in Gr-1<sup>+</sup> cells was evaluated by flow cytometry. B, BMC were cultured for 30 min in the presence of 1 μg/ml neutralizing antibody against TNF-α (TNF-α nAb) or IL-1β (IL-1β nAb) before stimulation with CM derived from macrophages. C, BMC were cultured in the presence of various doses (ng/ml) TNF-α, and analyzed as before. D, BMC were cultured for 30 min in the presence of 10 μg/ml TNF-α nAb before treatment for 24 h with different doses of LPS-serum. E, TNFR1 null mice were injected intraperitoneally with 50 μg of LPS; 24 h later their BMC were collected and analyzed by flow cytometry. Control animals (CTR) received intraperitoneal injections of sterile saline alone. F, BMC obtained from TNFR1 null mice were cultured in vitro for 24 h with increasing doses of TNF-α (gray bars) or in the presence of 10% control or 10% LPS-serum (black bars) and examined as before. G, wild-type BMC were cultured in vitro for 24 h in the presence of 10% serum obtained from TNFR1 null mice previously treated with either LPS or sterile saline alone (CTR). *, p < 0.05 (A and B); **, p < 0.01 (A and B); ***, p < 0.001 (A, F, and G), and ***, p < 0.01 (B) compared with untreated groups, and #, p < 0.05 compared with CM LPS alone; n ≥ 3.

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FIGURE 3. TGF-β and glucocorticoids concur to regulate PrP<sup>C</sup> in vitro. Bone marrow cells were cultured in vitro for 24 h with various concentrations of either TGF-β (A) or DEX (B) and analyzed by flow cytometry for PrP<sup>C</sup> content at the surface of Gr-1<sup>+</sup> cells. In some cases, the glucocorticoid receptor antagonist RU486 was added to the cultures 30 min before DEX treatment (B). *, p < 0.01, and **, p < 0.001 compared with untreated groups, and #, p < 0.01 compared with each dose of DEX alone; n ≥ 5 (A) and n ≥ 4 (B).

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flammary cytokines (33). Interestingly, GC increase the half-life of human neutrophils (34, 35) and selectively increase the number of neutrophils in both blood and bone marrow in mice, without impairing cell functions (36). Thus, we tested whether GC would affect the regulation of neutrophil PrP<sup>C</sup>. Treatment of bone marrow cell cultures for 24 h with DEX massively up-regulated PrP<sup>C</sup> in neutrophils. This effect was dose-dependent and was abrogated by pretreatment of cells with the GC receptor antagonist mifepristone (RU486) (Fig. 3B).

To directly test for an in vivo role of glucocorticoids, mice received intraperitoneal injections of DEX. In agreement with the results obtained in vitro, DEX strongly up-regulated neutrophil PrP<sup>C</sup> in vivo in a dose-dependent (Fig. 4, A and B) and time-dependent manner (Fig. 4C). The results were confirmed both by laser scanning confocal microscopy (Fig. 4D) and in Western blots, where we found up-regulation of PrP<sup>C</sup> in the bone marrow but not in the brain of wild-type mice of two distinct strains, as shown both with a polyclonal anti-PrP<sup>C</sup> (Fig. 4, E and F) and with the commercially available monoclonal antibody SAF32 (Fig. 4, G and H). Treatment of isolated neutrophils with DEX also up-regulated PrP<sup>C</sup> (Fig. 4H), a finding that excludes the requirement of other cell types for this effect, and strongly suggests action upon neutrophils. The specificity of the antibodies to PrP<sup>C</sup> was confirmed by the lack of staining of protein isolated from tissues of PrP<sup>C</sup> knock-out mice (Fig. 4, F–H). Similar to neutrophils from LPS-treated mice (Fig. 1F), as well as human platelets (27), immunoblots of DEX-treated bone marrow cells included predominantly protein species with molecular masses higher than those of brain tissue. Notwithstanding, deglycosylation of protein extracts reduced the immunodetected protein to a single band of ~25 kDa (Fig. 4I). This is consistent with the exclusive detection of PrP<sup>C</sup> in our Western blots.
We next examined the role of TGF-β and GC upon the up-regulation of neutrophil PrP<sup>C</sup> induced by LPS. Bone marrow cells were cultured in the presence of a neutralizing antibody to TGF-β/H9252 and/or RU486 before adding the LPS-serum. Pretreatment of cells with either anti-TGF-β or RU486 inhibited 60–65% of the effect of LPS-serum, although simultaneous treatment with both drugs completely blocked the effect of the serum (Fig. 5A). In addition, in vitro treatment of cells with both TGF-β and DEX led to a synergistic effect on PrP<sup>C</sup> content (Fig. 5B). Finally, when animals were pretreated with RU486 prior to the injection of LPS, there was a profound inhibition of the effect of endotoxemia on neutrophil PrP<sup>C</sup> content (Fig. 5C).

**FIGURE 4. Glucocorticoid up-regulates PrP<sup>C</sup> in vivo.** A, mice received intraperitoneal injections of 2 mg of DEX, and 24 h later their BMC were collected and analyzed by flow cytometry. Control animals (CTR) received intraperitoneal injection of sterile saline alone. NIS, nonimmune serum. B, mice were injected intraperitoneally with varying doses of DEX, and BMC were analyzed as above. C, mice were injected intraperitoneally with 2 mg of DEX and had their BMC collected at various time points for analysis. D, neutrophils purified from the bone marrow of animals either treated or not with 2 mg of dexamethasone for 24 h were fixed, permeabilized, labeled with anti-PrP<sup>C</sup>, counterstained with TO-PRO-3, and examined by confocal microscopy. Scale bar, 5 μm. E–H, immunoblots showing PrP<sup>C</sup> content of lysates from brain and bone marrow cells (E–G and I) or isolated neutrophils (H) from C57/BL6 (E, H, and I) or C57/BL10 (F and G) mice either treated or not with DEX as before. A polyclonal antiserum raised in Prnp<sup>0/0</sup> mice was used in E and F, and a monoclonal anti-PrP<sup>C</sup> SAF32 was used in G–I. Erk2 was used as a loading control. Molecular mass standards are indicated at the left in kDa. In I we show the analysis of deglycosylated PrP<sup>C</sup>. BMC obtained from DEX-treated C57/BL6 mice were treated or not with N-glycosidase F (PNGase) and probed for PrP<sup>C</sup>. *<sup>,</sup> p < 0.001 (B); *, p < 0.05, and **, p < 0.001 (C) compared with untreated groups; n > 3.
These data indicate that TGF-β and GC concur to up-regulate neutrophil PrP<sub>C</sub> during systemic inflammation in vivo.

**Behavioral Stress Increases PrP<sub>C</sub> Content in Neutrophils**—The experiments above suggested that the main proinflammatory cytokines were dispensable for the LPS-induced up-regulation of PrP<sub>C</sub>. Because TGF-β and GC appear to jointly mediate neuroendocrine stress responses in the absence of an inflammatory insult (37), we examined the content of PrP<sub>C</sub> in neutrophils following a behavioral restraint stress protocol, which is known to increase the serum levels of both TGF-β and GC (26, 37).

Animals were physically restrained in well ventilated polypropylene tubes for 15 h, after which they were euthanized and their bone marrow cells were collected for analysis. Strikingly, the amount of PrP<sub>C</sub> in neutrophils obtained from stressed animals was more than three times higher than in unstressed animals. Pretreatment by oral gavage of mice for 2 h with RU486 inhibited ~70% of the stress effect on neutrophil PrP<sub>C</sub> (Fig. 6A).

Furthermore, the serum obtained from restrained animals (“RS-serum”) also induced up-regulation of PrP<sub>C</sub> in vitro (Fig. 6B). Finally, as expected, serum obtained from animals stressed by either restraint or LPS contained 2–3 times more corticosterone than that of nonstressed animals (Fig. 6C). Taken together, these findings show a clear effect of stress-induced release of at least GC upon the regulation of neutrophil PrP<sub>C</sub> both in vitro and in vivo.

**Increased PrP<sub>C</sub> Is Associated with Enhanced Gene Expression**—Because flow cytometric and Western blot analyses cannot determine whether regulation of PrP<sub>C</sub> occurs at the transcriptional or post-transcriptional level, we examined gene expression using real time reverse transcriptase-PCR (real time RT-PCR). Cells were taken from the bone marrow of animals treated or not with either LPS (5 μg/animal) or DEX (2 mg/animal) for 12 h, as well as from restrained animals. We found a 2.5–7-fold increase in Prnp mRNA (Fig. 7A), indicating transcriptional regulation of the prion protein by stressful conditions.

**Evidence of Functional Impact of Up-regulated Prion Protein in Neutrophils**—As a preliminary approach to the functional impact of the up-regulation of PrP<sub>C</sub>, we compared phagocytic and cytotoxic activities of neutrophils from either wild-type (WT) or Prp-null mice (KO) following GC treatment with that of corresponding neutrophils of untreated mice. No differences were found among phagocytosis of zymosan particles by neutrophils from either GC-treated or untreated mice of either...
genotype (data not shown). However, an unexpected effect was observed on neutrophil cytotoxicity to bAEc. These cells were incubated in the presence of conditioned media prepared from cultures of PMA-treated neutrophils obtained from either WT or KO mice previously treated or not with DEX. Treatment with DEX led to increased cytotoxicity of CM obtained from wild-type but not PrP^C-null neutrophils (Fig. 7B). The effect was completely abrogated by incubation of CM with catalase, indicating a direct role for hydrogen peroxide (H_2O_2) in the process. Notably, CM derived from PrP^C-null neutrophils treated or not with DEX and/or PMA exhibited a remarkably higher peroxide-dependent cytotoxicity than untreated WT cells. Neutrophils were, in turn, tested for both spontaneous and H_2O_2-induced cell death. Interestingly, there was no difference in the sensitivity of either wild-type or PrP^C-null neutrophils to peroxide. In addition, whereas pretreatment with corticosteroid hormone resulted in the expected cytoprotection and H_2O_2-induced cell death. bAEc obtained from WT or KO mice previously treated or not with DEX were cultured for 24 h in RPMI without serum, containing H_2O_2 at various concentrations. Cells were stained with 1 μg/ml propidium iodide (PI) and analyzed by flow cytometry. *, p < 0.05; **, p < 0.001 compared with untreated group; n = 3 (B and C).

**DISCUSSION**

The prion protein is present in several distinct cell types outside the nervous system, including almost all lineages of hematopoietic origin (reviewed in Isaacs et al. (12)). In hematopoiesis-derived cell types, PrP^C content seems to be tightly regulated along differentiation or maturation, but its primary function in the immune system is still controversial. Previous studies have suggested that PrP^C is a component of the T cell receptor (38, 39), modulates lymphocyte activation (40), and stabilizes the immunological synapse between lymphocytes and dendritic cells (41). These functions are reminiscent of the proposed role of PrP^C in the formation and activity of neuronal synapses (42, 43) and add to the suggested roles of PrP^C in phagocytosis both within and outside the nervous system and in inflammatory responses (13).

Here, we provide evidence of a major neuroimmunoendocrine regulation of the content and function of the prion protein in neutrophils, which has eluded previous studies. It is interesting to note that, so far, we observed such stress-induced up-regulation of the prion protein only in neutrophils. No other cell type in the bone marrow cells up-regulated PrP^C in the present experimental conditions, including related myeloid cells, such as resident or inflammatory monocytes. The magnitude and selectivity of the up-regulation of PrP^C in neutrophils argues for an important role of PrP^C in physiological properties of these cells.

Previous studies have identified promoter regions of the Prnp gene both in the 5’-flanking region and within the first intron, containing several Sp1-binding sites, as well as a number of elements potentially involved in transcriptional regulation, such as AP1, AP2, MZF-1, MEF2, MyT1, Oct-1, NFAT, POZ (BCL6); RP58 (ZNF238); NEUROG1; EGR4, Oct-1/Oct-2, POZ (BCL6); RP58 (ZNF238); NEUROG1; EGR4, Oct-1/Oct-2, NF-IL6, MyoD, p53, HSE, MRE, and MLS but not CRE, NF-κB, or OTF-1 (1). Our results strongly suggest a direct action of glucocorticoids and TGF-β upon neutrophils, leading to up-regulation of PrP^C at the transcriptional level. Indeed, we located both a candidate glucocorticoid-response element and a possible Smad-responsive element in the second intron of the mouse Prnp gene (data not shown). Strikingly, neutrophils obtained from restrained animals showed an augmented content of PrP^C, demonstrating that behavioral stress alone, without inflammation, is sufficient to induce the Prnp gene, a finding consistent with the simultaneously elevated levels of corticosteroids and TGF-β in stressed animals. Collectively, these results place the regulation of the expression of a prion gene under the control of the hypothalamic-pituitary-adrenal axis, thus revealing a novel aspect of the systemic control of prion biology.

In previous studies, despite the widespread expression of the prion protein in the immune system, no bulky defects were found in PrP-null mice concerning the number or state of maturation of immune cells nor the expression of their typical surface antigens (12). This suggests that immunomodulatory functions of PrP^C are either subtle or may be critical only in...
particular circumstances, such as in the hematopoietic engraf-ment of lethally irradiated mouse recipients (44) or in the response of macrophages to proinflammatory mediators (13, 45).

In our initial assessment of the impact of up-regulated PrP\textsuperscript{C} upon neutrophil functions, a selective effect on cytotoxic activity was noted. The higher cytotoxic activity of conditioned media from PrP\textsuperscript{C}-null neutrophils, as compared with CM from wild type, suggests that deletion of PrP\textsuperscript{C} may have a significant functional impact upon these cells. However, treatment with corticosteroid in vivo did not affect cytotoxic activity of PrP\textsuperscript{C}-null neutrophils, whereas the cytotoxicity of neutrophils from wild-type mice was greatly enhanced in parallel with the massive up-regulation of PrP\textsuperscript{C} induced by the hormone. The data strongly suggest that the prion protein is involved in the control of hydrogen peroxide-dependent cytotoxicity, a physiological property of neutrophils associated with the fundamental role of the latter in host defense against pathogens (46). The effect of PrP\textsuperscript{C} upon neutrophil cytotoxicity was nevertheless neither all-or-none nor linear, which has also been observed in other experimental models (47). In contrast, both the sensitivity to cell death induced by peroxide and the protection provided by corticosteroid were identical in neutrophils of both genotypes. Thus, although both the presence and the up-regulation of PrP\textsuperscript{C} affected the capacity of neutrophils to induce peroxide-dependent damage on endothelial cells, their own resistance to peroxide was unaffected. The latter result differs from the association of PrP\textsuperscript{C} with resistance to oxidative stress in the nervous system (48, 49), but the discrepancy is not surprising, given the variety of other mechanisms involved in the resistance of neutrophils to the substantial amounts of reactive oxygen species produced by these cells during oxidative burst (50).

The neuroimmunoendocrine regulation of PrP\textsuperscript{C} in neutrophils and its impact upon the cytotoxic potential of this cell may help explain previous observations that behavioral stress, which modifies both the risk and the course of various diseases (51–55) and strongly modulates the immune system (56–58), also affects the cytotoxic functions of neutrophils (59). Notably, the cytotoxicity of neutrophils often swings toward the production of tissue damage (15, 16). This warrants the examination of the hypothesis that the neuroimmunoendocrine regulation of PrP\textsuperscript{C} may be involved in the deregulation of vascular permeability concurrent with inflammatory components of various pathological conditions (16, 59, 61–63). Interestingly, disruption of endothelial tight junctions also follows exposure to hydrogen peroxide (64, 65).

In conclusion, the overall data both unveiled a novel neuroimmunoendocrine pathway of regulation of the prion protein, as well as evidence that the content of PrP\textsuperscript{C} at the plasma membrane affects neutrophil effector functions. Similar to the multiple roles of PrP\textsuperscript{C} in the nervous system, the current data add to the diversity of immunomodulatory functions proposed for PrP\textsuperscript{C} which, together with the nonlinearity of the effect of PrP\textsuperscript{C} upon neutrophil cytotoxicity, is consistent with the view that the prion protein functions as a cell surface scaffold protein involved in the assembly of signaling modules (1, 60). The current demonstration of a close interaction of the nervous, endothelial, and immune systems upon PrP\textsuperscript{C} in neutrophils brings a fundamental advance to our understanding of how systemic control of the expression and function of the prion protein modulates cellular physiology and pathology.

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