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A role of cellular prion protein in programming T-cell cytokine responses in disease

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ABSTRACT **The cellular prion protein (PrP^C) is widely expressed in neural and non-neural tissues, but its function is unknown. Elucidation of the part played by PrP^C in adaptive immunity has been a particular conundrum: increased expression of cell surface PrP^C has been documented during T-cell activation, yet the functional significance of this activation remains unclear, with conflicting data on the effects of *Prnp* gene knockout on various parameters of T-cell immunity. We show here that *Prnp* mRNA is highly inducible within 8–24 h of T-cell activation, with surface protein levels rising from 24 h. When measured in parallel with CD69 and CD25, PrP^C is a late activation antigen. Consistent with its up-regulation being a late activation event, PrP deletion did not alter T-cell-antigen presenting cell conjugate formation. Most important, activated $PrP^{0/0}$ T cells demonstrated much reduced induction of several T helper (Th) 1, Th2, and Th17 cytokines, whereas others, such as TNF-α and IL-9, were unaffected. These changes were investigated in the context of an autoimmune model and a bacterial challenge model. In experimental autoimmune encephalomyelitis, PrP-knockout mice showed enhanced disease in the face of reduced IL-17 responses. In a streptococcal sepsis model, this constrained cytokine program was associated with poorer local control of infection, although with reduced bacteremia. The findings indicate that PrP^C is a potentially important molecule influencing T-cell activation and effector function—Ingram, R. J., Isaacs, J. D., Kaur, G., Lowther, D. E., Reynolds, C. J., Boyton, R. J., Collinge, J., Jackson, G. S., Altmann, D. M. A role of cellular prion protein in programming T-cell cytokine responses in disease. *FASEB J.* 23, 000–000 (2009)**

Key Words: T lymphocyte . knockout mouse . Th17

PRION PROTEIN (PRP) HAS BEEN the subject of intense study because it is the only factor known to be essential in the pathogenesis of the transmissible spongiform encephalopathies or prion diseases (1). However, the constitutive functions of the cellular isoform (PrP^C) remain unclear. Whereas PrP^C expression levels are highest in neural tissues, PrP^C expression is also readily detected in the lymphoid system (2, 3). Prion replication occurs in lymphoid tissues after peripheral infection, a process that is at least partly dependent on lymphoid PrP^C expression (4). There is a need to refine understanding of PrP^C function and the mechanisms regulating PrP^C expression, as PrP^C levels can be correlated with prion disease susceptibility (5, 6) as well as with other pathological features, such as neoplastic transformation (7). Furthermore, therapeutic strategies in prion disease may involve targeting or silencing of PrP, with the accompanying potential for inadvertent modulation of immune function.

Although PrP is constitutively expressed by human T lymphocytes, its expression is influenced by the activation state of the cell with reported up-regulation of cell surface PrP^C after T-cell activation (8, 9). PrP^C is subject to endocytic recycling (10) and is present in secretory cytoplasmic granules (11) that, at least in platelets, can facilitate increases in surface membrane PrP^C levels (12). Thus, surface expression levels may be influenced by post-translational processing as well as by transcriptional up-regulation. However, the extent to which the considerable increase in surface PrP^C expression caused by lymphocyte activation is dependent on transcriptional up-regulation has not been studied. PrP^C can be detected at the immune synapse, but attempts to colocalize it with other key synapse proteins have been inconclusive (13). Whether this failure to detect colocalization might be due to temporal dissociation between up-regulation and surface mi-

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gration of PrP^C and that of other synapse molecules is unknown. Indeed, neither the temporal relationship between PrP^C up-regulation and that of classical lymphocyte activation markers nor the specificity of PrP up-regulation compared with that of other glycosyl phosphatidylinositol (GPI)-anchored proteins has been elucidated.

The gene encoding PrP^c, *Prnp*, has been knocked out, and the mice show normal major histocompatability complex (MHC) class I and II expression, dendritic cell (DC) maturation, and numbers of hematopoietic stem, $CD4^+$, $CD8^+$, and B cells (13–16), with no signs of any spontaneous immunopathology (14, 17). However, data on responses of $PrP^{0/0}$ T cells to activation are somewhat conflicting: whereas some have reported reduced proliferative and cytokine responses to mitogens, others found no significant differences from wild-type controls (9, 14, 18–20). Furthermore, whereas PrP is strongly expressed in regulatory T (Treg) cells, studies with knockout mice give no clear evidence as to the specific functional implications of this expression (21). Mice lacking both PrP and its downstream partial homolog Doppel have normal $CD8^+$ expansions after lymphocytic choriomeningitis virus (LCMV) infection and specific antibody production after infection with LCMV or vesicular stomatitis virus (22). However, the range of infectious and immunological challenges to which PrP knockouts have been exposed is limited. The precise role of PrP^C in lymphocyte activation and during functional immune responses thus remains unclear.

In addition to its expression within the adaptive immune system, there are data linking PrP^C with innate immunity. PrP^C has been proposed to modulate phagocytosis by macrophages and promote neutrophil recruitment in zymosan-induced peritonitis (23). PrP^C is expressed in myeloid cells and is up-regulated during maturation of circulating monocytes and bone marrow-derived DCs (13, 24). PrP^C may also influence the capacity of DCs to stimulate T cells. In mixed lymphocyte reaction (MLR) experiments, PrP knockout in DCs impaired allogeneic responses whereas knockout in responder T cells did not (13). The highly conserved nature of PrP^C during evolution suggests that it may mediate a phylogenetically relatively ancient function, such as pathogen pattern recognition (25). Indeed, the range of ligands to which PrP^C has been demonstrated to have affinity is impressive (26) and includes certain polysaccharides that potently stimulate PrP^C internalization (27). Thus, in acting as a pathogen recognition molecule and playing a role in T-cell-DC signaling or activation, PrP^C may serve as a link between innate and adaptive immunity.

The alternative differentiation fates of CD4 T cells, an aspect of immune activation that is highly susceptible to the influence of small differences in the innate-adaptive interface, has received little attention in the context of PrP^C function. The fate of a CD4 T cell to adopt different cytokine transcriptional profiles with very different phenotypic characteristics and implications for immune and pathological phenotype is determined by a number of factors. Th1, Th2, Th17, and Treg cells develop their different cytokine profiles through the activation of decisive lineage-determining transcription factors, T-bet, GATA-3, RORγt, and Foxp3, respectively (28). Among the factors that may influence these branch points are cytokine milieu, antigen-presenting cell (APC) type and activation state, antigen dose, and T-cell receptor (TCR) affinity. There is currently particular interest in the nature of interactions predisposing to Th17 function, especially in the context of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) (29). The observation that Th17-defective $p19^{-/-}$ or $p40^{-/-}$ mice are EAE-resistant and Th1-defective $p35^{-/-}$ mice are conventionally susceptible forced a shift in the paradigm of cytokines in autoimmunity (29–31).

In this study, we examined the expression of PrP at transcriptional and surface protein levels during T-cell activation. We then compared T-cell effector functions between wild-type and $PrP^{0/0}$ FVB/N mice. We demonstrated that PrP^C is a late T-cell activation antigen, controlled by increases in *Prnp* transcription. Whereas *Prnp* deletion in our model does not alter T-cell to APC conjugation, $PrP^{0/0}$ T cells demonstrate altered ability to enter normal differentiation programs for release of several cytokines. We here go on to characterize the consequences of this defect in two models, EAE as an example of autoimmune disease and streptococcal sepsis as an example of infectious disease.

MATERIALS AND METHODS

Mice

Adult, wild-type FVB/N and C57/BL6 mice were used where described. $PrP^{0/0}$ mice were originally made on a C57BL/6 × 129 Sv background (14) and crossed onto an FVB/N background for 10 generations. All experiments on $PrP^{0/0}$ mice used age-matched wild-type FVB/N mice as controls. Transgenic mice carrying a human TCR specific for HLA-DR15/MBP 85-99 (line 7) mice were made as described (32). These mice develop spontaneous demyelinating disease. However, all mice used here were free from clinical disease (score 0) at the time of sacrifice. All mice were housed in accordance with institutional and UK Home Office requirements.

Peptide activation of T cells

For *in vitro* activation of splenocytes from line 7 mice, a peptide corresponding to mouse mylin basic protein (MBP) 85-99 was synthesized by the Advanced Biotechnology Centre (Imperial College London, London, UK) and used in *in vitro* culture at 2 μg/ml.

EAE induction

EAE was induced in young female $PrP^{0/0}$ (n=8) mice with age-matched wild-type FVB/N mice as controls (n=8). On d 0 and 7, mice received 200 μg of proteolipoprotein 95-116 in complete Freund's adjuvant supplemented with killed *Myco-*

bacterium butyricum (4.5 mg/ml) (33). On d 0, 2, 7, and 9, mice were given 200 ng of pertussis toxin (i.p.). Mice were weighed daily and scored for clinical signs of disease as follows: 0, normal; 1, limp tail; 2, impaired righting reflex or waddling gait; 3, partial hind limb paralysis; 4, total hind limb paralysis; 5, total limb paralysis; and 6, moribund.

Analysis of CNS T cells

Wild-type or $PrP^{0/0}$ mice in which EAE had been induced as above (n=8 of each strain) were sacrificed on d 13, and their brains were individually harvested into RPMI 1640 medium before being minced with sterile scissors and further homogenized before adding DNase/collagenase and incubating at 37°C for 1 h. Single cell suspensions were washed once in tissue culture medium. Mononuclear cells were prepared from the interface of a 30%:70% discontinuous Percoll gradient after centrifugation for 20 min at 850 *g*. The cells were washed 3 times with HL-1 serum-free medium (Cambrex, East Rutherford, NJ, USA), supplemented with 1% L-glutamine (Invitrogen, Paisley, UK) and 0.5% penicillin-streptomycin (Invitrogen), and resuspended at 2×10^6 cells/ml in HL-1. The cells were stimulated with PMA/ionomycin (5 and 200 ng/ml, respectively) for 2 h, Golgi stop (BD Biosciences, Oxford, UK) (1 μl/ml) was then added, and the cells were incubated for a further 4 h. The cells were then stained and fixed. Absolute numbers of T cells infiltrating the brain of each animal were calculated using the total viable cell counts, and the percentage of $CD3^+$ cells was detected by flow cytometry.

Streptococcal sepsis model

Young male $PrP^{0/0}$ (n=10) mice and sex- and age-matched wild-type FVB/N control mice (n=10) were infected intramuscularly in the thigh with a PBS suspension of 1×10^7 *Streptococcus pyogenes* (M1 serotype, reference stain NCTC8198). The mice were culled at 6 h (n=5 of each strain) and 24 h (n=5 of each strain) postinfection. At both time points a segment of spleen from each mouse was placed in RNAlater (Sigma-Aldrich, Poole, UK) and stored at −20°C until used for real-time PCR analysis. At 24 h postinfection, blood was collected by cardiac puncture, and tissue was collected from the site of infection (thigh) and the spleen. Five microliters of the blood was diluted in 45 μl of PBS containing 5 U of sodium heparin. The remaining blood was allowed to clot at room temperature for 2 h. The serum fraction was collected and stored at −20°C until used for Luminex cytokine analysis. Spleen and thigh tissue samples were weighed and homogenized before these and the anticoagulated blood were appropriately diluted with PBS and plated onto Columbia blood agar plates (Oxoid, Cambridge, UK). After 24 h at 37°C with 5% CO_2, β-hemolytic colonies were counted and the numbers of colony forming units per milligram of tissue were calculated.

Stimulating antibodies and cytokines

For *in vitro* Th subset differentiation, combinations of anti-CD3 (5 μg/ml; eBioscience, San Diego, CA, USA), anti-CD28 (2 μg/ml; eBioscience), recombinant IL-2 (20 IU/ml; Teceleukin, NCI-Frederick Biological Resources Branch, Frederick, MD, USA), and IL-23 (50 ng/ml; R&D Systems, Minneapolis, MN, USA) were used as indicated. For analysis of PrP^C, CD25, and CD69 expression, wild-type FVB/N splenocytes were stimulated with anti-CD3 (0.6 μg/ml) and anti-CD28 (2 μg/ml).

Lymphocyte activation

Cells were cultured in U-bottom 96-well plates at 3×10^5 cells/well, except for line 7 and Th subset activation studies in which cells were cultured in 24-well plates at a density of 2×10^6 or 3×10^6/ml, respectively, in HL-1 medium. Mitogens and/or cycloheximide (Sigma-Aldrich) was added to wells as described for individual experiments. All incubations were performed at 37°C with 5% CO_2. Where relevant, 1 μCi of [^{3}H]thymidine was added after 48 h of culture, and plates were incubated overnight for analysis of isotope uptake with a beta counter (Wallac, Turku, Finland).

In intracellular cytokine staining (ICCS) experiments, after either 24 or 72 h, 1 ml of supernatant was removed from cultures in 12-well plates. Supernatants taken at 72 h were stored at −20°C until used for analysis of secreted cytokines by Luminex. The removed supernatant was replaced with 1 ml of fresh medium containing PMA/ionomycin (5 and 200 ng/ml, respectively) and Golgi stop (1 μl/ml). Cells were incubated for 6 h before being stained and fixed.

Quantification of cytokines

Cytokines in mice sera taken at 24 h after *S. pyogenes* infection (n=5 for each mouse strain) and supernatants recovered at 72 h from *in vitro* lymphocyte activation studies (n=5 for each strain) were quantified using a Bioplex assay (Bio-Rad, Hercules, CA, USA). This enumeration was carried out following the manufacturer's protocol. In brief, this is a bead assay based on a capture sandwich immunoassay. A mixture of antibodies targeting specific cytokines, coupled to internally dyed beads, were reacted with the samples. After a series of washes to remove unbound protein, biotinylated detection antibodies were added. Streptavidin-phycoerythrin was then added, which bound to the detection antibodies on the bead surface. Data from the reaction were then acquired using the Luminex 200 system (Luminex Corporation, Austin, TX, USA). The individual dyed bead populations as well as the fluorescent signal on the bead surface were detected. This allowed identification of each cytokine and reported the level of target protein in the well, extrapolated from a standard curve.

Antibodies and flow cytometry

Anti-PrP mouse IgG1 monoclonal ICSM18 (D-Gen Ltd., London, UK) was FITC-conjugated using the FluoroTag-FITC kit (Sigma-Aldrich) according to the manufacturer's instructions. FITC-conjugated mouse IgG1 (eBioscience) was used as a control for ICSM18-FITC. Other fluorophore-conjugated antibodies (and isotype controls) were purchased from eBioscience as follows: FITC-conjugated: anti-mouse CD4, anti-mouse Qa2, and anti-mouse Thy1.2; PE-conjugated: anti-mouse CD4 and anti-mouse CD69; PECy5-conjugated: anti-mouse CD4 and anti-mouse CD8; and APC-conjugated: anti-mouse CD25 and anti-mouse CD3. For intracellular staining of cytokines, cells were stained with PE-conjugated anti-IL-10 or anti-IL-17 (BD Biosciences) or FITC-conjugated anti-IFNγ antibodies (BD Biosciences) and isotype controls according to the manufacturer's protocols at 24 and 72 h.

All flow cytometry was performed using either a FACSCalibur or FACSAria machine (BD Biosciences), and data were analyzed using FlowJo (Treestar, Ashland, OR, USA) or CellQuest software (BD Biosciences). For analysis of PrP expression, the geometric mean values for PrP and an isotype control (measuring autofluorescence and nonspecific binding) were obtained. PrP expression was defined as the difference between

these two values (Δ geometric mean). In some experiments using murine splenocytes, cells were split into PrP^{high} and PrP^{low} populations. The cutoff between PrP^{high} and PrP^{low} was determined using a FITC-conjugated IgG1 control antibody.

Quantitative real-time PCR

RNA was prepared by the acid phenol method using TRIzol (Invitrogen). RNA concentration and the nucleic acid/protein ratio were analyzed using a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE, USA). RNA was diluted to 60–200 ng/ml. cDNA was prepared using SuperScript III RNase HRT (Invitrogen) according to the manufacturer's instructions and stored at −20°C until further use.

Quantitative analysis of *Prnp* and 18S transcript levels was performed using TaqMan Assays-on-Demand, with TaqMan MGB probes (FAM dye-labeled) (Applied Biosystems, Foster City, CA, USA). The T-cell transcription factors and the housekeeping genes *Rpl-13a* and *YWHAZ* were enumerated using the in-house designed primer and probe set detailed in **Table 1**.

Values for each transcript were obtained by running monoplex PCR reactions (in triplicate) for each sample containing cDNA equivalent to 200 ng of RNA. Reactions were performed in a 20-μl volume on a Mx3000P real-time PCR thermocycler (Stratagene, LA Jolla, CA, USA), programmed at 50°C for 2 min, followed by 10 min at 95°C and then 50 cycles of 15 s at 95°C and 1 min at 60°C.

Prnp transcript in each sample was calculated relative to 18S quantity using the $\Delta\Delta C_T$ method. The C_T values generated from the T-cell transcription amplifications were analyzed using the Relative Expression Software Tool (REST) (34).

Conjugate analysis

Splenocytes were suspended at 1×10^8/ml in PBS with 0.1% BSA (Sigma-Aldrich). T or B cells were negatively selected using the appropriate kit for T or B isolation (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. Purity of isolated T and B cells was determined by flow cytometry to be >96%. T cells were suspended at 5×10^6/ml in HL-1 medium containing 1 μM 6-carboxyfluorescein diacetate (Molecular Probes, Invitrogen). B cells were suspended at 5×10^6/ml in HL-1 medium containing 10 μM Snarf-1 (Molecular Probes). Cells were incubated at 37°C for 30 min and then washed in HL-1 medium. CFDA-stained cells were rested for 1 h at 37°C before further use. CFDA-stained T cells and Snarf-1-stained B cells were mixed at a ratio of 1:2 in HL-1 medium to a total cellular density of 5×10^6/ml. staphylococcal enterotoxin A (SEA) (Sigma-Aldrich) was added at a final concentration of 5 μg/ml. Cells were incubated at 37°C for 2–4h. At the end of the incubation an equal volume of 2× CellFix (BD Biosciences) was gently laid over the cell suspension. CFDA-stained T cells were detected in the FL-1 channel and Snarf-1-stained B cells in the FL-2 channel. The efficiency of conjugate formation was measured as a percentage of total acquired T cells, *i.e.*, total number of conjugates/(total number of conjugates + total number of unconjugated T cells) × 100.

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between $PrP^{-/-}$ or wild-type FVB/N mice were performed using Student's *t* test or ANOVA as stated. The exception is the flow cytometric analysis of cells expressing a specific marker because percent data are intrinsically nonparametric and therefore were analyzed using the Mann-Whitney test.

RESULTS

PrP^C up-regulation in activated CD4 splenocytes follows that of CD69 and CD25

Splenocytes from FVB/N mice were stimulated with soluble anti-CD3 and anti-CD28, and the expressions of PrP^C, CD69, and CD25 in $CD4^+$ lymphocytes were measured during the first 72 h of activation. The first marker to be up-regulated was CD69, with ~70% of $CD4^+$ cells expressing this marker within 4 h of activation, rising to more than 80% by 24 h and more than 90% by 48 h (**Fig. 1*A***). This was followed by CD25, which was expressed by ~25% of $CD4^+$ cells after 8 h of stimulation, ~50% after 24 h of stimulation, and universally by 48 h (Fig. 1*B*). PrP^C was first elevated from baseline by 24 h with an ~9- to 10-fold increase in expression at 72 h compared with starting basal levels and nonactivated cells cultured in parallel (Fig. 1*C*). At 24 h the rise in PrP^C expression was greater in the $CD69^+CD25^+$ population compared with that in the $CD69^+CD25^-$ population (Fig. 1*D*). Thus, we concluded that PrP^C is an activation antigen, although it is more slowly up-regulated than CD69 and CD25 after CD3 and CD28 ligation.

TABLE 1. *T-cell transcript and housekeeping control real-time PCR primers*

Gene	Sense primer	Antisense primer	6FAM-probe-TAMRA
Rpl-13a	5′-CCTACCAGAAAGTTTGCTT ACCTG-3′	5′-GATCTGCTTCTTCTTCCGAT AGTG-3′	5′-TGACAGCCACTCTG GAGGAGAAACG-3′
YWHAZ	5′-AACTTAACATTGTGGACAT CGGATA-3′	5′-TGGATGACAAATGGTCTACT GTGTA-3′	5′-TGAAGCAGAAGCAG GAGAAGGAGGG-3′
T-bet	5′-ACTTTGAGTCCATGTACG CATCT-3′	5′-AGGATACTGGTTGGATAGAA GAGGT-3′	5′-CACCTGGACCCAACT GTCAACTGCTT-3′
RORγt	5′-GTCTGCAAGTCCTTCC GAGAG-3′	5′-ATCTCCCACATTGACTTCC TCTG-3′	5′-CTGCGACTGGAGGA CCTTCTACGGC-3′
GATA3	5′-TACCACCTATCCGCCC TATGT-3′	5′-ACACACTCCCTGCCTTC TGT-3′	5′-ACAGCTCTGGACTCT TCCCACCCAG-3′
FoxP3	5′-ATAGTTCCTTCCCAGAGTT CTTCC-3′	5′-ATGGTAGATTTCATTGAGTGT CCTC-3′	5′-CACCTATGCCACCCT TATCCGATGG-3′

Sequences of the primer/probe sets used to quantify the control housekeeping genes (*Rpl-13a* and *YWHAZ*) and the transcription factors used to define Th1 (*T-bet*), Th2 (*GATA3*), Th17 (*RORγT*), and Treg (*FOXP3*) differentiation.

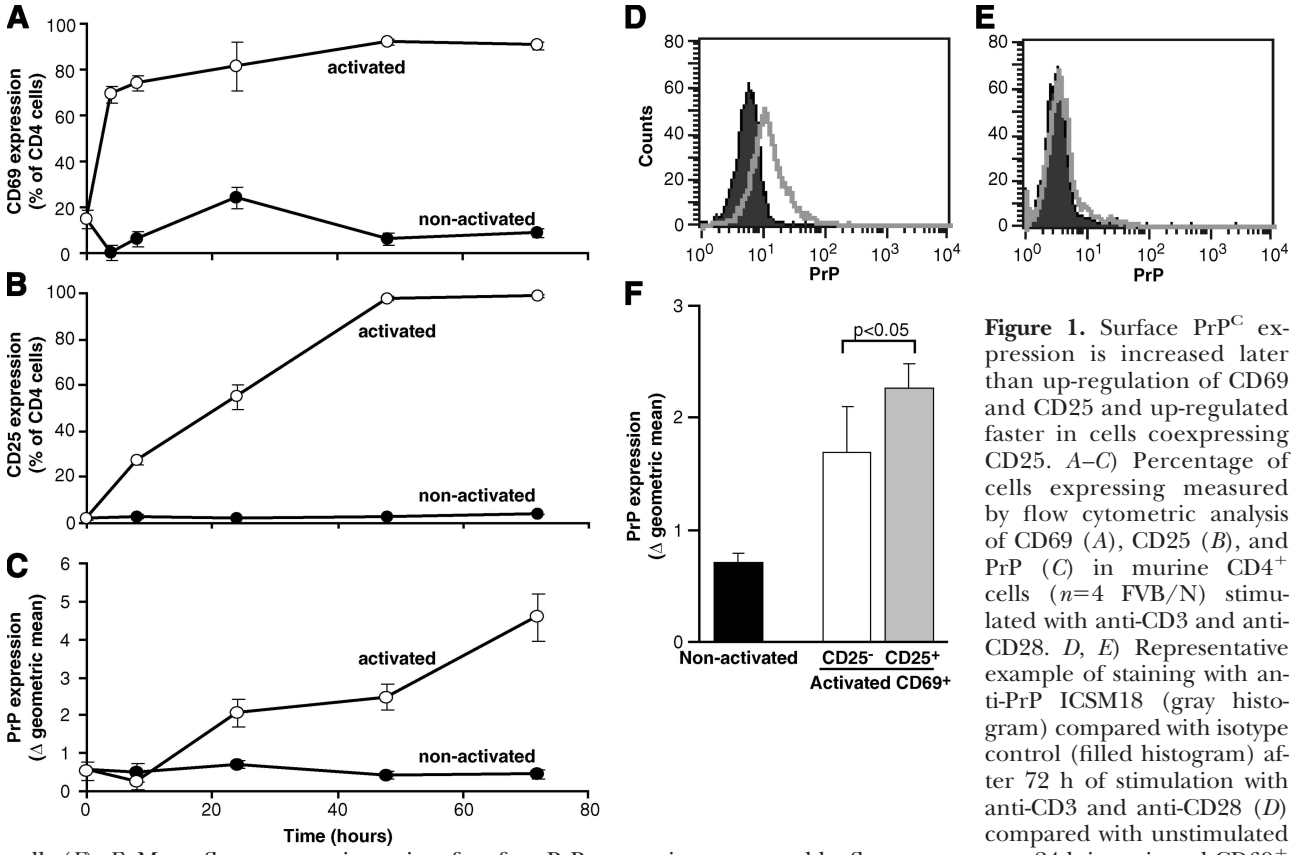

Figure 1. Surface PrP^C expression is increased later than up-regulation of CD69 and CD25 and up-regulated faster in cells coexpressing CD25. *A–C*) Percentage of cells expressing measured by flow cytometric analysis of CD69 (*A*), CD25 (*B*), and PrP (*C*) in murine $CD4^+$ cells (n=4 FVB/N) stimulated with anti-CD3 and anti-CD28. *D, E*) Representative example of staining with anti-PrP ICSM18 (gray histogram) compared with isotype control (filled histogram) after 72 h of stimulation with anti-CD3 and anti-CD28 (*D*) compared with unstimulated cells (*E*). *F*) Mean fluorescence intensity of surface PrP expression measured by flow cytometry at 24 h in activated $CD69^+$ cells is higher in cells coexpressing CD25 (ANOVA with Tukey-Kramer multiple comparisons *post hoc* test).

PrP^C expression at transcriptional and translational levels in response to peptide-MHC stimulation of TCR transgenic lymphocytes

To characterize the expression of PrP^C during a more physiological model of *in vitro* T-cell activation, we used splenocytes from transgenic mice expressing human HLA-DR15 and a TCR specific for MBP 85-99 (line 7). Approximately 97% of the mature $CD4^+$ lymphocytes from these mice carry the transgenic TCR and recognize this myelin epitope (32). When cultured with MBP 85-99 (2 μg/ml), we saw increases in *Prnp* mRNA from line 7 splenocytes as measured by real-time RT-PCR (**Fig. 2*A***). Mean *Prnp* expression was 6-fold higher after

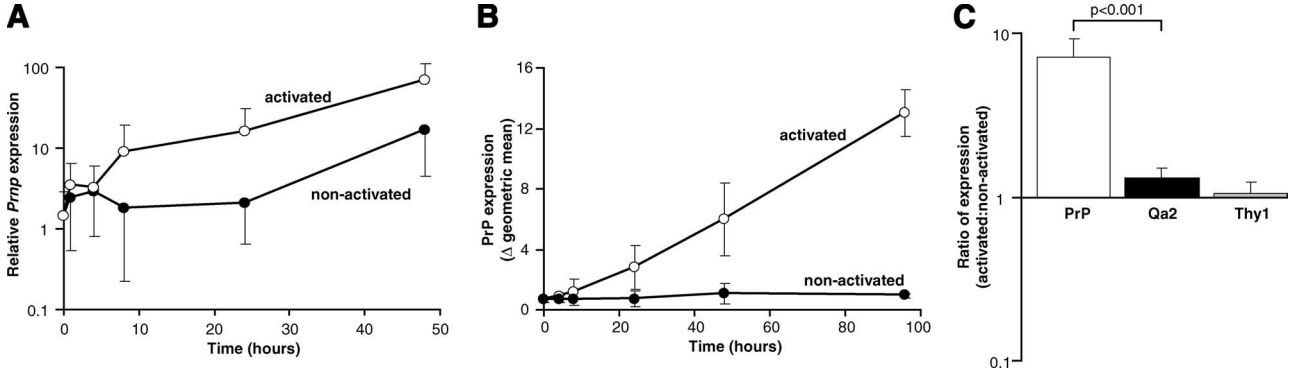

Figure 2. Up-regulation of surface PrP^C expression is preceded by increased abundance of *Prnp* mRNA and is not a nonspecific effect on GPI-anchored proteins. *A*) *Prnp* expression measured by real-time RT-PCR in TCR transgenic splenocytes stimulated with MHC-peptide (n=6 line 7 mice at each time point, except 48 h, where n=3). *B*) Surface PrP expression measured by flow cytometry in $CD4^+$ cells activated with specific MHC-peptide (line 7 mice; 0 h, n=10; 4, 8, and 24 h, n=7; 48 h, n=12; and 96 h, n=3). *C*) Change in surface expression of PrP, Qa2, and Thy1 measured by flow cytometry in $CD4^+$ T cells (n=4 line 7 mice) on MHC-peptide stimulation for 48 h. Both PrP (P<0.001) and Qa2 (P=0.011) expression are significantly increased by activation, whereas there is no significant change in expression of Thy1 (P=0.662) (log-transformed values, Student's t test). However, the magnitude of increase in surface PrP expression is significantly higher than that of Qa2 (log-transformed values, P<0.001, Student's t test).

8 h and ~50-fold increased compared with baseline at 48 h. These changes were reflected in surface PrP^C levels, with an upward trend at 8 h and an ~20-fold increase on baseline expression at 96 h (Fig. 2*B*). One possible explanation for the rise in PrP^C expression is that this is a general feature of GPI-anchored proteins during T-cell activation and not a specific property of PrP^C. We therefore compared changes in cell surface PrP^C expression with those of GPI-anchored proteins Thy1 and Qa2 during *ex vivo* stimulation of line 7 splenocytes. Although there was a slight increase (~30%) in surface Qa2 at 48 h, this was significantly less than the >7-fold increase observed for PrP^C, whereas there was no significant change in the level of Thy1 (Fig. 2*C*).

Surface PrP^C up-regulation requires *de novo* protein synthesis

To determine whether increased surface PrP^C seen on T-cell activation required *de novo* protein synthesis, we performed the *ex vivo* stimulation in the presence of cycloheximide. This treatment almost completely inhibited the increase in PrP seen at 24 h, even though *Prnp* transcript up-regulation was not entirely abolished (**Fig. 3*A*, *B***), which implies that surface changes in PrP during lymphocyte activation

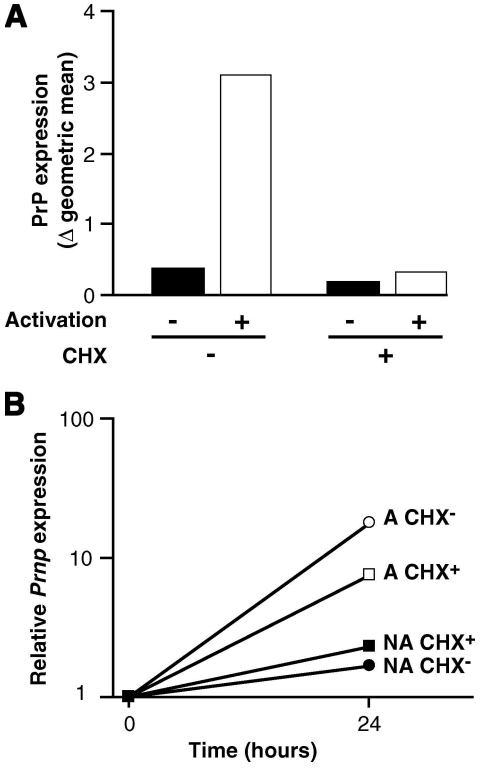

Figure 3. Up-regulation of cell surface PrP^C expression during T-cell activation requires intact ribosomal function. *A*) Surface PrP measured by flow cytometry in line7 $CD4^+$ cells stimulated with MHC-peptide for 24 h with or without cycloheximide (CHX) (10 μg/ml). *B*) *Prnp* levels measured by real-time RT-PCR in activated (A) and nonactivated (NA) splenocytes cultured for 24 h with or without CHX.

are dependent on new protein synthesis and cannot be mediated by cycling of preformed protein to the cell surface.

Absence of PrP^C does not affect formation of T-cell-APC conjugates

Increased expression of PrP^C during T-cell activation and DC maturation and migration of PrP^C to points of T-cell-DC contact have been proposed to reflect a role for PrP^C in formation or stabilization of the immune synapse (13). Thus, lack of PrP^C expression might be expected to impede T-cell-APC conjugate formation. We modeled the effect on T-cell-APC interaction in a polyclonal system by using the superantigen SEA to cross-link the TCR Vβ chain with MHC class II on B cells. We saw no significant difference in the proportion of $PrP^{+/+}$ or $PrP^{0/0}$ T cells able to form stable conjugates with syngeneic B cells in the presence of SEA (**Fig. 4**); ~10% of T cells in each group formed conjugates after incubation with SEA for 2 or 4 h.

$PrP^{0/0}$ mice show altered susceptibility in a model of bacterial sepsis

Although we and others have attempted to elucidate a role of PrP in model systems of immune function of the type described above, there has been little work done to look at more global measures of immune function, such as host defense against bacterial infection. We therefore compared knockout and wild-type mice in a murine model of infection with *S. pyogenes* (35, 36) (**Fig. 5**). This model allows investigation of the control of local infection at the inoculation site, which is mainly dependent on neutrophils, as well as control by the adaptive immune response of systemic spread. The ability to control the local bacterial infection in the thigh was impaired, as bacterial counts at this site were significantly higher at this site at 24 h. This finding may indicate a failure to traffic neutrophils to the site or a functional impairment in the ability to kill bacteria (Fig. 5*A*). However, despite the exacerbated local infection, $PrP^{0/0}$ mice showed a considerable decrease in bacteremia with respect to colony counts from the spleen and peripheral blood. In agreement with this result, the serum IL-6 level, which we find to be a reliable surrogate marker of sepsis in this model, was substantially reduced in knockout mice (Fig. 5*B*). Serum levels of the neutrophil chemoattractant, KC, were significantly reduced, offering a potential explanation for the poor control of infection in the thigh. A number of other serum cytokines here indicating the early stages of the adaptive immune response were also diminished. These included IL-2, IL-3, IL-5, IL-10, IL-13, and IL-17, although there were no detectable changes in the levels of IFNγ, TNF-α, and IL-9 (Fig. 5*B*). Although a reduction in T-cell cytokines seen in concert with seeming protection from bacteremia may at first seem paradoxical, it should be remembered that *S. pyogenes* carries a number of superantigens thought

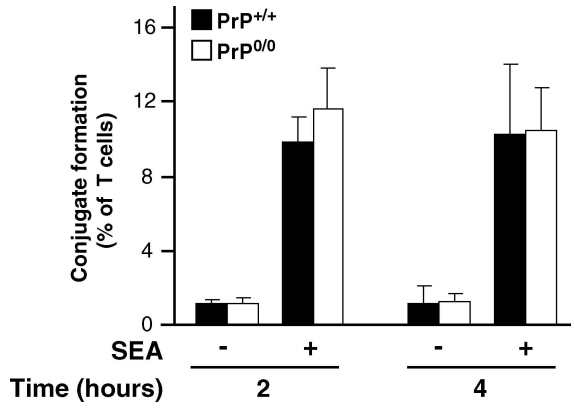

Figure 4. Embryonic deletion of PrP does not alter superantigen-induced T-cell-B-cell conjugate formation. Efficiency of $PrP^{+/+}$ and $PrP^{0/0}$ T-cell-B cell conjugation induced by superantigen SEA measured by flow cytometry. T and B cells (n=3/strain) were mixed at a 1:2 ratio with or without SEA for 2 or 4 h.

to be involved in the cytokine storm implicated in bacterial shock (37). Thus, the reduction in cytokines may ensue partly from reduced systemic bacterial infection leading to reduced superantigen stimulation of T cells. However, this theory did not offer any resolution as to the nature of any enhanced mechanism that could explain the reduction in systemic spread in the $PrP^{0/0}$ mice. We therefore decided to look at T-cell subset-specific transcription factors during the early response to infection, starting at 6 h (Fig. 5*C*). In keeping with the notion of some form of enhanced host response to infection in the knockouts, we found large changes in the levels of T-bet, RORγt, and GATA-3 relative to those in wild-type mice. That is, there were 8- to 50-fold changes in expression of the hallmark transcription factors for Th1, Th17, and Th2 cells, respectively. However, because this response is not sustained, with expression levels of each transcription factor falling to well below the level seen in wild-type mice by 24 h, we concluded that some component of the T-cell activation program necessary to follow through to a fully effective superantigen or antigen response is aborted in the knockout mice.

$PrP^{0/0}$ lymphocytes show an altered pattern of cytokine secretion

To pursue the issue of an abortive effector program in T-cell subsets in a more clearly defined system, we explored induction of a wide range of cytokines from cultured splenocytes activated with panels of defined stimuli. We chose to look at IL-2 as a simple driver of IL-2 receptor α-positive cells, IL-2 with anti-CD3 as a minimal "signal-1" trigger of activation, IL-2, anti-CD3, and anti-CD28 as an example encompassing costimulation "signal-2" as well, and then IL-2, anti-CD3, and anti-CD28 with the further addition of IL-23, on the assumption that this combination would be able to sustain and expand any preexisting Th17 cells (38). Splenocytes from wild-type or $PrP^{0/0}$ mice were cultured for 24 or 72 h in the presence of IL-2, IL-2 and anti-CD3, IL-2, anti-CD3, and anti-CD28, and IL-2 and IL-23 or the combination of IL-2, IL-23, anti-CD3, and anti-CD28, and then cells were analyzed by ICCS (**Fig. 6*A***), with supernatants collected for analysis by Luminex (Fig. 6*B*). Following on from the substantial changes we had seen in the more complex setting of bacterial challenge, we here saw major shifts in cytokine profiles between wild-type and $PrP^{0/0}$ mice. By ICCS, the proportion of cells making IL-17 and IL-10 is significantly reduced in knockout mice, whereas the proportion of cells making IFNγ is increased (Fig. 6*A*), supporting the notion of a shift from Th17 to Th1 differentiation. However, comprehensive analysis of actual cytokine yields by Luminex shows a rather widespread impairment in cytokine release; this encompasses reduced IFNγ release, despite the increased proportion of Th1 cells (Fig. 6*B*). Thus, many of the cytokines were significantly reduced in knockout mice, including IFNγ, IL-10, IL-17, IL-4, IL-5, IL-13, and IL-3, but not TNF-α or IL-9. Taken together with the studies above showing normal proliferation, conjugate formation, and initial cytokine transcription factors, this result supports the notion of impaired ability to sustain a full program of cytokine effector function after initial activation in knockout mice.

Susceptibility to EAE in $PrP^{0/0}$ mice

In light of the differences we had seen in the bacterial challenge model and the alteration in Th1 and Th17 populations we had seen in $PrP^{0/0}$ mice, it seemed likely that there might be an influence on susceptibility to EAE. This had once been considered a prototypic Th1-mediated disease until considerable evidence was accrued in recent years for a predominant role of Th17 cells in many murine EAE models (28–31); this conceptual model was recently modified to accommodate the findings that Th1 and Th17 cells may each be able to transfer disease of different histopathological features and that Th1 cells may be required to facilitate CNS ingress of Th17 cells (39, 40). We thus immunized knockout mice or wild-type controls with the encephalitogenic epitope, proteolipoprotein 95-116. This epitope was selected for its ability to induce EAE in $H\text{-}2^q$ strains (33). In the absence of the >12-generation breeding program to cross our mice onto a fully C57BL/6 background, it was not possible here to use the MOG 35-55 EAE model often exploited for Th17 investigations. We nevertheless reasoned that any fundamental lessons about cytokine profiles in EAE should be equally relevant irrespective of the choice of encephalitogenic peptide or mouse strain. We found that EAE in knockout mice was somewhat exacerbated (**Fig. 7*A***). This exacerbation was associated with a greatly increased number of CNS infiltrating T cells in the knockout animals (Fig. 7*B*). On ICCS analysis of the

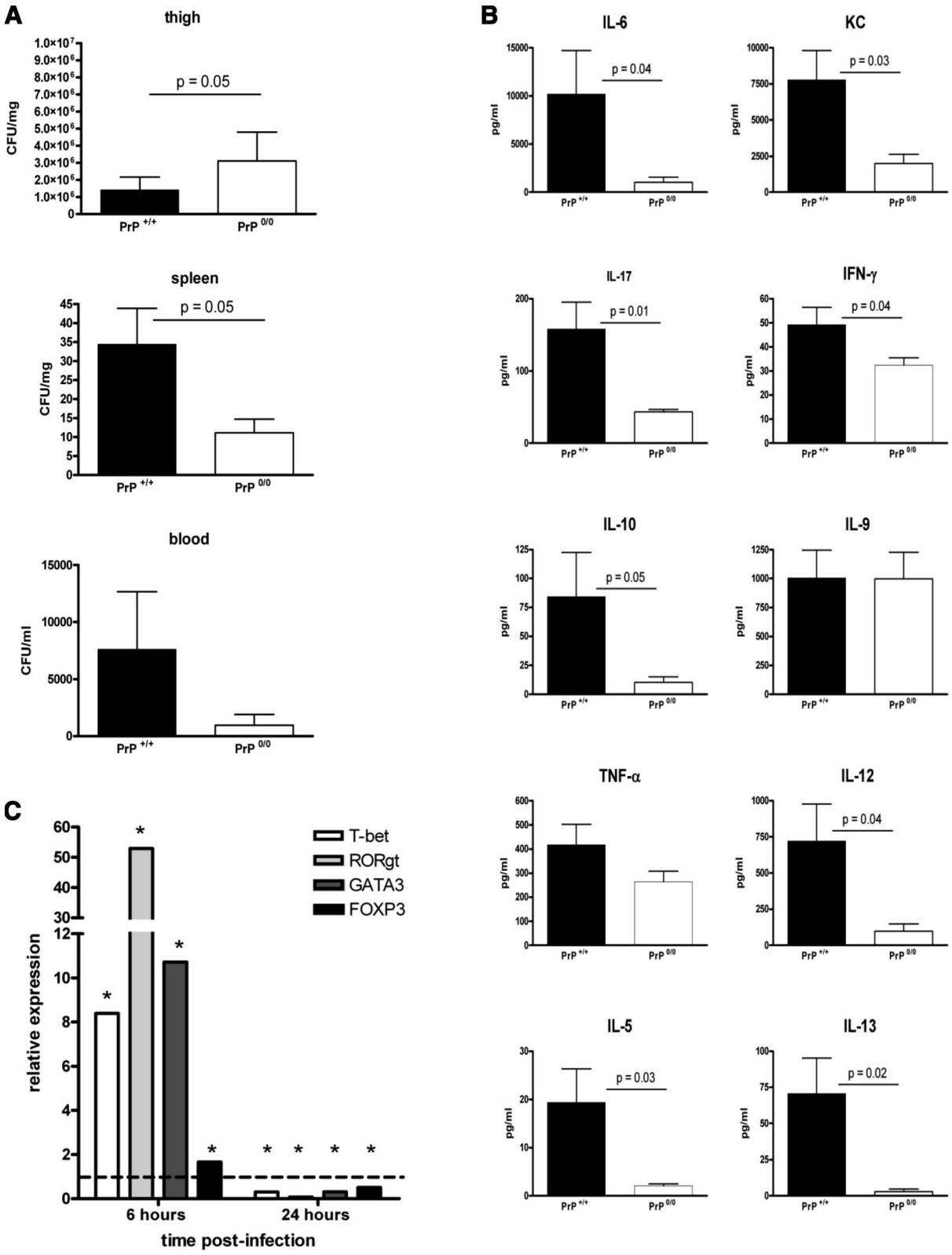

Figure 5. *S. pyogenes* infection experiment in $PrP^{+/+}$ (■) and $PrP^{0/0}$ (□) mice. *A*) Colony-forming units (CFU) of *S. pyogenes* detected 24 h postinfection (n=5 each; $PrP^{0/0}$ and FVB/N mice) at the site of infection (thigh) and systemic spread of bacteria in the blood and spleen. *B*) Serum cytokine levels 24 h postinfection (n=5/strain). Statistical analysis was performed with Student's t test. *C*) RT-PCR showing the relative expression of the transcription factors in $PrP^{0/0}$ mice compared with that in the wild-type control (samples normalized using the expression of two housekeeping genes *Rpl-13a* and *YWHAZ*). $^*P < 0.05$; REST analysis.

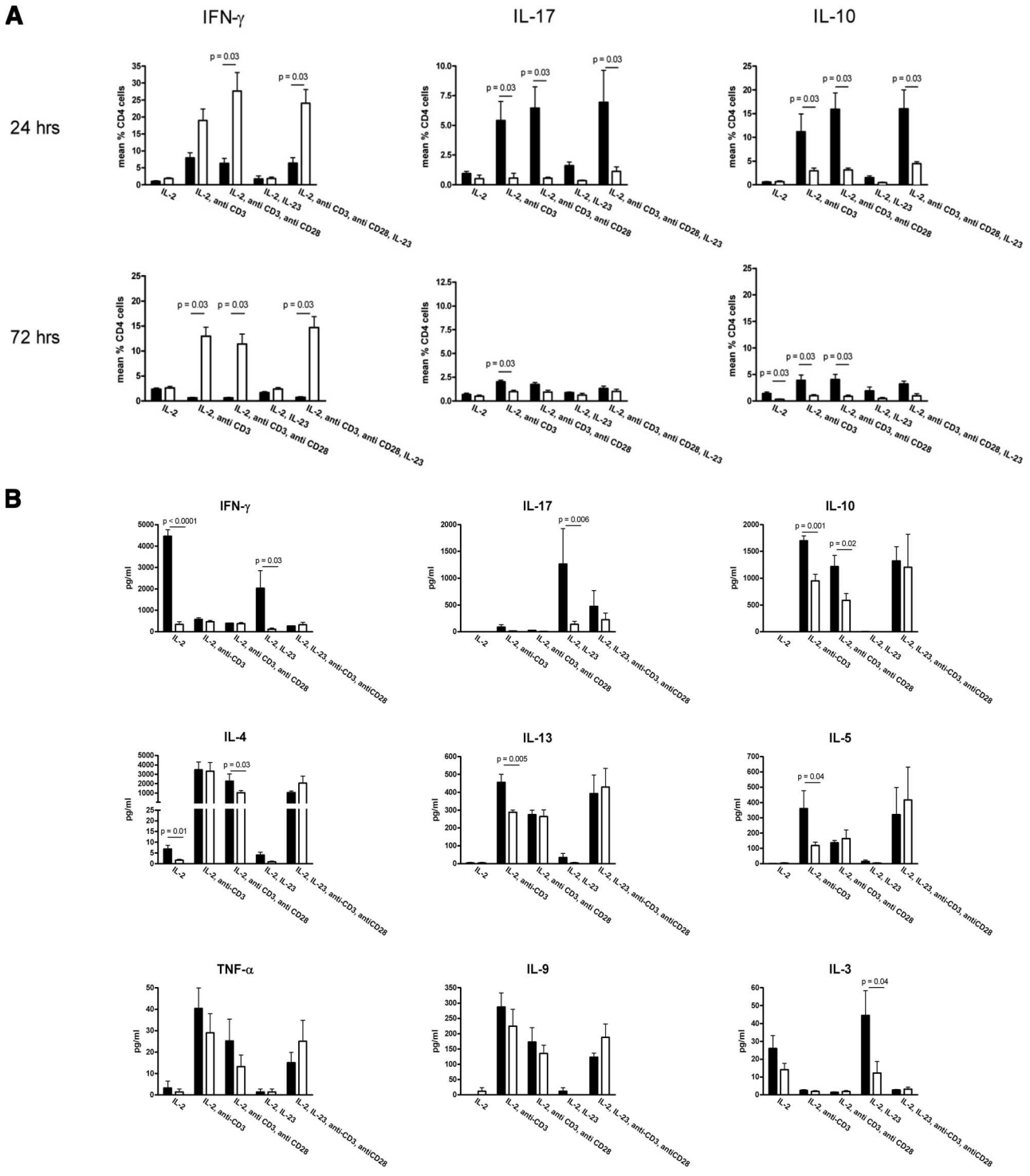

Figure 6. *In vitro* induction of Th17 responses is impaired in PrP-knockout mice. *A*) ICCS staining of PMA/ionomycin-stimulated $PrP^{0/0}$ or control FVB/N splenocytes (n=5/strain) after culture in IL-2 and combinations of anti-CD3, anti-CD28, and IL-23 for either 24 or 72 h. There is a significant reduced induction of IL-10 and IL-17 but increased percentage of $IFN\gamma^+$ cells (Mann-Whitney test). *B*) Cytokine levels in culture medium from cells stimulated as above for 72 h, demonstrating significant reductions in a broad range of Th1 and Th2 cytokines, but not IL-9 or TNF-α (Student's t test, with correction for multiple comparisons).

CNS-infiltrating T cells we found that the frequency of cells that were either $IL\text{-}10^+$-positive, $IFN\gamma^+$-positive, or IL-17/IFNγ-dual positive was largely unaltered. However, this disease exacerbation was associated with a significant deficit in $IL\text{-}17^+$ Th17 cells (Fig. 7*C*); the disease profile here may thus be reminiscent of the findings from the laboratory of Segal and others in which a distinctive EAE disease profile is attained in the absence or relative absence of Th17 cells (39, 40).

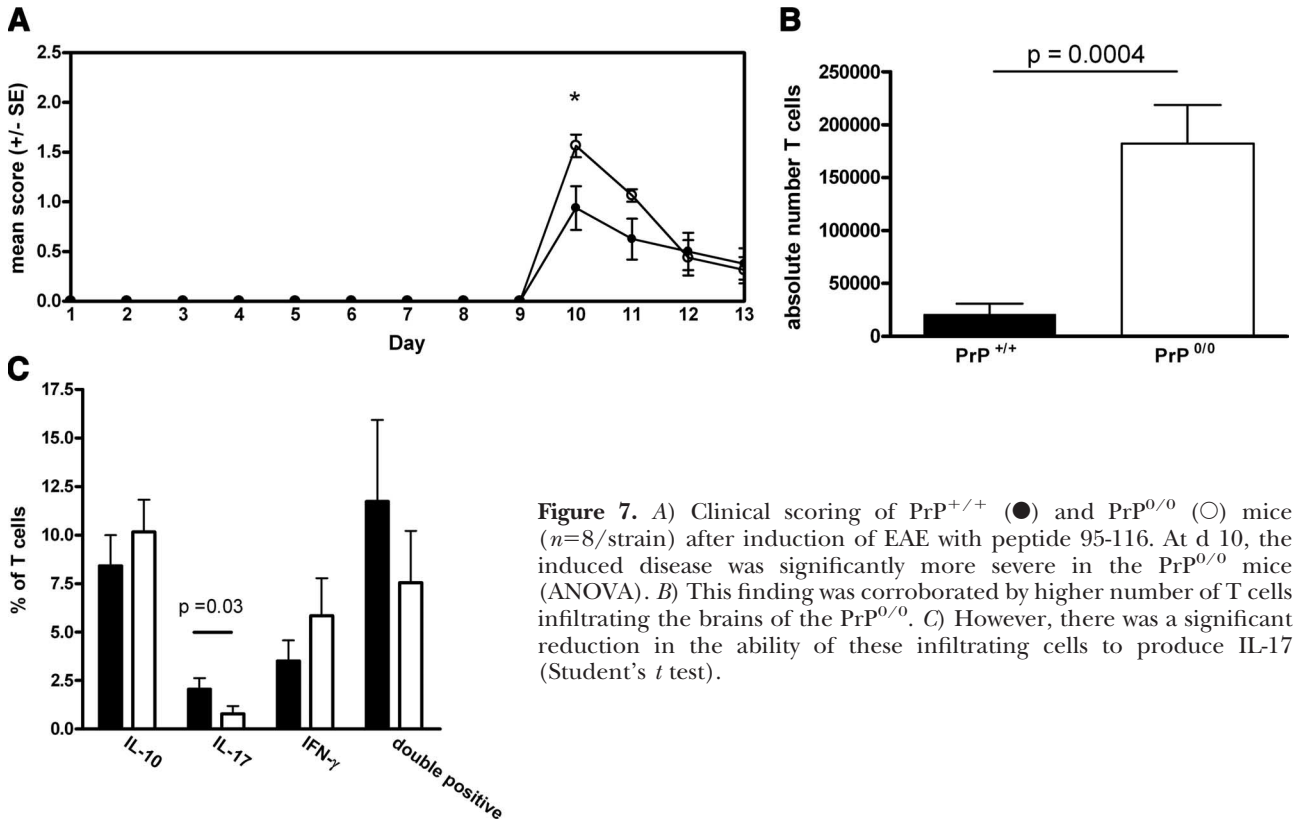

Figure 7. *A*) Clinical scoring of $PrP^{+/+}$ (●) and $PrP^{0/0}$ (○) mice (n=8/strain) after induction of EAE with peptide 95-116. At d 10, the induced disease was significantly more severe in the $PrP^{0/0}$ mice (ANOVA). *B*) This finding was corroborated by higher number of T cells infiltrating the brains of the $PrP^{0/0}$. *C*) However, there was a significant reduction in the ability of these infiltrating cells to produce IL-17 (Student's t test).

DISCUSSION

PrP^C is highly expressed by human lymphocytes (24, 41, 42), yet its function remains unclear. Elucidation of its role in lymphocyte biology will lead to a clearer understanding of the lymphoid-dependent phase of prion disease progression from the gut to the CNS and of the systemic effects of therapeutic targeting of PrP. In this study, we have characterized several aspects of PrP^C expression and function in the immune system; the most striking effect demonstrated is the markedly impaired ability of PrP-knockout mice to sustain a full cytokine effector program (despite initial activation and proliferation), with functional implications demonstrated here in both infection and autoimmune models.

PrP^C has for some time been considered a T-cell activation antigen (8, 9). However, the transcriptional regulation of PrP expression and its relationship to classic T-cell activation markers have not previously been characterized. We observed similar kinetics of surface PrP^C up-regulation in the line 7 TCR transgenic model and on polyclonal stimulation of wild-type murine $CD4^+$ cells. PrP^C up-regulation does not therefore appear to be dependent on specific MHC-peptide-TCR signaling but at a minimum may require nonspecific TCR stimulation. Indeed, cell surface PrP^C in lymphoid cells does not appear to be increased by treatment with PMA (43). These findings suggest that up-regulation of PrP^C is linked to T-cell signaling events upstream of protein kinase C and calcineurin.

The observed increase in surface PrP^C is dependent on *de novo* synthesis, as activation in the presence of cycloheximide blocked surface up-regulation, a phenomenon previously observed in monocytes that up-regulate PrP in response to IFNγ (24). Thus, although release to the surface of preformed PrP^C has been demonstrated in activated platelets (12), such mechanisms can be discounted as sources of increased surface expression in activated T cells. Moreover, under identical activation conditions, other GPI-anchored proteins are not robustly up-regulated. Thus, this phenomenon cannot be explained by nonspecific effects of T-cell activation on synthesis and trafficking of GPI-anchored proteins.

Following anti-CD3 and anti-CD28 stimulation of wild-type $CD4^+$ splenocytes, PrP^C was up-regulated more slowly than CD69 or CD25. In this model nearly all $CD4^+$ cells had become $CD69^+$ before up-regulation of PrP^C. These observations place PrP^C as a late T-cell activation antigen. Our results are in general agreement with previous reports in observing PrP up-regulation over a matter of hours rather than minutes (8, 9). The kinetics of *Prnp* transcription and the specificity of surface PrP^C up-regulation suggest that PrP levels during T-cell activation are regulated *via* specific preformed transcription factors. Indeed, the *PRNP* promoter contains a putative NFAT binding site (44), and PrP mRNA expression is increased during Th0 to Th2 differentiation (45), a process that is dependent on induction of NFATc2 expression by IL-6 (46).

Having established PrP^C as a late activation antigen, we then sought to elucidate more precisely its role in induction of T-cell immune responses. On the basis of

its localization in lymphocyte lipid rafts and coimmunoprecipitation with key proximal signaling molecules (47–49), we wondered whether PrP^C was implicated in function of the immunological synapse. As a model for T-cell/antigen/APC synapse formation we used SEA, which promiscuously binds MHC class II on B cells and activates the TCR on a significant proportion of T cells. We found that T-cell-APC conjugation caused by SEA was identical between PrP-deficient and wild-type cells. This finding is not unexpected in light of our observation that surface PrP^C is not appreciably up-regulated until at least 8 h of activation. The role, if any, of PrP^C in the immunological synapse thus remains unclear. Ballerini *et al.* (13) reported that despite its presence at the synapse during antigen-driven T-cell-DC conjugation, PrP^C did not colocalize with CD3, CD43, LAT, Thy-1, or LFA-1. Further, it has recently been shown that small interfering RNA-mediated silencing of PrP expression had no effect on immunological synapse formation by Jurkat T cells (42).

Thus, up to this point, we had concluded from our studies and those of others that PrP^C is a late activation antigen of T cells, present at the immune synapse, yet without an obvious functional requirement in that event. We therefore sought to examine the immunological phenotype of PrP null cells at later stages of immune activation in various models: *in vitro* activation of splenocytes using stimulatory cytokines and antibodies or *in vivo* models of bacterial infection and autoimmunity. The *in vitro* studies showed quite profound alterations in the cytokine programs of knockout cells. Whereas ICCS staining showed a clear switch in the response in favor of Th1 differentiation in preference to Th17, quantification of actual cytokine release showed a more general impairment. Specifically, production of several Th1, Th2, and Th17 cytokines was significantly reduced in knockout mice. Because of the diversity of cytokines involved, this result places the likely functional defect in knockout mice at the level of impairment in the TCR signaling cascade. Because our experiments encompass those in which activation is APC-independent, through anti-CD3/anti-CD28, it is relatively unlikely that the differences found here relate to the presence or absence of PrP^c on APC rather than T cells.

Recently, it has been demonstrated that in addition to classic Th1 and Th2 differentiation, there is a third class of effector $CD4^+$ T cell that is characterized by IL-17 production (28–31). These cells, termed Th17, are implicated in the development of certain autoimmune diseases and may also be important in defense against fungal and other pathogens (50). We initially looked at the ability of wild-type or knockout mice to resist bacterial infection. We have previously described a murine model of streptococcal sepsis that mimics many aspects of pathogenesis and immunity in the human disease, including superantigen-dependent septic shock (35–37). Interestingly, we found that although the knockout mice had impaired ability to clear the *S. pyogenes* at the site of inoculation in the thigh (an event that is largely neutrophil dependent), they were substantially protected from bacteremia and sepsis, both with respect to colony counts in the spleen and blood and to serum levels of IL-6, the major surrogate marker of disease severity. This result was accompanied by a substantial decrease in levels of most serum cytokines except IL-9 and IFNγ. One complex aspect of bacterial sepsis models is that it is difficult to unravel the protective, host immune response from the potentially pathogenic host response to bacterial superantigens. It is tempting to speculate that PrP-knockout mice may here be afforded some protection from full bacterial sepsis through their inability to mount a full-blown cytokine response. Our findings are noteworthy in the context of the impaired neutrophil response to zymosan-induced peritonitis in $PrP^{0/0}$ mice (23), may which, in light of our findings, may be due to deficient IL-17 production leading to inadequate neutrophil migration.

We predicted that mice showing perturbation of Th17 and Th1 responses might show altered susceptibility to EAE. Several pieces of evidence indicate an important role of Th17 cells in disease: mice lacking the p19 subunit of the IL-12/IL-23 receptor are EAE-resistant, implicating the IL-23/IL-17 pathway in disease (30). Furthermore, IL-17A-knockout mice show delayed disease onset and reduced severity (51) and IL-17R-Fc or anti-IL-17 antibody treatment can ameliorate EAE (52). Thakker *et al.* (53) argued that IL-23 was critical for the induction but not for the effector phase of EAE as disease induction by adoptive transfer to $p19^{-/-}$ mice was normal. More recently, as discussed by Steinman (31), the model has become somewhat more complex again with respect to the relative contributions of Th1 and Th17 cells. For example, Kroenke *et al.* (39)found that either IL-12 or IL-23 polarized (that is, Th1 or Th17) could transfer EAE, but whereas disease pathology in the former was more neutrophilic, that in the latter was more monocytic. A possible explanation for these seemingly contradictory observations across EAE experiments was offered by a recent study showing that Th1 cells, but not Th17 cells, have the ability to access the noninflamed CNS and thus a contribution from each cell-type is required for full-blown disease, with Th1 cells allowing CNS ingress of Th17 cells (40). In view of the rather diminished cytokine responses found in knockout mice in our earlier assays, we had expected to find reduced susceptibility to EAE. In fact, we found enhanced disease with a greatly increased number of CNS-infiltrating cells. This finding may indeed support the notion that, as long as both Th1 and Th17 cells are present (as was the case here), the relative shift to Th1 cells facilitates disease through allowing an increased number of encephalitogenic cells to access the CNS.

In conclusion, PrP^C expression appears to be robustly associated with the induction of immunological effector mechanisms. Although its precise function in these processes remains unclear, we have demonstrated

that it may play a role in sustaining an effector cytokine program after TCR stimulation, with some evidence that Th17 responses may be more PrP^c-dependent than Th1 responses. However, many cytokine responses are impaired in the knockout mice, encompassing also Th2 cytokines. Further *in vivo* challenges to PrP-null models and conditional knockout models and *in vivo* administration of PrP-ligating agents may assist in refining knowledge of the lymphoid function of PrP^C and predicting the effects of anti-PrP therapeutics on the immune system. Our data suggest that such anti-PrP therapeutics could have profound effects on both autoimmunity and resistance to infection through modulation of responses. FJ

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